Renal and mammary PD excretion in Holstein/Friesian dairy cows

Its potential as a non-invasive index of protein metabolism

by

Kevin John Shingfield B.Sc. (Nottingham)

A Thesis submitted to the Faculty of Science of the University of Glasgow

for the degree of Ph D

June, 1996

Grassland and Ruminant Science Department

The Scottish Agricultural College

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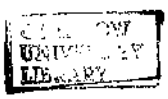

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Acknowledgements

I am indebted to many people who have made this project possible. I would like to express my gratitude to Dr. N.W. Offer, Dr. R.J. Dewhurst and Dr. D.J. Roberts for their assistance and supervision. I would like to thank Nick Offer in particular, for his encouragement, good humour and intellectual stimulation during this study.

I am also indebted to the following:- Angela Mitton, Anne Dowdeswell, Irene Yuill and Ian Wilson for experimental assistance, John McAuslan and his staff for the care of experimental animals, Dr. Jim Dixon and Dr. Ivy Barclay and their technicians, and Kenny McIssac for technical and analytical expertise, Alistair Sword and David Hitchcock of the Scottish Biomathematics and Statistics Service for advice on data analysis and the library staff at SAC, Auchincruive for assistance in researching references. In addition, I would like to thank Mr Stuart Robertson for expertise and training in the fitting of bladder catheters.

I would also like to acknowledge the Ministry of Fisheries and Food for provision of the current studentship.

Finally, I would like to express my sincere gratitude to my parents, for their unfailing support throughout my education and friends and colleagues at SAC, Auchincruive for their continual support and sense of humour.

Declaration

For the animal experiments described in chapters 6, 7 and 8, I had occasional assistance from Angela Mitton, Anne Dowdeswell, John McAuslan, Irene Yuill and Ian Wilson of the Grassland and Ruminant Science department, SAC, Auchincriuve. For all experiments technical staff of the Analytical Services Unit and Dairy technology department, SAC, Auchincruive, conducted routine feed and milk analysis. Experimental designs, day to day management of experiments, analytical development, sample analysis, statistical evaluation and thesis preparation were all my own work. Sources of information have been acknowledged in the form of references.

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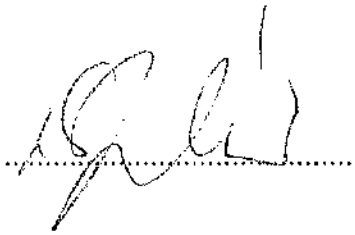


K.J. Shingfield

Abbreviations

AA	Amino acid
A/c	Molar ratio of allantoin to creatinine
ADOMR	Organic Matter Apparently Digested in the Rumen
AFRC	Agricultural and Food Research Council
AHEE	Acid Hydrolysed Ether Extract
AMP	Adenosine Monophosphate
ARC	Agricultural Research Council
ATP	Adenosine Triphosphate
CDM	ethanol Corrected toluene Dry Matter
CP	Crude Protein
CPI	Crude Protein Intake
CV	Coefficient of Variation
DDM	Digestible Dry Matter
DM	Dry Matter
DMI	Dry Matter Intake
DNA	Deoxyribonucleic Acid
DOMD	Degradable Organic Matter Digestibility
DOMI	Digestible Organic Matter Intake
UDP	Digestible Undegradable Protein
EMPS	Energetic efficiency of MCP Synthesis
eRDP	Effective Rumen Degradable Protein
FME	Fermentable Metabolisable energy
FMEI	Fermentable Metabolisable energy Intake
GFR	Glomerular Filtration Rate
GMP	Guanosine Monophosphate
HPLC	High Performance Liquid Chromatography
IMP	Inosine Monophosphate
IVOMD	*In-Vitro* Organic Matter Digestibility
k'	Compound capacity factor
$k1$	Rumen-reticulum outflow rate
$k2$	Hindgut outflow rate
MCP	Microbial Crude Protein
ME	Metabolisable Energy
MEI	Metabolisable Energy Intake
M-N	Microbial Nitrogen
MP	Metabolisable Protein
mRNA	Messenger Ribonucleic Acid
NA	Nucleic Acid
NCGD	Neutral Cellulase and Gammanase Digestibility

NPN	Non-Protein Nitrogen
NRC	National Research Council
OM	Organic Matter
P	Probability (of differences being due to random variation)
PD	PDs
PD/c	Molar ratio of PDs to creatinine
Ps	Pseudouridine
Ps/c	Molar ratio of Pseudouridine to creatinine
r	Pearson correlation coefficient
r^2	Regression coefficient
RNA	Ribonucleic Acid
SAC	Scottish Agricultural College
SD	Standard Deviation
SE	Standard Error
SED	Standard Error of the Differences of the means
tRNA	Transfer Ribonucleic Acid
VFA	Volatile Fatty Acid

Abstract

The significance and contribution of rumen synthesised MCP (MCP) in the context of current metabolisable protein (MP) systems and variations in the energetic efficiency of MCP synthesis reported in the literature are reviewed. The estimation of MCP supply from urinary PD (PD) excretion is discussed and reviewed in detail, with the conclusion that it is a reliable non-invasive technique. HPLC methodologies were developed to determine PD, pseudouridine and creatinine in bovine urine and allantoin in bovine milk. A series of experiments were conducted to evaluate the potential of the PD technique using spot urine samples or measurements of allantoin in milk as on-farm diagnostics of MCP supply. Prediction of daily mean urinary molar ratios of PDs to creatinine (PD/c) ratios from spot urine samples was poor due to diurnal variations, the extent of which was influenced by feeding regimen. Furthermore, prediction of urinary PD excretion from daily mean PD/c ratios was poor due to between-cow variations in urinary creatinine excretion. On this basis the spot urine sampling technique was considered unreliable and a total urine collection proved necessary. Variabilty of urinary creatinine excretion precludes its use as a urinary output marker for individual cows. Urinary pseudouridine excretion was independent of nutrient supply but appeared to be influenced by metabolic changes occurring during lactation. In two experiments, dietary fermentable metabolisable energy (FME) supply was manipulated during early and late lactation. For both experiments, individual cow urinary PD excretion was poorly predicted from calculated FME intake or MCP supply. Based on mean treatment values, urinary PD excretion was accurately predicted from calculated MCP. Individual cow milk allantoin excretion or concentration were poorly correlated with urinary PD excretion, calculated FME intake or MCP. Relationships derived using mean treatment values indicated that milk allantoin excretion or concentration were strongly correlated with urinary PD excretion or calculated MCP. Variabilty precludes the use of milk allantoin as an index of MCP supply for individual cows, but it appears as reliable as urinary PD excretion when used on a herd or group feeding basis.

Introduction

Ruminant animals possess the ability to utilise fibrous feedstuffs due to the presence of micro-organisms located in a highly specialised region of the gastro-intestinal tract, the reticulo-rumen. Host ruminant protein requirements are satisfied by MCP synthesised in the rumen, dietary protein that escapes ruminal degradation and to a lesser extent endogenous protein reaching the duodenum. MP systems adopted in many countries describing protein requirements for ruminant animals consider both the requirements of the host ruminant and its microbial population. While quantification of MCP supply is crucial to these systems, factors influencing its magnitude are poorly defined. Traditionally MCP supply available to the ruminant host has been assessed using internal markers e.g. diaminopimelic acid, RNA and purine bases or external isotopic markers e.g. ^{15}N or ^{35}S. Estimates derived from such techniques are inconsistent and all of these procedures require surgically modified animals restricting their use due to concerns over animal welfare, cost and scale of such studies. Over recent years, urinary PD excretion has been proposed as a non-invasive alternative. The technique assumes that purines derived from feedstuffs are degraded in the rumen so that purines leaving the rumen are essentially microbial in origin and quantitatively related to their derivatives (allantoin, uric acid, xanthine and hypoxanthine) excreted in the urine. Although there are uncertainties regarding the detail of the technique, it has the potential to provide an non-invasive index of MCP supply. Unfortunately the technique is limited in its application due to the requirement for a total urine collection. Since there are no reliable predictive models of MCP yield, the accuracy of practical protein rationing for dairy cows is limited. Extension of the PD method has the potential to be developed as a on-farm diagnostic test of MCP supply. Experiments conducted in this thesis have evaluated the potential of spot urine sampling technique and secretion of allantoin in milk as on-farm diagnostics of MCP supply.

Chapter One

General introduction and literature review

Summary

Rumen energy and nitrogen metabolism is outlined. Variation in the energetic efficiency of microbial protein synthesis (EMPS) reported in the literature is reviewed. The significance and contribution of ruminally derived microbial protein (MCP) to the ruminant animal is documented in the context of current metabolisable protein systems. Assessment of MCP supply in surgically modified animals utililising internal and external microbial markers is evaluated. Estimation of MCP supply from urinary purine derivative (PD) excretion (termed the PD method) is discussed and reviewed in detail. Experimental evidence obtained from numerous studies indicates the PD method is a viable non-invasive alternative to existing techniques for estimating MCP supplies in ruminant animals.

Section one

1.1.1. Rumen microflora

It has long been recognised that the ruminant possesses the ability to utilise fibrous feedstuffs. The digestive enzymes required to digest structural plant components are absent in mammals, although are known to be present in micro-organisms. The gastro-intestinal tract of ruminants has developed to accomodate a large population of microorganisms in order to facilitate the digestion of plant material prior to entering the stomach. Micro-organisms residing in the rumen can be divided into three distinctive groups, bacteria, protozoa and fungi. Strained rumen fluid contains over 10^{10} bacteria ml^{-1} with over 200 bacterial species being identified but only 30 of these are present at a concentration above 10^{7} per ml (Hungate, 1966; Czerkawski, 1986; Murphy, 1989 and

Orskov and Ryle, 1990). Protozoal species are less diverse with the 10^6 ml^{-1} present containing more than 20 species (Czerkawski, 1986), while fungi account for up to 8% of the rumen biomass (Orskov and Ryle, 1990). Rumen micro-organisms are primarily involved in the degradation and subsequent fermentation of dietary carbohydrates ingested with the end products VFAs being utilised by the ruminant animal.

1.1.2. Nitrogen metabolism in the rumen

The major pathways of nitrogen metabolism in the rumen, and subsequent digestion and utilisation of protein in the ruminant are well documented in reviews by Thomas and Rook (1977), Satter and Roffler (1977), Bondi (1987), Orskov (1992 and 1994) and Beever (1993). Only a brief outline of rumen nitrogen metabolism is presented in this review (refer to Figure 1.1.).

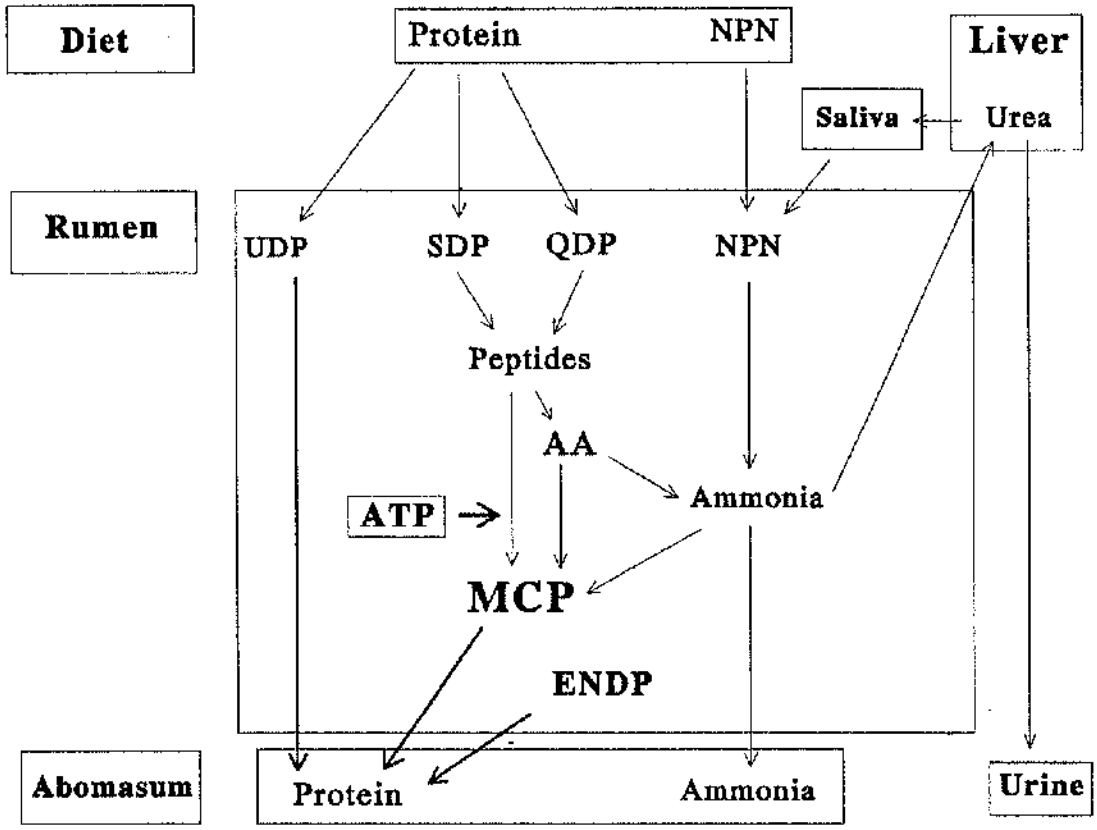
Nitrogen requirements of the rumen microbes are primarily satisfied by the diet. Dietary protein entering the rumen is hydrolysed into peptide and AAs by microbial proteases with the majority of the AAs being deaminated to yield ammonia. The extent of feed nitrogen degradation is primarily influenced by the physical structure of the protein, and its resistance to proteolysis and also by rumen residence time.

Ammonia and AAs form the major substrates utilised by rumen microbes to synthesise microbial cellular proteins. Incorporation of nitrogenous substrates into MCP is dependent on microbial energy availability, derived from the fermentation of dietary carbohydrates to the rumen microbes. Ammonia not incorporated into MCP, can be absorbed across the rumen wall and converted into urea in the liver. While, urea can be recycled back into the rumen via saliva or directly through the rumen wall (and subsequently converted to ammonia), the majority is lost and excreted in the urine. Unincorporated ammonia and non-protein nitrogen (NPN) passes out of the rumen into the intestines and ultimately excreted in the urine.

Protein available for digestion is determined by the quantity of ruminally synthesised MCP, the amount of undegraded dietary protein (UDP) and to a lesser extent, by endogenous protein (ENDP) reaching the duodenum (Satter, 1986). On most

diets, MCP is the major component of duodenal protein, with UDP considered to make a small and variable contribution (Beever, 1993).

Figure 1.1. Schematic representation of nitrogen metabolism in the rumen



Key:-

QDP	Quickly degradable protein
SDP	Slowly degradable protein

1.1.3. Importance of MCP

MCP contributes a significant part (42-93%) of the total protein flux entering the small intestine in ruminants (Stern, 1986), but can be insufficient to satisfy an animal's protein requirements under many physiological conditions, such as early growth, late pregancy, and during lactation. Reports have indicated that high yielding dairy cows

require protein in excess of that available from MCP (Clark, 1975; Oldham, 1984 and Orskov and Reid, 1985). Attempts to manipulate post-ruminal protein supplies by enhancing MCP have been limited, while greater research interest has centered around providing protein sources which are poorly degraded in the rumen and therefore largely escape into the duodenum. A number of studies have investigated production responses to increasing UDP supply in the form of fishmeal, or physically (heat) or chemically (formaldehyde) treated vegetable and animal based proteins.

More direct attempts to increase AA availability in the duodenum have involved supplementing rations with rumen protected AA, lysine and methionine. Manipulation of duodenal protein supply in ruminants is beyond the scope of this review. Recent reviews of Orskov (1992) and Beever (1993), provide a particularly useful insight.

Since the protein content of a ruminant diet is often the most expensive component, there is a need for an accurate prediction of protein requirement and response to protein supplements, in terms of growth rate, milk yield or milk protein yield. Traditionally, digestible crude protein systems (e.g. ARC, 1965) were used to calculate apparent digested crude protein (N in feed minus N in faeces) as a measure of AAs available to the animal. Considerable research effort over the last two decades has lead to an increase in our knowledge of protein nutrition in the ruminant and lead to the development of newer metabolisable protein (MP) systems which distinguish between microbial and dietary protein escaping degradation in the rumen in the prediction of AAs available for absorption. Quantifing MCP supplies are fundamental in all the new MP systems proposed by ARC (1984), Madsen (1985), Ausschuss fur Bedarfsnormen (1986), Jarrige (1989), National Research Council (1989) and AFRC (1992).

1.1.4. Energetic efficiency of MCP synthesis

The understanding of rumen MCP production under various feeding conditions is far from complete, despite substantial research. ARC (1984) MP system formerly adopted in the UK assigned an estimate of 32g microbial-N (M-N)/kg ADOMR, based on 262 observations in sheep and cattle (a large proportion of which were obtained from

sheep at maintenance), omitting estimates derived from experiments with rumen ammonia concentrations below 3.5mM. Although it is generally recognised that MCP yields are proportional to the energy available for fermentation, large variations in energetic efficiency of microbial protein synthesis (EMPS) exist. Data used by ARC (1984) indicated that gM-N/kg ADOMR ranged from 19.3-44.7 (CV 39%), consequently the use of a single value (32g M-N/kg ADOMR) will not accurately predict MCP yield for most situations. Theurer (1979) cited in the review of Dewhurst (1989) discussed a number of factors influencing EMPS and concluded in addition to real sources of variation, problems associated with microbial and digesta markers in particular would also affect its determination. It has not been the intention of this review to explore variations in EMPS. Currently it is generally accepted that dietary nutrient type and level, synchrony of nutrient release in the rumen, defaunation, rumen outflow rate and dietary supplements of various agents such as ionophores and branch chain fatty acids influence EMPS. ARC (1984) summarised the information available at the time, while Dewhurst (1989), Orskov (1992) and Broderick and Merchen (1992) have reviewed this topic subsequently.

Criticisms of the ARC (1984) system are partly addressed in the new MP system proposed by the AFRC (1992). Feedstuffs are characterised by their fermentable metabolisable energy (FME) content (MJ/kg) which is defined as the metabolisable energy (ME) content of the feed less the ME contained in oils, fats and fermentation acids. EMPS is expressed in terms of g MCP/MJ FME and calculated according to a level of feeding (i.e. multiples of maintenance) correction. Typically a EMPS of 11g MCP/MJ FME is used to calculate MCP (calculated as the product of EMPS and FME intake) in lactating dairy cows.

The Cornell net carbohydrate and protein system (Russell *et al*, 1992; Fox *et al*, 1992 and Sniffen *et al*, 1992) incorporates semi-mechanistic models of protein utilisation by rumen microbes and subsequent outflow from the rumen to predict fermentation end products and materials that escape ruminal degradation. EMPS (on a truly fermented organic matter basis) are based on rumen liquid-phase outflow rates. Clearly this model does take account of microbes leaving the rumen attached to feed particles (McAllan,

1980; Craig *et al*, 1987 and Faichney and White, 1988). At present, the Cornell model predictions of MCP supply are probably the most accurate, compared to other MP systems.

However it is important to stress that none of the modern MP systems reliably predict MCP yields and they are limited in the information they provide concerning protein nutrition decisions. A much more satisfactory approach might be to directly assess rumen function from measurements obtained from animals in a given production situation to allow appropriate modifications of diet.

1.1.5. Quantification of MCP

Numerous techniques have been employed in order to assess MCP supplies to the duodenum. Generally, microbial markers used can be classified as being either internal or external. Internal markers are typically present in the microbial cell as integral structural components such as 2,6-diaminopimelic acid (DAPA) in bacteria, 2-aminoethylphosphonic acid (AEPA) in protozoa and D-Alanine or as intra-cellular components such as nucleic acids (NA), RNA, DNA or individual pyrimidine and purine bases. External markers are compounds which are introduced externally into the microbial cell during its growth. ^{35}S, ^{15}N, ^{32}P and ^{3}H radio-isotopes have been used to label microbial cells for this purpose. Irrespective of type an ideal microbial marker should satisfy the following criteria:- i) easily measured, ii) unique to the microorganisms, iii) present at a constant ratio under specific experimental conditions and iv) biologically stable (Horigane and Horiguchi, 1990).

Although the choice of marker is reasonably wide, the principle is the same for all methods. The ratio of marker:nitorgen in rumen microbes is compared to that in the duodenum. Assuming the same marker:nitrogen ratio in duodenal digesta, the proportion of duodenal-N of microbial origin can be estimated. Provided measurements of digesta flow have been made, total M-N entering the duodenum can be calculated.

Digesta flow through the duodenum can be measured directly or more commonly indirectly using single or preferably dual-phase markers (Faichney and White, 1988) such

as chromium and cobalt (Uden *et al*, 1980). Modelling of changes in duodenal marker concentratons allow digesta flow to be calculated.

Only a brief outline of the advantages and problems associated with more commonly used microbial markers is reviewed here, while more detailed assessment of individual microbial markers has been reported by Broderick and Merchen (1992).

2,6-diaminopimelic acid (DAPA) a component of oligopeptides that crosslink peptidoglycan units in bacterial cell walls (Czerkawski, 1986) was first used as a bacterial marker by Weller *et al* (1958). While DAPA:N ratios in mixed rumen bacteria were reported to be relatively constant (Czerkawski, 1974), DAPA content in rumen bacterial strains has been found to be highly variable (Purser and Buechler, 1966; Ling and Buttery, 1978; Dufva *et al*, 1982 and Arambel *et al*, 1987). Clark *et al* (1992) reported variations ranging between 21.1 and 44.5 (mean 27.6) in the N:DAPA ratio in rumen bacteria based on 20 recorded measurements in the literature, serving to highlight the necessity of collecting representative samples of the rumen bacterial population. DAPA the most common internal marker of microbial (bacterial) protein has been used successfully by determining DAPA:N ratios under specific experimental conditions (Czerkawski, 1974; Ling and Buttery, 1978 and Rahnema and Theurer, 1986). Presence of DAPA in feedstuffs (Dufva *et al*, 1982 and Rahnema and Theurer, 1986) is a cause for concern despite the attempts made to correct for dietary DAPA intakes. Recent experimental evidence has indicated differences in DAPA catabolism between gram-positive and gram-negative bacteria by rumen protozoa and bacterial species *in-vitro* (Denholm and Ling, 1989) implying that not all of the DAPA leaving the rumen will be associated with intact microbial cells, leading to an overestimation of MCP supply.

2-aminoethylphosphonic acid (AEPA) has often been reported in conjunction to DAPA in order to assess the proportion of M-N supplied by protozoa. The assumption that AEPA is specific to protozoa (Abou Akkada *et al*, 1968) has subsequently found to be flawed as AEPA is also present in feedstuffs and bacteria (Ling and Buttery, 1978; Whitelaw *et al*, 1984 and Horigane and Horiguchi, 1990).

High concentrations of DNA and RNA in particular in unicellular organisms indicated their potential as rumen microbial markers. RNA entering the duodenum is

assumed to be essentially of microbial origin, following observations indicating complete degradation of dietary NAs in the rumen (Smith and McAllan, 1970 and McAllan and Smith, 1973a). MCP yields were subsequently estimated using RNA (Smith and McAllan, 1970 and Ling and Buttery, 1978), total NA (Coelho da Silva *et al*, 1972a and Zinn and Owens, 1982 and 1986), individual pyrimidine base (Koenig *et al*, 1980; Schelling *et al*, 1982 and Sinclair *et al*, 1991) and total purine base (Zinn and Owens, 1986) concentrations in duodenal digesta. The use of NAs as microbial markers is well established and has been reviewed by Schelling *et al* (1982). Concerns exist over their use, due to escape of dietary NAs entering the duodenum (Smith *et al*, 1978 and Ling and Buttery, 1978). Furthermore, differences in the NA-N:total-N ratios between rumen bacteria and protozoa associated with fluid and particulate phases have been identified (Smith and McAllan, 1974; Smith, 1975 and Broderick and Merchens, 1992). Variations in purine:N ratios in bacterial and protozoal cells are reviewed in section 1.2.3.

Widescale use of NAs, and RNA in particular, has been limited due to laborious and relatively inaccurate procedures involved in its determination (Schelling *et al*, 1982 and Schelling and Byers, 1984) and appreciable losses during sample storage (Harrison and McAllan, 1980). The advent of high performance liquid chromatography (HPLC) techniques has facilitated more convienient and accurate determinations of individual purine and pyrimidine bases compared to less robust techniques used to determine DNA and RNA (Schelling *et al*, 1982). Consequently, greater research effort in recent years has concentrated on the use of purine and pyrimidine bases as microbial markers. Illg and Stern (1994) compared DAPA and purine bases in 12 *in-vitro* and 4 *in-vivo* studies and concluded that using purines as a microbial marker resulted in fewer unreasonable estimates of microbial activity compared to DAPA. Less complicated and expensive procedures involved in purine measurement lead Illg and Stern (1994) to suggest their use as the marker for estimating MCP. Estimation of MCP yield from the absorption of microbial purine bases entering the duodenum and subsequent metabolism and excretion as purine derivatives is reviewed extensively in section 1.2.

Henderickx (1961) suggested ^{35}S incorporation as a means of measuring MCP. Typically $Na_2{}^{35}SO_4$ is infused into the rumen, where it is incorporated into bacterial

protein in the form of methionine and cysteine. Numerous reports have documented this approach (Beever *et al*, 1974; Kennedy *et al*, 1976 and Mathers and Miller, 1980). Estimates of MCP yield using ^{35}S are subject to less variation compared to alternative microbial markers (Ling and Buttery, 1978), although representative sampling of the rumen microbial population or duodenal digesta is essential to its accuracy. Removal of unincorporated ^{35}S in duodenal digesta is also critical (Kennedy and Milligan, 1978). Although subsequent methodologies have accounted for this (Mathers and Miller, 1980 and Kennedy *et al*, 1980) routine use of ^{35}S is inconvenient in practice due to waste disposal problems associated with accumulation of radioactivity in the tissues and milk in addition to the production of $H_2{}^{35}S$ (Broderick and Merchens, 1992). The merits and demerits of ^{35}S as a microbial marker have been considered in more detail by Dewhurst (1989).

Inorganic ^{15}N has also been employed extensively *in-vivo* to label MCP in an analgous manner to ^{35}S. Measurements performed using ^{15}N are theoretically sounder as its not found in feedstuffs above natural enrichment, while ruminal infusions of ^{15}N-labelled ammonium salts label all microbial pools. Inorganic ^{15}N is relatively stable, and therefore its use is extremely attractive. However, uncertainties do exist in estimates of microbial matter in faunated animals using this technique due to differences in ^{15}N enrichment in bacterial and protozoal pools (Broderick and Merchens, 1992). Unfortunately high costs and complex analytical techniques have limited its use in practice (Ling and Buttery, 1978).

A number of comparative studies using different markers have been reported (Ling and Buttery, 1978; Mercer *et al*, 1980; Whitelaw *et al*, 1984; Lindberg *et al*, 1989; Sinclair *et al*, 1991 and Illg and Stern, 1994). Data from Ling and Buttery (1978), Lindberg *et al* (1989) and Sinclair *et al* (1991) is presented in Tables 1.1., 1.2. and 1.3., respectively and serve to highlight discrepancies that exists in their estimation of MCP.

Table 1.1. Proportions of M-N in duodenal digesta-N assessed by three methods

Dietary nitrogen source	RNA	^{35}S	DAPA
Urea	0.98	0.92	0.80
Soyabean meal	0.70	0.64	0.47
Fishmeal	0.56	0.54	0.42

Data from Ling and Buttery (1978)

Table 1.2. Estimates of duodenal M-N flow (mg/kg live weight $^{0.75}$) assessed using three microbial markers

Microbial Marker	Experimental diet						
	I	II	III	AI	AII	BI	BII
RNA	305	532	670	446	889	335	755
Cytosine	324	526	641	412	759	328	704
Guanine	361	570	605	447	805	359	766

Data from Lindberg *et al* (1989)

Table 1.3. Estimates of M-N flow (g/d) obtained with four markers and using rumen and duodenally derived microbes

Bacterial isolate	DAPA	[^{3}H] leucine	Total purines	Cytosine
Rumen	10.90	11.20	9.19	11.17
Duodenum	11.89	10.02	7.43	6.30

Data from Sinclair *et al* (1991)

Dewhurst (1989) reviewed a number of studies using a number of internal and external microbial markers, and reported the most favorable comparison between RNA and DAPA. However, values derived using RNA were on average 0.42 times greater than those for DAPA, although this was variable. Measurements made using ^{15}N and ^{35}S were in good agreement, suggesting both techniques are reliable, or at least the errors involved in both techniques are consistent. Latterly, intra-ruminal ^{15}N infusions (Broderick and Merchen, 1992) and measuring purines entering the duodenum

(Broderick and Merchen, 1992 and Illg and Stern, 1994) have been recommended as the most appropriate means of assessing MCP available to the ruminant.

Assessment of MCP requires a marker which can account for bacterial and protozoal pools associated with liquid and particulate phases. No marker has been completely satisfactory in this respect, and hence MCP supplies are relative rather than absolute (Broderick and Merchen, 1992) confirming earlier arguments of Ling and Buttery (1978) who proposed simultaneous evaluation of marker methodologies.

It is important to stress that estimates of MCP using the microbial markers described in this section all require surgically modified animals (rumen fistulas and post-rumen cannulas) and this has remained the largest drawback with existing microbial marker techniques. In addition to welfare concerns of experimentation with surgically modified animals, the actual conduct of experiments with fistulated ruminants, high yielding dairy cows in particular, is technically involved. Furthermore uncertainities also exist as to how closely surgically modified animals reflect their physiologically normal counterparts.

Existing techniques employed to assess MCP are therefore unsuitable for large scale multi-factorial experiments. Indeed, it could be argued the inability to assess the influence of several factors on EMPS simultaneously represents the largest constraint in accurately predicting MCP and hence MP supply. This inevitably leads to a lack of necessary information required to enable appropriate ruminant protein feeding decisions to be made.

Section two

1.2. Urinary PD excretion as a index of MCP production

The first section of this review has concentrated on the importance of MCP entering the duodenum outlining the problems and uncertainties that exist in its measurement. Urinary PD excretion has commanded considerable research interest in recent years in an attempt to validate its use as a viable non-invasive alternative to

existing internal and external microbial markers. Assessing MCP supply using the PD method is dependent on the following assumptions:-

i) NAs entering the duodenum are essentially microbial in origin

ii) Small variations in purine-N:total-N ratio in rumen microbes

iii) Small variations in microbial purine digestibility

iv) Endogenous purine losses in sheep are met by salvage of microbial and to a lesser extent dietary purines under normal situations

v) Salvage of microbial and dietary purines is limited in cattle and therefore endogenous PD excretion needs to be quantified

vi) Endogenous purine losses are independent of nutrient supply

vii) Quantity of absorbed microbial purines can be accurately predicted from urinary PD excretion

viii) Proportion of PDs excreted via renal and non-renal routes is constant

Each is critically appraised in the context of experimental findings in the following section.

1.2.1. Introduction

Allantoin, uric acid, xanthine and hypoxanthine, collectively termed PDs are excreted in the urine of mammalian species as a result of purine degradation (Watts, 1980). Allantoin, the major PD excreted, has been suggested as an indicator of rumen MCP production (Topps and Elliot, 1965 and Rys *et al*, 1975). Early observations of Terrione and Mourot (1931) indicated a close correlation between allantoin excretion and protein intake in sheep. Morris and Ray (1939) noted excretion of allantoin and uric acid declined in sheep, goats and cows during a 7 day starvation period, suggesting allantoin excretion was associated with certain aspects of the diet. Later studies have shown allantoin and uric acid excretion in sheep (Topps and Elliot, 1967) and steers (Ellliot and Topps, 1963) to increase with concommitant increases in protein intake.

Close correlations between rumen NA concentrations and urinary allantoin excretion (Topps and Elliot, 1965; Mudgal and Taneja, 1977; Turchinski, 1980; Turchinski and Sorokin, 1983 and Antoniewicz, 1983) have provided further indirect evidence to support urinary allantoin excretion as an indicator of MCP supply. A large number of studies have reported close relationships between DMI, DOMI and energy intake with urinary allantoin excretion in ruminant species, (summarised in Table 1.4.) which are consistent with previous findings, providing gross evidence to indicate the potential of PD excretion to reflect MCP production.

Table 1.4. Relationships between DM, DDM, DOM (kg/d) and energy intakes (MJ/d) and urinary allantoin excretion in ruminant species reported in the literature

Species	Parameter	Correlation	Reference
Sheep	DOM	0.930	Antoniewicz *et al* (1981)
Sheep	DM	0.950	Antoniewicz (1983)
Sheep	DOM	0.790	Balcells *et al* (1993)
Sheep	DM	0.850	Chen *et al* (1995)
Goats	DOM	0.830	Lindberg (1985)
Buffalo	DDM	0.728	Vercoe (1976)
Buffalo	DDM	0.806	Liang *et al* (1994)
Cattle	DDM	0.812	Vercoe (1976)
Cattle	DDM	0.872	Liang *et al* (1994)
Cows	Energy	0.840	Giesecke *et al* (1994)
Cows	DOM	0.529	Gonda *et al* (1995)
Cows	DOM	0.735	Gonda *et al* (1995)

1.2.2. Fate of NAs in the rumen

In common with RNA and total purine microbial marker techniques, utilisation of the PD method is dependent on NAs entering the duodenum being essentially microbial in origin. Therefore the fate of dietary NAs has to be considered. Ingested feedstuffs provide the major source of NAs entering the rumen. The amount of NA present in most

commonly used ruminant feedstuffs varies between 1-50 g NA/kg DM (McAllan, 1982). Reports of NA content of feedstuffs in the literature are summarised in Table 1.5. Very small amounts of purines, uric acid and allantoin are also present in some forages (Ferguson and Terry, 1954). In addition to dietary NAs, endogenous NAs present in mucosal secretions and sloughed epithelia cells will also contribute to the non-microbial NAs in the rumen, although in negligible amounts (McAllan, 1982).

Table 1.5. NA content of ruminant feedstuffs

Feedstuff	NA-N:Total-N (%)	Reference
Fishmeal	10	Smith and McAllan (1970)
Extracted groundnut meal	12	Smith and McAllan (1970)
Flaked maize	2	Smith and McAllan (1970)
Dried lucerne	26	Coelho da Silva (1972a)
Dried ryegrass	5	Coelho da Silva (1972b)
Protein concentrates and cereals	1-4	McAllan (1982)
Hays	8-11	McAllan (1982)
Grasses	5-15	McAllan (1982)
Straws	13-19	McAllan (1982)
Legumes	15-25	McAllan (1982)
Fresh grass	5-7	McAllan and Braithwaite (1988)
Silage	3-4	McAllan and Braithwaite (1988)

Substantial experimental evidence exists indicating NAs are extensively degraded in the rumen. Smith and McAllan (1970) reported the ratio of RNA:DNA ratios in rumen fluid was similar to that in rumen bacteria and was independent of diet, indicating negligible amounts of dietary NA in rumen fluid. The RNA:DNA ratio in duodenal digesta has been shown to be similar to the particularly distinctive ratio in rumen bacteria, suggesting NAs entering the duodenum are essentially microbial in origin (Mugdal *et al*, 1978 and McAllan, 1982). Further indirect evidence is provided by the observations of McAllan and Smith during the early seventies. Infusions of yeast RNA and thymus DNA into the rumen of calves were found to disappear by 100 and 85%, respectively after one hour (McAllan and Smith, 1973a). These observations were

subsequently confirmed by *in-vitro* incubations of purine bases, nucleosides and nucleotides with bovine rumen bacteria. Virtually all of the compounds incubated were found to be absent in the incubating medium after 4 hours. Degradation of free purine bases such as adenine were variable, with xanthine being the most resistant (McAllan and Smith, 1973b).

While rumen bacteria can digest most purine and pyrimidine bases, Jurtshuk *et al* (1958) reported adenine was neither decarboxylated nor deaminated after *in-vitro* incubation with a washed cell suspension of bovine rumen bacteria. Schelling and Byers (1984) while substantiating the use of the pyrimidine base cytosine as a microbial marker, reported that some dietary adenine can escape rumen degradation.

In common with dietary NAs, PDs contained in feedstuffs are generally thought to be degraded in the rumen. Urinary allantoin excretion has been shown to be independent of intra-ruminal infusions of allantoin in sheep and steers maintained by intra-gastric infusion (Chen *et al*, 1990d), suggesting allantoin is degraded by the activities of the microbial population associated with the rumen epithelium, in the absence of functional rumen microflora.

It is important to recognise that some dietary NA present in the undegraded feed fraction will escape rumen degradation and enter the duodenum. Escaped dietary NAs will inevitably lead to an overestimate of MCP supply using the PD method. However it is also important to recognise that this criticism is not confined to the PD method but equally applies to the use of RNA, cytosine and total purine microbial markers (refer to section 1.1.5). Studies with radio-labelled NAs in steers receiving equal proportions of hay and concentrate indicated that non-microbial NA may contribute to up to 15% of the RNA entering the duodenum (Smith *et al*, 1978). Ruminal escape of NAs contained in alfalfa hay has been reported to be relatively minor, except immediately after feeding (Koenig, cited by Schelling *et al*, 1982). More recent observations in sheep fed ammonia treated straw supplemented with fish meal or soyabean meal indicates that escaped dietary purines can contribute to between 11-29% of the total purines present in duodenal digesta (Perez *et al*, 1995). Employing the nylon bag technique and using ^{15}N to correct for microbial contamination indicated effective disappearances of purines

contained in fishmeal of around 93 and 85% for rumen outflow rates of 2 and 8%, respectively (Perez *et al*, 1995). In contrast, degradation of fishmeal proteins would typically be 38 and 25 % at rumen outflow rates of 2 and 8%, respectively (AFRC, 1992).

If we apply the findings of Perez *et al* (1995) to a 600kg dairy cow and assume urinary PD excretion to be 200mmoles/d, duodenal purines available for absorption would be 180 mmoles/d estimated according to the model of Verbic *et al* (1990). Supplementing a dairy ration with 2 kg DM of fishmeal with a purine content of 59 mmol/kg DM (16% of which escapes rumen degradation), escaped purines from fishmeal would contribute 19 mmoles/d, leading to a 10 % overestimate of MCP supply (according to the model proposed by Chen *et al*, 1992a). Replacing fishmeal with soyabean meal with a purine content of 19 mmol/kg DM (13% of which escapes rumen degradation) escaped purines from soyabean meal would contribute 5 mmoles/d, leading to 3% overestimate of MCP supply.

On balance it appears that non-microbial NA contributes a small but significant proportion to the total NA pool entering the duodenum. Recent findings of Perez *et al* (1995) tend to suggest this contribution is potentially much larger when ruminant diets contain high proportions of undegraded protein derived in particular from animal sources such as fishmeal.

1.2.3. Variations in the NA content of rumen microbes

As described in section 1.1.5., one of the characteristics of an ideal microbial marker is that it is present at a constant marker:total N ratio in rumen microbes. Mixed rumen microbes typically contain 8% of NAs (Czerkawski, 1986) and between 7-11% N (Smith and McAllan, 1974; Czerkawski, 1976 and Storm and Orskov, 1983). Accurate prediction of MCP supply by the total purine method and PD method is based on the assumption that the purine content of rumen microbes is constant. *In-vitro* studies have shown the total-N:purine-N (T-N:P-N) ratio in bacteria to vary markedly according to culture growth conditions (Nikolic and Jovanovic, 1973; Bergen *et al*, 1982 and Bates

and Bergen, 1984). However variations observed using isolated bacteria strains *in-vitro* are likely to be much greater than those encountered for mixed rumen microbes. Cecava *et al* (1990a and 1990b) assessed relative T-N:P-N ratios in fluid associated bacteria (FAB), particulate associated bacteria (PAB) and mixed rumen bacteria, and found marked differences particularly between FAB and PAB, but little difference between PAB and mixed bacteria (refer to Table 1.6.).

Table 1.6. Nitrogen:purine ratios of rumen bacteria isolated from steers fed at two energy levels and feeding frequencies

Nitrogen:purine ratios	Energy level MJ/kg DM		Feeding frequency	
	9.4	12.3	2 x	12 x
Mixed bacteria	0.77	0.77	0.76	0.78
FAB	0.64	0.64	0.61	0.66
PAB	0.72	0.75	0.72	0.74

Data from Ceceva *et al* (1990b)

Table 1.7. Estimates of bacterial-N flows at the duodenum (g/d) of steers fed two energy levels and feeding frequencies using T-N:P-N ratios measured for different bacterial isolates

Nitrogen:purine ratios	Energy level MJ/kg DM		Feeding frequency	
	9.4	12.3	2 x	12 x
Frozen mixed	122.2	106.4	109.7	120.5
Fresh mixed	113.4	104.1	101.0	118.5
FAB	94.7	87.7	81.4	101.0
PAB	107.8	102.3	95.7	114.4

Data from Ceceva *et al* (1990b)

Estimates of bacterial-N flows in the duodenum calculated using T-N:P-N ratio of bacteria from different fractions were similar, with the exception of those based on FAB which significantly ($P<0.05$) underestimated bacterial-N entering the duodenum (refer to Table 1.7.).

Significant changes in the T-N:P-N ratio of FAB and PAB have been observed over the feeding cycle, with the lowest ratios occuring immediately post-feeding (Craig *et al*, 1987). These findings confirm those of John (1984), who demonstrated that RNA and DNA concentrations in FAB varied during a 24 hour period in sheep fed once daily. Ceceva *et al* (1990b) reported small changes in the T-N:P-N ratio of bacterial fractions when collected from steers fed at two-hourly intervals, while much larger changes were observed in steers fed twice daily. Conclusions drawn from this work indicate that the use of mixed rumen bacteria or PAB isolated from digesta over the entire feeding cycle is the most appropriate way of assessing the T-N:P-N ratio of rumen bacteria. Clark *et al* (1992) reviewed the literature from 50 studies and reported large variation in the T-N:P-N ratio of rumen bacteria ranging between 0.61-2.13 (mean 1.06; CV 29.8%). In order to underline the significance of this variability, nitrogen supply calculated on the basis of the minimum and maximum T-N:P-N ratio reported in the literature assuming a duodenal purine supply of 250g/d, varied between 152-533 g/d. Whilst some of the variability reported was probably due to genuine differences in the T-N:P-N ratio for different bacteria combined with variation in microbial populations, a large proportion would inevitably be a consequence of random experimental errors between laboratories.

Czerkawski (1976) reported little difference in the purine-N:Total-N ratio between rumen bacteria and protozoa. In contrast, Firkins *et al* (1987) found the T-N:P-N ratio in protozoa to be typically half those for rumen bacteria. Broderick and Merchens (1992) suggested that the use of bacterial T-N:P-N could lead to underestimates of M-N entering the duodenum due to an underestimation of protozoal-N. Quantifying true protozoal protein is problematic due to possible selective retention of protozoa within the rumen (Harrison *et al*, 1979) and therefore ruminal bacterial and protozoal pool sizes may not be indicative of protozoal protein flows in the duodenum.

Large variations in the T-N:P-N ratio of rumen bacteria and protozoa reported in the literature require careful consideration. It is essential when using any microbial marker that a representative samples of rumen microbes and duodenal digesta are obtained in order to assess post-rumen marker flows. It is possible that a large proportion of variations in rumen microbe T-N:P-N ratio could be due to

unrepresentative sampling of rumen microbes or duodenal digesta. The observations of Siddons *et al* (1982) and Punia *et al* (1985) serve to highlight differences between marker-N:total-N ratios of rumen and duodenally isolated bacteria. Further sources of sampling error could be due to differences between small and large bacteria. The ratio of DAPA:total-N has been shown to be higher in large compared to small rumen bacteria (Czerkawski and Faulds, 1974 and Coto *et al*, 1985).

On balance it appears that the total-N:purine-N ratio in individual rumen bacterial species is subject to considerable variation, although the contribution due to sampling error is unclear. The soundest approach for the assessment of MCP supply using the total purine or PD methods would be to measure the T-N:P-N ratio of mixed rumen microbes for each experimental investigation. In situations where this is not practical the use of a mean T-N:P-N ratio based on observations reported from PAB and mixed rumen microbes would appear to be a satisfactory compromise. A correction for possible underestimates of protozoal contribution to MCP also needs to be considered in the future.

1.2.4. Digestion of NAs

Microbial and dietary purines escaping ruminal degradation are generally absorbed in the form of nucleosides (Wilson and Wilson, 1962 and McAllan, 1980). Duodenal NA bases, nucleosides and nucleotides in ruminants are degraded to varying degrees by pancreatic ribonuclease, pancreatic nucleases, phosphodiesterases I and II, nucleotidase and nucleosidase secreted in the small intestine. Pancreatic ribonuclease activity is particularly high in the ruminant (Barnard, 1969) indicating their capacity to digest large quantities of nucleic acids. Nucleosides, NAs and free bases are subsequently absorbed from the intestinal lumen (Fox, 1978). Free NAs entering the small intestine are almost entirely digested and absorbed in sheep (Ellis and Blechner, 1969b and Jackson *et al*, 1976) and in cattle (McAllan, 1980).

Studies in ruminants have shown that NAs in the small intestine are readily digested and absorbed. Apparent NA digestibilities reported in the literature range

between 72-89%, with RNA being more readily digested than DNA (Condon *et al*, 1970; Smith and McAllan, 1971; Coelho da Silva, 1972b; Storm and Orskov, 1983; Storm *et al*, 1983 and Chen *et al*, 1990a). Storm and Orskov (1983) reported true digestibilites of microbial NA-N and RNA-N of 0.859 and 0.871 respectively, while Chen *et al* (1990a) calculated microbial purine digestibility to be 0.913. These experiments are particularly important, and remain the only direct measurements of microbial purine digestibility in ruminant species reported in the literature. NAs, synthesised as a result of microbial activites in the hindgut, are poorly digested (Ellis and Bleichner, 1969a) and are virtually all excreted in the faeces. It appears that purine degradation products such as uric acid and allantoin are not subsequently absorbed from the lower gut (Sorenson, 1960 and Chen *et al* 1990a).

1.2.5. Fate of absorbed purines

Microbial and escaped feed purines absorbed within the small intestine are either degraded (refer to section 1.2.6.) or enter *salvage* pathways (refer to section 1.2.8.) to be re-utilised in the synthesis of tissue NAs in the ruminant host. The fate of absorbed purines is described schematically in Figure 1.3. Absorbed purines undergo degradation during their passage through intestinal mucosa (Wilson and Wilson, 1962), the extent of which determines the substrate pool available to enter *salvage* pathways. Saviano *et al* (1980) demonstrated that only adenine was able to cross the intestinal mucosal membrane. *Guanine deaminase* (EC. 3.5.4.3; Henderson and Paterson, 1973), *adenosine deaminase* (EC. 3.5.4.4; Barman, 1969), which catalyse the deamination of purine nucleosides and free bases into hypoxanthine and xanthine have also been shown to be present in mucosal cells. Hypoxanthine and xanthine can be further catabolised into uric acid in the presence of *xanthine oxidase* (EC 1.2.3.2). Uric acid produced as a result of *xanthine oxidase* activities is catabolised by the action of the enzyme *uricase* (EC 1.7.3.3) to allantoin.

The intestinal mucosa is rich in *xanthine oxidase* in cattle (Roussos, 1963) which reduces the potential for *salvage* of purine synthesis precursors from absorbed

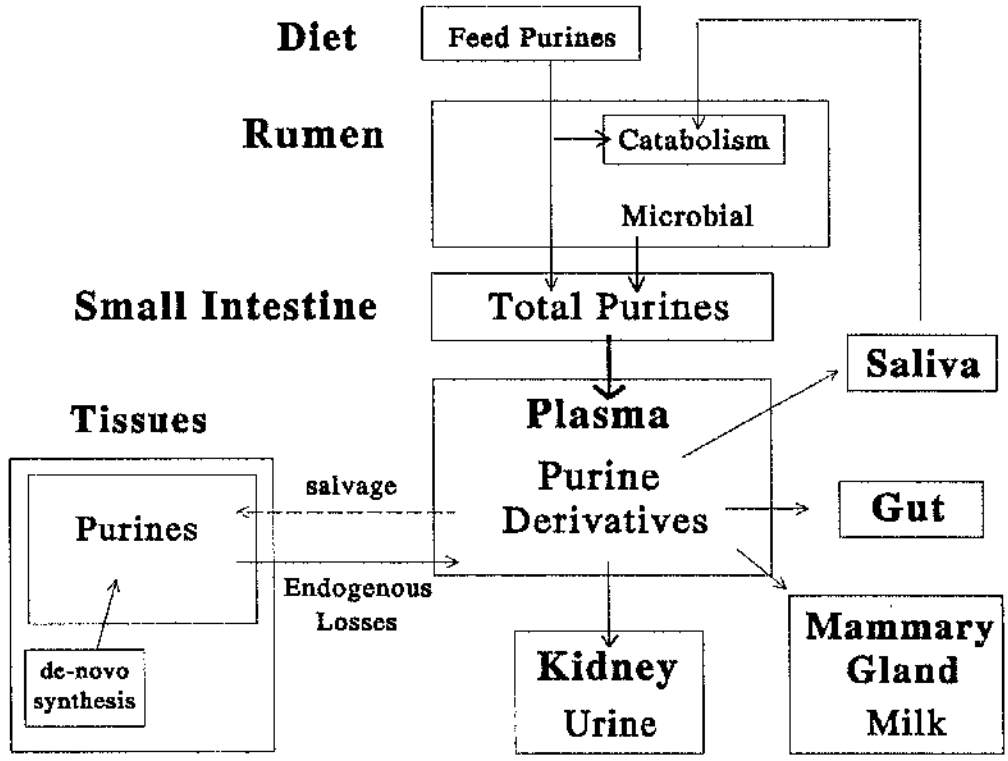
exogenous sources. In contrast, intestinal mucosa in sheep contains only trace amounts of *xanthine oxidase* (Al-Khalidi and Chaglassian, 1965) which limits mucosal degradation of absorbed purines and therefore increases the potential of re-utilisation by *salvage* pathways. *Xanthine oxidase* is present in most animal tissues, but its distribution varies between tissues and animal species (Al-Khalidi and Chaglassian, 1965, also refer to section 1.2.7.). Absorbed purines entering the bloodstream are subjected to further degradation by *xanthine oxidase* and *uricase*. *Xanthine oxidase* has been shown to be present in bovine plasma but absent in ovine plasma, while low *uricase* activities have been demonstrated in ovine plasma, but completely absent in bovine plasma (Chen *et al*, 1990c) which potentially explains why levels of uric acid are higher in the blood of cattle than sheep. *Uricase* activity in intestinal mucosal cells in ruminant species has not been documented in the literature, consequently it is unclear whether purines absorbed from the gut can be catabolised to allantoin within the mucosal cell.

PDs in portal blood of sheep are likely to be predominately hypoxanthine and xanthine and to a lesser extent uric acid due to the absence or low activity of catabolic enzymes in extra-hepatic cells and blood in sheep (Al-Khalidi and Chaglassian, 1965). Ovine hepatic cells contain large quantities of *xanthine oxidase* and *uricase* (Chen *et al*, 1990a) and therefore the liver is the major site of purine catabolism to allantoin in the sheep. Observations in normally fed sheep have confirmed similar jugular and portal blood allantoin and uric acid concentrations, while xanthine and hypoxanthine concentrations were significantly ($P<0.01$) higher (mean of 60%) in portal compared to jugular blood (Chen *et al*, 1990a).

It appears that the fate of absorbed purines in cattle is distinctly different to that in sheep. Uric acid is thought to predominate in portal blood due to high *xanthine oxidase* activities in the blood and intestinal mucosa (Al-Khalidi and Chaglassian, 1965 and Chen *et al*, 1990c). Therefore, utilisation of absorbed purines has been suggested to occur only in the intestinal mucosa, while *non-salvaged* purines are assumed to be catabolised to allantoin and uric acid before entering the liver (Verbic *et al*, 1990). Urinary excretion of hypoxanthine and xanthine is negligible in cattle (Chen *et al* 1990b; Verbic *et al* 1990; Susmel *et al* 1994b and Dewhurst *et al* 1995), as a result of high

xanthine oxidase activity in most bovine tissues. Allantoin predominates in bovine urine presumably due to high *uricase* activities in bovine tissues. As a consequence of enzyme catabolic activities, allantoin is the principle PD circulating in peripheral blood of ruminant species due to high levels of hepatic purine catabolism.

Figure 1.2. Schematic representation of purine transformations in the ruminant

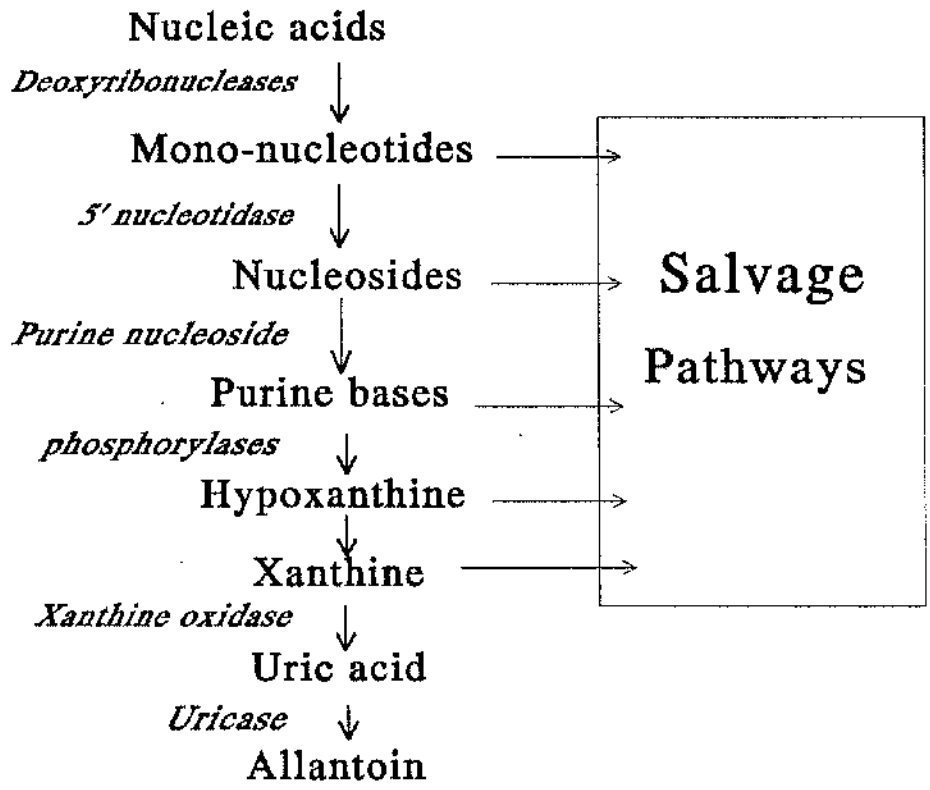


1.2.6. Purine catabolism

Turnover of cellular DNA tends to be relatively low, while turnover rates of certain RNA molecules such as mRNA is particularly rapid. During cellular NA turnover, DNA and RNA are hydrolysed by nucleases and diesterases to yield mono-nucleotides and nucleosides, of which the later can be re-utilised and incorporated into NAs or further catabolised. Degradation of nucleoside molecules involves enzymatic cleavage of glycosidic bonds between purine bases and sugar moieties, yielding purine bases which

are either salvaged or degraded further and excreted. The catabolism of purine (adenine and guanine) compounds is described schematically in Figure 1.3.

Figure 1.3. Biochemical pathways of purine catabolism



Adenosine monophosphate (AMP) and adenine are degraded to hypoxanthine which is oxidised to xanthine and then uric acid. Guanine nucleosides and guanine bases enter the catabolic pathway as xanthine, which is subsequently catabolised to uric acid. Uric acid, produced as a result of adenine and guanine degradation, is further catabolised to allantoin, the major purine catabolite in ruminant species. In most mammals, allantoin is the end product of purine catabolism and is excreted mainly in the urine. Although allantoin is the major purine catabolite excreted in ruminants, hypoxanthine, xanthine and uric acid are also excreted in variable proportions, as reviewed in section 1.2.14. Purine

catabolic pathways are limited to uric acid in primates (including man) and can proceed beyond allantoin to glyoxylic acid and urea (via allantoic acid) in evolutionary less developed animals such as fish.

1.2.7. Tissue distribution of *xanthine oxidase*

Xanthine oxidase is present in tissues and body fluids as either a dehydrogenase (EC 1.2.1.3) or as an oxidase (EC 1.2.3.2) while its activity is not specific to hypoxanthine and xanthine, but has the capability to oxidise aldehydes (Zittle, 1964). *Xanthine oxidase* is intergral in diverting potentially salvagable hypoxanthine and xanthine purine substrates into the formation of uric acid, which is subsequently excreted mainly as allantoin. Distribution of *xanthine oxidase* has been shown to be species specific (refer to Table 1.8.).

Table 1.8. Tissue distribution of *xanthine oxidase*

Organ	*Xanthine oxidase* activity milliunits/ g wet tissue	
	Sheep	Cow
Liver	12	29
Lung	0.032	48
Kidney	0.0035	1.6
Heart	0.007	0.22
Muscle	0.022	0.33
Intestine	0.058	12

(Data from Al-Khalidi and Chaglassian, 1965)

In cattle and sheep *xanthine oxidase* has been demonstrated to be concentrated in the liver. Activities in non-hepatic tissues have been found to be much higher in cattle, especially in the lung compared to sheep, for all but the pancreas and adrenal glands (Al-Khalidi and Chaglassian, 1965). Of all the farm species only the bovine has appreciable *xanthine oxidase* activities in the blood, which have been quantified *in-vitro* based on the

rate of uric acid formation as 0.142 μmol min^{-1} l^{-1} (Al-Khalidi and Chaglassian, 1965) and 1.13 μmol min^{-1} l^{-1} (Chen *et al*, 1990c).

In-vitro studies by Roussos, (1963) with *xanthine oxidase* isolated from the bovine small intestine has indicated that its activity can be inhibited by adrenalcorticoid hormones, oestrogens and progesterone, and stimulated by testosterone.

Species with *xanthine oxidase* present in plasma also have *xanthine oxidase* in their milk (Modi *et al*, 1959) which exist in either free or membrane bound forms, the majority of which in bovine milk is associated with the milk fat globule membrane (Briley and Eisenthal, 1974). Its activity in bovine milk has been shown to be related to the molybdenum content of the forage consumed (Kiermeier and Capellari, 1958, cited in a review by Zittle, 1964), stage of lactation (Rajan *et al*, 1962) and breed (Cerbulis and Farrell, 1977). Intracardial injections of ^{125}I labelled *xanthine oxidase* in lactating rats and subsequent milking tentatively suggests *xanthine oxidase* in milk is derived from the blood (Blaikstone *et al*, 1978). The role of *xanthine oxidase* secreted in milk is unclear. Hypoxanthine secreted in bovine milk has been suggested to be derived primarily from mammary purine metabolism (Rosskopf, 1989). During milk protein synthesis, turn-over rates of NA and in particular RNA molecules would be expected to be high, and presumably hypoxanthine secretion increases accordingly. It is interesting to speculate that milk *xanthine oxidase* secretion occurs to facilitate catabolism of endogenous mammary hypoxanthine.

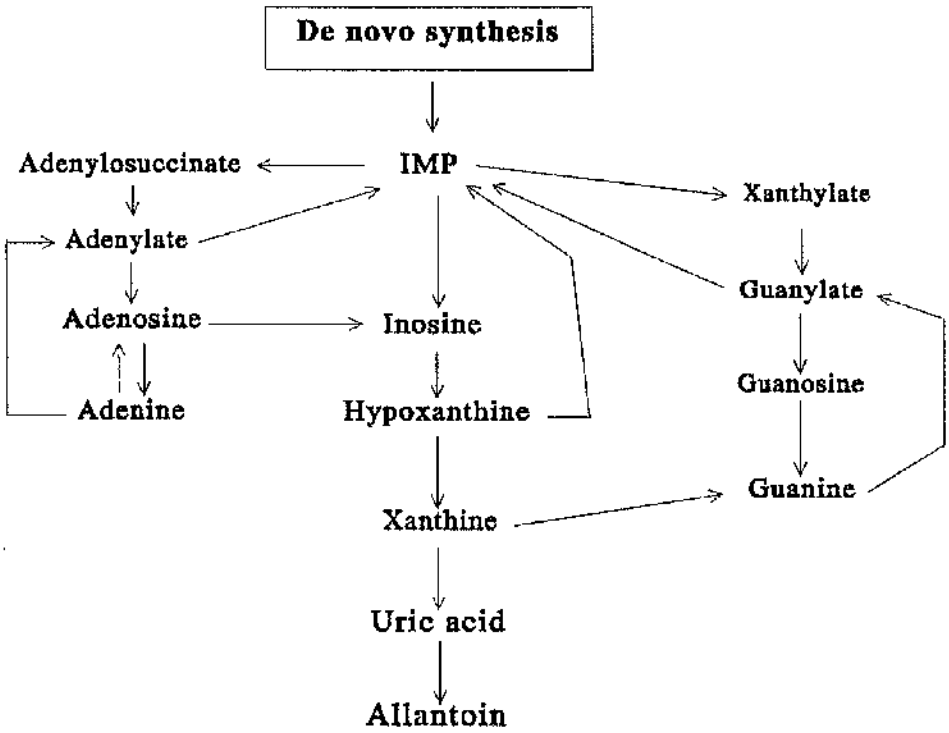
1.2.8. Purine synthesis

Purines are synthesised in ruminant tissues to replace obligatory purine losses incurred during cellular NA turnover and to meet purine accretion requirements during cellular growth. Synthesis of purine ribonucleotides occurs through two distinctly different routes, commonly refered to as the *de novo* and *salvage* pathways.

1.2.8.1. Purine *de novo* synthesis

Purine synthesis has been extensively reviewed by Hartman (1970), Gots (1971) and Henderson and Paterson (1973) and only a brief outline is presented here (refer to Figure 1.4.).

Figure 1.4. Pathways of purine metabolism in ruminant tissues



De novo purine synthesis is an extremely energetically demanding process (Lehninger, 1982 and Mura *et al*, 1987) and proceeds using 5-phospho-ribosyl-1-pyrophosphate (PRPP) as a building block onto which glycine, glutamine, aspartate, one carbon unit and carbon dioxide precursors are incorporated to yield a purine ring. On completion of the purine ring, inosinic acid (the nucleotide of hypoxanthine) is produced which can be converted to guanine or adenine nucleosides. It is important to recognise that not all tissues have the ability to perform *de novo* purine synthesis. Experimental observations of rabbit bone marrow (Smellie *et al*, 1958; Henderson and LePage, 1959

and Thomson *et al*, 1960) and human leukocytes (Scott, 1962 and Williams, 1962) have indicated *de novo* purine synthesis to be absent. Tissue purine requirements are therefore met by other purine sources, such as the liver which has been shown to be a major site of purine synthesis (Murray, 1971).

1.2.8.2. Purine *salvage*

Purines derived from tissue NA degradation or purine nucleosides and free bases absorbed from the gut can be salvaged and re-utilised for nucleotide synthesis (refer to Figure 1.4.). Studies in sheep using radio-labelled exogenous purines have confirmed free purine bases and their corresponding nucleosides can be salvaged and re-utilised in the synthesis of nucleotides and NAs (Smith *et al*, 1974 and Razzaque *et al*, 1981). Even in the absence of absorbed purines, *salvage* pathways still operate. In humans, up to 90% of the free purines produced during tissue NA turnover may be recycled via *salvage* pathways (Lehninger, 1982), hence avoiding the considerable energetic costs associated with *de novo* synthesis. Purine *salvage* catalysed by *phosphoribosyltransferases (PRTase)* results in the formation of nucleotide units from adenine, guanine and hypoxanthine precursors (refer to Table 1.9.).

Table 1.9. Enzymes involved in purine *salvage*

Substrate	Enzyme	Product
Adenine	*Adenine-PRTase* (EC.2.4.2.7)	Adenosine 5'-phosphate
Guanine	*Hypoxanthine-guanine PRTase* (EC. 2.4.2.8)	Guanosine 5'-phosphate
Hypoxanthine	*Hypoxanthine-guanine PRTase* (EC. 2.4.2.8)	Inosine 5'-phosphate

Xanthine may be salvaged by *hypoxanthine-guanine PRTase* to form xanthine monophosphate (Gots, 1971), but this reaction is very slow due to a low affinity of xanthine for this enzyme (Hitchings, 1978). It appears likely that xanthine would be oxidised in the presence of xanthine oxidase to uric acid rather than enter *salvage*

pathways in the ruminant. Further products of purine catabolism, uric acid and allantoin are not able to salvaged, but are excreted primarily in the urine.

Purine bases can also be salvaged by combining with ribose-1-phosphate to yield their respective nucleosides. The reactions catalysed by *nucleoside phosphorylases* (EC. 2.4.2.1) are quantitatively much less important than those proceeding via *PRTases* (Lehninger, 1982). Purine nucleotides derived from *de novo* synthesis or *salvage* pathways can be converted into nucleotides of other purine bases via a common intermediate inosine 5'-phosphate (IMP). This mechanism enables cells to maintain the desired nucleotide pool composition.

Ruminant animals receive an abundant supply of purines from the absorption of nucleosides and free bases derived from microbial NAs from the gut. The energetic cost of a mole of purine mononucleotides via salvage is 2 ATP, while *de novo* synthesis of AMP and GMP requires 8 and 7 ATP, respectively (Lehninger, 1982). Leleiko *et al* (1979) demonstrated that dietary purines enhanced *salvage* enzyme activities, while switching off those involved in *de novo* synthesis. D'Mello (1982) indicated that administration of allopurinol (an allostearic inhibitor of xanthine oxidase), lead to an accumulation of hypoxanthine, which stimulated purine *salvage* enzymes. D'Mello (1982) concluded that it was reasonable to assume that tissues receiving a supply of absorbed purine bases will conserve resources by switching off *de novo* synthesis. In the light of the considerable energetic advantage of salvaging purine bases compared to their *de novo* synthesis, *salvage* of absorbed purines would be expected to be fully exploited in the ruminant.

Purine *salvage* is subject to feed-back control of purine nucleotides. Once cellular levels of purine nucleotides have been attained, further nucleotide loading will inhibit *adenine-PRTase* and *hypoxanthine-guanine PRTase* activities (Murray, 1971). Regulation of enzymatic activities also influences the cellular uptake of *salvage* substrates due to their uptake being proportional to *PRT-ase* activities (Gots, 1971). Once cellular purine nucleotide requirements are met, surplus nucleosides and free bases are diverted towards catabolic pathways leading to the formation of allantoin and uric acid.

Assessment of MCP supply using urinary PD excretion proposed by Chen *et al* (1992a) assumes that purines absorbed from the gut enter *salvage* pathways in sheep. At feeding levels of 0.8 x maintenance and above, Chen *et al* (1990a) argued that *salvage* pathways operate at a maximal rate, leading to the assumption that under normal feeding situations, tissue purine requirements would be expected to be met entirely from the *salvage* of absorbed purines, rather than by *de-novo* synthesis.

In contrast, bovine intestinal mucosa is rich in *xanthine oxidase*, although Verbic *et al* (1990) suggested purine *salvage* could occur within the mucosal cell, the majority of absorbed purines are assumed to be catabolised beyond the point at which they can be salvaged during their absorption from the gut. Verbic *et al* (1990) suggested *de novo* purine synthesis would contribute greatly to tissue purine requirements in cattle. It appears that further investigations of the relative contributions of purine *salvage* and *de novo* purine synthesis to tissue purine requirements are required for a range of dietary situations to further our understanding, particularly in lactating ruminants.

1.2.9. Endogenous urinary PD excretion

Not all of the PDs excreted in the urine by the ruminant originate from the absorption of microbial purines. During the turnover of tissue NAs, a proportion of purine bases are not salvaged and re-utilised, but are catabolised constituting an endogenous loss. Assessment of MCP supply using the PD method requires quantification of endogenous contributions in cattle and sub-maintenance fed sheep. Estimating endogenous urinary PD excretion in conventionally fed ruminants is complicated by large quantities of ruminally derived microbial purines entering the small intestine. Consequently, several approaches have been adopted to overcome this.

Measurements of urinary PD excretion in sheep have been performed during periods of fasting, when the supply of essentially microbial purines is much reduced. Numerous observations have confirmed dramatic decreases in urinary PD excretion after fasting for several days (Morris and Ray, 1939; Walker, 1967 and Matsuoka *et al*, 1988) while Rys *et al* (1973 and 1975) reported negligible allantoin and uric acid excretion in

sheep after five days. These findings, tentatively suggest purines catabolised during normal feeding and excreted as PDs are conserved, presumably via *salvage* pathways. However it is also possible that the extremely low nutrient supply reduces rates of tissue NA turnover, thereby reducing endogenous purine losses and consequently reducing PD excretion as fasting continues. Assessment of endogenous PD excretion from fasting experiments has to be questioned further, as it has been obtained under abnormal physiological conditions.

The use of the intra-gastric infusion technique of Orskov *et al* (1979) and Macleod *et al* (1982) has enabled energy and nitrogen requirements of ruminant animals to be met by infusion of VFAs and casein into the rumen and abomasum, respectively. This is a useful methodology enabling ruminant metabolism to be investigated in isolation from the influence of rumen microfloral fermentation. However concerns of gut wall abnormalities exist in animals nourished by this technique. The infusion technique has been used extensively in sheep (Antoniewicz and Pisulewski 1982; Sibanda *et al*,1982; Giesecke *et al*, 1984; Fujihara *et al*, 1987; Lindberg and Jacobssen 1989 and Chen *et al*, 1990a), and in cattle (Sibanda *et al*, 1982; Fujihara *et al*, 1987; Verbic *et al*, 1990 and Chen *et al*, 1990c), while Balcells *et al* (1991) working with sheep, took the useful approach of replacing duodenal digesta with a purine-free nutrient solution, which has the advantage of maintaining rumen function.

Alternatively, PD excretion has been measured in milk fed pre-ruminant animals when it can be assumed to be entirely due to endogenous losses of purines. Urinary allantoin excretion has been measured in milk fed calves (Blaxter and Wood, 1951), lambs (Antoniewicz, 1983) and goat kids (Lindberg, 1989 and 1991). However, this approach does assume that milk is essentially purine-free, and that purine *salvage* pathways are well developed and do not significantly differ with age or rumen function. This approach has to a certain extent been validated by Chen *et al* (1990c) who concluded there was no difference in the endogenous urinary PD excretion in milk fed preruminant calves and mature animals.

Further estimates of endogenous urinary PD have been derived by extrapolation of regression equations derived from observations of the response of allantoin excretion

to digestible organic matter intake in sheep and goats (Laurent and Vignon, 1983), duodenal microbial NA flows in sheep (Puchala and Kulasek, 1992) and cattle (Beckers and Thewis, 1994). Susmel *et al* (1993) estimated endogenous PD excretion by extrapolation of the response of urinary PD excretion and estimated digestible MCP in the small intestine of Simmental cows. Endogenous PD (or allantoin alone) excretion derived from data reported in the literature for sheep and goats, and cattle is presented in Tables 1.10. and 1.11., respectively. Data from Blaxter and Wood (1951) has not been included due to the confounding effect of reducing sugars in the determination of urinary allantoin (Walker and Faichney, 1964). Reports in the literature (see Tables 1.10 and 1.11) have tended to indicate that cattle excrete approximately three times more PDs per unit metabolic liveweight than other ruminant species. This distinct species difference has been suggested to be due to differences in tissue and particularly blood *xanthine oxidase* profiles, which reduce the potential for purine *salvage* (Chen *et al*, 1990c).

Assessing endogenous PD excretion in lactating dairy cows is particularly uncertain due to difficulties in its measurement and also because it is likely to vary due to changes in metabolic activity throughout lactation. Further investigations assessing endogenous PD excretion in the lactating dairy cow appear necessary to allow accurate and reliable estimates of MCP supply to be made using the PD method.

1.2.10. Influence of nutrient supply on endogenous urinary PD excretion.

Current experimental evidence is conflicting concerning the influence of nutrient intake on urinary endogenous PD excretion. Observations of Fujihara *et al* (1987) in steers and lambs maintained by intra-gastric infusion showed endogenous allantoin excretion to be independent of protein infusions.

Table 1.10. Endogenous urinary PD and allantoin excretion in sheep and goats expressed in μmol/$W^{0.75}$/d

Species	PD excretion	Allantoin excretion	No.	Nutrient supply	Reference
Sheep	-	22-44	10	Intra-Gastric Infusion	Antoniewicz and Pisulewski (1982)
Sheep	-	161	3	Intra-Gastric Infusion	Sibanda *et al* (1982)
Sheep	202	176	2	Intra-Gastric Infusion	Giesecke *et al* (1984)
Sheep	165	-	3	Intra-Gastric Infusion	Fujihara *et al* (1987)
Sheep	136-217	68-109	4	Intra-Gastric Infusion	Lindberg and Jacobssen (1989)
Sheep	168	93	29	Intra-Gastric Infusion	Chen *et al* (1990c)
Sheep	191	73	4	Purine-free nutrient supply	Balcells *et al* (1991)
Sheep	-	46	-	Conventionally fed	Laurent and Vignon (1983)
Sheep	-	140	10	Conventionally fed	Puchala and Kulasek (1992)
Sheep	-	156	4	Milk fed pre-ruminant lambs	Antoniewicz (1993)
Goats	-	46-152	-	Conventionally fed	Laurent and Vignon (1983)
Goats	217	150	3	Milk fed pre-ruminant kids	Lindberg (1989)
Goats	253	161	3	Milk fed pre-ruminant kids	Lindberg (1991)

- indicate measurements not reported

Table 1.11. Endogenous urinary PD and allantoin excretion in cattle expressed in μmol/$W^{0.75}$/d

Species	PD excretion	Allantoin excretion	No.	Nutrient supply	Reference
Steers	-	375-447	2	Intra-Gastric Infusion	Sibanda *et al* (1982)
Steers	443-468	-	2	Intra-Gastric Infusion	Fujihara *et al* (1987)
Steers	428	365	2	Intra-Gastric Infusion	Verbic *et al* (1990)
Steers	401-571	373-500	6	Intra-Gastric Infusion	Chen *et al* (1990c)
Steers	531	-	2	Conventionally fed	Beckers and Thewis (1994)
Cows	-	536-840	2	Intra-Gastric Infusion	Sibanda *et al* (1982)
Cows	513	424	1	Intra-Gastric Infusion	Chen *et al* (1990c)
Cows	429	-	3	Conventionally fed	Susmel *et al* (1993)
Calves	443-613	329-494	3	Milk fed calves	Chen *et al* (1990)

- indicate measurements not reported

This finding was later confirmed by Chen *et al* (1990c) who found no differences in endogenous PD excretion between sheep maintained on a nitrogen-free infusion or abomasal casein infusion supplying 1-1.2 times nitrogen maintenance requirements. Further studies in sheep maintained by intra-gastric infusion indicated that endogenous urinary PD excretion was largely unaffected by moderate changes in energy intake and large changes in protein intake (Lindberg and Jacobsson, 1990).

In contrast to these findings, Sibanda *et al* (1982) found endogenous allantoin excretion to be influenced by energy and protein supply in steers and cows maintained by intra-gastric infusion, with the highest values being reported when no nutrients were infused. Similarly, Giesecke *et al* (1984) reported increases in endogenous allantoin excretion during VFA infusions supplying 25% of maintenance requirements, which were reduced when energy supply was restored to maintenance levels. These findings have also been confirmed by Lindberg (1989) working with milk-fed goat kids, who found endogenous allantoin excretion increased with decreasing energy and nitrogen intakes. Lindberg (1989) concluded that in the young growing ruminant endogenous PD excretion was only marginally affected by large variations in protein supply and milk intake.

Further work in lambs maintained by intra-gastric infusion has shown changes in endogenous allantoin excretion to be closely related to changes in cumulative nitrogen balance rather than being directly affected by daily nitrogen intake (Fujihara *et al*, 1988 and Chen *et al*, 1992c). It was suggested from these studies that endogenous allantoin excretion reflects the metabolic state of the animal during periods of fluctuating protein supply.

The majority of the studies assessing the influence of protein and energy supplies on endogenous urinary PD or allantoin excretion have used the method of intra-gastric infusion, it is unclear whether the effects observed in these experimental animals are paralleled in physiologically normal animals. On balance it appears that large changes in energy and protein supply are needed to influence greatly endogenous PD excretion.

1.2.11. Recovery of infused purines

In order to utilise the PD model, the relationship between duodenal NA supply and urinary PD excretion needs to be quantified, such that any variability in this relationship is known or at least predictable. A number of observations in sheep have indicated that absorbed purines can be utilised for NA synthesis (Ellis and Bleichner, 1969b; Condon *et al*, 1970; Smith *et al*, 1974 and Razzaque *et al*, 1981). Ellis and Bleichner (1969b) reported 70% of absorbed purines (as estimated using chromium oxide as a digesta flow marker) could not be accounted for by urinary PD excretion.

Further studies in sheep have demonstrated variable recoveries of radioactivity in urine over a 24 hour period as PDs following the administration of radio-labelled adenine. Condon *et al* (1970) reported urinary recoveries of 32% following infusion of ^{14}C adenine into the abomasum. Urinary recoveries of 15% (Smith *et al*, 1974) and 34% (Razzaque *et al*, 1981) have been observed following ruminal injections of mixed rumen bacteria labelled with [8-^{14}C] adenine. Kahn, 1991 (cited by Kahn and Nolan, 1993), intravenously injected ^{14}C adenine in sheep and reported 9% of total absorbed radioactivity was recovered in the urine. Presumably the lower recovery reported by Kahn (1991) is a consequence of by-passing gut wall metabolism. The extent of incorporation of radio-labelled exogenous purines into tissue NAs in these experiments was found to vary considerably (approximately 25% by Smith *et al*, 1974 and 46% Razzaque *et al*, 1981) suggesting differences in the extent of purine *salvage* between experiments, which could account for variations in the proportion of labelled purines excreted as PDs in the urine. Low urinary recoveries of radioactivity tentatively suggest that in sheep, absorbed labelled purines enter a large tissue purine pool (via *salvage* pathways) which is turned-over at a low rate, reducing the probability of urinary excretion. Interpretation of radio-labelling studies in the context of quantifying recovery of exogenous purines as urinary PD is compounded by the fact that no observations have been made in ruminants with guanine containing compounds.

Studies in monogastrics has demonstrated that exogenous guanine is not incorporated into tissue NAs but is extensively catabolised to allantoin (Burridge *et al*,

1976; Savaiano and Clifford, 1978 and Ho *et al*, 1979), presumably as a consequence of guanine deaminase in the gut wall (Henderson and Paterson, 1973) and poor absorption of guanine (Savaiano *et al*, 1980).

Further investigations have involved manipulating duodenal purine supply and measuring urinary PD excretion. Duodenal purine supplies have been manipulated by duodenal infusions of yeast RNA in conventionally fed sheep (Condon and Hatfield, 1970; Antoniewicz *et al*, 1980 and Balcells *et al*, 1991), milk fed goat kids (Matsumoto and Itabashi, 1988 and Lindberg, 1991) and cattle (Laurent and Vignon, 1987; Puchala *et al*, 1993 and Beckers and Thewis, 1994). Studies in ruminants nutritionally maintained by intra-gastric infusion have utilised RNA (Giesecke *et al*, 1984) or microbial NAs (Sibanda *et al*, 1982; Fujihara *et al*, 1987; Chen *et al*, 1990a and Verbic *et al*, 1990) as a source of purines. Recoveries of exogenous purines calculated as urinary PDs or allantoin reported in the literature range between 36 to 118% and 22 to 88 %, respectively as shown in Table 1.12. Studies in sheep have indicated urinary recovery of labelled purines to be much lower than unlabelled sources. Discrepancies between these observations can be explained by labelled purines entering a large tissue purine pool where they are able to be salvaged and retained in tissue NAs. In contrast, *salvage* of unlabelled purines would be compensated by endogenous purine losses. Reports in the literature have shown recovery of exogenous purines in sheep to be highly variable and dependent on exogenous purine supply. Data from early experiments (Antoniewicz *et al*, 1981; Sibanda *et al*, 1982; Giesecke *et al*, 1984 and Fujihara *et al*, 1987) was interpreted on the basis of the assumption that the relationship between purine (or NA) supply and PD excretion was linear, implying that endogenous PDs is constant across a range of exogenous purine supplies.

Many researchers have argued that in the sheep (Dewhurst and Webster, 1989; Chen *et al*, 1990a; Balcells *et al*, 1991 and Puchala and Kulasek, 1992) and cattle (Verbic *et al*, 1990) the relationship between purine absorption from the gut and PD excretion is curvi-linear. Chen *et al* (1990a) suggested the non-linearity is a result of *de novo* purine synthesis occuring when the absorption of purine *salvage* precursors from

the gut is low which would lead to a net positive endogenous contribution to purine losses.

Table 1.12. Recovery of exogenous purines as urinary purine derivatives

Animal	Nutrient supply	Purine source	Recovery (%) as		Reference
			Allantoin	PD	
Lambs	Mixed diet	RNA	71-79	92-110	Condon and Hatfield (1970)
Ewes	Mixed diets	RNA	22	-	Antoniewicz *et al* (1980)
Sheep	I-G infusion	RNA	48	52	Giesecke *et al* (1984)
Lambs	I-G infusion	M-NA	-	96-118	Fujihara *et al* (1987)
Lambs	I-G infusion	RNA	-	36-76	Chen *et al* (1990a)
Sheep	Mixed diets	RNA	88	93	Balcells *et al* (1991)
Sheep	Mixed diet	Adenine	-	73	Kahn (1991) cited by Kahn and Nolan (1993)
Goat kids	Milk	RNA	51	-	Matsumoto and Itabashi (1988)
Goat kids	Milk	RNA	49-78	53-85	Lindberg (1991)
Cattle	Mixed diets	RNA	32-76	-	Laurent and Vignon (1987)
Steers	I-G infusion	M-NA	-	77	Verbic *et al* (1990)
Cows	Mixed diets	RNA	82	87	Puchala *et al* (1993)
Steers	Mixed diets	RNA	-	73	Beckers and Thewis (1994)

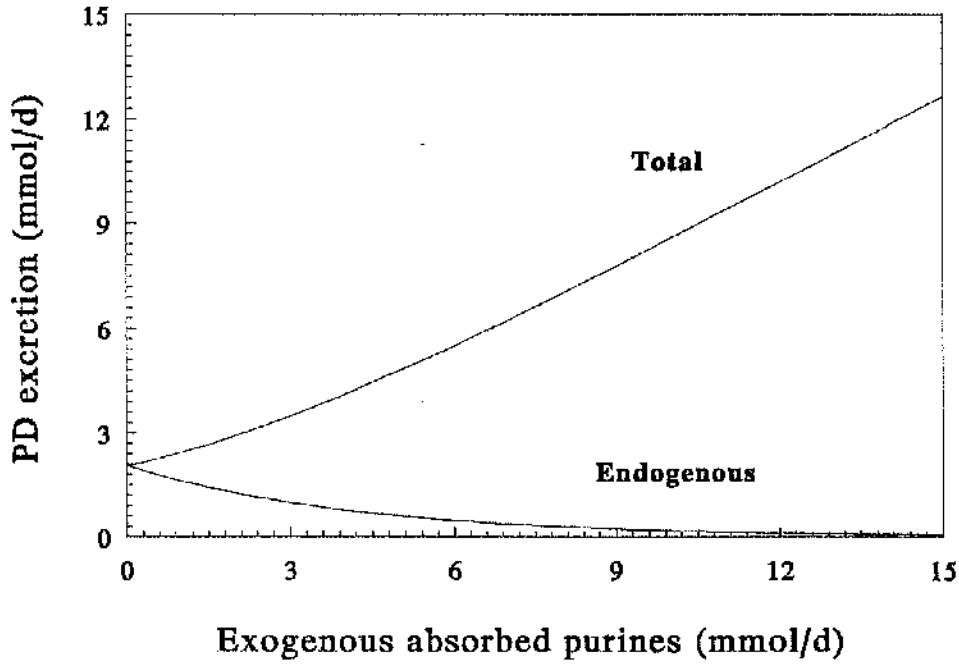
M-NA refers to microbial NAs
RNA refers to yeast RNA preparations
I-G refers to intra-gastric

Experimental data obtained from duodenal infusion of microbial NAs in intra-gastrically maintained sheep was used to construct a quadratic mathematical model relating urinary PD excretion (Y mmol/d) to absorbed exogenous purines (x mmol/d) by the following equation:-

$$Y = bx + cW^{0.75} \exp^{(-kx)}$$

Based on experimental data the model indicated that coefficient b=0.84, implying 16% of absorbed purines are excreted via non-renal routes. The rate constant (k= -0.25) describes the replacement of *de novo* purine synthesis by *salvage* of purine precursors. The coefficient c = 2.06 mmol/d, represents endogenous PD excretion at zero purine absorption. The model proposed by Balcells *et al* (1991) to describe their data, based on replacing duodenal digesta with nutritive solutions containing variable amounts of purines in sheep uses the same mathematical form as described by Chen *et al* (1990a) but assigns different values for the various coefficients (b = 0.938, k = -0.14 and c = 3.29 mmol/d). Non-renal allantoin and PD excretion is assumed to be 12 and 6% respectively.

Figure 1.5. Model of urinary PD excretion in relation to exogenous microbial purine absorption in sheep as proposed by Chen *et al* (1990a)



Mathematical models recently proposed have advanced the PD method considerably, allowing estimates of microbial protein rather than relative empirical indices to be calculated. In spite of the research effort applied to quantifying the

relationships between absorbed purines and urinary PD excretion, caution should be applied in universally accepting the derived estimates of MCP supply. Recent experimental observations of Chen *et al* (1991a) indicated that, in sheep, mean urinary recovery of intravenous allantoin infusions was 72% which ranged between 62-105%, suggesting that the models proposed underestimate non-renal PD excretion, leading to an overestimation of MCP supply. If the models proposed are correct, then the corollary is that, in the sheep, complications of determining endogenous PD excretion are removed when fed diets supplying maintenance and above energy requirements. Once salvage of purines operates at a maximal rate the relationship between absorbed purines and PD excretion is assumed to be linear over a wide range of exogenous purine supplies (refer to Figure 1.5.).

The situation with cattle is different, based on experimental observations in steers maintained by intra-gastric infusion and manipulating duodenal NA supplies, Verbic *et al* (1990) proposed a model relating purine derivative excretion (Y, mmol/d) to exogenous purine supply (x, mmol/d) described by the following equation:-

$$Y = cx + (a + b \exp^{-kx})$$

Where:- a + b is the endogenous PD excretion at no exogenous input

a is the endogenous PD excretion derived from *de novo* purine biosynthesis

b is the endogenous PD excretion that can be replaced by *salvage*

c is the proportion of exogenous purines recovered in the urine

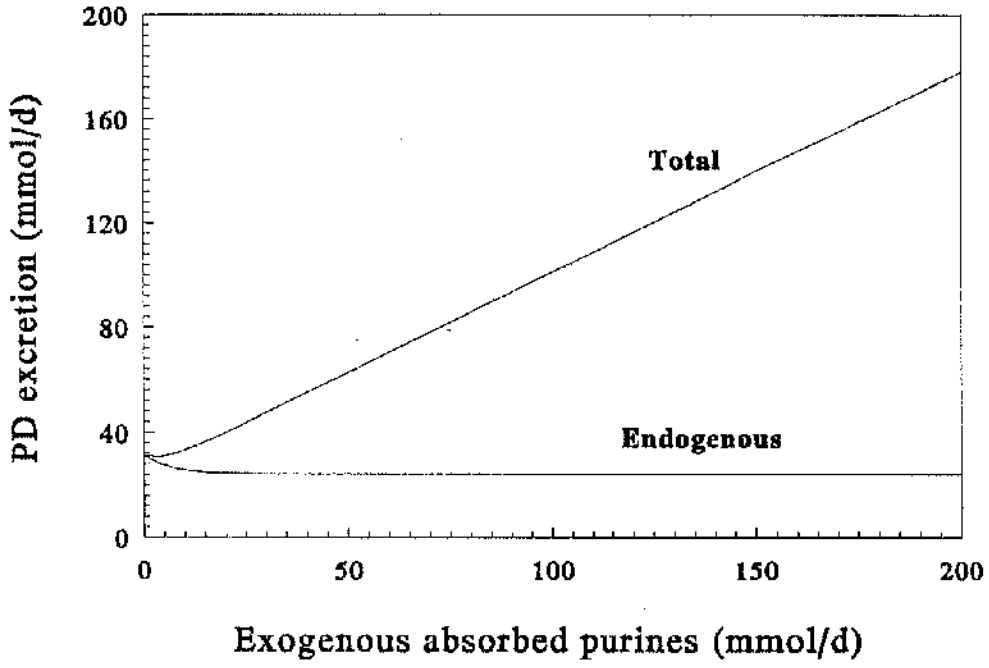
Fitting experimental values becomes:-

$$Y = 0.77\ x + (24.2 + 6.9 \exp^{-0.16x})$$

In sheep, salvage is assumed to be complete when a is zero, while in cattle, Verbic *et al* (1990) proposed that up to 6.9 mmol/d of total endogenous PD excretion

(total 31.1 mmol/d) could be replaced by salvage. These researchers argued that purine salvage can occur within bovine intestinal mucosa but not in other tissues due to high levels of *xanthine oxidase*. While utilisation of absorbed purines via *salvage* pathways has been suggested in cattle, it is nevertheless small compared to total endogenous losses (refer to Figure 1.6.). Once salvage pathways are saturated, endogenous PD excretion is assumed to be independent of exogenous purine supply.

Figure 1.6. Model of urinary PD excretion in relation to exogenous microbial purine absorption in cattle as proposed by Verbic *et al* (1990)



In cattle, unlike sheep an estimate of endogenous PD excretion is required to enable supplies of absorbed exogenous purines to be calculated. Verbic *et al* (1990) suggested that 22% of endogenous PD excretion could be salvaged and proposed a corrected endogenous PD excretion value of 385μmol/kg $w^{0.75}$/d. Verbic *et al* (1990) reported that 77% of infused microbial purines were recovered as PDs excreted renally. Assuming a true purine digestibility of 0.91, recovery of absorbed purines as PD in the

urine was calculated to be 0.85. Thus in cattle, urinary PD excretion (Y, mmol/d) is related to absorbed exogenous purines (x, mmol/d) by the following equation:-

$$Y = 0.85\ x + 0.385\ W^{0.75}$$

Where:- W is liveweight

The coefficient of 0.85, is similar to the coefficient (0.84) employed in the model proposed for sheep by Chen *et al* (1990a), implying that the relationship between absorbed purines and PD excretion is linear over a wide range of exogenous supplies and that 15% of absorbed purines are excreted as PDs via non-renal routes. In common with the model of Chen *et al* (1990a), non-renal PD excretion appears to be underestimated. Observations in steers indicated that only 70% of allantoin intravenously injected was recovered in the urine (Giesecke *et al*, 1993). Further research quantifying non-renal PD excretion in ruminant species, over a range of experimental diets appears to be necessary.

Differences in models relating purine absorption and PD excretion between those for sheep or cattle are due to the differences between the two species in the ability of many tissues to utilise exogenous purines. While the models proposed in sheep (Chen *et al*, 1990a and Balcells *et al*, 1991) and cattle (Verbic *et al*, 1990) seem plausible, Kahn and Nolan (1993) critically pointed out that the feedback control of *de novo* purine synthesis has not been experimentally verified and further questioned why mammals in general, and ruminants in particular, need to synthesise any purines *de novo*, in light of the large exogenous purine supplies and high energetic costs of purine biosynthesis.

Mathematical models proposed relating purine absorption and urinary PD excretion are particularly important as they enable estimates of MCP supply in intact ruminant species. Discrepancies concerning non-renal PD excretory routes are difficult to reconcile, tentatively suggesting that the proposed models underestimate this excretory pathway. Further investigations in cattle particularly, appear necessary to validate existing models.

1.2.12. Renal clearance of PDs

Appearance of hypoxanthine, xanthine and uric acid in ovine (e.g. Lindberg *et al*, 1989; Chen *et al*, 1990a; Chen *et al*, 1990c; Lindberg and Jacobssen 1990 and Balcells *et al*, 1991) and caprine (Lindberg, 1985 and 1991) urine suggests a high renal clearance of these compounds. Greger *et al* (1976) reviewed the available literature and concluded that the renal clearance of allantoin occured within the glomerulus of the mammalian kidney without subsequent reabsorption or secretion along the nephron. Renal allantoin clearances have been documented as being rapid, approaching that of creatinine (Greger *et al*, 1976). More recently, quantitative investigations into renal allantoin clearance suggest that allantoin is reabsorbed within the kidney. Chen *et al* (1991a) continously infused intravenously allantoin into four lambs maintained by intra-gastric infusion. Using creatinine clearance as an estimate of glomerular filtration rate (GFR), maximal tubular allantoin reabsorbtion was calculated to be 1.28 mM/d. Faichney and Welch, (1994) used ^{51}CrEDTA to measure GFR in sheep and found that renal clearances of plasma allantoin were marginally, but significantly ($P<0.05$) less than 100%. They concluded that in normally fed sheep, allantoin tubular reabsorption is probably less than 10% of the filtered load, an estimate much lower than that reported by Chen *et al* (1991a). Studies in sheep (Chen *et al*, 1991a) and cattle (Giesecke *et al*, 1993) have described clearances of allantoin plasma loads by quadratic mathematical models. Both models assigned a first order rate constant of 0.3, implying that approximately a third of plasma allantoin is cleared renally per unit time. Estimates of allantoin GFR in steers ranged between 588-802 ml min^{-1} (mean 683 ml min^{-1}) and were found to be independent of a functional rumen, implying renal clearances are unaffected by the supply of purine catabolites entering the bloodstream. Allantoin GFR in sheep was measured as 99 ml min^{-1} (Chen *et al*, 1991a). Assuming kidney mass is related to metabolic liveweight, then the value reported by Chen *et al* (1991a) is equivalent to 608 ml min^{-1} in steers (as described in the work of Giesecke *et al*, 1993) suggesting allantoin GFR is similar between ruminant species.

Renal clearance of uric acid involves complicated post-filtration secretion and reabsorption of uric acid processes occuring in the nephron and the Loop of Henle in the mammalian kidney (Greger *et al*, 1976). GFR measurements in steers during conventional feeding and after rumen emptying, suggest renal clearances of 670 ml min^{-1} for uric acid and 683 ml min^{-1} for allantoin (Giesecke *et al*, 1993). Little information is available in the literature concerning renal clearances of xanthine and hypoxanthine in the ruminant. Studies with mammalian kidneys tend to suggest that they are cleared at similar rate to creatinine (Greger *et al*, 1976). On balance, it appears renal clearance of uric acid, xanthine and hypoxanthine are similar to that of allantoin in the ruminant. Renal clearance of allantoin has been shown to be rapid implying changes in the rate of urinary allantoin excretion would closely reflect changes in plasma allantoin concentrations.

1.2.13. Non-renal PD excretory routes

Incomplete recoveries of exogenous purine sources (e.g. Chen *et al*, 1990a; Verbic *et al*, 1990; Balcells *et al*, 1991 and Beckers and Thewis, 1994) suggest that typically 16% of PDs are excreted via non-renal routes. Incomplete recoveries of intravenous allantoin injections in the urine (Chen *et al*, 1991a and Giesecke *et al*, 1993) has confirmed the existence of non-renal PD excretory routes. Although allantoin is secreted in milk in lactating dairy cows, accounting for between 1-10% of that excreted in the urine (Giesecke *et al*, 1994 and Susmel *et al*, 1995), indicating the presence of other non-renal excretory routes, such as excretion of PDs into the gut particularly as allantoinase is thought to be absent in ruminant tissues. Experimental evidence suggests PDs can enter the gut via salivary secretions in ruminant species. Other losses might also occur via gastro-intestinal secretions (Kahn and Nolan, 1993), or by direct passage across the gut wall with subsequent degradation by bacteria associated with the gut wall as observed for uric acid in humans (Sorenson, 1978).

1.2.13.1. PD recycling via saliva

Studies in sheep have shown saliva can contain higher concentrations of allantoin and uric acid than plasma, tentatively suggesting these compounds can be actively transported (Chen *et al*, 1990d). Using an estimate of salivary outflow of 10 l/d, the researchers estimated PD excretion into the gastro-intestinal tract via saliva was equivalent to 10% of urinary PD excretion. Kahn (1991) cited by Kahn and Nolan (1993) reported that 2.5% of the ^{14}C derived from intravenously administered ^{14}C-adenine entered saliva in a 24 hour period. Assuming the same salivary outflow as Chen *et al* (1990d) salivary PD excretion accounted for 27% of urinary excretion during the same period. In contrast, Surra *et al* (1993), reported PD concentrations in sheep saliva to be approximately 10% of normally observed plasma concentrations. Assuming a mean salivary flow of 7 l/d (estimated using a Co-EDTA marker), PD recycling via saliva was calculated to be approximately 1% of that excreted in the urine. Such large discrepancies reported in the literature are difficult to reconcile and only serve to highlight uncertainties of PD partitioning.

1.2.13.2. Fate of PDs recycled via saliva

Accepting uncertainties concerning the proportion of PDs absorbed that are recycled via saliva, the PD model proposed by Chen *et al* (1992a) assumes no subsequent re-absorption into the blood. *In-vitro* incubations with ovine rumen liquor have shown that rumen microbes degrade allantoin at a rate of 15 $\mu mol\ h^{-1}\ l^{-1}$ (Chen *et al*, 1990d). Assuming a rumen volume of 5-10 litres for the sheep, the capacity of the rumen microflora to degrade salivary allantoin would range between 1.8-3.6 mmoles/day. It is possible that, at high rates of salivary secretion e.g. during the ingestion of fibrous feedstuffs, that the rate of PDs entering the rumen via saliva could exceed the degradative capacity of the rumen microflora. Intra-ruminal infusions of allantoin in sheep and steers maintained by intra-gastric infusion and in conventionally fed lambs, did not increase urinary allantoin excretion (Chen *et al* 1990d). The study

indicated that the rate of allantoin disappearance from the rumen was twice that of polyethylene glycol and the researchers suggested that this was caused by degradation of allantoin by the activities of microbes associated with the rumen epithelium rather than by allantoin absorption through the rumen wall. Uric acid also enters the gut and is assumed to be degraded to allantoin. Furthermore, the capacity of the small intestine to absorb allantoin has been shown to be limited. Only 6-8% of abomasal allantoin infusions in sheep maintained by intra-gastric infusion was recovered in the urine (Chen *et al*, 1990d) implying allantoin recycled via saliva which escaped ruminal degradation is unlikely to significantly increase urinary allantoin excretion.

Purine compounds entering the rumen via salivary recycling are likely to be rapidly degraded and are unlikely to reappear in the urine. Allantoin entering the gut is rapidly degraded to glyoxylate and urea, with the later being catabolised to ammonia and carbon dioxide by bacterial *urease* (McAllan, 1982). Early observations of Belasco (1954) indicated that allantoin and uric acid could be utilised as nitrogen sources by rumen bacteria *in-vitro*. Purine derivatives recycled into the rumen by saliva would appear to make a significant contribution to total recycled endogenous nitrogen in the ruminant animal.

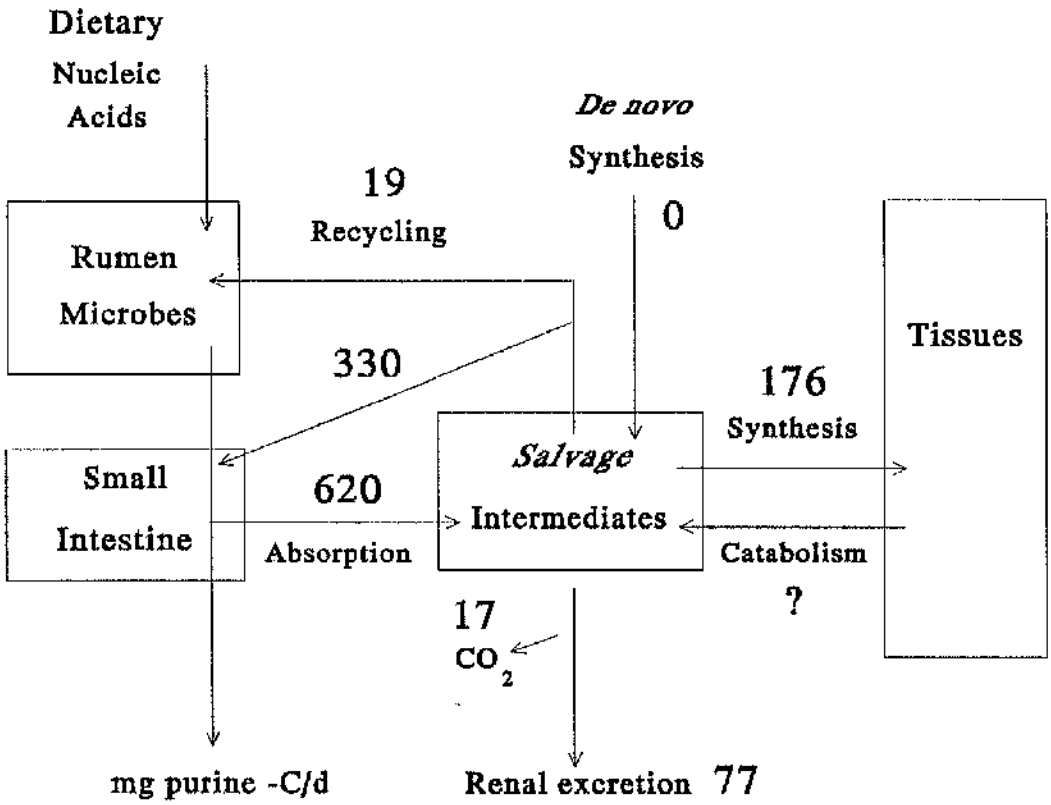
1.2.13.3. PD excretion via gastro-intestinal secretions

Observations in sheep with intravenously injected labelled U-^{14}C- adenine, lead Kahn and Nolan (1993) to propose a model describing the major pathways of purine metabolism in ruminants, shown schematically in Figure 1.7.

Twenty four hours after the administration of labelled adenine tracer, 13% was excreted in the urine. Accounting for known losses of $^{14}CO_2$ as a result of hepatic catabolism of uric acid to allantoin and ruminal fermentation of salivary recycled purine compounds, indicated total urinary recovery to be 19%. Urinary recovery of adenine-C (19%) fell short of the 48% of administered tracer that passed through blood bicarbonate during the same period. Kahn and Nolan (1993) hypothesised that $^{14}CO_2$ not recovered in the urine was most likely to be liberated as a result of microbial catabolism of purines

contained in bile and other gastro-intestinal secretions, rather than purine catabolism by host tissues.

Figure 1.7. Model of purine transactions in ruminants proposed by Kahn and Nolan (1993)



Values (mg purine C/d) are means of estimates derived from four sheep given straw based diets supplemented with formaldehyde treated casein

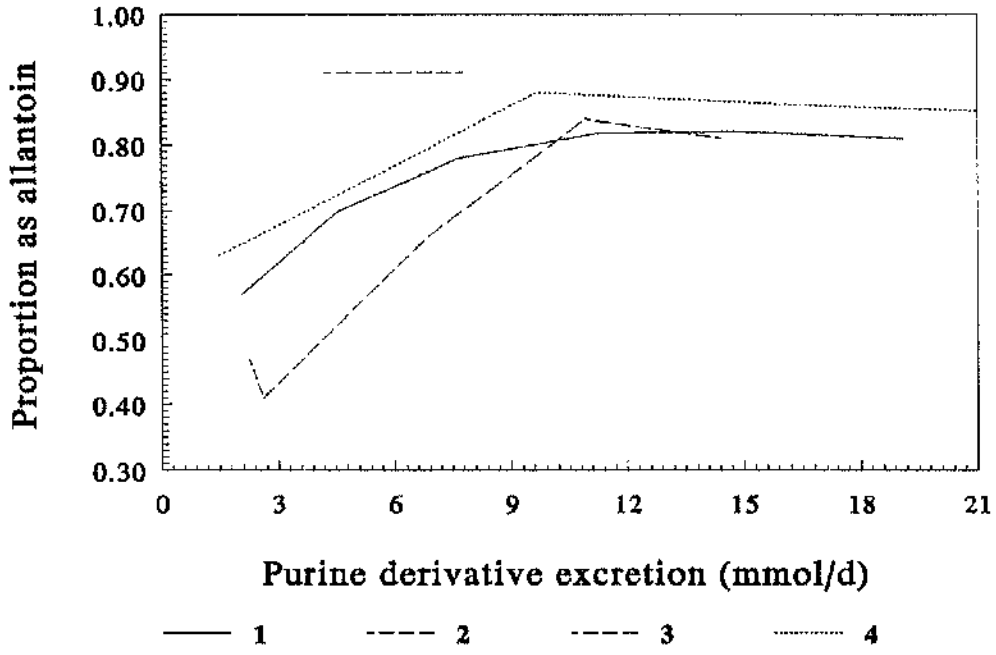
Accounting for $^{14}CO_2$ assumed to be derived by microbial catabolism of recycled purines indicated that total recovery of ^{14}C-adenine carbon was 64% of that administered. The remaining radioactivity (36%) was suggested to be incorporated in tissue purine pools, being similar to 25 and 46% reported by Smith *et al* (1974) and Razzaque *et al* (1981), respectively. Assuming the hypothesis of Kahn and Nolan (1993) is correct, PD excretion via gastro-intestinal secretions appears to be quantitatively much more significant that recycling via saliva and requires further investigation.

1.2.14. Proportions of urinary PDs excreted in the urine

Prediction of absorbed purines from measurements of urinary allantoin excretion alone in sheep and goats is complicated by changes in the relative proportions of allantoin, uric acid, xanthine and hypoxanthine excreted. Observations in sheep have indicated relative increases in proportions of PD excreted as allantoin with increasing exogenous purine loads, suggesting differences between sites of derivative formation within the body (Chen *et al*, 1990a). Ovine non-hepatic cells contain only traces of *xanthine oxidase*, while blood contains no *xanthine oxidase* and only traces of *uricase* (Al-Khalidi and Chaglassian, 1965 and Chen *et al*, 1990c). Thus endogenous purines can be catabolised to hypoxantine, xanthine and uric acid, while further catabolism to allantoin requires derivative transport to the liver. It is likely that PDs derived from endogenous purine catabolism will be excreted in the urine due to high renal clearances rather than enter the liver. In contrast, absorbed purines enter portal blood and are readily catabolised by the liver to allantoin. Lindberg (1991) demonstrated with goat kids, differences in the proportion of PDs excreted from catabolism of endogenous and exogenous purines, supporting the arguments of Chen *et al* (1990a). If the sites of purine catabolism are different for endogenous and exogenous purines, it seems reasonable to expect the proportion of PDs excreted as allantoin to increase with increased exogenous purine supply. Despite these suggestions, experimental observations are conflicting. Condon and Hatfield (1970) reported increases in non-allantoin derivatives at high duodenal RNA infusions, while Giesecke *et al* (1984) detected significant increases in hypoxanthine excretion with RNA infusions. Balcells *et al* (1991) reported that only allantoin excretion increased in response to incremental inputs of duodenal purines. In contrast, Lindberg (1991) and Kahn and Nolan (1993) working with goats and sheep respectively, have reported the proportions of PDs excreted were constant over a range of exogenous purine supplies, with allantoin accounting for 89 and 91% repectively, of the total PDs excreted. In experiments with sheep receiving much lower purine supplies, allantoin accounts for between 49% (Lindberg and Jacobssen, 1990) and 55% (Chen *et al*, 1990c) of total PD excretion. The proportion of PDs excreted as allantoin appears to

be influenced by the proportion of absorbed:endogenous purines catabolised and consequently absorbed purine supply (refer to Figure 1.8.).

Figure 1.8. Variations in the proportion of allantoin:total PDs excreted in ruminant urine with increasing rates of exogenous purine supply reported in the literature



References:-

1) Observations in sheep of Chen *et al* (1990a)
2) Observations in sheep of Kahn (1991), cited by Kahn and Nolan (1993)
3) Observations in sheep from experiment 1 of Balcells *et al* (1991)
4) Observations in goat kids from experiment 1 of Lindberg (1991)

Differences between sheep and goat breeds could also influence the proportion of PDs excreted as allantoin (Kahn and Nolan, 1993). It should also be remembered that various analytical methodologies employed will also contribute to the differences between experimental reports. Currently there is some debate as to which derivatives to

measure. Balcells *et al* (1991) and Puchala and Kulasek (1992) have suggested measurement of allantoin alone provides an accurate assessment of urinary PD excretion in ovine and caprine species. In contrast, Giesecke *et al* (1984), Fujihara *et al* (1985 and 1987) and Lindberg *et al* (1989) have suggested all derivatives need to be taken into account.

Observations of Verbic *et al* (1990) have indicated that the proportions of allantoin to uric acid (the principle PDs in bovine urine) were similar for endogenous and exogenous sources, and unaffected by exogenous purine supply. In cattle, the proportion of PDs excreted as allantoin (0.82, Chen *et al*, 1990c; 0.85, Verbic *et al*, 1990; 0.89, Giesecke *et al*, 1994; 0.93, Gonda and Lindberg, 1994; 0.90, Dewhurst *et al*, 1995 and 0.88, Dewhurst *et al*, 1996) appears to be relatively constant over a wide range of urinary PD excretion. Experimental observations indicate that cattle generally excrete a higher proportion of PDs as allantoin than other ruminant species. Bovine endogenous and absorbed purines are catabolised to yield similar PD proportions due to high *xanthine oxidase* activities in most tissues (Al-Khalidi and Chaglassian, 1965). Consequently, the proportions of PDs excreted as allantoin is largely unaffected by absorbed purine supplies (Verbic *et al*, 1990).

1.2.15. Typical urinary PD excretion in ruminant species

Reports of urinary PD excretion by ruminant species in the literature over the last two decades have been considerable. Typical urinary PD excretion in conventionally fed ruminants, calculated from data reported in the literature is presented in Table 1.13.

Table 1.13. Typical urinary PD and allantoin excretion (mmoles/d) in conventionally fed ruminant species

Species	Lwt (kg)	Diet	DMI (kg/d)	PD	Allantoin	Reference
Sheep	40.5	Straw based	0.71-1.05	5.7-10.6	-	Chen *et al* (1992a)
Sheep	44	Straw based	0.27-0.63	4.0-11.7	-	Balcells *et al* (1993)
Sheep	61.3	Silage based	0.85-1.05	11.8-17.1	-	Chamberlain *et al* (1993)
Sheep	15.8	Various	0.58-0.61	5.1-5.8	3.8-4.3	Osuji *et al* (1993)
Sheep	57.4	Semi-synthetic	0.88-1.35	12.0-20.4	9.5-16.2	Djouvinov and Todorov (1994)
Goats	48	Straw based	0.80-1.80*	-	12.0-41.0	Lindberg (1985)
Cows	NQ	Straw based	4.3-7.8*	97-200	-	Daniels *et al* (1994)
Cows	614	Maize silage based	13.1-19.5	-	234-354	Giesecke *et al* (1994)
Cows	604	Hay and concentrate	13.0-15.1	185-208	166-202	Susmel *et al* (1994a)
Cows	662	Various	7.3-10.3	117-295	106-286	Susmel *et al* (1994b)
Cows	577	Silage based	14.9-16.2	203-226	183-204	Dewhurst *et al* (1995)

* DMI not quoted and calculated on the assumption of feed OM content = 950g/kg DM

NQ:- not quoted

Ranges described are based on treatment means

1.2.16. Assessment of MCP using the PD model

Several models have been proposed over a number of years to calculate MCP supply based on urinary PD excretion (Rys *et al*, 1975; Balcells *et al*, 1991 and Chen *et al*, 1992a). The model described by Chen *et al* (1992a) has more frequently been employed to assess MCP supplies in sheep, (Chamberlain *et al*, 1993; Osuji *et al*, 1993 and Djouvinov and Todorov, 1994) and cattle (Khalili *et al*, 1994 and Susmel *et al*, 1994b). It must be emphasised that this model takes no account of feed purines escaping rumen degradation, and appears to underestimate non-renal PD excretion (refer to section 1.2.11.). Therefore estimates using this model from urinary PD excretion are likely to overestimate MCP supply. The model proposed by Chen *et al* (1992a):-

$$\textbf{M-N supply (g/d)} = \frac{\textbf{Absorbed purines (mmol/d) x purine-N (mg/mmol)}}{\textbf{Microbial purine digestibility x purine-N:total-N x 1000}}$$

Assumptions made:-

Microbial purine digestibility	= 0.83
Nitrogen content of purines (mg/mol)	= 70
Purine-N:Total-N ratio in microbes	= 0.116:1.00

Absorbed purines (ABP) in **sheep**:-

PD excretion = 0.84 x ABP (derived by Chen *et al*, 1990a)

(valid for PD excretion over 0.6mmol/$kg^{0.75}$/d or feeding levels above 0.8 x maintenance)

Absorbed purines (ABP) in **cattle**:-

PD excretion = 0.85 x ABP + 0.385 Liveweight $^{0.75}$

(derived by Verbic *et al*, 1990)

1.2.17. Comparisons of urinary PD excretion with other microbial markers

It is essential that the PD method gives reliable and accurate estimates of MCP supplies. Djouvinov and Todorov (1994) compared estimates of MCP in sheep by urinary PD excretion using the model of Chen *et al* (1992a) with the total purine method proposed by Zinn and Owens (1986) and DAPA. They concluded that assessment of MCP entering the duodenum based on urinary PD excretion was not significantly different to measuring total purine-nitrogen in duodenal digesta. DAPA gave comparable results, but tended to underestimate MCP with diets which stimulated high numbers of protozoa in the rumen. Data reported by Djouvinov and Todorov (1994) is shown in Table 1.14. In a similar study in sheep, Perez *et al* (1994) reported good agreement between estimates derived from urinary allantoin excretion (although the model used to calculate this was not cited) and ^{15}N measurements (refer to Table 1.15.) concluding that urinary allantoin excretion provides reliable estimates of MCP synthesis, although this may not be the case in diets containing a high proportion of concentrates.

Table 1.14. Comparison of M-N (g/d) entering the duodenum assessed by urinary PD excretion and total purine and DAPA flows

Microbial Marker	Experimental diet					
	ST 10	ST 30	ST 50	PV0	PV 24	PV 32
Urinary PD excretion	13.7	15.2	17.6	10.1	12.0	13.9
Total Purines	14.1	16.4	19.3	10.7	12.8	14.3
DAPA	13.3	14.2	15.9	9.9	11.8	14.0

Data from Djouvinov and Todorov (1994)

Table 1.15. Comparison of duodenal M-N flow (g/d) assessed by urinary allantoin excretion and ^{15}N flow.

Microbial Marker	Concentrate level (g/d)			
	0	220	400	550
Urinary allantoin excretion	5.92	8.63	13.82	9.84
^{15}N	6.21	8.98	14.04	14.36

Data from Perez *et al* (1994)

1.2.18. Urinary pseudouridine excretion

Pseudouridine, a modified pyrimidine nucleoside is present within RNA and in humans is excreted in the urine (Gehrke *et al*, 1979). Pseudouridine produced as the result of tissue RNA degradation is not further metabolised or re-utilised but excreted in the urine (Borek and Keer, 1972). In monogastric animals urinary pseudouridine excretion increases with cell accretion, while experiments in cows has indicated that urinary pseudouridine excretion is not influenced by duodenal yeast RNA infusions, but is influenced by growth rate. Indeed pseudouridine excretion is higher in young growing animals compared to the mature adult (Puchala *et al*, 1993). Currently pseudouridine is thought to originate from tissue catabolism of RNA, representing an possible index of RNA turnover, and could potentially be used as a index of nitrogen status in the ruminant (Puchala *et al*, 1993).

1.2.19. Summary

Experimental evidence reported in the literature suggests that dietary NAs are extensively degraded by rumen microbes, and as a consequence NAs entering the small intestine are essentially microbial in origin. In ruminant species, NAs are degraded and absorbed as nucleosides and their free pyrimidine and purine bases. Excretion of purine catabolites allantoin, uric acid, xanthine and hypoxanthine, collectively termed PDs, is highly correlated with NA content in the rumen and duodenum, and with purine base absorption from the duodenum. Numerous studies have quantified inevitable endogenous losses of purines associated with tissue NA turn-over in farm species. Reports in the literature indicate that endogenous PD excretion reported for cattle is approximately three times greater than that for sheep and goats. Differences in the activities of *xanthine oxidase* in tissues and particularly blood have been suggested to account for observed differences between farm ruminant species. Studies investigating the recovery of radio-labelled exogenous purines as PDs excreted in the urine of sheep indicates that absorbed purines can enter *salvage* pathways and subsequently be re-utilised in the synthesis of

three times greater than that for sheep and goats. Differences in the activities of *xanthine oxidase* in tissues and particularly blood have been suggested to account for observed differences between farm ruminant species. Studies investigating the recovery of radio-labelled exogenous purines as PDs excreted in the urine of sheep indicates that absorbed purines can enter *salvage* pathways and subsequently be re-utilised in the synthesis of tissue NAs. Reports in the literature indicate that the recovery of unlabelled exogenous purines is highly variable. Recent experimental evidence has promoted the hypothesis of a curvi-linear relationship between purine absorption and PD excretion as a consequence of progressive replacement of *de novo* purine synthesis by *salvage* of exogenous purines in ruminant species. Mathematical models describing the curvi-linear relationships, imply in sheep and presumably goats, that endogenous PD excretion is satisfied by *salvage* of absorbed purines under normal feeding regimens. In cattle only a proportion (0.22) of absorbed purines can be salvaged and therefore endogenous PD excretion needs to taken into account. Current, models allow absorbed purines to be estimated from urinary PD excretion in ruminant species. Assuming a constant purine-N:total-N ratio in rumen microbial cells (0.116) and microbial purine digestibility (0.83), MCP supply available to the ruminant animal can be estimated. The PD method appears to give accurate estimates of MCP supply when compared to existing microbial markers, and represents the only available non-invasive alternative. Routine use of the PD method is restricted to research activities at present due to the requirement of a total urine collection to assess urinary PD excretion.

Chapter Two

Analysis of creatinine, pseudouridine and purine derivatives in bovine urine by High Performance Liquid Chromatography

Summary

Current methodologies of measuring PDs and creatinine in urine are reviewed. A HPLC method developed specifically for simultaneous determination of creatinine, pseudouridine and PDs in bovine urine is described. Optimum mobile phase composition, urine sample preparation and storage were investigated.

2.1. Methodology

HPLC apparatus:-	Isocratic pump, autosampler fitted with a 20 µL loop, UV detector and integrator
Mobile Phase :-	7.5 mM Ammonium dihydrogen phosphate ($NH_4H_2PO_4$) 10 mM Sodium 1-heptanesulphonic acid (Na-HSA; $CH_3(CH_2)_6SO_3Na$) 1.0 mM Triethylamine (TEA; $(C_2H_5)_3N$)
pH	3.00
Stationary Phase:-	5 micron Spherisorb ODS II 250mm x 4.6 mm C-18 column

Chromatographic Conditions:-

Flow rate	1ml /min

Detection wavelength	218 nm
Temperature	20°C
Analysis time	60 minutes
Calibration Standard:-	0.5 mM allantoin and creatinine, 0.125 mM uric acid, xanthine and hypoxanthine and 25 µM pseudouridine
Sample Preparation:-	500 µl of sample diluted in 4.5 mls of diluent, filtered and 20 µl injected onto the column

2.2. Introduction

Numerous methods have been described for the determination of PDs and creatinine in biological fluids. Although ion-exchange chromatography has been used (Razzaque and Topps, 1978), the majority of the methods are based on measuring allantoin following a series of reactions leading to the production of a chromophore. Traditional colorimetric analysis of allantoin in urine is based on the Rimini-Schryver reaction as described by Young and Conway (1942). The principle involved is the conversion of allantoin to glyoxylic acid by sequential hydrolysis under alkaline and acidic conditions. Glyoxylic acid reacts with phenylhydrazine to form a hydrazone which is quantified colorimetrically. In order to achieve good reproducibility the timing of the addition of reagents and termination of the chemical reactions is critical. Pentz (1969) automated the Rimini-Schryver reaction, which Lindberg and Jansson (1989) and Chen *et al* (1990b) have subsequently modified. Borchers (1977) proposed determining allantoin based on measuring the 2,4-dinitrophenylhydrazone of glyoxylic acid, which has the advantage of increasing reagent and reaction product stability. Colorimetric methodologies can lead to an overestimation of allantoin due to their lack of specificity (Young and Conway, 1942). Interfering compounds such as formaldehyde can potentially complicate urinary allantoin measurements, particularly those of ruminants

fed formaldehyde treated feedstuffs (Chen *et al*, 1993a). Chen *et al* (1993a) addressed these criticisms by separating glyoxylic hydrazone from interfering hydrazones by HPLC.

Determination of urinary concentrations of uric acid, xanthine and hypoxanthine has been achieved by measuring allantoin production following the addition of *xanthine oxidase* and *uricase* (Razzaque and Topps, 1978 and Fujihara *et al*, 1987). However this approach is unsatisfactory as recoveries of xanthine and uric acid as allantoin were incomplete. Chen *et al* (1990b) measured urinary xanthine and hypoxanthine from uric acid production, following the addition of *uricase*. Uric acid was determined using a phosphotungstic method, which has been reported to be unreliable due to ascorbic acid interference (Hatch and Sevanian, 1984).

HPLC determination of allantoin in urine represents a rapid and sensitive alternative to colorimetric methods. Tiermeyer and Giesecke (1982) described an isocratic technique to determine allantoin in urine, using a 5 mM phosphate buffer mobile phase at pH 3.5 eluting through two μ-Bondupak C-18 columns connected in series. More recently Diez *et al* (1992) reported a similar isoocratic method which had the advantage of measuring allantoin and creatinine simultaneously. Separation was achieved with a 10 mM phosphate buffer mobile phase at pH 4.0 eluting through a Novapak C-18 column. Balcells *et al* (1992) described an alternative method which measured all four purine derivatives simultaneously using a pair of ODS II C-18 columns and a gradient 100mM phosphate/100mM phosphate:acetonitrile (80:20) mobile phase.

HPLC techniques used to determine urinary PD concentrations are not beyond criticism, due in particular to poor retention and separation of allantoin. Ideally a compound capacity factor (described as the ratio of compound retention time to mobile phase elution time) for a particular HPLC method should fall within a 1-10 range (Dolan and Synder, 1989). Published HPLC techniques for measurement of urinary PDs of which that reported by Tiermeyer and Giesecke (1982) is typical, describe allantoin capacity factors which fall outside the ideal range leading to incomplete separation of allantoin from similarly polar compounds present in urine. Allantoin retention has been demonstrated to be improved by the inclusion of an ion-pairing agent in the mobile phase, sodium 1-heptanesulphonic acid in particular (Lux *et al*, 1992). Despite the

criticisms of both HPLC and colorimetric determinations of allantoin, comparisons have indicated a good agreement between these methodologies (Lindberg and Jansson, 1989 and Resines *et al*, 1993).

Creatinine is widely determined by the Jaffe alkaline picrate procedure described by Peters and Slyke (1958). However this method involves several steps and can result in overestimates due to the interference of endogenous and exogenous psuedo-creatinine chromogens (Narayanan and Appleton, 1980). More recently, HPLC techniques have been developed to determine creatinine concentrations in biological fluids of which the procedure outlined by Xue *et al* (1988) is typical.

At the start of this project, urine samples were routinely analysed by the method of Balcells *et al* (1992), modified to measure creatinine. However, poor compound separation in urine samples (standard and sample chromatograms are shown in Figures 2.1 and 2.2, respectively) and the limited availability of a binary HPLC pump initiated development of an isocratic HPLC technique. Development proceeded with the aim of measuring pseudouridine in addition to PDs and creatinine in bovine urine.

Figure 2.1. Standard chromatogram using the method of Balcells *et al* (1992)

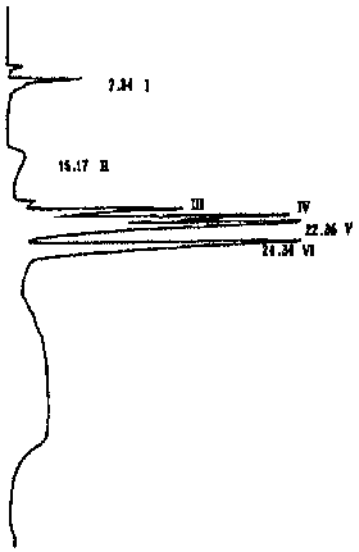
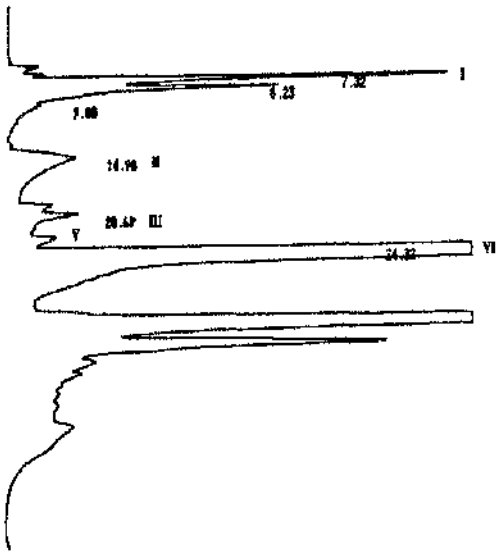


Figure 2.2. Ovine urine sample chromatogram using the method of Balcells *et al* (1992)



Compound Identification:-

I	Allantoin
II	Creatinine
III	Uric acid
IV	Hypoxanthine
V	Xanthine
VI	Allopurinol

2.3. Materials and methods

2.3.1. HPLC apparatus

Analytical apparatus comprised of an isocratic pump model P100, UV detector model 200, data-jet integrator (Spectra Physics Limited, Hemel Hempsted, Herts, England), Gilson Bio-Autosampler model No. 232 (Anachem, Luton, Bedfordshire, England). Chromatography was performed using 5 micron Spherisorb C 18 ODS II packed reversed phase HPLC column measuring 250mm x 4.6mm internal diameter (Phase Separations Limited, Deeside, Clwyd, Wales).

2.3.2. pH measurement

Eluant and diluting solution pH was measured using a general purpose combination gelplas electrode, (BDH limited, Poole, Dorset, England) and Cranwell CR 99 digital pH meter. The meter was calibrated twice weekly using pH 7.0 ($\pm$ 0.02) and pH 4.0 ($\pm$ 0.02) at 20^{0}C colourkey buffer solutions (BDH Limited, Poole, Dorset, England).

2.3.3. Reagents

Allantoin (Phase Separations Limited, Deeside, Clwyd, Wales), pseudouridine, allopurinol, sodium salts of uric acid and xanthine (Sigma Chemical Company, Poole, Dorset, England), creatinine, hypoxanthine, triethylamine (Aldrich Chemical Company, Gillingham, Dorset, England), ammonium dihydrogen phosphate (Fluka Chemicals Limited, Gillingham, Dorset, England) and sodium 1-heptane sulphonic acid (BDH Laboratory Supplies, Poole, Dorset, England) were all assayed at 98% purity or above. Double-deionised water was obtained from a laboratory Fistreem water filtration unit (Fisons Scientific Equipment, Loughborough, Leicestershire, England).

2.3.4. Method development

An initial mobile phase of 5 mM ammonium dihydrogen phosphate ($NH_4H_2PO_4$) at pH 3.20 was based on the conditions described by Tiermeyer and Giesecke (1982) incorporating an ion-pairing reagent, 5 mM sodium 1-heptane sulphonic acid (Na-HSA) as suggested by Lux *et al* (1992). Unacceptable creatinine retention of 45 minutes was reduced by the inclusion of triethylamine (TEA) in the mobile phase. A range of mobile phase compositions of 5-15 mM ($NH_4H_2PO_4$), 1-30 mM (TEA), 1-10 mM (Na-HSA) pH of 3.0-3.2 were tested. Mobile phase containing 7.5mM ($NH_4H_2PO_4$), 1.0mM (TEA) and 10.0mM (Na-HSA) at pH 3.00 provided the best separation of PDs, pseudouridine and creatinine.

2.3.4.1. Compound identification

Compounds were initially identified by injecting a compound standard onto the column in isolation and determining peak retention times. Comparison of peak retention times obtained with authentic standards allowed each compound to be indentified in the urine. This was subsequently confirmed by spiking urine samples with compound standards and observing peak area increases. Peak purity was not assessed.

2.3.4.2. Chromatographic conditions

Chromatography was achieved under isocratic conditions at a flow rate of 1ml /min. The eluted mobile phase was monitored at 218 nanometers. Total analytical run time was 60 minutes, with PDs, pseudouridine and creatinine being eluted by 29 minutes. The flow of mobile phase was reversed for 29 minutes to remove later eluting compounds from the column. The direction of flow was restored and the mobile phase eluted through the column for 2 minutes before the next sample injection. Representative standard and sample chromatograms are presented in Figures 2.3. and 2.4., respectively. Column temperature was maintained at 20^{0}C during analysis.

Figure 2.3. Calibration standard chromatogram

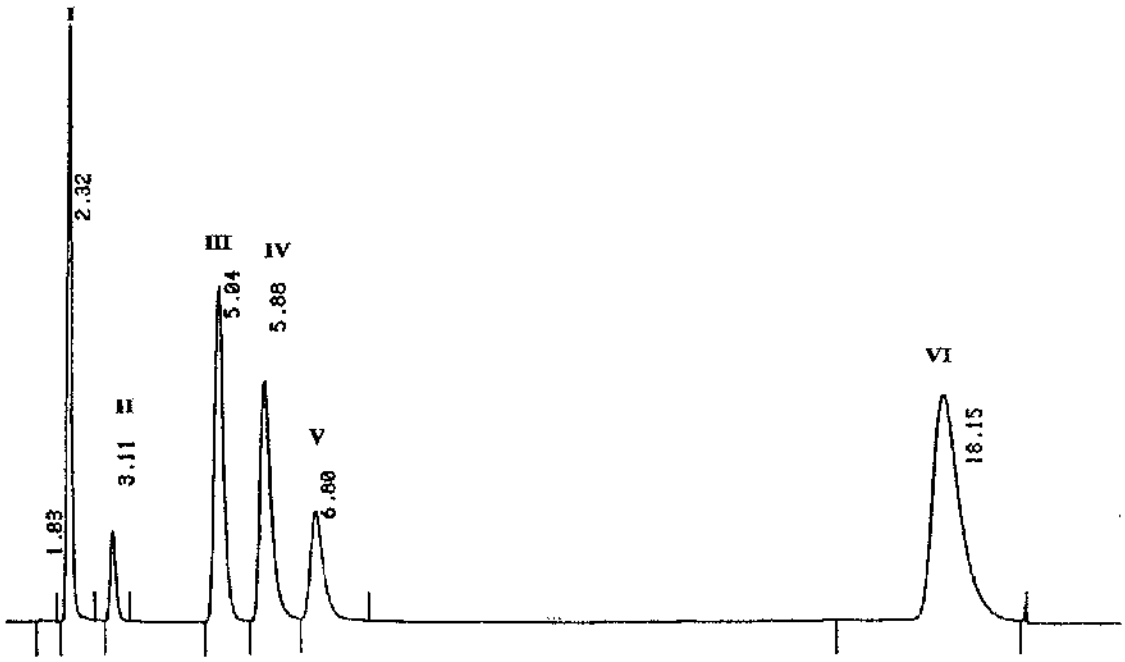
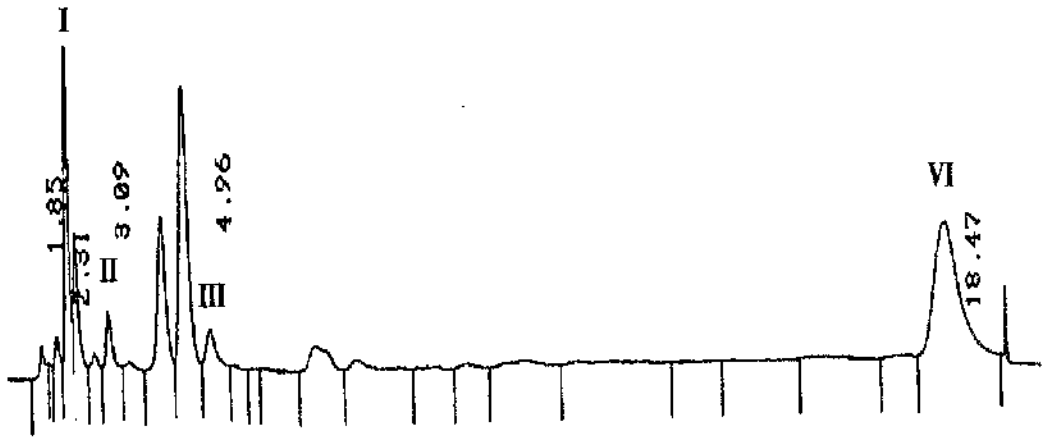


Figure 2.4. Bovine urine sample chromatogram



Compound Identification :-

I	Allantoin
II	Pseudouridine
III	Uric acid
IV	Xanthine
V	Hypoxanthine
VI	Creatinine

2.3.4.3. Peak area response to the addition of compound standard

Responses of peak area to addition of compound standards to a urine sample was assessed over a range of allantoin (0-8 nanomoles), pseudouridine (0-400 picomoles), uric acid (0-2 nanomoles), xanthine (0-2 nanomoles), hypoxanthine (0-2 nanomoles) and creatinine (0-8 nanomoles) additions.

2.3.4.4. Urine diluent

Retention of a compound on a HPLC column is dependent on the pH of the mobile phase. Dolan and Snyder (1989) quote that pH changes of $\pm$ 0.01 can on average result in a $\pm$ 1% change in compound retention times in reversed phase HPLC analytical systems. As the range in urine concentrations are likely to be wide, it is essential that the pH of the urine sample approaches the mobile phase pH. The effects of a range of sample diluents (7.5, 15, 30 and 60 mM $NH_4H_2PO_4$ and 10.0mM Na-HSA) and dilution rates (1:20, 1:50 and 1:100) on urine (n=10) pH were measured.

2.3.4.5. Method precision

Analytical precision was determined by performing five replicate standard and sample injections for five consecutive days.

2.3.5. Analytical methodology

2.3.5.1. Mobile phase preparation

Mobile phase was prepared from 2.022g Na-HSA and 0.8625g $NH_4H_2PO_4$ dissolved in one litre of double deionised water. After the addition of 0.14 mls of TEA, pH of the solution was adjusted to 3.00 using a 10% (v/v) hydrochloric acid solution (HCl).

To avoid potential problems of microbial contamination the mobile phase was passed through a 0.2 micron nylon 66 filter (Anachem, Luton, Bedfordshire, England) into a HPLC mobile phase reservoir. The use of an in-line 0.2 micron nylon membrane filter/degasser assembly (Whatman International Limited, Maidstone, England), connected between the HPLC reservoir and pump, ensured problems of microbial contamination were minimised. Mobile phase was thoroughly degassed under a vigorous stream of helium for five minutes before purging. The flow of helium was reduced to a steady stream during sample analysis.

2.3.5.2. Urine diluent preparation

Diluent for preserved urine samples was prepared as described for the mobile phase without the addition of TEA. Diluent for unpreserved urine samples was prepared by the addition of 2 mls of 10% (v/v) HCl to 488 mls of diluent.

2.3.5.3. Calibration standard preparation

Calibration standard was prepared by dissolving 0.1581g allantoin, 0.1131g creatinine, 0.0475g uric acid (sodium salt), 0.0435g xanthine (sodium salt), 0.0340g hypoxanthine and 0.0122g pseudouridine in two litres of preserved urine diluent. Resulting in a final standard concentration of 0.5 mM allantoin and creatinine, 0.125 mM uric acid, xanthine and hypoxanthine and 25 µM pseudouridine.

2.3.5.4. Sample preparation

A 100 µl aliqot of urine was diluted with 4.9 mls, of the appropriate diluent dispensed from a 5 millilitre Distrivar dispenser (Anachem, Luton, Bedfordshire, England). In the experiment described in chapter 7, 500 µl of preserved urine was diluted with 3.45 mls of diluent. After mixing using a whirlimixer (Fisons Scientific Equipment, Loughborough, Leicestershire, England) samples were passed through a 13mm diposable

syringe filter containing a 0.45 micron polysulfane membrane (Whatman International Limited, Maidstone, England). A 20µl sample volume was injected onto the column.

2.3.5.5. Sample storage

Based on the observations of Rocks (1977), Chen *et al* (1993a) and R.J. Dewhurst (personal communication) urine samples from the experiments described in chapters 5 and 6 were frozen unpreserved at -20°C. The influence of sample freezing at -20°C compared to cold storage at 4°C on urinary PD, pseudouridine and creatinine concentrations was determined for ten randomly collected urine samples. Four replicate analyses were performed for each sample on four consecutive days.

2.4. Results

2.4.1. Method development

During the initial stages of method development problems of microbial contamination of the mobile phase were encountered. Addition of 0.04% (v/v) of sodium azide commonly used to inhibit microbial growth in aqueous mobile phases (Dolan and Snyder, 1989), resulted in inadequate sample separation of allantoin, pseudouridine and uric acid. Filtration of the mobile phase during preparation and before elution through the column resolved problems of microbial contamination. Fitting a C-18 guard column (30mm x 4.6mm I.D.) resulted in a deterioration in peak shape for all compounds which improved after its removal. Further development was conducted with the main column alone.

2.4.1.1. Mobile phase composition

Influence of mobile phase composition on the separation of compound standards is shown in Table 2.1.

Table 2.1. The effect of mobile phase composition on the separation of PDs, pseudouridine and creatinine in urine

$[NH_4H_2PO_4]$ mM	[Na-HSA] mM	[TEA] mM	pH	Effect on chromatography
7.5	5	30	3.2	Uric acid, xanthine and hypoxanthine coeluted
7.5	5	20	3.2	Xanthine and hypoxanthine coeluted
7.5	5	10	3.2	Xanthine and hypoxanthine coeluted
7.5	5	5	3.2	Xanthine and hypoxanthine coeluted
15.0	5	5	3.2	Xanthine and hypoxanthine co-eluted
15.0	-	5	3.2	Poor separation of the allantoin and uric acid peaks
7.5	-	30	3.2	The uric acid and allantoin peaks partially co-elute
7.5	-	20	3.2	Poor separation of the allantoin and uric acid peaks
7.5	-	10	3.2	Incomplete separation of the allantoin and uric acid peaks
7.5	-	5	3.2	Allantoin and uric acid peak separated by only 0.5 min
7.5	1	20	3.2	Uric acid, xanthine and hypoxanthine coelute
7.5	20	1	3.2	Compounds well separated in standards and samples
7.5	20	1	3.0	Improved sample allantoin and pseudouridine peak resolution

2.4.1.2. Compound capacity factors

Under various separation conditions a compound elutes from the column with a particular retention time. The affinity of the stationary phase for a compound is independent of flow rate through the column which determines retention times, and is best described by the term capacity factor. Compound capacity factors for a HPLC method would ideally fall between 1 and 10, although 0.5-20 is tolerated (Dolan and Snyder, 1989). Compound capacity factors are presented in Table 2.2. and are calculated as:-

$$k' = (t_r - t_o) / t_o$$

where:-

k' is the compound capacity factor

t_o is the column dead time equal to the retention time of the mobile phase /min

t_r is the retention time of the compound /min

Table 2.2. Compound capacity factors

Compound	Capacity factor (± SD)
Allantoin	0.311 (±0.004)
Pseudouridine	0.868 (±0.01)
Uric acid	2.185 (±0.04)
Xanthine	2.749 (±0.04)
Hypoxanthine	3.405 (±0.08)
Creatinine	12.328 (±0.02)

2.4.1.3. Peak area response to compound addition

Peak area responses to the addition of all compounds to urine was found to be highly linear. Responses for allantoin and creatinine, uric acid, xanthine and hypoxanthine and pseudouridine are shown in Figures 2.5., 2.6. and 2.7., respectively. The range of

compound additions, regression coefficients of the peak area responses and compound recoveries are summarised in Table 2.3. The response factor indicates the relative increase of peak area units to the addition of one nanomole of compound (PAUs/nm). Compound recoveries were determined by comparison of the standard and sample response factors.

Figure 2.5. Peak area response to the addition of allantoin to bovine urine

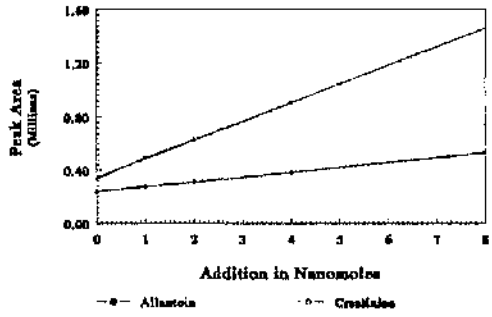


Figure 2.6. Peak area response to the addition of creatinine to bovine urine

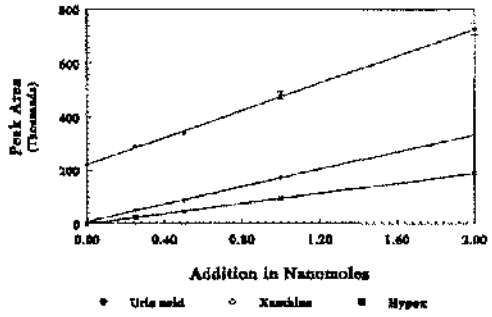
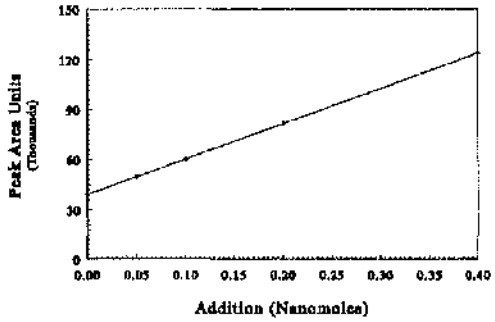


Figure 2.7. Peak area response to the addition of pseudouridine to bovine urine



Each point indicates the mean of 25 measurements
Error bars indicate the standard error of each measurement (where detected)

Table 2.3. Peak area response regression equations and compound recoveries

Compound	Addition range (nanomoles)	Response factor (PAUs/nm)		n	r^2	P	Recovery %
		Standard	Urine				
Allantoin	0-8	39664	36785	125	0.99	<0.001	92.7
Creatinine	0-8	152877	139534	125	1.00	<0.001	91.3
Hypoxanthine	0-2	226631	212563	125	1.00	<0.001	93.8
Pseudouridine	0-0.4	98604	95543	125	1.00	<0.001	96.9
Uric acid	0-2	231312	233883	125	0.99	<0.001	101.1
Xanthine	0-2	161899	162556	125	1.00	<0.001	100.4

2.4.2. Sample diluent

In order to match sample pH to that of the mobile phase as injected onto the column, several sample diluents were tested on ten randomly collected urine samples. Mean urine pH of 8.20 was reduced to 6.19 after a 1:50 dilution with 7.5 mM $NH_4H_2PO_4$ at pH 3.0. Further acidification was required. Addition of 0.2 mls of 10% (v/v) HCl ml^{-1} urine reduced mean urine pH to 3.18. As the mobile phase contained only 7.5 mM $NH_4H_2PO_4$ at pH 3.00, attempts to reduce sample pH still further were investigated. The influence of $NH_4H_2PO_4$ concentration (pH 3 0) on mean urine pH of samples diluted 1:50 is shown in Table 2.4. The effect of dilution rate with a 7.5 mM $NH_4H_2PO_4$ at pH 3.0 on mean urine pH is shown in Table 2.5.

Table 2.4. Influence of $NH_4H_2PO_4$ concentration on mean urine sample pH

$[NH_4H_2PO_4]$ mM	0	7.5	15	30	60
pH	3.18	3.16	3.13	3.07	3.03

Table 2.5. Effect of dilution rate on mean urine sample pH

Dilution rate	0	1:20	1:50	1:100
pH	3.18	3.17	3.08	3.03

2.4.3. Method precision

Method precision was tested by performing five replicate sample and standard analyses for five consecutive days. Between-day and mean within-day (n=5) compound peak area coefficients of variation (CV%) are shown in Table 2.6.

Table 2.6. Between-day and mean within-day peak area coefficients of variation

Compound	Between-day (CV%)		Within-day (CV%)	
	Urine	Standard	Urine	Standard
Allantoin	0.45	2.32	0.52	0.40
Creatinine	1.38	2.56	1.59	0.39
Uric acid	1.87	0.15	2.75	0.56
Xanthine	6.92	0.38	2.70	0.39
Hypoxanthine	ND	2.03	ND	0.80
Pseudouridine	2.10	2.86	3.31	0.42

ND:- not detected

2.4.4. Determination of urinary PD, pseudouridine and creatinine concentrations

Samples collected from each experiment were analysed randomly to minimise analytical bias. A calibration standard was injected at the begining of each analysis and after every ten samples. Peak area within-day variations for each compound standard were found to be asystematic and therefore a mean standard response factor for each compound was used to calibrate each sample batch. Sample concentrations were calculated by comparing peak areas to standard response factor (SRF) incorporating a recovery factor (RCF) calculated as 1/compound recovery (refer to section 2.4.2.) sample dilution factor (SDF) and a sample storage factor (SSF) which accounted for the effects of -20^{0}C storage on uric acid and pseudouridine (refer to section 2.4.7.).

$$\textbf{[Sample compound] (mM)} = \frac{\text{Sample compound peak area}}{\text{Mean compound RF}} * \text{RCF} * \text{SDF} * \text{SSF}$$

2.4.5. Column regeneration

Column regeneration was performed after a hundred sample injections. Regeneration involved sequential column rinsing with double-deionsed water, 50% (v/v) aqueous acetonitrile and 100% acetonitrile. The process was reversed prior to the mobile phase being eluted through the column. Chromatography was restored after a long conditioning period of approximately 24 hours.

2.4.6. Detection limits

Method detection limits were determined at a signal to noise ratio of 3:1, which corresponded to concentrations of 3 μm creatinine, 1.1 μm allantoin, 1.0 μm xanthine, hypoxanthine and uric acid and 0.4 μm pseudouridine.

2.4.7. Sample storage

The effect of freezing on urinary PD, pseudouridine and creatinine concentrations was tested by comparing peak areas of urine samples stored at 4^0C with those frozen at -20^0C for 48 hours. Effects of freezing on allantoin, creatinine and uric acid content of urine are shown in Figures 2.8., 2.9. and 2.10., respectively. Regression equations derived relating peak areas in cold stored (x) and frozen samples (y) are shown in Table 2.7. Incomplete recoveries of uric acid and pseudouridine were accounted for in determinations performed with previously frozen samples (refer to section 2.4.4).

Table 2.7. Regression equations relating peak areas in cold stored (x) and frozen samples (y)

Compound	Regression equation	r^2	No.	Prob.
Allantoin	y = 0.99 x +3722	1.00	40	P< 0.001
Creatinine	y = 1.00 x + 1081	1.00	40	P< 0.001
Uric acid	y = 0.92 x + 11971	0.96	40	P< 0.001
Xanthine	y = 1.00 x + 328	0.83	40	P< 0.001
Pseudouridine	y = 0.93 x - 4267	0.83	40	P< 0.001

Figure 2.8. Effect of freezing on urinary allantoin

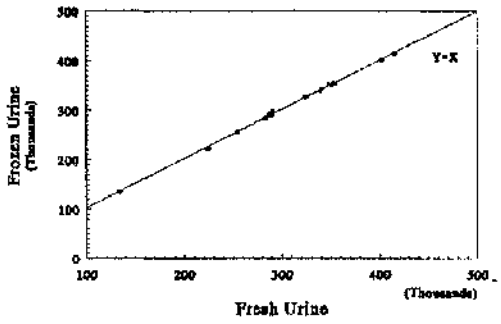


Figure 2.9. Effect of freezing on urinary creatinine

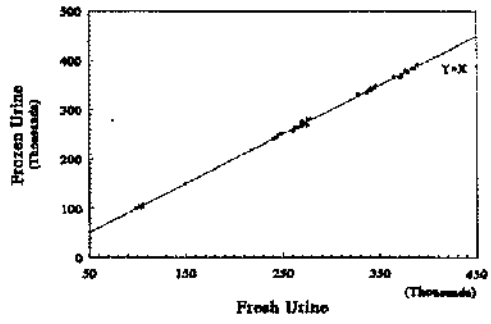
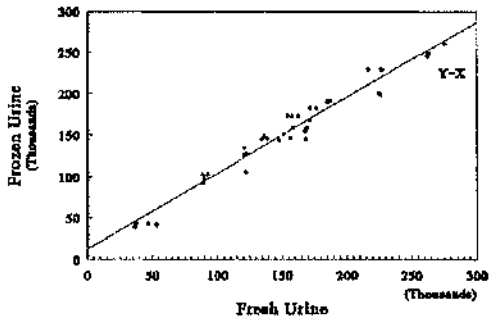


Figure 2.10. Effect of freezing on urinary uric acid



For Figures **2.8., 2.9.** and **2.10:-**
Each point is the mean of four replicate measurements
Fresh urine was stored at 4^0C, frozen urine stored at -20^0C for 48 hours
The line y=x, is plotted

2.5. Discussion

Biological fluids contain compounds with a similar polarity and UV absorbance as allantoin. In common with problems reported with other HPLC techniques, separation of allantoin was difficult to achieve. Allantoin retention times were independent of changes in mobile phase composition confirming the observations of Tiermeyer and Giesecke (1982) and Diez *et al* (1992). The allantoin capacity factor of 0.31 for this method compares favorably to 0.24 reported by Tiermeyer and Giesecke (1982), but falls outside the acceptable range of 0.5-20 (Dolan and Synder, 1989) and is lower than 0.9-1.2 reported by Diez *et al* (1992). Despite a lower capacity factor the separation of the allantoin in this method is an improvement over most of the methods documented in section 2.2.

Separation of allantoin appears to be a function of the stationary phase. Octadecyl (C-18) bonded silica columns differ with respect to the number of alkyl chains attached to the silica matrix (carbon loading) and the efficiency of chain bonding during manufacture (end-capping). Any silanol groups (Si-OH) on the silica matrix which are not attached to an alkyl group are free to interact with polar compounds while interactions between the polar analytes and the alkyl chains would not be expected to be significant. Hicks *et al* (1993) evaluated three C-18 columns to separate allantoin, uric acid and parabanic acid. Compound retention times were found to be column dependent. Assessment of relative Si-OH numbers from methyl red binding indicated that stationary phases which resulted in better allantoin separation correspondingly bound more dye. These results suggest that the presence of free Si-OH on the stationary phase is critical for resolution of polar compounds. Column selection would appear to be critical in achieving satisfactory allantoin separation.

The presence of silanol groups on the stationary phase were assumed to be responsible for the strong retention of creatinine observed when the mobile phase contained $NH_4H_2PO_4$ and Na-HSA. Creatinine retention times were reduced in direct proportion to TEA (which is known to preferentially occupy the finite silanol groups on

the column, Dolan and Synder, 1989) concentration in the mobile phase, which presumably reduces Si-OH-creatinine interactions.

Optimum chromatography was achieved by the inclusion of an ion-pairing agent (Na-HSA) and TEA to the mobile phase. Inclusion of Na-HSA ion-pairing reagent improved the separation of allantoin and uric acid confirming the observations of Lux *et al* (1993). Inclusion of TEA into the mobile phase reduced creatinine retention and improved peak shape for all compounds. It also reduced uric acid retention times resulting in co-elution with allantoin. The effects of TEA in some respects were antagonistic to those of Na-HSA and therefore fine tuning of the mobile phase composition was needed to achieve the required chromatography.

Peak area responses to the addition of purine derivatives, pseudouridine and creatinine were all found to be highly linear, indicating the suitability of the method to analyse a wide range of experimental samples.

Method precision in the determinations of all compounds was comparable to that reported by Tiermeyer and Giesecke (1982), Diez *et al* (1992) and Resines *et al* (1992) and was a distinct improvement on the methods described by Balcells *et al* (1992) and Lux *et al* (1992).

Recovery of allantoin of 92.7% while acceptable, is lower than reported recoveries of 98% (Tiermeyer and Giesecke, 1982), 97.9% (Diez *et al*, 1992 and Resines *et al*, 1992) and 96.7% (Balcells *et al*, 1992). In common with allantoin, the recovery of creatinine of 91.3% was acceptable but lower than the 99.1% found by Diez *et al* (1992) and Resines *et al* (1992). With the exception of hypoxanthine, the recovery of the other compounds approached or slightly exceeded 100%. The preparation of standard solutions used to determine compound recoveries was scrutinised and found to be correct. Consequently, the reasons for incomplete recoveries of allantoin and creatinine particularly, remain unclear.

Compound retention times were extremely consistent and is presumably a reflection of urine sample pH approaching that of the mobile phase. Alternative methods have diluted the sample with water (Tiermeyer and Giesecke, 1982; Diez *et al*, 1992 and Resine *et al*, 1992) or buffer (Balcells *et al*, 1992) with little account of variations in

sample pH. Considering the difficulty of separating allantoin, highly reproducible retention times would appear to be a clear advantage of this method.

The method described relies on the flow of mobile phase being reversed (backflushed) after creatinine has been eluted from the column. The process of backflushing removes longer eluting compounds off the column, but also decreases the accumulation of contaminants on the stationary phase. Consequently, column regeneration is required after a hundred samples have been analysed compared to fifty reported by Tiermeyer and Giesecke (1982), which would inevitably prolong column lifetime.

Effects of sample storage indicated that urinary allantoin, creatinine and xanthine concentrations remained unaffected after storage at -20^{0}C agreeing with the observations of Chen *et al* (1993a). Recovery of uric acid and pseudouridine after freezing was 92 and 93%, respectively compared to 4^{0}C cold storage, tentatively suggesting both compounds are lost through precipitation out of solution during the freezing and thawing processes. Attempts to recover uric acid and pseudouridine were not investigated further, while a freezing correction factor was incorporated into the calculation of uric acid and pseudouridine concentrations in previously frozen samples.

Chapter Three

Determination of allantoin in bovine milk by High Performance Liquid Chromatography

Summary

Current methods of measuring purine and pyrimidine derivatives in bovine milk are reviewed. An HPLC method developed specifically for the determination of allantoin in bovine milk using an amino column and aqueous acetonitrile mobile phase is described. Method precision is comparable to existing techniques, while retention and separation of allantoin is improved.

3.1. Methodology

HPLC Apparatus:-	Isocratic pump, autosampler fitted with 60µl loop UV detector and integrator
Mobile Phase:-	90% acetonitrile:water (v/v)
Stationary Phase:-	5 micron Spherisorb 250mm x 4.6 mm amino column

Chromatographic Conditions:-

Flow rate	2mls / min
Detection wavlength	214 nm
Temperature	20°C
Analysis time	10 minutes

Calibration standard: 0.1 mM allantoin

Sample preparation: 500µl of sample diluted in 4.5 mls of acetonitrile mixed, filtered and 60µl injected onto the column

3.2. Introduction

The presence of PDs in bovine milk has been reported as early as 1959 by Deutsch and Mattsson, using ion and paper chromatography. More recent reports of PDs in bovine milk have used a colorimetric technique or HPLC. Kirchgessner and Kreuzer (1985) and Kirchgessner and Windisch (1989) reported milk allantoin concentrations using a colorimetric method based on the method of Christman *et al* (1944), the principle of which is described in section 2.2. Serious criticisms of this technique exist due to problems of interfering compounds (refer to section 2.2). Consequently, HPLC methodologies have been developed to determine PDs in bovine milk.

Tiemeyer *et al* (1984) described an isocratic HPLC method for the determination of PDs in bovine milk using a phosphate buffer mobile phase eluting through a µ-Bondapak C-18 column. Measurement of allantoin required a separate analysis using the method of Tiermeyer and Giesecke, (1982). More recently, Rosskopf *et al* (1991) outlined a HPLC method for measuring PDs in bovine milk. Chromatography was achieved using a phosphate buffer eluting through a column (500 x 4.6 mm) containing ODS II C-18 packing. Determination of allantoin required a separate analysis, using a modified phosphate buffer and a change in the detection wavelength from 254 to 214 nanometers. Reports in the literature indicate measurement of milk PDs would require two analyses. Allantoin is quantitatively the most important PD excreted in the milk (Giesecke *et al*, 1994 and Susmel *et al*, 1995). Furthermore, Tiermeyer and Giesecke (1982) and Rosskopf *et al* (1991), have suggested milk allantoin excretion alone, has potential as an index of MCP. Therefore in the present study only measurement of allantoin was considered.

Dennis *et al* (1987) reported a sensitive and accurate HPLC method for the determination of allantoin in comfrey roots which utilised a 70% acetonitrile:water (v/v)

mobile phase eluting through an amino column. Amino columns are similar to C-18 columns with the exception that amino groups are attached to a silica matrix instead of alykl groups.

Initially, development of a technique to quantify allantoin in milk was based on the method described in chapter 2. Changes in mobile phase composition and pH did not allow satisfactory separation of allantoin from other compounds present in milk. Use of the method of Tiermeyer and Giesecke (1982) also lead to unsatisfactory retention and separation of allantoin. Attempts to establish the method of Rosskopf *et al* (1991) by coupling two 250mm x 4.6 mm ODS II columns in series, resulted in excessive pressure (above 4000 PSI) across the column. Long term use would inevitably lead to pump failure, column packing voiding and peak tailing (K. McIssac, personal communication). High column pressures could have been alleviated by reducing flow rate, but would result in unacceptably long analysis times. Further considerations of the intensive sample preparation procedures and criticisms of C-18 columns (refer to sections 2.2. and 2.5.) initiated development of an alternative method to measure allantoin in milk. Initial development proceeded as outlined by Dennis *et al* (1987).

3.3. Materials and methods

3.3.1. HPLC apparatus

HPLC isocratic pump, detector, integrator and auto-sampler are as described in section 2.3.1. Changing the injection loop to 60 µl enabled more sample to be injected onto the column, overcoming potential detection problems. Chromatography was achieved using a 5 micron Spherisorb amino normal phase HPLC column measuring 250mm x 4.6 mm internal diameter preceeded by a normal phase guard column, internal diameter, 10mm x 4.6 mm (both supplied by Phase Separations Limited, Deeside, Clwyd, Wales).

3.3.2. Reagents

Allantoin assayed above 98% was supplied by Phase Separations Limited, Deeside, Clwyd, Wales. Far UV HPLC grade acetonitrile was supplied by Rathburn Chemicals Limited, Walkerburn, Peeblesshire, Scotland. Double-deionised water was obtained from the same source as described in section 2.3.3.

3.3.3. Method development

Initially a 70% acetonitrile:water (v/v) mobile phase as outlined by Dennis *et al*, (1987) was tested. Separation of allantoin was not completely satisfactory. Therefore a range of 30-100% (v/v) aqueous acetonitrile mobile phases were evaluated.

3.3.3.1. Compound identification

Allantoin was initially identified in milk by comparison of peak retention times with an allantoin standard. Further confirmation was provided by spiking milk with allantoin standard and noting the peak area increase. Peak purity was not assessed.

3.3.3.2. Chromatographic conditions

Chromatography was achieved under isocratic conditions at a flow rate of 2.0 ml / min. Eluted mobile phase was monitored at 214 nms, with analysis completed within 10 minutes. Allantoin eluted from the column within 4 minutes. Mobile phase flow through the column was reversed (backflushed) for 4 minutes to remove longer eluting compounds. Mobile phase flow was restored for 2 minutes before the next sample injection.

3.3.3.3 Peak area response to the addition of allantoin standard

Peak area responses to allantoin additions in standard solutions and milk samples were assessed over 120-480 and 600-3000 picomole ranges.

3.3.3.4. Method precision

Analytical precision was determined by performing five replicate sample and standard injections for three consecutive days.

3.3.4. Analytical methodology

3.3.4.1. Mobile phase preparation

Mobile phase was prepared from 900 mls of acetonitrile made upto one litre with double-deionised water. The solution was thoroughly mixed and transfered to a HPLC reservoir. After 10 minutes degassing under a vigorous stream of helium the mobile phase was pumped through the column and the flow of helium reduced to a steady stream throughout analysis.

3.3.4.2. Allantoin standard preparation

Allantoin calibration standard was prepared by dissolving 0.0158g of allantoin in one litre of 90% acetonitrile:water (v/v) resulting in a final allantoin concentration of 0.1mM.

3.3.4.3. Sample preparation

Previously frozen milk samples were homogenised using a T1500 homogeniser (Ystral, Dottingen, Germany). Fresh milk samples were thoroughly shaken. A 500 µl

milk aliqot was diluted with 4.5 mls of acetonitrile dispensed from a 5 millilitre Distrivar dispenser (Anachem, Luton, Bedfordshire, England) and mixed using a whirlimixer (Fisons Scientific Equipment, Loughborough, Leicestershire, England). A sub-sample was passed through a 13mm disposable syringe filter containing a 0.45 micron PTFE membrane (HPLC Technology, Macclesfield, Cheshire, England) into a HPLC vial. 60µl of sample was injected onto the column. Giesecke *et al* (1994) indicated little evidence to support existence of *uricase* activity within the mammary gland. Consequently overestimation of allantoin arising from the oxidation of milk uric acid was considered to be negligible. Consequently, sample temperatures were not maintained below 4°C during preparation. No internal standard was used.

3.3.4.4. Sample storage

Samples collected from experiments described in chapters 6 and 7 were stored frozen at -20°C. Once thawed, samples were prepared for analysis as described in section 3.3.4.3. Effects of freezing on milk allantoin content were not investigated.

3.4. Results

3.4.1. Method development

During the initial stages of development the main column was used without a guard column. As the number of samples analysed increased, peak shape was found to deteriorate. Closer inspection revealed that the packing material had become contaminated and the main column had to be replaced. Fitting of the guard column preserved the lifetime of the main column. Each guard column was replaced after 100 injections. Representative standard and milk sample chromatograms are shown in Figures 3.1 and 3.2, respectively.

Figure 3.1. Allantoin calibration standard chromatogram

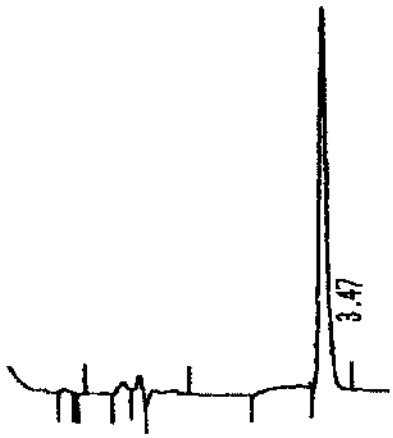
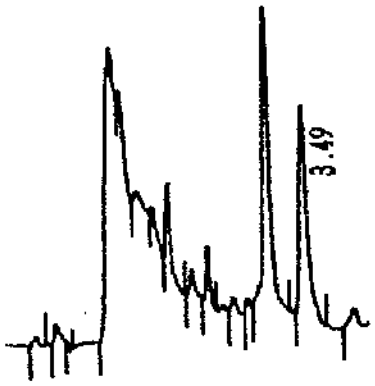


Figure 3.2. Bovine milk sample chromatogram



The effects of mobile phase composition on allantoin retention are shown in Table 3.1. Increasing the proportion of acetonitrile in the mobile phase resulted in increases in allantoin retention and consequently allantoin capacity factor (defined in section 2.4.1.2.). Mobile phase containing 90% acetonitrile:water (v/v) resulted in the best compromise between allantoin retention and analytical run time.

Table 3.1. The influence of mobile phase composition on allantoin retention

Mobile phase composition % acetonitrile:water (v/v)	Retention time (minutes)	Capacity factor
30	1.55	0.57
50	1.87	0.89
70	1.98	1.0
80	2.73	1.76
90	3.49	2.53
95	4.34	3.38

3.4.2. Peak area response to the addition of allantoin

Peak area responses to allantoin addition were found to be highly linear for both standards and milk samples over 120-480 and 600-3000 picomole ranges, as described in Figures 3.3. and 3.4., respectively. Relationships between allantoin addition and peak area units, standard and milk allantoin response factors and allantoin recoveries are shown in Table 3.2.

Table 3.2. Peak area response to the addition of allantoin

Allantoin addition (picomoles)	n	r^2		Response factor PAUs/picomole		Recovery %
		Standard	Sample	Standard	Sample	Sample
120-480	20	1.0	0.96	49.5	48.1	97.2
600-3000	20	1.0	0.99	44.2	44.1	99.6

n refers to the total number of injections performed

The response factor is defined as the increase in peak area units (PAUs)/picomole allantoin. Recovery of allantoin added to milk was calculated from comparison of the standard and sample response factors.

Figure 3.3. Peak area response to 120 -480 picomole allantoin addition to bovine milk

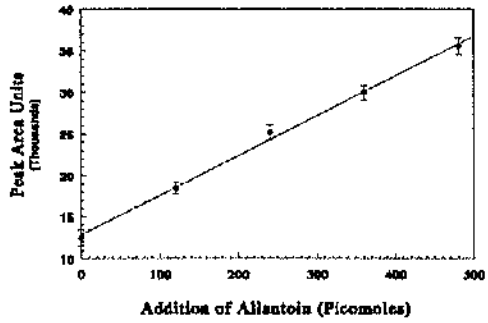
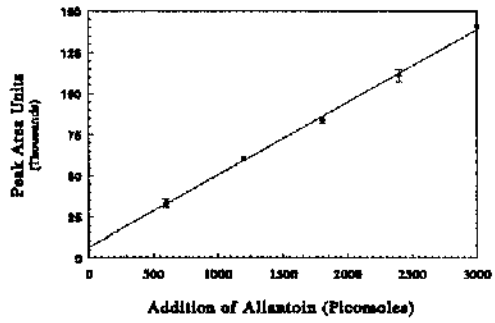


Figure 3.4. Peak area response to 600 -3000 picomole allantoin addition to bovine milk



For Figures **3.3.** and **3.4.**:-
Each point represents the mean of four measurements
Error bars indicate standard deviation

3.4.3. Method precision

Method precision was assessed by performing five replicate injections of a calibration standard and milk sample within a day, for three consecutive days. Standard and sample allantoin peak area between-day and within-day CV % are shown in Table 3.3.

Table 3.3. Between-day and within-day peak area variability

Within-day variability (CV %)		Between-day variability (CV %)	
Standard	Sample	Standard	Sample
2.51	2.15	3.79	1.66

3.4.4. Determination of milk allantoin concentration

Each batch of milk samples were analysed randomly to minimise analytical bias. An authentic standard was injected at the begining of each analytical run and after every ten milk samples. Within-day variability in the standard response factor (mean CV of 2.5%) was found to be asystematic. Therefore it was appropriate to calculate the daily mean standard response factor and compare this to each sample peak to calculate the final milk allantoin concentration, accounting for sample dilutions during preparation for analysis. Recovery of allantoin from milk was assumed to be 100%.

3.4.5. Column regeneration

Column regeneratioon was performed after two hundred injections, by rinsing the column with double-deionised water followed by 20 mM acetic acid for thirty minutes, respectively. The process was reversed before the mobile phase was passed through the column. Chromatography was restored several hours later.

3.4.6. Detection limits

Detection limits were determined at a signal:noise ratio of 3:1, corresponding to an allantoin concentration of 1 μM.

3.5. Discussion

The method developed represents an alternative to previous published methods to determine allantoin in bovine milk, with several advantages. Allantoin was well separated from other compounds present in milk while analysis was completed within 10 minutes. Method precision is comparable to published methods utilising C-18 stationary phase. Dennis *et al* (1987) and Rosskopf *et al* (1991) reported within-day sample variabilities of less than 1%. Within-day sample variation based on five injections was

found to be slightly higher with this method of 2.15%. Variability between-days was larger than within-day variations for the calibration standard and smaller for the milk sample tested. Differences between days were not reported in the published methods. In order to overcome within and between-day variability, milk samples were analysed randomly avoiding analytical bias.

Recovery of allantoin was found to be 97.2 and 99.6% over 120-480 and 600-3000 picomole ranges, respectively. This is a substantial improvement over the 91.9% reported by Rosskopf *et al* (1991) and compares well with the 99.6% reported by Tiermeyer *et al* (1984). The use of allopurinol as an internal standard as suggested by Balcells *et al* (1992) was investigated but problems with coellution with unidentified compounds in the milk samples prevented its use. Almost complete recovery of allantoin in milk would tend to suggest that its use is not essential, particularly as sample preparation does not involve extraction of allantoin. Linearity of peak area responses to allantoin over an extremely wide range indicates that the current method would be suitable for allantoin determinations over a range of concentrations.

Utilisation of an amino stationary phase and a non-polar mobile phase gives similar precision as using non-polar C-18 stationary phase and a polar mobile phase. However, using an amino column for the analysis of allantoin does result in increased retention of allantoin compared to C-18 variants. Considering the criticisms raised in sections 2.2. and 2.5., regarding poor retention and separation of allantoin achieved using C-18 stationary phases this represents a significant improvement. Allantoin capacity factor of 2.53 reported for this method, falls within the ideal 1-10 range (Dolan and Snyder, 1989), and could potentially be improved further by increasing the proportion of acetonitrile in the mobile phase. In a comparison of C-18 columns, Hicks *et al* (1993) noted an Ultro-pak TSK ODS-120T C-18 column gave the best retention of allantoin, which assuming a column dead volume of 2.0 mls, results in an allantoin capacity factor of 0.65. Comparison of allantoin capacity factors indicates the advantages of using an amino stationary phase in the determination of allantoin. Other significant advantages exist over techniques described by Tiermeyer and Giesecke (1982) and Rosskopf *et al* (1991). Mobile phase and sample preparation in this method are less

intensive. Current methods require longer mobile phase preparation and complicated sample defatting and deproteinisation procedures prior to analysis. In contrast, sample preparation reported for this method involves simple dilution and filtration. Furthermore, adoption of column backflushing reduces problems associated with stationary phase contamination, prolonging column lifetime compared to published methods.

Chapter Four

General materials and methods

4.1. Introduction

Materials and methods common to more than one experiment are documented, including feedstuff evaluation, milk analysis and methods of urine collection. In addition methods for ovine rumen fluid VFA and ammonia determinations and for dairy cow rate of passage studies (chapter 7, only) are described.

4.2. Feedstuff evaluation

Feedstuffs submitted to the Analytical Services Unit, Auchincruive, were analysed as either fresh samples or after freezing at -20^{0}C. Dry matter (DM) content was determined after overnight drying at 100^{0}C in a fan assisted oven. All subsequent measurements were corrected to a 100^{0}C DM basis. Forage samples were milled through a 2 mm screen (Crypto-Peerless Ltd, London) ensuring sample uniformity prior to analysis.

4.2.1. Organic matter *in-vitro* digestibilty

In-vitro organic matter digestibility (OMD) determinations were performed on silage samples from the experiment described in chapter 6, based on the method outlined by Tilley and Terry (1963) incorporating modifications of Alexander (1969). Samples were anaerobically incubated in filtered ovine rumen liquor at 38.5^{0}C for 24 hours. The incubation pH was adjusted to 6.9 by the addition of HCl and continued for another 24 hours. After a final 48 hour digestion with aqueous pepsin solution at pH 1.2, the residue was filtered, dried at 100^{0}C, weighed, ashed at 480^{0}C and re-weighed. The use of

appropriate control incubations of rumen liquor enabled the residual OM of the sample to be measured and hence OMD could be calculated.

4.2.2. Determination of neutral cellulase plus gamanase digestibility

Neutral cellulase and gamanase digestibilities (NCGD) were performed for all concentrate feedstuffs according to the procedure outlined by MAFF (1993). Samples were passed through a 1mm mesh followed by fat extraction using petroleum spirit. Fat-free material was extracted with neutral detergent solution to remove the soluble cell contents, while the starch was removed by conversion to sugars by the action of α - amylase. The insoluble material was incubated with a buffered solution of cellulase and gamanase and the organic fraction of the remaining insoluble matter designated NCGD.

4.2.3. Determination of crude protein

A Kjeldahl digestion was performed on 0.25 g of dried sample using 7.5 mls of acid digestion solution, (containing 40g selenium dioxide dissolved in two litres of 95% (v/v) sulphuric acid) on a Tecator 1006 heating block (Tecator Ltd, Bristol). Three mls of hydrogen peroxide was used as an oxidant and the resultant solution made up to 75mls. Nitrogen content was measured using the indophenol blue method, with absorbance measured at 584 nm and the N content calculated from authentic standards. Protein content was calculated as 6.25 x N.

4.2.4. Determination of acid hydrolysis ether extract

Samples allocated for acid hydrolysis ether extract (AH-EE) were dried at 60°C overnight, with subsequent determinations corrected for 100°C DM. After extraction with petroleum ether the residue was heated with HCl. After cooling the solution was filtered. The residue was washed, dried and subjected to a second petroleum ether extraction, with the solvent being removed by distillation and the residue dried and

weighed. A more detailed description of the procedure is reported by MAFF (1993).

4.2.5. Determination of starch

Following the extraction of soluble sugars with 40% (v/v) ethanol, starch was gelatinised and incubated with amyloglucosidase. The production of glucose was determined spectrochemically using the glucose oxidase procedure (Feedstuffs Evaluation Unit, 1981).

4.2.6. Electrometric titration

Silage samples collected from the experiment described in chapter 8 were submitted for routine analysis at SAC, Aberdeen. Analysis primarily based on the method of Moisio and Heikonen (1989) involved the automatic titration of juice squeezed from the silage to pH 2.0 with HCl, followed by incremental titration with NaOH to pH 12.0. Lactic acid, acetic plus butyric acids (VFA), sugars, soluble and ammonia N were predicted from the buffering capacities measured over segments of the titration curve.

4.2.7. Prediction of metabolisable energy

Metabolisable energy (ME) of silage fed in the experiment described in chapter 6 was predicted from the OMD in the dry matter (DOMD), derived from the *in-vitro* organic matter digestibility (IVOMD) measurements (refer to 4.2.1.). Silage ME was calculated as 0.16 x DOMD (Thomas and Chamberlain, 1982). In contrast, sillage fed in the experiment described in chapter 8, was analysed by near infra-red spectroscopy (NIRS) allowing an accuate prediction of silage true digestibility. Predictions are based on a calibration dataset (n=180) which relates NIR spectra of dried milled silage samples with measured *in-vivo* silage digestibility (N.W. Offer, personal communication). Silage ME was calculated as 0.16 x true DOMD. Further analysis by electrometric titration enabled silage volatiles to be predicted.

For concentrate feeds, dry matter NCGD and AHEE expressed as g/100g were used to predict concentrate ME according to the E3 equation (Thomas *et al*, 1988), where:-

$$ME_{(E3)} = NCGD_{(g/100g)} \times 0.14 + AH\text{-}EE_{(g/100g)} \times 0.25$$

4.3. Milk analysis

Samples were submitted to the Dairy Technology Department, SAC, Auchincruive for milk fat, protein, lactose and urea nitrogen determinations.

4.3.1. Determination of milk fat, protein and lactose

Milk samples for milk fat, protein and lactose measurements were preserved with one Lactab (potassium dichromate/sodium chloride tablet; supplied by Thompson and Capper Ltd, Runcorn, Cheshire) and stored at 4°C. Analysis was performed using the Milko-Scan 203 analyser (Foss Electric, Denmark). The near infra-red detection of milk constituents was calibrated using milk samples collected from SAC Crichton Royal Farm, Dumfries for which reference measurements had previously been made. Estimation of crude protein was based on the assumption that protein:NPN ratio was constant.

4.3.2. Determination of milk urea nitrogen

Unpreserved milk samples were bulked for each sampling day according to yield and stored at 4°C. Milk urea nitrogen was determined using a sigma test kit No. 640, following the procedure outlined for blood serum measurements.

4.4. Rumen liquor analysis

Rumen liqour was collected from suffolk wethers fitted with rumen fistulas.

Rumen contents were collected using a suction pump and flexible polythene tubing as outlined by Alexander and McGowan (1969). Aliqots (8 ml) were transfered into centrifuge tubes. Following the addition of 2 mls of preservative 25% (w/w) metaphosphoric acid, the tubes were stoppered, shaken and centrifuged for 10 minutes at 3000 rpm at a temperature of 4°C. The supernatant was transfered to 1.5 ml Ependorf tubes and stored at -20°C.

4.4.1. Determination of volatile fatty acids

After defrosting, 2.5 mls of rumen liquor was transfered into a 10 ml centrifuge tube. After the addition of 1.1 mls of 1M NaOH (BDH, Ltd, Poole), 0.5 mls of pivalic acid (0.22g dissolved in 100 mls of distilled water) internal standard and 2.0 mls of oxalic acid (4.725g dissolved in 500 mls of distilled water) samples were centrifuged at 4°C and 3000 rpm for 10 minutes. 1µl of supernatant was injected onto a glass gas chromatography column (2mm x 2mm I.D. packed with 4% Carbowax on Carbopack B-DA 80/120 mesh (Supelchem UK Ltd, Essex). Determinations were performed isothermally at 175°C using N_2 as the carrier gas at a flow rate of 24 ml/min. This process was repeated for a calibration standard containing 50mM acetic, 20mM propionic, 20mM butyric, 2mM isobutyric, 2mM isovaleric and 2mM valeric acids (all supplied by BDH, Ltd, Poole). VFA concentrations were calculated by comparison with of sample and calibration standard peak areas.

4.4.2. Determination of rumen liquor ammonia

Preserved rumen liquor samples were defrosted, diluted 1:10 with distilled water and vortex mixed. Sample volumes (20µl) were transfered into a 96 hole microplate. 80µl of caustic phenol reagent (prepared from dissolving 2.4g NaOH, 20g phenol and 0.1g sodium nitroprusside in 1600 ml of distilled water) was transfered to each well. After thorough mixing and the addition of 200µl of buffer (prepared from 10g NaOH, 7.48g of anhydrous Na_2HPO_4, 63.6g $Na_3PO_4.12H_2O$ and 20 mls of sodium hypochlorite

made up to 4l with distilled water) the plate was incubated at room temperature for 1 hour. Absorbance at 570 nm was read using a Dynatech MR5000 microplate reader (Dynatech Laboratories Ltd, Sussex). Ammonia concentrations were calculated by calibration with ammonium sulphate standard (0-360 mM) solutions.

4.4.3. Determination of *in-sacco* DM degradability

Approximately 4-8g freshweight (depending on substrate type) of dry material was placed into nylon bags of known weight measuring 150mm x 60mm with a pore size of 40μm with an open area of approximately 25% (supplied by Henry Simons, Stockport, Cheshire). Bags were re-weighed and secured to a nylon cord measuring 25cm which was subsequently sheathed with polythene tubing. Sets of bags (between 3 and 9, depending on substrate type) were placed in the rumen with the bag strings attached to the fistula bung. After removal, incubation bags were washed in cold water for approximately ninety minutes using an automatic washing machine (Zanussi model Z915T-program based cold). After drying at 60^0C for 48 hours the bags were re-weighed and the dry matter disappearance calculated. Zero time dry matter losses were assessed in duplicate for each substrate using the washing procedure alone.

4.5. Determination of rumen solid and liquid outflow rates

In the dairy cow experiment described in chapter 7, solid (defined as small particle) and liquid outflow rates were estimated using chromium mordanted hay and Lithium Cobalt EDTA (LiCo-EDTA.$3H_20$) complex markers, respectively. Computer software based on the model of Grovum and Williams (1973) was used to calculate solid and liquid rumen and hindgut outflow rates based on spot faecal sample chromium (Cr) and cobalt (Co) concentrations (mg marker/100g faecal DM).

4.5.1. Preparation of chromium mordanted hay

A 7 kg batch of hay was prepared for chromium mordanting using the method of Uden *et al* (1981). The hay used was chopped, rinsed thoroughly with water to remove small fibre particles and dried at 60°C for 48 hours. The fibre was subsequently mordanted with four volumes of a sodium dichromate solution containing Cr equivalent to 12-14% of the hay weight. After incubation at 60°C for 24 hours the hay was washed in tap water containing ascorbic acid (equivalent to half the hay weight) and left for several hours. Following thorough washing with water, the hay was dried at 60°C. Each cow received 100g of Cr mordanted hay during each experimental period.

4.5.2. Preparation of Lithium Cobalt EDTA complex

Estimates of rumen fluid outflow was assessed using Lithium Cobalt-EDTA (LiCo-EDTA.$3H_20$) complex as a marker. LiCo-EDTA.$3H_20$ was prepared as described by Uden *et al* (1981). An oral drench was prepared by diluting 156.25g LiCo-EDTA.$3H_20$ in 3.125 l of distilled water. Cows received 250 mls of drench representing 12.5g of LiCo-EDTA.$3H_20$, per experimental period.

4.5.3. Spot faecal sampling

Faecal spot samples were collected from each cow during a sampling period at the time of dosing (0), 4, 6, 10, 13, 21, 24, 28, 34, 37, 45, 48, 52, 58, 61, 69, 72, 76, 82, 85, 95, 105 hours post-dosing based on the sampling regimens adopted by Uden *et al* (1981) and Pearson and Lawrence (1992).

4.5.4. Faecal cobalt and chromium determinations

Faecal samples were dried at 60°C for 96 hours prior to milling through an 1mm screen. Chromium (Cr) and Cobalt (Co) determinations were performed using the method of

Stevenson and Clare (1963). Sample organic matter was removed during ashing and the trivalent chromic form is oxidised to the hexavalent form by the action of potassium bromate. Chromium and cobalt concentrations were measured simultaneously using an inductively coupled plasma spectrometer (ICP). A brief outline is presented here.

Reagents:-

10% (w/w) manganese (II) sulphate ($MnSO_4.4H_2O$)

4.5% (w/w) potassium bromate (KBr)

0.45%(w/w) potassium bromate (KBr)

Digestion acid:- prepared by carefully adding 250 mls of orthophosphoric acid to 500 ml of distilled water. After cooling, 250 mls of sulphuric acid was carefully added while constantly stirring followed by 50 mls of 10% $MnSO_4.4H_2O$.

Nitric/Triton solution:- prepared by dissolving 60 mls of 5% (w/w) Triton x in 300mls of water followed by the addition of 60 mls of nitric acid. The solution was made up to 500 mls.

25ppm Chromium and 2ppm Cobalt standard solution:- 5 mls of 1000 ppm Cr solution and 4 mls of 100ppm Co solution were transfered into a 200 ml volumetric flask. After the addition of 24 mls of digestion acid, 6 mls of 4.5% KBr and 40 mls of 0.45% KBr the solution was made up to 200 mls with distilled water.

Procedure:-

1.0g (+0.001g) of dried milled sample was placed into a 50 ml beaker and covered with a watchglass, placed in furnace and ashed at 480°C overnight. Once cool, the ash was moistened with a few drops of water. The ashed sample was heated with 12 mls of digestion acid, to which 3 mls of 4.5% KBr was added. Once the production of bromine ceased, the solution was cooled for 10 minutes followed by the addition of 20 mls of 0.45% KBr. The solution was boiled for 5 minutes, cooled and quantitatively transfered to a 100 ml volumetric flask. The solution was shaken and left to stand

overnight. The liquid was carefully decanted into a polystyrene test tube, volume adjusted to 5 mls and 1 ml of nitric/trition solution added. After capping and mixing the solution was analysed using an inductively coupled plasma spectrometer (ICP). ICP conditions were established at 205.55nm, with a torch height of 14mm. The ICP was calibrated using blank and standard solutions to report the results in ppm Cr and Co in the dry matter. Due to large sample numbers analytical replication was restricted to 1 in 5 samples selected at random. Samples were analysed in batches of fifty. An internal control containing 1.0 ml of 1000 ppm Cr and 1.0 ml of 100 ppm Co solutions was prepared in duplicate for each sample batch.

4.5.5. Rate of passage determinations

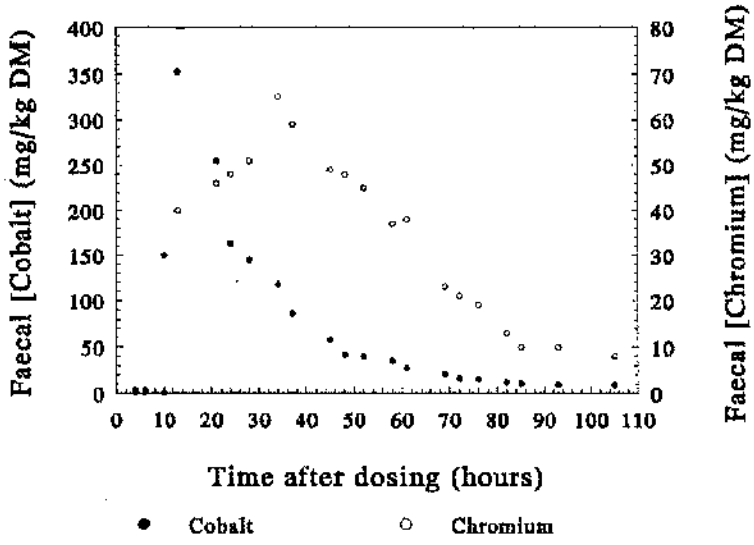
Estimation of solid and liquid outflow rates were performed by fitting the two compartmental mathematical curve-fitting model of Grovum and Williams (1973) to faecal chromium and cobalt concentrations, respectively using computer software kindly provided by N.W. Offer. Estimates of rumen and hindgut outflow were obtained by expressing the natural logarithim of marker concentrations in the dry matter as a function of time post-dosing. The model calculates the rate of decline in marker concentrations after the point of inflection corresponding to rumen-reticulum outflow rate (*k1*). Regression analysis involving the ascending and peak marker concentrations allow hindgut (caecum and proximal colon) transit rates (*k2*) to be calculated.

Modeling involves an iterative procedure to sequentially alter the rate constant estimates (*k1* and *k2)* by assigning different numbers of data points to the ascending and descending parts of the excretion curve so as to minimise a weighted variance about the curve. Correlation coefficients of predicted compared to measured values are also calculated for *k1* and *k2* estimates. Weighted variance in particular, and calculated correlation coefficients indicate the goodness of fit of experimental data to the model. Rate constant estimates were selected for iterations giving minimum weighted variance.

Initially all 22 samples collected from cow 71 during the co-variance period were analysed for Cr and Co (refer to Figure 4.1.). In an attempt to reduce sample numbers

the last data entry was sequentially removed until the weighted variance approached an arbitrary maximum of 5.0. This process enabled samples collected after 69 hours post-dosing to be excluded from analysis.

Figure 4.1. Changes in faecal cobalt and chromium concentrations for cow 71 during the co-variance period



4.6. Urine collection

4.6.1. Spot urine sample collection

Spot urine samples were collected using the vulval stimulation technique. The area around the vulva was gently stroked until urination had been induced. Once an animal had become accustomed to the technique urine samples could typically be collected within two minutes, unless the animal had recently urinated before sampling.

4.6.2. Total urine collection

Total urine collections were performed for the experiments described in chapters 7 and 8 using bladder catheters according to the following procedure. The vulva and

surrounding area was thoroughly washed with disinfectant. A sterile human folatex balloon catheter (Arterial Medical Ltd, Southgate, London) was liberally covered with Lubrel lubricating gel, (Arnolds Veterinary Products Ltd, Harlescott, Shrewsbury) and inserted into the vulva. Once the cow had relaxed the catheter was guided along the urethra into the bladder. If this process induced agitation to the cow, a few drops of Xylocaine anaesthetic gel (Astra Pharmaceuticals, Kings Langley) was placed on the tip of the catheter and the process repeated. Once correctly located, the tip of the catheter was inflated with 50 mls of Aquapharm, sterile bovine saline (Veterinary Drug Company, Falkirk). The catheter was connected up to 1m of 9mm bore silicon tubing (secured to the right hind leg). The silicon tubing was connected to 4m of 9mm bore PVC tubing, terminating into 30 litre containers. Tubing and connectors were supplied by Fisons Scientific Equipment (Loughborough, Leicestershire). At the end of each sampling period catheters were removed at the earliest opportunity, by draining the saline from the catheter tip and carefully withdrawing the catheter from the vulva.

4.6.2.1. Urine preservation

Collected urine was preserved with sulphuric acid (H_2SO_4) to prevent purine derivative degradation. The first series of collections (performed in the experiment described in chapter 7), urine was preserved with 200mls of 1M H_2SO_4 (S. Robertson, personal communication). However for the second series of collections (performed in the experiment described in chapter 8) a more accurate approach was adopted. The level of acid preservation required was estimated as 4.5 mls 4M H_2SO_4 l urine^{-1}, from measuring the volume of 4M H_2SO_4 required to reduce the pH of one litre of urine to below 3. Assuming a urinary output of 40 l, 200 mls of 4M H_2SO_4 was used to ensure the pH of collected urine was maintained below 3.

Chapter Five

Variations in the molar ratio of purine derivatives and pseudouridine to creatinine in spot urine samples

Summary

The literature concerning diurnal variation of PDs in urine is reviewed. Five spot urine samples were collected within each of six consecutive days from lactating Holstein/Friesian cows. Urinary PD, allantoin, pseudouridine and creatinine concentrations were significantly different ($P<0.001$) between sampling times and followed a diurnal pattern. Observed variations in urinary concentrations, between sampling times and days were reduced by expressing urinary PD, allantoin (A) and pseudouridine (Ps) as a ratio to creatinine (c) concentration. PD/c, A/c and Ps/c ratios varied markedly between sampling times ($P<0.01$, $P<0.01$ and $P<0.001$, respectively), with no evidence of a consistent diurnal pattern for an individual cow. Within-day variation in PD/c, A/c and Ps/c ratios (CV of 25.1, 23.6 and 24.8%, respectively) were large compared to that between-days (CV of 9.6, 9.2 and 12.1%, respectively). The extent of within-day variations in PD/c, A/c and Ps/c ratios suggested that several samples are required per day, from each animal and that, even then this approach may be subject to unacceptable errors. It is suggested more intensive investigations of the variation in PD/c, A/c and Ps/c ratios are required before spot sampling can be widely adopted.

5.1. Introduction

Previous work has shown that the urinary excretion of purine derivatives (PD) reflects the supply of microbial NAs acids absorbed in the small intestine and has potential as a non-invasive technique to assess the MCP (refer to chapter 1). Currently,

the most accurate means of assessing urinary PD excretion is to perform a total urine collection (Grubic *et al*, 1992), restricting the application of the PD technique to use in metabolism units. Collection of spot urine samples using creatinine as an internal marker for urinary output is a possible alternative to performing a total urine collection. Widescale adoption of spot sampling of urine is problematic due to uncertainities concerning the existence of diurnal variability in the molar ratio of PDs to creatinine (PD/c) ratios and the quantitative relationship between this ratio and urinary PD output.

5.1.1. The use of spot urine samples as an alternative to a total urine collection

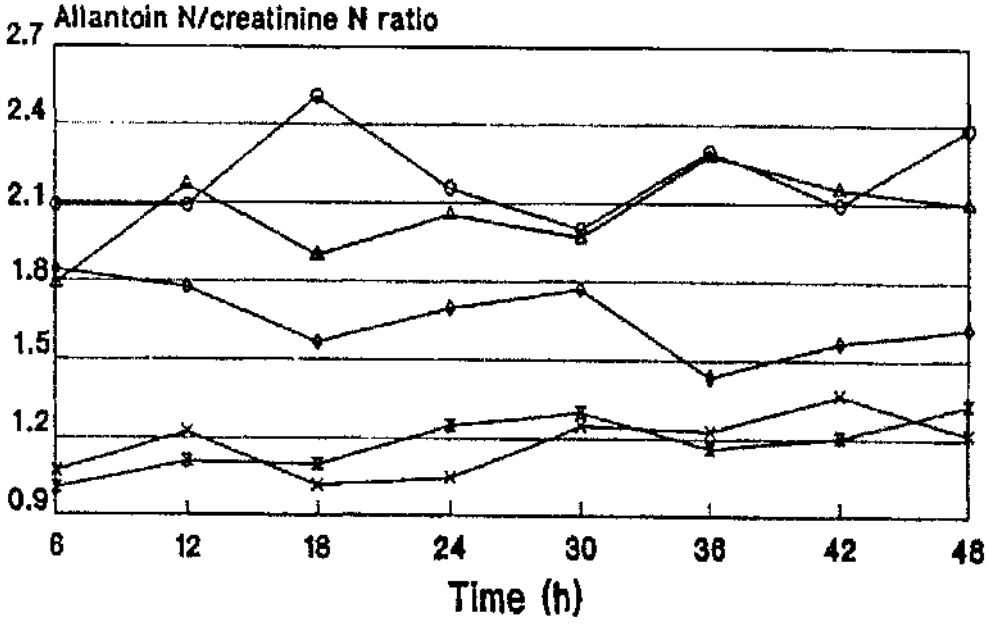
Collection of spot urine samples is dependent on accounting for variations in PD concentration and therefore urine volume. Ultimately this requires the use of a marker which is excreted in the urine at a constant rate hour by hour and day to day. Creatinine is often used as a reference for evaluating metabolite levels in blood and has been used as an internal marker for urine volume (de Groot and Aafjes, 1960; Albin and Clanton,1966 and Erb *et al*, 1977). Observations of renal clearances of allantoin approaching that of creatinine (Greger *et al*, 1976), led Antoniewicz *et al* (1981) to propose the molar ratio of allantoin to creatinine (A/c) in spot urine samples as an index of MCP supply to ruminants. Constancy of urinary creatinine excretion is reviewed in chapters 6 and 9. Ultimately, use of spot urine sampling depends on the existence and extent of the diurnal variability in spot sample PD/c ratios, and how closely these are related to PD output.

5.1.2. Diurnal variation in the PD/c ratio in spot urine samples

If spot urine sample PD/c ratios are to serve as a meaningful index of MCP, diurnal variations in the ratio must be small, or consistent over a range of diets and feeding frequencies. Hourly spot urine samples collected from sheep fed *ad libitum*, indicated that PD/c ratios were not significantly affected by sampling time (Chen *et al*, 1993b and 1995), confirming earlier observations of small changes in the A/c ratio of

two hourly spot samples (Antoniewicz *et al*, 1981). In contrast, Puchala and Kulasek (1992) found considerable variation in the A/c ratio of six hourly spot samples collected over a 48 hour period from sheep (refer to Figure 5.1.).

Figure 5.1. Variations in the A/c ratio of 6-hour urine collections in sheep (n=8) from Puchala and Kulasek (1992)



Lines represents diets differing in protein:energy ratio

Urinary PD concentrations and excretion have been shown to vary diurnally between five sampling times ($P<0.05$) in steers fed once or twice daily (Chen *et al*, 1992d). Diurnal variability in PD/c ratios however, was small and reduced by increased feeding frequency. Two-hourly spot samples collected over a 24 hour period fiom lactating beef cattle have indicated no systematic variation in the PD/c ratio (Daniels *et al*, 1994 and Gonda and Lindberg, 1994, refer to Figure 5.2.). Moorby and Dewhurst (1993b) working with high yielding dairy cows, observed differences in the PD/c ratio of spot samples collected at 10.30 and 15.00 hours which were inconsistent between experiments and diets. Dewhurst *et al* (1996), using the same sampling regimen with lactating Holstein/Friesian cows reported small and generally insignificant effects on PD/c ratios between sampling times. Furthermore, Moorby and Dewhurst (1993b) and

Dewhurst *et al* (1996) have suggested mean PD/c ratios are sufficiently sensitive to detect changes in the outflow of MCP, although this had been not measured. In contrast, Iriki *et al* (1994) suggested PD/c ratios to be an unreliable indicator of MCP due to protein-energy nutrition effects on endogenous urinary allantoin and creatinine excretion.

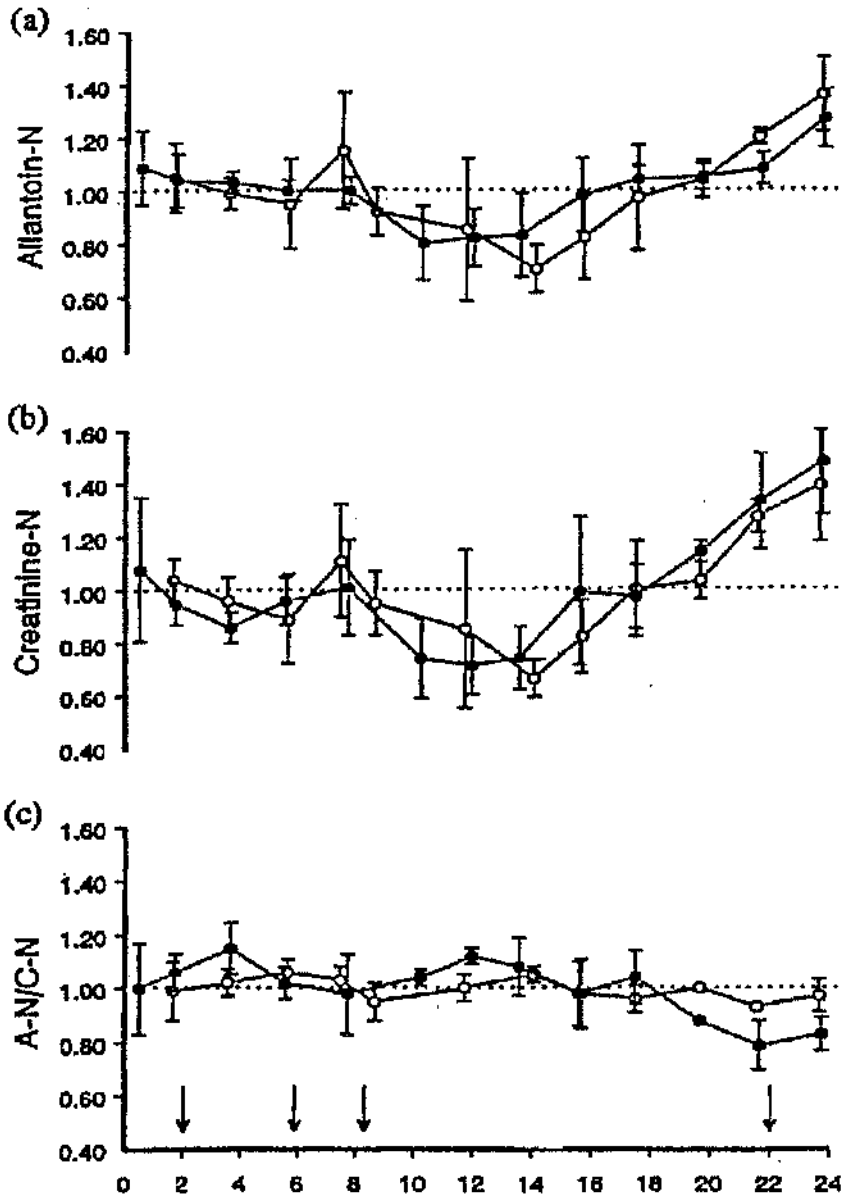
Numerous reports have indicated variation in PD/c ratios collected at different sampling times. It is essential to establish, particularly in the dairy cow whether this variation is random due to experimental error or follows a diurnal pattern. Existence of diurnal variation in PD/c ratios would have serious implications on the sampling regimen required.

In conclusion, collection of spot urine samples has been suggested as potential index of MCP supply in sheep (Antoniewicz, 1981; Chen *et al*, 1993b and 1995), steers (Chen *et al*, 1992d), lactating beef cattle (Daniels *et al*, 1994 and Gonda and Lindberg, 1994) and lactating Holstein/Friesian dairy cows (Moorby and Dewhurst, 1993b and Dewhurst *et al*, 1996). The sensitivity and reliability of this approach needs to be thoroughly assessed, particularly in the dairy cow before it can be universally applied. Measuring PD or allantoin excretion by total urine collection allows a more accurate estimate of MCP than PD/c and A/c ratios of spot samples (Puchala and Kulasek,1992), but the potential benefits of spot urine sampling in providing a practical index of MCP under farm conditions, must not be overlooked.

5.1.3. Relationship between urine spot sample PD/c ratios and urinary PD excretion

In order to adopt the spot urine sampling as an alternative to performing a total urine collection, it is critical that spot sample PD/c ratios are closely related to daily total urinary PD excretion. This relationship is reviewed and discussed in chapters 6 and 9, respectively.

Figure 5.2. Diurnal variation of the concentration of allantoin-N (a), creatinine-N (b) and allantoin-N to creatinine-N ratio (c) in spot urine samples collected throughout a 24 hour period from two groups of cows (from Gonda and Lindberg, 1994)



Creatinine and allantoin concentrations and the A-N/C-N ratio are expressed as a proportion of the daily mean. Each value is the mean (with standard deviation) of 4 cows. Arrows represent feeding times. Open and closed symbols indicate different groups of cows.

5.2. Experimental aims

The aim of this preliminary experiment was to investigate the extent of diurnal variation in the PD/c ratio in spot urine samples, collected from Holstein/Friesian dairy cows.

5.3. Materials and methods

5.3.1. Animals and animal management

Eight multiparous Holstein/Friesian cows were selected from the Crichton dairy herd and fed individually behind Calan-Broadbent gates. Water was available *ad libitum.* Experimental cows were walked into the milking parlour and milked alongside the main herd at 07.00 and 15.00 hours.

5.3.2 Experimental design

The primary aim of the experiment was to evaluate the use of n-alkanes as a herbage intake marker. The opportunity to spot urine sample each animal became available, enabling a small scale investigation in the variability of PD/c, A/c and Ps/c ratios. Consequently, only a brief description of the experimental design is reported. Cows were paired according to stage of lactaction and bodyweight, and randomly allocated to group one or two. Cows in group two received a 2 kg DM barley supplement, while herbage intake was fixed for each cow. Experimental diet allocation is shown in Table 5.1. Following a 20 day adjustment period all experimental measurements were made during a 6 day sampling period.

Due to the confounding effects of cow, herbage level and supplement, the influence of dry matter intake and barley supplement on spot urine sample PD/c, A/c and Ps/c ratios could not be statistically evaluated. Analysis of variance for sampling time was performed within Genstat 5.3 (Lawes Agricultural Trust, 1993) using the following

model:- diet/cow/sampling day blocking structure and sampling hour treatment structure. Effects of diet within a cow were evaluated using the following model:- sampling day/time blocking structure and diet/cow treatment structure. Regression analysis was undertaken using Minitab statistical package (Minitab, Inc.,1980).

Table 5.1. Allocation of experimental diets

Cow	Group	Herbage intake (kg DM/d)	Barley intake (2 kg DM/d	Total DM intake (kg DM/d)
567	1	8	-	8
421	1	10	-	10
871	1	12	-	12
836	1	14	-	14
571	2	8	2	10
631	2	10	2	12
582	2	12	2	14
624	2	14	2	16

5.3.3. Diet Formulation

Herbage offered was harvested daily from a perennial ryegrass/clover sward. Herbage and barley chemical analysis is shown in appendix 1.

5.3.4. Animal Feeding

Herbage was offered as five approximately equal meals from 09.00 to 20.00 hours. Herbage dry matter, determined at 60°C for 24 hours was monitored throughout the experiment allowing the fresh weight of herbage offered to be adjusted to maintain dry matter intakes. Each cow received a daily 60g general purpose vitamin and mineral supplement and 25g paloxalene bloat guard at 09.00 hours. The barley supplement was offered at 11.00 hours.

5.3.5. Experimental sample collection

Spot urine samples were collected by vulval stimulation as described in section 4.6.1, at 08.00, 11.00, 14.00, 17.00 and 20.00 hours for six consecutive days. Once collected, urine samples were immediately frozen and stored at -20°C. At the end of the experiment samples were analysed in random sequence using the method described in chapter 2.

5.4. Results

5.4.1. Variations in urinary PD, pseudouridine and creatinine concentrations

Urinary PD, allantoin and creatinine concentrations were found to fluctuate between sampling times ($P<0.001$) for individual cows, although the diurnal variation observed was not consistent between sampling days (refer to Figures 5.3.-5.10.). Variations in mean (for all cows and sampling days) PD, allantoin, pseudouridine and creatinine concentrations between sampling times followed diurnal patterns, as illustrated in Figures 5.11. to 5.14. Diurnal changes in mean (for all cows and sampling days) pseudouridine concentrations followed a distinctly different pattern between sampling times compared to those for PD, allantoin and creatinine. Mean sampling time and day coefficients of variation (CV%) for urinary PD, allantoin, pseudouridine and creatinine concentrations are shown in Table 5.2.

Table 5.2. Mean sampling time and day urinary PD, allantoin, pseudouridine and creatinine concentration coefficients of variation (CV%)

	PD	Allantoin	Pseudouridine	Creatinine
Sampling time (CV%)	38.3	39.3	44.4	46.5
Sampling day (CV%)	18.2	18.2	19.3	18.1

Allantoin concentrations were highly correlated to total PD concentration (r^2=0.970, n=240, P<0.001), and accounted for 85% of the total PD excreted, with the remainder mostly as uric acid.

5.4.2. Variations in molar ratio of PDs and Pseudouridine to creatinine

Expressing urinary PD, allantoin and pseudouridine concentrations as a ratio to creatinine concentration (PD/c, A/c and Ps/c, respectively) reduced the variation due to sampling time and day compared to absolute PD, allantoin and pseudouridine concentrations. Mean sampling time and day coeffcients of variation (CV%) for PD/c, A/c and Ps/c ratios are shown in Table 5.3.

Table 5.3. Mean sampling time and day PD/c, A/c and Ps/c ratio coefficients of variation

CV(%)	PD/c	A/c	Ps/c
Sampling time	25.1	23.6	24.8
Sampling day	9.6	9.2	12.1

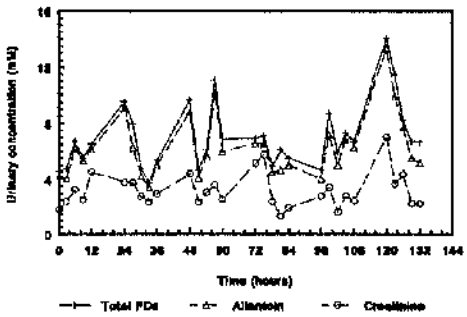
Variations in urinary PD, allantoin and creatinine concentrations and PD/c and A/c ratios for an individual cow did not appear to conform to a consistent diurnal pattern (refer to Figures 5.3.-5.10.). Mean (for all cows and sampling days) urinary PD, allantoin, pseudouridine and creatinine concentrations were significantly (P<0.001) different between sampling times. Diurnal variations in urinary PD, allantoin, pseudouridine and creatinine concentrations are shown in Figures 5.11., 5.12., 5.13. and 5.14., respectively. Mean (for all cows and sampling days) PD/c, A/c and Ps/c ratios were significantly different between sampling times (P<0.01, P<0.01 and P<0.001, respectively) but did not appear to follow consistent diurnal patterns (refer to Figures 5.15., 5.16. and 5.17., respectively). Urinary PD/c ratios were closely correlated with A/c ratios (r^2=0.970, n=240, P<0.001).

5.4.3. Influence of dietary treatment on mean PD/c, A/c and Ps/c ratios

Spot urine sample PD/c, A/c and Ps/c ratios collected from individual cows fed different experimental diets were significantly different ($P<0.001$) As discussed in section 5.4.2., differences due to experimental diet could not be separated from between-cow effects. Mean PD/c and A/c ratios ranged from 1.5 to 2.1 and 1.4 to 2.0 respectively, for herbage diets, and 1.7 to 2.3 and 1.5 to 2.1 respectively, for barley supplemented diets. The influence of diet on mean PD/c ratios is shown in Figure 5.18. Mean Ps/c ratios varied from 0.101 to 0.135 and 0.106 to 0.120 for the herbage and supplemented diets, respectively. The influence of diet on mean Ps/c ratios is shown in Figure 5.19.

Figure 5.3. Cow 624 (14 kg DM herbage + 2 kg DM barley)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration



ii) Diurnal variation in spot urine sample PD/c and A/c ratios

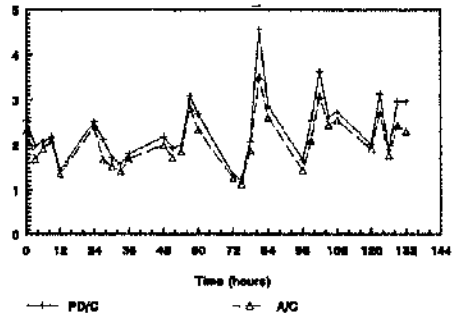


Figure 5.4. Cow 836 (14 kg DM herbage)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

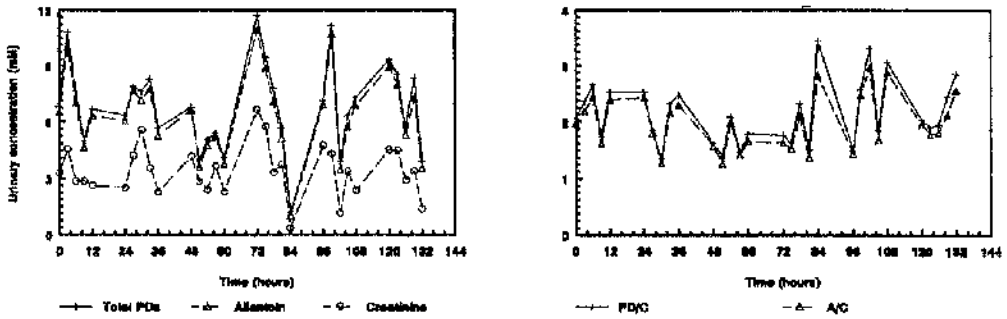


Figure 5.5. Cow 582 (12 kg DM herbage + 2 kg DM barley)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

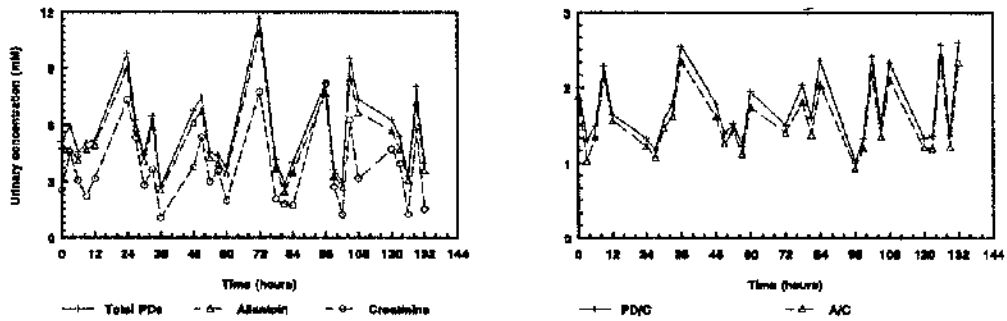


Figure 5.6. Cow 871 (12 kg DM herbage)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

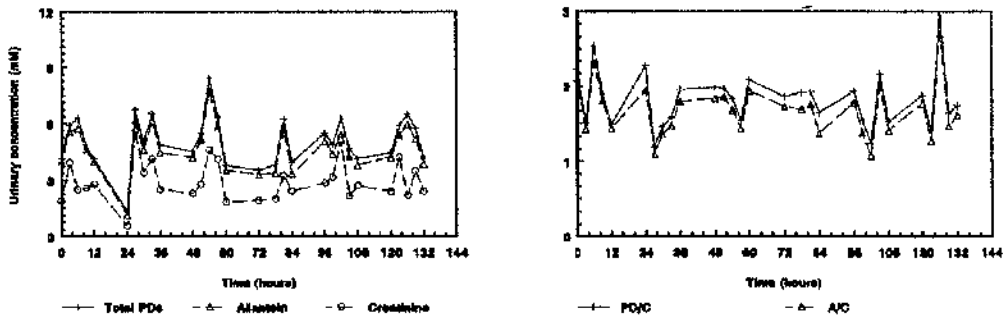


Figure 5.7. Cow 631 (10 kg DM herbage + 2kg DM barley)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

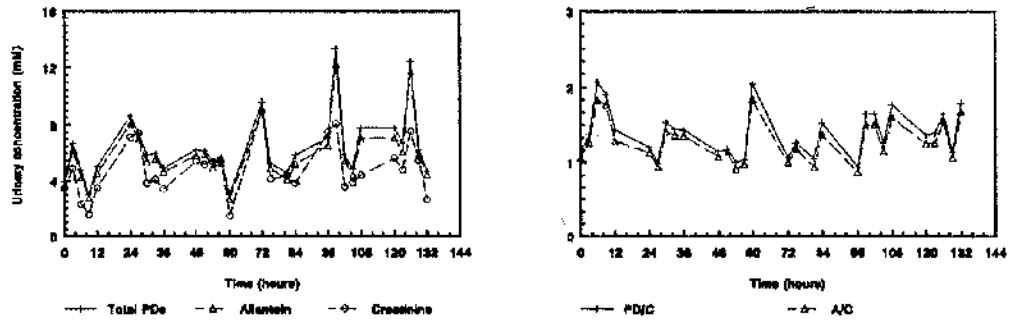


Figure 5.8. Cow 421 (10 kg DM herbage)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

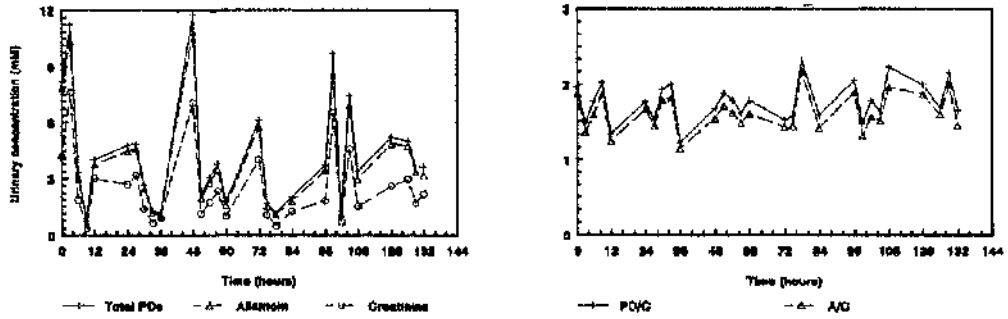


Figure 5.9. Cow 571 (8 kg DM herbage + 2 kg DM barley)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios

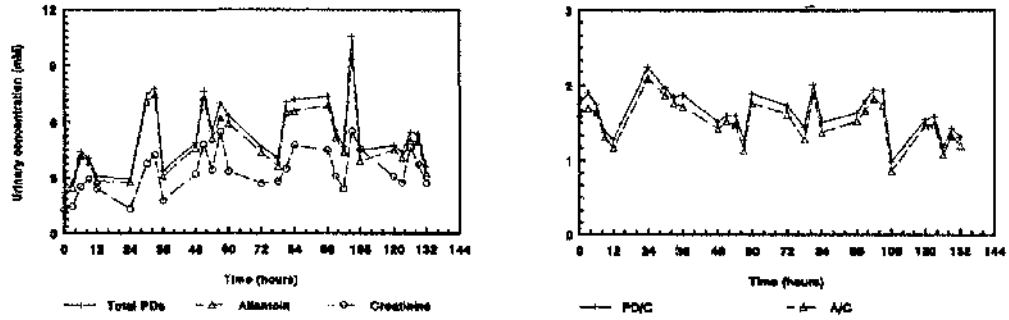
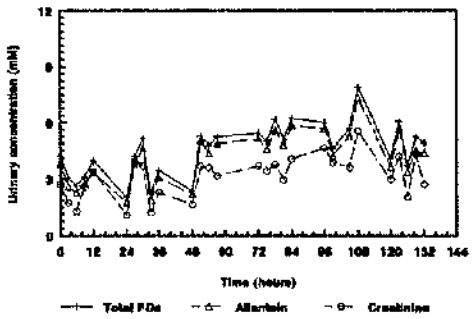


Figure 5.10. Cow 567 (8 kg DM herbage)

i) Diurnal variation in urinary PD, allantoin and creatinine concentration

ii) Diurnal variation in spot urine sample PD/c and A/c ratios



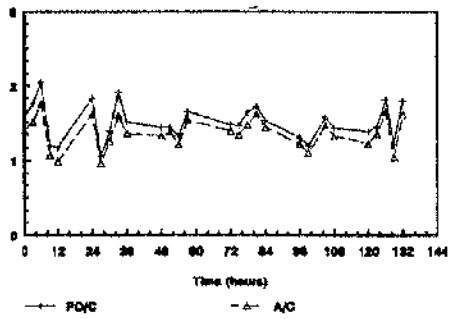
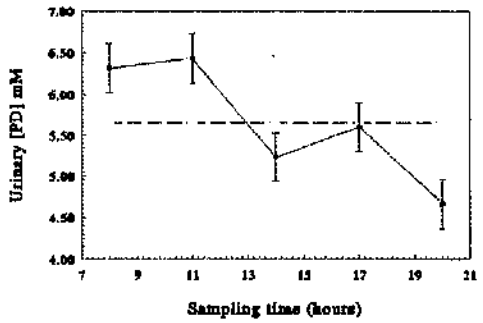
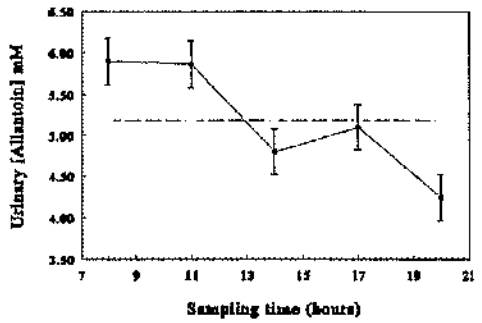


Figure 5.11. Diurnal variation in mean urinary PD concentration

Figure 5.12. Diurnal variation in mean urinary allantoin concentration





For Figures **5.11.** and **5.12.**:- Each point is the mean of 48 measurements

Error bars indicate between sampling time SE

Dotted line indicates the daily mean

Figure 5.13. Diurnal variation in mean urinary pseudouridine concentration

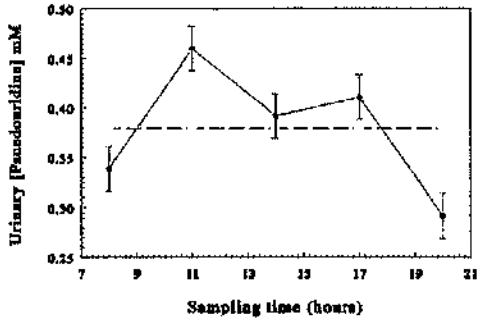


Figure 5.14. Diurnal variation in mean urinary creatinine concentration

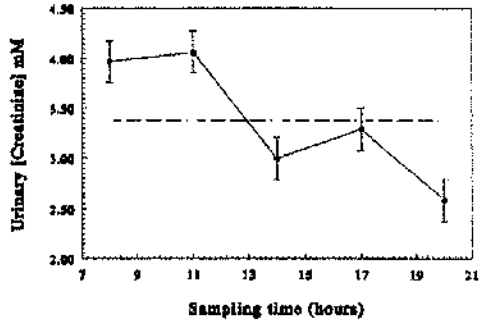


Figure 5.15. Diurnal variation in mean PD/c ratios

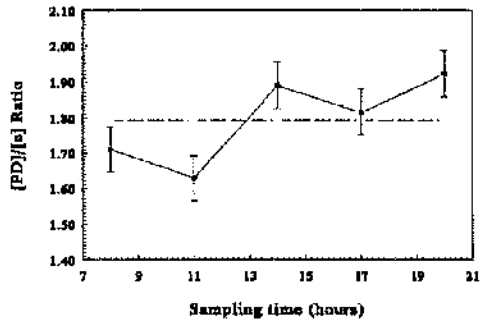
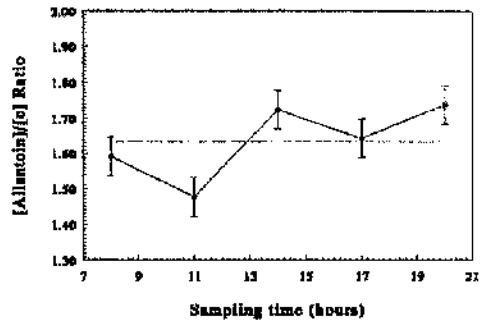
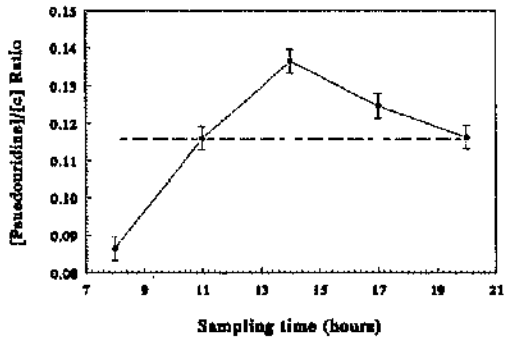


Figure 5.16. Diurnal variation in mean A/c ratios



For Figures **5.13., 5.14., 5.15.** and **5.16.**:- Each point is the mean of 48 measurements
Error bars indicate between sampling time SE
Dotted line indicates the daily mean

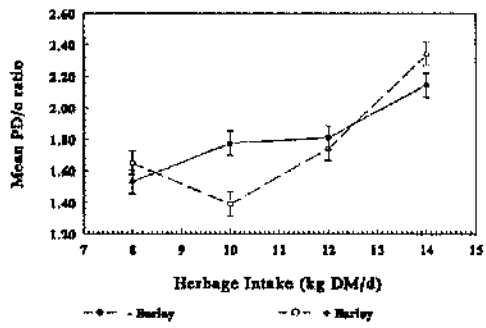
Figure 5.17. Diurnal variation in mean Ps/c ratios

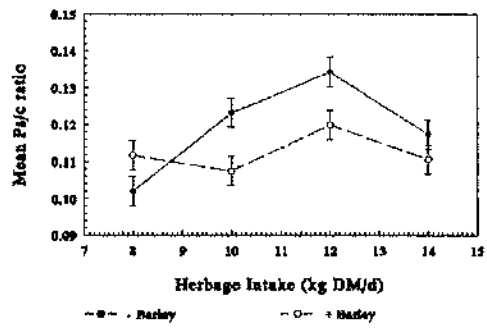


For Figure **5.17.**:- Each point is the mean of 48 measurements
Error bars indicate between sampling time SE
Dotted line indicates the daily mean

Figure 5.18. Influence of experimental treatment on mean PD/c ratios

Figure 5.19. Influence of experimental treatment on mean Ps/c ratios





For Figures **5.18.** and **5.19.**:- Each point is the mean of 30 measurements
Error bars indicate between treatment SE

5.5. Discussion

Urinary PD concentration was highly correlated with allantoin concentration, which accounted for 85% of total PD excreted, with the remainder as uric acid. Urinary concentrations of xanthine and hypoxanthine were found to be neglible as reported by Chen *et al* (1990b), Verbic *et al* (1990), Susmel *et al* (1994b) and Dewhurst *et al* (1995 and 1996).

Urinary PD, allantoin, psudouridine and creatinine concentrations for individual cows exhibited diurnal variations, which were inconsistent between sampling days. Changes in mean PD, allantoin, and creatinine concentrations between sampling times followed similar patterns, suggesting that the renal clearance of PDs approaches that of creatinine as reported by Greger *et al* (1976). Diurnal variations in mean pseudouridine concentrations were distinctly different from those observed for creatinine. It is unclear whether this is a result of true differences in the renal clearance of pseudouridine compared to creatinine, or possibly a reflection of relatively large changes in plasma pseudouridine concentration or changes in the reabsorption and secretory processes in the renal tubule.

Using urinary creatinine excretion to account for changes in urine volume as suggested by Antoniewz *et al* (1981) and expressing PD and allantoin concentrations as a ratio to creatinine concentrations dramatically reduced the variability due to sampling time and day confirming the observations of Antoniewicz *et al* (1981), Chen *et al* (1992d) and Gonda and Lindberg (1994). Individual cow PD/c and A/c ratios varied markedly between sampling times which did not appear to follow a consistent diurnal pattern. These observations confirm previous observations in sheep (Antoniewicz *et al*, 1981 and Chen *et al* 1993b and 1995) and cattle (Chen *et al*, 1992d; Daniels *et al*, 1994 and Gonda and Lindberg 1994). The extent of within-day variation indicated that collection of a single sample from an individual cow would not yield useful information on rumen function. Calculating mean PD/c and A/c ratios (for all cows and sampling days) indicated a diurnal variation existed between sampling times. In order to estimate reliably daily mean PD/c ratios from spot samples the sampling regimen employed would

have to be capable of accounting for this variation. In this experiment, collection of spot samples at 6 hour intervals 08.00 and 14.00 compared to 11.00 and 17.00 hours would give estimates of the daily mean as 1.80 and 1.72, respectively. Alternatively samples collected at 9 hourly intervals at 08.00 and 17.00 and 11.00 and 20.00 would have estimated the daily mean PD/c as 1.76 and 1.78, respectively. Influence of diet and cow (combined effects) on PD/c and A/c ratios ($P<0.001$) suggests that determining the optimum sampling times to estimate daily mean PD/c ratios from spot samples will also be dependent upon the diet offered.

In the current experiment when any one sample is taken, the measured PD/c or A/c ratio has coefficients of variation (CV) of 25.1 and 23.6%, respectively. This is larger than the 5-10 % CV due to sampling time reported for A/c ratios in sheep (Antoniewicz *et al*, 1981 and Chen *et al*, 1993b and 1995). Later observations in sheep reported a CV of 16% associated with one sample, which was reduced to 8% when the mean of four samples was used (Chen *et al*, 1995), indicating the need of multiple samples to estimate reliably the daily mean PD/c ratio. Further observations in other ruminant species have reported sampling time coefficients of variation in PD/c ratios as 2% in steers (Chen *et al*, 1992d) and 11.4% in lactating beef cattle (Daniels *et al*, 1994). Discrepancies between these findings and the current experiment are diffcult to reconcile, particularly as all the samples in this experiment were analysed at random reducing analytical bias between sampling times. The small diurnal variation in the PD/c ratio reported elsewhere, has been suggested to be a consequence of a relatively constant composition and flow of digesta into the small intestine in sheep (Chen *et al*, 1993b and 1995) steers (Chen *et al*, 1992d) and lactating beef cattle (Daniels *et al*, 1994). The use of lactating dairy cattle in this study, resulted in generally higher dry matter intakes, compared to other studies working with cattle. The higher level of feeding could potentially promote greater diurnal changes in microbial protein and hence purine supply entering the small intestine, resulting in larger variations in PD/c ratios.

In the present study, reasons for variations in PD/c and A/c ratios remain unclear. Changes in the rate of PD absorption within the small intestine or the proportion of plasma PDs excreted renally, could affect the rate of urinary PD excretion and possibly

account for the observed variations. Alternatively, the variability in the PD/c ratio could be a reflection of changes in the rate of urinary creatinine excretion as suggested by Daniels *et al* (1994).

Despite differences observed in the pattern of mean creatinine and pseudouridine concentrations, expressing pseudouridine concentration as a ratio to creatinine concentrations, reduced sampling time and day variation. Variations in mean sampling time (for all cows and sampling days) Ps/c ratios followed a distinct diurnal pattern, implying that estimates of daily mean Ps/c ratios from spot urine samples would be heavily dependent upon time of sampling. The effect of diet and cow (combined effects in this experiment, $P<0.001$) on Ps/c ratios tentatively suggests that use of a particular sampling regimen to determine daily mean Ps/c ratio would be further compounded by diet.

On the basis of the current experiment, it appears that variations in the PD/c and A/c ratios are larger in lactating dairy cows than other ruminant species. Considerations of sampling time and day coefficients of variation indicate that a more reliable estimate of the daily mean PD/c ratio would be achieved by collecting several samples within a day than by using a single sample collected over consecutive days. Large variations associated with sampling time raises concerns about the reliability of the spot sampling technique. More experimental work concentrating on the lactating dairy cow is required to assess the sensitivity of several sampling regimens and their accuracy in estimating daily mean PD/c, A/c and Ps/c ratios. Further information is also required on the quantitive relationships between daily mean PD/c, A/c and Ps/c to urinary PD, allantoin and pseudouridine excretion. Furthermore, the use of urinary creatinine excretion as a urine volume marker requires more considered evaluation.

excretion.

Chapter Six

Diurnal variations in purine derivative excretion in milk and urine

Summary

The literature concerning variation of urinary creatinine excretion, diurnal variations in urinary PD excretion in ruminant species and the reliability of the spot urine sampling technique is reviewed. The potential of spot urine sampling as an alternative to performing a total urine collection was evaluated in the experiment described in this chapter. Twelve multiparous Holstein/Friesian cows were fed two experimental diets in a complete change-over design using two 14 day experimental periods. Experimental diets comprised either silage fed *ad libitum* with a 7 kg concentrate supplement offered as a single meal, or a complete diet formulated from the same ingredients with a similar forage:concentrate ratio. Total urine collections were performed every two hours on days 11 and 14 of each experimental period, with a subsample taken, stored at -20^{0}C and subsequently analysed by HPLC. Daily PD and allantoin (A) excretion were highly correlated ($r=0.995$, $n=48$, $P<0.001$). Daily PD, pseudouridine (Ps) and creatinine (c) excretion during each two hour period depended on time of collection (PD, $P<0.001$; Ps, $P<0.001$ and c, $P<0.05$) and on cow ($P<0.01$) but were unaffected by sampling day or treatment. Diurnal variations in PD/c, A/c and Ps/c ratios followed similar diurnal patterns as observed for PD, allantoin and pseudouridine excretion. Experimental data was used to assess the error of three spot sampling regimens, based on the collection of four samples collected at four hour intervals, three samples collected at eight hour intervals and two samples collected at twelve hour intervals. Evaluation of all sampling regimens indicated collection of multiple samples within a day was more reliable than collecting fewer samples over several days. Even the most intensive sampling regimens did not allow acceptable prediction of daily mean PD/c, A/c or Ps/c ratios. Furthermore, daily mean PD/c, A/c and Ps/c ratios proved poor predictors of daily PD, allantoin and

pseudouridine excretion (r values of 0.69, 0.72 and 0.70, respectively). Total urine collection appears necessary to assess accurately daily PD, allantoin and pseudouridine excretion in dairy cows.

Cows were also milked at four-hour intervals on days 11 and 14. Milk sub-samples were stored at -20^{0}C, prior to HPLC analysis. Milk yield during each four hour period depended on time of collection ($P<0.001$), cow ($P<0.001$) and sampling day ($P<0.001$). Milk allantoin excretion during each period depended on time of milking ($P<0.01$) and cow ($P<0.01$) but was unaffected by diet and sampling day. In contrast, milk allantoin concentrations were unaffected by time of collection, cow, diet or sampling day. Based on individual cow observations, no significant relationships existed between milk and urinary allantoin excretion.

6.1. Experimental aims

The current experiment was designed to:-

i. Investigate the influence of feeding system (complete diet or silage and concentrate) on diurnal variations in urinary PD, pseudouridine and creatinine and milk allantoin excretion.

ii. Evaluate urinary creatinine excretion as an internal marker of urine volume.

iii. Determine the accuracy of three spot sampling regimens to predict daily mean PD/c, A/c and Ps/c ratios from spot urine samples.

iv. Quantify the relationships between daily mean PD/c, A/c and Ps/c ratios and daily urinary PD, allantoin and pseudouridine excretion, respectively.

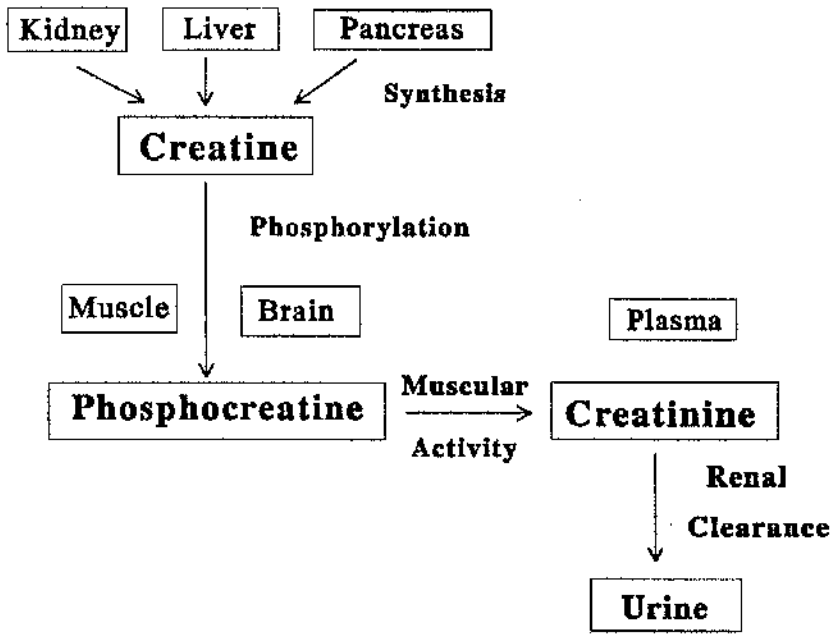
v. Investigate the relationship between renal and mammary allantoin excretion.

6.2. Introduction

6.2.1. Urinary creatinine excretion

Creatine is synthesised in the kidney, liver and pancreas and transported via the blood to other organs such as brain and muscle where it is phosphorylated to phosphocreatine (Tietz, 1986). Creatinine is derived from phosphocreatine which serves as an energy storage compound in skeletal muscle. In humans, phosphocreatine accounts for 98% of muscle creatine reserves (Borsook and Dubnoff, 1947). During normal muscular activity, phosphocreatine is converted into its cyclic anhydride-creatinine. Creatinine is universally present in serum, erythrocytes and all body fluids (Schumann, 1931, cited in a review by Narayanan and Appleton, 1980). In addition to creatinine, creatine is also excreted renally. Generally, creatinine concentrations exceed those of creatine in ruminant urine (Church, 1976, cited by Bristow *et al*, 1992). Creatinine synthesis and excretion is described schematically in Figure 6.1.

Figure 6.1. Schematic representation of creatinine synthesis and excretion



Urinary creatinine excretion has commonly been used in human medicine to check the completeness of 24 hour urine collections. The validity of this approach is dependent on creatinine being excreted at a constant rate. Folin (1905) was the first to adopt this approach, and found little between-day variation in healthy human individuals. Although creatine conversion to creatinine occurs at a constant rate in man, the synthesis of creatine is variable affecting creatine pool size (Crim *et al*, 1976). This implies that creatinine excretion can be subject to considerable variation within an individual. Narayan and Appleton (1980), reviewed a number of studies and concluded that between-day variations in creatinine excretion within an individual precluded its use as a practical means of assessing the completeness of 24 hour urine collections. Further work with human subjects indicated ρ-amino benzoic acid (PABA) to be a more sensitive and reliable indicator of urine output than creatinine (Bingham and Cummings, 1985). Studies in ruminant species have demonstrated creatinine excretion to be relatively constant during a 24 hour period in an individual animal (deGroot and Aafjes, 1960 and Albin and Clanton, 1966). Consequently, urinary creatinine excretion has been proposed as an internal marker of urine volume (deGroot and Aafjes, 1960; Albin and Clanton, 1966 and Erb *et al*, 1977). Typical estimates of daily urinary creatinine excretion for sheep, goats and cattle are presented in Table 6.1. Values presented have been calculated from data reported in the literature and are expressed on a μmol/kg metabolic liveweight basis. The values reported in this review indicate that creatinine excretion is higher per unit metabolic liveweight for cattle than for sheep or goats. Lindberg (1989) tabulated values from much earlier reports in the literature which indicated small differences between goats, sheep or cattle when creatinine excretion was expressed on a liveweight basis.

Generally, nutritional restrictions or excesses do not seem to have a substantial effect on creatinine excretion in goats (Lindberg, 1985 and 1989), sheep (Fujihara *et al*, 1987 and Lindberg and Jacobsson, 1990) steers (Fujihara *et al*, 1987) or cows (Orskov and MacLeod, 1982 and Gonda and Lindberg, 1994). In other studies creatinine excretion has however, been reported to fluctuate with energy supply in lambs nutritionally maintained by intra-gastric infusion (Hovell *et al*, 1983 and 1987) and

protein and energy supplies in conventionally fed sheep (Matsuoka *et al*, 1988). It would appear from these observations that the sensitivity and reliability of urinary creatinine excretion as an index of urine output would be compromised by large differences in nutrient supply.

Table 6.1. Typical creatinine excretion in ruminant species

Species	No. of Animals	Creatinine excretion (μmol/kg $W^{0.75}$ /d)	Reference
Sheep	27	410-505	Hovell *et al* (1987)
Sheep	16	408-558	Dewhurst (1989)
Sheep	20	348-597	Lindberg and Jacobsson (1990)
Sheep	4	233-446	Balcells *et al* (1991)
Sheep	2	517	Chen *et al* (1991a)
Sheep	16	407-525	Chen *et al* (1995)
Goat kids	14	689	Lindberg (1985)
Goat kids	3	448-505	Lindberg (1989)
Goat kids	6	467-583	Lindberg (1991)
Steers	4	800-985	Chen *et al* (1992d)
Cows	2	923	Orskov and MacLeod (1982)
Cows	3	995-1048	Puchala *et al* (1993)
Cows	24	879-981	Gonda and Lindberg (1994)
Cows	8	957-1042	Susmel *et al* (1994a)

6.2.2. Renal clearance of creatinine

The use of creatinine to assess urine output is reliant on creatinine being excreted at a constant rate and that its clearance approaches that of the metabolites under investigation. Creatinine clearances have been shown in humans to approach that of inulin which is excreted by glomerular filtration and not augmented by secretion or reabsorption within the kidney tubule (Shannon, 1935). Creatinine clearances are not exact estimates of GFR, due to creatinine secretion by the kidney tubules (Narayanan and Appleton, 1980). Endogenous creatinine plasma clearance has commonly been used

in ruminant studies as a convenient estimator of GFR (e.g. Chen *et al*, 1991a). Studies in sheep have provided evidence of creatinine secretion and reabsorption within the kidney nephron. Comparisons with inulin clearance have indicated that creatinine is reabsorbed at low GFRs while secreted at high GFRs (Farningham, 1986). Further work has shown that about 16% more creatinine is excreted than is filtered by the kidney indicating the magnitude of creatinine secretion by the nephron (Faichney and Welch, 1994). Recent studies reporting rates of creatinine excretion in ruminant species are limited. Currently, it is unclear whether the rates of creatinine reabsorption and secretion occuring within the kidney tubule change significantly within a 24 hour period, which would ultimately influence the rate of urinary creatinine excretion.

6.2.3. Variation in urinary creatinine excretion

Early observations indicated creatinine excretion varied considerably between individual animals (deGroot and Aafjes, 1960 and Albin and Clanton, 1966). Urinary creatinine excretion in humans has been shown to be highly variable (CV 17-18%) between individuals (Bingham and and Cummings, 1985). Relatively large between-animal variations in creatinine excretion for ruminant species have been reported in the literature and these are summarised in Table 6.2.

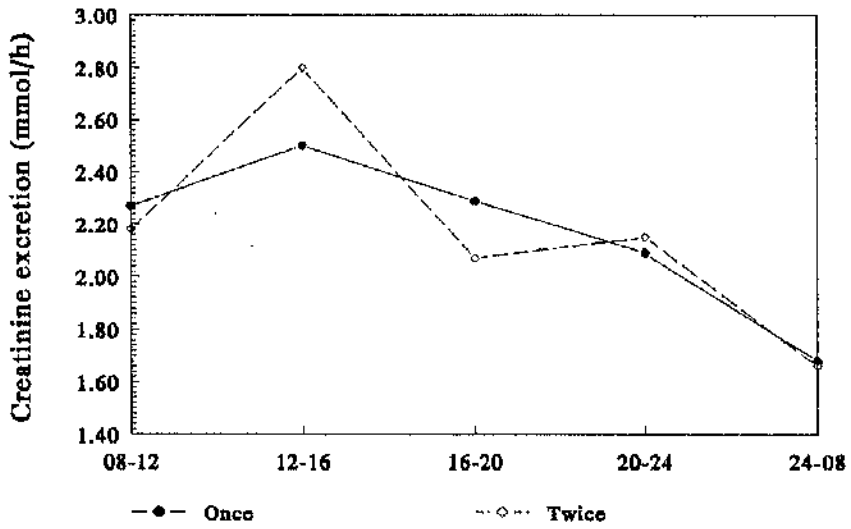
Table 6.2. Between-animal coefficients of variation (CV%) in urinary creatinine excretion reported in the literature

Species	Between-animal variability (CV%)	Reference
Sheep	5.7-9.1	Hovell *et al* (1987)
Sheep	15.5	Dewhurst (1989)
Sheep	12.0-18.6	Lindberg and Jacobsson (1990)
Sheep	12.4	Chen *et al* (1995)
Goat kids	14.9	Lindberg (1985)
Goat kids	2.4-3.2	Lindberg (1989)
Steers	8	Chen *et al* (1992d)
Cows	12	Daniels *et al* (1994)

Between-animal variability in creatinine excretion lead Dewhurst (1989) to suggest groups of 15 animals would be required in order to utilise creatinine excretion as an index of urine output.

Following the observations in cattle of deGroot and Aafjes (1960) and Albin and Clanton (1966) creatinine excretion in individual animals has been assumed to be constant from day to day. Studies in buffalo calves demonstrated creatinine excretion within an individual animal to be significantly different ($P<0.01$) from day to day (Chetal *et al*, 1975). Later studies have reported within-animal variations in creatinine excretion of 11% in steers (Chen *et al*, 1992d) and 15% in cows (Daniels *et al*, 1994), being similar to 7-11% reported for human subjects (Bingham and Cummings, 1985).

Figure 6.2. Variations in the rate of urinary creatinine excretion in steers fed once or twice daily reported by Chen *et al* (1992d)



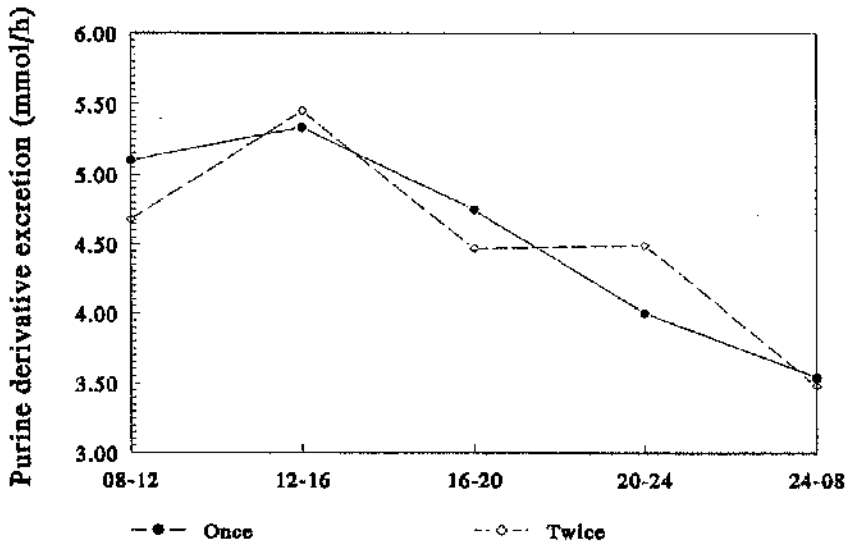
Studies investigating the rate of urinary creatinine excretion in ruminant species are poorly documented in the literature. However, Chen *et al* (1992d) using once and twice daily fed steers demonstrated variations in the rate of creatinine excretion (refer to

Figure 6.2). The use of urinary creatinine excretion as an index of urine output requires careful implementation in order to overcome the between- and within-animal variations.

6.2.4. Variation in urinary PD excretion

Although variations in spot urine sample PD or allantoin concentrations collected from sheep (Antoniewicz *et al*, 1981; Puchala and Kulasek, 1992 and Chen *et al*, 1993b and 1995), steers (Chen *et al*, 1992d) and cows (Daniels *et al*, 1994 and Gonda and Lindberg, 1994) are well documented, relatively little information exists concerning the rate of PD or allantoin excretion. Chen *et al* (1992d) provided the only measurement of variations in the rate of urinary PD excretion in steers fed once or twice daily (CV of 15 and 16%, respectively). The amount of PD excreted per hour varied with time of day, and was significantly higher ($P<0.05$) during 12.00 to 16.00 hour period and lower during 24.00-08.00 hour period for both feeding frequencies (refer to Figure 6.3.).

Figure 6.3. Variations in the rate of urinary PD excretion in steers fed once or twice daily reported by Chen *et al* (1992d)



The researchers argued that the variations observed were not a reflection of changes in purine absorption from within the small intestine but due to fluctuations in urine volume.

6.2.5. Prediction of urinary PD excretion from PD/c ratios

Observations of Balcells *et al* (1991) in sheep demonstrated duodenal yeast RNA infusions above 200μmol/ kg $W^{0.75}$ were highly correlated (r=0.983) with daily mean A/c ratios indicating the potential of spot urine sampling as an alternative to measuring daily PD excretion. Chen *et al* (1995) working with sheep, reported daily PD excretion to be highly correlated (r=0.92) with daily mean PD/c ratios corrected for metabolic liveweight. Earlier observations in dairy goats also indicated a close correlation (r=0.86) between allantoin excretion and daily mean A/c ratios scaled for liveweight and creatinine excretion (Lindberg, 1985). In contrast, Daniels *et al* (1994) found daily mean PD/c ratios were only moderately correlated (r=0.69) with daily PD excretion in beef cattle. These findings suggest estimates of MCP supply from spot urine sampling are likely to be more reliable for sheep and goats than for cattle.

Adoption of the spot sampling technique is dependent on the PD/c ratio of spot samples collected during the day reflecting daily mean PD/c ratio. Observations involving 24 hour urine collections from steers by Chen *et al* (1992d) only serve to highlight the necessity of collecting representative samples. While, mean PD/c ratios of urine collected between 12.00 and 16.00 hours were significantly correlated (r=0.83) with daily mean PD excretion, no clear relationships were observed with PD/c ratios of urine collected over other time periods.

6.2.6. Reliability of spot urine sampling

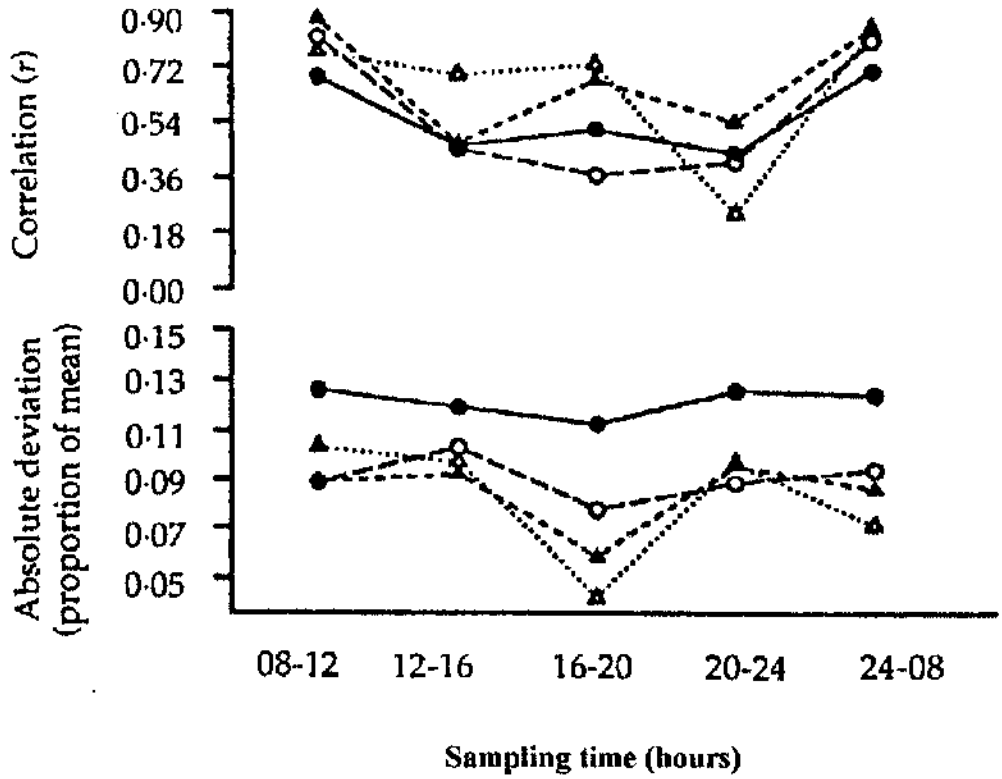
Diurnal variations in spot sample PD/c or A/c ratios collected over a 24 hour period have been demonstrated to be small in sheep (Antoniewicz *et al*, 1981 and Chen *et al*, 1993b and 1995) and in cattle (Chen *et al*, 1992d; Daniels *et al*, 1994 and Gonda

and Lindberg, 1994). Observations in sheep particularly, have demonstrated that daily mean PD/c ratios are closely correlated to PD excretion, impling urinary PD output could be predicted from spot sample PD/c ratios. Prediction of daily mean PD/c ratios from PD/c ratios of spot samples is poorly documented in the literature. Chen *et al* (1992d) assessed the sensitivity of the spot urine sampling technique, by calculating correlation coefficients between single-sample and daily mean (n=5) PD/c ratios in steers fed once or twice daily. Measurements of the PD/c ratio collected between 24.00-08.00 hours were more closely correlated (r=0.74) with the daily mean than PD/c ratios of urine collected between 08.00-12.00 or 12.00-16.00 or 16.00-20.00 or 20.00-24.00 hours. Mean deviations of spot sample PD/c ratios from the daily mean PD/c ratios ranged from 11 to 12%, being similar between sampling intervals and independent of feeding frequency. Absolute deviations from the mean and correlation coefficients reported by Chen *et al* (1992d) are shown in Figure 6.4.

Despite the poor correlations between the mean PD/c ratio and PD/c ratio of urine collected between 12.00-16.00 or 20.00-24.00 hours, the researchers concluded that single spot sample PD/c ratios could potentially be used as an alternative to daily PD excretion.

Measurement of PD/c ratios in spot urine samples has been proposed as a means of assessing MCP supply in sheep (Antoniewicz *et al*, 1981 and Chen *et al*, 1993b and 1995) and cattle (Chen *et al*, 1992d; Moorby and Dewhurst, 1993b; Daniels *et al*, 1994; Gonda and Lindberg, 1994 and Dewhurst *et al*, 1996). However, as Chen *et al* (1995) critically pointed out the spot urine sampling technique may be insufficiently sensitive to compare dietary treatments which result in small differences in MCP supply. In this case total urine collection and hence daily PD excretion would be more suitable.

Figure 6.4. Correlation and absolute deviation of the A/c ratio determined from a single spot urine sample compared to the daily mean (n=5)
Data from Chen *et al* (1992d)



Key:-

Open circles represent single sampling day measurements (n=32)
Closed circles represent mean of two sampling day measurements (n=16)
Open triangles represent mean of three sampling day measurements (n=8)
Closed triangles represent mean of four sampling day measurements (n=8)

6.3. Materials and methods

All experimental cows remained in good health during the experiment. Due to spontaneous urination between two hourly sampling intervals, 608 rather than 624 urine samples were collected. All other sampling procedures were performed as described.

6.3.1. Animals and animal management

Twelve multiparous Holstein/Friesian cows were selected from the Auchincruive main dairy herd and retained in individual stalls within a metabolism unit using de Boer yokes. Milking was performed *in-situ* at 7.00 and 15.00 hours, except on sampling days when cows were milked at four hour intervals from 09.00 hours, for a twenty four hour period.

6.3.2. Experimental design

The experiment was a 2 x 2 complete change over design. Cows were paired according to calving date, parity and bodyweight. Each set of paired animals were randomly blocked. Mean days in lactation, bodyweight (kg) and parity were 148, 634 and 4.33, and 148, 632 and 3.8, for blocks one and two, respectively. Each experimental period lasted for 14 days with measurements performed on days 11 and 14. The ten day adjustment period was considered adequate to enable rumen function to adjust to treatments, as all experimental animals had previously been fed the same silage and concentrate.

With experimental data collected at regular intervals within a period of time, the problem arises of dependence of a measurement on the previous observation. Experimental data were tested for ante-dependence, using the Antorder procedure within Genstat 5.3 (Lawes Agricultural Trust, 1993), which tests whether a model which allows dependence on the previous observation fits the data significantly better than the model with no dependence. The model accounting for dependence did not fit significantly better and therefore the data were considered to be independent. The experimental data were analysed by analysis of variance (Genstat 5.3, Lawes Agricultural Trust, 1993) rather than repeated measures analysis on the basis that analysis of variance is statistically well understood, straightforward to perform and allows a clear interpretation of the results (A.M. Sword, personal communication). Analysis of variance was performed using the

following model:- cow.period/day/hour blocking structure and cow.day + diet.hour treatment structure.

Coefficients of variation were calculated for hour, day, period and cow within Genstat 5.3 (Lawes Agricultural Trust, 1993). Initially PD/c and A/c ratios determined from spot samples at each two hour interval (n=13) compared with daily mean PD/c and A/c ratios, producing 52 correlations. Each correlation contained data for the 6 cows contained within a block for each experimental period i.e. 12 points. In practice however, more than one spot sample would be collected. Consequently it was possible to test several sampling regimens using mean PD/c and A/c ratios for each cow as the reference value. The sampling regimens estimated PD/c and A/c ratios from:- two samples collected at twelve hour intervals (12 hour scheme), three samples collected at eight hour intervals (8 hour scheme) and four samples collected at four hours intervals (4 hour scheme). Each sampling regimen was tested within the twenty four hour collection period. Sampling regimen estimates were correlated with the actual daily mean for each cow within a block for both sampling days within a period. To assess the increase in accuracy from using both sampling days jointly, the mean sampling regimen estimate for both sampling days was compared with the mean of both sampling days. Calculation of all sampling regimen estimates and correlations with calculated daily means were performed using Genstat 5.3 (Lawes Agricultural Trust, 1993).

6.3.3. Diet formulation

One of the aims of the experimental was to investigate variations in urinary PD, pseudouridine and creatinine excretion in dairy cows fed typical on-farm diets. Experimental diets were formulated from a first cut silage ensiled during may 1992 from grass swards in which perrenial ryegrass predominated supplemented with a standard dairy concentrate currently fed to the Auchincruive dairy herd.

Chemical analysis of the silage (described in chapter 4) did not include lactate, VFAs or alcohols produced during the ensiling process. In order to estimate silage FME, the ME contained in these organic compounds has to be taken into account. The

following equations were used based on a 107 silage dataset, kindly provided by N.W. Offer :- i) silage oven DM expressed as a function of ethanol corrected toulene dry matter was calculated using equation 1; ii) unfermentable ME (uFME) was calculated using equation 2; iii) silage DOMD determined on an oven DM basis was re-calculated on a CDM basis using equation 3; iv) silage ME was calculated using the equation of Thomas and Chamberlain (1982, equation 4); v) silage FME was calculated as the difference between ME and uFME (equation 5). Silage analysis including estimates of FME is shown in appendix 2.

Equations used:-

$$\text{oDM / CDM} = 0.921 + 0.000191\ \text{oDM} - 0.000253\ \text{oAsh} + 0.000357\ \text{oCP} - 0.00175\ \text{oAH-EE} \quad (r^2 = 0.425,\ n = 107) \qquad (1)$$

$$\text{uFME} = 4.57 + 0.00363\ \text{NH}_4\text{-N/ Tot-N} - 0.00250\ \text{oDM} - 0.643\ \text{pH} + 0.0342\ \text{oAH-EE} \quad (r^2 = 0.503,\ n = 107) \qquad (2)$$

$$\text{DOMD}_{\text{true}} = (1000-((1000-\text{oDOMD})*\ \text{oDM/CDM})) \qquad (3)$$

$$\text{ME} = 0.16 * \text{DOMD}_{\text{true}} \quad \text{(Thomas and Chamberlain, 1982)} \qquad (4)$$

$$\text{FME} = \text{ME} - \text{uFME} \qquad (5)$$

Key:- o prefix refers to oven

Dietary nutrient supply was manipulated by feeding silage and concentrates separately (treatment 1) or as a complete diet (treatment 2). Concentrate (formulation and chemical composition are shown in appendix 3 and 4, respectively) allocated was fixed at 7 kg F.Wt/d. SAC Advisory Service rationing software was used to predict *ad libitum* silage intake enabling complete diet formulation. Consequently,

forage:concentrate ratios (62:38, on a DM basis) were the same for separate and complete diets. The complete diet was prepared daily in 600kg F.Wt batches in a Keenan mixer wagon (composition shown in appendix 5). SAC rationing software predicted total DM intake as 16.3 kg/d, ME intake of 199 MJ/d, with eRDP and MP supplies of 119 and 104% of requirements, respectively. Predicted DM, ME, FME and CP intakes are shown in Table 6.3.

Table 6.3. Predicted DM, energy and nitrogen intakes

	Treatment 1		Treatment 2
	Silage	Concentrate	Complete Diet
Intake (kg F.Wt/d)	40	7	47
DM intake (kg/d)	11.92	6.13	18.1
CDM intake (kg/d)	12.8	6.13	17.9
CP intake (g/d)	2146	1269	3415
ME intake (MJ/d)	132.3	82.1	214.4
FME intake (MJ/d)	111.7	62.5	174.2

6.3.4. Animal feeding

Cows were individually fed fresh silage or complete diet at 08.00 hours each day. During the afternoon feed bins were topped up with silage and the complete diet ensuring all cows had enough feed in front of them. Concentrate offered at 10.00 hours was consumed rapidly, typically within 90 minutes. Water was available *ad libitum* throughout the experiment.

6.3.5. Experimental measurements and sample collection

6.3.5.1. Urine collection and sampling

On days 11 and 14 of each experimental period two hourly urine collections were performed for a twenty four hour period, starting at 08.00 hours. Cows were induced to

urinate by vulval stimulation and the urine voided was collected. Approximately three minutes later, stimulation was repeated to minimise end of collection errors. Urine volume was determined, with subsamples taken and stored at -20°C. Constant cow observation, ensured potential spontaneous urination between sampling intervals was taken account. Urine sample analysis was performed in random sequence using the HPLC methodology described in chapter two.

6.3.5.2. Water intake

Individual cow two-hourly water intakes were recorded simultaneously with urine sampling, using water meters installed in the metabolism unit.

6.3.5.3. Milk recording and sampling

During each sampling day, cows were milked at four-hourly intervals from 09.00 hours for a twenty four hour period, with milk yields being recorded. Samples collected for milk fat, lactose and protein determinations were preserved, stored at 4°C and analysed by Dairy Technology Department staff at SAC, Auchincruive. Unpreserved milk samples were also collected and immediately stored at -20°C. At the end of the experiment unpreserved milk samples were analysed in random sequence for allantoin using the HPLC methodology described in chapter three.

6.3.5.4. Feed sampling

Feed offered and refused was weighed on each sampling day. Refused feed was bulked for each sampling day with dry matter determinations performed at 60°C for 48 hours. Representative samples of silage, complete diet and concentrate were submitted to the Analytical Services Unit at SAC, Auchincruive for chemical analysis as described in chapter 4 .

6.3.5.5. Cow liveweights

Cows were weighed at the begining of the experiment and at the end of each experimental period at 10.00 hours.

6.4. Results

6.4.1 Animal production

6.4.1.1. DM, CP, ME and calculated FME intakes

Mean treatment intakes of true DM, CP, ME and FME are presented in Table 6.4. DM, CP and ME intakes were not significantly ($P>0.05$) different between experimental treatments. Calculated FME intakes were significantly higher ($P<0.05$), for separate (treatment 1), compared to complete diet feeding (treatment 2).

Table 6.4. Mean treatment true DM, ME, FME and CP intakes

	Treatment 1	Treatment 2	SED	P
Corrected DM intake (kg/d)	20.1	19.5	0.557	>0.05
DM intake (kg/d)	19.1	18.6	0.531	>0.05
ME intake (MJ/d)	246	243	7.1	>0.05
FME intake (MJ/d)	184	173	4.8	<0.05
CP intake (g/d)	3558	3569	98.5	>0.05

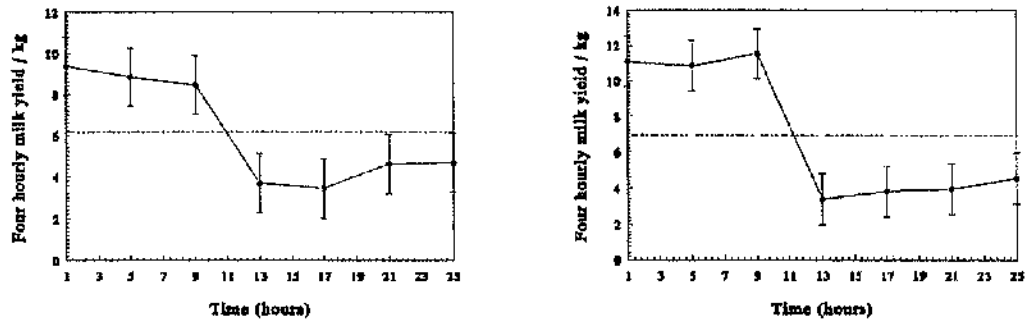
6.4.1.2. Milk yield

Daily milk yields were not significantly different between treatments (means of 28.8 and 27.5 kg/d for treatments 1 and 2, respectively), being significantly different between cows ($P<0.001$) and sampling days ($P<0.001$). Four-hourly milk yields were significantly different between milking times ($P<0.001$), but unaffected by treatment and

milking time interactions. Mean four hour milk yields ranged between 3.5-9.4 kg and 3.4-11.1 kg for treatments 1 and 2, respectively, according to diurnal patterns described in Figure 6.5.

Figure 6.5. Diurnal variations in milk yield

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



Each point represents the mean of 24 measurements
Dotted line represents the daily mean
Error bars indicate SE for diet and sampling time interactions

6.4.1.3. Milk composition

Mean treatment effects on daily mean milk lactose, protein and fat concentrations and daily milk lactose, protein and fat yields are presented in Table 6.5.

Milk lactose concentrations were significantly different between cows ($P<0.001$), marginally affected by sampling day ($P=0.083$), but there was no significant interaction between treatment and sampling hour ($P=0.779$). Mean lactose concentrations of milk collected at four-hour intervals varied markedly between 30.3-52.3 and 29.4-48.6 g/kg for treatments 1 and 2, respectively. Milk protein and fat concentrations were also found to be significantly different between cows ($P<0.001$) but unaffected by sampling day ($P=0.135$ and 0.216, respectively) and treatment and sampling time interactions

(P=0.828 and 0.732, respectively). Mean milk protein concentrations were relatively constant between sampling times ranging between 30.5-31.1 g/kg for treatment 1 and 30.4-31.4 g/kg for treatment 2. Similarly, mean milk fat concentrations were also found to be relatively constant varying between 46.1-47.5 and 46.0-47.0 g/kg for treatments 1 and 2, respectively.

Table 6.5. Mean treatment effects on daily mean milk lactose, fat and protein and fat concentrations (g/kg) and daily milk lactose, protein and fat yields (g/d)

Parameter	Treatment 1	Treatment 2	SED	P
[Lactose]	39.4	36.9	1.76	>0.05
[Protein]	30.8	31.0	0.22	>0.05
[Fat]	47.0	46.7	0.22	>0.05
Lactose yield	1149	1037	90.7	>0.05
Protein yield	756	725	56.7	>0.05
Fat yield	1162	1102	87.0	>0.05

Daily milk lactose yields were significantly different between cows (P<0.001), but unaffected by sampling day (P=0.774). Interactions between sampling times and treatments significantly (P<0.05) influenced four-hour lactose yields as shown in Figure 6.6. Mean four-hour lactose yields varied diurnally between 103-240g for treatment 1 and 112-198g for treatment 2.

Daily milk protein and fat yields were also significantly different between cows (P<0.001) and marginally affected by sampling day (P=0.078 and 0.083, respectively). Mean four-hour milk protein yields were significantly (P=0.01) influenced by treatment and sampling time interactions and ranged between 103-144 and 104-136 g for treatments 1 and 2, respectively (refer to Figure 6.7.). Mean four-hour milk fat yields were also significantly (P<0.05) influenced by sampling time and treatment interactions. Variations in mean four-hour milk fat yields followed a diurnal pattern as shown in Figure 6.8., ranging between 163-226 and 158-213g for treatments 1 and 2, respectively.

Figure 6.6. Diurnal variations in milk lactose yield

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

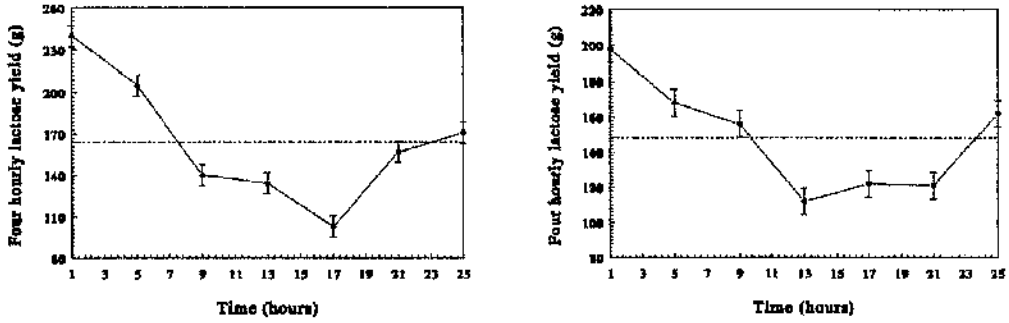
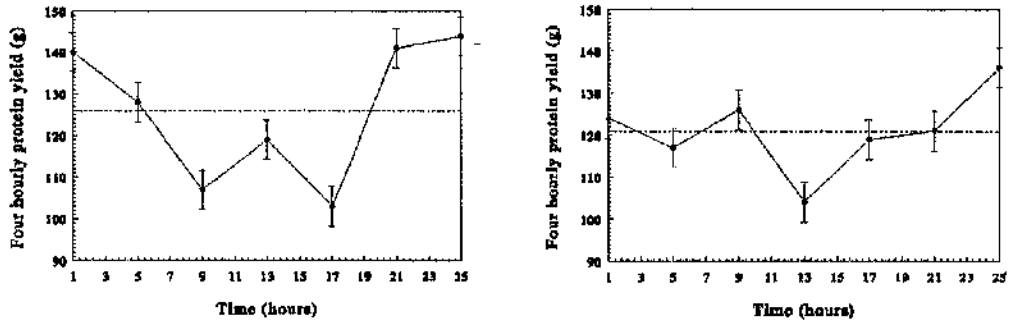


Figure 6.7. Diurnal variations in milk protein yield

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.6.** and **6.7.**:-

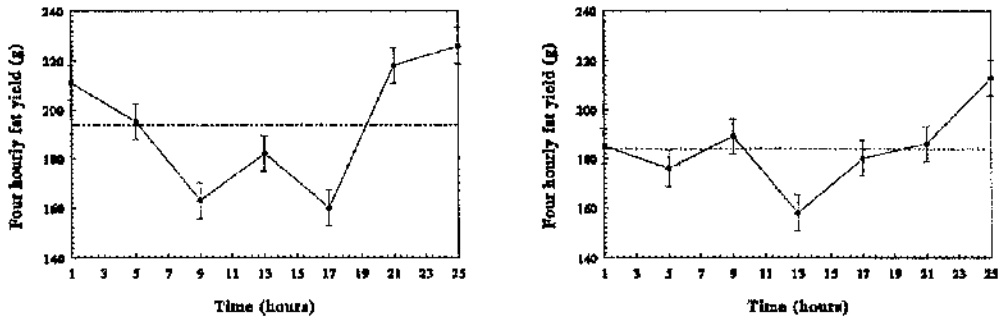
Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

Figure 6.8. Diurnal variations in milk fat yield

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time

6.4.1.4. Cow liveweights

Liveweights were significantly different ($P<0.001$) between individual cows, but unaffected by dietary treatment (means 615 and 617 kg, for treatment 1 and 2, respectively).

6.4.2. Water intake

Daily water intakes were significantly different between cows ($P<0.01$), while differences between treatments and sampling days were not statistically significant. Mean daily water intakes were 47.5 and 46.9 litres/d for treatments 1 and 2, respectively. Two-hour water intakes were significantly influenced by recording time ($P<0.001$) and treatment and recording time interactions ($P<0.001$). Water intakes varied (0.3-12.1 and

0.0-10.2 litres for treatments 1 and 2, respectively) between two hour recording intervals according to a diurnal pattern for both treatments as described in Figure 6.9.

Cows receiving treatment 1 tended to consume water over the first six hours of the experiment, reaching peak intakes during the period of concentrate feeding. Feeding treatment 2 resulted in lower peak water intakes, with water consumption being extended over a longer period.

6.4.3. Urine volume

Daily urinary outputs were not significantly different between treatments (19.1 and 19.9 litres for treatments 1 and 2, respectively), but were significantly different between cows ($P<0.001$) and sampling days ($P<0.001$). Two-hourly urine ouputs were significantly different between sampling intervals ($P<0.001$). Interactions between treatment and sampling time significantly ($P<0.001$) influenced the pattern of urination. For treatment 1, two-hour urinary outputs varied between 1.27-2.14 litres, according to a distinct diurnal pattern (refer to Figure 6.10.), dramatically increasing during the first six hours and then declining. Urine output remained relatively constant thereafter. In contrast, urine outputs for treatment 2 were less variable, ranging between 1.49-1.81 litres. The pattern of urination observed did not conform to a distinct diurnal pattern as observed for treatment 1. Instead urine output was extremely stable for the first twelve hours of sampling, but increased and subsequently decreased during the last twelve hours of sampling as described in Figure 6.10. No clear relationships were observed between daily water intake and daily urine output, or between two-hourly water intake and urine output.

Figure 6.9. Diurnal variations in water intake

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

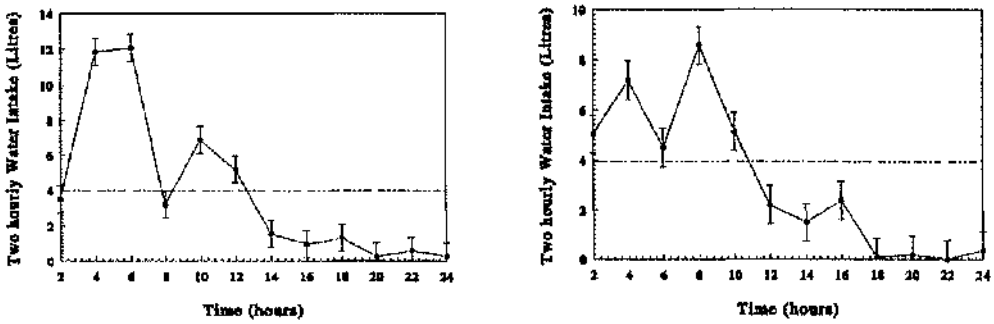
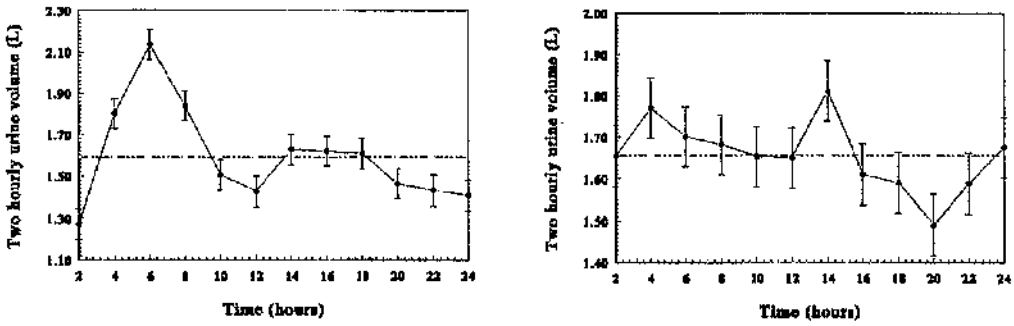


Figure 6.10. Diurnal variations in urinary output

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.9.** and **6.10.**:-

Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

6.4.4. Urinary PD, pseudouridine and creatinine concentrations

Mean treatment effects on urinary total PD, allantoin, uric acid, pseudouridine and creatinine concentrations are shown in Table 6.6. Urinary concentrations of all metabolites were not significantly influenced by experimental treatment.

Table 6.6. Mean treatment effects on urinary PD, allantoin, uric acid, pseudouridine and creatinine concentrations (mM)

Parameter	Treatment 1	Treatment 2	SED	P
Total PDs	10.4	10.6	0.91	>0.05
Allantoin	9.4	9.6	0.82	>0.05
Uric acid	0.91	0.91	0.146	>0.05
Pseudouridine	0.437	0.439	0.050	>0.05
Creatinine	7.11	7.05	0.19	>0.05

6.4.4.1. Urinary PD concentration

Purine derivative concentrations in two-hourly urine samples were highly correlated with allantoin concentrations (r^2=0.980, n=608, P<0.001). Allantoin accounted for 90% of the total PD excreted in urine with the remainder mainly as uric acid. Only trace amounts of xanthine and hypoxanthine were detected.

Absolute PD concentrations of urine samples collected at two-hour intervals were found to be significantly different between cows (P<0.001), sampling intervals (P<0.001) and significantly affected by sampling time and treatment interactions (P<0.05). Mean urinary PD concentrations varied diurnally during the sampling period ranging between 8.7-12.9 mM for treatment 1 (refer to Figure 6.11.). In contrast, mean urinary PD concentrations were less variable between sampling intervals for treatment 2, fluctuating between 9.2-12.0 mM and did not appear to change according to a diurnal pattern (refer to Figure 6.11.).

Changes in mean allantoin concentrations between sampling intervals reflected those of total PD concentrations, ranging between 8.0-11.2 and 9.3-10.8 mM for treatments 1 and 2, respectively. Variations in mean urinary allantoin concentrations between sampling intervals for treatments 1 and 2 are described in Figure 6.12.

Figure 6.11. Diurnal variations in urinary PD concentration

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

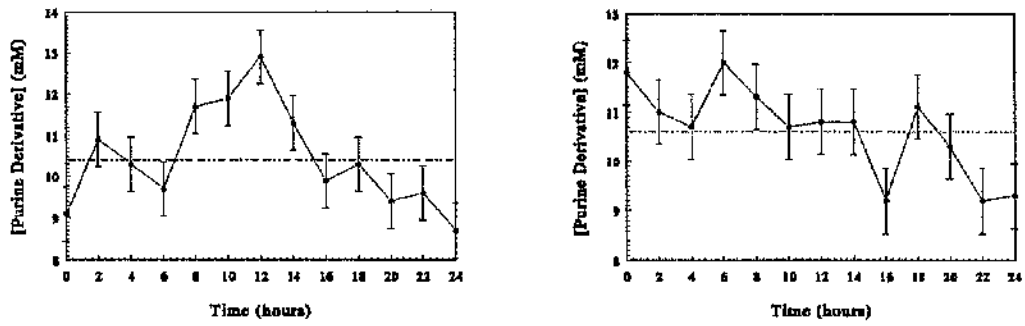
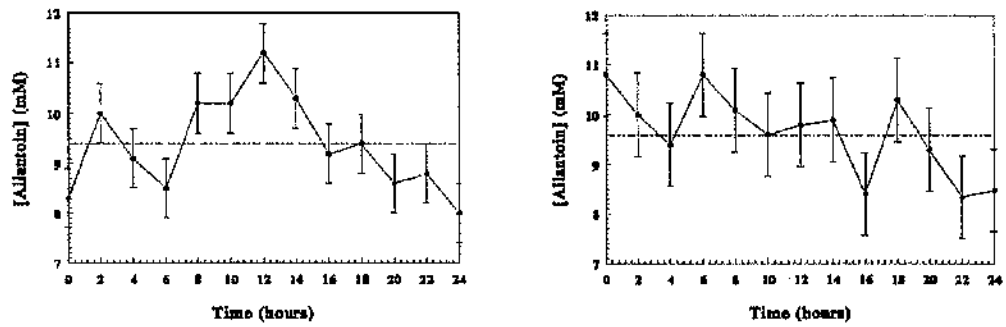


Figure 6.12. Diurnal variations in urinary allantoin concentration

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.11.** and **6.12.**:-

Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

In common with urinary PD and allantoin concentrations, uric acid concentrations were significantly different between cows ($P<0.001$) and sampling times ($P<0.001$). Interactions between treatment and sampling time also significantly ($P<0.01$) influenced urinary uric acid concentrations. Mean uric acid concentrations varied between 0.59-1.51 mM for treatment 1 and 0.69-1.17 mM for treatment 2, following similar patterns as observed for total PD and allantoin concentrations. Coefficients of variation for urinary PD, allantoin and uric acid concentrations due to cow, period, sampling day and sampling hour are presented in Table 6.7.

Table 6.7. Coefficients of variation (CV%) for urinary PD, allantoin and uric acid concentrations due to cow, period, sampling day and sampling hour

Variate	Cow	Period	Sampling day	Sampling hour
Total PD	16.9	6.8	12.2	31.2
Allantoin	16.5	6.8	12.6	30.8
Uric acid	23.9	9.3	16.3	61.2

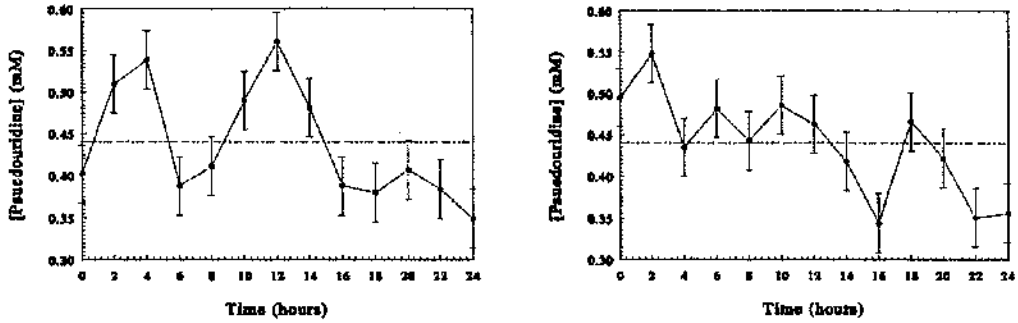
6.4.4.2. Urinary pseudouridine concentration

Urinary pseudouridine concentrations were found to be highly significantly different between cows ($P<0.001$) and sampling intervals ($P<0.001$). Interactions between treatments and sampling times significantly influenced ($P<0.05$) urinary pseudouridine concentrations. Mean pseudouridine concentrations varied considerably between sampling times, ranging between 0.346-0.561 and 0.351 and 0.561 mM for treatments 1 and 2, respectively. Changes in mean urinary pseudouridine concentrations between sampling times followed distinct diurnal patterns for both treatments as shown in Figure 6.13.

Coefficients of variation for urinary pseudouridine concentration due to cow, period, sampling day and sampling hour were 16.8, 8.1, 18.5 and 41.1 %, respectively.

Figure 6.13. Diurnal variations in urinary pseudouridine concentration

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

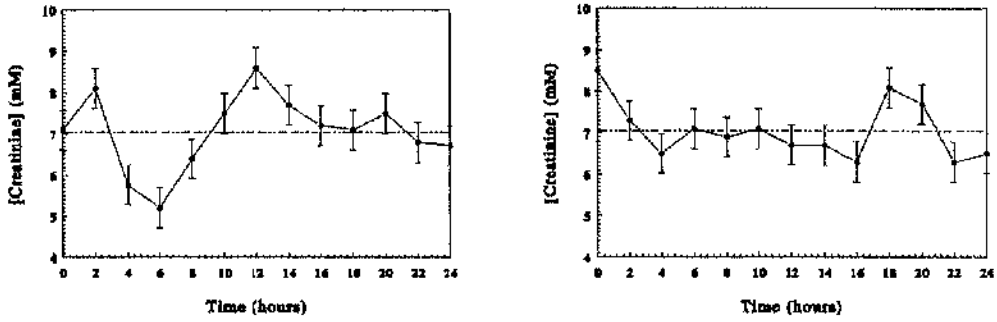
6.4.4.3. Urinary creatinine concentration

Urinary creatinine concentrations were found to be significantly different between cows ($P<0.001$), sampling days ($P<0.05$), sampling times ($P<0.001$). In common with other measured metabolites, interactions between treatments and sampling times significantly ($P<0.01$) influenced urinary creatinine concentrations. Mean urinary creatinine concentration fluctuated between sampling intervals (5.17-8.05 mM) for treatment 1, according to a distinct diurnal pattern. Variations in mean creatinine concentrations for treatment 2, were smaller ranging between 6.31-8.46 mM, and did not appear to conform to a distinct diurnal pattern as observed for treatment 1 (refer to Figure 6.14.).

CV% for urinary creatinine concentration due to cow, period, sampling day and sampling hour, were estimated as 18.3, 6.4, 13.0 and 34.5 %, respectively.

Figure 6.14. Diurnal variations in urinary creatinine concentration

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

6.4.5. Urinary PD, pseudouridine and creatinine excretion

Mean treatment effects on daily urinary PD, allantoin, pseudouridine and creatinine excretion are shown in Table 6.8. Excretion of these metabolites were not significantly (P>0.05) different between treatments. CV% for urinary PD, pseudouridine and creatinine excretion due to cow, period, sampling day and sampling hour are shown in Table 6.9.

Table 6.8. Mean treatment effects on daily urinary PD, allantoin, pseudouridine and creatinine excretion (mmoles/d)

Parameter	Treatment 1	Treatment 2	SED	P
PD	196	207	19.9	P>0.05
Allantoin	178	185	17.9	P>0.05
Pseudouridine	8.06	8.33	0.90	P>0.05
Creatinine	131	132	12.1	P>0.05

Table 6.9. CV% for urinary PD, allantoin, pseudouridine and creatinine excretion due to cow, period, sampling day and hour

Variate	Cow	Period	Sampling day	Sampling hour
PD	14.6	7.4	12.2	36.8
Allantoin	14.8	7.4	12.4	36.2
Pseudouridine	10.5	9.1	17.0	41.6
Creatinine	15.4	7.8	9.1	32.3

6.4.5.1. Urinary PD excretion

Daily PD excretion was highly correlated with daily allantoin excretion (r^2=0.990, n=48, P<0.001). Allantoin accounted for 90% of total PD excreted, with the remainder mostly as uric acid. PD and allantoin excretion was significantly different between cows (P<0.01) and sampling intervals (P<0.001), while interactions between treatments and sampling times and differences between sampling days were not significant (P>0.05).

Mean two-hourly PD excretion varied diurnally for both treatments as shown in Figure 6.15. The extent of variation in two-hourly PD excretion was greater for treatment 1 (12.3-20.9 mmoles) than for treatment 2 (13.6-19.8 mmoles). Mean two-hourly PD excretion was significantly correlated with daily mean PD concentration (r^2=0.390, n=48, P<0.001).

Changes in mean two-hourly allantoin excretion between sampling intervals varied according to a similar pattern observed for PD excretion (refer to Figure 6.16.). Mean two-hour allantoin excretion ranged between 11.3-18.4 and 12.4-17.7 mmoles for treatments 1 and 2, respectively. Mean two-hour allantoin excretion was significantly correlated with daily mean allantoin concentration (r^2=0.390, n=48, P<0.001).

Significant relationships between daily PD or allantoin excretion and corrected dry matter intakes were observed (r^2=0.160, n=48, P<0.01 and r^2=0.180, n=48, P<0.01, respectively).

Figure 6.15. Diurnal variations in urinary PD excretion

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

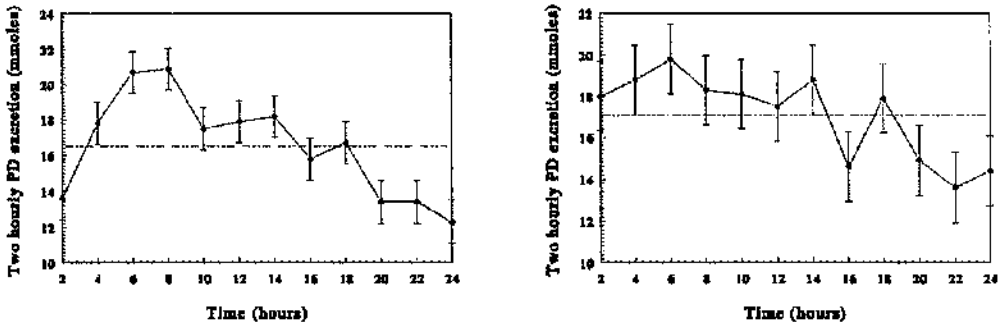
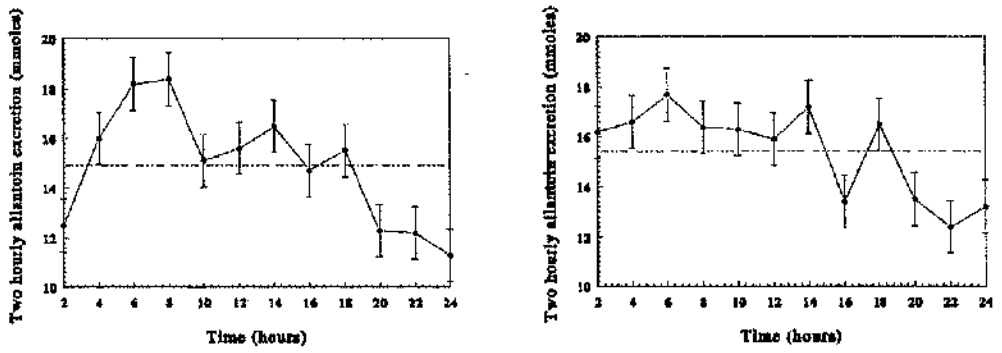


Figure 6.16. Diurnal variations in urinary allantoin excretion

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.15.** and **6.16.**:-

Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

6.4.5.2. Urinary pseudouridine excretion

Two-hour pseudouridine excretion was significantly different between cows ($P<0.01$) and sampling intervals ($P<0.001$). Interactions between treatments and sampling times also significantly influenced pseudouridine excretion ($P<0.05$). As observed for PD and allantoin, mean-two hour pseudouridine excretion varied diurnally between sampling intervals for both diets, while the extent of variation observed was greater for treatment 1 (refer to Figure 6.17.). Mean two-hour pseudouridine excretion varied between 0.46-0.91 mmoles for treatment 1 and 0.52-0.87 mmoles for treatment 2. Daily mean two-hour pseudouridine excretion was significantly correlated with daily mean urinary pseudouridine concentration ($r^2=0.250$, $n=48$, $P<0.05$).

6.4.5.3. Urinary creatinine excretion

Daily creatinine excretion was significantly correlated with daily mean urinary creatinine concentration ($r^2=0.370$, $n=48$, $P<0.001$), liveweight ($r^2=0.600$, $n=48$, $P<0.001$) and metabolic liveweight ($r^2=0.590$, $n=48$, $P<0.001$).

Mean two-hour creatinine excretion was significantly different between cows ($P<0.01$) and sampling time ($P<0.05$), while differences between sampling days and interactions between treatment and sampling times were not significant ($P>0.05$). Diurnal variations were observed in mean two-hour creatinine excretion between sampling times for both treatments. The pattern of variation was distinctly different for each treatment (refer to Figure 6.18.), with mean two-hour creatinine excretion fluctuating between 9.2-12.2 and 9.4-12.6 mmoles for treatments 1 and 2, respectively.

Figure 6.17. Diurnal variations in urinary pseudouridine excretion

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

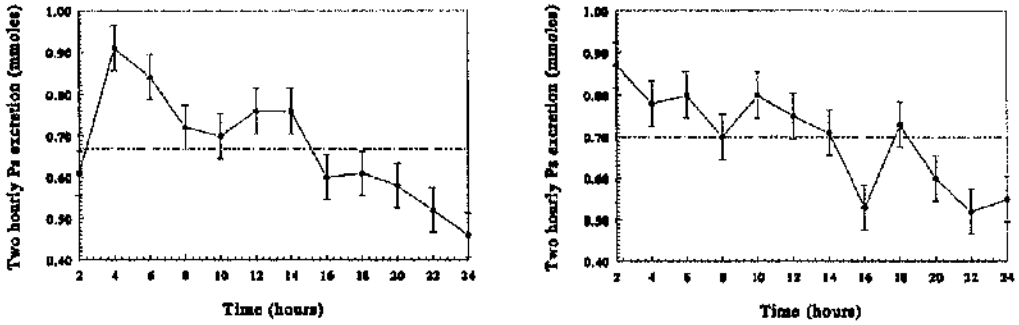
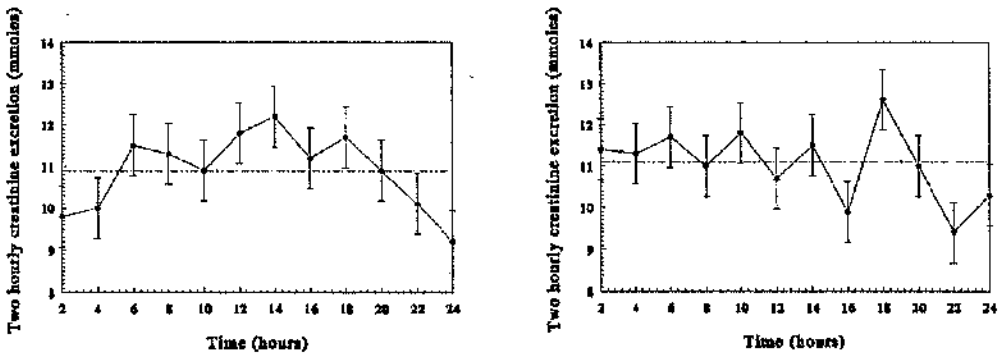


Figure 6.18. Diurnal variations in urinary creatinine excretion

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.17.** and **6.18.**:-

Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

6.4.6. PD/c and Ps/c ratios

Mean treatment effects on daily mean PD/c, A/c and Ps/c ratios are shown in Table 6.10. Daily mean PD/c, A/c and Ps/c ratios, were not significantly different (P>0.05) between treatments. CV% for PD/c, A/c and Ps/c ratios due to the effects of cow, period, sampling day and sampling time are shown in Table 6.11.

Table 6.10. Mean treatment effects on daily mean PD/c, A/c and Ps/c ratios

Parameter	Treatment 1	Treatment 2	SED	P
PD/c	1.55	1.58	0.108	P>0.05
A/c	1.39	1.43	0.096	P>0.05
Ps/c	0.062	0.053	0.0041	P>0.05

Table 6.11. CV% for PD/c, A/c and Ps/c ratios due to cow, period, sampling day and hour

Variate	Cow	Period	Sampling day	Sampling hour
PD/c	7.8	6.3	9.5	26.0
A/c	8.1	6.5	9.5	25.1
Ps/c	10.8	6.5	10.8	27.3

6.4.6.1. Diurnal variation in the PD/c ratio

Daily mean PD/c ratios were highly correlated with A/c ratios (r^2=0.980, n=48, P<0.001). Both daily mean PD/c and A/c ratios were significantly different between cows (P=0.05) and sampling days (P<0.01). PD/c and A/c ratios of two-hourly spot urine samples were significantly different between sampling times (P<0.01). Interactions between sampling times and treatment significantly (P<0.05) influenced two-hour spot sample PD/c and A/c ratios. Mean spot sample PD/c ratios were found to fluctuate between sampling intervals for both treatments (refer to Figure 6.19.). The extent of

variation was greater for treatment 1, with PD/c ratios ranging between 1.31-2.02 and 1.2-1.76 for treatments 1 and 2, respectively. Similar diurnal variations were observed in mean A/c ratios (refer to Figure 6.20.). Mean A/c ratios varied between 1.39-1.74 for treatment 1 and 1.26-1.58 for treatment 2.

Figure 6.19. Diurnal variation in the PD/c ratio

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

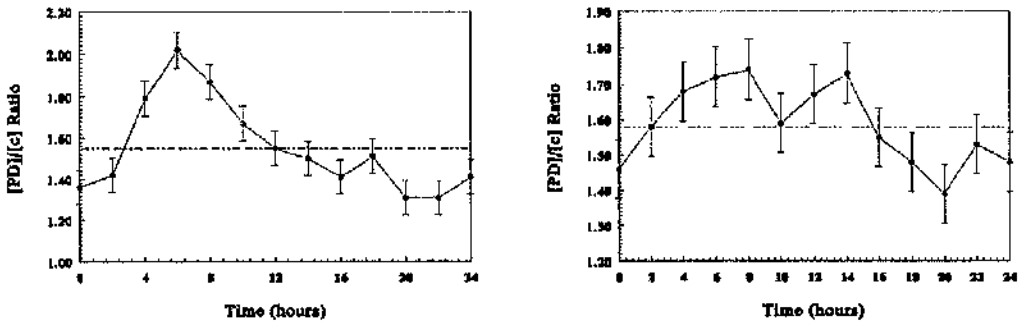
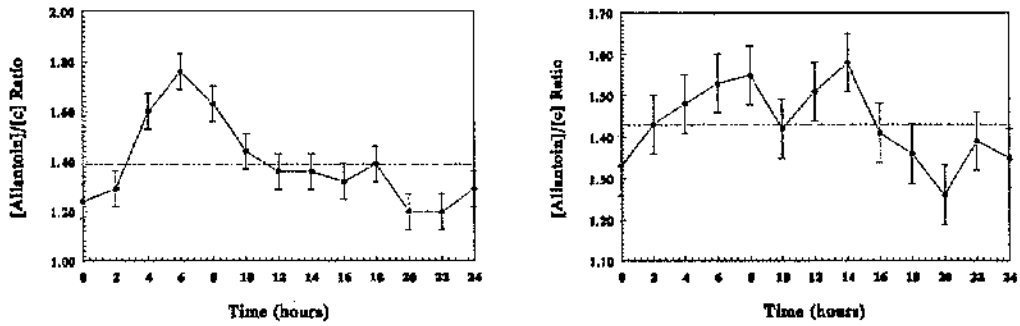


Figure 6.20. Diurnal variation in the A/c ratio

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.19.** and **6.20.**:-

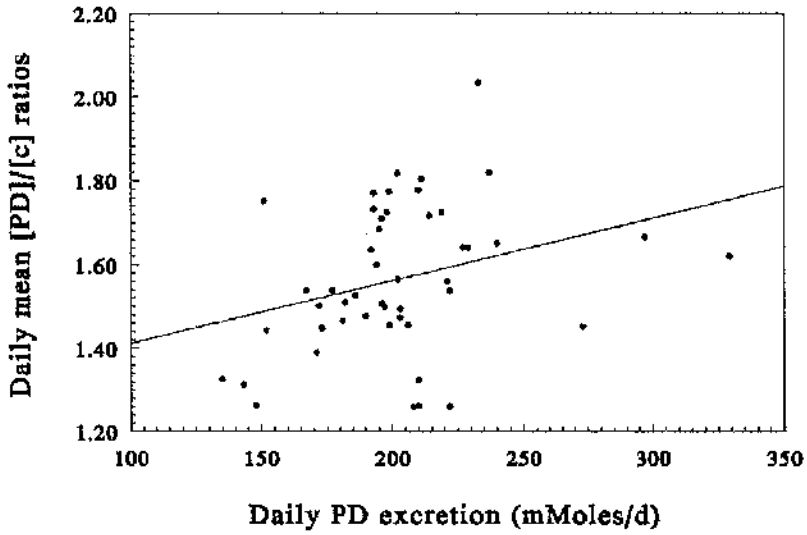
Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

Daily mean PD/c ratios determined from 13 spot samples were significantly related to daily PD excretion (r^2=0.090, n=48, P<0.05). The relationship between daily mean PD/c ratios and daily PD excretion is shown in Figure 6.21. A similar relationship was observed between daily mean A/c ratios and daily allantoin excretion (r^2=0.120, n=48, P<0.05).

Figure 6.21. Relationship between daily mean PD/c ratios and daily PD excretion (r^2=0.090, n=48, P<0.05)



Low r^2 values indicated these relationships would be of little use for predicting daily PD and allantoin excretion from daily mean PD/c and A/c ratios, respectively. Improvements in the prediction of PD and allantoin excretion by accounting for between-cow variations in creatinine excretion was investigated. Daily mean PD/c and A/c ratios were scaled for urinary creatinine concentration, metabolic liveweight, liveweight and daily creatinine excretion. Relationships between scaled daily mean PD/c and A/c ratios and daily PD and allantoin excretion respectively, are presented in Table 6.12.

Table 6.12. Relationships between scaled daily mean PD/c and A/c ratios with daily PD and allantoin excretion

Scaling Factor	PD/c		A/c	
	r^2	P	r^2	P
None	0.09	<0.05	0.12	P<0.05
[Creatinine]	0.39	<0.001	0.39	<0.001
Metabolic liveweight	0.42	<0.001	0.48	<0.001
Liveweight	0.48	<0.001	0.52	<0.001
Liveweight squared	0.46	<0.001	0.49	<0.001
Liveweight and [creatinine]	0.10	<0.05	0.10	<0.05
Creatinine excretion	0.94	<0.001	0.95	<0.001

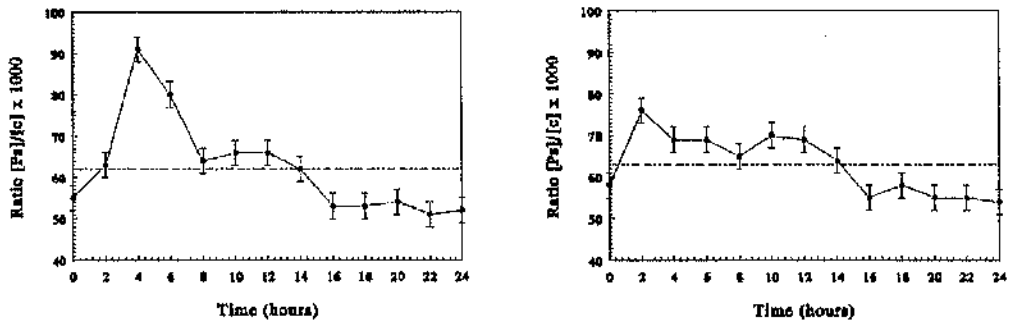
The best prediction of PD excretion was attained by scaling daily mean PD/c ratios for daily creatinine excretion, indicating that between-cow variability in daily creatinine excretion was responsible for the poor prediction of PD excretion. However, this is of no practical value as assessment of creatinine excretion requires a total urine collection to be performed, in which case PD excretion would be measured. Relationships derived using other measured parameters indicated the best prediction of PD excretion was obtained by scaling daily mean PD/c ratios by liveweight, but even then this relationship could only account for half the variation observed in PD/c ratios.

6.4.6.2.Diurnal variation in the Ps/c ratio

Daily mean Ps/c ratios were found to be significantly different between cows ($P<0.01$) while differences between sampling days were not significant ($P>0.05$). Mean Ps/c ratios of urine samples collected at two-hour intervals were significantly different between sampling times ($P<0.001$) and significantly influenced by sampling time and treatment interactions ($P<0.05$). Mean two-hour Ps/c ratios fluctuated diurnally for both treatments, as shown in Figure 6.22. Mean two-hour Ps/c ratios ranged between 0.051-0.091 and 0.054-0.076 for treatments 1 and 2, respectively.

Figure 6.22. Diurnal variations in the Ps/c ratio

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

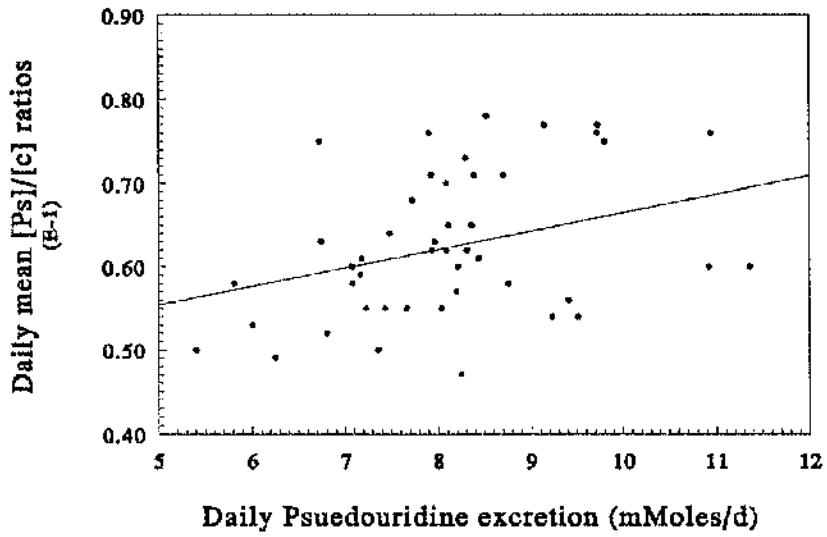


Each point represents the mean of 24 measurements

Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

Figure 6.23. Relationship between daily mean Ps/c ratios and daily pseudouridine excretion (r^2=0.113, n=48, P<0.05)



Daily mean Ps/c ratios determined from 13 spot samples were significantly related to daily pseudouridine excretion (r^2=0.113, n=48, P<0.05) and are shown in Figure 6.23. Improvements in the prediction of pseudouridine excretion by accounting for between-cow variations in creatinine excretion were investigated. Daily mean Ps/c ratios were scaled for urinary creatinine concentration, metabolic liveweight, liveweight and daily creatinine excretion. Relationships between scaled Ps/c ratios and daily pseudouridine excretion are presented in Table 6.13.

Table 6.13. Relationships between scaled daily mean Ps/c ratios and daily pseudouridine excretion

Scaling Factor	r^2	P
None	0.11	<0.05
[Creatinine]	0.37	<0.001
Metabolic liveweight	0.32	<0.001
Liveweight	0.38	<0.001
Liveweight squared	0.48	<0.001
Liveweight and [creatinine]	0.14	<0.01
Creatinine excretion	0.96	<0.001

The best prediction of pseudouridine excretion was attained by scaling daily mean PD/c ratios for daily creatinine excretion, indicating between-cow variability in daily creatinine excretion was responsible for the poor prediction of psuedouridne excretion. However, assessment of creatinine excretion requires a total urine collection to be performed, in which case pseudouridine excretion would be measured.

Relationships derived using other measured parameters indicated that the best prediction of pseudouridine excretion was obtained by scaling daily mean PD/c ratios by liveweight squared, but even then this relationship could only account for half the variation observed in Ps/c ratios.

6.4.7. Sensitivity of spot urine sampling

In order to estimated daily PD, allantoin and pseudouridine excretion using spot urine sampling technique, it is essential that PD/c, A/c and Ps/c ratios of collected samples reflect the daily mean. Estimates of PD/c, A/c and Ps/c from spot samples collected at each sampling interval were compared to the daily mean, for each sampling day resulting in a total of 52 correlations. Each correlation contained 12 data points. Mean correlation coefficients for each sampling interval are presented in Table 6.14. Overall mean correlation coefficients between daily mean PD/c, A/c and Ps/c ratios and single spot sample estimates were 0.478, 0.486 and 0.558, respectively.

Table 6.14. Mean correlation coefficients (n=4) between single sample and daily mean PD/c, A/c and Ps/c ratios

Sampling time (hours)	PD/c	A/c	Ps/c
0	0.458	0.457	0.473
2	0.581	0.622	0.507
4	0.415	0.391	0.522
6	0.564	0.559	0.633
8	0.612	0.619	0.361
10	0.382	0.414	0.486
12	0.548	0.565	0.636
14	0.410	0.393	0.620
16	0.461	0.459	0.495
18	0.445	0.478	0.576
20	0.517	0.573	0.640
22	0.427	0.402	0.747
24	0.401	0.390	0.559

In practice, more than one spot sample would be used to assess daily mean PD/c, A/c and Ps/c ratios. Estimates of PD/c, A/c and Ps/c based on the mean of two samples collected at 14.00 and 20.00 hours were compared to daily mean ratios, resulting in four correlations each based on 12 data points. Mean correlation coefficients between two

sample estimates and daily mean PD/c, A/c and Ps/c ratios were 0.706, 0.701 and 0.782, respectively. Accuracy of spot sampling was assessed further by comparing estimates obtained from either the mean of four spot samples collected at 4 hour intervals or three spot samples collected at 8 hour intervals or two samples collected at 12 hour intervals to daily mean ratios for each sampling day. Sampling regimens estimates were derived from all the spot samples collected. Correlation coefficients between sampling regimen estimates and daily means are summarised in Table 6.15.

Table 6.15. Summary of correlation coefficients between sampling regimen estimates and daily mean PD/c, A/c and Ps/c ratios

Variate	Sampling regimen (hours)	n	Mean	Median	Minimum	Maximum
PD/c	12	28	0.60	0.68	0.17	0.87
	8	20	0.72	0.76	0.19	0.92
	4	28	0.75	0.75	0.43	0.95
A/c	12	28	0.62	0.68	0.27	0.87
	8	20	0.72	0.75	0.22	0.92
	4	28	0.76	0.77	0.48	0.95
Ps/c	12	28	0.70	0.69	0.35	0.92
	8	20	0.77	0.75	0.58	0.96
	4	28	0.82	0.84	0.68	0.93

This process was repeated using estimates based on spot samples obtained over two sampling days to investigate whether accuracy could be improved by extending the observation period. Correlation coefficients between sampling regimen estimated and daily means for the two day collection period are summarised in Table 6.16.

Table 6.16. Summary of correlation coefficients between sampling regimen estimates and daily mean PD/c, A/c and Ps/c ratios based on the mean of two 24 hour urine collections

Variate	Sampling regimen (hours)	n	Mean	Median	Minimum	Maximum
PD/c	12	8	0.636	0.697	0.117	0.969
	8	16	0.802	0.888	0.199	0.979
	4	24	0.787	0.796	0.537	0.951
A/c	12	8	0.673	0.718	0.162	0.973
	8	16	0.828	0.887	0.411	0.973
	4	24	0.813	0.822	0.701	0.955
Ps/c	12	8	0.756	0.839	0.233	0.963
	8	16	0.840	0.887	0.403	0.981
	4	24	0.852	0.887	0.649	0.998

Collection of multiple spot samples improved the correlation between sampling regimen estimates and the daily mean. Collection of spot samples over two days improved the accuracy of spot sampling further. Even under the most intensive sampling regimen (4 hourly sampling) over two day periods, only modest correlations between sampling estimates and two day mean PD/c, A/c and Ps/c ratios existed (means of 0.787, 0.813 and 0.852, respectively). More importantly minimum correlations between sampling estimates and two day means remained poor (0.537, 0.701 and 0.649 for PD/c, A/c and Ps/c ratios, respectively).

6.4.8. Milk allantoin concentration and excretion

Mean treatment effects on daily mean milk allantoin excretion and allantoin concentration are presented in Table 6.17. Daily mean milk allantoin excretion and concentration were not significantly different between treatments ($P>0.05$).

Table 6.17. Mean treatment effects on daily mean milk allantoin concentration and excretion

Parameter	Treatment 1	Treatment 2	SED	P
Concentration (mM)	0.129	0.141	0.0161	P>0.05
Excretion (mmoles/d)	3.14	3.34	0.443	P>0.05

6.4.8.1. Milk allantoin concentration

Daily mean milk allantoin concentrations were not significantly different between cows (P>0.05) or sampling day (P>0.05). Mean milk allantoin concentration of samples collected at four hour intervals were not significantly different between sampling times (P>0.05). No clear relationship was observed between milk yield and milk allantoin concentration during each four hour period.

Variations in mean milk allantoin concentrations between sampling times were found to be small and did not conform to a distinct diurnal pattern. The extent of variation in mean milk allantoin concentrations was greater for treatment 1 (0.115-0.143 mM) than for treatment 2 (0.134-0.147 mM) as shown in Figure 6.24.

6.4.8.2. Milk allantoin excretion

Daily milk allantoin excretion was significantly different between cows (P<0.01), but not between sampling days (P>0.05). Mean four-hour milk allantoin excretion was found to be significantly different between sampling times (P<0.01) but was unaffected by interations between treatments and sampling times (P>0.05). The extent of diurnal variation in four-hour allantoin excretion was greater for treatment 1 (refer to Figure 6.25.). Mean four-hour allantoin excretion ranged between 0.413-0.664 and 0.469-0.655 mmoles for treatments 1 and 2, respectively. Diurnal variations in mean allantoin excretion between sampling intervals were distinctly different from the diurnal variations observed in milk yield, for both treatments.

Figure 6.24. Diurnal variations in milk allantoin concentration

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)

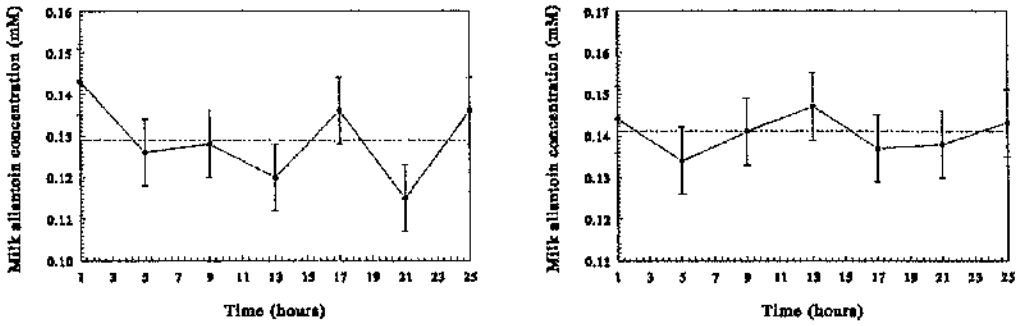
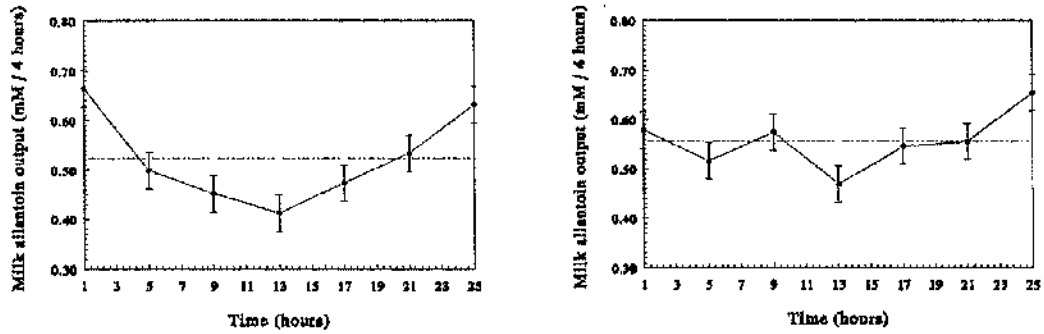


Figure 6.25. Diurnal variations in milk allantoin output

i) Treatment 1 (separate feeding) ii) Treatment 2 (complete diet)



For Figures **6.24.** and **6.25.**:-

Each point represents the mean of 24 measurements

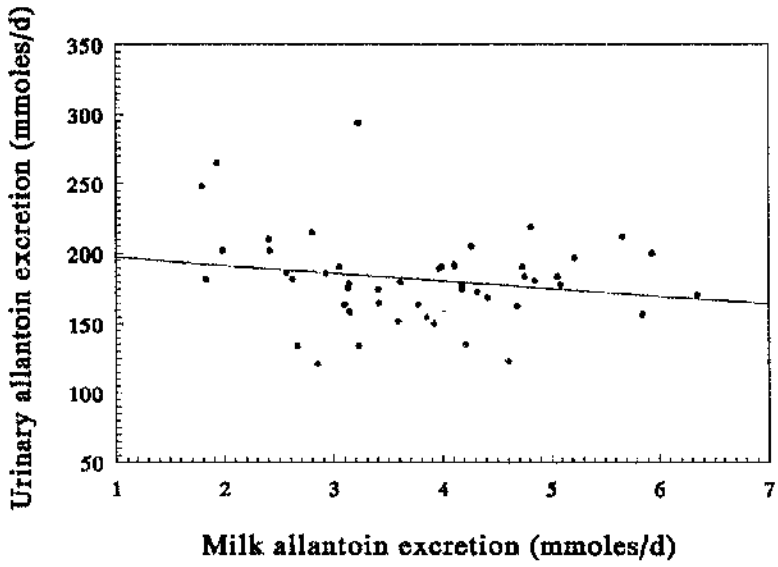
Dotted line represents the daily mean

Error bars indicate SE for diet and sampling time interactions

Based on four-hour measurements, allantoin excretion was found to be closely correlated with allantoin concentration (r^2=0.610, n=331, P<0.001) and to a lesser extent with milk yield (r^2=0.330, n=331, P<0.001).

Mean daily milk allantoin excretion represented 1.76 and 1.81% of daily mean urinary allantoin excretion for treatments 1 and 2, respectively. Based on individual cow observations, no significant relationship (r^2=0.035, n=48, P=0.204) existed between daily milk and urinary allantoin excretion (refer to Figure 6.26.).

Figure 6.26. Relationship between daily urinary and milk allantoin excretion (r^2=0.035, n=48, P=0.204)



6.5. Discussion

No significant differences in DM intake, milk yield or milk composition were observed between separate and complete feeding regimens. These observations confirm much earlier work of Stanley and Morita (1967). Daily milk yields were marginally higher for cows fed silage and concentrates separately, and can be partially explained by their slightly higher ME intakes. Based on treatment means, efficiency of milk production

(expressed as kg milk/per MJ ME) appeared to be higher for separate (0.117) compared to complete diet feeding (0.113). This difference may be explained by altered mobilisation of energy (fat) stores which was not measured in this experiment.

Daily milk yields measured in this experiment are likely to be artifically high due frequent milking. Numerous experiments have established that milk yields of cows milked twice-daily are lower than those milked thrice-daily (as documented in the review of Elliot, 1959). More recently, Morag (1973) demonstrated a significant 11% increase in milk yield in Friesian cows milked three-times, over twice-daily milking. In the current experiment, milk yields were not measured on non-sampling days during which cows were milked twice daily. Consequently, assessing the effects of very frequent (seven times) milking on daily milk yield is not possible.

Milk fat, protein and lactose yields were found to vary diurnally between milking intervals. Changes in milk yield between milking intervals can to some extent explain these observations. However, it is recognised that, for instance milk fat concentration, is largely influenced by fermentation patterns occuring in the rumen (Sutton, 1989). It would therefore seem feasible that changes in rumen fermentation patterns, absorption of fermentation products and post-rumen nutrient supplies could influence the quantity of substrates available for the synthesis of milk fat, protein and lactose influencing milk fat, protein and lactose yields.

Daily urinary PD excretion was greater for the complete diet, compared to separate feeding. Employing the models proposed by Verbic *et al* (1990) and Chen *et al* (1992a) enables M-N supply to be estimated from PD excretion as 127 and 136 g/d for separate and complete diets, respectively. As DM intakes were slightly lower for the complete diet, this implies that the EMPS was improved by feeding silage and concentrate as a complete diet (6.97g M-N/kg DM) compared to separate feeding (6.32g M-N/kg DM). It has been recognised that decreases in rumen pH below 6.0 can result in partial washout of rumen microflora (Mould and Orskov, 1983) and inhibition of rumen cellulolytic bacteria (Mould and Orskov, 1983 and Mould *et al*, 1983) while further reductions below 5.5 may lead to serious disturbances in rumen fermentation (Counotte and Prins, 1979). Rapid reduction in rumen pH are likely to reduce EMPS by inhibiting

cellulolytic bacterial growth and increasing the energetic cost of maintaining the integrity of bacterial cells (Malestein *et al*, 1981). Feeding concentrates as a single meal results in a rapid intake of easily fermentable compounds, stimulating the production of rumen VFAs causing a strong decrease in rumen pH (Malestein *et al*, 1981). Depressions in rumen pH tend to be smaller during frequent compared to less frequent concentrate feeding in dairy cows (e.g. McCullough and Smart, 1968 and Rohr and Daenicke, 1973) and occur for shorter periods (Malestein *et al*, 1981). The duration of pH depression is an important factor determining the extent of inhibition of rumen bacteria cellulolytic activity (Istasse and Orskov, 1983). Differences observed in EMPS in the current experiment would appear to be a consequence of diet induced rumen pH changes.

Allantoin accounted for 90% of total urinary PD excretion. This is in good agreement with observations of Chen *et al* (1990c), Verbic *et al* (1990), Giesecke *et al* (1994), Gonda and Lindberg (1994), Dewhurst *et al* (1995 and 1996) and also agrees with the results reported in chapter 5. The remaining PD was excreted as uric acid. Hypoxanthine and xanthine excretion was negligible, confirming the observations of Chen *et al* (1990), Verbic *et al* (1990), Susmel *et al* (1994b) and Dewhurst (1995).

Total urinary PD and allantoin excretion were found to vary diurnally between two-hour collections, with the extent of variation being greater for the separate feeding regimen. Only the work of Chen *et al* (1992d) has previously reported diurnal changes in PD excretion, the extent of which was little affected by feeding frequency. Chen *et al* (1992d) argued that the observed variation was due to end-of-urine collection errors, rather than variations in purine absorption. In an attempt to reduce end-of-collection errors in this experiment, cows were induced to urinate twice at each collection interval to ensure all urine stored in the bladder was voided. Thus end-of-collection errors are unlikely to account for observed diurnal variations in urinary PD and pseudouridine excretion.

Purines entering the ruminant small intestine are essentially microbial in origin (McAllan, 1982), being readily digested and absorbed (Ellis and Bleichner 1969b; Condon *et al*, 1970; Smith and McAllan, 1971; Coelho da Silva *et al*, 1972b; Jackson *et al*, 1976; McAllan, 1980; Storm and Orskov, 1983 and Chen *et al*, 1990a). Renal

clearances of plasma PDs have been shown to be rapid (Greger *et al*, 1976; Chen *et al*, 1991a and Gisecke *et al*, 1993), implying that rates of urinary PD excretion would closely reflect variations in purine absorption. *In-vitro* studies have demonstrated that purine content of rumen microbes varies markedly according to growth conditions (Nikolic and Javonovic, 1973; Bergen *et al*, 1982 and Bates and Bergen, 1984) while *in-vivo* studies have indicated variations in purine concentration of rumen bacteria over the entire feeding cycle (Craig *et al*, 1987 and Cecava *et al*, 1990b). Both plasma PD concentrations and urinary PD excretion respond rapidly and concurrently with changes in duodenal exogenous purine supplies (Chen and Fujihara, unpublished, cited by Chen *et al*, 1995). It therefore seems plausible that variations in PD excretion could be a reflection of changes in the relative proportions of microbial species entering the duodenum. However, it is unlikely that changes in purine concentration of rumen bacteria entering the small intestine alone could account for observed variations in urinary PD excretion. Several studies have indicated that EMPS assessed by urinary PD excretion, increases with feed intake due to increases in rumen fractional outflow rates (Chen *et al*, 1992b; Dewhurst and Webster 1992a and 1992b and Djouvinov and Todorov, 1994). In the current experiment, daily DM intakes were not constant with the consequence that flows of rumen microbes entering the duodenum might also vary. Variations in flow of microbes leaving the rumen and changes in the purine content of rumen bacteria would inevitably lead to fluctuations in microbial purines avaliable for absorption and may explain variations in urinary PD excretion. In retrospect, it would have been desirable to have assessed variations in duodenal purine flows. Direct measurement would not have been possible due to requirement for experimental cows to be fitted with duodenal re-entrant cannulae. Rapid renal clearances of plasma PDs (Greger *et al*, 1976; Chen *et al*, 1991a and Giesecke *et al*, 1993) indicate that regular measurements of plasma PD concentrations could have been employed. If as is suggested, diurnal variations in purine absorption account for variations in PD excretion, these changes would have been reflected by fluctuations in plasma PD concentration which have been demonstrated in other experiments (Rosskopf *et al*, 1991; Rosskopf and Giesecke, 1992; Giesecke *et al*, 1993 and Irki *et al*, 1994).

Although the majority of PDs are excreted renally in cattle (Verbic *et al*, 1990 and Beckers and Thewis, 1994), PDs are also excreted in milk (Rosskopf *et al*, 1991; Rosskopf and Giesecke, 1992 and Giesecke *et al*, 1994), saliva (Chen *et al*, 1990d; Kahn and Nolan, 1993 and Surra *et al*, 1993), gastro-intestinal secretions (Kahn and Nolan, 1993) or by direct passage into the gut (Sorenson, 1978). Diurnal changes in non-renal PD excretion could influence the magnitude of the plasma PD pool, thereby influencing the quantity of PDs cleared by the kidney. Thus, physiological changes within an animal could potentially have contributed to the observed variations in the rate of urinary PD excretion.

Pseudouridine, a modified nucleoside present in RNA molecules (Gehrke *et al*, 1979), is produced as a result of tissue RNA degradation (Borek and Keer, 1972) and is not re-utilised or catabolised further (Weissman *et al*, 1962). RNA and transfer-RNA (the main source of pseudouridine) are directly involved in protein synthesis and therefore urinary pseudouridine excretion has been suggested as a potential indicator of nitrogen status in ruminant species (Puchala *et al*, 1993). Daily pseudouridine excretion was not significantly different between feeding regimens indicating that RNA turnover and presumably protein synthesis was similar in cows fed both experimental diets. However, rates of urinary pseudouridine excretion varied diurnally for both separate and complete diet feeding, the extent of variation being greater for separate feeding. These findings suggest that, not only do rates of protein synthesis change within an animal, but appear to be influenced by nutrient supply. The reasons for this are unclear, but it is interesting to speculate that protein synthetic activities might be related to the supply of amino acid substrates absorbed from the small intestine. Generally, pseudouridine excretion was higher during the first 14 hours of sampling for both experimental diets, which is concurrent with higher rates of PD excretion. Furthermore, variations in the rate of pseudouridine excretion followed similar patterns as observed for PD excretion. These findings tentatively suggest that, during periods of more rapid RNA turnover, more MCP was available for absorption from the small intestine.

Mean daily urinary creatinine excretion of 131 and 132 mmoles/d for separate and complete diets, respectively were slightly higher than previous reports of between

112-117 mmoles/d (Puchala *et al*, 1993; Susmel *et al*, 1994a and Gonda and Lindberg, 1994). Although cow liveweights in the current experiment were lower than those in the studies of Susmel *et al* (1994a) and Gonda and Lindberg (1994) it is conceivable that differences in musculature between breeds used could account for discrepancies in creatinine excretion. Studies in humans have indicated that urinary creatinine excretion is more closely correlated with muscle mass than bodyweight (Clark *et al*, 1951, cited by Narayanan and Appleton, 1981). Between-day variability in creatinine excretion was not significant (CV 9.1%), being lower than the values of 11 and 15 % previously reported by Chen *et al* (1992d) and Daniels *et al* (1994), respectively. Significant ($P<0.05$) differences in daily creatinine excretion were observed between cows ($P<0.05$), confirming earlier observations of Chetal *et al* (1975). The extent of this variation (CV 15.4%) was larger than the 8% reported in steers (Chen *et al*, 1992d) and 12% reported in cows (Daniels *et al*, 1994) but is similar to 12-18.6% (Lindberg and Jacobssen, 1990) and 15.5% (Dewhurst, 1989) reported in sheep.

Variations in urinary creatinine excretion were found to be larger between two-hour intervals (CV 32.3%), than between individual cows (CV 15.4%) or sampling days (CV 9.1%). Use of creatinine excretion as a urine output marker as proposed by deGroot and Aafjes (1960), Albin and Clanton (1966) and Erb *et al* (1977) assumes that its excretion rate is constant. Current work indicated creatinine excretion varied diurnally, confirming the observations of Chen *et al* (1992d). The extent of within-day and between-cow variations in urinary creatinine excretion observed in the current experiment precludes its use as an index of urinary output in individual animals, confirming the findings of Dewhurst (1989) who also rejected the use of creatinine excretion as a marker of urinary output due to large between-animal variations.

In common with urinary PD and pseudouridine concentration and excretion, greater variations in PD/c and Ps/c ratios were observed between sampling intervals, than between sampling days or cows. Sampling time variations in urinary PD and pseudouridine concentrations (CV% 31.2 and 41.1, respectively) and excretion (CV% 36.8 and 41.6%, respectively) were dramatically reduced by expressing PD and pseudouridine concentrations as a molar ratio to creatinine (CV% 26.0 and 27.3,

respectively). The observed reduction of variation due to sampling time by using urinary creatinine excretion to account for changes in urinary output as suggested by Antoniewicz *et al* (1981) confirms observations reported by Chen *et al* (1992d) and Gonda and Lindberg (1994) and in chapter 5. Variations in PD/c and A/c ratios between sampling times (26.0 and 25.1%, respectively) observed in the current experiment were much larger than sampling time coefficients of variation found by other researchers for sheep of 5-10% (Antoniewicz *et al*, 1981), 6.6% (Chen *et al*, 1993b) and 16.0% (Chen *et al*, 1995) or for cattle of 2% (Chen *et al*, 1992d) and 11.4% (Daniels *et al*, 1994). In contrast, sampling time variations reported here are very similar to those reported in chapter 5 (CV% of 25.1 and 23.6%, for PD/c and A/c ratios, respectively).

Variations in urinary PD/c and A/c ratios between sampling intervals followed diurnal patterns for both experimental diets, the extent of which was greater when cows were offered silage and concentrate separately. Observed diurnal variations in PD/c and A/c ratios support the findings of Puchala and Kulasek (1992) and Moorby and Dewhurst (1993b), while numerous other experiments have reported little or no diurnal variation in PD/c ratios (Antoniewicz *et al*, 1981; Chen *et al*, 1992d, 1993b, 1995; Daniels *et al*, 1994; Gonda and Lindberg, 1994 and Dewhurst *et al*, 1996). Variations in PD/c and A/c ratios in this experiment were due to diurnal variations in the rates of PD, allantoin and creatinine excretion. The current experiment has shown that the pattern of urinary PD excretion may be influenced by nutrient supply. Therefore it would seem plausible that different experimental feeding regimens would precipitate variations in the rate of urinary PD excretion, the extent of which would be dependent on the type and amount of feedsuff fed. Consequently, variations in rates of PD excretion would influence PD/c ratios of spot urine samples collected at any given sampling time, and would make it difficult to establish a valid spot sampling protocol.

Diurnal variations were also observed in two-hourly spot sample Ps/c ratios due to diurnal variations in the rate of urinary pseudouridine and creatinine excretion.

The assessment of urinary PD or pseudouridine excretion from the collection of spot urine samples is dependent on:- i) diurnal variations in PD/c and Ps/c ratios being small and ii) spot sample PD/c and Ps/c ratios being highly correlated with daily PD and

pseudouridine excretion (Chen *et al*, 1995). The existence of diurnal variation complicates the use of spot sampling due to difficulty of selecting the optimum sampling schedule to collect representative samples. Mean, median, minimum and maximum correlation coefficients of mean PD/c and Ps/c ratios collected at two-hour intervals with daily mean PD/c and Ps/c ratios (0.48, 0.53, 0.01, 0.83 and 0.56, 0.57, 0.08, 0.92, repectively) indicated that single spot samples would poorly predict daily mean PD/c and Ps/c ratios. These observations agree with those of Chen *et al* (1992d), who reported correlations between spot sample A/c ratios with daily mean A/c ratios to be dependent on time of sample collection.

Chen *et al* (1995) showed that variations in PD/c ratios due to sampling time for single spot samples of 16% was reduced to 8% by taking the mean of four or more samples. Following these findings the researchers argued that multiple spot samples are required to assess daily mean PD/c ratios. In the current investigation, the data was used to assess the accuracy of three sampling regimens, based on collecting two spot samples collected at 12 hour intervals, three at 8 hour intervals and four at 4 hour intervals. Correlations between sampling regimen PD/c and Ps/c estimates with daily mean PD/c and Ps/c ratios indicated that more reliable estimates of daily mean ratios were derived from more frequent sampling. It is important to recognise that, while mean correlation coefficients for any given sampling regimen, may be acceptable, it is the minimum correlation coefficient that should be closely scrutinised. In any given situation, variations in PD/c and Ps/c ratios are unknown in advance. Consequently, collection of a spot sample might be highly or poorly representative of the daily mean. Accepting these uncertainties, it is would be sensible to consider the worst-case scenario. Based on this criteria, none of the sampling regimens gave acceptable estimates of the daily mean PD/c and Ps/c ratios. In an attempt to improve the accuracy of spot sampling further, sampling regimen estimates were derived based on the mean of two sampling days. In most cases correlations between sampling regimen estimates and daily mean ratios were improved. Of all the sampling regimens tested, collection of four spot samples collected at 4 hour intervals over two days proved to be the most accurate approach. These findings support the arguments of Daniels *et al* (1994) and Chen *et al* (1995) advocating multiple spot

sample collection to accurately estimate daily mean PD/c ratios. However, minimum correlations coefficients between sampling regimen PD/c, A/c and Ps/c estimates and the daily mean PD/c, A/c and Ps/c ratios were found to be 0.537, 0.701 and 0.649, respectively, indicating that none of the estimates derived even for the most intensive sampling regimens would always allow reliable predictions of daily mean PD/c, A/c and Ps/c ratios. Furthermore it is questionable whether collection of large numbers is feasible.

The experiment showed that, if total urine collection facilities are not available and spot urine sampling is the only means of assessing urinary PD and pseudouridine excretion, then multiple samples need to be collected over several sampling days. If the quantity of spot samples able to be collected was constrained to four per cow, the experimental data indicated that a more reliable estimation of daily mean PD/c and Ps/c ratio would be obtained by collecting four samples at 4 hour intervals on a single sampling day than by collecting two spot samples at 12 hour intervals over two sampling days, supporting the arguments made in chapter 5.

Prediction of daily PD from daily mean PD/c was best achieved by scaling the ratios for liveweight (r^2=0.48, n=48, P<0.001) agreeing closely with the relationships observed in cattle by Daniels *et al* (1994). Closer correlations of 0.92 (Chen *et al*, 1995) and 0.86 (Lindberg, 1985) have been reported in sheep and goats, respectively. It appears that daily PD/c ratios are less closely related to daily PD excretion in cattle than in sheep and goats. The best prediction of daily pseudouridine excretion from daily mean Ps/c ratio was obtained by scaling the ratios by liveweight squared (r^2=0.480, n=48, P<0.001). The quantitative relationships derived between mean daily PD/c and Ps/c ratios and daily PD and pseudouridine excretion indicate that, even if sufficient spot samples were collected reliably to estimate daily mean PD/c and Ps/c ratios, prediction of daily PD and pseudouridine outputs would be subject to unacceptable errors. In contrast to the observations of Daniels *et al* (1994) and Gonda and Lindberg (1994), current experimental findings indicate that a total urine collection is needed to accurately assess urinary PD and pseudouridine excretion in cattle.

No significant relationships were observed between urinary and milk allantoin excretion for an individual cow. Mean daily milk allantoin excretion amounted to 1.76

and 1.81% of mean daily urinary allantoin excretion for separate and complete diet feeding regimens respectively, confirming the 1-4% range reported by Giesecke *et al* (1994). Tiermeyer *et al* (1984), suggested that allantoin appears in milk as a consequence of diffusion from plasma into mammary secretory epithelial cell. If these suggestions are correct then allantoin secretion in milk is presumably dependent on plasma allantoin concentrations and mammary blood flow. Allantoin appears in urine as a result of filtration processes occuring in the kidney (Greger *et al*, 1976). As physiological processes involved in renal and mammary allantoin excretion are completely different, it is perhaps not surprising that no clear relationships between milk and urinary allantoin excretion were observed.

Observed diurnal variations in milk allantoin excretion between milking intervals are almost certainly due to variations in milk yield, mammary blood flow and plasma allantoin concentration. Milk allantoin excretion was found to be moderately correlated with milk yield (r=0.574), while other experiments have indicated the existence of much closer relationships, r=0.949 (Rosskopf *et al*, 1991), r=0.936 (Rosskopf and Giesecke, 1992) and r=0.950 (Giesecke *et al* 1994). It seems plausible that mammary blood flow would increase as a result of increased milk production, resulting in increased transport of plasma allantoin to the mammary gland which would potentially explain the close relationships observed between milk allantoin excretion and milk yield.

The extent of diurnal variation observed in milk allantoin excretion was larger than that observed for milk allantoin concentration. Milk allantoin concentrations have been demonstrated to be positively correlated (r values ranging between 0.76-0.78) with plasma allantoin concentrations (Rosskopf *et al*, 1991; Rosskopf and Giesecke, 1992 and Giesecke *et al*, 1994). However interpreting variations in milk allantoin concentrations as a reflection diurnal variations in plasma allantoin concentration is confounded by variations in milk yield. It seems most likely that variations in milk allantoin concentration are a result of variations in allantoin transport to the mammary gland and circulating plasma allantoin concentrations.

Chapter Seven

The influence of fermentable energy supply on purine derivative excretion in milk and urine during late lactation

Summary

The literature concerning milk allantoin and uric acid excretion is reviewed. The potential of milk allantoin as an index of MCP supply is evaluated in the current experiment. Two experiments are described in the following chapter.

In the first experiment, twelve late lactation cows received 14.3 kg DM/d of a basal ration consisting of (g/kg DM) chopped barley straw (415), soyabean meal (322), molassed sugar beet pulp (197), molasses (33), urea (17) and a vitamin and mineral supplement (17) for a 21 day co-variance period. During four 16 day periods, six dietary treatments allocated randomly to cows, consisting of potato starch (1, 2 or 3 kg DM/d) or Megalac (0.64, 1.27 or 1.91 kg DM/d) were evaluated. Daily urinary PD excretion, pseudouridine and creatinine excretion, milk yield and milk concentrations of fat, protein, lactose, urea-N and allantoin were measured during the last two days of each period. Experimental treatments did not significantly ($P>0.05$) influence urinary pseudouridine excretion. Urinary PD excretion was highly correlated with urinary allantoin excretion ($r^2=0.987$, $n=133$, $P<0.001$). For individual cows relationships between milk allantoin excretion and urinary allantoin excretion ($r^2=0.003$, $n=58$, $P=0.680$), FME intake ($r^2=0.019$, $n=58$, $P=0.153$) or calculated MCP supply ($r^2=0.016$, $n=58$, $P=0.172$) were not significant. Individual cow daily mean milk allantoin concentrations were poorly correlated with urinary allantoin excretion ($r^2=0.003$, $n=58$, $P=0.692$), FME intake ($r^2=0.135$, $n=58$, $P<0.01$) or calculated MCP supply ($r^2=0.139$, $n=58$, $P<0.01$). Responses of milk allantoin excretion and concentration to changes in FME intake within individual cows were variable, which would limit its use as an on-farm diagnostic index of MCP supply. Problems with this approach are discussed.

The use of mean treatment values, indicated both urinary PD and allantoin excretion were highly correlated with FME intake (r^2=0.707, n=6, P<0.05, r^2=0.721, n=6, P<0.05, respectively). Strong relationships also existed between mean milk allantoin excretion and daily mean concentration with urinary allantoin excretion (r^2=0.639, n=6, P<0.05 and r^2=0.624, n=58, P<0.05, respectively), calculated FME intake (r^2=0.822, n=6, P<0.01 and r^2=0.981, n=6, P<0.01, respectively) and calculated MCP supply (r^2=0.944, n=6, P<0.001 and r^2=0.981, n=6, P<0.01, respectively). Experimental results suggest that, milk allantoin excretion and concentration would be of little value as a microbial marker for individual cows, but may have potential on a herd basis, although this observation may hold only when variations in milk yield are relatively small.

In the second experiment, three wether sheep fitted with rumen cannula were used in a complete-change-over design to evaluate the effects of potato starch and Megalac on rumen fermentation and basal diet DM degradation. Basal diet was fed as the sole feed (0.84 kg DM/d) or supplemented with 0.18kg DM of potato starch or 0.11 kg DM of Megalac corresponding to the extreme diets of the dairy cow experiment. Dietary inclusion of Megalac significantly (0.10>P>0.05) increased rumen liqour VFA concentrations, while inclusion of potato starch depressed rumen basal diet DM degradation.

7.1. Experimental aims

The experiment was conducted to evaluate the potential of milk allantoin as an alternative to urinary PD excretion for estimating MCP supply in late lactation Holstein/Friesian cows.

7.2. Introduction

Deutsch and Mattson (1959) reported the presence of purine and pyrimidine derivatives in bovine milk. Due to the ease of milk collection and sampling, interest in

milk PD excretion has increased. Recent experiments have been conducted to evaluate the potential of milk PD excretion as a non-invasive index of MCP (e.g. Rosskopf *et al*, 1991; Lebzien *et al*, 1993 and Giesecke *et al*, 1994).

7.2.1. Milk allantoin excretion

Allantoin present in bovine milk is thought to be a consequence of its diffusion from plasma into the mammary alveolar lumen (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994). Oxidation of uric acid derived from plasma or that present as a result of mammary purine metabolism (Rosskopf, 1989) to allantoin is not thought to occur within mammary secretory cells (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994). These suggestions have been tentatively confirmed in rat studies which were unable to identify *uricase* or *uricase* mRNA activity in mammary tissue (Motojima and Goto, 1990). Rosskopf *et al* (1991) working with dairy cows identified significant ($P<0.001$) positive correlations between plasma allantoin concentration and milk allantoin concentration ($r=0.758$). Other studies have confirmed significant ($P<0.001$) correlations between plasma allantoin concentration, milk allantoin concentration $r=0.74$, and milk allantoin excretion, $r=0.80$ (Giesecke *et al*, 1994).

Early experimental evidence supporting the use of milk allantoin excretion as a potential index of MCP supply was provided by Kirchgessner and Kreuzer (1985) who demonstrated a 14% reduction in milk allantoin excretion in cows fed a diet deficient in crude protein. Further work indicated that milk allantoin excretion increased with dietary energy intake (Kirchgessner and Kaufmann, 1987). More recently, significant ($P<0.001$) positive correlations have been demonstrated between energy intake and milk allantoin excretion, $r=0.942$ (Rosskopf *et al*, 1991), $r=0.783$ (Lebzien *et al*, 1993) and $r=0.800$ (Giesecke *et al*, 1994). However, these relationships are difficult to interpret due to the close correlations that exist between milk allantoin excretion and milk yield, $r=0.949$ (Rosskopf *et al*, 1991) and $r=0.950$ (Giesecke *et al*, 1994). Increases in energy intake are likely to stimulate MCP synthesis due to an increase in fermentable substrates available to rumen microbes, but energy intake is also a major determinant of milk yield.

It is unclear from these experiments, whether the response of milk allantoin excretion to increases in energy intake are a consequence of maintaining milk allantoin concentration or a true reflection of MCP supply.

Milk allantoin concentrations have been shown to be largely independent of large changes in dietary energy (Kirchgessner and Kaufmann, 1987 and Kirchgessner and Windisch, 1989) and protein supply (Kirchgessner and Kreuzer, 1985 and Kirchgessner and Windisch, 1989) which highlights the uncertainities of interpreting milk allantoin excretion. Moorby and Dewhurst (1993a) attempted to overcome auto-correlations between milk yield and milk allantoin excretion by manipulating dietary ME and FME supplies. Milk PD (allantoin and uric acid) output was found to be independent of milk yield but related to FME intake. However the results are equivocal due to a crude estimation of FME intakes. Furthermore, milk uric acid excretion, which typically accounts for between 10 to 21% of milk PD excretion (Giesecke *et al*, 1994 and Susmel *et al*, 1995) is thought to be derived from mammary purine metabolism which is dependent on milk yield (Rosskopf, 1989), and not therefore an accurate index of MCP supply.

7.2.2. Proportion of allantoin excreted in milk

Early experiments indicated that milk allantoin excretion amounted to 6-7% of urinary allantoin excretion (Kirchgessner and Kreuzer, 1985 and Kirchgessner and Windisch, 1989). These estimates have subsequently been criticised due to the insensitive analytical technique used to determine milk allantoin concentrations (Giesecke *et al*, 1994). More recent work based on HPLC determinations of milk allantoin, has indicated that milk allantoin excretion accounts for between approximately 1-4% (Giesecke *et al*, 1994) or 8-9% (Susmel *et al*, 1995) of total, milk plus urinary allantoin excretion depending on milk yield. Giesecke *et al* (1994) noted that milk allantoin excretion increased from 0.6-2.4% of total allantoin excretion with concurrent increases in milk yield from 8-40 kg/d. These increases were assumed to be a consequence of elevated plasma allantoin concentrations at higher milk yields.

7.2.3. Comparison of milk allantoin excretion with ^{15}N estimates of duodenal MCP supply

Lebzien *et al* (1993) compared estimates of MCP supply based on milk allantoin excretion or ^{15}N over a wide range of energy intakes and diets. Estimates of duodenal MCP supply were significantly ($P<0.001$) correlated with milk allantoin excretion ($r=0.711$), while a closer correlation existed with energy intake ($r=0.916$). These results suggest that measurement of milk allantoin excretion would yield less information on duodenal MCP supplies than measuring energy intake. However, Giesecke *et al* (1994) pointed out that, ^{15}N microbial marker measurements in duodenal digesta take no account of subsequent digestion and absorption of MCP, while plasma PD concentrations and consequently milk allantoin concentrations reflect the digestion and absorption of microbial purines from the small intestine. Giesecke *et al* (1994) argued that differences in digestion and subsequent absorption of MCP between experimental diets could account for the moderate correlation observed between milk allantoin excretion and ^{15}N estimates of duodenal MCP supply. Milk allantoin might therefore provide a more biologically valid estimate of available protein.

7.2.4. Milk uric acid excretion

Early observations of Wolfschoon-Pombo (1982) indicated that uric acid concentrations in bovine milk were dependent on cow breed, parity and stage of lactation. Further work lead Rosskopf (1989) to suggest that, uric acid present in bovine milk was derived as a result of purine metabolism within the mammary gland. More recently, Giesecke *et al* (1994) demonstrated that milk uric acid concentrations in milk (mean 56.2 µmol/l) were 65% higher than those in plasma (mean 34.0 µmol/l). Uric acid excreted in milk accounted for 3.1% of total uric acid excretion, while milk allantoin excretion accounted for only 1.6% of total allantoin excretion, implying that a proportion of uric acid secreted in milk originated from the mammary gland. The experiment demonstrated that milk uric acid excretion was significantly ($P<0.05$)

correlated to energy intake, (r= 0.61), which was considered a consequence of increased mammary purine turnover at higher milk yields. Typically, milk uric acid excretion amounts to 10-12% of that observed for allantoin (Susmel *et al*, 1995).

In conclusion, the potential of milk uric acid excretion to reflect MCP supply appears to be limited due to the secretion of uric acid derived from mammary purine metabolism. In contrast, significant correlations between milk allantoin excretion and energy intake, have lead to the suggestion that its measurement represents a non-invasive index of MCP supply (Giesecke *et al*, 1994). However, uncertainties exist in these relationships due to auto-correlations between milk allantoin excretion and milk yield.

7.3. Materials and methods

Variations in FME intake were achieved by supplementing a basal ration with either potato starch or Megalac. Megalac is a commercially available protected fat containing calcium saponified C-16:0, C-18:0, C-18:1 and C-18:2 fatty acids and is typically used to increase the ME content of the diet without adversely affecting rumen fibre digestion.

7.3.1. Dairy cow experiment

All cows remained in good health during the experiment, with the exception of cow 270 which was removed after suffering from rumen acidosis during the last experimental period. Problems in fitting a bladder catheter to cow 273 during the co-variance period and leaking connections between catheters and collection vessels resulted in 113 of a possible 120 total urine collections being performed. All other experimental measurements were made as described.

7.3.1.1. Animals and animal management

Twelve late lactation multiparous Holstein/Friesian cows were selected from the Auchincruive main dairy herd and housed for the duration of the experiment in a dairy cow metabolism unit. Cows were restrained in individual stalls fitted with de Boer yokes and milked in *situ* at 07.15 and 15.00 hours.

7.3.1.2. Experimental design

The experiment was of an incomplete 2 x 3 factorial (4 period) change over design with each animal receiving four experimental diets, preceeded by a 21 day co-variance period, during which the cows received a basal diet without supplement. Each experimental period lasted 16 days. Dietary treatments were either supplements of potato starch (1.0, 2.0, 3.0 kg DM for S1, S2 and S3 diets, respectively) or Megalac (0.625, 1.250, 1.875 kg DM, for F1, F2 and F3 diets, respectively) added to the basal diet. Selected cows of similar calving date, parity and bodyweight were allocated randomly to treatments according to two 6 x 6 latin squares.

Due to the unbalanced nature of the experimental design, analysis of variance for DM, ME, FME and CP intakes, milk urea-N concentration and output, urinary PD, allantoin, pseudouridine and creatinine excretion and PD/c, A/c and Ps/c ratios were performed using Residual Maximum Likelihood (REML, Patterson and Thompson, 1971) directive within Genstat 5.3 (Lawes Agricultural Trust, 1993) with the following model:- [fixed = covariate + period + carry over effects of diet type, level and their interactions (cofs.colev) + interactions between diet type and level (fs.level); absorb=cow] random=cow/period/day. Milk yield, fat, protein, lactose and allantoin concentrations and fat, protein, lactose and allantoin yields were analysed using the following model:- [fixed = covariate + period + hr + cofs.colev + fs.level; absorb=cow] random=cow/period/day/hr.

Exploration of the experimental data and regression analysis were undertaken using Minitab statistical package (Minitab Inc., 1980; Minitab Data Analysis Software, Pennsylvania State University, Pennsylvania).

7.3.1.3. Diet formulation

SAC advisory rationing software was used to formulate a semi-synthetic basal diet, which consisted of 7 kg fresh weight (F.Wt) chopped barley straw supplemented with 10 kg F.Wt of concentrate, which consisted of soyabean meal, molassed sugar beet pulp, molasses, urea and a mineral and vitamin supplement. Formulation was performed to ensured eRDP supply from the basal diet would be in excess at the highest starch inclusion (S3). Consequently rumen MCP synthesis would be expected to be constrained by FME supply for all dietary treatments. In order to overcome potential phosphorus deficiencies with the experimental diets, a high phosphorus cattle mineral and vitamin supplement (supplied by Bowie and Aram, Renfrew, composition shown in appendix 6) was incorporated into the basal diet concentrate, which was prepared in 250 kg F.Wt batches to minimise urea losses. Basal diet formulation is shown in appendix 7. Chemical analysis of the basal diet, potato starch and Megalac are shown in appendix 8.

Predicted daily ME, FME, DUP and eRDP intakes for dietary treatments are shown in Table 7.1. DUP and eRDP supplies were calculated from published values (AFRC, 1992) assuming a mean rumen outflow rate of 5%.

Table 7.1. Predicted ME, FME, DUP and eRDP intakes

Daily intake	Experimental diet						
	Basal	S1	S2	S3	F1	F2	F3
eRDP /g	2276	2276	2276	2276	2276	2276	2276
DUP /g	821	821	821	821	821	821	821
ME /MJ	143	158	173	189	162	180	198
FME /MJ	137	152	167	182	137	137	137

7.3.1.4. Animal feeding

Chopped barley straw and 7 kg F.Wt of concentrate were thoroughly mixed and fed as five equal meals at 08.00, 10.00, 12.30, 15.00 and 20.00 hours. During each milking (07.15 and 15.00 hours) cows received 1.5 kg F.Wt of concentrate. Megalac was fed as two equal meals by incorporating with the concentrate offered at milking. Potato starch was fed as five equal meals concurrently with the basal ration in an attempt to prevent bloating. Water was available *ad libitum* throughout the experiment.

7.3.1.5. Experimental measurements and sample collection

Rate of passage measurements were performed during the last four days of each experimental period, with all other measurements and sampling procedures performed during the last two days of each experimental period.

7.3.1.5.1. Urine collection and sampling

The day prior to urine collections, bladder catheters were fitted to each cow. Two consecutive 24 hour urine collections were made starting at 07.15. Collections were weighed, urine density determined and two subsamples collected and immediately stored at -20^{0}C. At the end of the experiment samples were thawed and analysed in random sequence using the method described in chapter 2.

7.3.1.5.2. Milk recording and sampling

Milk yields were recorded over the last two days of each sampling period. Milk samples were collected at each milking for milk fat, protein and lactose determinations, stored with lactab preservative at 4^{0}C prior to analysis. Samples collected for milk allantoin analysis were stored with no preservative at -20^{0}C, prior to analysis at the end of the experiment as outlined in chapter 3. Samples destined for urea-N determination

were collected and stored at 4^{0}C. Am and pm samples collected from each cow on each sampling day were bulked according to yield before being submitted for analysis.

7.3.1.5.3. Total faecal collection

Two consecutive total faeces collections were performed concurrently with urine sampling. Collections were weighed with representative subsamples taken and stored at -20^{0}C. At the end of the experiment, samples were thawed and DM content determined at 60^{0}C for 96 hours. *In-vivo* dry matter digestibilities were calculated as [((DM intake-DM faeces)/DM intake) x 100].

7.3.1.5.4. Rate of passage determinations

Rumen small particle and liquid outflow rates were determined using chromium mordanted hay (containing 59g Cr/kg DM) and lithium cobalt-EDTA complex ((LiCo-EDTA.3$H_2$0) containing 116mg Co/g, dry weight), respectively. 100g of mordanted hay was offered at 10.00 on day 13 of each experimental period to each cow and was consumed within a hour. LiCo-EDTA.3$H_2$0 was administered as a 250 ml oral drench concurrently with the mordanted hay. Spot faecal samples were collected from each cow at 0, 4, 6, 10, 13, 21, 24, 28, 34, 37, 45, 52, 58, 61, 69, 72, 76, 82, 85, 95 and 105 hours post-dosing. After drying at 60^{0}C for 96 hours, faecal samples were milled and analysed for Cr and Co. Rates of passage were determined by fitting the model of Grovum and Williams (1973) to the experimental data.

7.3.1.5.5. Feed sampling

Representative samples of straw, concentrate, potato starch and Megalac offered on each sampling day were bulked together for each experimental period and submitted to the Analytical Services Unit, SAC Auchincruive for analysis. Individual cow feed intakes were recorded on each sampling day while refusals were separated into straw,

concentrate, potato starch and Megalac components and weighed. Each component was bulked together for each sampling day with DM determined at 60^0C for 48 hours.

7.3.1.5.6. Cow Liveweights

At the begining of the experiment all cows were weighed before entering the metabolism unit. Cows were weighed at 10.00 hours at the end of each experimental period.

7.3.2. Sheep experiment

In addition to the dairy cow experiment, the effects of dietary treatments, S3 and F3 on rumen fermentation and basal diet DM degradation were assessed in Suffolk wethers each fitted with rumen fistulas.

7.3.2.1. Animals and their management

Three Suffolk wethers, mean liveweight of 65.8 (SD $\pm$ 0.66) kg were housed in individual pens bedded with sawdust. The abdominal region surrounding the rumen fistula of each sheep was routinely checked for leaks and cleaned when necessary.

7.3.2.2. Experimental design

The experiment was a three period complete change over design. Wethers were randomly allocated to treatments according to a 3 x 3 latin square. Treatments corresponded to basal, S3 and F3 diets fed in the dairy cow experiment. The first period was 21 days to ensure rumen microfloral adaptation to the experimental diets. Subsequent periods lasted 14 days. Statistical analysis of experimental data was performed using the analysis of variance directive within Genstat 5.3 (Lawes

Agricultural Trust, 1993) using sheep.period as the blocking structure and diet.hour as the treatment structure.

7.3.2.3. Diet formulation

Basal diet formulation is described in section 7.3.1.3., with the exception that the cattle mineral and vitamin supplement was replaced by a sheep alternative (supplied by Scotmin Nutrition Limited, Ayr, refer to appendix 9). Basal diet was offered at 1kg F.Wt/d (0.840 kg DM; refer to appendix 10). Daily dietary supplements, corresponding to diets S3 and F3, respectively (on a basal diet DM: treatment DM basis), consisted of 0.220 kg/d F.Wt (0.179 kg/d DM) potato starch (S3) and 0.116 kg/d F.Wt (0.112 kg/d DM) of Megalac (F3). Basal diet concentrate consisting of soyabean meal, molassed sugar beet pulp, molasses, urea and a vitamin and mineral supplement was prepared in weekly 12 kg F.Wt batches to minimise urea losses.

7.3.2.4. Animal feeding

Experimental diets were offered as two equal meals at 08.30 and 16.45 hours. All feed offered during the course of the experiment was consumed. Water was avaliable *ad libitum* throughout the experiment.

7.3.2.5. Experimental measurements and sample collection

In-sacco DM degradability determinations were performed during the last four days of each experimental period, while rumen liqour samples were collected on the last sampling day.

7.3.2.5.1. In-*sacco* rumen DM degradability

Four replicate 4g straw (length 1.5cm), 5g concentrate samples (passed through a 2.5 mm screen using a Christie and Norton hammer mill) and 4g of Megalac were placed in nylon bags and incubated in the rumen at 3, 5, 8, 12, 16, 24, 48 and 72 hour intervals as outlined in chapter 4.

7.3.2.5.2. Rumen liquor sampling

Rumen liquor samples (80 mls) were collected from each sheep at 10.00 and 16.30 hours and stored at -20°C. Unfortunately rumen liquor pH was measured after frozen storage and not immediately after collection. Measured pH was corrected using following equation (kindly provided by N.W. Offer) relating rumen liqour pH at collection and after frozen storage, where:-

$$pH_{collection} = 0.256 + 0.970\ pH_{stored} \qquad (r^2 = 0.975,\ n = 204)$$

Subsamples (8 ml) of rumen liquor were immediately prepared for VFA and ammonia analysis, stored at -20°C and subsequently analysed.

7.4. Results

7.4.1. Animal production

7.4.1.1. DM, ME, FME and CP intakes

Mean treatment effects, based on individual cow measurements, on basal diet, potato starch and Megalac dry matter intakes are shown in Table 7.2. Mean treatment effects on true DM, ME, FME and CP intakes are presented in Table 7.3.

Table 7.2. Mean treatment basal diet, potato starch and Megalac DM intakes (kg/d)

DMI (kg/d)	Experimental treatment						Mean
	F1	F2	F3	S1	S2	S3	SED
Chopped barley straw	5.75	5.55	5.08	5.34	5.43	5.01	0.266
Concentrate	7.49	7.56	7.43	7.49	7.51	7.43	0.089
Potato starch	-	-	-	1.01	1.86	2.98	0.124
Megalac	0.62	1.15	1.21	-	-	-	0.124

Table 7.3. Mean treatment true DM, ME, FME and CP intakes

Daily Intake	Experimental treatment						Mean
	F1	F2	F3	S1	S2	S3	SED
DM (kg)	14.18	15.09	15.10	14.18	15.09	14.71	0.60
ME (MJ)	158.5	174.3	173.8	144.9	156.6	160.0	7.89
FME (MJ)	140.0	139.3	134.0	143.5	155.1	158.3	7.67
CP (g)	3149	3167	3080	3135	3143	3140	37.9

Table 7.4. Mean treatment effects on milk yield, milk fat, protein, lactose and urea-N concentrations and milk fat, protein, lactose and urea-N yields

	Experimental treatment						Mean	FS	Lev	FS *
	F1	F2	F3	S1	S2	S3	SED			Lev
Yield (kg/d)	12.2	13.3	12.1	12.2	13.7	13.4	0.86	NS	#	NS
[Fat] (g/kg)	47.1	50.3	48.4	42.2	41.8	41.8	0.146	***	NS	NS
[Prot] (g/kg)	33.9	33.4	32.5	35.6	35.9	38.0	0.094	***	NS	**
[Lactose] (g/kg)	41.1	41.3	40.9	42.8	42.3	41.4	0.063	**	#	NS
[Urea-N] (mg/kg)	336	343	322	342	312	292	14.0	**	**	*
Fat yield (g/d)	345	392	368	331	358	341	22.6	NS	*	NS
Prot yield (g/d)	199	217	191	215	243	253	13.5	**	*	*
Lact yield (g/d)	254	288	251	264	289	279	19.4	NS	NS	NS
Urea-N output (g/d)	4.31	4.34	3.83	4.10	4.05	3.88	0.319	NS	NS	NS

Key:-

FS denotes differences between Megalac and starch diets
Lev denotes differences between treatment levels
FS*Lev denotes interactions between treatment type and level
NS denotes Not Significant
#,*, **, *** indicate significance at the 0.10>P>0.05, P<0.05, P<0.01 and P<0.001 levels, respectively

Table 7.5. Correlation coefficients between milk fat, protein and lactose and urea-N concentrations, milk fat, protein, lactose and urea-N yields and DM, ME, FME and CP intakes, based on individual cow measurements (n=58)

	DMI	MEI	FMEI	CPI	Yd	[Fat]	[Prot]	[Lact]	[Urea-N]	Fat Yd	Prot Yd	Lact Yd
MEI	0.813											
FMEI	0.783	0.487										
CPI	0.210	-0.065	0.252									
Yd	-0.132	-0.245	-0.093	0.235								
[Fat]	0.218	0.326	-0.030	-0.327	-0.300							
[Prot]	0.421	0.335	0.492	-0.059	-0.584	0.347						
[Lact]	-0.376	-0.506	-0.122	0.193	0.584	-0.358	-0.390					
[Urea-N]	-0.143	-0.269	-0.215	-0.042	-0.209	-0.075	-0.133	0.107				
Fat Yd	0.070	0.103	-0.018	-0.461	0.180	0.243	-0.003	-0.036	0.135			
Prot Yd	0.030	-0.153	0.130	0.242	0.890	-0.207	-0.170	0.481	-0.182	0.237		
Lact Yd	-0.206	-0.329	-0.113	0.239	0.977	-0.325	-0.585	0.735	-0.022	0.138	0.852	
Urea-N Yd	-0.199	-0.362	-0.204	0.201	0.839	-0.120	-0.604	0.593	0.476	0.213	0.672	0.854

7.4.1.2. Milk yield

Mean treatment effects on milk yield are shown in Table 7.4. Daily milk yields were not significantly ($P>0.05$) different between starch and Megalac diets (means of 13.1 and 12.5 kg/d, respectively). Correlation coefficients between individual cow (mean of two measurements) milk yields, DM, ME, FME and CP intakes are presented in Table 7.5.

7.5.1.3. Milk composition

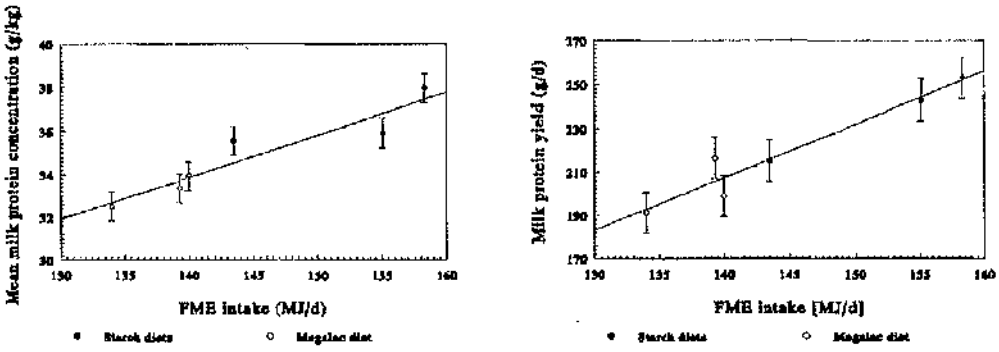
Mean treatment effects on milk fat, protein and lactose concentrations and milk fat, protein and lactose yields are presented in Table 7.4. Correlation coefficients between individual cow (mean of two measurements) milk fat, protein and lactose concentrations and milk fat, protein and lactose yields and DM, ME, FME and CP intakes are shown in Table 7.5.

Milk fat concentrations were significantly ($P<0.001$) increased following the inclusion of Megalac (means of 48.6 and 41.9 g/kg, for Megalac and starch diets respectively). Accordingly, mean milk fat yields were also significantly ($0.10<P>0.05$) higher for Megalac diets (370 and 344 g/d for Megalac and starch diets, respectively). In contrast, milk lactose concentrations were significantly ($P>0.01$) higher for starch compared to Megalac diets (means of 42.2 and 41.1 g/kg, respectively). No significant ($P<0.05$) differences in milk lactose yields were observed between starch and Megalac diets (means of 278 and 261 g/d, respectively). Milk protein concentrations were significantly higher ($P<0.001$) for starch compared to Megalac diets (means of 36.5 and 33.3 g/kg, respectively). Significant ($P<0.01$) increases in milk protein yield were observed following the inclusion of starch to the basal ration (means of 237 and 202 g/d, for starch and Megalac diets respectively). Based on individual cow measurements, milk protein concentration was significantly correlated with FME intake ($r^2=0.229$, $n=59$, $P<0.001$), which was greatly improved by the use of mean treatment milk protein concentrations ($r^2=0.856$, $n=6$, $P<0.01$) as shown in Figure 7.1. In contrast, no significant correlation existed between individual cow (mean of two measurements) milk

protein yields and FME intakes. However, the use of co-variate data to calculate treatment means gave a close correlation between milk protein yield and FME intake (r^2=0.917, n=6, P<0.01), shown in Figure 7.1.

Figure 7.1. Relationship between mean treatment milk protein concentration and yield with calculated FME intake

i) Protein concentration ii) Protein yield



Each point is the mean of 16 measurements

Error bars indicate between treatment mean SE

7.4.1.3. Milk urea-N concentration and output

Mean treatment effects on milk urea-N concentration and milk urea-N output are shown in Table 7.4. Milk urea-N concentrations were signifcantly (P<0.01) higher for Megalac compared to starch diets (means of 333 and 315 mg/kg, respectively). Milk urea-N outputs were not significantly (P>0.05) different between Megalac and starch diets (means of 4.16 and 4.01 g/d, respectively). Correlation coefficients between individual cow (mean of two measurements) milk urea-N concentrations, urea-N outputs, DM, ME, FME and CP intakes are shown in Table 7.5.

7.4.1.4. Cow liveweights

Mean treatment effects on cow liveweights are presented in Table 7.6. Differences between starch and Megalac diets were not significant (P>0.05), means were 557 and 558 kg, respectively.

Table 7.6. Mean treatment effects on cow liveweight

	Experimental treatment						Mean
	F1	F2	F3	S1	S2	S3	SED
Liveweight (kg)	562	557	553	555	561	556	6.5

7.4.2. Whole tract *in-vivo* DM digestibility

Mean treatment effects on whole tract *in-vivo* DM digestibilities are presented in Table 7.7. No significant differences (P>0.05) were observed in whole tract DM digestibility between Megalac and starch diets (means of 0.695 and 0.708, respectively).

7.4.3. Rumen and hindgut liquid and small particle outflow rates

Mean treatment effects on rumen and hindgut liquid and small particle outflow rates are presented in Table 7.7. Rumen small particle outflow rates were not significantly (P>0.05) different between Megalac and starch diets (means of 5.14 and 5.79% hour, respectively). In contrast, rumen liquid outflow rates were significantly (P<0.05) higher for starch compared to Megalac diets (means of 6.80 and 6.05 % hour, respectively). Hindgut small particle and liquid outflow rates for starch diets (11.3 and 20.7 % hour, respectively) were not significantly (P>0.05) different from Megalac diets (11.2 and 15.8 % hour, respectively). Individual cow rumen and hindgut small particle and liquid outflow rates are tabulated in appendix 12.

Table 7.7. Mean treatment effects on *in-vivo* whole tract DM digestibility, rumen and hindgut small particle and liquid outflow rates

	Experimental treatment						Mean SED	FS	Lev	FS * Lev
	F1	F2	F3	S1	S2	S3				
DM digestibility	0.691	0.694	0.701	0.701	0.717	0.705	0.0016	NS	NS	NS
Rumen small particle outflow rate, *k1* (% hour)	5.84	4.93	4.66	5.81	5.56	6.00	0.725	NS	NS	NS
Hindgut small particle outflow rate, *k2* (% hour)	12.24	10.91	10.38	11.26	13.11	9.45	2.427	NS	NS	NS
Rumen liquid outflow rate, *k1* (% hour)	6.18	6.12	5.85	6.40	7.07	6.93	0.488	*	NS	NS
Hindgut liquid outflow rate, *k2* (% hour)	17.31	13.42	16.68	16.31	24.00	21.89	4.067	NS	NS	NS

Key:-

FS denotes differences between Megalac and starch diets
Lev denotes differences between treatment levels
FS*Lev denotes interactions between treatment type and level
NS denotes Not Significant
*, indicates significance at the P>0.05 level

7.4.4. Calculated MCP supply

MCP supply was calculated using the current MP system adopted in the UK (AFRC, 1992). EMPS Y (g MCP/MJ FME), was calculated according to the following equation:-

$$Y = 7.0 + 6.0 \{1-e^{(-0.35L)}\} \qquad \text{AFRC (1992)}$$

where:- L is the level of feeding (i.e. multiples of maintenance)

MCP supplies were derived as a product of energetic efficiency of MCP synthesis and FME intake. MCP supply based on a level of feeding correction (lMCP) was calculated for each cow on each sampling day. Mean treatment effects on lMCP are shown in Table 7.8.

The estimation of MCP based on level of feeding is unavoidable in situations where available information on factors affecting EMPS is lacking. However, rumen liquid and small particle outflow rates were measured in the current experiment and were used to calculate a rumen outflow corrected estimate of MCP supply (oMCP). Calculations were based on the broad assumption that 50% of rumen microbial mass was present in the liquid phase, while the remaining 50% was associated with small feed particles. Consequently, rumen outflow rate (r) was taken as the mean measured rumen liquid and small particle outflow rates. EMPS Y (g MCP/ MJ FME) was calculated using the model of Cottrill (1991) described by the following equation:-

$$Y = 6.25/(0.362 + 0.019/r) \qquad \text{Cottrill (1991)}$$

oMCP derived from the product of Y and FME intake, was calculated for all cows. Mean treatment effects on oMCP are presented in Table 7.8.

Table 7.8. Mean treatment effects on lMCP and oMCP supply (g/d)

Variate	Experimental treatment						Mean
	F1	F2	F3	S1	S2	S3	SED
lMCP	1603	1631	1577	1613	1783	1820	100.9
oMCP	1286	1224	1171	1300	1448	1487	80.4

7.4.5. Effects on rumen fermentation

Mean treatment effects on rumen fermentation characteristics are shown in Table 7.9.

Table 7.9. Mean treatment effects on rumen fermentation characteristics

Rumen fermentation parameters	Experimental treatment			SED	P
	Basal diet	S3	F3		
pH_m	7.56	7.59	7.62	0.211	0.952
pH_c	7.58	7.62	7.65	0.203	0.951
NH_3 (mg/l)	276	427	364	141	0.634
Total [VFA] mM	83.0	84.5	88.9	1.26	0.078
Acetate mM (A)	57.2	59.6	61.7	1.29	0.141
Propionate mM (P)	16.9	16.25	17.61	1.33	0.658
Butyrate mM (B)	6.09	6.00	6.53	0.2	0.203
Isobutyrate mM	1.21	1.17	1.25	0.07	0.321
Valerate mM	0.66	0.57	0.75	0.04	0.098
Isovalerate mM	0.95	0.857	0.982	0.11	0.582
Acetate (molar %)	69.3	70.7	69.5	1.30	0.596
Propionate (molar %)	20.2	19.1	19.8	1.23	0.715
Butyrate (molar %)	7.42	7.17	7.25	0.07	0.125
Isobutyrate (molar %)	1.42	1.33	1.25	0.00	<0.001
Valerate (molar %)	0.83	0.75	1.00	0.18	0.500
Isovalerate (molar %)	1.08	1.08	1.00	0.07	0.500
A + B / P	3.82	4.09	3.91	0.27	0.650

7.4.5.1. Rumen pH

Mean treatment effects on measured and corrected rumen liquor pH are presented in Table 7.9. Both measured and corrected pH values were abnormally high. Consequently, further investigation of the effect of sampling time on rumen pH values was not pursued.

7.4.5.2. Rumen ammonia concentration

Mean treatment effects on rumen ammonia concentrations are shown in Table 7.10. Differences between experimental treatments or sampling times (means 274 and 437 mg/l for am and pm samples, respectively) were not significant (P>0.05).

Table 7.10. Mean treatment effects on rumen ammonia concentration (mg/l)

Sampling time	Experimental treatment		
	Basal diet	S3	F3
am	192	212	418
pm	359	641	310

SED between treatments 141
SED between sampling times 121

7.4.5.3. Rumen fermentation end products

7.4.5.3.1. Total VFA concentrations

Mean total VFA concentrations were significantly (0.10>P>0.05) higher for treatment F3 (means of 83.0, 84.5 and 88.9 mM for basal, S3 and F3 diets, respectively), while differences between sampling times were not significant (P>0.05, means of 85.7 and 85.2 mM for am and pm sampling times, respectively). Mean treatment effects on total VFA concentrations are shown in Table 7.11.

Table 7.11. Mean treatment effects on total VFA concentrations (mM)

Sampling time	Experimental treatment		
	Basal diet	S3	F3
am	81.9	86.5	88.7
pm	84.0	82.5	89.1

SED between treatments 1.26
SED between sampling times 6.77

7.4.5.3.2. Major VFA concentrations

Mean treatment effects on rumen acetate, propionate and butyrate concentrations are shown in Table 7.12. Differences between treatments and sampling times were not significant (P>0.05). Mean treatment effects on major VFA proportions are presented in Table 7.13. In common with major VFA concentrations, differences in major VFA proportions were not significantly (P>0.05) different between experimental treatments or sampling times. Mean treatment effects on major VFA molar ratios are presented in Table 7.14.

Table 7.12. Mean treatment effects on major VFA concentrations (mM)

Major VFA	Sampling time	Experimental treatment			SED Treatment	SED Sampling time
		Basal	S3	F3		
Acetate	am	56.9	60.3	61.1	1.29	4.30
	pm	57.5	58.9	62.3		
Propionate	am	16.4	17.2	17.7	1.33	1.67
	pm	17.3	15.3	17.5		
Butyrate	am	5.93	6.03	6.61	0.200	0.503
	pm	6.26	5.98	6.44		

Table 7.13. Mean treatment effects on major VFA molar proportions (molar %)

VFA	Sampling time	Experimental treatment			SED Treatment	SED Sampling time
		Basal	S3	F3		
Acetate	am	70.0	70.0	68.8	1.30	0.98
	pm	68.5	71.3	70.2		
Propionate	am	19.8	19.7	20.2	1.23	0.54
	pm	20.5	18.5	19.3		
Butyrate	am	7.0	7.0	7.5	0.68	0.25
	pm	7.8	7.3	7.0		

Table 7.14. Mean treatment effects on major VFA molar ratios

VFA	Sampling time	Experimental treatment			SED Treatment	SED Sampling time
		Basal	S3	F3		
A / P	am	3.54	3.59	3.44	0.249	0.142
	pm	3.36	3.84	3.64		
A + B / P	am	3.90	3.94	3.82	0.265	0.145
	pm	3.73	4.23	4.00		

A:- acetate; B:- butyrate; P:- propionate

7.4.5.3.3. Minor VFA concentrations

Table 7.15. Mean treatment effects on minor VFA concentrations (mM)

Minor VFA	Sampling time	Experimental treatment			SED Treatment	SED Sampling time
		Basal	S3	F3		
Isobutyrate	am	5.93	6.03	6.61	0.069	0.166
	pm	6.26	5.98	6.44		
Isovalerate	am	0.908	0.948	1.077	0.109	0.167
	pm	0.992	0.765	0.888		
Valerate	am	0.527	0.693	0.787	0.041	0.176
	pm	0.788	0.455	0.710		

Mean treatment effects on minor VFA concentrations are shown in Table 7.15. Differences in minor VFA concentrations between treatments and sampling times were not significant (P>0.05).

7.4.6. *In-sacco* rumen DM degradabilty

Measured rumen DM disappearances were fitted to the model of Orskov and McDonald (1979) where DM degradability = a + b ($1-e^{-ct}$), using dedicated computer software (Sherrod, 1992). Degradability constants a, b and c indicate the soluble fraction, degradable fraction and the fractional rate of degradation for DM, respectively. Mean treatment effects on basal diet straw and concentrate degradation constants are shown in Table 7.16. Fitting measured Megalac DM losses to the model of Orskov and McDonald (1979) resulted in b values in excess of 100. Since this model is clearly inappropriate for megalac, degradation coefficients are not reported. Measured basal diet straw, concentrate and Megalac dry matter losses within the rumen are described in Figures 7.2, 7.3. and 7.4., respectively. Effective degradability calculated as:- effective Dg = a + [(bc)/c + *k*)] for basal diet concentrate and straw is shown in Table 7.17.

Table 7.16. Mean treatment effects on basal diet concentrate and straw DM degradability constants

Incubation substrate	Degradation constant	Experimental treatment			SED Treatment	P
		Basal	S3	F3		
Concentrate	a	44.6	45.5	44.5	0.18	<0.05
	b	54.0	53.8	54.1	1.21	0.964
	c	0.077	0.060	0.076	0.0088	0.290
Straw	a	6.7	7.6	6.6	0.67	0.405
	b	53.4	85.3	53.1	17.6	0.313
	c	0.030	0.014	0.302	0.004	0.010

Figure 7.2. *In-sacco* chopped straw DM degradability

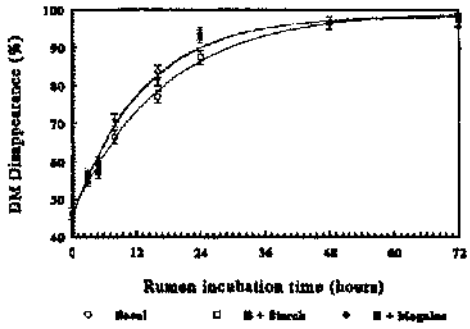
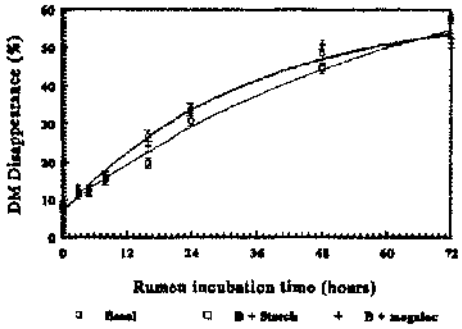


Figure 7.3. *In-sacco* concentrate DM degradability



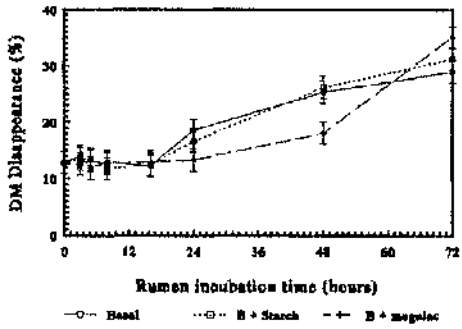
For Figures **7.2.** and **7.3.**:-

Each point is the mean of 12 measurements

Error bars indicate between diet SE

Lines indicate model of Orskov and McDonald (1979)

Figure 7.4. *In-sacco* Megalac DM degradability



Each point is the mean of 12 measurements

Table 7.17. Basal diet concentrate and straw effective degradability

Incubation substrate	Rumen outflow rate (%)	Experimental treatment		
		Basal	S3	F3
Concentrate	5	45.42	46.14	45.31
	8	45.11	45.90	45.01
Straw	5	7.02	7.84	9.62
	8	6.90	7.75	8.53

7.4.7. Urinary PD excretion

Daily urinary PD excretion was highly correlated with allantoin excretion (r^2=0.987, n=113. P<0.001), with allantoin accounting for 82 % of all PDs excreted. Mean treatment effects on urinary PD and allantoin excretion are presented in Table 7.20. Urinary PD excretion was significantly (P<0.001) higher for starch diets (means of 296 and 215 mmoles/d for starch and Megalac diets, respectively). Correspondingly, allantoin excretion was significantly (P<0.001) higher for starch compared to Megalac diets (means of 242 and 172 mmoles/d, respectively). Correlation coefficients between individual cow (mean of two measurements) urinary PD, allantoin and pseudouridine excretion, PD/c, A/c, and Ps/c ratios, DM, ME, FME, CP intakes and rumen and hindgut small particle and liquid outflow rates are shown in Table 7.19.

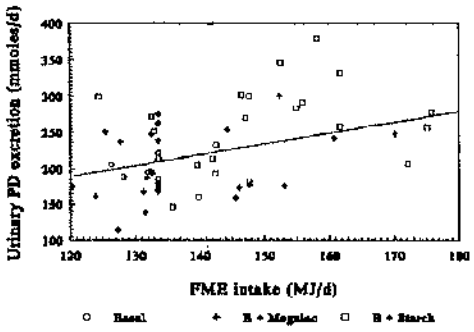
Based on individual cow data (mean of two measurements) a significant relationship existed between FME intake and urinary PD excretion (r^2=0.108, n=58, P<0.01). Co-variate data were used to calculate treatment mean values, the use of which dramatically improved the relationship between FME intake and urinary PD excretion (r^2=0.707, n=6, P<0.05). Relationships between urinary PD excretion based on individual cow measurements and mean treatment values are described in Figure 7.5. Similar relationships were also observed between urinary allantoin excretion and FME intake based on individual cow measurements (r^2=0.117, n=58, P<0.01) and mean treatment values (r^2=0.721, n=6, P<0.05). Relationships between urinary PD and allantoin excretion, with calculated MCP supply based on level of feeding (lMCP) or rumen outflow rates (oMCP) are shown in Table 7.18.

The relationships between calculated MCP supply (lMCP) and urinary PD excretion based on individual cow data and treatment means are described in Figure 7.6.

Further investigations were performed by expressing PD and allantoin excretion as a ratio to creatinine excretion (PD/c and A/c, ratios, respectively). Daily mean PD excretion was poorly related to daily mean PD/c ratios (r^2=0.088, n=113, P<0.001), but the relationship improved by scaling the PD/c ratio by creatinine concentration (r^2=0.698, n=113, P<0.001).

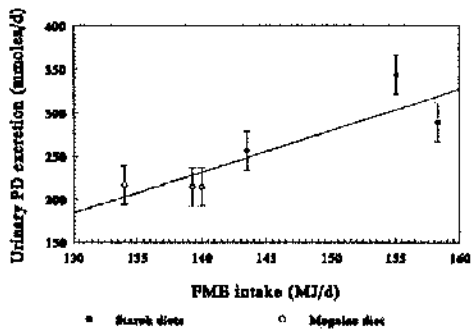
Figure 7.5. Relationship between PD excretion and calculated FME intake

i) Individual cows



Each point is the mean of 2 measurements

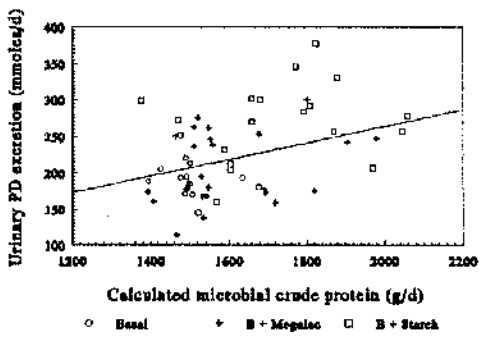
ii) Treatment means



Each point is the mean of 16 measurements
Error bars indicate between treatment SE

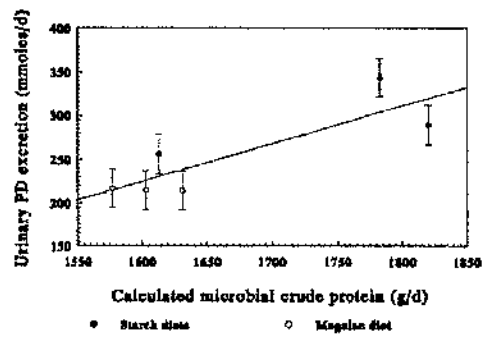
Figure 7.6. Relationship between urinary PD excretion and calculated MCP supply

i) Individual cows



Each point is the mean of 2 measurements

ii) Treatment means



Each point is the mean of 16 measurements
Error bars indicate between treatment SE

Table 7.18. Relationships between urinary PD and allantoin excretion and calculated MCP supply

Variate	Dataset	lMCP			oMCP		
		n	r^2	P	n	r^2	P
PD Excretion	Individual cow	58	0.099	<0.01	58	0.084	<0.05
	Treatment means	6	0.646	<0.05	6	0.670	<0.05
Allantoin Excretion	Individual cow	58	0.101	<0.01	58	0.085	<0.05
	Treatment means	6	0.649	<0.05	6	0.687	<0.05

Similar relationships existed between daily allantoin excretion and daily mean A/c ratios (r^2=0.120, n=113, P<0.001), which also improved by scaling for creatinine concentration (r^2=0.703, n=113, P<0.001). Mean treatment effects on urinary PD/c and A/c ratios are shown in Table 7.20. Relationships between FME intake and calculated MCP supply with PD/c and A/c ratios, and those scaled for creatinine concentration are presented in Table 7.21. Figure 7.7. describes the relationships between PD/c ratios and FME intake based on individual cow and treatment mean values. Removing three obvious outliers improved the relationships between calculated FME intake and PD/c and A/c ratios (r^2=0.309, n=58, P<0.001 and r^2=0.297, n=58, P<0.001, respectively).

Further investigations were conducted to establish sources of variation of urinary PD and allantoin excretion. Multiple regression analysis was performed on individual cow, diet, milk, outflow measurements and calculated MCP supply. Regression equations which accounted for the largest variation in urinary PD excretion using single and multiple terms are summarised in Table 7.22. Equations derived for urinary allantoin excretion were very similar to those derived for urinary PD excretion and are therefore not presented. Multiple regression analysis was also performed to identify the best predictors of urinary PD/c and A/c ratios. Regression equations which accounted for the largest variation in urinary PD/c ratios using single and multiple terms are presented in Table 7.23. Equations derived based on urinary A/c ratios which were very similar to those derived for urinary PD/c ratios are not shown.

Table 7.19. Correlation coefficients between individual cow urinary PD, allantoin (All), pseudouridine (Ps), creatinine (Crt) excretion, PD/c, A/c and Ps/c ratios, milk allantoin excretion (Maout), DM, ME, FME, CP intakes, rumen and hindgut small particle and liquid outflow rates

	DMI	MEI	FMEI	CPI	PD	All	Ps	Crt	PD/c	A/c	Ps/c	*k*1s	*k*1l	*k*2s	*k*2l
MEI	0.813														
FMEI	0.783	0.487													
CPI	0.210	-0.065	0.252												
PD	0.237	0.082	0.352	0.181											
All	0.242	0.050	0.364	0.186	0.992										
Ps	0.093	0.194	-0.007	-0.070	0.532	0.498									
Crt	0.081	0.177	-0.007	-0.022	0.677	0.644	0.856								
PD/c	0.184	-0.094	0.449	0.227	0.316	0.348	-0.385	-0.435							
A/c	0.187	-0.117	0.444	0.227	0.300	0.348	-0.388	-0.439	0.990						
Ps/c	0.031	0.080	-0.029	-0.087	0.161	0.147	0.743	0.316	-0.138	-0.135					
*k*1s	-0.009	-0.110	0.135	0.176	-0.000	-0.014	-0.209	-0.150	0.198	0.174	-0.157				
*k*1l	0.093	0.026	0.106	0.208	0.215	0.212	-0.233	-0.049	0.278	0.250	-0.282	0.475			
*k*2s	-0.200	-0.307	0.057	0.236	0.206	0.175	0.236	0.201	0.001	-0.022	0.191	0.381	-0.027		
*k*2l	0.238	0.206	0.355	-0.203	0.233	0.221	0.130	0.143	0.107	0.089	0.065	0.052	0.217	-0.29	
Maout	0.132	-0.040	0.190	0.182	-0.093	-0.057	-0.471	-0.517	0.511	0.520	-0.213	0.035	-0.28	0.018	0.005

Table 7.20. Mean treatment effects on urinary PD, allantoin, pseudouridine and creatinine excretion and PD/c, A/c and PS/c ratios

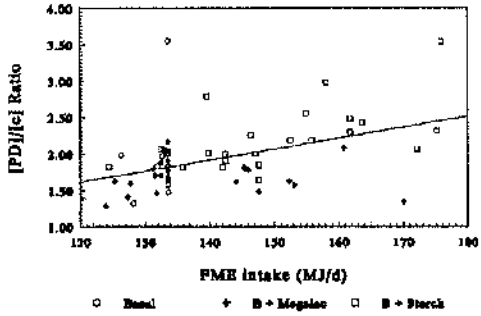
	Experimental treatment						Mean	FS	Lev	FS *
	F1	F2	F3	S1	S2	S3	SED			Lev
PD excretion (mmoles/d)	215	214	217	256	344	289	31.6	***	NS	NS
Allantoin excretion (mmoles/d)	173	172	171	208	282	235	26.0	***	NS	NS
Pseudouridine excretion (mmoles/d)	9.80	10.14	10.00	10.15	10.25	8.14	1.654	NS	NS	NS
Creatinine excretion (mmoles/d)	129	127	131	135	146	121	16.7	NS	NS	NS
PD/c	1.67	1.73	1.63	1.98	2.39	2.54	0.106	***	***	***
A/c	1.34	1.39	1.27	1.63	1.98	2.08	0.085	***	**	***
Ps/c	0.075	0.081	0.076	0.074	0.066	0.062	0.006	*	NS	NS

Key:-

FS denotes differences between Megalac and starch diets
Lev denotes differences between treatment levels
FS*Lev denotes interactions between treatment type and level
NS denotes Not Significant
*, **, *** indicate significance at the $P<0.05$, $P<0.01$ and $P<0.001$ levels, respectively

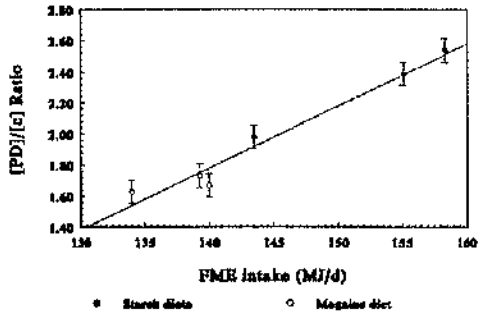
Figure 7.7. Relationship between urinary PD/c ratios and calculated FME intake

i) Individual cows



Each point is the mean of 2 measurements

ii) Treatment means



Each point is the mean of 16 measurements
Error bars indicate between treatment SE

Table 7.21. Relationships between urinary PD/c and A/c ratios and those scaled for creatinine concentration with FME intake and calculated MCP

Variate	Dataset	PD/c			Scaled PD/c		
		n	r^2	P	n	r^2	P
FME Intake	Individual cow	58	0.187	<0.01	58	<0.001	0.873
	Treatment means	6	0.960	<0.001	6	0.797	<0.05
lMCP	Individual cow	58	0.160	<0.01	58	0.001	0.798
	Treatment means	6	0.903	<0.01	6	0.709	<0.05
oMCP	Individual cow	58	0.203	<0.001	58	<0.001	0.989
	Treatment means	6	0.900	<0.01	6	0.788	<0.05
		A/c			Scaled A/c		
FME Intake	Individual cow	58	0.183	<0.01	58	<0.001	0.944
	Treatment means	6	0.974	<0.001	6	0.807	<0.01
lMCP	Individual cow	58	0.148	<0.01	58	<0.001	0.885
	Treatment means	6	0.884	<0.01	6	0.706	<0.05
oMCP	Individual cow	58	0.211	<0.001	58	<0.001	0.907
	Treatment means	6	0.909	<0.01	6	0.796	<0.05

7.4.8. Urinary pseudouridine excretion

Mean treatment effects on urinary pseudouridine excretion are presented in Table 7.20. Urinary pseudouridine excretion was not significantly ($P>0.05$) different between starch and Megalac diets (means of 9.51 and 9.57 mmoles/d, respectively). Daily mean pseudouridine excretion was significantly correlated with daily mean Ps/c ratios ($r^2=0.512$, $n=113$, $P<0.001$). Prediction of daily mean pseudouridine excretion from daily mean Ps/c ratios was improved by scaling Ps/c ratios by mean creatinine concentration for each cow ($r^2=0.781$, $n=113$, $P<0.001$). Mean treatment effects on Ps/c ratios are shown in Table 7.20. In contrast to urinary pseudouridine excretion, Ps/c ratios were significantly ($P<0.05$) higher for Megalac diets (means of 0.077 and 0.067 for Megalac and starch diets, respectively).

Correlation coefficients between individual cow pseudouridine excretion, Ps/c ratios, DM, ME, FME, CP intakes, rumen and hindgut small particle and liquid outflow rates are shown in Table 7.19. Relationships between mean treatment pseudouridine excretion and Ps/c ratios with DM, ME, FME and CP intakes and calculated MCP supply are presented in Table 7.24.

7.4.9. Urinary creatinine excretion

Mean treatment effects on urinary creatinine excretion are shown in Table 7.20. Differences between starch and Megalac diets (means of 134.0 and 129.0 mmoles/d) were not significant ($P>0.05$), while creatinine excretion was significantly ($P<0.05$) different between cows, ranging between 39 to 233 mmoles/d.

Table 7.22. Prediction of urinary PD excretion

Source of variation	No. of terms	Regression equation	n	r^2	P
Diet	Single	Y = 9.5 + 1.5 FMEI	58	0.108	<0.01
	Multiple	Same as single term			
Milk	Single	Y = 31.2 + 56.3 [Prot]	58	0.108	<0.01
	Multiple	Y = 185 + 946 Prot Yd -601 Lact Yd	58	0.143	<0.01
Outflow rates	Single	Y = 88.8 + 55.4 *k*2s	58	0.050	0.051
	Multiple	Y = 88 - 55.1 *k*1s + 80.9 *k*2s + 113 *k*1l	58	0.113	<0.05
Estimated MCP	Single	Y = 37.7 + 0.113 lMCP	58	0.099	<0.01
Diet and outflow rates	Multiple	Y = -258 + 1.43 FMEI -62.6 *k*1s + 73.9 *k*2s	58	0.212	<0.01
Milk and outflow rates	Multiple	Y = -362 + 65.5 [Prot] -54.4 *k*1s + 86.9 *k*2s + 132 *k*1l	58	0.271	<0.001
All sources	Multiple	Y = -556 + 507 DMD + 1273 Prot Yd - 1075 Lact Yd + 67.5 *k*2s + 104 *k*1l + Urea-N Yd	58	0.311	<0.001

Table 7.23. Prediction of urinary PD/c ratios

Source of variation	No. of terms	Regression equation	n	r^2	P
Diet	Single	Y = 0.201 + 0.0151 FMEI	58	0.187	<0.001
	Multiple	Same as single term			
Milk	Single	Y = 0.825 + 5.1 ProtYd	58	0.196	<0.001
	Multiple	Y = 2.03 + 0.109 Yd - 0.24 [Fat] + 11.1 Prot Yd	58	0.398	<0.001
Outflow rates	Single	Y = 0.406 + 0.83 *k*1l	58	0.075	<0.05
	Multiple	Y = 0.125 + 0.746 *k*1l + 0.16 *k*2l	58	0.076	<0.05
Estimated MCP	Single	Y = 0.148 + 0.0011 lMCP	58	0.160	0.001
Diet and outflow rates	Multiple	Y = 0.201 + 0.0151 FMEI	58	0.187	<0.001
Milk and outflow rates	Multiple	Y = -1.85 -0.239 [Fat] + 0.504 [Prot] + 5.34 Prot Yd + 0.729 *k*1l + 0.00191 [Urea-N]	58	0.453	<0.001
All sources	Multiple	Y = -2.5 - 0.226 [Fat] + 9.22 Prot Yd + 0.714 *k*1l + 0.00513 [Urea-N] - 0.00226 Urea-N Yd + 0.00854 lMCP	58	0.533	<0.001

Table 7.24. Relationships between mean treatment pseudouridine excretion, Ps/c ratios and DM, ME, FME, CP intakes and calculated MCP supply

Variate	Pseudouridine			Ps/c		
	n	r^2	P	n	r^2	P
DM	6	<0.01	0.836	6	<0.01	0.998
ME	6	<0.01	0.959	6	0.023	0.400
FME	6	0.166	0.231	6	0.795	<0.05
CP	6	<0.01	0.913	6	<0.01	0.813
lMCP	6	0.205	0.195	6	0.743	<0.05
oMCP	6	0.171	0.227	6	0.826	<0.01

7.4.10. Milk allantoin excretion

Mean treatment effects on milk allantoin excretion are shown in Table 7.26. Daily milk allantoin excretion was moderately correlated with daily mean milk allantoin concentration (r^2=0.468, n=109, P<0.001) and milk yield (r^2=0.630, n=109, P<0.001).

No direct measurements of MCP were made in the current study, consequently, milk allantoin excretion and concentration responses were compared to:- i) urinary PD excretion, ii) calculated FME intake and iii) calculated MCP supply.

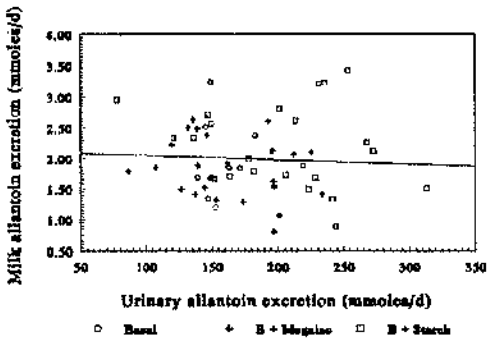
Based on individual cow measurements, milk allantoin excretion was not significantly related to urinary PD or allantoin excretion (r^2=0.009, n=58, P=0.494 and r^2=0.003, n=58, P=0.680, respectively). The use of treatment mean values indicated milk allantoin excretion was moderately correlated with urinary PD and allantoin excretion (r^2=0.639, n=6, P=0.056 and r^2=0.639, n=6, P=0.056, respectively). Figure 7.8. describes the relationships between urinary and milk allantoin excretion based on individual cow and mean treatment values. Relationships between milk allantoin excretion and urinary PD/c and A/c ratios and the same ratios scaled for creatinine concentration were also investigated and are summarised in Table 7.25. Figure 7.9. describes the relationships between individual cow and mean treatment milk allantoin excretion and urinary A/c ratios.

Table 7.25. Relationships between urinary PD and allantoin excretion and urinary PD/c and A/c ratios and those scaled for creatinine concentration with milk allantoin excretion

Variate	Dataset	n	r^2	P
Urinary PD excretion	Individual cow	58	0.009	0.494
	Treatment means	6	0.639	0.056
Urinary allantoin excretion	Individual cow	58	0.003	0.680
	Treatment means	6	0.639	0.056
Urinary PD/c ratio	Individual cow	58	0.248	<0.001
	Treatment means	6	0.851	<0.01
Scaled urinary PD/c ratio	Individual cow	58	0.022	0.276
	Treatment means	6	0.570	0.051
Urinary A/c ratio	Individual cow	58	0.257	<0.001
	Treatment means	6	0.825	<0.01
Scaled urinary A/c ratio	Individual cow	58	0.023	0.274
	Treatment means	6	0.573	0.050

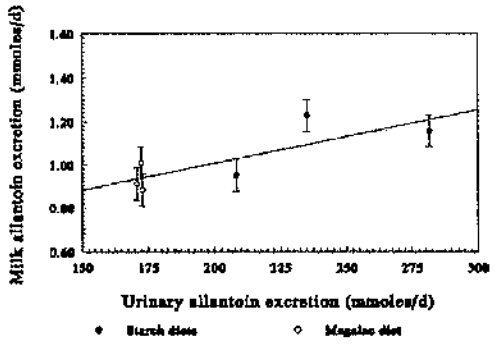
Figure 7.8. Relationships between urinary and milk allantoin excretion

i) Individual cows



Each point is the mean of 2 measurements

ii) Treatment means

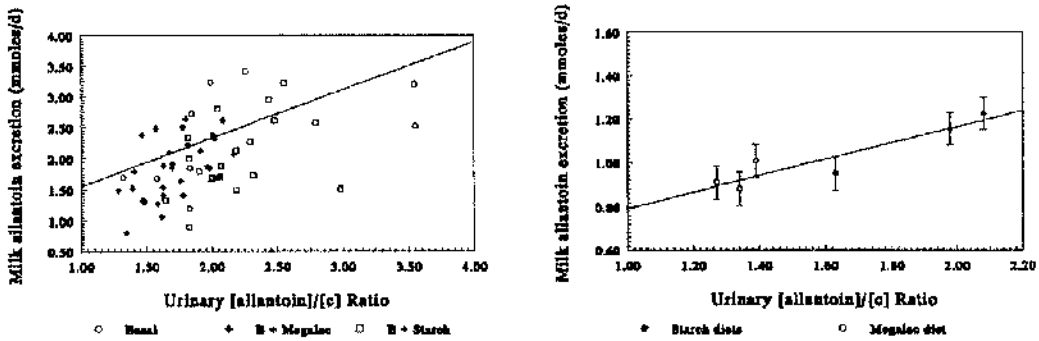


Each point is the mean of 16 measurements

Error bars indicate between treatment SE

Figure 7.9. Relationship between urinary A/c ratios and milk allantoin excretion

i) Individual cows ii) Treatment means

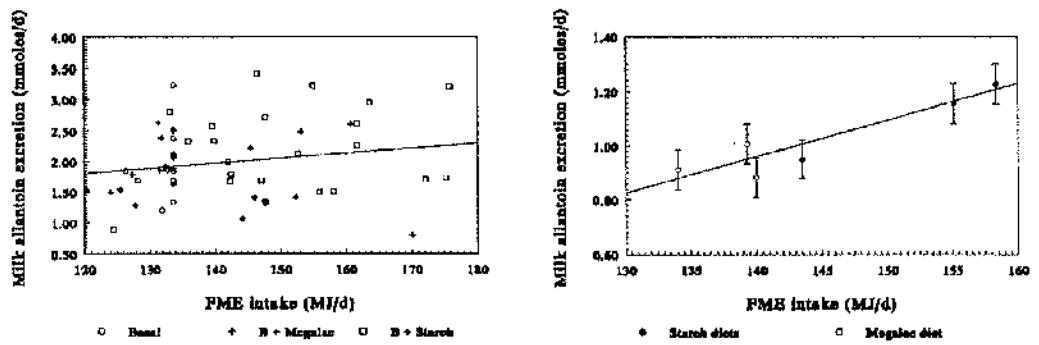


Each point is the mean of 2 measurements Each point is the mean of 16 measurements

Error bars indicate between treatment SE

Figure 7.10. Relationship between milk allantoin excretion and calculated FME intake

i) Individual cows ii) Treatment means



Each point is the mean of 2 measurements Each point is the mean of 16 measurements

Error bars indicate between treatment SE

Based on individual cow observations (mean of two measurements), milk allantoin excretion was poorly correlated with FME intake (r^2=0.019, n=58, P=0.153), but dramatically improved when mean treatment values were used (r^2=0.822, n=58,

P<0.01). Figure 7.10 describes the relationships between milk allantoin excretion and FME intake based on individual cow and treatment mean values. In order to evaluate the potential of milk allantoin excretion as an indicator of MCP further investigations were conducted to establish the response of milk allantoin excretion to incremental changes in FME intake within individual cows, using co-variate data as a reference measurement. Responses of milk allantoin excretion to incremental changes in FME intake derived for each cow are tabulated in Table 7.27. Pooling all the individual cow data indicated a poor response of milk allantoin excretion to incremental changes in FME intake (r^2=0.001, n=45, P=0.308). Relationships between indivdual cow and mean treatment milk allantoin excretion with calculated oMCP and lMCP supplies are shown in Table 7.28. Correlation coefficients between individual cow milk allantoin excretion, urinary PD, allantoin, pseudouridine, creatinine excretion, PD/c, A/c, Ps/c ratios, DM, ME, FME, CP intakes, rumen and hindgut small particle and liquid outflow rates are presented in Table 7.19.

Multiple regression analysis was performed on individual cow dietary, milk, rumen and hindgut outflow measurements and calculated MCP to establish the best predictors of milk allantoin excretion. Regression equations which accounted for the largest variation using single and multiple terms are presented in Table 7.30.

Table 7.26. Mean treatment effects on milk allantoin excretion (mmoles/d) and concentration (mM)

	Experimental Treatment						Mean	FS	Lev	FS *
	F1	F2	F3	S1	S2	S3	SED			Lev
Milk allantoin excretion	0.883	1.008	0.911	0.950	1.156	1.227	0.104	#	NS	NS
Arithmetic mean allantoin concentration	0.120	0.121	0.114	0.123	0.137	0.143	0.0098	#	NS	NS
Weighted mean allantoin concentration	0.117	0.117	0.122	0.120	0.132	0.140	0.0093	#	NS	NS
AM allantoin concentration	0.113	0.115	0.123	0.118	0.132	0.139	0.0112	NS	NS	NS
PM allantoin concentration	0.126	0.127	0.108	0.127	0.142	0.149	0.0140	NS	NS	NS
% of urinary allantoin excreted in milk	1.18	1.39	1.64	0.89	0.78	1.04	0.312	*	NS	NS

Key:-

FS denotes differences between Megalac and starch diets
Lev denotes differences between treatment levels
FS*Lev denotes interactions between treatment type and level
NS denotes Not Significant
and * indicates significance at the 0.10>P>0.05 and P<0.05 levels, respectively

Table 7.27. Individual cow milk allantoin excretion responses to changes in calculated FME intake

Cow	n	r^2	P
33	4	0.017	0.413
71	4	0.683	0.112
127	4	0.351	0.247
139	3	0.999	<0.01
140	4	<0.001	0.644
186	4	<0.001	0.906
195	4	<0.001	0.665
246	4	0.909	<0.05
255	4	0.288	0.275
270	3	<0.001	0.507
273	4	0.946	<0.05
285	4	<0.001	0.811

Table 7.28. Relationships between milk allantoin excretion and calculated MCP

Variate	Dataset	Milk allantoin excretion		
		n	r^2	P
FME Intake	Individual cow	58	0.019	0.153
	Treatment means	6	0.801	0.01
lMCP	Individual cow	58	0.016	0.172
	Treatment means	6	0.944	<0.001
oMCP	Individual cow	58	0.010	0.222
	Treatment means	6	0.699	<0.05

Table 7.29. Prediction of milk allantoin excretion

Source of variation	No. of terms	Regression equation	n	r^2	P
Diet	Single	Y = 0.081 + 0.00831 FMEI	58	0.019	0.153
	Multiple	Same as single term			
Milk	Single	Y = - 0.715 + 12.3 ProtYd	58	0.705	<0.001
	Multiple	Y = - 0.045 - 0.178 [Prot] + 12.0 Prot Yd	58	0.711	<0.001
Outflow rates	Single	None			
	Multiple	None			
Estimated MCP	Single	Y = 0.98 + 0.000621 iMCP	58	0.016	0.172
Diet and outflow rates	Multiple	Y = 0.081 + 0.00831 FMEI	58	0.019	0.153
Milk and outflow rates	Multiple	Y = - 0.045 - 0.178 [Prot] + 12.0 Prot Yd	58	0.711	<0.001
All sources	Multiple	Y = - 0.546 + 0.00929 FMEI - 0.373 [Prot] + 0.246 Fat Yd + 10.9 Prot Yd	58	0.744	<0.001

7.4.11. Milk allantoin concentration

Mean treatment effects on am and pm milk allantoin concentrations are shown in Table 7.26. Allantoin concentrations of am and pm samples were not significantly ($P>0.05$) different between starch (means of 0.129 and 0.139 mM, respectively) and Megalac diets (means of 0.117 and 0.120 mM, respectively). Mean daily allantoin concentrations derived from an arithmetic mean of am and pm samples, and a mean weighted for milk yield were calculated for each cow. Mean treatment effects on arithmetic and weighted mean milk allantoin concentrations are presented in Table 7.26. Daily arithmetic mean milk allantoin concentrations were significantly ($0.10>P>0.05$) higher for starch compared to Megalac diets (means of 0.134 and 0.118 mM, respectively). Mean milk allantoin concentrations, weighted for milk yield were also significantly ($0.10>P>0.05$) higher for starch diets (means of 0.131 and 0.118 mM for starch and Megalac diets, respectively).

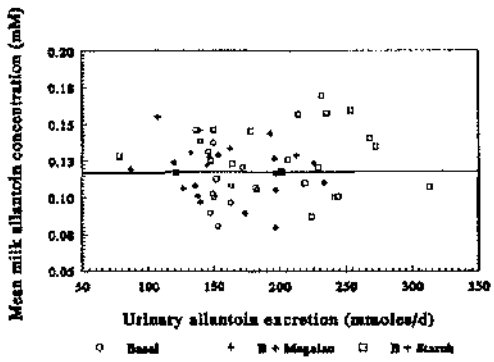
Relationships derived between arithmetic and weighted mean milk allantoin concentration with urinary PD and allantoin excretion and urinary PD/c and A/c ratios and those scaled for creatinine concentration are summarised in Table 7.30. The relationships between urinary and milk allantoin excretion based on individual cow and mean treatment values are described in Figure 7.11. Figure 7.12. describes the relationships between urinary A/c and milk allantoin excretion based on individual cow and mean treatment values.

Relationships between am, pm, arithmetic and weighted mean allantoin concentrations and FME intake and calculated MCP supply, based on individual cow and treatment mean values are shown in Table 7.31. Based on individual cow measurements, correlations between FME intake and am, arithmetic and weighted mean milk allantoin concentrations were similar. Using treatment mean values dramatically improved these relationships, indicating that arithmetic mean milk allantoin concentrations more closely reflected variations in FME intake than am, pm or weighted mean allantoin concentrations. Relationships between arithmetic mean milk allantoin concentration and

FME intake based on individual cow and treatment mean values are describe in Figure 7.13.

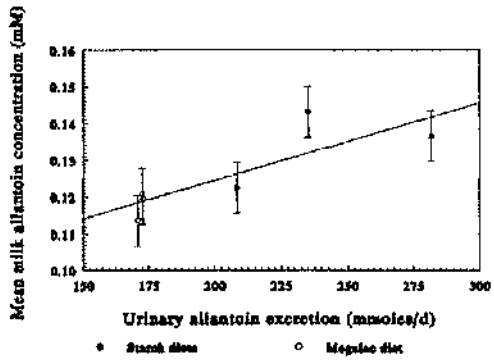
Figure 7.11. Relationship between urinary allantoin excretion and mean milk allantoin concentration

i) Individual cows



Each point is the mean of 4 measurements

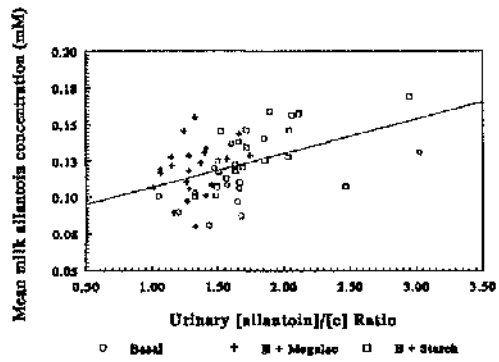
ii) Treatment means



Each point is the mean of 32 measurements
Error bars indicate between treatment SE

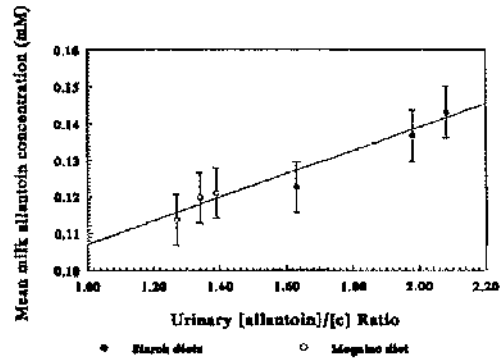
Figure 7.12. Relationship between urinary A/c ratios and mean milk allantoin concentration

i) Individual cows



Each point is the mean of 4 measurements

ii) Treatment means



Each point is the mean of 32 measurements
Error bars indicate between treatment SE

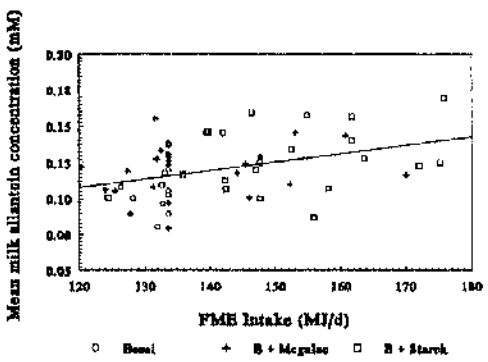
Table 7.30. Relationships between urinary PD and allantoin excretion and urinary PD/c and A/c ratios and those scaled for creatinine concentration with arithmetic and weighted mean milk allantoin concentration

Variate	Dataset	Arithmetic mean			Weighted mean		
		n	r^2	P	n	r^2	P
Urinary PD	IC	58	<0.001	0.931	58	<0.001	0.822
excretion	TM	6	0.613	<0.05	6	0.534	0.061
Urinary allantoin	IC	58	0.004	0.647	58	0.006	0.594
excretion	TM	6	0.624	<0.05	6	0.516	0.066
Urinary PD/c	IC	58	0.177	<0.001	58	0.199	<0.001
ratio	TM	6	0.942	<0.001	6	0.834	<0.01
Scaled urinary PD/c	IC	58	<0.001	0.898	58	<0.001	0.916
ratio	TM	6	0.701	0.051	6	0.519	0.065
Urinary A/c	IC	58	0.196	<0.001	58	0.218	<0.001
ratio	TM	6	0.825	<0.01	6	0.784	<0.05
Scaled urinary A/c	IC	58	<0.001	0.984	58	<0.001	0.847
ratio	TM	6	0.573	0.050	6	0.512	0.067

Key:-
IC denotes individual cow measurements
TM denotes treatment mean values

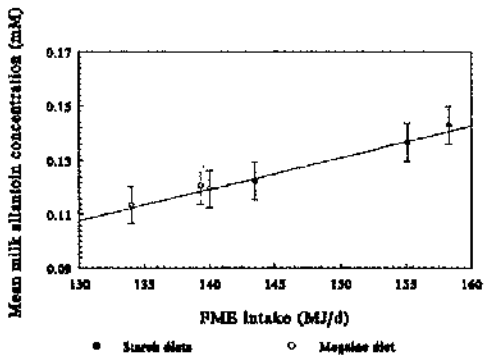
Figure 7.13. Relationship between mean milk allantoin concentration and FME intake

i) Individual cows



Each point is the mean of 4 measurements

ii) Treatment means



Each point is the mean of 32 measurements

Error bars indicate between treatment SE

In order to evaluate the potential of milk allantoin concentration as an indicator of MCP supply, further investigations were conducted to establish am, pm, arithmetic and weighted mean allantoin concentration responses to incremental changes in calculated FME intake within individual cows, using co-variate data as a reference measurement. Responses of am, pm, arithmetic and weighted mean milk allantoin concentrations to incremental changes in FME intake derived for each cow are tabulated in Tables 7.32., 7.33., 7.34. and 7.35., respectively. Pooling all the individual cow data indicated poor responses of am, pm arithmetic and weighted mean milk allantoin concentrations to incremental changes in FME intake shown in Table 7.36.

Table 7.31. Relationships between am, pm, arithmetic and weighted mean allantoin concentrations and calculated FME intake and MCP supply

	Dataset	FME intake			lMCP			oMCP		
		n	r^2	P	n	r^2	P	n	r^2	P
am	IC	58	0.158	<0.01	58	0.161	<0.01	58	0.043	0.068
	TM	6	0.598	<0.05	6	0.729	<0.05	6	0.526	0.063
pm	IC	58	0.068	<0.05	58	0.160	<0.01	58	<0.01	0.439
	TM	6	0.899	<0.01	6	0.903	<0.01	6	0.880	<0.01
Art	IC	58	0.135	<0.01	58	0.139	<0.01	58	0.017	0.165
Mean	TM	6	0.981	<0.01	6	0.919	<0.01	6	0.936	<0.001
Wt	IC	58	0.158	<0.01	58	0.160	<0.01	58	0.030	0.103
Mean	TM	6	0.743	<0.05	6	0.825	<0.01	6	0.685	<0.05

Key:-

IC denotes individual cow measurements
TM denotes treatment mean values
Art mean denotes arithmetic mean of am and pm allantoin concentrations
Wt mean denotes mean allantoin concentration weighted by milk yield

Table 7.32. Individual cow am milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
33	4	<0.001	0.729
71	3	<0.001	0.833
127	3	<0.001	0.578
139	3	0.956	0.095
140	4	0.627	0.133
186	4	<0.001	0.451
195	4	<0.001	0.913
246	4	0.995	<0.01
255	4	<0.001	0.651
270	3	0.236	0.424
273	4	<0.001	0.491
285	4	<0.001	0.709

Table 7.33. Individual cow pm milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
33	4	<0.001	0.597
71	3	<0.001	0.928
127	3	0.992	<0.05
139	2	-	-
140	3	<0.001	0.627
186	4	0.098	0.369
195	4	<0.001	0.465
246	4	0.611	0.139
255	4	<0.001	0.828
270	3	<0.001	0.635
273	4	0.729	0.095
285	4	<0.001	0.886

Table 7.34. Individual cow arithmetic mean milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
33	4	<0.001	0.582
71	4	0.850	0.051
127	4	<0.001	0.580
139	3	0.821	0.194
140	4	0.774	0.079
186	4	0.059	0.390
195	4	<0.001	0.561
246	4	0.833	0.057
255	4	<0.001	0.454
270	3	0.061	0.481
273	4	0.859	<0.05
285	4	<0.001	0.960

Table 7.35. Individual cow weighted mean milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
33	4	<0.001	0.461
71	4	0.452	0.204
127	3	0.974	0.072
139	3	0.998	<0.05
140	4	0.903	<0.05
186	4	<0.001	0.429
195	4	<0.001	0.603
246	4	0.900	<0.05
255	4	0.432	0.212
270	3	0.058	0.482
273	4	0.555	0.161
285	4	<0.001	0.867

Table 7.36. Relationships between individual cow am, pm, arithmetic and weighted mean milk allantoin concentration responses to changes in calculated FME intake

Concentration	n	r^2	P
am	44	0.017	0.194
pm	42	0.011	0.237
Arithmetic mean	46	0.018	0.140
Weighted mean	44	0.027	0.186

7.5. Discussion

The current experiment was designed to evaluate the potential of milk allantoin as an index of MCP supply. Consequently the effects of potato starch and Megalac dietary supplements on milk production and composition, rumen basal diet DM degradation and rumen fermentation are not discussed in detail.

Rumen VFA concentrations

Rumen liquor total VFA concentrations were found to be significantly higher ($0.10>P>0.05$) for the Megalac supplemented diet which could indicate either a reduced rate of VFA absorption or increased production rate. *In-sacco* degradability measurements indicated that increases in rumen VFA concentration were not due to increased fermentation of the basal diet. Dietary inclusion of Megalac which contains a very small proportion of the potentially fermentable substrate glycerol (typically 1%, S. Taylor, personal communication) would not be expected to stimulate VFA production, suggesting increases in rumen VFA concentration are likely to be accounted for by a reduction in VFA absorption from the rumen.

Rumen pH

Mean rumen pH values ranged between 7.56 to 7.62. Previous observations in sheep have demonstrated that rumen pH typically varies between 6.6 to 6.9 for urea

supplemented straw based diets (Balcells *et al*, 1993b). Discrepancies between rumen pH values reported in the current study and those reported in the literature are difficult to reconcile. Rumen pH was not measured directly after rumen liquor collection, but after frozen storage. Although a correction was used to account for rumen sample storage it is conceivable that losses of VFAs prior to storage and during subsequent thawing could result in an artifically high rumen pH.

Rumen ammonia concentrations

Experimental diets were formulated to ensure MCP synthesis would not be constrained by dietary eRDP supply, achieved by dietary inclusion of soyabean meal and urea. Consequently mean rumen ammonia concentrations (276-427 mg/l) were higher than reported concentrations of between 5.6-128 mg/l observed in sheep fed a urea supplemented straw diet (Balcells *et al*, 1993b). Mean rumen ammonia concentrations were found to be higher for the potato starch supplemented diet. Further investigations indicated that high rumen ammonia concentrations were only observed eight hours post-feeding, suggesting microbial capture of dietary nitrogen was limited by rumen energy availablity. Assuming potato starch was almost completely fermented eight hours post-feeding which combined with a depressed basal diet fermentation would lead to a reduction in microbial energy supply which could account for increased rumen ammonia concentrations.

In-sacco DM degradability

Dietary inclusion of potato starch resulted in a depression of basal diet DM degradation within the rumen, which may be accounted for by increases in rumen outflow rate which would be expected to lead to a reduction in rumen retention of feed particles depressing dry matter degradation (Orskov, 1994). Measurements of dairy cow rumen outflow rates reported in this chapter and those performed in sheep by Huque (1991) tend to confirm this suggestion. Depressions in basal diet DM degradation could also be a result of changes in rumen fermentation induced by feeding potato starch. Feeding readily fermentable carbohydrates in the form of concentrates generally

stimulates a rapid production of VFAs causing a strong decrease in rumen pH (Malestein *et al*, 1981). Rumen cellulolytic activity is known to be highly dependent on rumen pH (Itasse and Orskov, 1983), being completely inhibited at rumen pH below 5.9 (Itasse *et al*, 1986). Decreases in rumen cellulolytic activity could be responsible for depressions in basal diet DM degradation, despite rumen pH measurements indicating it to be above 7.0. However, the rumen liqour sampling used in the current experiment is subject to considerable criticism. Ovine rumen pH has been shown to vary diurnally (e.g. Chamberlain *et al*, 1993), while further observations have demonstrated feeding of a starch based diet resulted in depressions in rumen pH below 6.0 four hours post feeding (Huntington, 1994). If similar variations in rumen pH occured in the current experiment they would be inadequately detected due to the limited sampling. Adverse effects of potato starch on rumen pH can not be precluded as a possible explanation for depressed basal diet DM degradation.

In-vivo studies have demonstrated that calcium saponified fatty acids are not completely stable during rumen incubation (Brinkmann and Abel, 1992). Measured *in-sacco* Megalac DM losses are most likely to be accounted for as a result of dissociation of calcium saponified fatty acids contained in Megalac.

Rumen and hindgut outflow rates

Rumen small particle and liquid outflow rates tended to increase with dietary inclusion of potato starch and decrease with Megalac. Numerous reports in the literature have documented concurrent increases in rumen outflow rates with DM intake (Evans, 1981a and b; Chen *et al*, 1992b; Dewhurst and Webster, 1992b; Gomes *et al*, 1993 and Djouvinov and Todorov, 1994). However, variations in mean DM intakes (ranging 14.18-15.10 and 14.18-15.09 kg/d for Megalac and starch diets, respectively) were relatively small, suggesting that differences in rumen outflow rates can be attributed to supplement type. This supports the observations of Castrillo *et al* (1993) who noted in sheep that similar intakes of a range of diets resulted in different rumen outflow rates.

Milk yield and composition

Increasing ME intakes with potato starch or Megalac resulted in significant increases in milk yield and induced significant changes in milk composition. Increasing the fat availability from the diet by inclusion of Megalac significantly increased milk fat concentrations confirming observations with other protected fats documented in the reviews of Storry (1981) and Palmquist (1984) and the study of Garnsworthy (1990).

Inclusion of potato starch with the basal diet resulted in significant increases in milk protein concentration and milk protein yield. Since potato starch and Megalac provide negligible amounts of dietary nitrogen, DUP supplies between experimental treatments can be assumed to be relatively constant. Variations in MP available for absorption and subsequent milk protein synthesis are likely to be a result of changes in the quantity of rumen MCP, reaching the small intestine. Based on AFRC (1992) recommendations, the basal diet was formulated to supply eRDP in excess of predicted requirements, therefore FME intake would be expected to be the main constraint on rumen MCP synthesis. Close correlations between mean treatment FME intake and milk protein concentration (r^2=0.930, n=6, P<0.01) and milk protein yield (r^2=0.870, n=6, P<0.01) suggest that increased milk protein concentrations and yields observed for starch diets are a result of increased supply of MCP and hence MP. Other experiments have also demonstrated the dependence of milk protein yield (Dewhurst, 1989 and Dewhurst *et al*, 1996) and milk protein concentration (Moorby, 1993) on MP supply.

Milk urea nitrogen concentration and output

Numerous experiments (Oltner and Wiktorsson, 1983; Ropstad *et al*, 1989; Miettinen and Juvonen, 1990; Spain *et al*, 1990; Lindberg and Murphy, 1991; Gustavsson and Palmquist, 1993; Roseler *et al*, 1993 and Gonda and Lindberg, 1994) provide strong evidence to support the use of milk urea as an index of dietary nitrogen utilisation, based on the assumption that urea secreted in milk is essentially derived from rumen ammonia. Intakes of the basal diet, the sole source of dietary nitrogen were very similar between treatments, so milk urea-N concentrations may have reflected differences in dietary nitrogen utilisation. Increasing FME supply in the form of starch significantly

(P<0.01) depressed milk urea-N concentrations, indicating an increase in the incorporation of dietary nitrogen into MCP. These findings confirm those of Erbersdobler *et al* (1980), Oltner and Wiktorsson (1983) and Gustavsson (1993, cited by Gonda and Lindberg, 1994) which demonstrated the effect of dietary protein and energy balances on milk urea in dairy cows.

Urinary creatinine excretion

Mean daily urinary creatinine excretion ranged between 121-146 mmoles/d, confirming earlier observations of 132 mmoles/d reported in chapter 6. Daily urinary creatinine excretion reported in both chapters is higher than previous reports of 112-117 mmoles/d (Puchala *et al*, 1993; Susmel *et al*, 1994a and Gonda and Lindberg, 1994). Differences in musculature between breeds as discussed in chapter 6 could potentially explain this discrepancy. Creatinine excretion was found to be significantly (P<0.05) different between-cows confirming observations of Chetal *et al* (1975) and those reported in chapter 6, but was found to be independent of nutrient supply confirming earlier observations in cows (Orskov and MacLeod, 1982 and Gonda and Lindberg, 1994), steers (Fujihara *et al*, 1987), sheep (Fujihara *et al*, 1987 and Lindberg and Jacobssen, 1990) and goats (Lindberg, 1985 and 1989).

Urinary pseudouridine excretion

Daily urinary pseudouridine excretion was not significantly (P>0.05) different between treatments confirming earlier observations which indicated that pseudouridine excretion was similar in non-lactating cows fed at 1 and 1.5 times maintenance (Puchala *et al*, 1993). Mean daily pseudouridine excretion ranged between 8.14-10.25 mmoles/d, confirming similar values reported in chapter 6 (8.06-8.33 mmoles/d). In contrast, Puchala *et al* (1993) reported pseudouridine excretion varied between 2.2-2.45 and 1.9-2.25 mmoles/d in non-lactating cows and heifers, respectively. Pseudouridine excreted in the urine has been demonstrated to be unrelated to pseudouridine content of the diet (Weissman *et al*, 1962 and Puchala *et al*, 1993) and is therefore assumed to be derived mainly from RNA and t-RNA, representing an index of tissue RNA turnover. Urinary

pseudouridine excretion (expressed on a metabolic weight basis) has been shown to be higher in young compared to mature animals suggesting that RNA turnover and possibly protein synthesis increase during protein accretion (Puchala *et al*, 1993). Discrepancies between current experimental observations and those of Puchala *et al* (1993) are difficult to reconcile, but are possibly due to elevated tissue RNA turnover and protein accretion during lactation.

Urinary PD excretion

Urinary allantoin excretion was highly correlated with urinary PD excretion (r^2= 0.987, n=113, P<0.001) and accounted for 82% of all PDs excreted, with the remainder as uric acid. This finding agrees closely with observations reported in chapter 6. Urinary xanthine and hypoxanthine excretion was negligible confirming earlier observations of Chen *et al* (1990b), Verbic *et al* (1990), Susmel *et al* (1994b) and Dewhurst *et al* (1995). Due to the their close correlation further discussions are confined to urinary PD excretion, but apply equally to urinary allantoin excretion.

The use of a calculated value for the FME of feeds may be criticised because inadequacy of the techniques for its estimation may lead to flawed assumptions. This problem has been minimised in the current experiment by the use of potato starch (assumed to be completely fermented) and Megalac (assumed to be unfermented) to manipulate FME supply. This approach ensured a reliable estimate of incremental changes of FME although its absolute level was not accurately known.

MCP supply was not directly measured but assumed to be reflected by urinary PD excretion on the basis of literature evidence (e.g. Chen *et al*, 1990a; Verbic *et al*, 1990; Djouvinov and Todorov, 1994 and Perez *et al*, 1994). Correlations between urinary PD excretion with calculated FME intake (r^2=0.108, n=58, P<0.01) suggest that calculated FME intake does not allow an accurate prediction of MCP supply for an individual cow.

Renal clearances of creatinine are assumed to approach that of PDs (Greger *et al*, 1976) and therefore improved correlation between urinary PD/c ratios with calculated

FME intake (r^2=0.187, n=58, P<0.01) are almost certainly due to better accounting for between-cow variations in the proportion of PDs excreted renally.

Correlations between urinary PD excretion or PD/c ratios with calculated FME intake (r^2=0.707, n=6, P<0.05 and r^2=0.960, n=6, P<0.001, respectively), improved dramatically when mean treatment values were used.

Similar relationships were observed between urinary PD excretion or PD/c ratios with MCP supply calculated by either level of feeding or measured rumen outflow methods as those reported for calculated FME intake. This is to be expected as both calculations are based on calculated FME intake. Experimental data indicates that current AFRC (1992) predictions of MCP are reasonably accurate on a group basis, in this case eight cows, but poor for an individual cow, highlighting the requirement for an on-farm diagnostic marker of MCP supply. Lack of relationships between individual cow urinary PD excretion or PD/c ratios with calculated FME intake are almost certainly due to between-cow variations in EMPS which are taken into account when mean treatment values were used. It is well documented that sources of FME can lead to differences in EMPS (e.g. Offer *et al*, 1978, ARC, 1984, Balcells *et al*, 1993a, Chamberlain *et al*, 1993). Using AFRC (1992) calculations, EMPS values were 11.5, 11.6, 11.7, 11.3, 11.5 and 11.5 g M-N/ MJ FME for diets F1, F2, F3, S1, S2 and S3, respectively, suggesting relatively small differences in EMPS between experimental diets. However, using the models of Verbic *et al* (1990) and Chen *et al* (1992a) to estimate M-N supply from measured urinary PD excretion gives values of EMPS (g M-N/MJ FME) as 12.6, 16.5, 13.2, 10.4, 10.5 and 11.0 for diets S1, S2, S3, F1, F2, F3, respectively, indicating higher efficiencies for starch diets. EMPS values for the three starch diets were relatively similar confirming studies in sheep which demonstrated EMPS to be unaffected by increases in FME supply in the form of maize or barley starch (Gomes *et al*, 1993).

Current experimental findings are in complete agreement with those of Moorby and Dewhurst (1993a) who reported urinary PD excretion to be related to the FME content of the diet. Experiments in sheep have also demonstrated that increases in FME in the form of sugars (Chamberlain *et al*, 1993), wheat starch (Chamberlain *et al*, 1993), barley and maize starch (Gomes *et al*, 1993) result in elevated urinary PD excretion.

Milk allantoin excretion and concentration

Previous studies have indicated that variations in milk allantoin concentration are small (Kirchgessner and Kreuzer, 1985; Kirchgessner and Windisch, 1989; Rosskopf *et al*, 1991 and Giesecke *et al*, 1994) The current study has demonstrated that milk allantoin concentrations are not constant, but appear to be related to FME intake confirming the observations of Moorby and Dewhurst (1993a).

Direct measurements of MCP supply were not made and therefore evaluation of milk allantoin responses was investigated using three sets of measurements as references namely, i) urinary PD excretion and PD/c ratios, ii) calculated FME intake or iii) calculated MCP supply based on level of feeding or rumen outflow rate. Which of the three sets of measurements is the most reliable reference of MCP supply is uncertain. Since urinary PD excretion has been demonstrated to be closely correlated with duodenal microbial NA supplies (Chen *et al*, 1990a and Verbic *et al*, 1990) and comparisons between urinary PD excretion and other microbial markers have been shown to be in good agreement (Djouvinov and Todorov, 1994 and Perez *et al*, 1994), urinary PD excretion was considered to be the preferred reference.

i) Relationships with urinary PD excretion and PD/c ratios

Based on individual cow measurements, milk allantoin excretion or concentration were found to be poorly correlated with urinary PD excretion (r^2=0.009, n=58, P=0.680 and r^2<0.001, n=58, P=0.931, respectively), but improved when urinary PD/c ratios were used (r^2=0.248, n=58, P<0.001 and r^2=0.177, n=58, P<0.001, respectively). These relationships tend to indicate that partitioning of allantoin between renal and mammary excretory routes is highly variable between individual animals. Allantoin excreted by both excretory routes is derived from the plasma as a result of simple diffusion in the case of milk (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994) or by renal clearance in the case of urine (Chen *et al*, 1991a and Giesecke *et al*, 1993). Lack of relationships between urinary and milk allantoin excretion for individual cows suggests that these processes are not quantitiatively related and that even when between-cow variations in the proportion of PDs excreted renally by using urinary PD/c are taken

into account, the relationships remained poor. Since allantoin secreted in milk is thought to be due to diffusion from plasma (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994), it appears plausible that between-cow variations in plasma allantoin concentration and mammary blood flow could account for these observations.

The use of mean treatment values dramatically improved the correlations between urinary PD excretion or PD/c ratios with milk allantoin excretion (r^2=0.639, n=6, P=0.056 and r^2=0.851, n=6, P<0.01, respectively) or concentration (r^2=0.613, n=6, P<0.05 and r^2=0.942, n=6, P<0.001, respectively), presumably as a result of accounting for between-cow variations in allantoin partitioning which is discussed further in chapter 9.

ii) Relationships with calculated FME intake

Based on individual cow measurements daily milk allantoin excretion was not significantly correlated with calculated FME intake, while significant relationships existed between FME intake and am, pm, arithmetic and weighted mean allantoin concentration. Further investigations of individual cow reponses of milk allantoin excretion and concentration to changes in FME intake indicated responses varied considerably between cows, the vast majority of which were not significant (P>0.05). Furthermore, closer correlations were observed based on absolute rather than relative values.

The lack of relationship between individual cow milk allantoin excretion or concentration with calculated FME intake can potentially be accounted for primarily as a result of variations in allantoin partitioning and also factors influencing EMPS. Using co-variate data to calculate mean treatment values dramatically improved the relationships between milk allantoin excretion and concentrations with calculated FME intake suggesting that between-cow variations in allantoin partitioning and to a lesser extent EMPS have largely been accounted for.

iii) Relationships with calculated MCP supply

Calculations of MCP supply using level of feeding (AFRC, 1992) or rumen outflow (Cottrill, 1991) corrections for variations in EMPS, are primarily based on calculated FME intake and explain why relationships between milk allantoin excretion or concentration with calculated MCP supply were very similar to those with calculated FME intake. The use of calculated MCP supply as a reference of true MCP reaching the ruminant intestine is subject to the same criticisms of using calculated FME intake.

Current experimental observations indicated that, measurements of milk allantoin in an individual cow were poorly correlated with either urinary PD excretion, calculated FME intake or MCP supply. These results suggest that measuring allantoin in milk would be of little value as an index of MCP supply for an individual cow. Responses based on mean treatment values, tentatively suggest that milk excretion and concentration could be used to assess MCP supplies on a herd basis, although these observations may only hold when variations in milk yield are relatively small. However, the biological significance of the observed responses of milk allantoin excretion to urinary PD excretion, calculated FME intake or MCP supply based on mean treatment values can be criticised on two main grounds. Firstly, only a limited number of data points was used which hides the true variability of the relationship and secondly, no primary measurement of MCP synthesis was made. A far more satisfactory approach would have been to compare milk and urinary PD excretion with measurements of MCP supply using several microbial markers and duodenal digesta collection. However these measurements of MCP supply by this approach are subject to considerable error and are made in abnormal surgically modified animals.

Chapter Eight

The influence of fermentable energy supply on purine derivative excretion in milk and urine during early lactation

Summary

This experiment evaluates the potential of milk allantoin as an index of MCP supply under conditions causing variations in milk yield. Four experimental diets were offered to twelve multiparous Holstein/Friesian cows in an incomplete change-over design. Diets comprised of 40 kg (F.wt) silage (307 g/kg corrected DM, 149 g/kg CP and 12.4 MJ/kg ME) supplemented with 4.1 (L1), and 8.1 (L2) kg F.wt of a low fat concentrate (DM, CP and AH-EE of 860, 200 and 45 g/kg, respectively) or 3.8 (H1) and 7.5 (H2) kg F.wt of a high fat concentrate (880g/kg DM, 235g/kg CP and 110g/kg AH-EE). Milk recording, milk sampling and total urine collections were performed during the last three days of each experimental period. Subsamples of milk and urine were immediately stored at -20^{0}C prior to HPLC analysis. Individual milk yields varied between 16.8-46.8 kg/d. Prediction of individual cow urinary PD excretion from calculated FME intake (r^2=0.180, n=35, P<0.01) or calculated MCP supply (r^2=0.121, n=35, P<0.05) was poor. The use of mean treatment values dramatically improved the prediction of urinary PD excretion (r^2=0.963, n=4, P<0.05 and r^2=0.991, n=4, P<0.01, respectively). Based on individual cow measurements, milk allantoin excretion or concentration was poorly correlated with urinary PD excretion, calculated FME intake or MCP supply. Lack of relationships suggest that variations in milk allantoin excretion or concentration preclude the use of milk allantoin as a microbial marker for individual cows. Strong relationships existed between milk allantoin excretion or concentration with urinary PD excretion, calculated FME intake or MCP supply when mean treatment values were used. These results suggest that milk allantoin could potentially be used as an alternative to urinary PD excretion to assess MCP supply on a herd basis.

8.1. Experimental aims

The experiment was conducted to evaluate the potential of milk allantoin as an index of MCP under conditions causing relatively large variations in milk yield in early lactation cows.

8.2. Introduction

Observations documented in chapter 7 indicated that, based on individual cow measurements daily mean milk allantoin excretion or concentration was poorly correlated with urinary PD excretion, calculated FME intake or calculated MCP supply. The use of mean treatment values improved these relationships and suggested that, in conditions where variations in milk yield are relatively small, milk allantoin could potentially be used to assess MCP supply in dairy cows on a herd basis. The current experiment evaluates the potential of milk allantoin as a diagnostic marker of MCP supply under conditions causing relatively large variations in milk yield.

8.3. Materials and methods

All experimental cows remained in good health during the experiment, with the exception of cow 388 which was removed during the last experimental period due to a compound fracture of the right metacarpus and had to be humanely destroyed. Due to problems associated with connections between bladder catheters and urine collection vessels, eight total urine collections were unable to be performed. All other sampling procedures were performed as described.

8.3.1. Animals and animal management

Twelve early lactation mulitparious Holstein/Friesian cows were selected from the Auchincruive main dairy herd taking into account previous health history and housed

for the duration of the experiment in a dairy cow metabolism unit. Cows were restrained in individual stalls fitted with de Boer yokes and milked in *situ* at 05.00 and 15.30.

8.3.2. Experimental design

The experiment was of an incomplete (3 periods) change-over design with each animal receiving three experimental diets. Cows were assigned to three groups of four, according to calving date, parity and bodyweight. Mean days in lactation, bodyweight and parity prior to the begining of the experiment was 42, 41, 71; 570, 573, 558; 2.8, 2, 2.3 for groups one, two and three, respectively. Treatments were allocated randomly to cows within a group according to a 4 x 4 latin square. Samples were collected during the last week of each twenty one day experimental period.

Due to the unbalanced nature of the experimental design, analysis of variance was performed using residual maximum likelihood (REML, Patterson and Thompson, 1971) directive within Genstat 5.3 (Lawes Agricultural Trust, 1993) with the following model:- [fixed = type.level (interactions between treatment type and level) random = cow/period/day].

Exploration of the experimental data and regression analysis were undertaken using Minitab statistical package (Minitab Inc., 1980; Minitab Data Analysis Software, Pennsylvania State University, Pennsylvania).

8.3.3. Diet formulation

Silage intake was fixed at 40 kg F.wt/d throughout the experiment in an attempt to reduce the technical difficulties in estimating FME intake. SAC advisory rationing software was used to establish silage and concentrate allowances.

Silage fed was ensiled during late may 1994 from grass swards in which perennial ryegrass predominated. Silage was analysed by electrometric titration at SAC, Aberdeen (Chemical composition and titration analysis are shown in appendix 13).

FME intake was manipulated by feeding two concentrates, containing 11 (high fat, H) and 4.5% (low fat, L) fat, on a DM basis, at two inclusion levels (1 and 2). Concentrate chemical composition and formulation is shown in appendix 14 and 15, respectively. Concentrates were allocated as 4.1, 8.1, 3.8 and 7.5 kg F.wt/d for treatments L1, L2, H1 and H2, respectively, ensuring iso-ME and CP supplies from high and low fat sources at each inclusion level.

Predicted daily energy and protein intakes calculated from silage and concentrate intakes and measured chemical compositions are shown in Table 8.1.

Table 8.1. Predicted DM, energy and nitrogen intakes

	Experimental diet			
	L1	L2	H1	H2
Silage intake kg F.Wt/d	40	40	40	40
Silage oven DM intake kg/d	11.26	11.26	11.26	11.26
Silage CDM intake kg/d	12.28	12.28	12.28	12.28
Concentrate intake kg F.Wt/d	4.1	8.1	3.8	7.5
Concentrate DM intake kg/d	3.54	6.99	3.34	6.59
Total DM intake kg/d	15.82	19.27	15.62	18.87
CP intake g/d	2547	3247	2613	3377
ME intake MJ/d	195	236	198	242
FME intake MJ/d	137	174	134	166

8.3.4. Animal feeding

Silage was offered at 09.00 hours, with concentrates fed as two equal meals at approximately 05.30 and 15.30. Water was available *ad libitum* throughout the experiment.

8.3.5. Experimental measurements and sample collection

Experimental measurements and sampling procedures were performed over the last three days of each period.

8.3.5.1. Urine collection and sampling

The day before collections were made, bladder catheters were fitted to each cow. Total urine collections were made for three consecutive 24 hour periods starting at 05.30 hours. Collections were weighed and urine density was determined. Two subsamples were collected and immediately analysed in random sequence using the methodology described in chapter 2.

8.3.5.2. Milk recording and sampling

Milk yields were recorded for each milking over the three day sampling period. Milk samples collected at each milking for milk fat, protein and lactose determinations were stored with lactab preservative and submitted to the Dairy Technology Department, SAC, Auchincruive for analysis. Further milk samples were collected and stored at 4^0C, prior to milk allantoin analysis described in chapter 3 and urea-N determinations. Determination of milk allantoin was performed within several hours of collection. Samples destined for urea-N measurements were bulked according to yield and submitted to the Dairy Technology Department, SAC, Auchincruive for analysis.

8.3.5.3. Feed sampling

Representative samples of the silage offered on each sampling day were bulked together for each experimental period and stored at -20^0C. At the end of the experiment, silage samples were analysed by electrometric titiration and near infra-red reflectance spectroscopy. Silage intakes were recorded for each cow on all sampling days. Daily

silage refusals were weighed, sub-sampled and stored at -20°C. At the end of the experiment, silage refusals were thawed and bulked together for each experimental period. After thorough mixing, oven DM determinations were performed by drying at 80°C for 48 hours. Two represenative samples of each concentrate were submitted to the Analytical Services Unit, SAC, Auchincruive during each sampling period for chemical analysis.

8.3.5.4. Cow liveweights

At the begining of the experiment, all cows were weighed at 10.00 hours before being housed in the metabolism unit. Cows were weighed at 10.00 hours at the end of each sampling period.

8.4. Results

8.4.1. Animal production

8.4.1.1. DM, ME, FME and CP intakes

True DM, ME, FME and CP intakes were calculated for each cow for all sampling days based on measured feed intakes and the compositions of silage and concentrate. Mean treatment true DM, ME, FME and CP intakes are shown in Table 8.2. Although the CDM content of silage fed during period 1 was lower than that for the silage fed during periods 2 and 3, no significant ($P>0.05$) differences were observed in silage CDM between experimental periods (means 11.8, 12.2 and 11.9 kg DM/d for periods 1, 2 and 3, respectively).

Table 8.2. True DM, ME, FME and CP intakes

Intake	Experimental diet				Mean
	L1	L2	H1	H2	SED
Silage (kg F.Wt/d)	39.5	39.3	37.6	39.39	1.48
Silage oven DM (kg/d)	10.4	10.4	10.0	10.4	0.30
Silage CDM (kg/d)	12.1	12.1	11.5	12.1	0.46
Total DM (kg/d)[1]	15.7	19.1	14.9	18.7	0.46
ME (MJ/d)[1]	193	233	188	239	5.7
FME (MJ/d)[1]	136	172	128	165	3.6
CP (g/d)[1]	2526	3217	2500	3350	70.9

[1] Calculated on the basis of silage corrected DM values

8.4.1.2. Milk yield

Mean treatment effects on daily milk yields are shown in Table 8.3. Daily milk yields ranged between 16.8 and 46.8 kg/d. Milk yields were significantly higher ($P<0.05$) for high fat (mean 29.7 kg/d) than low fat treatments (mean 28.0 kg/d). Significant ($P<0.001$) increases in milk yields were observed with increasing concentrate intake (means 26.5 and 31.2 kg/d, for levels 1 and 2, respectively).

Correlation coefficients for the relationships between daily milk yield and DM, ME, FME and CP intakes based on individual cow mean experimental period measurements are presented in Table 8.4.

8.4.1.3. Milk composition

Mean treatment effects on milk fat, protein and lactose concentrations and milk fat, protein and lactose yields are shown in Table 8.6. Correlation coefficients for the relationships between daily mean milk fat, protein and lactose concentrations and milk fat, protein and lactose yields with DM, ME, FME and CP intakes based on individual cow mean experimental period measurements are presented in Table 8.7.

Table 8.3. Mean treatment effects on milk yield, fat, protein, lactose and urea-N concentrations and fat, protein, lactose and urea-N yields

	Experimental Treatment				Mean	LH	Lev	LH*
	L1	L2	H1	H2	SED			Lev
Yield (kg/d)	26.5	29.5	26.6	32.9	0.98	*	***	*
[Fat] (g/kg)	38.0	35.5	38.8	32.8	0.90	NS	***	**
[Protein] (g/kg)	29.1	30.9	28.5	30.0	0.04	*	***	NS
[Lactose] (g/kg)	48.2	47.8	48.0	47.7	0.23	NS	*	NS
[Urea-N] (mg/kg)	194	187	198	202	7.0	*	NS	NS
Fat yield (g/d)	999	1038	1021	1068	35.7	NS	#	NS
Protein yield (g/d)	770	893	755	976	32.4	NS	***	*
Lactose yield (g/d)	1277	1416	1283	1566	48.3	*	***	*
Urea-N output (g/d)	5.13	5.34	5.28	6.58	0.34	**	**	*

Key:-

LH denotes differences between low and high fat treatments
Lev denoted differences between treatment levels
LH*Lev denotes interactions between treatment type and level
NS denotes Not Significant
#,*,**,*** indicate significance at the 0.10<P>0.05, P<0.05, P<0.01 and P<0.001 levels, respectively

Table 8.4. Correlation coefficients for the relationships between milk fat, protein and lactose and urea-N concentrations, milk fat, protein, lactose and urea-N yields and DM, ME, FME and CP intakes, based on individual cow mean experimental period measurements (n=35)

	DMI	MEI	FMEI	CPI	Yd	[Fat]	[Prot]	[Lact]	[Urea-N]	Fat Yd	Prot Yd	Lact Yd
MEI	0.986											
FMEI	0.954	0.933										
CPI	0.962	0.987	0.906									
Yd	0.438	0.478	0.503	0.468								
[Fat]	-0.539	-0.562	-0.564	-0.547	-0.291							
[Prot]	0.394	0.362	0.316	0.345	-0.400	-0.162						
[Lact]	-0.214	-0.228	-0.004	-0.262	0.206	-0.14	-0.81					
[Urea-N]	0.026	0.057	-0.128	0.113	-0.204	0.149	0.156	-0.505				
Fat Yd	0.076	0.093	0.108	0.078	0.733	0.416	-0.479	0.143	-0.80			
Prot Yd	0.616	0.648	0.657	0.629	0.931	-0.375	-0.049	0.217	-0.159	0.615		
Lact Yd	0.403	0.438	0.497	0.421	0.989	-0.294	-0.387	0.343	-0.271	0.718	0.928	
Urea-N Yd	0.341	0.403	0.289	0.436	0.691	-0.182	-0.221	-0.144	0.545	0.518	0.671	0.639

Milk fat concentrations ranged between 13.6-66.4 g/kg. Daily mean milk fat concentrations were not significantly ($P>0.10$) different between high fat and low fat treatments (means 35.8 and 36.7 g/kg, respectively), but were significantly ($P<0.001$) depressed with increased concentrate intake (mean 38.4 and 34.1 g/kg for levels 1 and 2, respectively). Daily milk fat yields were not significantly ($P>0.10$) different between concentrate type (means 1045 and 1019 g/d for high and low fat treatments, respectively), but significantly ($0.10<P>0.05$) increased with concentrate intake (means 1010 and 1053 g/d for levels 1 and 2, respectively).

Milk lactose concentrations ranged between 43.9 and 51.3 g/kg. Daily mean milk lactose concentrations were not significantly ($P>0.10$) different between concentrate type (means 47.8 and 48.0 g/kg for high and low fat treatments) or intake (means of 48.1 and 47.7 g/kg for levels 1 and 2, respectively). In contrast, daily milk lactose yields were significantly ($P<0.05$) higher for high (mean 1424 g/d) than low fat (mean 1346 g/d) concentrates. Increases in concentrate intake resulted in significant ($P<0.001$) increases in daily milk lactose yields (means 1280 and 1491 g/d for levels 1 and 2, respectively).

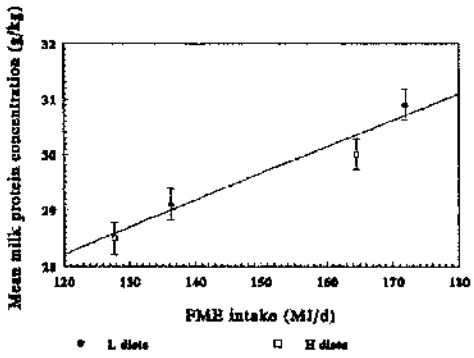
Daily milk protein concentrations varied between 25.1 and 37.3 g/kg. Daily mean milk protein concentrations were significantly ($P<0.05$) higher for low (mean of 30.0 g/kg) than high fat (mean of 29.3 g/kg) concentrates. Increases in concentrate intake significantly ($P<0.001$) increased daily mean milk protein concentration (means 28.8 and 30.4 g/kg for levels 1 and 2, respectively). In contrast, daily milk protein yields were not significantly ($P>0.10$) different between low and high fat concentrates (means 832 and 865 g/d, respectively), while increases in concentrate intake stimulated significant ($P<0.001$) increases in daily milk protein yields (means 762 and 935 g/d for levels 1 and 2, respectively).

Based on individual cow values, means for each experimental period, milk protein concentration was poorly correlated with calculated FME or ME intake ($r^2=0.073$, $n=35$, $P=0.060$ and $r^2=0.106$, $n=35$, $P<0.05$, respectively) while closer correlations existed between calculated FME and ME intakes with milk protein yield ($r^2=0.415$, $n=35$, $P<0.001$ and $r^2=0.404$, $n=35$, $P<0.001$, respectively). The use of

treatment mean values dramatically improved the relationships between calculated FME intake and milk protein concentration (r^2=0.920, n=4, P<0.05) and FME intake and protein yield (r^2=0.682, n=4, P=0.112) as described in Figure 8.1.

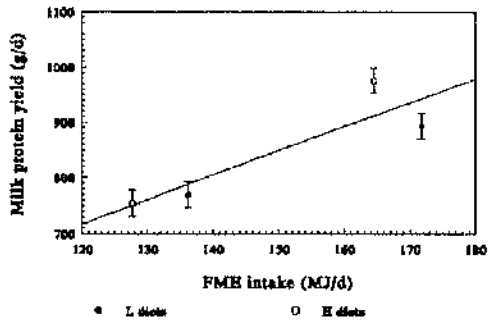
Figure 8.1. Relationships between mean treatment milk protein concentration and yield with calculated FME intake

i) Milk protein concentration



Each point is the mean of 54 measurements
Error bars indicate between treatment SE

ii) Milk protein yield



Each point is the mean of 27 measurements
Error bars indicate between treatment SE

8.4.1.4. Milk urea-N concentration and output

Mean treatment effects on milk urea-N concentrations and output are shown in Table 8.3. Correlation coefficients for the relationships between daily mean concentrations and output of milk urea-N with DM, ME, FME and CP intakes based on mean experimental period measurements from all cows are presented in Table 8.4. Daily mean milk urea-N concentrations ranged between 101-259 mg/kg, and were significantly (P<0.05) higher for high fat (mean 200 mg/kg) than low fat (mean 191 mg/kg) treatments. Differences between concentrate levels were not significant (P>0.10), means 196 and 195 mg/kg for levels 1 and 2, respectively. Daily mean milk urea-N outputs were significantly (P<0.01) higher for high fat (mean 5.93 g/d) than low fat (mean 5.23

g/d) treatments and significantly (P<0.001) increased with concentrate level (means of 5.21 and 5.96 g/d for levels 1 and 2, respectively).

8.4.1.5. Cow liveweights

Mean treatment effects on cow liveweights are shown in Table 8.5. Cow liveweights ranged between 436-615 kg, and were not significantly (P>0.10) different between concentrate types (means 549 and 554 kg for low and high fat treatments, respectively) or levels (means 542 and 561 kg, for levels 1 and 2, respectively).

Table 8.5. Mean treatment effects on cow liveweights

	Experimental Treatment				Mean
	L1	L2	H1	H2	SED
Cow liveweight (kg)	531	568	552	555	18.46

8.4.2. Calculated MCP supply

Mean treatment effects on calculated MCP supply are shown in Table 8.6. Individual cow calculated MCP supply ranged between 1337-2808 g/d. Although FME intakes were significantly (P<0.05) higher for low fat concentrates, this was not the case for calculated MCP supply (P>0.10), means 2439 and 2422 g/d for low and high fat treatments, respectively. Calculated MCP supply increased significantly (P<0.001) with concentrate intake (means 2122 and 2739 g/d, for levels 1 and 2, respectively).

Table 8.6. Mean treatment effects on calculated MCP supply

	Experimental treatment				Mean
	L1	L2	H1	H2	SED
Calculated MCP supply (g/d)	2166	2712	2078	2765	74.21

8.4.3. Urinary PD excretion

Daily urinary PD excretion was highly correlated with urinary allantoin excretion (r^2=0.960, n=100, P<0.001), with allantoin accounting for 82% of all PDs excreted. Urinary hypoxanthine and xanthine excretion was negligible. Prediction of daily PD excretion from daily mean PD/c ratios was poor (r^2=0.240, n=100, P<0.001), but improved by scaling for creatinine concentration (r^2=0.590, n=100, P<0.001). Scaling each ratio by cow liveweight had a detrimental effect on the prediction of PD excretion (r^2=0.130, n=100, P<0.001). Similarly, prediction of daily allantoin excretion from daily mean A/c ratios was also poor (r^2=0.230, n=100, P<0.001), but improved by scaling for creatinine concentration (r^2=0.590, n=100, P<0.001).

Mean treatment effects on urinary PD and allantoin excretion and urinary PD/c and A/c ratios are presented in Table 8.7. Daily urinary PD excretion was not significantly (P>0.10) different between low and high fat treatments (means 396 and 384 mmoles/d, respectively), but significantly (P<0.001) increased with concentrate level (means 333 and 447 mmoles/d for levels 1 and 2, respectively). In common with daily urinary PD excretion, daily mean PD/c ratios were not significantly (P>0.10) different between low and high fat concentrates (means 3.36 and 3.26, respectively), but significantly (P<0.001) increased with concentrate level (means 2.88 and 3.74 for levels 1 and 2, respectively). Correlation coefficients for the relationships between daily urinary PD and allantoin excretion and PD/c and A/c ratios with DM, ME, FME and CP intakes based on mean experimental period measurements for individual cows are presented in Table 8.8.

Based on mean experimental period values for individual cows, daily urinary PD excretion was significantly correlated with calculated FME intake (r^2=0.180, n=35, P<0.01). The use of mean treatment values dramatically improved this relationship (r^2=0.963, n=4, P<0.05). In an attempt to overcome between-cow variations, the responses of urinary PD excretion for individual cows to changes in calculated FME intake were determined. Responses between-cows varied considerably, as shown in Table 8.9.

Table 8.7. Mean treatment effects on urinary PD/c, A/c, Ps/c ratios and urinary PD, allantoin, pseudouridine and creatinine excretion

	Experimental Treatment				Mean	LH	Lev	LH*
	L1	L2	H1	H2	SED			Lev
PD excretion mmol/d	346	445	319	448	44.6	NS	***	NS
Allantoin excretion mmol/d	306	374	275	392	37.9	NS	***	NS
Pseudouridine excretion mmol/d	12.5	14.0	11.0	12.8	1.84	NS	NS	NS
Creatinine excretion mmol/d	119	122	120	122	14.0	NS	NS	NS
PD/c	2.90	3.82	2.85	3.67	0.146	NS	***	NS
A/c	2.62	3.17	2.41	3.24	0.161	NS	***	NS
Ps/c	0.102	0.119	0.099	0.104	0.0129	NS	NS	NS

Key:-

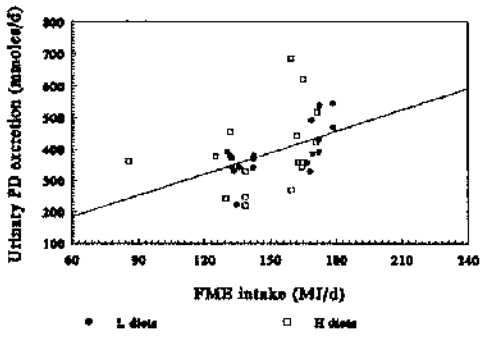
LH denotes differences between low and high fat treatments
Lev denoted differences between treatment levels
LH*Lev denotes interactions between treatment type and level
NS denotes Not Significant
*** indicate significance at the P<0.001 level

Table 8.8. Correlation coefficients for the relationships between urinary PD, allantoin (All), pseudouridine (Ps), creatinine (Crt) excretion, PD/c, A/c and Ps/c ratios, milk allantoin excretion (Maout) with DM, ME, FME and CP intakes based on individual cow mean experimental period measurements (n=35)

	DMI	MEI	FMEI	CPI	PD	All	Ps	Crt	PD/c	A/c	Ps/c
MEI	0.986										
FMEI	0.954	0.933									
CPI	0.962	0.987	0.906								
PD	0.392	0.401	0.452	0.429							
All	0.399	0.412	0.460	0.430	0.969						
Ps	0.132	0.113	0.312	0.074	0.368	0.390					
Crt	-0.123	-0.116	-0.044	-0.105	0.629	0.638	0.346				
PD/c	0.614	0.616	0.582	0.648	0.474	0.406	-0.005	-0.360			
A/c	0.616	0.627	0.584	0.649	0.422	0.449	0.022	-0.378	0.921		
Ps/c	0.156	0.139	0.287	0.097	-0.081	-0.064	0.782	-0.296	0.185	0.223	
Maout	0.346	0.369	0.356	0.411	-0.006	0.076	0.008	-0.431	0.466	0.622	0.273

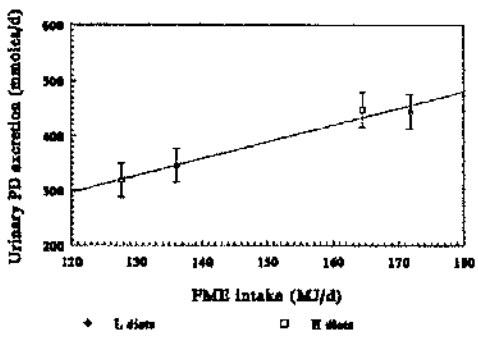
Figure 8.2. Relationships between urinary PD excretion and calculated FME intake

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

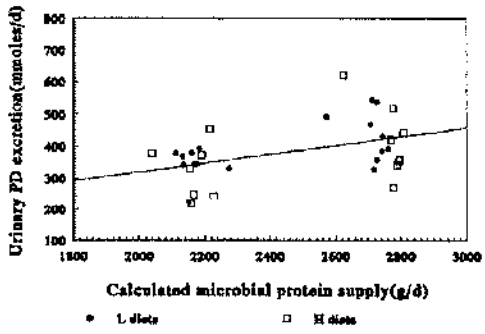


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

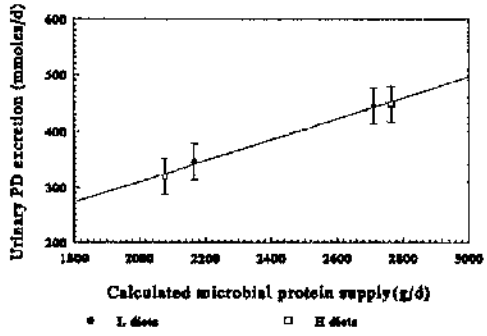
Figure 8.3. Relationships between urinary PD excretion and calculated MCP supply

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

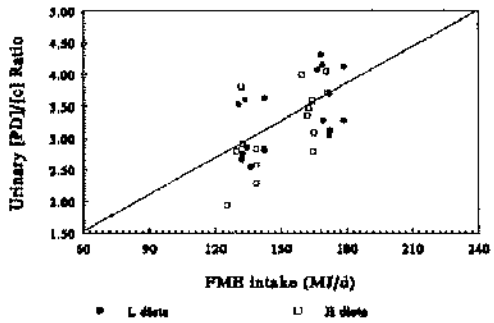


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

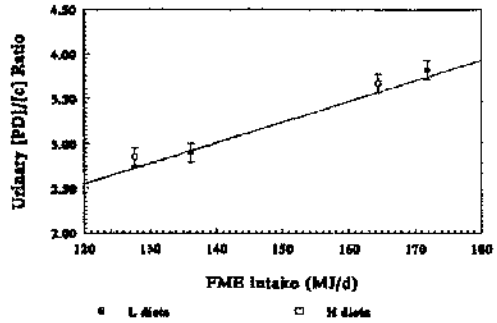
Figure 8.4. Relationships between urinary PD/c ratios and calculated FME intake

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

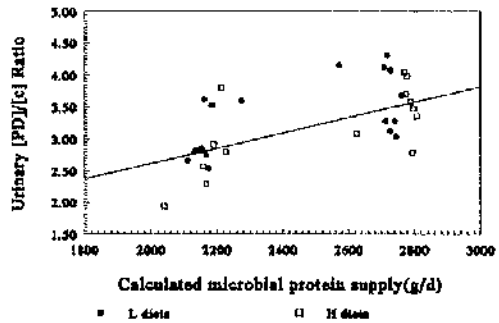


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

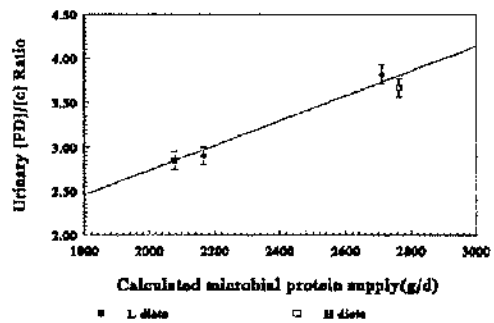
Figure 8.5. Relationships between urinary PD/c ratios and calculated MCP supply

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means



Each point is the mean of 27 measurements

Error bars indicate between treatment SE

Based on individual cow mean experimental period values, daily urinary PD excretion was less well correlated with calculated MCP supply (r^2=0.121, n=35, P<0.05), than calculated FME intake.

The relationship between urinary PD excretion and calculated MCP supply dramatically improved when mean treatment values were used (r^2=0.991, n=4, P<0.01).

Table 8.9. Responses of urinary PD excretion for individual cows to changes in calculated FME intake

Cow	n	r^2	P
291	9	0.530	0.063
294	7	0.621	0.422
297	9	0.125	0.492
307	8	0.042	0.697
372	8	0.596	0.042
373	9	0.691	0.011
375	9	0.430	0.078
378	7	0.020	0.822
383	8	0.190	0.328
385	9	0.331	0.310
388	9	0.382	0.076
412	8	0.171	0.489

Expressing daily urinary PD excretion as a ratio to urinary creatinine excretion (PD/c) improved the relationships with calculated FME intake (r^2=0.319, n=35, P<0.001) and calculated MCP supply (r^2=0.399, n=35, P<0.001), which both improved when mean treatment values were used (r^2=0.977, n=4, P<0.01 and r^2=0.946, n=4, P<0.05, respectively).

In order to examine between-cow variations in urinary PD/c ratios, responses of PD/c ratios to changes in calculated FME intake were determined for individual cows and are presented in Table 8.10. Variations in the responses between-cows were large, but indicated that, for most cows, urinary PD/c ratios reflected changes in calculated FME intake more closely than urinary PD excretion.

Table 8.10. Responses of urinary PD/c for individual cows to changes in calculated FME intake

Cow	n	r^2	P
291	9	0.480	0.084
294	7	0.984	0.082
297	9	0.729	0.030
307	8	0.005	0.894
372	8	0.437	0.106
373	9	0.579	0.028
375	9	0.581	0.028
378	7	0.629	0.109
383	8	0.329	0.178
385	9	0.980	<0.001
388	9	0.530	0.026
412	8	0.964	<0.001

8.4.4. Urinary pseudouridine excretion

Mean treatment effects on daily urinary pseudouridine excretion are shown in Table 8.7. Correlation coefficients for the relationships between daily urinary pseudouridine excretion and DM, ME, FME and CP intakes based on individual cow mean experimental period measurements are presented in Table 8.8. Daily urinary pseudouridine excretion was poorly correlated with DM, ME, FME and CP intakes. Use of multiple regression analysis indicated that the best prediction of daily urinary pseudouridine excretion was achieved with two term model using FME and DM intakes (r^2=0.379, n=35, P<0.001).

Daily pseudouridine excretion was not significantly (P>0.10) different between concentrate types (means 13.2 and 11.9 mmoles/d for low and high fat treatments, respectively) or levels (means 11.7 and 13.4 mmoles/d, for levels 1 and 2, respectively).

Prediction of daily pseudouridine excretion from daily mean Ps/c ratios was poor (r^2=0.230, n=100, P<0.001), but improved by scaling for creatinine concentration (r^2=0.590, n=100, P<0.001). In common with pseudouridine excretion, Ps/c ratios were

not significantly (P>0.10) different between concentrate type (means 0.110 and 0.101 for low and high fat treatments, respectively) or level (means 0.100 and 0.111, for levels 1 and 2, respectively).

8.4.5 Urinary creatinine excretion

Mean treatment effects on urinary creatinine excretion are shown in Table 8.7. Correlation coefficients for the relationships between daily urinary creatinine excretion and DM, ME, FME and CP intakes based on individual cow mean experimental period measurements are presented in Table 8.8. Daily creatinine excretion varied between 61 and 229 mmoles/d, being significantly (P<0.05) different between cows. Daily creatinine excretion was not significantly (P>0.10) different between concentrate types (means 120 and 121 mmoles/d for low and high fat treatments, respectively) or levels (means 119 and 122 mmoles/d, for levels 1 and 2, respectively).

8.4.6. Milk allantoin excretion

Milk allantoin excretion ranged between 1.24 and 8.45 mmoles/d, and was moderately correlated with daily mean milk allantoin concentration (r^2=0.620, n=105, P<0.001). Correlation coefficients for the relationships between daily milk allantoin excretion and DM, ME, FME and CP intakes based on cow mean experimental period measurements are presented in Table 8.8. Mean treatment effects on milk allantoin excretion are presented in Table 8.11. Daily milk allantoin excretion was not significantly (P>0.10) different between low and high fat concentrates (means 4.27 and 4.26 mmoles/d, respectively), but significantly (P<0.01) increased with concentrate intake (means 3.86 and 4.87 mmoles/d for levels 1 and 2, respectively).

Table 8.11. Mean treatment effects on am, pm, arithmetic and weighted mean milk allantoin concentrations and milk allantoin excretion

	Experimental Treatment				Mean SED	LH	Lev	LH* Lev
	L1	L2	H1	H2				
Milk allantoin excretion (mmoles/d)	3.99	4.54	3.72	4.80	0.414	NS	**	NS
Am allantoin concentration (mM)	0.155	0.157	0.149	0.158	0.0109	NS	NS	NS
Pm allantoin concentration (mM)	0.142	0.158	0.151	0.170	0.0092	NS	**	NS
Arithmetic mean allantoin concentration (mM)	0.153	0.163	0.146	0.158	0.0087	NS	#	NS
Weighted mean allantoin concentration (mM)	0.153	0.161	0.147	0.158	0.0091	NS	NS	NS
% of urinary allantoin excreted in milk	1.41	1.32	1.47	1.45	0.265	NS	NS	NS

Key:-

LH denotes differences between low and high fat treatments
Lev denoted differences between treatment levels
LH*Lev denotes interactions between treatment type and level
NS denotes Not Significant
#,**, indicate significance at the 0.10<P>0.05 and P<0.01 levels, respectively

Direct measurements of MCP supply were not performed in the current experiment, and therefore milk allantoin responses were evaluated using:- i) urinary PD excretion, ii) calculated FME intake and iii) calculated MCP supply.

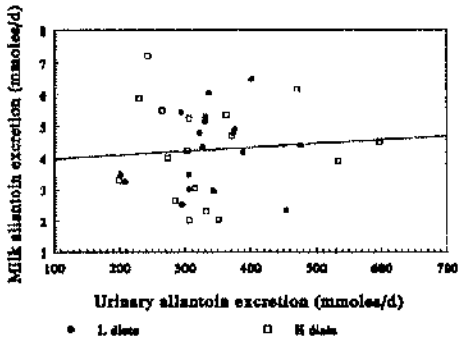
Using mean experimental data for individual cows indicated that milk allantoin excretion was poorly correlated with urinary PD or allantoin excretion (r^2<0.001, n=35, P=0.973 and r^2=0.006, n=35, P=0.665, respectively). These relationships dramatically improved when mean treatment values were used (r^2=0.936, n=4, P<0.05 and r^2=0.990, n=4, P<0.01, respectively). Figure 8.6. describes the relationships between milk and urinary allantoin excretion based on individual cow and mean treatment values. Closer correlations were found to exist between milk allantoin excretion with urinary PD/c or A/c ratios (refer to Table 8.12.). Figure 8.7. describes the relationships between milk allantoin excretion and urinary A/c ratios based on individual cow and mean treatment values.

Table 8.12. Relationships between urinary PD and allantoin excretion and urinary PD/c and A/c ratios and those scaled for creatinine concentration with milk allantoin excretion

Variate	Dataset	n	r^2	P
Urinary PD excretion	Individual cow	35	<0.001	0.973
	Treatment means	4	0.936	<0.05
Urinary allantoin excretion	Individual cow	35	0.006	0.665
	Treatment means	4	0.990	<0.01
Urinary PD/c ratio	Individual cow	35	0.193	<0.01
	Treatment means	4	0.787	0.074
Scaled urinary PD/c ratio	Individual cow	35	<0.001	0.997
	Treatment means	4	0.770	0.080
Urinary A/c ratio	Individual cow	35	0.368	<0.001
	Treatment means	4	0.968	<0.05
Scaled urinary A/c ratio	Individual cow	35	0.007	0.634
	Treatment means	4	0.922	<0.05

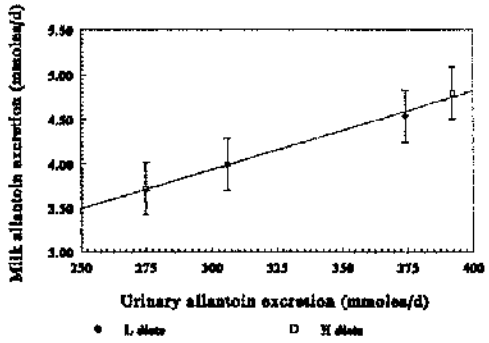
Figure 8.6. Relationships between milk and urinary allantoin excretion

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

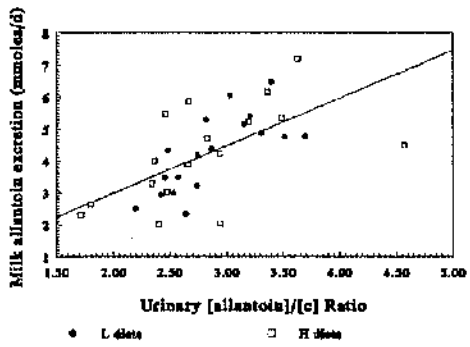


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

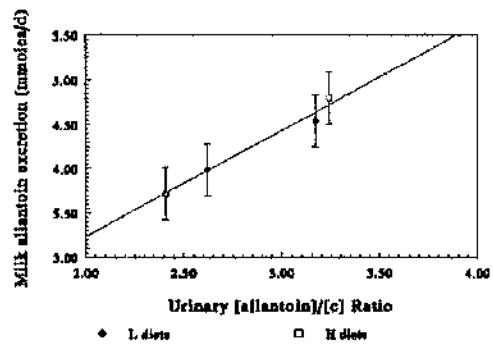
Figure 8.7. Relationships between milk allantoin excretion and urinary A/c ratios

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

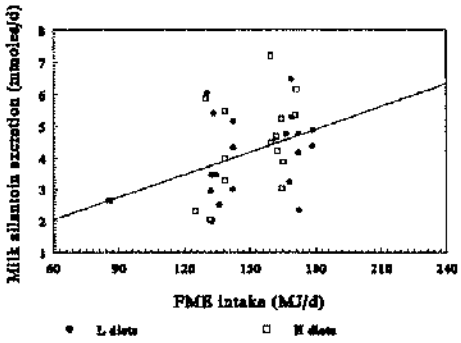


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

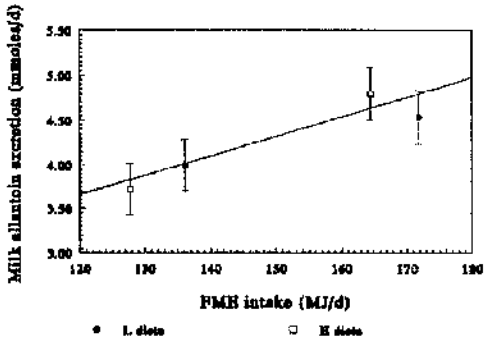
Figure 8.8. Relationships between milk allantoin excretion and calculated FME intake

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means

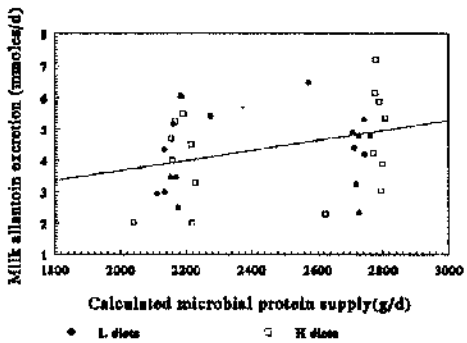


Each point is the mean of 27 measurements

Error bars indicate between treatment SE

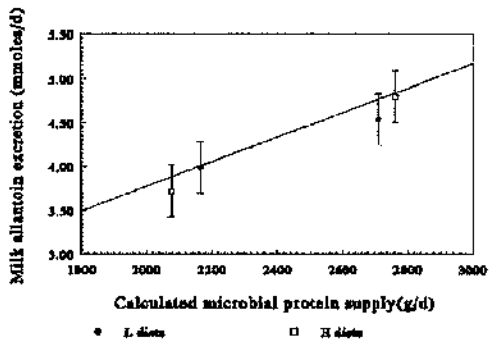
Figure 8.9. Relationships between milk allantoin excretion and calculated MCP supply

i) Individual cows



Each point is the mean of 3 measurements

ii) Treatment means



Each point is the mean of 27 measurements

Error bars indicate between treatment SE

Based on mean experimental period values for individual cows, daily milk allantoin excretion was poorly correlated with calculated FME intake (r^2=0.101, n=35, P<0.05), but improved when mean treatment values were used (r^2=0.812, n=4, P=0.065). In an attempt to investigate between-cow variations, responses of milk allantoin excretion to changes in calculated FME intake were determined for individual cows. Responses between-cows varied considerably, as shown in Table 8.13.

Table 8.13. Individual cow milk allantoin excretion responses to changes in calculated FME intake

Cow	n	r^2	P
291	8	0.204	0.261
294	9	0.050	0.565
297	9	0.295	0.131
307	9	0.019	0.745
372	9	0.229	0.192
373	9	0.297	0.129
375	9	0.252	0.168
378	9	0.445	0.050
383	9	0.039	0.612
385	9	0.540	0.024
388	9	0.175	0.263
412	9	0.612	0.013

Daily milk allantoin excretion (mean experimental period values for individual cows) was poorly correlated with calculated MCP supply (r^2=0.128, n=35, P<0.05), which dramatically improved when mean treatment values were used (r^2=0.940, n=4, P<0.05).

8.4.7. Milk allantoin concentration

Daily mean milk allantoin concentrations were calculated as an arithmetic mean of am and pm concentrations, or as a mean weighted by milk yield. Mean treatment

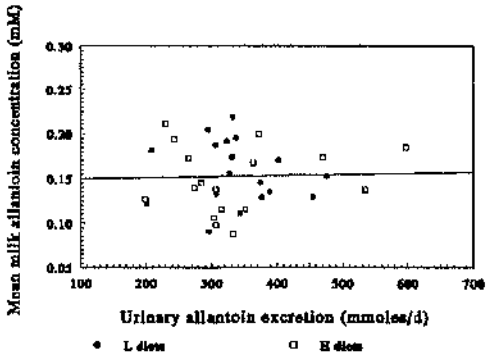
effects on am, pm, arithmetic and weighted mean milk allantoin concentrations are presented in Table 8.11. Differences in am, pm, arithmetic and weighted mean milk allantoin concentrations between concentrates were not significant ($P>0.10$). Effects of concentrate level on am and weighted mean milk allantoin concentrations were not significant ($P>0.10$). In contrast, pm and daily mean milk allantoin concentrations increased significantly ($P<0.01$ and $0.10<P>0.05$, respectively) with increases in concentrate intake.

Relationships between arithmetic and weighted mean milk allantoin concentrations with urinary PD and allantoin excretion, urinary PD/c and A/c ratios and those scaled for creatinine concentration are shown in Table 8.14. Relationships between arithmetic mean milk allantoin concentration and urinary allantoin excretion based on individual cow and mean treatment values are described in Figure 8.10. Figure 8.11. describes the relationships between arithmetic mean milk allantoin concentration and urinary A/c ratios based on individual cow and mean treatment values. Relationships between am, pm, arithmetic and weighted mean milk allantoin concentrations with calculated FME intake and MCP supply are shown in Table 8.15. Figures 8.12. and 8.13. describe individual cow and treatment mean arithmetic mean milk allantoin concentration responses to calculated FME intake and MCP supply, respectively.

Individual cow responses of arithmetic and weighted mean milk allantoin concentrations to changes in calculated FME intake are presented in Tables 8.16. and 8.17., respectively. Responses between-cows were highly variable, confirming observations of responses of urinary PD/c ratios and PD excretion, and milk allantoin excretion to changes in calculated FME intake.

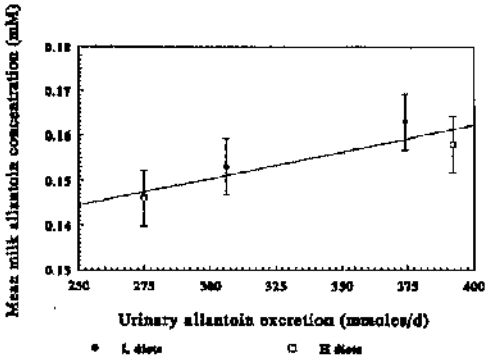
Figure 8.10. Relationships between mean milk allantoin concentration and urinary allantoin excretion

i) Individual cows



Each point is the mean of 6 measurements

ii) Treatment means

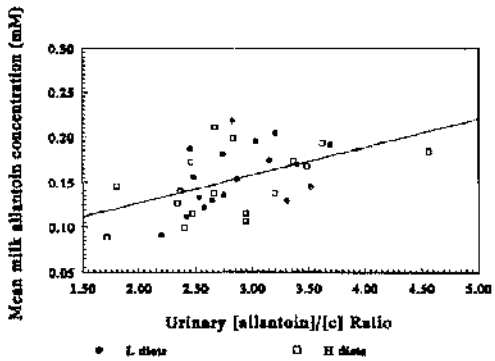


Each point is the mean of 54 measurements

Error bars indicate between treatment SE

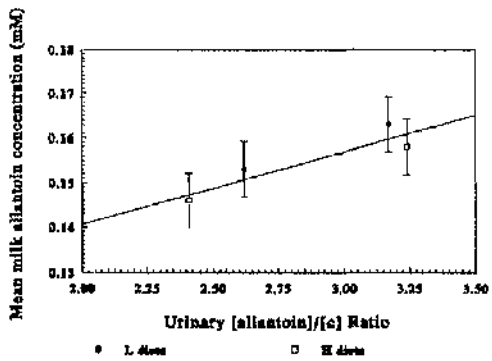
Figure 8.11. Relationships between mean milk allantoin concentration and urinary A/c ratios

i) Individual cows



Each point is the mean of 6 measurements

ii) Treatment means

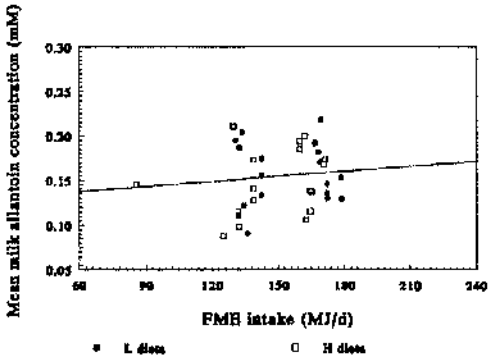


Each point is the mean of 54 measurements

Error bars indicate between treatment SE

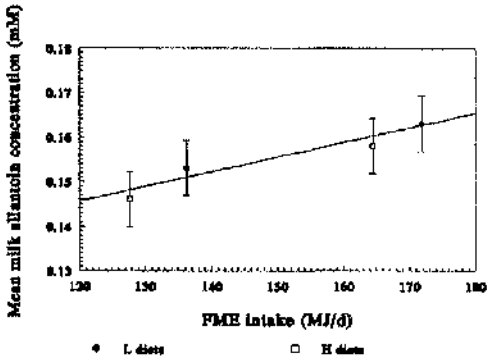
Figure 8.12. Relationships between mean milk allantoin concentration and calculated FME intake

i) Individual cows



Each point is the mean of 6 measurements

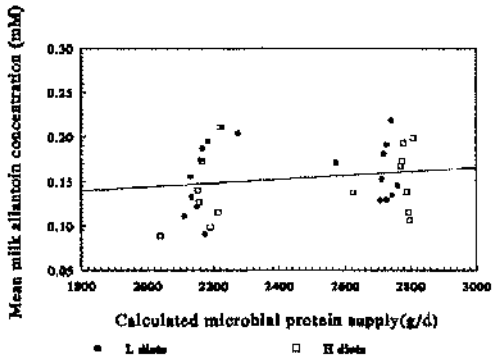
ii) Treatment means



Each point is the mean of 54 measurements
Error bars indicate between treatment SE

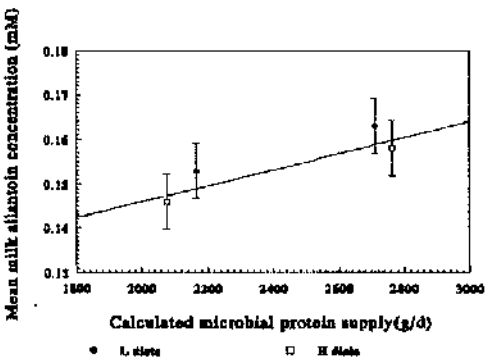
Figure 8.13. Relationships between mean milk allantoin concentration and calculated MCP supply

i) Individual cows



Each point is the mean of 6 measurements

ii) Treatment means



Each point is the mean of 54 measurements
Error bars indicate between treatment SE

Table 8.14. Relationships between urinary PD and allantoin excretion and urinary PD/c and A/c ratios and those scaled for creatinine concentration with arithmetic and weighted mean milk allantoin concentration

Variate	Dataset	Arithmetic mean			Weighted mean		
		n	r^2	P	n	r^2	P
Urinary PD	IC	35	<0.001	0.983	35	<0.001	0.895
excretion	TM	4	0.771	0.077	4	0.822	0.061
Urinary allantoin	IC	35	<0.001	0.871	35	<0.001	0.970
excretion	TM	4	0.706	0.103	4	0.783	0.075
Urinary PD/c	IC	35	0.161	<0.01	35	0.169	<0.01
ratio	TM	4	0.764	0.082	4	0.783	0.075
Scaled urinary PD/c	IC	35	0.016	0.470	35	0.017	0.461
ratio	TM	4	0.508	0.180	4	0.542	0.167
Urinary A/c	IC	35	0.212	<0.01	35	0.228	<0.01
ratio	TM	4	0.760	0.084	4	0.813	0.064
Scaled urinary A/c	IC	35	0.007	0.623	35	0.007	0.633
ratio	TM	4	0.561	0.159	4	0.612	0.139

Table 8.15. Relationships between am, pm, arithmetic and weighted mean allantoin concentrations with calculated FME intake and MCP supply

	Dataset	Calculated FME intake			MCP supply		
		n	r^2	P	n	r^2	P
am	IC	36	<0.010	0.851	36	<0.010	0.659
	TM	4	0.625	0.134	4	0.609	0.140
pm	IC	36	<0.010	0.351	36	0.028	0.170
	TM	4	0.843	0.054	4	0.607	0.141
Art	IC	36	<0.010	0.538	36	<0.010	0.331
Mean	TM	4	0.876	<0.05	4	0.692	0.108
Wt	IC	36	<0.010	0.619	36	<0.010	0.447
Mean	TM	4	0.895	<0.05	4	0.742	0.090

Key for Tables **8.17.** and **8.18.** :-

IC denotes individual cow mean experimental period measurements

TM denotes treatment mean values

Art mean denotes arithmetic mean of am and pm allantoin concentrations

Wt mean denotes mean allantoin concentration weighted by milk yield

Table 8.16. Individual cow arithmetic mean milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
291	8	0.062	0.554
294	9	0.367	0.084
297	9	0.544	0.023
307	9	0.410	0.063
372	9	0.335	0.102
373	9	0.097	0.415
375	9	0.558	0.021
378	9	0.358	0.089
383	9	0.280	0.143
385	9	0.456	0.046
388	9	0.288	0.140
412	9	0.045	0.584

Table 8.17. Individual cow weighted mean milk allantoin concentration responses to changes in calculated FME intake

Cow	n	r^2	P
291	8	0.073	0.516
294	9	0.413	0.085
297	9	0.552	0.022
307	9	0.354	0.091
372	9	0.347	0.095
373	9	0.254	0.308
375	9	0.368	0.110
378	9	0.349	0.094
383	9	0.307	0.122
385	9	0.464	0.044
388	9	0.158	0.435
412	9	0.062	0.554

8.5. Discussion

The current experiment was designed to evaluate the potential of milk allantoin as a potential index of MCP supply under conditions causing relatively large variations in milk yield. Manipulation of FME intake was achieved using two concentrates of different fat content, involving the inclusion of vegetable fat at the expense of wheat.

Milk yield

Inclusion of supplementary fat in dairy cow rations has received considerable research attention in recent years and aspects of the use of fats for ruminants and lactating dairy cows has been reviewed in detail by Palmquist and Jenkins (1980), Storry (1981), Czerkawski and Clapperton (1984), Moore and Christie (1984), Palmquist (1984) and Coppock and Wilks (1991). In the current experiment, daily ME intakes were very similar between high and low fat concentrates (means of 214 and 213 MJ/d, respectively), while milk yields were significantly ($P<0.05$) higher for high fat diets, suggesting inclusion of dietary fat improved the efficiency of energy utilisation for milk production. Increases in efficiency of energy utilisation could be achieved by optimising the ratio of fat to other nutrients (Krönfield, 1976; Baldwin and Smith, 1983 and Baldwin *et al*, 1985) or as a result of reducing rapidly fermentable carbohydrate content of the diet facilitating a more stable rumen fermentation leading to improved fibre digestion.

Generally, inclusion of unprotected fat, such as vegetable fat used in the current experiment is thought to inhibit fibre digestion in the rumen (Palmquist and Jenkins, 1980 and Coppock and Wilks, 1991). Possible mechanisms are physical coating of fibre, modification of microbial populations due to selective toxicity, interference with the microbial cell membrane or reduced availability of cations by the formation of insoluble complexes (Devendra and Lewis, 1974). *In-vivo* DM digestibilites in the rumen or the whole gastro-intestinal tract were not measured in the current experiment and it is uncertain whether dietary inclusion of vegetable fat depressed rumen fibre fermentation.

Milk composition

Added fats in the ruminant diet have the propensity to alter rumen fermentation, reduce fibre digestion and ultimately cause a milk fat depression (Czerkawski and Clapperton, 1984 and Palmquist, 1984). In the current experiment high fat concentrate intake did not significantly depress milk fat concentrations or fat yields, suggesting potential milk fat depressions due to changes in rumen fermentation were balanced by increased supply of dietary triglycerides for direct incorporation into milk fat (Moorby, 1993).

The current experiment demonstrated that milk protein concentrations, but not protein yield were significantly higher for low fat diets. Milk yields were significantly lower for low fat diets, indicating that milk volume effects accounted for the observed differences in milk protein concentrations.

Milk protein yield and concentration have been shown to be dependent on MP supply (Dewhurst, 1989; Moorby, 1993 and Dewhurst *et al*, 1996). Both MCP and UDP contribute to MP supply. The use of mean treatment values indicated that both milk protein concentration and yield were closely correlated with calculated FME intake (r^2=0.920, n=4, P<0.05 and r^2=0.682, n=4, P=0.112, respectively). Interpreting these relationships as milk protein responses to increases in MCP supply takes no account of variations in UDP supply which will be determined by the extent of rumen dietary protein degradation. Based on mean experimental period measurements for individual cows, calculated FME intake was closely correlated with crude protein intake (r=0.906). It is interesting to speculate that FME intake was also correlated with UDP supplies, suggesting the relationships between milk protein concentration and yield and calculated FME intake, were a reflection of responses to total MP supply.

Milk urea nitrogen concentration and output

Milk urea has been proposed as an indicator of utilisation of dietary nitrogen (Oltner and Wiktorsson, 1983; Ropstad *et al*, 1989; Miettinen and Juvonen, 1990; Spain *et al*, 1990; Lindberg and Murphy, 1991; Gustavsson and Palmquist, 1993; Roseler *et al*, 1993 and Gonda and Lindberg, 1994). CP intakes were not significantly (P>0.10)

different between high and low fat diets (means 2925 and 2872 g/d, respectively) and therefore do not account for the significantly lower milk urea-N concentrations and outputs observed for low fat diets. This suggests that utilisation of dietary nitrogen was higher for low than for the high fat diets. Numerous experiments have shown that urea secretion in the milk of dairy cows reflects dietary energy and protein balance (Erbersdobler *et al*, 1980; Oltner and Wiktorsson, 1983 and Gustavsson 1993, cited by Gonda and Lindberg, 1994). The current MP system (AFRC, 1992) predicts that increases in FME supply would increase MCP supply provided eRDP supplies are in excess of microbial requirements. Improvements in utilisation of dietary nitrogen are almost certainly a consequence of increased incorporation of dietary nitrogen into MCP. Increases in microbial capture of dietary nitrogen could be achieved as a direct consequence of increasing FME supply or as a result of improved synchronisation of dietary energy and nitrogen release in the rumen. Previous work has demonstrated that a diet which promoted a synchronous energy and nitrogen release in the rumen resulted in a 27% increase in MCP supply compared to an asynchronous diet (Sinclair *et al*, 1993).

Urinary creatinine excretion

Mean daily urinary creatinine excretion ranged between 119-122 mmoles/d agreeing with variations of 112-117 mmoles/d reported in the literature (Puchala *et al*, 1993; Susmel *et al*, 1994a and Gonda and Lindberg, 1994) and confirm observations reported in chapters 6 and 7 which ranged between 121-146 mmoles/d. Daily urinary creatinine excretion was significantly different between-cows confirming observations of Chetal *et al* (1975) and those reported in chapter 6 and 7. Large between-cow variations in urinary creatinine excretion indicate that its use as a urine volume marker, as suggested by deGroot and Aafjes (1960), Albin and Clanton (1966) and Erb *et al* (1977) would be limited. Urinary creatinine excretion was found to be independent of nutrient supply confirming observations documented in chapters 6 and 7 and those reported by the researchers, Orskov and MacLeod (1982) and Gonda and Lindberg (1994) for cows, Fujihara *et al* (1987) for steers, Fujihara *et al* (1987) and Lindberg and Jacobssen (1990) for sheep and Lindberg, (1985 and 1989) for goats.

Urinary pseudouridine excretion

Daily urinary pseudouridine excretion was found to be largely independent of nutrient supply confirming observations reported in chapters 6 and 7 and those of Puchala *et al* (1993). Mean daily pseudouridine excretion ranged between 11.0-14.0 mmoles/d, being higher than variations of 8.06-8.33 and 8.14-10.25 mmoles/d reported in chapters 6 and 7, respectively and variation of 2.2-2.45 and 1.9-2.25 mmoles/d in non-lactating cows and heifers, respectively (Puchala *et al*, 1993). Urinary pseudouridine excretion has been demonstrated to be independent of pseudouridine content of the diet (Weissman *et al*, 1962 and Puchala *et al*, 1993) and is thought to represent an index of tissue RNA turnover (Puchala *et al*, 1993). Urinary pseudouridine excretion (expressed on a metabolic weight basis) has been shown to be higher in young compared to mature animals suggesting that RNA turnover and possibly protein synthesis increase during protein accretion (Puchala *et al*, 1993). Discrepancies between experimental observations in this thesis and those of Puchala *et al* (1993) are difficult to reconcile, but could potentially be accounted for by changes in tissue RNA turnover and protein accretion during lactation.

Urinary PD excretion

Urinary allantoin excretion was highly correlated with urinary PD excretion accounting for 82% of all PDs excreted, with the remainder as uric acid. This finding agrees closely with reports in the literature which indicated allantoin accounted for between 82 and 93% of urinary PD excretion (Chen *et al*, 1990c; Verbic *et al*, 1990; Giesecke *et al*, 1994; Gonda and Lindberg, 1994 and Dewhurst *et al*, 1995) and confirms the observations documented in chapters 5, 6 and 7. Urinary xanthine and hypoxanthine excretion was negligible. This finding is in complete agreement with the observations of Chen *et al* (1990b), Verbic *et al* (1990), Susmel *et al* (1994b) and Dewhurst *et al* (1995) and confirms those reported in chapters 6 and 7. Due to the their close correlation further discussions are confined to urinary PD excretion, but apply equally to urinary allantoin excretion.

The use of calculated FME values for feeds may be criticised because of errors in its prediction. This problem has been minimised in the current experiment by restricting silage intake and formulating concentrates of different fat content to manipulate FME supply. Based on individual cow observations, urinary PD excretion was poorly correlated with calculated FME intake or MCP supply, confirming the observations reported in chapter 7. These poor relationships suggest either one or both predictions of MCP supply based on FME intake (AFRC, 1992) or on urinary PD excretion are inaccurate. Since urinary PD excretion has been shown to closely reflect microbial NA supply entering the duodenum (Chen *et al*, 1990a and Verbic *et al*, 1990) and comparisons with other microbial markers have been reported to be in good agreement (Djouvinov and Todorov, 1994 and Perez *et al*, 1994), the current findings strongly suggest that FME intake is a poor predictor of MCP supply. Since the current MP system adopted in the UK (AFRC, 1992) predicts MCP supply based primarily on FME intake these observations raise concerns over the accuracy of such an approach.

The lack of relationships between urinary PD excretion and FME intake or calculated MCP supply for individual cows could be accounted for by:- i) random experimental errors, ii) inherent errors of the PD technique or iii) between-cow variations in EMPS.

Random experimental errors in the calculation of FME intake or in the determination of PD excretion inevitably contribute to prediction errors. Such errors were minimised in this experiment by the use of dietary fat to manipulate FME intake. Since fat content of feedstuffs can be measured reliably, calculations of individual cow FME intakes would be expected to be reasonably accurate at least in incremental if not absolute terms. Errors in the assessment of urinary PD excretion were also minimised by performing measurements in duplicate using a HPLC method with comparable precision to the published methods of Tiermeyer and Giesecke (1982) and Diez *et al* (1992).

Errors associated with the use of PD technique are likely to arise as a result of between-cow variations in endogenous urinary PD excretion, the proportion of plasma PDs excreted renally, dietary purines escaping rumen degradation or the purine content of rumen microbes. The proportion of plasma allantoin excreted renally has been

demonstrated to vary considerably (0.62-1.05) between individual animals (Chen *et al*, 1991a). Assuming renal clearances of allantoin, uric acid, xanthine and hypoxanthine approach that of creatinine as suggested by Greger *et al* (1976), between-cow variations in the proportion of PDs excreted renally could be accounted for by expressing PD excretion as a ratio to creatinine. Even accounting for these variations, urinary PD/c ratios were only moderately correlated with calculated FME intake (r^2=0.319, n=35, P<0.001) or MCP supply (r^2=0.399, n=35, P<0.001). Assuming that endogenous urinary PD excretion is relatively independent of nutrient supply (Fujihara *et al*, 1987; Chen *et al*, 1990c and Lindberg and Jacobssen, 1990), variations in responses of urinary PD excretion or PD/c ratios to calculated FME intake are probably mainly due to variations in EMPS rather than variations in the extent of dietary purines escaping rumen degradation.

Responses of urinary PD excretion and PD/c ratios to changes in calculated FME intake within individual cows varied considerably. PD/c ratios generally reflected changes in calculated FME intake more closely than urinary PD excretion. Between-cow variations in EMPS are likely to account for varied responses of urinary PD excretion to calculated FME intake. Numerous studies have shown that rumen outflow rates increase with DM intake (Evans 1981a and 1981b; Chen *et al*, 1992b; Dewhurst and Webster 1992b; Gomes *et al*, 1993 and Djouvnov and Todorov, 1994), while increases in rumen outflow rates have been reported to increase EMPS (Chen *et al*, 1992b and Dewhurst and Webster, 1992a) due to a reduction in microbial maintenance coefficient i.e. the proportion of energy associated with maintenance of the microbial population in the rumen. Variations in rumen outflow rate could account for variations in EMPS, although dietary effects on rumen fermentation are also likely to influence EMPS.

AFRC (1992) predicts values of EMPS as 11.3, 11.6, 11.0 and 11.6 g MN/MJ FME for diets L1, L2, H1 and H2, respectively. The models proposed by Verbic *et al* (1990) and Chen *et al* (1992a) to estimate M-N supply from mean treatment urinary PD excretion indicated values of EMPS of 19.1, 19.9, 18.4 and 21.0 g MN/MJ FME for diets L1, L2, H1 and H2, respectively. Formulation of the high fat concentrate involved substituting readily fermentable carbohydrates sourced from wheat for vegetable fat.

Although it is well recognised that dietary inclusion of unprotected fats generally depress rumen fibre digestion the extent of which will be dependent on level of fat supplementation and level, type and source of carbohydrate in the diet (Palmquist and Jenkins, 1980 and Coppock and Wilks, 1991), EMPS was not depressed by the highest fat intakes. Feeding readily fermentable carbohydrates in the form of concentrates generally stimulates a rapid production of VFAs causing a strong depression in rumen pH (Malestein *et al*, 1981) ultimately inhibiting rumen cellulolytic activity (Itasse and Orskov, 1983 and Itasse *et al*, 1986) and reducing fibre fermentation (Orskov, 1994). Despite higher DM intakes for diet L2, estimates of EMPS were lower than for diet H2 possibly as a consequence of an unstable rumen fermentation induced by feeding of relatively large quantities of readily fermentable carbohydrates.

The use of mean treatment values which are the mean of 27 observations from 9 cows, indicated urinary PD excretion was strongly related to calculated FME intake or MCP supply. Although the use of mean treatment values is subject to criticism as discussed in section 7.5., the strength of these relationships suggest MCP supply is accurately predicted from FME intake. However prediction of MCP supply based on FME intake for an individual cow is subject to large errors. Based on experimental observations, prediction of MCP within the current MP system appears to be good on a group basis but poor on an individual cow basis, highlighting the requirement for a diagnostic test to assess MCP supply in the dairy cow.

Milk allantoin excretion and concentration

Direct measurements of MCP supply were not performed in the current study and therefore milk allantoin responses were examined using three sets of reference measurements namely:- i) urinary PD excretion, ii) calculated FME intake and iii) calculated MCP supply. As discussed in section 7.5., the lack of information concerning true MCP supply introduces uncertainities of which set of measurements should be used as a reference to evaluate milk allantoin responses. Numerous studies documented in chapter one have indicated that urinary PD excretion closely reflects MCP supply and consequently this was considered to be the preferred reference measurement.

i) Relationships with urinary PD excretion and PD/c ratios

Based on individual cow measurements urinary PD excretion was poorly correlated with milk allantoin excretion (r^2<0.001, n=35, P=0.895) or mean concentration (r^2<0.001, n=35, P=0.983). Lack of relationships could be accounted for by either random experimental error or due to between-cow variations in allantoin partitioning between renal and mammary excretory routes. Experimental errors were minimised by determining urinary PD and milk allantoin concentrations in duplicate using HPLC methodologies with comparable precision to published methods.

Individual cow allantoin secretion accounted between 1.41 and 3.37 % of urinary excretion, but was not significantly different (P>0.05) between concentrate types (1.46 and 1.36 % for high and low fat concentrates, respectively). Variations in allantoin partitioning could explain the poor relationships between milk and urinary allantoin excretion. Accounting for between-cow differences in the proportion of plasma PDs excreted in the urine by expressing urinary PD excretion as a ratio to creatinine (PD/c) resulted in closer correlations with milk allantoin excretion (r^2=0.193, n=35, P<0.01) or mean concentration (r^2=0.161, n=35, P<0.01). Urinary PD excretion is governed by the renal clearance of plasma PDs (Chen *et al*, 1991a and Giesecke *et al*, 1993), while allantoin secretion in milk is thought to be due to the diffusion from plasma into the mammary alveolar tissue (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994). As discussed in section 7.5., it seems plausible that between-cow and within-cow variations in plasma allantoin concentration and mammary blood flow could be responsible for the observed variations in allantoin partitioning between mammary and renal excretory routes.

Presumably strong relationships between urinary and milk allantoin excretion (r^2=0.990, n=4, P<0.01) or mean concentration (r^2=0.706, n=4, P=0.082) derived when using mean treatment values are a result of accounting for between-cow variations in allantoin partitioning. Derivation of these relationships based on mean treatment values does raise concerns as to whether allantoin renal and mammary excretory routes are truly related or due to an artefact of correlation as a consequence of using a limited number of datapoints. Partitioning of allantoin is discussed further in chapter nine.

ii) Relationships with calculated FME intake

Milk allantoin excretion and mean concentration were poorly correlated with calculated FME intake for individual cows (r^2=0.101, n=35, P<0.05 and r^2<0.010, n=35, P=0.538, respectively). Milk allantoin responses to calculated FME intake within individual cows were highly variable as observed for urinary PD excretion. In certain cows, milk allantoin concentration or excretion reflected changes in calculated FME intake more closely than urinary PD excretion while the reverse was true in other cows. Interpretation of these observations is difficult due to the lack of information concerning true MCP supplies. Sources of error which accounted for the poor prediction of urinary PD excretion from calculated FME intake in addition to variations in mammary blood flow and milk volume potentially explain the lack of relationships between milk allantoin excretion or concentration with calculated FME intake.

iii) Relationships with calculated MCP supply

Relationships between individual cow milk allantoin excretion and concentration with calculated MCP supply (r^2=0.128, n=35, P<0.05 and r^2<0.010, n=35, P=0.172, respectively) were similar to those derived using calculated FME intake. Since AFRC (1992) calculations are based on FME intakes, sources of error in the prediction of milk allantoin excretion or concentration would be expected to be common to both.

The use of mean treatment values indicated that, milk allantoin excretion and concentration were strongly related to calculated FME intake confirming earlier observations of Moorby and Dewhurst (1993a) and those reported in chapter 7. Close correlations between mean treatment milk allantoin excretion and concentration with urinary PD excretion, calculated FME intake and MCP supply suggests that measurements of allantoin in milk could be used to assess MCP supply in studies with large groups of cows, but not on an individual basis.

Chapter Nine

General discussion

The quantification of MCP supply is fundamental to all the new MP systems proposed by Madsen (1985), Ausschuss fur Bedarfsnormen (1986), Jarridge (1989), NRC (1989) and AFRC (1992). The current MP system adopted in the UK (AFRC, 1992), predicts MCP based on FME supply, with a correction for the effects of level of feeding on EMPS. Assuming no other limitations, EMPS, the microbial yield per unit of fermented energy, is influenced by the ratio of microbial outflow (yield):total synthesis and the maintenance energy costs of the rumen population (Dewhurst and Webster, 1992b). Microbial maintenance costs include those of motility, uncoupling, lysis, inefficient phosphorylation, secretion and active transport (Harmeyer, 1986). Large variations (19.3-44.7 gMN/ADOMR) in EMPS have been reported in the literature ARC (1984). Assuming that energy is the limiting factor for microbial yield, this variability is due to differences in rumen outflow rates and microbial maintenance coefficient (Dewhurst and Webster, 1992b). Clearly, calculated FME can not be a good predictor of MCP supply for all animals and feeding situations, due to the influence of the factors described above which cannot be measured on farm. Since information of MCP supply is central to accurate protein feeding, errors in its prediction can lead to a wastage of supplementary dietary protein. The dairy industry urgently requires an on-farm diagnostic test of MCP supply to indicate to farmers and advisers how well the rumen is performing and to act as a feeding decision support system (Dewhurst *et al*, 1996).

Urinary PD excretion has been proposed as an index of MCP supply (Topps and Elliot, 1965 and Rys *et al*, 1975). Although there are uncertainties surrounding the detail of the PD technique, it has the potential as a non-invasive predictor of MCP supply which could lead to development of an on-farm diagnostic test (Dewhurst *et al*, 1996). Currently, the most accurate means of assessing urinary PD excretion is to perform a total urine collection (Grubic *et al*, 1992), which restricts the PD method to detailed nutritional studies. Relatively little research has been conducted to investigate the

potential of the PD method for use in the commercial farm situation (Susmel *et al*, 1995). Experiments documented in this thesis have evaluated the potential of the PD method to provide an on-farm diagnostic marker of MCP supply in the dairy cow based on either spot sampling of urine or milk analysis.

9.1. Evaluation of the spot urine sampling technique

Urinary creatinine excretion has been proposed as an internal marker of urinary output (DeGroot and Aafjes, 1960; Albin and Clanton, 1966 and Erb *et al*, 1977). Assuming renal clearances of PDs approach that of creatinine (Greger *et al*, 1976), Antoniewicz *et al*, 1981, suggested that A/c ratios in spot urine samples could be used to indicate MCP supply. In order to utilise spot urine sampling it is essential that:- i) diurnal variations in the PD/c ratio is small or at least consistent and ii) PD/c ratios are closely correlated with daily PD excretion (Chen *et al*, 1995). Variations in spot urine sample PD/c ratios followed a diurnal pattern (chapters 5 and 6), as a consequence of diurnal variations in the rates of urinary PD and creatinine excretion (chapter 6). While Moorby and Dewhurst (1993b) reported significant differences in the PD/c ratio of am and pm spot samples, the consensus of the most recent research has indicated that variations in spot sample PD/c ratios do not show a diurnal pattern in bovine species (Chen *et al*, 1992d; Daniels *et al*, 1994; Gonda and Lindberg, 1994 and Dewhurst *et al*, 1996). Discrepancies between experimental findings could largely be explained by differences in feeding regimen. Comparisons of feeding silage and concentrate separately or as a complete diet indicated that feeding regimen influenced the extent of diurnal variation in spot sample PD/c ratios, being greater during separate feeding (chapter 6). The existence of diurnal variation in spot sample PD/c ratios and its dependence on feeding regimen strongly indicates that the on-farm application of the spot sampling technique would be limited due to uncertainites regarding timing and frequency of urine sampling. Evaluation of the accuracy of 4, 8 and 12 hour urine sampling regimens to predict the daily mean PD/c ratio indicated that collection of multiple samples within a day was a more reliable strategy than collection of fewer samples over several days (chapter 6). Even the most

intensive sampling regimen based on collecting four spot samples at four-hour intervals over two days did not allow an acceptable prediction of the daily mean PD/c ratio, with correlation coefficients between sampling estimates and daily mean PD/c ratios ranging between 0.537-0.951. The lack of sensitivity and precision of the spot sample technique to allow an accurate prediction of daily mean PD/c ratios has been demonstrated previously by Chen *et al* (1992d).

Accuracy of the spot sampling technique is also dependent on a close correlation between daily mean PD/c ratios and daily urinary PD excretion (Chen *et al*, 1995). In all cases (chapters 6,7 and 8), daily mean PD/c ratios, derived from a total urine collection were poorly correlated with daily PD excretion. Significant between-cow variations in urinary creatinine excretion largely account for the poor correlation, although random variation associated with the measurement of creatinine will inevitably contribute as suggested by Daniels *et al* (1994). Significant between-animal variations in urinary creatinine excretion have been reported previously (Chetal *et al*, 1975) and lead Dewhurst (1989) to suggest that groups of 15 animals would be required to utilise urinary creatinine excretion as an index of urinary output. Scaling daily mean PD/c ratios by cow liveweight or by the overall mean urinary creatinine concentration generally improved the prediction of urinary PD excretion to values similar to that reported by Daniels *et al* (1994), presumably by accounting for between-cow variation in creatinine excretion. However, the effects of scaling factor were inconsistent between experiments as documented in Table 9.1. Pooling data from all experiments indicated only a moderate correlation between daily mean PD/c ratios and urinary PD excretion (r^2=0.565, n=261, P<0.001), confirming the relationship described by Daniels *et al* (1994).

In the absence of diurnal variations in spot urine sample PD/c ratios, Dewhurst *et al* (1996) suggested that collection of two daily spot samples over two consecutive days from eight cows would yield useful information enabling dietary differences in MCP supply to be identified, with sufficient accuracy to assist diet formulation.

Table 9.1. Prediction of daily urinary PD excretion from daily mean PD/c ratios

Chapter	Scaling factor	n	r^2	P
6	None	48	0.090	<0.05
	Liveweight	48	0.480	<0.001
	[Creatinine]	48	0.390	<0.001
7	None	113	0.088	<0.001
	Liveweight	113	0.062	<0.01
	[Creatinine]	113	0.698	<0.001
8	None	100	0.240	<0.001
	Liveweight	100	0.130	<0.01
	[Creatinine]	100	0.590	<0.001
All data	None	261	0.565	<0.001

However, the lack of sensitivity and precision of spot urine sampling identified in chapters 5 and 6, suggest that the technique adopted by Dewhurst *et al* (1996) would only identify diets which caused extremes of MCP supply and in some cases could yield misleading information. These suggestions are confirmed by Iriki *et al* (1994), who argued that spot urine sampling would result in unreliable estimates of MCP supply due to protein-energy nutrition effects on endogenous urinary PD and creatinine excretion. Furthermore, the practicality of spot urine sampling of a relatively large group of cows under normal farm conditions is highly questionable. In contrast to the suggestions of Dewhurst *et al* (1996), experimental findings documented in this thesis suggest that the usefulness of the spot urine sampling technique as an on-farm diagnostic of MCP supply is limited.

The lack of precision and sensitivity of the spot urine sampling technique (chapter 6) indicates that a total urine collection is necessary to assess accurately daily urinary PD excretion, which confirms earlier suggestions of Grubic *et al* (1992) and Puchala and Kulasek (1992). As Chen *et al* (1995) suggested, only a total urine collection has the potential to detect difference in MCP supply with sufficient accuracy and precision to be of practical use.

9.2. Proportions of urinary PDs excreted in the urine

Allantoin is quantitatively the most important PD excreted in the urine, and constituted 82% (chapters 7 and 8), 85% (chapter 5) and 90% (chapter 6) of all PDs excreted confirming reports in the literature which range between 82 and 93% (Chen *et al*, 1990c; Dewhurst *et al*, 1995 and 1996; Giesecke *et al*, 1994; Gonda and Lindberg, 1994 and Verbic *et al*, 1990). The remainder of PDs were excreted as uric acid, while xanthine and hypoxanthine excretion was negligible supporting earlier findings of Chen *et al* (1990b), Verbic *et al* (1990), Susmel *et al* (1994b) and Dewhurst *et al* (1995). Increases in urinary PD excretion, have been demonstrated to decrease the proportion of PDs excreted as allantoin presumed to be due to saturation of *uricase*, (Stefanon *et al*, 1995 cited by Susmel *et al*, 1995 and Dewhurst *et al*, 1996). As cows used in all experiments were of a similar genetic background, variations in the proportion of PDs excreted as allantoin observed between experiments are almost certainly a consequence of *uricase* activity. Pooling experimental data from chapters 6, 7 and 8, indicated that urinary PD excretion was highly correlated (r^2=0.977, n=261, P<0.001) with urinary allantoin excretion as described in Figure 9.1. This relationship indicates that urinary PD excretion can be accurately predicted from measurements of allantoin alone using the following equation:-

$$\mathbf{Y = 1.13\ (\pm 0.01)\ x + 10.79\ (\pm 2.88)} \qquad \mathbf{r^2=0.977,\ n=261,\ P<0.001}$$

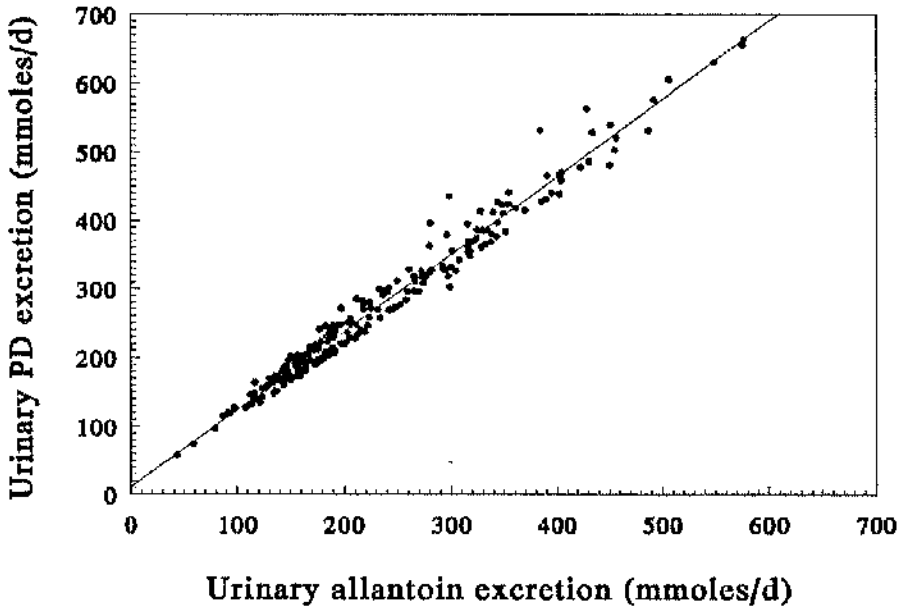
Where:-

Y is urinary PD excretion
X is urinary allantoin excretion
Values in parenthesis represent standard errors

Currently, controversy exists over the prediction of urinary PD excretion from measurements of allantoin. Giesecke *et al* (1984), Fujihara *et al* (1985 and 1987), Lindberg *et al* (1989) have suggested that all derivatives are required to predict PD excretion in sheep and goats and recent work of Stefanon *et al* (1995; cited by Susmel *et*

al, 1995) has supported these suggestions for dairy cows. However, current observations documented in this thesis tend to support those of Balcells *et al* (1991) and Puchala and Kulasek (1993) in sheep and those of Dewhurst *et al* (1996) in dairy cows, which indicated that measurements of allantoin alone gives an accurate prediction of assessment of urinary PD excretion.

Figure 9.1. Relationship between urinary PD and allantoin excretion (r^2=0.977, n=261, P<0.001)



9.3. Prediction of urinary PD excretion from calculated FME intake and MCP supply

Individual cow urinary PD excretion was poorly predicted from calculated FME intake (r^2=0.108, n=58, P<0.01 and r^2=0.180, n=35, P<0.01, for chapters 7 and 8, respectively) or calculated MCP supply (r^2=0.099, n=58, P<0.01 and r^2=0.121, n=35, P<0.05, for chapters 7 and 8, respectively). Random experimental errors, errors inherent

in the PD technique and between-cow variations in EMPS are likely to account for the poor prediction on an individual cow basis.

As discussed in sections 7.5. and 8.5., FME intake was manipulated using dietary fat which can be measured reliably. Therefore errors in the calculation of FME intake are likely to have been minimised in both experiments at least in incremental if not absolute terms. Errors associated with the assessment of urinary PD excretion are also likely to be small due to the fact that measurements were performed in duplicate using a HPLC technique with comparable precision to published methods.

Prediction errors due to errors associated with the PD technique are likely to arise as a result of between-cow variations in endogenous urinary PD excretion, proportion of plasma PDs excreted renally, dietary purines escaping rumen degradation or the purine content of rumen microbes. In a recent study designed to cause relatively large changes in MCP supply of 66%, urinary allantoin excretion was found to vary by only 20%, which increased to 42% when endogenous PD excretion was taken into account (Susmel *et al*, 1995). The lack of sensitivity of the PD technique identified by Susmel *et al* (1995) was attributed to metabolic factors described above. No attempts were made to account for between-cow variations in endogenous PD excretion in this thesis due to the uncertainties of its magnitude for a lactating dairy cow. Assuming renal clearances of creatinine approach those of purine derivatives (Greger *et al*, 1976), between-cow variations in the proportion of plasma PDs excreted renally, a main source of error of the PD technique, can be taken into account by the use of urinary PD/c ratios. Even when this potential source of error was taken into account, prediction of urinary PD/c ratios from calculated FME intake (r^2=0.187, n=58, P<0.01 and r^2=0.319, n=35, P<0.001, for chapters 7 and 8, respectively) or MCP supply (r^2=0.160, n=58, P<0.01 and r^2=0.399, n=35, P<0.001, for chapters 7 and 8, respectively) remained poor. These relationships suggest that other sources of error contributed to the variability in urinary PD responses to calculated FME observed for individual cows.

Using AFRC (1992) predictions gave values ranging between 11.0 to 11.7 g M-N/ MJ FME for experimental diets described in chapters 7 and 8. Using the models proposed by Verbic *et al* (1990) and Chen *et al* (1992a) to calculate EMPS based on

measured urinary PD excretion indicated a far greater range in EMPS of between 10.4 to 21.0 g M-N/ MJ FME than was predicted by AFRC (1992) calculations. Although calculations of EMPS based on urinary PD excretion involve crude assumptions concerning endogenous urinary PD excretion, they serve to highlight that between-cow variations in EMPS are almost certainly the major source of prediction error.

The use of mean treatment values indicated a good prediction of urinary PD excretion or urinary PD/c ratios from calculated FME intake or MCP supply in particular, as shown in Tables 9.2. and 9.3., respectively. Presumably the improved prediction when mean treatment values were used, compared to individual cow measurements, is due to taking into account random experimental errors, errors in the PD technique and between-cow variations in EMPS.

Table 9.2. Prediction of urinary PD excretion from calculated FME intake and MCP supply

Chapter	Dataset	FME intake			MCP supply		
		n	r^2	P	n	r^2	P
7	IC	58	0.108	<0.01	58	0.160	<0.01
	TM	6	0.612	<0.05	6	0.903	<0.01
8	IC	35	0.180	<0.01	35	0.399	<0.001
	TM	4	0.963	<0.05	4	0.946	<0.05
7 and 8	TM	10	0.424	<0.05	10	0.882	<0.001

Table 9.3. Prediction of urinary PD/c ratios from calculated FME intake or MCP supply

Chapter	Dataset	FME intake			MCP supply		
		n	r^2	P	n	r^2	P
7	IC	58	0.187	<0.01	58	0.160	<0.01
	TM	6	0.960	<0.001	6	0.903	<0.01
8	IC	35	0.319	<0.001	35	0.399	<0.001
	TM	4	0.977	<0.001	4	0.946	<0.05
7 and 8	TM	10	0.336	<0.05	10	0.936	<0.001

Key for Tables **9.2.** and **9.3.**:-
IC denotes individual cow measurements
TM denotes treatment mean values

However, as discussed in sections 7.5. and 8.5., prediction of urinary PD excretion or PD/c ratios from calculated FME intake or MCP supply based on mean treatment values is subject to criticism as only a limited number of data points was used which hides the true variability of the relationship. In an attempt to overcome these criticisms mean treatment values from chapters 7 and 8, were pooled together to investigate the prediction of urinary PD excretion and PD/c ratios. Data from chapter 6 was excluded due to the lack of reliable estimates of FME intake. Based on pooled mean treatment values, urinary PD excretion and urinary PD/c ratios were predicted more accurately by calculated MCP supply (r^2=0.882, n=10, P<0.001 and 0.936, n=10, P<0.001, respectively) than calculated FME intake (r^2=0.424, n=10, P<0.05 and 0.336, n=10, P<0.05). Figures 9.2. and 9.3. describe the relationships between urinary PD excretion with calculated FME intake and MCP supply, respectively based on pooled mean treatment values.

Presumably calculated MCP supply gives a better prediction of urinary PD excretion than calculated FME intake due to the fact that variations in EMPS are partly taken into account by a level of feeding correction.

Experimental observations documented in this thesis indicate that calculated FME intake or MCP supply (AFRC, 1992) poorly predict urinary PD excretion and therefore true MCP supply for an individual cow. Current experimental findings highlight the necessity for an on-farm diagnostic test to assess MCP supply to provide information on rumen function and therefore to act as a basis for refining dairy cow protein feeding.

9.4. Evaluation of milk allantoin as an index of MCP supply

Due to technical limitations, on-farm measurements of urinary PD excretion are not practical. Secretion of allantoin in milk could represent a potential alternative as it is quantitatively collected and easily sampled (Giesecke *et al*, 1994). Furthermore, milk samples are routinely collected on a large scale in many countries as part of a national milk recording scheme and have for many years been used as a medium for diagnostic tests (Emanuelson *et al*, 1989).

Figure 9.2. Relationship between mean treatment urinary PD excretion and calculated FME intake (r^2=0.424, n=10, P<0.05)

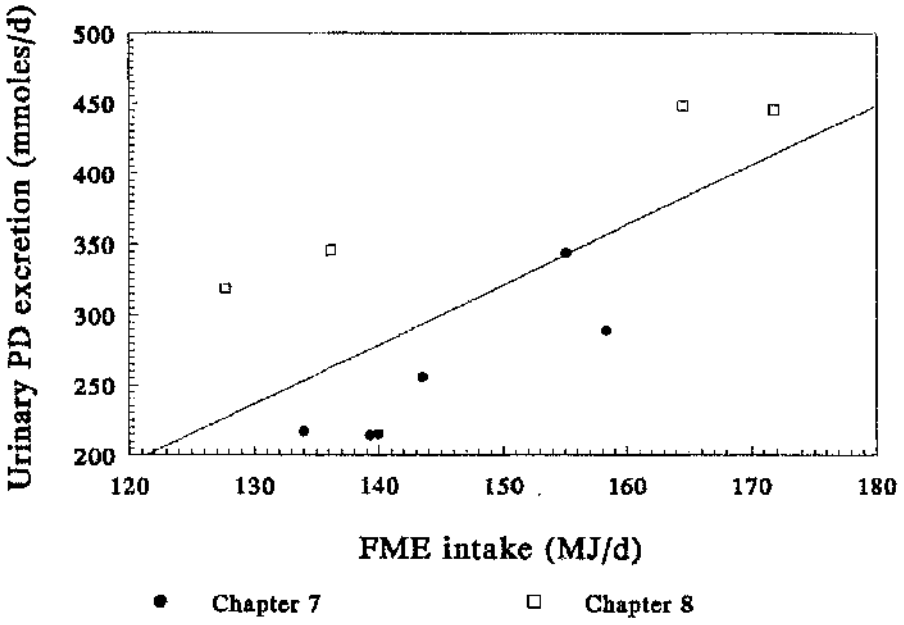
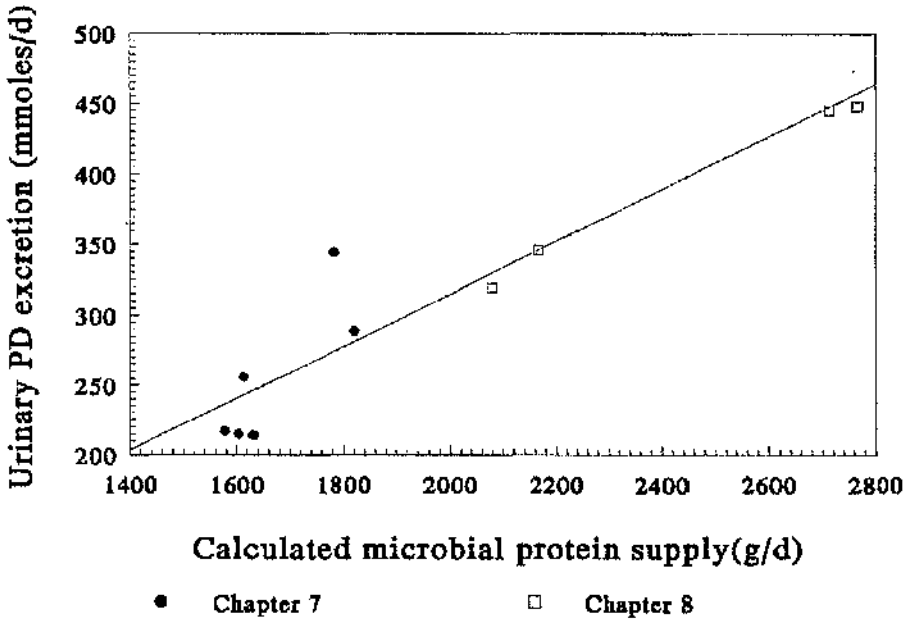


Figure 9.3. Relationship between mean treatment urinary PD excretion and calculated MCP supply (r^2=0.882, n=10, P<0.001)



While secretion of allantoin in milk is well documented in the literature (e.g. Rosskopf *et al*, 1991; Lebzien *et al*, 1993; Giesecke *et al*, 1994 and Susmel *et al*, 1995) little research has been conducted to evaluate critically its use as an on-farm diagnostic of MCP supply. Experiments documented in chapters 7 and 8 were designed to evaluate the potential of milk allantoin.

No direct measurements of MCP supply were made for three reasons. Firstly, comparative studies of microbial markers have highlighted large discrepancies in their estimation of MCP supply (Ling and Buttery, 1978; Mercer *et al*, 1980; Whitelaw *et al*, 1984; Lindberg *et al*, 1989; Sinclair *et al*, 1991 and Illg and Stern, 1994). Secondly, measurements of MCP supply using either internal or external markers require surgically modified animals, typically fitted with a rumen fistula and duodenal cannula, which i) confine investigations to small scale studies and ii) lead to uncertainties as to how closely surgically modified animals reflect their physiologically normal counterparts. Thirdly, due to considerations of animal welfare and the fact that experimental cows were obtained from the SAC, Auchincruive dairy herd.

Since no direct measurements were made, the problem arises for both experiments (chapters 7 and 8), as to which experimental measurements could serve as the most reliable measure of MCP supply. Potentially, urinary PD excretion, calculated FME intake or MCP supply (AFRC, 1992) could be used. Since urinary PD excretion has been shown to be closely correlated with duodenal microbial NA supply (Sibanda *et al*, 1982; Fujihara *et al*, 1987; Chen *et al*, 1990a and Verbic *et al*, 1990) and comparisons with other microbial markers have been shown to be in good agreement (Djouvinov and Todorov, 1994 and Perez *et al*, 1994), urinary PD excretion was considered to give the most reliable estimate of MCP supply.

Table 9.4. Relationships between milk allantoin excretion with urinary PD and allantoin excretion and PD/c and A/c ratios

Variate	Dataset	Chapter 7			Chapter 8			Chapters 7 and 8		
		n	r^2	P	n	r^2	P	n	r^2	P
Urinary PD excretion	IC	58	0.009	0.494	35	<0.001	0.973	93	0.276	<0.001
	TM	6	0.639	0.056	4	0.936	<0.05	10	0.713	<0.01
Urinary allantoin excretion	IC	58	0.003	0.680	35	0.006	0.665	93	0.346	<0.001
	TM	6	0.639	0.056	4	0.990	<0.01	10	0.777	<0.001
Urinary PD/c ratio	IC	58	0.248	<0.001	35	0.193	<0.01	93	0.596	<0.001
	TM	6	0.851	<0.01	4	0.787	0.074	10	0.826	<0.001
Urinary A/c ratio	IC	58	0.257	<0.001	35	0.368	<0.001	93	0.685	<0.001
	TM	6	0.825	<0.01	4	0.968	<0.05	10	0.865	<0.001

Key:-

IC denotes individual cow values

TM denotes treatment mean values

Table 9.5. Relationships between arithmetic mean milk allantoin concentration with urinary PD and allantoin excretion and PD/c and A/c ratios

Variate	Dataset	Chapter 7			Chapter 8			Chapters 7 and 8		
		n	r^2	P	n	r^2	P	n	r^2	P
Urinary PD excretion	IC	58	<0.001	0.931	35	<0.001	0.983	93	0.119	<0.001
	TM	6	0.613	<0.05	4	0.771	0.077	10	0.859	<0.001
Urinary allantoin excretion	IC	58	0.004	0.647	35	<0.001	0.871	93	0.149	<0.001
	TM	6	0.624	<0.05	4	0.706	0.103	10	0.880	<0.001
Urinary PD/c ratio	IC	58	0.177	<0.001	35	0.161	<0.01	93	0.377	<0.001
	TM	6	0.942	<0.001	4	0.764	0.082	10	0.944	<0.001
Urinary A/c ratio	IC	58	0.196	<0.001	35	0.212	<0.01	93	0.404	<0.001
	TM	6	0.825	<0.01	4	0.760	0.084	10	0.951	<0.001

Key:-

IC denotes individual cow values

TM denotes treatment mean values

Table 9.6. Relationships between mean milk allantoin concentration weighted by milk yield with urinary PD and allantoin excretion and PD/c and A/c ratios

Variate	Dataset	Chapter 7			Chapter 8			Chapters 7 and 8		
		n	r^2	P	n	r^2	P	n	r^2	P
Urinary PD excretion	IC	58	<0.001	0.882	35	<0.001	0.973	93	0.115	<0.001
	TM	6	0.534	0.061	4	0.936	<0.05	10	0.838	<0.001
Urinary allantoin excretion	IC	58	0.006	0.594	35	0.006	0.665	93	0.146	<0.001
	TM	6	0.516	0.066	4	0.990	<0.01	10	0.865	<0.001
Urinary PD/c ratio	IC	58	0.199	<0.001	35	0.193	<0.01	93	0.386	<0.001
	TM	6	0.834	<0.01	4	0.787	0.074	10	0.939	<0.001
Urinary A/c ratio	IC	58	0.218	<0.001	35	0.368	<0.001	93	0.416	<0.001
	TM	6	0.784	<0.05	4	0.968	<0.05	10	0.945	<0.001

Key:-

IC denotes individual cow values

TM denotes treatment mean values

Based on individual cow observations, milk allantoin excretion, arithmetic or weighted mean concentration were poorly correlated with urinary PD excretion in both experiments (refer to Tables 9.4., 9.5. and 9.6.), but dramatically improved when mean treatment values were used as shown in Tables 9.4., 9.5. and 9.6. Relationships derived from mean treatment values are subject to criticism due to a limited number of data points. In attempt to address this criticism, mean treatment values from both experiments (chapters 7 and 8) were pooled together, while data from chapter 6 were excluded due to the use of a different milking regimen. Based on pooled mean treatment values, milk allantoin excretion or mean concentrations were strongly related to urinary PD or allantoin excretion (refer to Figures 9.4. and 9.5.), which recognising the fact that these relationships were derived from experiments in which mean milk yields ranged between 12 to 33 kg/d, suggest that milk allantoin could be used to assess MCP supply on an herd basis, while between-cow variability precludes its use for an individual cow.

The lack of relationships between urinary PD excretion and milk allantoin excretion or concentration based on individual cow observations are potentially explained by either i) random experimental errors or ii) between-cow variations in the partitioning of plasma allantoin to renal and mammary excretory routes.

Experimental errors associated with determination of milk and urine PD excretion were minimised by performing duplicate measurements using precise HPLC methods (refer to chapters 2 and 3). Consequently, between-cow variations in allantoin partitioning would appear to be the main source of error in the relationships described above.

Allantoin secreted in milk is thought to be derived from plasma as a result of diffusion into mammary alveolar cells (Tiermeyer *et al*, 1984 and Giesecke *et al*, 1994) while excretion in the urine is determined by renal clearances (Chen *et al*, 1991a and Giesecke *et al*, 1993). Consequently, variations in the proportion of plasma PDs excreted renally will contribute to the poor relationships between urinary PD excretion and milk allantoin excretion or concentration.

Figure 9.4. Relationship between mean treatment milk and urinary allantoin excretion (r^2=0.777, n=10, P<0.001)

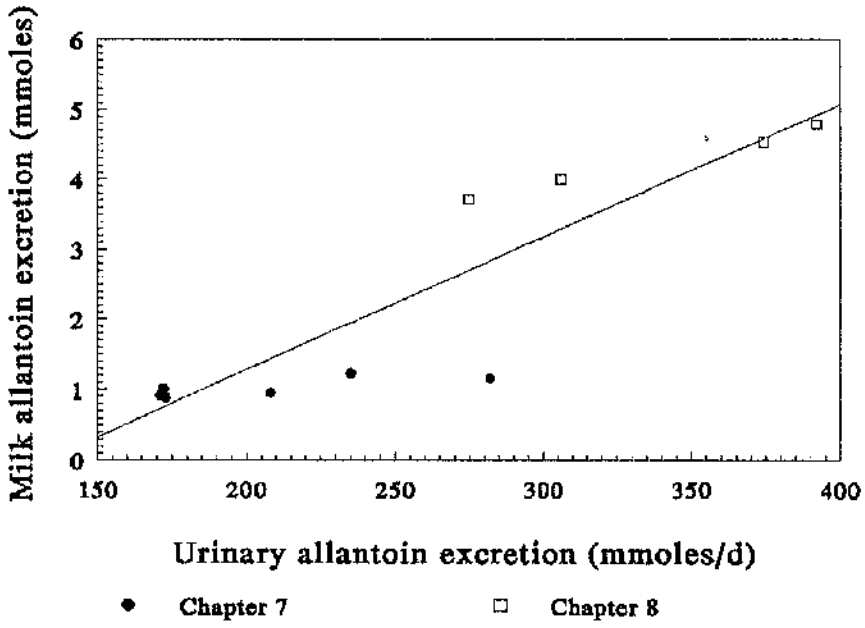
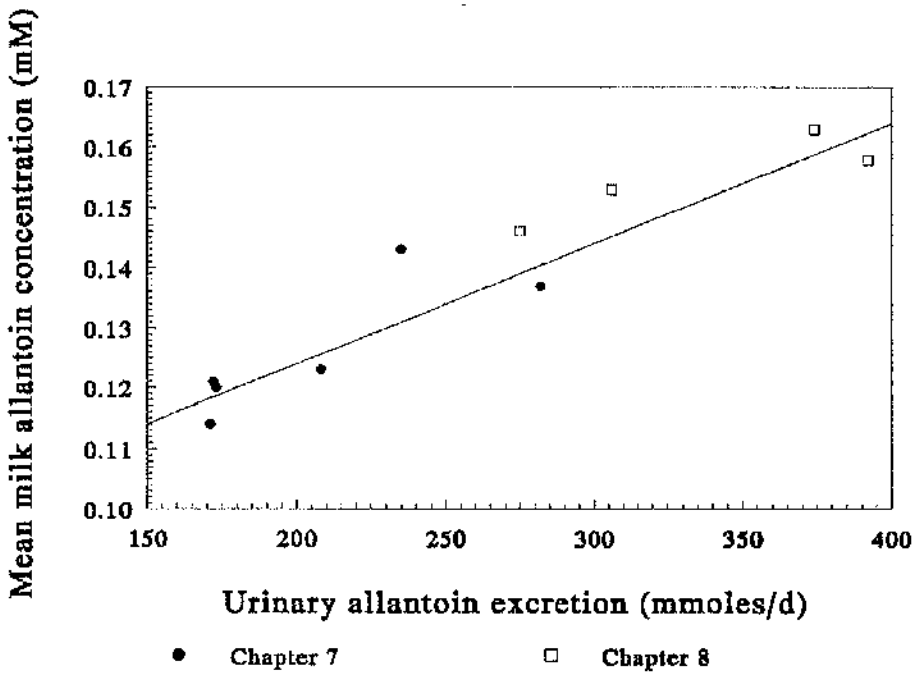


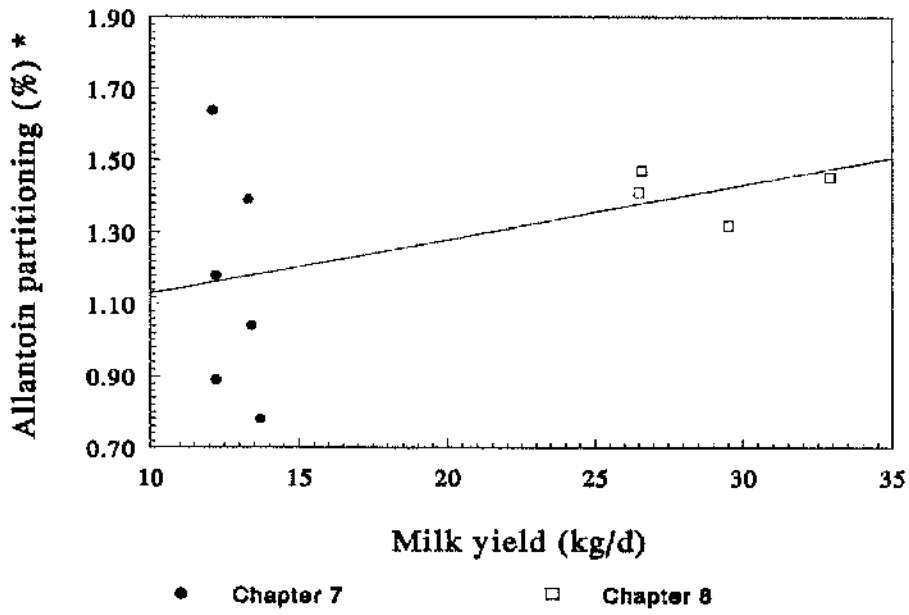
Figure 9.5. Relationship between mean treatment mean milk allantoin concentration and urinary allantoin excretion (r^2=0.880, n=10, P<0.001)



Assuming renal clearances of PDs approach that of creatinine (Greger *et al*, 1976), use of PD/c ratios would account for such between-cow variations. In all cases, individual cow milk allantoin excretion or concentration was more closely correlated with urinary PD/c or A/c ratios than with urinary PD or allantoin excretion.

Since diffusion into the mammary gland is thought to account for allantoin secretion in milk, it seems plausible that variations in plasma allantoin concentrations or mammary blood flow could contribute to variations in allantoin partitioning. Measurements of mammary blood flow were not made, but could potentially be related to milk yield. The use of pooled (chapters 7 and 8) mean treatment values indicated that milk yield accounted for only 10% of the observed variation in allantoin partitioning (r^2=0.101, n=10, P=0.194; refer to Figure 9.6.).

Figure 9.6. Relationship between allantoin partitioning and milk yield (r^2=0.101, n=10, P=0.194)



* Refers to allantoin secretion in milk as a percentage of urinary excretion

While it is accepted that the assumption that mammary blood flow and milk yield are closely related is unreliable, the experimental data suggests that use of an internal marker such as creatinine whose excretion in milk is directly proportional to milk yield (Susmel *et al*, 1995), is unlikely to account for between-cow variations in allantoin partitioning.

Based on mean treatment values, milk allantoin excretion accounted for between 0.78 to 1.64 % of urinary excretion for all experimental diets reported in this thesis, which is within the range reported by Giesecke *et al* (1994). In contrast, other studies have reported that secretion in milk accounts for between 6-10% of urinary allantoin excretion (Kirchgessner and Kreuzer, 1985; Kirchgessner and Windisch, 1989 and Susmel *et al*, 1995). In the absence of more detailed physiological measurements, these discrepancies are difficult to reconcile but could be accounted for by analytical methodology (Giesecke *et al*, 1994) or may reflect breed differences.

Since experimental cows were of similar genetic background, observed between-cow variations in allantoin partitioning are difficult to reconcile. While studies in rats have demonstrated *uricase* or *uricase* mRNA to be absent in mammary tissue (Motojima and Goto, 1990), it is conceivable that oxidation of plasma or mammary derived uric acid or xanthine and hypoxanthine could contribute to allantoin secretion in milk. Endogenous secretion of allantoin in milk could potentially account for between-cow differences in the proportion of allantoin excreted in milk compared to urine.

Based on individual cow measurements, mean milk allantoin concentration was more closely correlated with calculated FME intake than milk allantoin excretion, under conditions causing small variations in milk yield, while the reverse was true when variations in milk yield were much greater. Milk allantoin concentrations have been shown to be closely correlated with those in plasma (Rosskopf *et al*, 1991). Presumably when variations in milk volume are relatively small, plasma allantoin concentrations closely reflect those in milk.

Responses of milk allantoin excretion to changes in calculated FME intake for an individual cow were found to vary considerably and are potentially explained by the factors influencing the responses of urinary PD excretion to calculated FME intake and

those influencing allantoin partitioning between renal and mammary excretory routes. Relationships between milk allantoin excretion and concentration with calculated FME intake are shown in Table 9.7.

Table 9.7. Relationships between milk allantoin excretion and concentration with calculated FME intake

Chapter	Dataset	Parameter	n	r^2	P
7	IC	Milk allantoin excretion	58	0.019	0.153
7	TM		6	0.801	0.01
7	R		10	0.586	<0.01
8	IC		35	0.101	<0.05
8	TM		4	0.812	0.065
7 and 8	TM		10	0.003	0.341
7	IC	Arithmetic mean milk	58	0.135	<0.01
7	TM	allantoin concentration	6	0.981	<0.01
7	R		10	0.694	<0.01
8	IC		36	<0.010	0.538
8	TM		4	0.876	<0.05
7 and 8	TM		10	0.253	0.079
7	IC	Mean milk allantoin	58	0.158	<0.01
7	TM	concentration weighted	6	0.743	<0.05
7	R	by milk yield	9	0.756	<0.01
8	IC		36	<0.010	0.619
8	TM		4	0.895	<0.05
7 and 8	TM		10	0.184	0.120

Key:-

IC denotes mean experimental period measurements for all cows

TM denotes treatment mean values

R denotes dataset restricted to cows which achieved expected range in FME intakes

Figure 9.7. Relationship between mean treatment milk allantoin excretion and calculated FME intake (r^2=0.003, n=10, P=0.341)

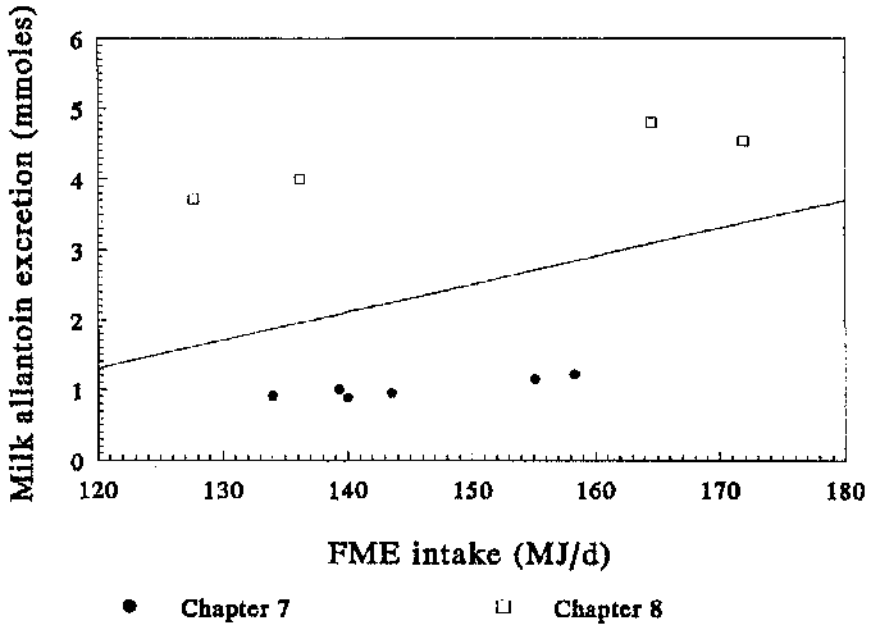
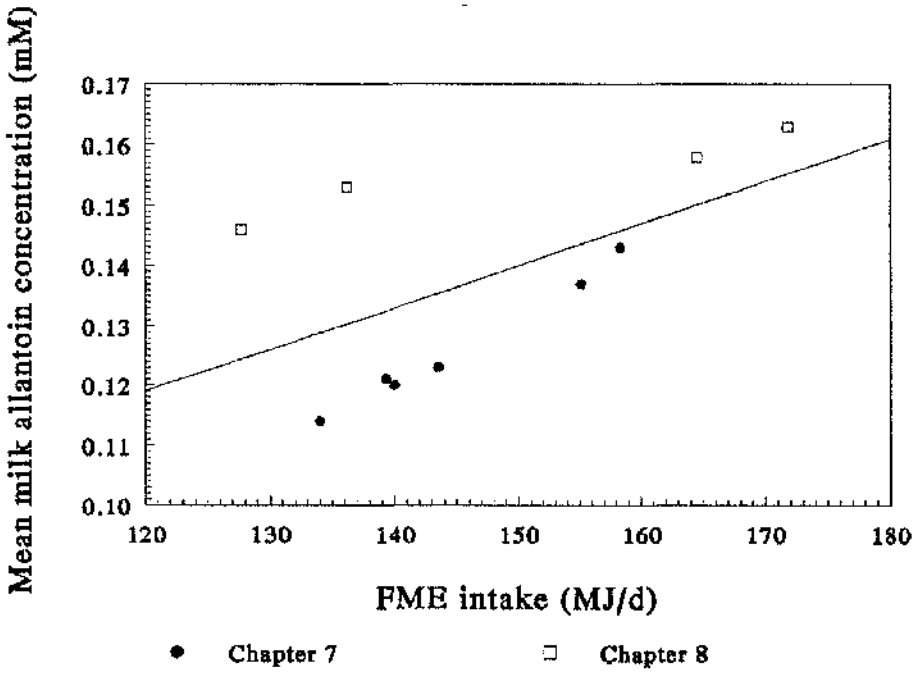


Figure 9.8. Relationship between mean treatment mean milk allantoin concentration and calculated FME intake (r^2=0.253, n=10, P=0.079)



Generally, responses of urinary PD excretion to changes in calculated FME intake were stronger than milk allantoin excretion or concentration responses, confirming observations of Susmel *et al* (1995) who reported variations in milk allantoin excretion to be approximately twice as large as those for urinary allantoin excretion. Pooling mean treatment values from chapters 7 and 8, indicated that milk allantoin excretion or mean concentration were poorly correlated with calculated FME intake (r^2=0.003, n=10, P=0.341 and r^2=0.253, n=10, P=0.079, respectively) as described by Figures 9.7. and 9.8., respectively.

Both individual cow milk allantoin excretion or concentration were poorly correlated with calculated MCP supply (refer to Table 9.8.). Relationships were similar to those derived with calculated FME intake, which is to be expected as AFRC (1992) calculations are based primarily on FME intake.

As observed for urinary PD excretion, use of pooled mean treatment values indicated that calculated microbial supply was a better predictor of milk allantoin excretion or concentration than FME intake (refer to Figures 9.9. and 9.10., respectively). These findings are almost certainly due to the fact that variations in EMPS are partly taken into account by a level of feeding correction. Use of mean treatment values for each experiment indicated that mean milk allantoin concentration was more closely correlated with calculated MCP supply under conditions causing relatively small changes in milk yield.

Figure 9.9. Relationship between mean treatment mean milk allantoin excretion and calculated MCP supply (r^2=0.875, n=10, P<0.001)

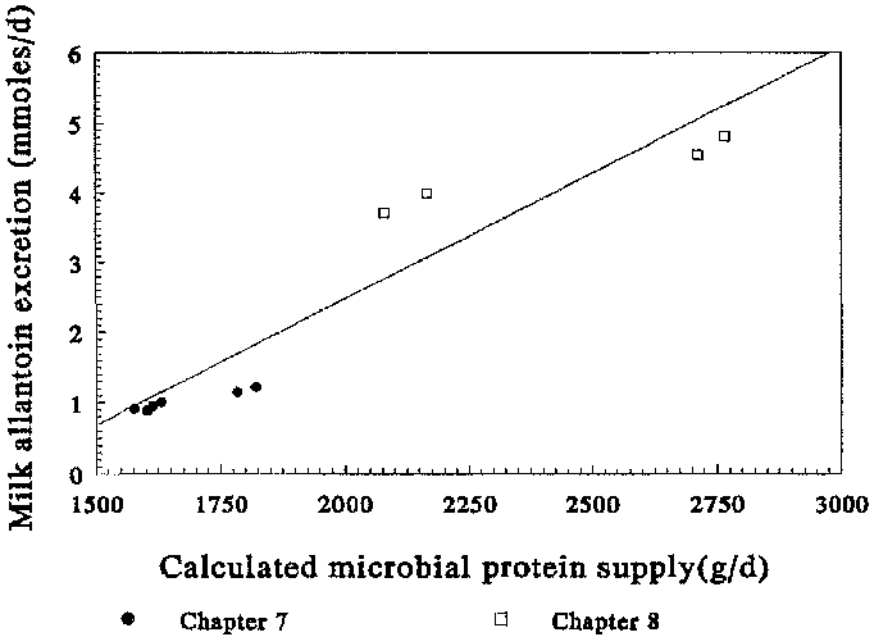


Figure 9.10. Relationship between mean treatment mean milk allantoin concentration and calculated MCP supply (r^2=0.819, n=10, P<0.001)

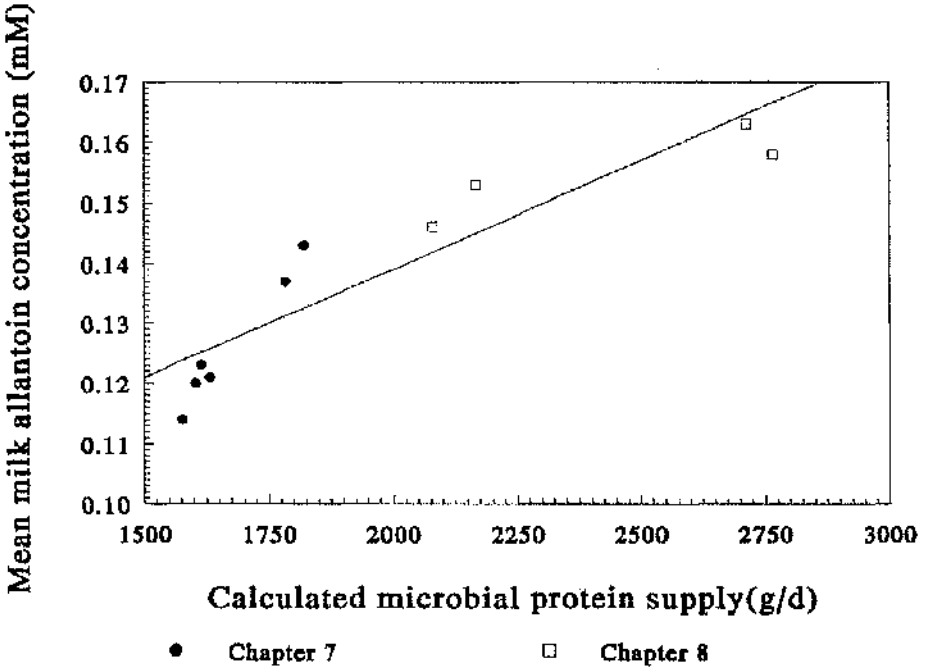


Table 9.8. Relationships between milk allantoin excretion and concentration with calculated MCP supply

Chapter	Dataset	Parameter	n	r^2	P
7	IC	Milk allantoin excretion	58	0.016	0.172
7	TM		6	0.944	<0.001
7	R		10	0.558	<0.01
8	IC		35	0.128	<0.05
8	TM		4	0.940	<0.05
7 and 8	TM		10	0.875	<0.001
7	IC	Arithmetic mean allantoin	58	0.139	<0.01
7	TM	concentration	6	0.919	<0.01
7	R		10	0.679	<0.01
8	IC		36	<0.010	0.331
8	TM		4	0.692	0.108
7 and 8	TM		10	0.819	<0.001
7	IC	Mean allantoin concentration	58	0.160	<0.01
7	TM	weighted by milk yield	6	0.825	<0.01
7	R		9	0.738	<0.01
8	IC		36	<0.010	0.447
8	TM		4	0.742	0.090
7 and 8	TM		10	0.855	<0.001

Key:-

IC denotes mean experimental period measurements for all cows
TM denotes treatment mean values
R denotes dataset restricted to cows which achieved expected range in FME intakes

In conclusion, pooling data from chapters 7 and 8 indicated that measurements of milk allantoin excretion or concentration poorly predicted urinary PD excretion or calculated MCP supply for an individual cow (refer to Table 9.9.). Based on pooled mean treatment values:- i) milk allantoin excretion and concentration in particular, were highly correlated with urinary PD excretion and ii) milk allantoin excretion and concentration, were as strongly correlated with calculated MCP supply as urinary PD

excretion (refer to Table 9.9.). Based on observations reported in this thesis, variability precludes the use of milk allantoin as an index of MCP for individual cows, but it appears as reliable as urinary PD excretion when used on a herd or group feeding basis.

Table 9.9. Relationships between urinary PD excretion, calculated MCP supply and milk allantoin excretion and concentration based on pooled experimental data

Variate	Dataset	Urinary PD excretion			Calculated MCP		
		n	r^2	P	n	r^2	P
Calculated	IC	93	0.589	<0.001	-	-	-
MPS	TM	10	**0.882**	<0.001	-	-	-
Milk allantoin	IC	93	0.276	<0.001	93	0.572	<0.001
excretion	TM	10	**0.713**	<0.01	10	**0.875**	<0.001
Art mean milk	IC	93	0.119	<0.001	93	0.286	<0.001
allantoin concentration	TM	10	**0.859**	<0.001	10	**0.819**	<0.001
Wt mean milk	IC	93	0.115	<0.001	93	0.278	<0.001
allantoin concentration	TM	10	**0.838**	<0.001	10	**0.855**	<0.001

9.5. Development of an on-farm diagnostic test of nitrogen metabolism in dairy cows

Inclusion of supplementary feed protein into dairy cow ration is expensive, while subsequent inefficient utilisation can contribute to enviromental contamination and in ceratin cases have a negative impact on health and reproduction (Hibbitt, 1984; Confield *et al*, 1990 and Ferguson *et al*, 1991). Consequently the ability to monitor and therefore improve the efficiency of nitrogen utilisation on dairy farms would benefit producers and consumers (DePeters and Ferguson, 1992). Excretion of urea and PDs in various biological fluids have potential as diagnostic markers of different aspects of nitrogen metabolism in the dairy cow.

In healthy animals dietary protein intake and the ratio of dietary energy to protein influences the concentration of urea in plasma, urine and milk (Gonda and Lindberg,

1994), while milk urea concentrations have been shown to be correlated with rumen ammonia concentrations (Oltner and Wiktorsson, 1983; Ropstad *et al*, 1989; Roseler *et al*, 1990; Miettinen and Juvonen, 1990 and Gustavsson and Palmquist, 1993). Although mammary amino acid catabolism can contribute up to a third of urea excretion in lactating cows (Bruckental *et al*, 1980), Refsdal *et al* (1985) suggested measurements of urea concentrations of bulk tank milk could indicate the adequacy of protein and energy ratio of the diet. Recent studies reviewed by DePeters and Ferguson (1992) have indicated considerable variations in milk urea concentrations. Experiments documented in this thesis have also demonstrated that milk urea-N concentrations can vary between 187 to 343 mg/kg. In order to utilise this milk component to evaluate dairy cow rations, normal values need to be established in order to establish confidence limits to identify nitrogen efficient and inefficient diets. Measurements of bulk tank milk may not be particulary useful as this could disguise inefficiencies within feeding groups. However, measurements based on individual or small groups of cows separated on the basis of milk production or genetic merit could yield useful information on protein feeding.

In conjunction with urea concentrations, DePeters and Ferguson (1992) have suggested that measurements of milk true protein could be used as the basis of a system evaluating dietary protein and energy status of the lactating dairy cow. Milk protein yield and concentration have been shown to be dependent on MP supply (Dewhurst, 1989; Moorby, 1993 and Dewhurst *et al*, 1996) suggesting its measurement would give an indication of whole-animal nitrogen utilisation. Currently, milk protein concentrations are measured routinely in many countries as part of a national milk recording scheme (Emanuelson *et al*, 1989), indicating that this information could be incorporated into a diagnostic test of dairy cow nitrogen metabolism.

Measurements of urea and true protein concentrations in milk do not however provide information on the magnitude of MCP available to the host ruminant. Experiments conducted in this thesis have suggested that milk allantoin would not provide useful information on MCP supply for an individual cow, but could be used to monitor groups of animals. Assuming variations in allantoin between renal and mammary excretory routes between-breeds are relatively small, measurement of milk allantoin has

the potential to provide information concerning rumen function. The possibility exists that measurements of milk allantoin, urea-N and true protein could be used as the basis of a herd diagnostic test of nitrogen metabolism. Development of an on-farm diagnostic test of dairy cow nitrogen metabolism based upon measurements of urea, true protein and allantoin milk components should be pursued in the future.

9.6. Future research proposals

Determinations of PDs in milk and urine are typically performed by HPLC based on a method using a C-18 stationary phase. Development of an HPLC method to quantify allantoin in milk (chapter 3) indicated advantages of improved retention and separation of allantoin by using an amino stationary phase. Accuracy of HPLC methods used to assess urinary PD concentrations are likely to be improved by developing techniques based on amino stationary phases.

Non-invasive assessment of rumen MCP based on urinary PD excretion has several advantages of lower cost, improved welfare and increases in scale over traditional methods. A substantial proportion of PDs excreted in urine are not derived from absorption of microbial or escaped feed purines, but occur as a result of tissue NA turnover. Quantification of endogenous urinary PD excretion in bovine species has largely been confined to non-lactating dairy cows and steers. More research is required to quantify the influence of metabolic changes during lactation on endogenous urinary PD excretion which would allow more accurate estimates of MCP supply to be made.

The use of the spot urine sampling technique as a practical on-farm diagnostic marker of individual cows requires further evaluation using alternative urinary output markers to creatinine. Numerous compounds are excreted in bovine urine and the possibility exists that one of these metabolites originates solely from an endogenous source. Other compounds such as salicylic acid when incorporated into the diet have been shown to be quantitatively recovered in the urine.

Variability in allantoin excretion and concentration precluded its use for individual cows. Further studies are required to investigate alternative non-invasive

microbial markers. Rumen microbial cell walls contain odd branched chained fatty acids which are unique to rumen bacteria and protozoa and are therefore unlikely to be encountered in typical ruminant rations. Recent advances in Gas-Liquid Chromatography have enabled the identification and quantification of odd and branched-chain fatty acids, indicating that the potential exists for assessing MCP supply from milk fatty acid content.

Biological mechanisms involved in milk secretion are poorly understood. Use of radio-labels could be used to elucidate such mechanisms and the factors which govern it. It is suspected that a proportion of the allantoin secreted in milk originates from catabolism of mammary and plasma derived uric acid, xanthine and hypoxanthine. Detailed studies are required to identify *uricase* activities or *uricase* mRNA content in bovine mammary tissue to establish whether endogenous mammary purine catabolism contributes to milk allantoin secretion. Endogenous mammary PD excretion could potentially be quantified by PD arterio-venous differences across the mammary gland or by radio-isotope studies. It is plausible that endogenous mammary PD excretion closely reflects mammary RNA turnover due to prolific protein synthetic activities within the gland. Currently it is thought urinary pseudouridine excretion closely reflects tissue RNA turnover. Consequently, variations in endogenous mammary PD excretion could be accounted for by either milk pseudouridine or true protein secretion.

The use of milk allantoin excretion as an on-farm diagnostic marker of MCP supply is largely constrained due to variations in the partitioning of allantoin between mammary and urinary excretory routes. Investigations are required to establish the extent of these variations over a range of diets and between dairy cow breeds and whether metabolites such as creatinine could be used to account for such variations.

Finally, observations documented in this thesis tentatively suggest measurement of allantoin in conjunction with urea and true protein milk components could form part of an on-farm dairy cow herd diagnostic test of nitrogen metabolism. Further research is required to establish whether information yielded by such a diagnostic test leads to improvements in on-farm efficiency of nitrogen utilisation.

9.7. General conclusions

i) Feeding regimen influences the extent of diurnal variations of PD/c ratios in spot urine samples.

ii) Prediction of daily mean urinary PD/c ratios from spot urine samples is unreliable.

iii) Daily urinary PD excretion is poorly predicted from daily mean PD/c ratios.

iv) Lack of sensitivity and precision of the spot sampling technique precludes its use as an on-farm diagnostic marker of MCP supply.

v) A total urine collection is necessary to accurately assess urinary PD excretion, while measurements of allantoin alone can be used to predict urinary PD excretion.

vi) Urinary creatinine excretion is independent of nutrient supply.

vii) Between-cow variations in urinary creatinine excretion precludes its use as a urinary output marker for individual cows

viii) Urinary pseudouridine excretion is independent of nutrient supply, but may be influenced by metabolic changes during lactation.

ix) Milk allantoin concentrations are not biologically maintained.

x) Milk allantoin excretion and concentration are poorly correlated with urinary PD or allantoin excretion within an individual cow.

xi) Variability in milk allantoin excretion or concentration precludes its use as an marker of MCP supply for an individual cow.

xii) Based on group mean values, milk allantoin excretion or concentration are highly correlated with urinary PD or allantoin excretion, suggesting that milk allantoin could be used as an index of MCP supply on a herd or group basis.

Chapter Ten

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Publications

Variations in the urinary excretion of Purine Derivatives and Creatinine in Holstein/Friesian dairy cows.

K.J. Shingfield, N.W. Offer and A.M. Sword (1995).

The estimation of rumen microbial protein output by urinary purine derivative (PD) excretion would have greater practical potential if spot samples could be used rather than a total collection. This was investigated using twelve multiparous Holstein/Friesian cows fed two diets in a changeover design using two 14-day periods. The diets were either silage *(ad libitum)* with 7 kg concentrate supplement (20% CP) fed at 10.00 or a complete diet *(ad libitum)* formulated from the same ingredients with a similar forage:concentrate ratio. Total urine collection was performed every 2 hours on days 11 and 14 of both periods and a subsample immediately frozen at -20°C prior to HPLC analysis. Daily allantoin (A) and daily PD excretion were highly correlated ($r=0.995$, $P<0.001$, $n=48$). Both PD and creatinine (C) excretion during each two hour period depended on time of collection (PD, $P<0.001$; C, $P<0.05$) and on cow ($P<0.01$) but were unaffected by diet and sampling day. Variations in PD/C and A/C ratios followed similar diurnal patterns as observed for PD and A. The data was used to assess the error of spot sampling regimens. None gave an acceptable prediction of mean daily PD/C (minimum median and maximum r values for 12hr, 8hr and 4hr sampling were 0.16 0.68 0.87, 0.19 0.76 0.92 and 0.43 0.75 0.95, respectively). Furthermore, the daily PD/C and A/C ratios proved poor predictors of daily PD and A output (r values of 0.69 and 0.72, respectively). Total urine collection appears necessary to assess total daily PD and A excretion in dairy cows.

Journal of Animal Science **60:** 18A.

The Influence of Fermentable Energy Supply on Urinary Purine Derivative and milk Allantoin excretion in Holstein/Friesian cows.

K.J.Shingfield, N.W. Offer and Sword, A.M. (1995).

Urinary purine derivative (PD) has been suggested as a non-invasive index of microbial protein, but widescale adoption of this technique is compromised by the requirement for a total urine collection. Purine derivatives are also secreted in the milk, mainly as allantoin. This experiment evaluated milk allantoin as a potential index of microbial protein. Twelve mid lactation cows received a 14.3 kgDM/d basal diet consisting of (g/kgDM) barley straw (415), soya (322), sugar beet pulp (197), molasses (33), urea (17) and vitamins and minerals (17) for a 21 day co-variance period. During four 16 day periods, six dietary supplements allocated randomly to cows and consisting of potato starch (1, 2 or 3 kgDM/d) or calcium palmitate (0.64, 1.27 or 1.91 kgDM/d), were evaluated. Daily urinary PD excretion, milk yield and the concentrations of fat, lactose, protein, urea-N and allantoin were measured during the last two days of each period. Urinary PD excretion was highly correlated with urinary allantoin excretion (r^2=0.99, n=133, p<0.001). Both were highly correlated to FME intake (r^2=0.69, n=6, p<0.001; r^2=0.69, n=6, P<0.001, respectively). However, there was a closer relationship between FME and either milk allantoin concentration or excretion (r^2=0.95, n=6, p<0.001; r^2=0.89, n=6, P<0.001, respectively). Milk allantoin output has potential as a microbial marker although this observation may hold only when variations in milk yield are relatively small.

Book of Abstracts of the 46th Annual meeting of the European Association for Animal Production, Wageningen Pers. **Paper N5.5.**, pp 113.

Milk allantoin excretion in relation to Fermentable Metabolisable Energy supply over a 26 to 33 kg/d range of milk yields in Holstein/Friesian dairy cows.

K.J. Shingfield and N.W. Offer (1996).

Widescale assessment of microbial protein supply by urinary purine derivative (PD) excretion in dairy cows is restricted by the necessity for a total urine collection. Attempts to utilise urinary creatinine (c) excretion as a marker of urinary output have proved to be unreliable. The current experiment evaluates the potential of milk allantoin excretion as a alternative. Four experimental diets were offered to twelve multiparous Holstein/Friesian cows in a incomplete change-over design. Diets comprised of 40 kg fresh weight (F.wt) silage (307 g/kg toluene corrected dry matter (TDM), 149 g/kg CP and 12.4 MJ/kg ME) supplemented with 4.1 (L1), and 8.1 (L2) kg F.wt of a low fat concentrate (DM, CP and acid hydrolysed ether extract, AH-EE of 860, 200 and 45 g/kg, respectively) or 3.8 (H1) and 7.5 (H2) kg F.wt of a high fat concentrate (880g/kg DM, 235g/kg CP and 110g/kg AH-EE). Milk recording, milk sampling and total urine collections were performed during the last three days of each experimental period. Subsamples of milk and urine were immediately stored at -20^{0}C prior to HPLC analysis. Individual milk yields varied between 16.8-46.8 kg/d. Based on individual cow measurements, urinary PD excretion was more closely correlated with estimated FME intake ($r=0.452$, $n=36$, $P<0.01$) than milk allantoin excretion ($r=0.356$, $n=36$, $P<0.05$) or milk allantoin concentration weighted by milk yield ($r=0.086$, $n=36$, $P=0.619$). The use of group mean values for each diet dramatically improved the correlation between FME intake and urinary PD excretion ($r=0.988$, $n=4$, $P<0.05$), FME and milk allantoin excretion ($r=0.935$, $n=4$, $P=0.065$) and FME and weighted mean allantoin concentration ($r=0.964$, $n=4$, $P<0.05$). The variability of milk allantoin output precludes its use for individual cows. In studies with large groups of cows, milk allantoin excretion or mean concentration are potential alternatives to urinary PD excretion, as a means of estimating microbial protein supply.

Book of Summaries, British Society of Animal Science, Winter meeting, **Paper 90.**

Appendix

Appendix 1

Herbage and barley chemical analysis (g/kg DM, unless stated)

Parameter	Herbage	Barley
DM (g/kg)	139	842
OM	895	973
CP	182	115
In-vitro D	682	-
NDF	415	-
ADF	247	-
NCGD	-	875
AH-EE	-	36
Starch	-	520
ME (MJ/kg)	11.7	13.0

Appendix 2

Silage chemical composition (g/kg oven DM, unless stated)

Parameter	Period 1	Period 2	Mean
Oven DM (g/kg F.wt)	353	243	298
Corrected DM (g/kg F.wt)	376	265	321
OM	917	917	917
OMD	772	756	764
CP	189	170	180
pH	3.8	4.0	3.9
NH_4-N/ Total-N (g/kg)	97	108	103
AH-EE	54.1	52.4	53.3
Starch	15	28	22
ME (MJ/kg)	11.2	11.0	11.1
ME (MJ/kg CDM)	12.4	12.3	12.4
FME (MJ/kg CDM)	8.69	8.70	8.7

Appendix 3

Concentrate formulation on a air-dry basis (g/kg)

Raw material	Inclusion (g/kg)
Wheat	200.0
White fishmeal	40.0
Extracted soyabean meal (hipro)	10.5
Extracted sunflower meal	100.0
Extracted rapeseed meal	100.0
Wheat distillers dark grains	125.0
Maize gluten	89.4
Malt by-product	58.0
Molassed sugar beet pulp	150.0
Sprayer fat	5.0
Protected fat	50.0
Molasses	60.0
Limestone	1.4
Calcined magnesite	3.4
Salt	4.8
Minerals and vitamins	2.5

Appendix 4

Concentrate chemical composition (g/kg oven DM, unless stated)

Parameter	Period 1	Period 2	Mean
Oven DM (g/kg F.wt)	869	882	876
OM	906	914	910
CP	202	212	207
NCGD	775	788	782
AH-EE	97.2	94.2	95.7
EE	81.7	81.2	81.5
Starch	107	118	113
ME (MJ/kg) (E3)	13.3	13.4	13.4
FME (MJ/kg DM)*	10.2	10.1	10.2

*calculated as ME -ME_{fat} (AH-EE (kg) x 33.0 MJ/kg, AFRC, 1992)

Appendix 5

Complete diet chemical composition (g/kg oven DM, unless stated)

Parameter	Period 1	Period 2	Mean
Oven DM (g/kg F.wt)	396	329	363
Corrected DM (g/kg F.wt)[1]	416	345	381
OM	921	917	919
CP	180	175	178
pH	4.1	4.3	4.2
NH_4 -N/ Total N (g/kg)	81	104	92.5
AH-EE	69.8	61.4	65.6
Starch	49.0	48.0	48.5
ME (MJ/kg CDM)[1]	12.47	12.44	12.46
FME (MJ/kg CDM)[1]	8.81	8.88	8.85

[1] Not measured but calculated from complete diet silage:concentrate fresh weight ratio

Appendix 6

High phosphorus cattle mineral and vitamin supplement composition

Compound	Inclusion rate /kg F.Wt
Calcium	170g
Phosphorus	100g
Magnesium	30g
Sodium	97.5g
Copper (as cupric sulphate)	1.6g
Cobalt (as cobaltous sulphate)	100mg
Iodine (as potassium iodide)	120mg
Iron (as ferric oxide)	1.25g
Manganese (as manganous oxide)	3.0g
Zinc (as zinc oxide)	3.0g
Selenium (as sodium selenite)	12mg
Vitamin A	300,000 IU
Vitamin D3	60,000 IU
Vitamin E	400 IU

Appendix 7

Basal diet formulation

Ingredient	F.Wt (kg/d)	DM (kg/d)	Inclusion (g/kg DM)
Chopped barley straw	7.00	5.95	415
Soyabean meal	5.10	4.62	322
Molassed sugar beet pulp	3.20	2.82	197
Molasses	0.63	0.47	33
Water	0.63	-	-
Mineral and vitamins	0.25	0.24	17
Urea	0.25	0.24	17
Total	17.10	14.34	1000

Appendix 8

Chemical analysis of the experimental diet components

(on a g/kg DM basis, unless otherwise stated)

Analysis	Ingredient			
	Barley Straw	Concentrate	Potato starch	Megalac
DM g/kg F.Wt	841	790	813	963
OM	943	900	996	743
CP	30	379	-	-
NCGD		863	-	-
DOMD	369	-	-	-
AH-EE	15.0	24.4		704
Starch	-	-	984	-
ME (MJ/kg)	5.9[1]	12.7[2]	14.9[3]	28.8[4]
FME (MJ/kg)	5.43	12.73	14.9	0.0

[1] Estimated as 0.16 x DOMD (Thomas and Chamberlain, 1982)
[2] Calculated using equation E3 (Thomas *et al*, 1988)
[3] calculated from first principles (refer to appendix 11)
[4] calculated from first principles (refer to appendix 11)

Appendix 9

Sheep mineral and vitamin supplement composition

Compound	Inclusion /kg F.wt
Calcium	190 g
Phosphorus	40 g
Sodium	80 g
Magnesium	50 g
Selenium (as sodium selenite)	15 mg
Iron	1500 mg
Manganese	4800 mg
Cobalt	200 mg
Iodine	200 mg
Zinc	3000 mg
Vitamin A	400,000
Vitamin D3	80,000
Vitamin E (as Alpha Tocopherol)	500

Appendix 10

Basal diet composition

Ingredient	g/kg F.Wt	g/kg DM
Chopped barley straw	400	340
Soyabean meal	304	275
Molassed SBP shreds	191	168
Molasses	37.6	28.2
Water	37.6	-
Minerals and Vitamins	14.9	14.2
Urea	14.9	14.2
Total	1000	840

Appendix 11

Calculation of potato starch ME (MJ/kg)

Assumptions:-

Starch G.E. [1]	= 17.7 MJ/kg
Energy lost through methane production [1]	= 1.1 MJ/kg
Energy lost in the urine [1]	= 0.8 MJ/kg
Starch digestibility [2]	= 950 g/kg
Starch D.E.	= 17.7 x 0.95
	= 16.8 MJ/kg
Starch M.E.	= 16.8 - (1.1 + 0.8)
	= 14.9 MJ/kg

Calculation of Megalac ME (MJ/kg)

Assumptions:-

Fat G.E. [1]	= 39.0 MJ/kg
Fat content of protected fat [2]	= 850 g/kg
Fat digestibility [2]	= 950 g/kg
Energy lost via urinary and methane losses [2]	= 1.0 MJ/kg
D.E. of protected fat	= 39 x 0.850 x 0.900
	= 29.8 MJ/kg
M.E. of protected fat	= 29.8 - (1.0)
	= 28.8 MJ/kg

[1] Data derived from M^{c}Donald *et al* (1981)

[2] N.W. Offer (personal communication)

Appendix 12

Rumen and hindgut liquid and solid outflow rates measured in individual cows as described in chapter 7 are presented in Tables A.1.-A.3. Measured faecal cobalt and chromium concentrations were fitted to the model of Grovum and Williams (1973) according to the procedure described in chapter 4. The goodness of model fit is described by correlation coefficients (measured compared to predicted values) and weighted variances for solid and liquid *k1* and *k2* values respectively.

Table A.1. Derived estimates of rumen (*k1*) and hindgut (*k2*) solid and liquid outflow rates

No.	Experimental period	Solid outflow (%/hr)					Liquid outflow rate (%/hr)				
		Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var	Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var
33	Co-variance	8.63	.993	13.3	.975	4.67	10.5	.862	19.7	.905	6.26
33	1	7.62	.986	16.7	.918	4.31	6.19	.929	13.4	.932	6.38
33	2	7.69	.985	17.9	.956	5.10	7.69	.985	18.1	.952	4.03
33	3	5.21	.975	11.9	.925	4.47	6.47	.997	7.12	.999	5.71
33	4	4.57	.963	11.1	.945	3.48	9.26	.988	17.6	.856	2.39
71	Co-variance	2.68	.951	10.0	.853	4.04	5.17	.990	5.56	.999	5.84
71	1	5.92	.935	10.1	.977	5.81	6.31	.973	25.1	.906	2.86
71	2	4.11	.969	9.08	.933	4.64	5.17	.989	13.1	.969	2.39
71	3	3.51	.980	10.2	.900	4.03	5.34	.982	12.7	.922	3.13
71	4	3.29	.974	7.04	.976	4.19	4.34	.975	25.3	.922	3.01
127	Co-variance	8.23	.927	10.1	.994	7.64	6.53	.933	18.0	.913	1.69
127	1	3.93	.942	6.52	.954	3.97	4.98	.973	11.63	.982	1.99
127	2	6.72	.990	9.51	.986	6.33	6.81	.989	17.9	.931	2.31
127	3	4.66	.971	7.62	.976	5.70	6.07	.985	14.7	.969	2.49
127	4	4.43	.982	10.5	.931	3.88	6.81	.978	11.2	.955	2.42
139	Co-variance	8.56	.991	11.1	.991	3.22	7.38	.989	13.2	.982	2.95
139	1	4.59	.984	8.95	.959	3.37	6.36	.975	15.6	.982	1.63
139	2	5.96	.990	11.3	.945	3.89	7.28	.988	14.9	.965	3.03
139	3	3.81	.957	9.61	.844	3.19	5.86	.978	9.83	.961	8.23
139	4	3.89	.985	8.77	.952	3.16	6.55	.989	11.5	.953	6.68

Table A.2. Derived estimates of rumen (*k1*) and hindgut (*k2*) solid and liquid outflow rates

Cow No.	Experimental period	Solid outflow (%/hr)					Liquid outflow rate (%/hr)				
		Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var	Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var
140	Co-variance	5.09	.957	9.27	.964	4.63	6.64	.989	12.3	.911	3.07
140	1	8.64	.894	12.3	.992	7.39	7.04	.929	10.7	.952	7.28
140	2	7.05	.986	9.36	.978	7.29	7.03	.978	11.2	.967	5.54
140	3	7.71	.997	10.6	.991	4.23	7.87	.985	32.9	.872	2.45
140	4	5.61	.979	10.3	.955	4.93	6.94	.989	39.1	.880	2.92
186	Co-variance	5.43	.976	13.1	.918	3.23	6.16	.978	16.6	.909	3.22
186	1	6.86	.884	11.3	.975	6.22	5.87	.992	10.4	.954	6.65
186	2	3.75	.880	7.71	.922	5.16	5.72	.975	17.6	.904	2.50
186	3	2.98	.976	8.29	.909	3.77	6.00	.974	11.0	.953	6.74
186	4	3.91	.955	13.4	.939	4.18	5.13	.979	7.35	.981	2.77
195	Co-variance	3.67	.954	9.40	.947	4.47	6.01	.985	14.9	.959	2.76
195	1	6.21	.823	13.1	.874	3.31	7.60	.993	14.9	.979	1.87
195	2	7.63	.936	9.86	.984	6.64	6.44	.985	17.5	.971	2.67
195	3	3.18	.951	7.68	.936	3.73	6.05	.969	16.2	.982	2.34
195	4	3.49	.975	7.35	.910	5.20	5.70	.992	24.4	.886	2.16
246	Co-variance	7.05	.973	11.5	.982	2.47	6.76	.976	14.0	.948	4.34
246	1	7.04	.998	11.3	.981	2.60	6.83	.974	17.4	.968	2.37
246	2	7.82	.975	11.6	.977	4.33	6.45	.997	20.7	.952	1.99
246	3	5.86	.959	12.6	.977	3.70	7.41	.984	16.3	.910	5.28
246	4	5.85	.974	17.0	.926	4.28	7.21	.990	31.0	.746	3.19

Table A.3. Derived estimates of rumen (*k1*) and hindgut (*k2*) solid and liquid outflow rates

No.	Experimental period	Solid outflow (%/hr)					Liquid outflow rate (%/hr)				
		Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var	Rumen (*k1*)	r	Hindgut (*k2*)	r	Wt. Var
255	Co-variance	6.21	.926	11.7	.972	3.83	7.57	.977	15.6	.988	2.34
255	1	4.90	.978	8.43	.966	5.55	6.33	.985	16.0	.987	1.93
255	2	4.69	.915	8.33	.981	2.74	5.24	.971	16.6	.942	1.79
255	3	7.21	.990	30.8	.760	2.45	5.32	.998	10.7	.979	4.07
255	4	3.00	.881	15.7	.945	2.74	5.57	.972	27.7	.877	2.42
270	Co-variance	4.77	.917	9.35	.951	4.58	5.75	.985	13.3	.927	3.39
270	1	4.93	.933	10.4	.962	4.90	7.10	.995	12.8	.953	4.80
270	2	5.70	.961	10.9	.967	4.44	6.66	.971	14.8	.931	3.57
270	3	4.14	.935	10.5	.939	4.27	5.82	.966	15.7	.910	3.44
270	4	*	*	*	*	*	*	*	*	*	*
273	Co-variance	6.41	.882	10.4	.972	8.17	7.47	.981	25.1	.945	1.61
273	1	7.12	.993	12.1	.945	4.48	7.46	.975	17.2	.938	2.74
273	2	9.29	.984	12.9	.988	5.09	6.54	.987	19.5	.834	2.37
273	3	5.50	.966	9.38	.974	3.28	6.01	.993	11.3	.947	3.78
273	4	7.16	.982	23.3	.891	2.81	3.58	.995	12.3	.929	3.37
285	Co-variance	5.40	.929	9.97	.969	4.67	6.66	.981	15.6	.924	2.24
285	1	5.57	.948	10.6	.981	4.16	6.20	.989	18.2	.900	2.63
285	2	6.70	.987	10.3	.986	3.66	7.88	.985	14.9	.998	1.53
285	3	3.58	.995	12.3	.929	3.37	6.34	.991	31.8	.861	2.19
285	4	3.53	.989	12.3	.917	3.71	6.00	.985	13.3	.907	5.30

Appendix 13

Silage composition (expressed as g/kg corrected DM (CDM), unless otherwise stated)

Parameter	Period 1	Period 2	Period 3	Mean
Oven DM (g/kg F.wt)	275	283	286	281
CDM (g/kg F.wt)	299	309	313	307
OM	940	932	932	935
OMD	773	770	775	773
ME (MJ/kg CDM)	12.4	12.3	12.4	12.4
FME/ME	0.71	0.65	0.62	0.66
FME (MJ/kg CDM)	8.8	8.0	7.69	8.16
uFME (MJ/kg CDM)	3.64	4.34	4.77	4.25
CP	147.9	144.7	154.2	148.9
Protein degradability	0.82	0.82	0.85	0.83
Ammonia-N (g/kg TN)	81.0	77.6	60.1	72.9
Soluble N	13.9	14.4	14.7	14.3
Amino acid N (g/kg F.Wt)	3.16	3.39	3.54	3.36
pH	3.9	3.7	3.7	3.8
NV[1]	250.3	344.7	389.0	328.0
Residual sugar	106.1	116.1	84.3	102.2
Lactic acid (g/kg F.Wt)	36.0	54.0	47.1	45.7
VFA (g/kg F.Wt)	6.43	5.54	4.95	5.64

[1] NV (Neutralising value from standing pH to pH 6.5, meq/kg F.Wt)
uFME denotes un-fermentable ME

Appendix 14

Concentrate chemical composition (g/kg DM, unless specified)

Parameter	Low fat (L)	High fat (H)
DM (g/kg F.Wt)	863	879
CP	203	235
AH-EE	44.9	109
NCGD	774	774
ME (MJ/kg)[1]	12.0	13.6
FME (MJ/kg)[2]	10.5	10.0

Results presented are the mean of six measurements
[1] calculated using equation E3 (Thomas *et al*, 1988)
[2] calculated as ME-ME $_{fat}$
ME $_{fat}$ calculated as AH-EE (g/kg DM) x ME $_{fat}$
ME $_{fat}$ assumed to be 33.0 MJ/kg DM (AFRC, 1992)

Appendix 15

Concentrate formulations (g/kg F.wt)

Ingredient	High fat (H)	Low fat (L)
Wheat feed	150	150
Wheat	50	190
Extracted rapeseed meal	150	110
Extracted sunflower meal	150	110
Extracted soyabean meal (Hipro)	85	65
Maize-gluten	150	150
Citrus pulp	50	75
Molasses	95	100
Vegetable fat	75	5
Limestone	25	25
Calcined magnesite	4	4
Salt	10	10
Minerals and vitamins	6	6