THE INFLUENCE OF DIET AND INTAKE LEVEL ON HEPATIC AMMONIA METABOLISM AND UREAGENESIS BY THE OVINE LIVER

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THE INFLUENCE OF DIET AND INTAKE LEVEL ON HEPATIC AMMONIA METABOLISM AND UREAGENESIS BY THE OVINE LIVER

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ABSTRACT

The New Zealand agricultural industry is based on the efficient utilisation of fresh forages, a characteristic of which is a high soluble protein content. A large proportion of the ingested protein is highly soluble in the rumen. A significant proportion of the ingested N is removed from the rumen as ammonia with the bulk of this ammonia being removed from the venous blood by the liver for detoxification to urea. Hepatic urea-N production, or ureagenesis, typically exceeds the rate of hepatic ammonia-N extraction, consequently it has been suggested that the shortfall in N required for ureagenesis is contributed by amino acid-N (Parker *et al.* 1995; Lobley *et al.*, 1995).

This study tested the hypothesis that elevated hepatic ammonia extraction would require a concomitant increase in hepatic amino acid catabolism to supply the additional N required for ureagenesis.

In order to evaluate the level of rumen ammonia production and consequently the rates of hepatic ammonia extraction, ureagenesis and amino acid catabolism, the following feeding regimens were tested in sheep held indoors in metabolism crates in three separate experiments; Firstly, lucerne pellets (*Medicago sativa*) were compared with fresh white clover (*Trifolium repens)*, secondly fresh white clover was offered at either a low or high intake and finally the daily allowance of fresh white clover was fed in two 2 hour periods per day.

In each experiment, silicone based catheters were surgically inserted into the posterior aorta and the mesenteric (2), portal and hepatic veins. Following a ten day dietary adjustment period and a ten day nitrogen balance, the sheep were infused with *para*-aminohippurate (*p*AH) and $^{15}NH_4Cl$ *via* the mesenteric vein. The *p*AH was infused to allow the blood flow across the splanchnic tissues to be estimated, whilst the $^{15}NH_4Cl$ was infused to trace hepatic ammonia metabolism to urea. Blood samples were collected to determine the ammonia, urea, oxygen and amino acid concentrations in the mesenteric, portal and hepatic veins, as well as the posterior aorta.

Despite similar DM intakes, the nitrogen intake of the sheep fed fresh white clover was 60% higher ($P < 0.001$) than that of the same animals fed lucerne

pellets. The difference in rumen protein fermentation in these two contrasting diets resulted in higher ($P < 0.001$) rumen ammonia production in the animals offered fresh white clover. There was, however, only a trend ($P = 0.072$) toward elevated hepatic ammonia extraction in these animals and urea production was not significantly different to the animals fed lucerne pellets. Hepatic amino catabolism was not elevated in the sheep fed fresh white clover, nor was there a significant difference in the proportion of ME intake that was utilised for ureagenesis between the two groups.

In the second experiment the DM intakes of the two groups were different ($P < 0.001$), with the sheep offered the low intake of fresh white clover consuming 807 g DM/d whilst the high intake group consumed 1118 g DM/d. Even with these differences in intake, portal vein ammonia and urea concentrations were similar. Therefore the rate of hepatic ammonia extraction and urea production were also similar between the two intake groups. However, hepatic extraction of ^{15}N-ammonia was higher ($P = 0.033$) in the high intake group compared to the low intake group. There was no evidence to suggest that the level of hepatic amino acid catabolism increased with intake level, consequently the proportion of ME intake attributed to urea synthesis was similar for the two intake groups.

When the experimental animals were restricted to two 2 hour feeding periods per day the DM and N intake decreased by 31% from that of the low intake group in the second experiment. There was no significant effect of time after the onset of feeding on portal ammonia or urea concentrations, hepatic ammonia extraction or hepatic urea production. However portal ammonia concentration and consequently hepatic ammonia extraction and urea production tended to be higher 4-6 hours after ingestion of fresh white clover. However this trend was not observed when the ^{15}N tracer data was used to calculate the hepatic ammonia transfer rate. The ammonia, urea and amino acid hepatic transfer values in this experiment were largely comparable to those recorded for the low and high intake treatments in the second experiment.

In these studies, there was no evidence of elevated hepatic amino acid catabolism occurring in response to elevated rates of hepatic ammonia extraction and hence ureagenesis. Additionally there was no suggestion that ammonia provided both of the N atoms of the urea molecule.

It is concluded that the liver adapted to the changes in dietary nitrogen supply without incurring significant increases in the metabolic cost of ammonia detoxification to urea. However the nutritional challenges presented to the liver may not have been severe enough to induce measurable changes in hepatic ammonia metabolism. A possible mechanism to account for these observations may be that the liver adapted to the changes in nitrogen supply by altering the activity of the primary regulator of the rate of ureagenesis, carbamoyl phosphate synthetase (CPS*I*).

THIS THESIS IS DEDICATED TO MY GREAT GRANDMOTHER
FLOSSY HOLYOAKE

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TABLE OF CONTENTS

1 CHAPTER 1. INTRODUCTION 1

1.1 General introduction 1

1.2 Objectives 1

2 CHAPTER 2. A REVIEW OF LITERATURE 3

2.1 Introduction 3

2.2 Plant protein structure 4

2.3 Plant protein content 5

2.4 Ruminant digestion 5
2.4.1 The contribution of the resident microbes to digestion 6
2.4.2 Carbohydrate digestion 7
2.4.3 Protein digestion 8
2.4.3.1 Rumen protein digestion 8
2.4.3.2 Intestinal protein digestion 11

2.5 Metabolite transport in the blood 12
2.5.1 The major blood vessels of the splanchnic region 13
2.5.2 Blood flows within the splanchnic region 15

2.6 Techniques for estimating the rate of metabolism 15
2.6.1 Methods used to estimate blood flow 16
2.6.2 The use of isotopes to estimate amino acid transfer rates 18
2.6.3 Techniques used to estimate the rate of protein synthesis 19
2.6.3.1 The arterio-venous difference technique 20
2.6.3.2 The large dose technique 22
2.6.4 Estimation of the rate of whole body protein synthesis 23
2.6.4.1 Estimation of the rate of hepatic protein synthesis 23

2.7 Liver metabolism 24
2.7.1 Liver structure 24
2.7.1.1 Acinus structure 27

2.8 Liver function 28
2.8.1 Periportal and perivenous hepatocyte functionality 28
2.8.2 Hepatic amino acid metabolism 29
2.8.3 Carbohydrate metabolism 31
2.8.4 Lipid metabolism 32
2.8.5 Mediators of liver metabolism 32
2.8.6 Nitrogen metabolism in the liver 33
2.8.6.1 The urea cycle 33
2.8.6.2 The function of ureagenesis 34
2.8.6.3 Spatial arrangement of the urea cycle 36
2.8.6.4 Amino acid requirement for ureagenesis 37
2.8.6.5 Pathways for nitrogen to enter the urea cycle 38
2.8.6.5.1 The first N of the urea molecule 38
2.8.6.5.2 The second N of the urea molecule 41
2.8.6.6 Regulation of ureagenesis 41
2.8.6.7 The energetic cost of ureagenesis 44
2.8.6.8 Variations in hepatic ammonia load 45
2.8.6.9 Variations in hepatic amino acid supply 46
2.8.6.10 Comparison with in vitro work 47

2.8.6.11 Ureagenesis versus glutamine synthetase activity 48
2.8.6.12 The inter-relationship between the urea and citric acid cycles 50

2.9 Conclusion 51
2.9.1 Objectives 52

3 CHAPTER 3. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) OR LUCERNE (*MEDICAGO SATIVA*) PELLETS 53

3.1 Abstract 53

3.2 Introduction 54

3.3 Materials and methods 55
3.3.1 Animals and surgical procedures 55
3.3.2 Diets and design 56
3.3.3 Infusion protocol 56
3.3.4 Analytical measurements 57
3.3.5 Calculations and statistics 59

3.4 Results 63
3.4.1 Feed intake and body weight gains 63
3.4.2 Blood flows and oxygen exchange 63
3.4.3 Ammonia and urea transfers 64
3.4.4 Amino acid transfers 66
3.4.5 Phenylalanine and methionine kinetics 69
3.4.6 ^{15}N transfers 69

3.5 Discussion 72
3.5.1 Hepatic ammonia metabolism 73
3.5.2 Urea metabolism 73
3.5.3 ^{15}N metabolism 74
3.5.4 Amino acid metabolism 75
3.5.5 Whole-body and hepatic protein metabolism 76
3.5.6 Energy metabolism 77

3.6 Conclusion 78

4 CHAPTER 4. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) AT TWO LEVELS OF INTAKE 79

4.1 Abstract 79

4.2 Introduction 80

4.3 Materials and methods 81
4.3.1 Animals and surgical procedures 81
4.3.2 Diets and design 81
4.3.3 Infusion protocol 82
4.3.4 Analytical measurements 82
4.3.5 Calculations and statistics 84

4.4 Results 85
4.4.1 Feed intake and body weight gains 85
4.4.2 Blood flows and oxygen exchange 85
4.4.3 Ammonia and urea transfers 86

4.4.4 Amino acid transfers 88
4.4.5 Cysteine kinetics 90
4.4.6 ^{15}N transfers 91

4.5 Discussion 92
4.5.1 Intake parameters 92
4.5.2 Blood flows 93
4.5.3 Hepatic ammonia metabolism 93
4.5.4 Amino acid metabolism 94
4.5.5 Urea metabolism 96
4.5.6 Energy metabolism 96
4.5.7 Whole body and hepatic protein metabolism 97

4.6 Conclusion 97

5 CHAPTER 5. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) IN TWO 2 HOUR PERIODS PER DAY 99

5.1 Abstract 99

5.2 Introduction 100

5.3 Materials and methods 101
5.3.1 Animals and surgical procedures 101
5.3.2 Diets and design 101
5.3.3 Infusion protocol 102
5.3.4 Analytical measurements 102
5.3.5 Terminal procedures 103
5.3.6 Calculations and statistics 104

5.4 Results 105
5.4.1 Feed intakes 105
5.4.2 Blood flows and oxygen exchange 105
5.4.3 Ammonia and urea transfers 105
5.4.4 Amino acid transfers 110
5.4.5 ^{15}N transfers 114

5.5 Discussion 116
5.5.1 Intake parameters 116
5.5.2 Blood flows 117
5.5.3 Hepatic ammonia metabolism 117
5.5.4 Amino acid metabolism 118
5.5.5 Urea metabolism 119
5.5.6 Energy metabolism 119

5.6 Conclusion 120

6 CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS 121

6.1 Introduction 121

6.2 Methodological considerations 123
6.2.1 Surgical methodology 123
6.2.2 The arterio-venous difference technique 123
6.2.3 Estimation of blood flow 124
6.2.4 Tracer methodology 125

6.3 Ammonia detoxification 125

6.4 Sources of N for ureagenesis 126

6.5 The energetic cost of ureagenesis 128

6.6 The influence of acidosis on hepatic N metabolism 128

6.7 Hepatic adaptation 129

6.8 Conclusions 129

6.9 Future work 130

7 CHAPTER 7. REFERENCES 133

8 CHAPTER 8. APPENDICES 164

8.1 Supplemental tables 164

8.2 Supplemental materials and methods 169

8.2.1 Surgical procedures 169
8.2.2 Catheter construction 169
8.2.3 $^{15}NH_4Cl$ infusion 170
8.2.4 Blood flow estimation 171
8.2.5 HPLC method 172
8.2.6 GCMS method 172
8.2.7 Ammonia method 174
8.2.8 Urea method 174

LIST OF TABLES

Table 3.1. Blood flow (g/min) and oxygen consumption (mmol/min) across the mesenteric drained viscera (MDV), portal drained viscera (PDV), rumen and the liver of sheep fed lucerne pellets (L) or fresh white clover (WC)..... 64

Table 3.2. Ammonia and urea concentrations and transfers across the mesenteric drained viscera (MDV), portal drained viscera (PDV), rumen and the liver of sheep fed fresh white clover (WC) or lucerne pellets (L)..... 65

Table 3.3. Summary of nitrogen (N) fluxes in sheep fed fresh white clover (WC) or lucerne pellets (L)..... 66

Table 3.4. Amino acid transfers (μmol/min) across the portal drained viscera (PDV) and liver and fractional removal of amino acids by the liver in sheep fed fresh white clover (WC) or lucerne pellets (L)..... 68

Table 3.5. Methionine and phenylalanine whole body and liver kinetics and protein metabolism in sheep fed fresh white clover (WC) or lucerne pellets (L)..... 70

Table 3.6. Hepatic ^{15}N transfers in sheep fed fresh white clover (WC) or lucerne pellets (L)..... 71

Table 4.1 The dry matter (DM) and nitrogen (N) intake and digestibility of fresh white clover offered at low or high intakes..... 85

Table 4.2. Blood flow (g/min) and oxygen consumption (mmol/min) across the portal drained viscera (PDV) and the liver of sheep fed fresh white clover at either low or high intake levels..... 86

Table 4.3. Ammonia and urea transfers across the portal drained viscera (PDV) and the liver of sheep fed fresh white clover at either low or high intake levels..... 87

Table 4.4. Summary of nitrogen (N) fluxes in sheep fed fresh white clover at either low or high intake levels..... 88

Table 4.5. Amino acid transfers (μmol/min) across the portal drained viscera (PDV) and liver, and fractional removal of amino acids by the liver in sheep fed fresh white clover at either low or high intake levels..... 89

Table 4.6. Cysteine whole body and liver kinetics, and protein metabolism in sheep fed fresh white clover at either low or high intake levels.....90

Table 4.7. Hepatic ^{15}N transfers in sheep fed fresh white clover at either low or high intake levels.....91

Table 5.1. Ammonia concentrations and transfers in sheep offered white clover in two 2 hour periods per day.....108

Table 5.2. Summary of nitrogen (N) fluxes in sheep offered white clover in two 2 hour periods per day.....109

Table 5.3. PDV amino acid transfers (μmol/min) in sheep offered fresh white clover in two 2 hour periods per day.....111

Table 5.4. Hepatic amino acid transfers (μmol/min) in sheep offered fresh white clover in two 2 hour periods per day.....112

Table 5.5. Fractional hepatic amino acid transfers in sheep offered fresh white clover in two 2 hour periods per day.....113

Table 5.6. Hepatic ^{15}N fluxes in sheep offered white clover in 2 two hour periods per day.....115

Table 8.1. Blood flows and oxygen transfers in sheep offered fresh white clover in two 2 hour periods per day.....164

Table 8.2. Ammonia concentrations and transfers in sheep offered white clover in two 2 hour periods per day.....165

Table 8.3. Urea concentrations and transfers in sheep offered fresh white clover in two 2 hour periods per day.....166

Table 8.4. Hepatic nitrogen (N) transfers in sheep offered white clover in two 2 hour periods per day.....167

Table 8.5. Summary of nitrogen (N) fluxes in sheep offered white clover in two 2 hour periods per day.....168

LIST OF FIGURES

Figure 1.1 The major organs and veins of the splanchnic region.....14

Figure 1.2 The portal triad and the liver ultra-structure.....26

Figure 1.3 The urea cycle.....35

Figure 3.1 Changes in arterial urea atoms % excess during infusion of $^{15}NH_4Cl$ into the mesenteric vein of sheep fed fresh white clover or lucerne pellets.....71

Figure 5.1 Blood flow in the portal and hepatic veins and hepatic artery in 6 sheep fed fresh white clover for two 2 hour periods per day.....106

Figure 5.2 Oxygen transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day.....106

Figure 5.3 Ammonia transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day.....107

Figure 5.4 Urea transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day.....107

Figure 5.5 Hepatic glutamine transfers and glutamine synthetase activity in sheep fed fresh white clover for two 2 hour periods per day.....110

Figure 5.6 Arterial ^{15}N-urea enrichment in 6 sheep fed fresh white clover for two 2 hour periods per day.....114

Figure 5.7 Hepatic ^{15}N-ammonia and ^{15}N-urea transfers in 6 sheep fed fresh white clover for two 2 hour periods per day.....114

LIST OF ABBREVIATIONS

AA	Amino acids
Ala	Alanine
APE	Atoms percent enrichment
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine tri-phosphate
AV	Arterio-venous difference
BF	Blood flow
BF_a	Blood flow in the hepatic artery
BF_h	Blood flow in the hepatic vein
BF_m	Blood flow in the mesenteric vein
BF_p	Blood flow in the portal vein
bwt	Body weight
C_a	Concentration *p*AH in the posterior aorta
C_h	Concentration *p*AH in the hepatic vein
Cit	Citrate
cm	Centimetre
C_p	Concentration *p*AH in the portal vein
CPS*I*	Carbamoyl-phosphate synthetase *I*
Cys	Cysteine
d	Day
DM	Dry matter
DTT	Dithiothreitol
E_a	APE in the posterior aorta
EC	European commission
EDTA	Ethylenediaminetetra-acetic acid
E_h	APE in the hepatic vein
E_p	APE in the portal vein
FHE	Fractional hepatic extraction rate
$Flow_h$	Blood flow in the hepatic vein
$Flow_p$	Blood flow in the portal vein
FSR	Fractional synthesis rate

g	Gram
G	Gravity
GCMS	Gas chromatography-mass spectrometry
GIT	Gastro-intestinal tract
GLDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GTP	Guanosine tri-phosphate
h	Hour
$H^{15}NT$	Hepatic ^{15}N transfer
Hepatic extraction $rate_h$	FHE based on venous SRA
Hepatic extraction $rate_{ha}$	FHE based on arterial SRA
His	Histidine
HMT	Hepatic mass transfer
HPLC	High performance liquid chromatography
I_r	Infusion rate of the *p*AH
ID	Internal diameter
Ile	Isoleucine
ILR	Irreversible loss rate
IR	Infusion rate
IU	International units
kg	Kilo gram
kJ	Kilo joule
l	Litre
IR_a	SRA infusion rate
L	Lucerne pellets
Leu	Leucine
Lys	Lysine
M	Maintenance level of intake
MBq	Mega becquerel
MDV	Mesenteric drained viscera
ME	Metabolisable energy
Met	Methionine

mg	Milligram
min	Minute
MJ	Mega joule
ml	Millilitre
μl	Microlitre
mm	Millimetre
mmol	Millimole
μm	Micrometer
μmol	Micro mole
mol/l	Mole per litre
mRNA	Messenger ribo-nucleic acid
m/z	Molecular weight
N	Nitrogen
NAD(P)	Nicotinamide adenine dinucleotide phosphate
$NAD(P)^+$	Nicotinamide adenine dinucleotide phosphate - oxidised form
NADPH	Nicotinamide adenine dinucleotide phosphate - reduced form
NAG	N-acetyl glutamate
nm	Nano-meter
nmol	Nanomole
NPN	Non-protein nitrogen
OM	Organic matter
Orn	Ornithine
P	Probability
P	Portal
*p*AH	*Para*- aminohippurate
PDV	Portal drained viscera
*p*H	Measure of acidity or alkalinity
Phe	Phenylalanine
Post. aorta	Posterior aorta
Pro	Proline
PVC	Poly-vinyl chloride
Q_a	SRA flux of in the posterior aorta

Q_h	SRA flux of in the hepatic vein
Q_p	SRA flux of in the portal vein
R^2	Correlation coefficient
RDP	Rumen degradable protein
Ser	Serine
SE	Standard error
sec	Second
SED	Standard error of the difference
SRA	Specific radio-activity
SRA_a	SRA in the posterior aorta
SRA_h	SRA in the hepatic vein
SRA_p	SRA in the portal vein
TBDMS	N-*tert*, butyldimethylsylil derivative
Thr	Threonine
tRNA	Transfer ribo-nucleic acid
Trp	Tryptophan
Tyr	Tyrosine
UDP	Undegradable protein
Val	Valine
VFA	Volatile fatty acid
WC	White clover
W:V	Weight : volume
W:W	Weight : weight
Z	Generic symbol for metabolites
Z_a	Generic metabolite concentration in the posterior aorta
Z_h	Generic metabolite concentration in the hepatic vein
Z_m	Generic metabolite concentration in the mesenteric vein
Z_p	Generic metabolite concentration in the portal vein

1 CHAPTER 1. INTRODUCTION

1.1 GENERAL INTRODUCTION

New Zealand's agricultural system is based on the efficient utilisation of fresh temperate pastures, which by virtue of the mild climate, are able to maintain a reasonable growth rate throughout the year. A characteristic of such fresh forage is a high soluble protein content (Mangan, 1982) that is poorly utilised by the ruminants grazing these pastures. Once ingested, the soluble protein is rapidly degraded in the rumen, predominantly to ammonia. Depending on the rate of production, a substantial proportion of this ammonia is incorporated into microbial protein (0.30-0.50 N intake; Huntington, 1982; Parker *et al.* 1995). If the rate of production exceeds the capacity of the microbes to utilise the ammonia, then the ammonia is either passively transported across the rumen wall into the venous blood or, to a lesser extent, passed with the digesta to the lower digestive tract (Nolan & Leng, 1972). The liver removes ammonia from the venous blood where it is metabolised into urea to maintain systemic ammonia concentrations below those toxic to the central nervous system (Remond *et al.* 1993).

The rate of hepatic ureagenesis typically exceeds the rate of ammonia extraction, leading to speculation that a significant quantity of amino acid-N is sequestered for catabolism to cover this apparent short fall in hepatic N requirements, particularly during periods of high rates of ammonia extraction (Parker *et al.,* 1995; Lobley *et al.*, 1995).

1.2 OBJECTIVES

This study tested the hypothesis that an increased rate of hepatic ammonia detoxification requires an increased rate of hepatic amino acid catabolism. Ultimately the animals productive capacity may be impaired if the losses of amino acids to hepatic catabolism were severe. The first experiment determined the rate of rumen ammonia production and the rates of hepatic ammonia extraction, ureagenesis and amino acid catabolism when the sheep were fed fresh white clover or lucerne pellets. These diets differ markedly in

protein solubility and hence, the fermentation of dietary protein to ammonia in the rumen. The second experiment sought to investigate the effect of fresh white clover intake level on the rate of rumen ammonia production and the resultant metabolism of that ammonia to urea. The third and final experiment restricted access to fresh white clover to two 2 hour periods per day and assessed the effect on the resultant pulses of rumen ammonia production and subsequent ammonia metabolism through to urea by the liver.

2 CHAPTER 2. A REVIEW OF LITERATURE

2.1 INTRODUCTION

The New Zealand agricultural industry is based on the efficient utilisation of fresh forages. Most improved New Zealand pastures are ryegrass (*Lolium perenne*) and white clover (*Trifolium repens)* mixes, with the latter used to enhance the supply of nitrogen (N) to ryegrass.

Seasonal variations in climate induce significant changes in the relative proportions of protein and carbohydrate in fresh forages. When protein content is high, for example, during the spring, the soluble carbohydrate content is relatively low, therefore there is a potential imbalance between the supply of energy from soluble carbohydrate and protein for microbial protein synthesis within the reticulo-rumen (rumen). This asynchrony is further accentuated by the fact that the soluble protein in fresh forage is rapidly digested within the rumen compared to structural carbohydrates which are more slowly digested.

During the digestion of fresh forage in the rumen, large quantities of ammonia are produced. The ammonia is absorbed into the blood and converted to urea by the liver and subsequently excreted. The process of ureagenesis requires an amino acid input, which may reduce the availability of amino acids for productive purposes.

This review will initially consider the structure of plant protein and how this affects its digestion by the ruminant. The techniques necessary to measure the rate of utilisation of absorbed nutrients will be discussed. The liver structure and function will be discussed before the process of ureagenesis is reviewed, with particular reference to the amino acid requirements for ureagenesis. Unless otherwise stated, the term ammonia refers to the sum of ammonium ions (NH_4^+) and ammonia (NH_3) free base (Haussinger, 1990; Meijer *et al.* 1990).

2.2 PLANT PROTEIN STRUCTURE

The protein content of fresh temperate forage consists of three main groups, designated fractions 1-3. The relative proportion of each is influenced by factors including the forage species, stage of growth and fertiliser applications (Mangan, 1982). The three protein groups have different nutritional values, predominantly based on degradability in the rumen and therefore their potential to provide N containing moieties for the synthesis of microbial protein (Beever, 1993).

Fraction 1 leaf protein consists of a single protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), which is the first enzyme in the photosynthetic cycle and is responsible for fixing atmospheric CO_2 (Bryant, 1964; Mangan, 1982). Chloroplasts contain approximately 75% of total leaf protein, of which approximately half is Rubisco (Mangan, 1982). Fraction 1 protein is a high biological value protein to the ruminant, although it is highly soluble (Mangan, 1982) and therefore rapidly degraded within the rumen (Nugent & Mangan, 1981).

Fraction 2 protein contributes approximately 25% of total leaf protein. It is a complex mixture of proteins predominantly derived from the chloroplasts and cytoplasm, with a lower biological value than fraction 1 protein (Mangan, 1982). Some of the components of fraction 2 contribute to the rumen by-pass fraction of ingested protein (Nugent & Mangan, 1981; Mangan, 1982).

Fraction 3 protein consists of the low biological value chloroplast membrane proteins which are composed of two main proteins; chlorophyll protein complexes 1 and 2, which together contribute approximately 70% of the chloroplast proteins (Mangan, 1982). The fate of fraction 3 within the rumen has not been investigated, although since these proteins are insoluble in water it is likely that this protein fraction predominates in the rumen by-pass protein (Mangan, 1982).

2.3 PLANT PROTEIN CONTENT

Temperate fresh forage is characterised by high crude protein (20-35%) and soluble carbohydrate contents (10-20%) with a low percentage of the less digestible structural carbohydrates (15-30%), and a low ratio of N to carbohydrate (MacRae, 1975). The composition of fresh forage leads to the suggestion that it may be a nearly ideal feed source for ruminants (MacRae, 1975).

A large proportion of the protein in fresh forage is highly soluble and is rapidly degraded within the rumen (Mangan, 1982).

2.4 RUMINANT DIGESTION

Digestion begins in the mouth where the mastication process ruptures many of the ingested plant cell membranes (Hogan & Hemsley, 1975). The process of ingesta particle size reduction is also initiated by the mastication process, effectively increasing the surface area of the plant material for digestion (Ulyatt *et al.* 1984). A large proportion (52-61%) of the soluble fraction 1 protein, Rubisco, is released during the mastication process (Reid *et al.* 1962; Bryant, 1964; Waghorn *et al.* 1989). The rumen is therefore presented with a rapid increase in soluble protein following the ingestion of fresh forage (Waghorn *et al.* 1989). The swallowing of fresh forage is accompanied by copious volumes of saliva (6-10 l/d; Waghorn & Barry, 1987).

In the rumen the ingesta is mixed with the resident microbes by the co-ordinated contractions of the rumen. The contractions also assist the passage of digesta to the oesophagus for regurgitation and of digesta to the subsequent compartments of the gastro-intestinal tract (GIT) (Phillipson, 1977). The probability of a feed particle leaving the rumen is greatly increased when that particle reaches a critical size of 1-2 mm (Reid *et al.* 1977), however there are other factors which influence particle retention time in the rumen, such as, particle density and the level of food intake (Waghorn *et al.* 1986).

The productivity of ruminants grazing fresh forage is below theoretical expectations (Preston & Leng, 1987b; Waghorn & Barry, 1987; Lobley, 1992), predominantly due to the large rumen N losses (MacRae & Ulyatt, 1974). The relatively low efficiency of conversion of dietary N to body tissue N (typically 13%; Lobley, 1992) suggests that the digestion and assimilation of fresh forage N is less than optimum (Waghorn & Wolff, 1984).

2.4.1 The contribution of the resident microbes to digestion

Ruminants rely on a symbiotic relationship with the microbes, resident in the rumen, to effectively digest their diet (Huntington, 1986; Lobley, 1992). The rumen microbial population consists of bacteria, protozoa and fungi (Preston & Leng, 1987a), which in total number approximately 8×10^{10}/ml (Hungate, 1968; Waghorn & Barry, 1987).

Bacteria represent the largest proportion of the microbial population in the rumen. Approximately 30% of the bacterial population is suspended in the rumen liquor, with the remainder of the bacterial population attached to the ingesta particles (Hungate, 1964). Bacteria breakdown the fibrous component of the digesta, such as cellulose, hemicellulose and pectin (Hungate, 1968).

Most of the rumen protozoa are ciliates and utilise predominantly carbohydrates (Hungate, 1968). Protozoa are preferentially retained in the rumen, possibly due to their adherence to large particles of digesta and to the wall of the rumen (Preston & Leng, 1987a). Protozoa also ingest the resident bacteria, which may reduce the effectiveness of the bacterial population (Waghorn & Barry, 1987).

The hyphae of fungi penetrate and open the plant cell wall fragments allowing further digestion by themselves and the other rumen microbes (Hungate, 1964; Preston & Leng, 1987a).

2.4.2 Carbohydrate digestion

Dietary carbohydrates are the major source of energy for the ruminant (Preston & Leng, 1987a; Lindsay *et al.* 1993). They can be divided into structural or soluble carbohydrates. The ratio of structural to soluble carbohydrates varies between fresh forages, for example; perennial ryegrass 1 : 2.4-3.8 and white clover 1 : 0.9-1.7 (Ulyatt & MacRae, 1974). Structural carbohydrates are predominantly celluloses or hemicelluloses which are slowly digested in the rumen (Ulyatt *et al.* 1984). The extent of structural carbohydrate digestion is further reduced by an increase in forage lignification, which generally occurs during plant maturation (Gill *et al.* 1989). In contrast, soluble carbohydrates are rapidly degraded by the rumen microbes, producing ATP (Isaacson *et al.* 1975) and volatile fatty acids (VFA's) (Beever, 1993). Therefore the major end products of rumen carbohydrate fermentation which are available to the ruminant host are the VFA's, mainly acetate (70%), propionate (20%) and butyrate (10%), methane, CO_2 and microbial biomass (Armstrong, 1965; Brockman & Laarveld, 1986; Beever, 1993).

The VFA's are predominantly absorbed from the rumen by passive diffusion (Lindsay *et al.* 1993). Acetate and propionate undergo relatively minor modifications during the absorption process, whereas butyrate is extensively metabolised to ketone bodies, β-hydroxybutyrate, acetoacetate, acetone and isopropanol (Armstrong, 1965). The VFA's provide the ruminant with approximately 60-70% of its digestible energy (McDowell & Annison, 1991).

Any glucose available to the rumen microbes is readily fermented with the result that little or no glucose *per se* is absorbed from the GIT (Beever, 1993). Consequently the major energy sources for the fresh forage fed ruminant are the VFA's which are derived from hemicellulose and cellulose (Beever, 1993). The cellulose and hemicellulose that escape digestion in the rumen is available for digestion by the caecal bacteria (Ulyatt *et al.* 1974). Depending on the diet, 5-30% of digestible cellulose is digested in the large intestine, with the large intestine as a whole accounting for 4-26% of the ruminants energy supply (Beever *et al.* 1971; Beever *et al.* 1972; Ulyatt & MacRae, 1974).

2.4.3 Protein digestion

The digestion of protein by the resident microbes in the rumen involves two major processes: proteolysis, which involves the hydrolysis of the peptide bonds between individual amino acids and the deamination of amino acids (Tamminga, 1979). Therefore peptides, amino acids and ammonia are produced from protein digestion within the rumen.

The extent of proteolysis is influenced by the size of the microbial population involved, digesta transit time through the rumen which in turn is influenced by the solubility of the protein and the level of food intake, the presence of inhibitors and the *p*H and temperature of the rumen (Hogan & Hemsley, 1975; Tamminga, 1979).

The bulk of the resident rumen microbial population can utilise the available peptides, amino acids and ammonia to synthesise their own protein (Sutherland, 1975). However, due to insufficient energy supplied from carbohydrate fermentation, the rate of microbial degradation of the ingested protein typically exceeds the capacity of the microbes to utilise the peptides, amino acids and ammonia produced (Walker, 1964; Sutherland, 1975; Mercer *et al.* 1980). This results in a large amount of ammonia being available for absorption directly into the blood from the rumen (MacRae, 1975). Proteolysis in the rumen and the subsequent fermentation of the peptides and amino acids has long been recognised as a serious loss of ingested energy and N (Chalmers *et al.* 1954).

2.4.3.1 Rumen protein digestion

Dietary N intake can be divided into non-protein-N (NPN), rumen degradable protein (RDP) and undegradable protein (UDP) (Beever, 1993).

The efficiency of NPN digestion within the rumen varies markedly depending on the form in which it is presented to the rumen (Cottrill *et al.* 1982). The NPN

content of plants, which is 4-32% of total N, predominantly consists of the amino acids; asparagine, aspartate, glutamine, glutamate and alanine (Mangan, 1982), all of which are readily degraded producing ammonia within the rumen (Ulyatt *et al.* 1984).

Microbial digestion of the RDP produces peptides, amino acids and ammonia, all of which are utilised by the rumen microbes to synthesise protein (Ulyatt *et al.* 1984). The extent of microbial utilisation of ammonia is governed by the availability of energy (ATP) (Firkins, 1996) produced during carbohydrate digestion (Isaacson *et al.* 1975). The efficiency of microbial protein synthesis within the rumen is impaired by the asynchrony of the N and energy supply (Beever *et al.* 1986a; Ulyatt *et al.* 1988; Beever, 1993; Firkins, 1996). The efficiency of microbial protein synthesis can be increased 11-20% by synchronising the supply of both carbohydrate and N within the rumen (Beever *et al.* 1986b; Sinclair *et al.* 1995). Microbial protein passes out of the rumen with UDP and is digested in the small intestine then absorbed as peptides, amino acids and ammonia (Ulyatt *et al.* 1984; Waghorn & Barry, 1987; Webb, 1990; Beever, 1993).

However, when the rumen energy supply is limiting, ammonia is absorbed directly from the rumen into the venous blood and is transported to the liver for detoxification to urea (Preston & Leng, 1987a; Waghorn & Barry, 1987). This represents a significant loss to rumen N metabolism, with up to 40% of the N intake lost in this manner (Ulyatt *et al.* 1984; Reynolds, 1992; van der Walt, 1993). Reducing the proportion of N lost as ammonia and increasing the proportion of N intake which escapes rumen degradation increases the efficiency of conversion of ingested N to protein deposition (Waghorn & Barry, 1987; Reynolds, 1992; Ulyatt, 1997).

Examples of techniques used to protect dietary protein from rumen degradation include chemically treating the protein with, for example, formaldehyde, feeding forages with a low content (1-4% of DM) of condensed tannins (Waghorn & Barry, 1987; McNabb *et al.* 1993), increasing the supply of N to the rumen with

supplements such as urea or fish meal (Mercer *et al.* 1980) or formulating diets with less soluble proteins (Tamminga, 1979; Mangan, 1982).

Rumen energy levels can be effectively supplemented with soluble sugars such as molasses, cereal grains or highly digestible by-products such as fish meal, or pulps (Preston & Leng, 1987b).

A significant proportion (up to 60%) of the urea produced by the liver (Egan *et al.* 1986; Huntington, 1989), or 10-42% of dietary N intake (Huntington, 1986) is recycled to the GIT *via* saliva or endogenous secretions (Lindsay & Armstrong, 1982; Huntington, 1986; Huntington, 1989; Firkins, 1996). This mechanism increases the N available to the rumen microbes for protein synthesis and is one adaptive feature that allows ruminants to survive on low N diets (Preston & Leng, 1987a; Beever, 1993).

In addition to the microbial protein produced in the rumen, the undegraded protein (UDP) which escapes digestion in the rumen may be broken down in the lower portions of GIT (Beever, 1993). The UDP fraction comprises protein which is passed out of the rumen before it is completely digested and protein which is indigestible within the rumen (Preston & Leng, 1987a).

The results of mass transfer experiments (Wilton & Gill, 1988; Huntington, 1989; Maltby *et al.* 1991; Reynolds *et al.* 1991) suggest that hepatic ammonia detoxification incurs an amino acid penalty (Reynolds, 1992; Parker *et al.* 1995). This is supported by isotopic studies in sheep infused with NH_4Cl, which demonstrated that the incremental increase in hepatic urea production was approximately twice the increase in ammonia load, which occurred in conjunction with an increase in hepatic leucine catabolism (Lobley *et al.* 1995). Therefore, it was suggested that high levels of rumen ammonia production following the ingestion of fresh forage (Ulyatt, 1997) may have a double negative influence on the animals productivity. Firstly, a large proportion of dietary-N intake is lost as ammonia from the rumen. Secondly, an increase in

hepatic amino acid catabolism is required to detoxify this ammonia to urea (Reynolds, 1992; Parker *et al.* 1995; Lobley & Milano, 1997).

However, the results obtained by Lobley *et al.* (1995) were compromised by a change in blood *p*H associated with the infusion of the acidic NH_4Cl (Lobley *et al.* 1996b; Milano *et al.*, 2000). A repeat experiment infusing NH_4HCO_3, which had no effect on blood *p*H, demonstrated an increase in hepatic urea production only 1.2 times the increase in ammonia load and showed no increase in hepatic leucine catabolism (Lobley *et al.* 1996b). This work was further extended to show that the increment in hepatic urea-N production is predominantly sourced from ammonia-N, rather than amino acid-N (Lobley & Milano, 1997; Milano *et al.*, 2000). These data confirm *in vitro* observations which showed that the oxidation of alanine and glutamate was not increased by incremental ammonia loads in ovine hepatocytes (Mutsvangwa *et al.* 1996). Therefore, current evidence suggests that increasing the hepatic ammonia detoxification load does not incur an increase in hepatic amino acid catabolism (Lobley *et al.* 1996b; Lobley & Milano, 1997; Milano *et al.*, 2000). However it should be noted that a small increase in hepatic amino acid catabolism could have severe negative ramifications on peripheral amino acid metabolism in an animal which may already be protein restricted, with the resultant limitation to productive output (Lobley & Milano, 1997; Milano *et al.*, 2000).

2.4.3.2 Intestinal protein digestion

As a result of rumen protein digestion, and the peptic digestion that occurs in the abomasum, the first section of the small intestine, the duodenum, is presented with rumen microbial protein, peptides, amino acids, undigested feed and endogenous secretions for either absorption or further digestion (Harrison *et al.* 1973; Mercer *et al.* 1980). The concentration of peptides and amino acids arriving at the duodenum is relatively constant despite changes in diet and intake levels (Harrison *et al.* 1973), however the peptide and amino acid fluxes increase with increased N intake (Bunting *et al.* 1987). There is significant metabolism of absorbed amino acids within the wall of the small intestine, such that the amino acid profile absorbed from the intestinal lumen by the epithelium

of the small intestine bears little resemblance to that entering the portal blood (Wolff *et al.* 1972a; Tagari & Bergman, 1978). The essential amino acids are preferentially absorbed relative to the non-essential amino acids (Armstrong & Hutton, 1975; Lindsay & Armstrong, 1982).

The quantity of peptides and amino acids entering the small intestine can be increased by either increasing the proportion of these compounds escaping degradation in the rumen, or by energy supplementation in the rumen, which effectively increases the rate of incorporation of these compounds into microbial protein and reduces the proportion of N intake lost from the rumen as ammonia (Mangan, 1982).

In the small intestine the microbial protein is digested by the hydrolytic secretions of the pancreas (trypsin and peptidases) which enter at the duodenum (Lindsay & Armstrong, 1982). Amino acids are absorbed from the mid-jejunum to lower-ileum (Johns & Bergen, 1973), although the highest rate of absorption is from the mid-jejunum (Ben-Ghedalia *et al.* 1974).

With the exception of ammonia absorption, it is generally assumed that the large intestine does not contribute significantly to GIT N absorption, that is, there is no evidence of amino acid absorption from the large intestine (Ulyatt & MacRae, 1974).

2.5 METABOLITE TRANSPORT IN THE BLOOD

The products of N digestion are transported in the blood as intact small proteins, peptides, amino acids and ammonia (Danilson *et al.* 1987). Amino acids were initially thought to be solely carried by the plasma component of whole blood (Brockman & Bergman, 1975). More recent evidence indicates that measurements of amino acid transport based on plasma alone were underestimates by approximately the volumetric contribution of the red blood cells (Bergman, 1986; Webb, 1986). However the precise role of the red blood cells in amino acid transportation still remains unclear (Heitmann & Bergman, 1980b; Webb, 1986).

Some amino acids transport ammonia *via* a couplet arrangement. The couplets of asparagine-aspartate and glutamine-glutamate donate an amine group from the former amino acid subsequently producing the latter amino acid (Krebs *et al.* 1976; Bergman & Pell, 1984).

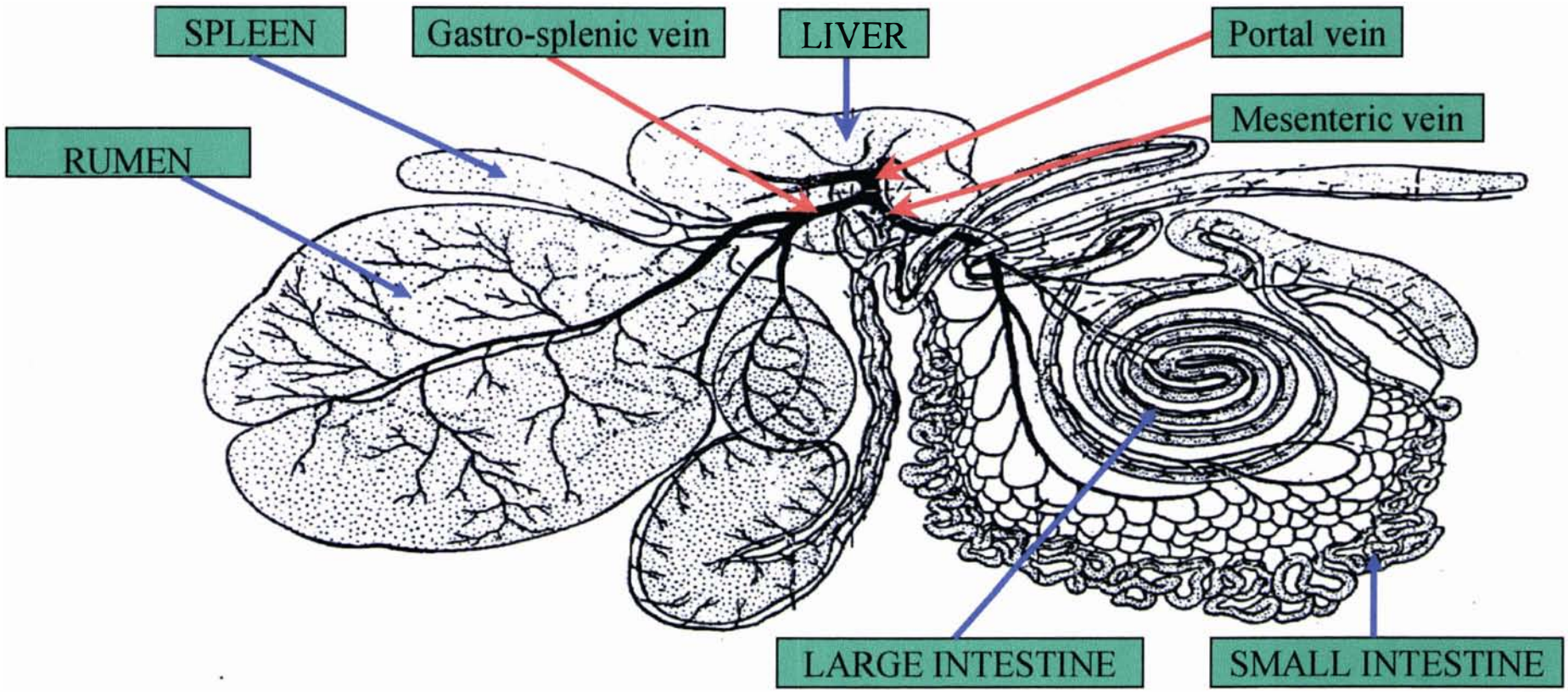
2.5.1 The major blood vessels of the splanchnic region

The mesenteric circulation (see Figure 1.1) is supplied with blood from the superior and inferior mesenteric arteries (Parks & Jacobson, 1987). The small and large intestines are drained by the cranial and caudal mesenteric veins which combine into the mesenteric vein before entering the portal vein 1.5 - 3 cm caudal to the ovine liver (Heath, 1968; Koch & Ghoshal, 1981). Therefore the mesenteric drained viscera (MDV) consists of the small intestine, caecum, large intestine, mesenteric fat, spleen and the pancreas (Reynolds & Huntington, 1988; Webb *et al.* 1993).

The blood supply to the rumen (including the reticulum), omasum and abomasum is supplied by branches of the celiac artery (Dyce *et al.* 1987), with drainage *via* the gastrosplenic vein which enters the mesenteric vein approximately 1 cm prior to this junction with the portal vein (Heath, 1968). Within the liver *per se* the portal blood is evenly distributed throughout the entire organ (Heath, 1968).

The portal drained viscera (PDV) consists of the rumen, omasum, abomasum, small intestine, caecum, large intestine, mesenteric fat, spleen and the pancreas (Reynolds & Huntington, 1988; Webb *et al.* 1993). Therefore by determining the difference in blood flow between the PDV and MDV, the contribution of the rumen, omasum and abomasum to the total blood flow from the PDV can be quantified (Reynolds & Huntington, 1988).

Figure 1.1. The major organs and veins of the splanchnic region



Source; Goshal (1975).

The liver is supplied with blood from both the portal vein (*vena portae communis*) and the hepatic artery (*arteria hepatica*) (Dyce *et al.* 1987). Blood from the liver drains *via* the hepatic vein into the *vena cava* for return to the heart (Dyce *et al.* 1987).

2.5.2 Blood flows within the splanchnic region

A major determinant of splanchnic blood flow is metabolic body weight (Barnes *et al.* 1983a), therefore blood flow rates will be quoted per kg metabolic body weight in this section. Typical ovine portal blood flows are 102-133 ml/min/kg$^{0.75}$ (Lush & Gooden, 1988; Mineo *et al.* 1991). The gastrosplenic and mesenteric veins each contribute approximately 53 ml/min/kg$^{0.75}$ to the portal blood flow (Reynolds & Huntington, 1988). Hepatic venous flows comprise approximately 80% portal and 20% hepatic arterial flows (Reynolds *et al.* 1988b; Mineo *et al.* 1991). The splanchnic blood flow at any one time is affected by such variables as the time after feeding, level of food intake and posture (Barnes *et al.* 1986).

2.6 TECHNIQUES FOR ESTIMATING THE RATE OF METABOLISM

There are several excellent reviews of the methods available to estimate the rate of amino acid and hence protein metabolism in ruminants (Bergman & Heitmann, 1978; Oddy & Neutze, 1991; Lobley, 1992; Reeds & Nissen, 1992; Kelly *et al.* 1993; Lindsay *et al.* 1993; Lobley *et al.* 1993; van der Walt, 1993; Lobley & Asplund, 1994; MacRae *et al.* 1995). However only the arterio-venous (AV) technique will be discussed here because this is currently the most widely accepted technique to estimate the rate of metabolism within a defined tissue (Lindsay *et al.* 1993). The AV technique requires the chronic placement of catheters in the arterial and venous vessels of a specific tissue, in conjunction with accurate determinations of both blood flow rates and metabolite concentrations (Boisclair *et al.* 1993; Lindsay *et al.* 1993; Lobley *et al.* 1993; Lobley & Asplund, 1994). This technique is limited by such technical difficulties as the accurate determinations of both the blood flow rate and metabolite concentrations, catheter patency, the potential for incomplete mixing

of both metabolites and tracers, precise isolation of both the supply and drainage of a homogenous tissue, and the need to integrate AV results over long periods, for example, 24 hours, to minimise diurnal variations (Boisclair *et al.* 1993; Lindsay *et al.* 1993). Despite these limitations, the AV technique has proven useful (Lobley & Asplund, 1994).

The rate of nutrient supply to a tissue is primarily influenced by the rate of blood flow to that tissue and the concentration of metabolites in the blood (Harris *et al.* 1991). Therefore, the precise determination of both blood flow and metabolite concentration is essential for accurate determinations of the rate of metabolism of a specific tissue (Katz & Bergman, 1969b; Reynolds & Huntington, 1988).

Subsequent discussion will be limited to a consideration of amino acids and their direct involvement in protein synthesis.

2.6.1 Methods used to estimate blood flow

Blood flow estimation techniques have been extensively reviewed (Linzell & Annison, 1975; Tepperman & Jacobson, 1982; Dobson, 1984; Barnes *et al.* 1986). Only the three major techniques which are most applicable to the present work will be discussed below, that is, dye-dilution, electronic probe, and tritiated water.

The dye-dilution technique for the estimation of blood flow rates is based on the Fick principle (Webster, 1974; Cant & McBride, 1995). By knowing the quantity of dye infused and the arterial and venous dye concentrations, use of the Fick equation (Cant & McBride, 1995) enables the blood flow rate to be calculated from the quotient of the infusion rate divided by the difference between the inflow and outflow dye concentrations (Tepperman & Jacobson, 1982). Many substances have been used as the dye, the most common of which is *para*-aminohippurate (*p*AH) (Webster, 1974). The appropriate dye must meet several requirements, such as being non-metabolisable by the target tissue and, preferably totally extracted by either the kidneys or the liver

to reduce the dye recirculation (Tepperman & Jacobson, 1982). This technique is relatively accurate and can be used repetitively, however poor dye mixing prior to the sampling point (streaming) and recirculation remain problems (Webster, 1974; Tepperman & Jacobson, 1982).

The dye-dilution technique using *p*AH is the most commonly used technique for estimating the blood flow rate of many splanchnic tissues (Tepperman & Jacobson, 1982). The determination of hepatic venous blood flow rates with the *p*AH dilution technique requires an additional acid deacetylation step to the normal methodology (Lobley *et al.* 1995). As a result of the difficulty involved with cannulating or isolating the hepatic artery, hepatic arterial blood flow rate is typically calculated by difference between portal and hepatic venous flow rates (Fischer, 1963; Campra & Reynolds, 1982).

There are a number of different electronic probes available for estimating the blood flow, including the electromagnetic and ultrasound probes (Tepperman & Jacobson, 1982). The former suffers from a requirement to be maintained perpendicular to the vessel and the need to accurately ascertain the vessel diameter, whereas the ultrasound probe is not as sensitive to the angle of incidence with the vessel, nor is knowledge of the vessel diameter required (Barnes *et al.* 1983b). A major advantage of these probes is that they give continuous estimates of blood flow (Tepperman & Jacobson, 1982; Barnes *et al.* 1983b).

Using the assumption that the absorption of tritiated water by a defined tissue is proportional to the blood flow rate, the clearance of $^{3}H_2O$ from the blood can provide an accurate estimate of blood flow (Tepperman & Jacobson, 1982). These calculations require measurements of the H_2O concentration in the arterial and venous blood, and the $^{3}H_2O$ activity in the tissues, to estimate the absorption rate (Oddy *et al.* 1981). The major disadvantage of this technique is that $^{3}H_2O$ clearance is probably not solely related to the blood flow at the absorptive site (Oddy & Lindsay, 1986). Other factors such as the metabolic

activity and filtration pressures can also influence the $^{3}H_2O$ clearance rate (Tepperman & Jacobson, 1982).

2.6.2 The use of isotopes to estimate amino acid transfer rates

Isotopes have a different relative atomic mass to that normally associated with a specific element. They are either stable (do not decay) or radio-active (decay releasing radio-activity; Waterlow *et al.* 1978c; Reeds & Nissen, 1992). This section concentrates on the use of isotopically-labelled amino acids to estimate whole body and specific tissue amino acid metabolism. Isotope labelling of amino acids may be used to trace the unidirectional metabolism of specific amino acids. The data from such studies can also be used to estimate the rate of protein synthesis (Reeds & Nissen, 1992) and to provide estimates of unidirectional amino acid metabolism which are not available from mass transfer data; for example, the net hepatic removal of glutamine was up to 2.6 times lower than the unidirectional removal of that amino acid, indicative of hepatic *de novo* glutamine synthesis (Wolff & Bergman, 1972b; Wolff *et al.* 1972; Reynolds, 1992).

The assumptions necessary to translate isotopic amino acid data into either whole body or specific tissue amino acid metabolism are;

- Steady state - the various amino acid pools are in steady state, so that the pool sizes remain constant. Attainment of a steady state may be assisted by hourly feeding intervals (Waterlow *et al.* 1978a). Pool divisions are based firstly on whether the amino acid is protein bound or free, then into extra- and intracellular divisions, and further based on such variables as the site of metabolism and the end fate of the amino acid (Riis, 1983).
- Homogenous mixing - from the assumptions that intra-pool mixing is both complete and rapid and that a constant fraction of a specific pool is transferred or exchanged per unit of time, it can be assumed that the various pools have the same specific activity (Waterlow *et al.* 1978b).
- Metabolism of the amino acid is not affected by the presence of the isotope (Reeds & Nissen, 1992).

When choosing the appropriate labelled amino acid to estimate turnover in a specific tissue, consideration should be given to the end fate of that amino acid and therefore in which pool the isotope will reside, and how does this affect the ease of measurement (Reeds & Nissen, 1992). It is preferable that the labelled amino acid not be metabolised by the tissue under consideration. However, if it is, the end-products of such metabolism should be known so that the specific activity associated with those end products can be quantified (Oddy & Neutze, 1991). Use of a labelled essential amino acid removes the requirement to estimate the rate of *de novo* amino acid synthesis (Lobley & Asplund, 1994). The amino acid used should be devoid of direct influences on the rate of metabolism; for example, both phenylalanine and leucine have been shown to affect the rate of insulin production (Kuhara *et al.* 1991; Southorn *et al.* 1992), whilst valine is presently thought to be free from such perturbations (Lobley & Asplund, 1994).

When calculating the rate of labelled amino acid infusion, the above variables should be considered in addition to factors such as the size of the pool into which the amino acid is being infused, what percentage of the pool must be labelled to provide accurate representation and the rate of infusion required to reach steady state (plateau specific activity). For steady state to be achieved, the specific activity leaving the given pool must equate to that entering the pool (Waterlow *et al.* 1978a; Waterlow *et al.* 1978b; Reeds & Nissen, 1992).

2.6.3 Techniques used to estimate the rate of protein synthesis

The techniques for estimating the rate of protein synthesis using isotopically labelled amino acids have been extensively reviewed (Waterlow *et al.* 1978a; Reeds & Lobley, 1980; Garlick & Fern, 1985).

Net protein gain is the difference between the rates of protein synthesis and protein degradation (Harris *et al.* 1992; Lobley *et al.* 1994). There is no method available for the accurate determination of the rate of protein degradation, however estimates are generally obtained by calculating the difference between isotopic estimations of protein synthesis and mass transfer estimates

of protein gain (Oddy *et al.* 1981). Estimates of ovine whole body protein synthesis range from 4.8-12.1 g/kg$^{0.75}$/d (Cronje *et al.* 1992). The fractional synthesis rate (FSR) measures the proportion of whole body or tissue protein mass synthesised per day, with typical ovine whole body FSR values of 2.7-4.9% (Harris *et al.* 1992).

A labelled amino acid (^{2}H-^{15}N-leucine) was first used by Schoenheimer *et al.* (1939) to estimate the rate of protein synthesis. However the lack of a non-isotopic method to estimate the rate of protein synthesis has prevented validation of this technique (Reeds & Nissen, 1992). It is generally accepted that estimates of protein synthesis using labelled amino acids should be compared with those obtained using uniformly labelled protein, as the best available technique (Grove & Jackson, 1995).

Direct estimates of protein synthesis are based on estimates of the dynamics of the protein pool *per se*, whereas indirect estimates are based on the degree of label incorporation into amino acids, or the amounts of label excreted in the end-products of amino acid metabolism (Reeds & Nissen, 1992). Both methods require the assumption that the metabolism of a single amino acid is representative of protein turnover (Reeds & Nissen, 1992) however this assumption may not be valid under some conditions (Bier *et al.* 1985).

Models used for the calculations of the rate of protein synthesis initially considered the simplest approach of just two amino acid compartments, the protein bound and free amino acids (Reeds & Lobley, 1980; Garlick & Fern, 1985) with a further extension to include a third compartment, the intracellular pool (Bier *et al.* 1985). This compartmental approach relies on the assumption that the intracellular free amino acid pool is kinetically and metabolically homogenous (Garlick & Fern, 1985; Oddy & Neutze, 1991).

2.6.3.1 The arterio-venous difference technique

Using the AV difference technique, protein synthesis is estimated by the difference between total labelled amino acid uptake and irrevocable amino acid

catabolism (to protein synthesis). Protein gain is estimated by the difference between net amino acid movement and irrevocable amino acid catabolism (to protein gain). The difference between the derived values of protein synthesis and gain estimates the rate of protein degradation (Reeds & Nissen, 1992).

Whilst this technique is relatively simple and accurate, there are a number of potential problems. Firstly, as with all AV difference methods, precise measurements of concentrations and activities are required to detect the small AV differences (Boisclair *et al.* 1993). Secondly, as with all isotopic amino acid methods for estimating protein synthesis, there is debate as to the validity of assuming that the specific activity in either the plasma, whole blood or tissue represents that of the true precursor pool, which is the respective aminoacyl-tRNA's (Oddy & Neutze, 1991; Reeds & Nissen, 1992). This relationship is derived from the assumption that protein synthesis draws from an homogenous pool of amino acids and therefore the aminoacyl-tRNA's have the same specific activity as the tracer amino acid. However, this is clearly an over-simplification of a complex biological system (Reeds & Lobley, 1980). For example, it has been suggested that the uncertainty associated with the choice of amino acid pool which most closely approximates the true precursor specific activity, may be responsible for the 3-4 fold variation in the rates of protein synthesis quoted in the literature (Lobley *et al.* 1994).

The specific activity of the true precursors of protein synthesis, the respective aminoacyl-tRNA's, are difficult to measure (Oddy & Neutze, 1991). The low concentration of the specific aminoacyl-tRNA in tissue, for example, 0.5-1 nmol valyl-tRNA/g rat liver; (Airhart *et al.* 1974), and the subsequent difficulty of extraction are both relatively minor problems compared to the rapid turnover (half life 0.3-2 sec) of the aminoacyl-tRNA complex (Airhart *et al.* 1974). Consequently direct measurements of the specific activity of aminoacyl-tRNA's, are a lengthy and expensive process (Lobley *et al.* 1980; Oddy & Neutze, 1991), and are not necessarily accurate (Airhart *et al.* 1974; Smith & Sun, 1995).

Therefore, due to the numerous difficulties involved with measuring the specific activity of the respective aminoacyl-tRNA, this specific activity is typically estimated from another, easier to sample pool, such as the arterial and venous plasma, blood or tissue homogenates. However, due to the increase in specific activity between samples of tissue homogenates, venous and arterial blood or plasma, the calculated values of protein synthesis decrease respectively (Airhart *et al.* 1974; McKee *et al.* 1978). Therefore the correct estimate of the rate of protein synthesis is within the range calculated from tissue homogenates and arterial blood or plasma specific activities (Schaefer *et al.* 1986; Lobley *et al.* 1988).

To provide accurate estimations of the rate of protein synthesis, the specific activity of an amino acid pool needs to be constant, which is indicative of a constant pool size (Waterlow *et al.* 1978a; Reeds & Nissen, 1992). The time taken to attain plateau specific activity depends on such variables as the infusion rate and the size of the pool (Waterlow *et al.* 1978b). However, there is a compromise between an infusion of sufficient duration to ensure plateau specific activity, whilst minimising label recycling (Boisclair *et al.* 1993).

2.6.3.2 The large dose technique

The precursor problem addressed above may be minimised by using the flood or large dose technique (Henshaw *et al.* 1971). As suggested by the name, this technique utilises a large dose of unlabelled amino acid, in conjunction with the labelled amino acid, to effectively equilibrate the specific activity of all amino acid pools in the body to that of the plasma or blood pool (Reeds & Nissen, 1992). This technique is particularly useful in tissue with a high protein turnover rate or with a high proportion of synthesised protein exported (Lobley *et al.* 1994).

The amount of amino acid given is usually 5-20 times the estimated size of the free amino acid pool (Lobley *et al.* 1990; Oddy & Neutze, 1991). Sample collection commences typically within 120 minutes of the amino acid injection, so that differences in specific activity between the various pools is sufficiently

small to allow plasma or blood values to represent all pools (Attaix *et al.* 1988; Lobley *et al.* 1990).

A possible concern with this technique is the reduction in the circulating concentrations of other amino acids (Schaefer *et al.* 1986; Lobley *et al.* 1990). The possible consequences of such alterations are as yet unknown, however they are unlikely to stimulate protein synthesis (Waterlow, 1984; Lobley *et al.* 1992; Reeds & Nissen, 1992). The effectiveness of the flood dose in achieving equivalent specific activities across the pools varies from 0.25 for the small intestine to 0.99 for skeletal muscle (Lobley *et al.* 1992).

2.6.4 Estimation of the rate of whole body protein synthesis

The rate of whole body protein synthesis may be estimated using either indirect or direct methods (Waterlow *et al.* 1978d). Indirect methods require prior knowledge of the protein pool size, allowing the rate of protein synthesis to be determined from the rate of decay in isotopic abundance (Reeds & Nissen, 1992). Using this method, the rate of amino acid catabolism can be estimated from the rate of label loss from the appropriately defined compartment (Garlick *et al.* 1980). This method relies on the assumption that the free amino acid pool is both kinetically and metabolically homogenous (Waterlow *et al.* 1978d).

Estimates of the rate of whole body protein synthesis from direct methods require measurements of the quantity of the labelled amino acid in the protein bound and free amino acid pools (Reeds & Nissen, 1992). This technique may also be used for a tissue with a slow rate of protein turnover, for example the muscle (Waterlow *et al.* 1978d).

2.6.4.1 Estimation of the rate of hepatic protein synthesis

Estimates of hepatic protein synthesis are confounded by two problems, the first is that the liver produces both constitutive and export proteins, and the second is that protein is assumed to be synthesised from an homogenous amino acid pool (Connell *et al.* 1997). Constitutive proteins are synthesised on

the polysomes from intracellular free amino acids (Smith & Sun, 1995), whereas export proteins are probably synthesised on the rough endoplasmic reticulum and therefore preferentially utilise amino acids as they enter the cell (Fern & Garlick, 1976). The recent use of hippuric acid, which is synthesised in the liver and rapidly excreted from the body, and apolipoprotien B100, are attempts to minimise these problems (Connell *et al.* 1997).

2.7 LIVER METABOLISM

The liver is located on the right of the abdomen against the diaphragm, under the protection of the lower ribs (Dyce *et al.* 1987). It is a soft flexible organ with its shape largely influenced by the space available within the peritoneal cavity. The liver is appropriately positioned between the site of nutrient absorption (GIT) and deposition (Huntington, 1990; Reynolds, 1992) to control the composition of systemic blood returning to the heart (Mutsvangwa *et al.* 1996; Connell *et al.* 1997). The major functions of the liver are to modify the composition of the blood from the PDV, nutrient storage, detoxification and waste removal, and to produce various secretions. The liver also participates in the regulation of blood flow (Campra & Reynolds, 1982) as a consequence of its large blood volume (25-30 ml/100 g) (Withrington & Richardson, 1986).

The liver receives large quantities of amino acids, carbohydrates, lipids, and vitamins in the portal blood (Arias *et al.* 1982). The degree to which each metabolite is extracted from the portal blood is dictated by the hepatic requirements for that metabolite. During passage through the liver, the blood is also enriched with metabolites, such as glucose and numerous proteins (Lindsay & Armstrong, 1982).

2.7.1 Liver structure

There are two major lobes to the liver, left and right, which are further divided into multiple sub-lobes (Elias & Sherrick, 1969b). The ovine liver contributes between 1.6 and 2.5% of live weight (Orzechowski *et al.* 1987; Lobley *et al.* 1995).

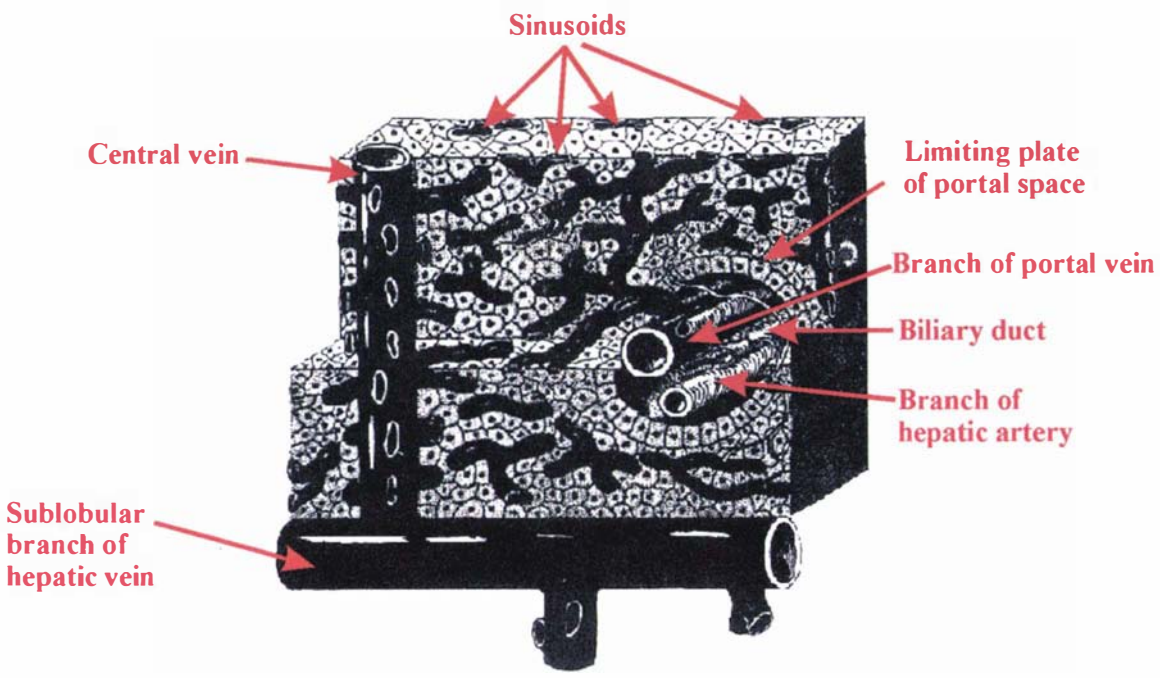
The portal vein bifurcates as it ascends into the hilus of the liver (*porta hepatis*), with numerous further divisions until it reaches capillary like dimensions (Elias & Sherrick, 1969a). Similarly the hepatic artery also divides into vessels which are ultimately of capillary like dimensions. The branches of the hepatic artery, portal vein, and the bile duct, in addition to lymph vessels and nerves, perfuse the entire liver in concert and are known as the "portal triad" (Figure 1.2) (Elias & Sherrick, 1969a).

The blood is drained from the liver by minute branches of the hepatic vein (*vena hepatica dextra*) which ultimately merge before exiting the liver as the hepatic vein.

The relative contribution from each of the portal vein and hepatic artery to the blood flow entering the metabolically active unit of the liver, the sinusoids, is controlled by pre-sinusoidal sphincters on each of these vessels (Aterman, 1963). Additionally the flow from the sinusoids is controlled by sphincters on the branches of the hepatic vein. These sphincters allow the net filtering pressure across the sinusoidal wall to be maintained whilst the blood flow to the liver is kept relatively constant (and hence the liver blood volume is maintained), which provides the hepatocytes with a steady supply of oxygen (Withrington & Richardson, 1986).

The portal blood flow rate is primarily determined by the relatively constant venous drainage from the GIT and spleen (Withrington & Richardson, 1986). The hepatic arterial blood flow rate alters reciprocally to the portal blood flow rate (Withrington & Richardson, 1986). The relative contribution from the hepatic artery to total hepatic venous blood flow is highly variable but is typically between 0.5 to 25% (Katz & Bergman, 1969a; Ortigues *et al.* 1994; Lobley *et al.* 1995; Goetsch *et al.* 1997).

Figure 1.2. The portal triad and the liver ultra-structure



Source; Harper (1973).

The liver is richly supplied with afferent nerves from the autonomic nervous system, with two communicating plexuses surrounding the portal vein and hepatic artery (Sasse, 1986). The influence of the nervous system on hepatic metabolism is *via* neural regulation of parenchymal blood flow (Arias *et al.* 1982).

2.7.1.1 Acinus structure

The parenchymal cells of the liver account for 80% of its volume and 60% of total cell number (Arias *et al.* 1982; Sasse, 1986). The acinus is the structural and functional unit of the liver (Arias *et al.* 1982; Gumucio & Miller, 1982; Sasse, 1986; Jungermann & Kietzmann, 1996). Structurally the acinus consists of sinusoids arranged radially around the terminal afferent vessels (the portal triad), with multiple terminal hepatic vessels in the periphery of the acinus (Figure 1.2) (Elias, 1963; Jungermann & Kietzmann, 1996).

The sinusoids are capillary like vessels surrounded by the functional cells of the liver. The hepatocytes are polarised parenchymal cells that form a trabecular network within the acinus, with their baso-lateral surface facing the sinusoid and bile ductules at their apex (Jungermann & Kietzmann, 1996).

The non-parenchymal cells are divided into four groups (Arias *et al.* 1982; Sasse, 1986);

1.The endothelial cells, which make up 44% of the non-hepatocyte cell volume, are evenly distributed throughout the liver parenchyma. These are digestive cells with limited endocytotic capabilities.

2.The Kupffer cells, which contribute 33% of the non-hepatocyte cell volume, are located predominantly in the periportal area of the sinusoid. These macrophagic cells are capable of phagocytosis of particles 100-800 nm in diameter. The digestive capacity of these cells is emphasised by the abundance of lysosomes.

3.The fat storage cells, which make up 10-25% of the non-hepatocyte cell volume, are the site of hepatic fat metabolism in addition to providing support to the sinusoid wall.

4.The pit cells, which account for 5% volume of the non-hepatocyte cell volume, and have a similar function to the lymphocytes.

The acinus is divided into three functional zones radiating from the afferent vessels which differ in enzyme activities and consequently have different metabolic rates and capacities (Sasse, 1986). The oxygen, insulin and glucagon gradients are thought to be important in the establishment of zonal heterogeneity (Jungermann & Kietzmann, 1996). These divisions are dynamic and functional rather than static and structural (Haussinger & Gerok, 1986; Jungermann & Kietzmann, 1996). These three zones are, the periportal zone which surrounds the axis, the intermediate zone which is minute or absent in the ovine, and the perivenous zone which receives blood from the two previous zones.

2.8 LIVER FUNCTION

2.8.1 Periportal and perivenous hepatocyte functionality

In general, metabolite concentrations decrease from the periportal to perivenous region of the sinusoid. There are also enzymatic gradients along the sinusoid length (Gumucio & Miller, 1982). Periportal cells account for the majority of the hepatocyte population (90%), leaving the perivenous cells to contribute the remaining 10% of hepatocytes in the form of a ring of one to three cells surrounding the terminal venule (Kilberg *et al.* 1993).

The periportal hepatocytes specialise in ureagenesis, gluconeogenesis, glutaminolysis, the synthesis of albumin and have a high rate of amino acid catabolism (Haussinger *et al.* 1992). The perivenous hepatocytes can synthesise glutamine and extract aspartate and glutamate (Stoll *et al.* 1992).

The oxygen concentration in the periportal zone is approximately twice that in the perivenous zone, indicative of the high metabolic activity throughout the sinusoid (Jungermann, 1986). There is diurnal variation in some enzyme concentrations and activities throughout the sinusoid, for example, tyrosine

aminotransferase in the periportal zone. However there is no apparent diurnal variation in the activity of this enzyme in the perivenous zone (Haussinger & Gerok, 1986).

Although ammonia is produced in the periportal zone during catabolism, its concentration decreases by approximately an order of magnitude between the periportal and perivenous zones (Jungermann, 1986) as a result of periportal ureagenesis (Haussinger & Gerok, 1986). Using the perfused rat liver, it has been demonstrated that glutaminase (EC 3.5.1.2) activity is restricted to the periportal zone, with glutamine synthetase (EC 6.3.1.2) similarly restricted to the perivenous zone (Haussinger & Sies, 1984). Thus periportal glutaminase and perivenous glutamine synthetase are simultaneously active in the liver (Haussinger & Sies, 1979; Haussinger *et al.* 1983; Haussinger & Sies, 1984), resulting in an energy consuming intercellular cycling of glutamine (Haussinger, 1983). The activity of this cycle is influenced by both the portal ammonia and glutamate concentrations and the blood *p*H (Haussinger, 1990).

The maintenance of blood *p*H requires a mechanism to control the ratio of HCO_3^- to CO_2 in the blood (Haussinger & Gerok, 1986). An important component of this regulation is HCO_3^- removal during ureagenesis, whereas HCO_3^- is not consumed during the removal of ammonia as glutamine. Thus the relative rate of these pathways can be used to control the HCO_3^- concentration, which is achieved *via* inhibition of either glutaminase or glutamine synthetase (Sies & Haussinger, 1984). This method can also assist the other metabolic functions which are used to control blood *p*H. Therefore during acidosis, there is a decreased rate of periportal urea synthesis and an increased rate of perivenous glutamine formation, whereas during alkalosis HCO_3^- removal by urea synthesis is increased (Haussinger & Gerok, 1986).

2.8.2 Hepatic amino acid metabolism

The liver is an important organ for protein metabolism and is the major site of amino acid catabolism (Elwyn, 1970; Lindsay & Armstrong, 1982; Seal & Reynolds, 1993; Lobley & Asplund, 1994; Connell *et al.* 1997). The rate of

hepatic amino acid extraction varies depending on hepatic requirements for a specific amino acid. For example, both alanine and glycine are almost entirely extracted from the portal blood by the liver in contrast to the branched chain amino acids which have small (0-3%) hepatic extraction rates (Lindsay & Armstrong, 1982).

On the basis of function, hepatic amino acid metabolism may be divided into five major categories: protein synthesis, the production of vital metabolites (such as enzymes), gluconeogenesis, ureagenesis and catabolism (Bergman & Heitmann, 1978; Lobley & Asplund, 1994).

Hepatic protein synthesis is an energetically expensive process which accounts for approximately 10% of whole body oxygen consumption (Lobley, 1991). Hepatic protein synthesis is divided into both constitutive and export protein synthesis. The extent of hepatic protein synthesis for export is large, with 10-12 g/d or a fractional synthesis rate of 10-50% (Lindsay & Armstrong, 1982; Lobley *et al.* 1992; Lobley & Asplund, 1994). Major export proteins synthesised are albumin, transferrin and various lipoproteins (Lindsay & Armstrong, 1982). Hepatic albumin production is 8-17% of the total hepatic protein synthesised in the ovine (Connell *et al.* 1997).

The rate of net hepatic protein output is minute relative to the rate of both hepatic protein synthesis and degradation, therefore small changes in the rate of either protein synthesis or degradation have a large impact on net hepatic protein production (Lobley *et al.* 1993; Lobley & Asplund, 1994). Alterations to the rate of protein degradation are thought to be the major controller of the net protein production rate, with the fractional synthesis rate (FSR) remaining constant (Lobley & Asplund, 1994). Increased protein intake increases the rate of hepatic amino acid absorption (Maltby *et al.* 1991) and induces a decrease in the rate of protein degradation, whilst the rate of protein synthesis is relatively unaffected (Natori, 1995). When protein intake is restricted, hepatic protein mass decreases as a result of decreased hepatic amino acid uptake (Maltby *et al.* 1991) which induces either a decrease in the rate of hepatic

protein degradation (Mortimore & Surmacz, 1984; Burrin *et al.* 1990; Natori, 1995) or protein synthesis (Pell *et al.* 1986) whilst the hepatic FSR remains relatively constant (Lobley *et al.* 1994).

Hepatic amino acid catabolism utilises absorbed amino acids to produce energy (ATP) *via* the citric acid cycle for both hepatic and whole body requirements (Elwyn, 1970; Lobley & Asplund, 1994; Lobley *et al.* 1996a; Connell *et al.* 1997). It is not known whether hepatic amino acid catabolism removes excess amino acids *per se* or whether there is selective amino acid catabolism to meet specific requirements (Lobley & Asplund, 1994).

The liver utilises small peptides in a comparable manner to small proteins and amino acids, however the magnitude of peptide metabolism by the liver is uncertain due to methodological problems determining peptide concentrations (Webb *et al.* 1993).

2.8.3 Carbohydrate metabolism

Propionate absorbed by the liver is either oxidised *via* the citric acid cycle or is used for gluconeogenesis (Brockman & Laarveld, 1986), to provide up to 50% of the glucose production in the fed ruminant (Baird *et al.* 1980). Acetate and the ketone bodies generated from butyrate are converted to acetylCoA in the liver and subsequently enter the citric acid cycle (Armstrong, 1965; Preston & Leng, 1987a).

Despite little glucose being absorbed from the GIT directly, glucose is still an important metabolite for ruminants (Linzell, 1960); for example the major milk precursors in a lactating ruminant are glucose (predominantly converted to lactose), free amino acids, acetate and β-hydroxybutyrate (Preston & Leng, 1987a).

2.8.4 Lipid metabolism

Lipids are absorbed from the GIT into the mesenteric venous drainage as well as the lymphatic vessels (Katz, 1986). Acetate is the major precursor for fatty acid synthesis (Hanson & Ballard, 1967). The liver of the non-lactating non-pregnant ovine has been estimated to produce triacylglycerol at a rate of approximately 0.5 μmol/h/g liver (Pullen & Ames, 1988).

2.8.5 Mediators of liver metabolism

Insulin is an important hormone in the control of ruminant metabolism (Brockman & Bergman, 1975; Brockman & Laarveld, 1986). Insulin inhibits hepatic gluconeogenesis (Wolff *et al.* 1972; Brockman & Laarveld, 1986). Plasma insulin concentrations are positively correlated with food intake levels, however, paradoxically, the insulin concentrations are highest in the fed state when hepatic glucose production rates are highest due to the increased availability of the substrates for gluconeogenesis (Brockman & Laarveld, 1986).

Glucagon stimulates both glycogenolysis and gluconeogenesis (Brockman & Bergman, 1975). Glucagon promotes hepatic utilisation of amino acids in gluconeogenesis (Wolff *et al.* 1972; Brockman & Laarveld, 1986). Glucagon and epinephrine are mainly involved in response to "fight or flight" reactions rather than the day-to-day regulation of metabolism (Brockman & Laarveld, 1986).

Some amino acids, including glycine, alanine and glutamine (Christensen, 1990) have been shown to directly regulate the rate of protein turnover in the liver by inducing changes in the cellular osmolarity, effectively altering (plus or minus 15-25%) cell volume (Haussinger *et al.* 1994; vom Dahl *et al.* 1995). Amino acids have also been shown to mediate the sensitivity of insulin control over protein degradation in ovine skeletal muscle (Lobley, 1998).

2.8.6 Nitrogen metabolism in the liver

The liver has a fundamental role in the integration of whole body N metabolism (Reynolds, 1992). Ruminants absorb a significant proportion of their dietary N in the form of ammonia (Huntington, 1986). The liver removes and detoxifies this ammonia, predominantly by converting it to urea, which is then released into the hepatic vein, for excretion *via* the kidney in the urine. Thus, urea is the major end product of N metabolism (Meijer & Hensgens, 1982).

2.8.6.1 The urea cycle

It has been suggested that the process of ureagenesis in sheep and possibly other ruminants is fundamentally different from that in non-ruminant mammals (Lobley *et al.* 1995; Luo *et al.* 1995). However, more recent work (Brosnan *et al.* 1996) suggests that the urea cycle is similar between various species. In the absence of definitive data, this review presents information for the ovine when available, but will also draw upon data from other species where appropriate.

The urea cycle was discovered by Krebs & Henseleit (1932), using the liver tissue-slice technique to investigate the rate of urea production in the presence of various potential precursors. Although the liver is the major organ of urea synthesis, the enzymes of ureagenesis are also present in some non-hepatic tissues, however none of these tissues are capable of synthesising urea from ammonia in significant quantities (Meijer & Hensgens, 1982).

Ammonia concentrations greater than 50 μmol in the peripheral circulation are toxic to the central nervous system Thus ureagenesis is regulated to maintain peripheral ammonia concentrations below this level (Meijer *et al.* 1990). Ammonia is also a precursor for the synthesis of pyrimidines, purines, glutamate and through glutamate, of all non-essential amino acids (Krebs *et al.* 1973).

Bicarbonate, which is produced during amino acid catabolism, is also removed by ureagenesis. However up to 5% of the total HCO_3^- produced by amino acid catabolism can be excreted directly in the urine (Meijer *et al.* 1990).

2.8.6.2 The function of ureagenesis

By definition, the major function of ureagenesis is urea production (Summerskill & Wolpert, 1970; Krebs *et al.* 1978). Secondary functions of ureagenesis include the removal of surplus amino acids (Meijer *et al.* 1990) and through the removal of bicarbonate, a role in *p*H homeostasis (Atkinson & Bourke, 1984).

Although the end point of ureagenesis is self evident, the starting point is less clearly defined (Watford, 1989). The production of carbamoyl-phosphate from ammonia and bicarbonate (catalysed by carbamoyl-phosphate synthetase *I*; CPS*I*) is generally taken as the first step of ureagenesis (Figure 1.3). Therefore nitrogen enters the urea cycle ultimately as ammonia, which is sourced from amino acids (especially glutamine and glutamate, and with the exception of the branched-chain amino acids) or as free ammonia produced by the GIT.

Ureagenesis comprises five reactions (Figure 1.3) (Krebs *et al.* 1978; Cohen, 1980; Meijer *et al.* 1990);

1) The first step of ureagenesis is catalysed by carbamoyl-phosphate synthetase *I* (CPS*I*; EC 6.3.4.16).

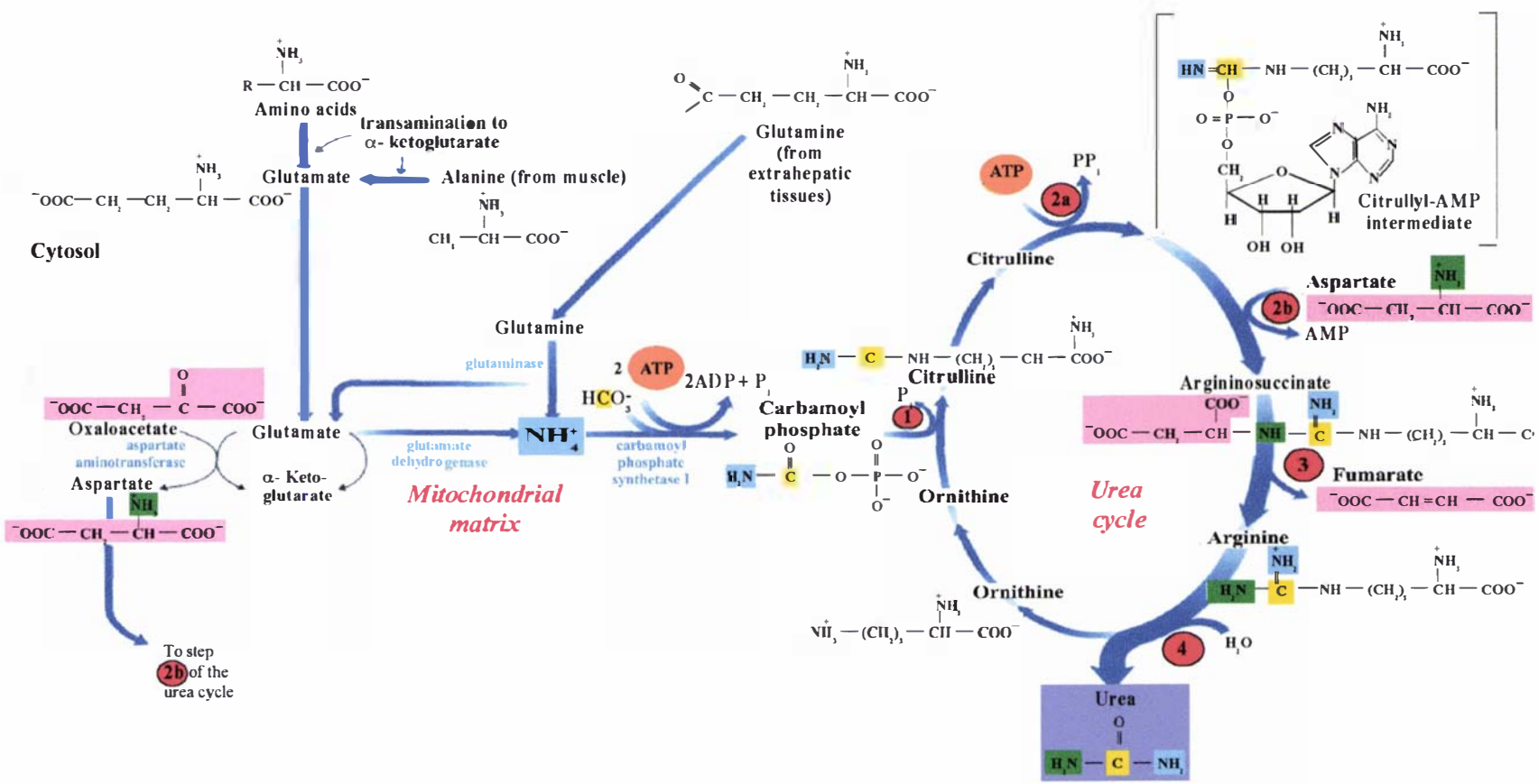
$NH_3 + HCO_3^- + 2ATP^{4-} \Rightarrow$ carbamoyl phosphate^{2-} + $2ADP^{3-}$ + Pi^{2-} + $2H^+$.

This reaction requires Mg^{2+} and K^+ as cofactors. N-acetylglutamate (NAG) is an allosteric regulator of CPS*I*. CPS*I* uses NH_3 rather than NH_4^+ (Cohen *et al.* 1985). This reaction provides the first N atom of the urea molecule.

2) Ornithine carbamoyltransferase (EC 2.1.3.3) catalyses the formation of citrulline.

Ornithine$^+$ + carbamoyl phosphate$^{2-} \Leftrightarrow$ citrulline + P^{2-} + H^+

Figure 1.3. The urea cycle.



Source; Lehninger *et al*. (1993).

3) Argininosuccinate synthase (EC 6.3.4.5) catalyses the following reaction, which requires Mg^{2+} as a cofactor. This reaction provides the second N atom of the urea molecule.

Citrulline + aspartate^{-} + ATP^{4-} $\Leftrightarrow$ argininosuccinate^{-} + AMP^{2-} + Ppi^{3-} + H^{+}

4) Argininosuccinate lyase (EC 4.3.2.1) catalyses the cleavage of argininosuccinate to arginine and fumarate.

Argininosuccinate^{-} $\Leftrightarrow$ arginine^{+} + fumarate^{2-}

5) Arginase (EC 3.5.3.10) catalyses the final step in ureagenesis

Arginine^{+} + H_2O $\Rightarrow$ urea + ornithine^{+}.

This reaction requires of Mn^{2+} as a cofactor.

2.8.6.3 Spatial arrangement of the urea cycle

The first two enzymes of the urea cycle (CPS*I* and ornithine carbamoyl transferase) are located within the mitochondria (Figure 1.3) (Gamble & Lehninger, 1973; Clarke, 1976; Cohen, 1981). Both of these enzymes have been shown to have a loose association with the inner mitochondrial membrane (Powers-Lee *et al.* 1987). This suggests that there is a degree of metabolite channelling between these two enzymes (Cohen *et al.* 1987; Powers-Lee e*t al.* 1987; Cheung *et al.* 1989; Watford, 1989).

The remaining three enzymes of the urea cycle (argininosuccinate synthase, argininosuccinate lyase and arginase) are located in the cytosol of the cell (Ratner, 1976). Early work suggested that these enzymes were soluble proteins, with no association to the mitochondrial membrane (Ratner, 1976; Cohen, 1981). Subsequent work has shown that these three enzymes are closely grouped around the outer mitochondrial membrane, with a spatial organisation that promotes efficient intermediate transfer between them (Cheung e*t al.* 1989).

The urea produced by the liver is released into the hepatic vein for subsequent removal by the kidneys and excretion in the urine (Gans & Mercer, 1984). The kidneys passively absorb urea from the blood, with the net rate of renal urea

absorption dependent on the rate of urine output and the final urea concentration in the urine (Schmidt-Nielsen *et al.* 1958).

The liver extracts arginine and releases citrulline and ornithine whereas the kidneys extract citrulline and ornithine and release arginine (Bergman & Heitmann, 1978). This constitutes a spatial separation of components of the urea cycle, in addition to providing another mechanism by which peripheral hyper-ammonaemia can be prevented (Bergman & Heitmann, 1978; Reynolds, 1992). The net renal and hepatic arginine metabolism is an example of inter-organ N transportation by an amino acid (Bergman & Heitmann, 1978).

2.8.6.4 Amino acid requirement for ureagenesis

Following the mitochondrial condensation of ornithine with carbamoyl phosphate, the resulting citrulline is transported into the cytosol as a neutral species (McGivan *et al.* 1977), therefore the transportation process does not require energy (Gamble & Lehninger, 1973).

The cytosolic aspartate required for condensation with citrulline to form argininosuccinate, is also of mitochondrial origin, *via* the malate-aspartate shuttle (De Bandt *et al.* 1995). Aspartate receives the bulk of alanine-N, which is especially important in catabolic states such as starvation, when alanine is the major substrate for ureagenesis (Meijer *et al.* 1990).

The cleavage of argininosuccinate produces arginine, which shares the same transporter as ornithine, and fumarate. The latter is therefore available for other cellular functions. Arginine and citrulline are also used to transport N to the kidneys for excretion (Bergman *et al.* 1974).

Ornithine enters the hepatocyte *via* system y^+ amino acid transporter (De Bandt *et al.* 1995), which exchanges ornithine for a proton, the capacity of which is limited (McGivan *et al.* 1977; Metoki & Hommes, 1984). However there is a non-saturable transport component (Metoki & Hommes, 1984), the contribution of which becomes increasingly important with higher amino acid

concentrations, or demands (White & Christensen, 1982; Medina *et al.* 1991). Excessive intracellular ornithine concentrations are reduced by the action of ornithine aminotransferase which deaminates ornithine producing glutamate (Gamble & Lehninger, 1973; McGivan *et al.* 1977).

The mitochondrial production of citrulline during ureagenesis requires an intra-mitochondrial supply of ornithine. Ornithine is transported into the mitochondria in exchange for citrulline (McGivan *et al.* 1977; Inoue *et al.* 1988) or a proton (Bradford & McGivan, 1980) *via* the y^+ system (White & Christensen, 1982), however there is a suggestion that another transport system is active at higher amino acid concentrations (Metoki & Hommes, 1984; Medina *et al.* 1991; De Bandt *et al.* 1995).

It has been suggested that the availability of ornithine for mitochondrial ureagenesis may be the primary regulator of the rate of ureagenesis (Gamble & Lehninger, 1973; Lund & Wiggins, 1986; Cohen *et al.* 1987). For example, under some *in vivo* conditions, carbamoyl phosphate is synthesised at rates which exceed the capacity of the mitochondria to synthesis citrulline (Hatchwell & Milner, 1978), in such cases the limiting factor may be the transport of ornithine into the mitochondria (Raijman, 1976; Cohen *et al.* 1980; Cohen *et al.* 1985; Cohen *et al.* 1987). However this suggestion has since been discarded (De Bandt *et al.*, 1995).

2.8.6.5 Pathways for nitrogen to enter the urea cycle

The form in which N enters the liver and, potentially, the urea cycle, is largely dependent on the nutritional and hormonal state of the animal (Meijer *et al.* 1990). However, for each urea molecule synthesised in the liver, half of the N must pass through ammonia (Figure 1.3, shaded blue) and the other half must pass through aspartate (Figure 1.3, shaded green) (Krebs *et al.* 1978; Nissim *et al.* 1992).

2.8.6.5.1 The first N of the urea molecule

The ammonia-N used in ureagenesis is produced by many different processes at various sites throughout the body and transported to the liver in numerous forms, including ammonia *per se.* As discussed above, proteolysis of ingested protein by the rumen resident microbes, followed by deamination of the resultant amino acids, produces large quantities of ammonia, a proportion of which is absorbed directly into the blood and transported to the liver (Reynolds, 1992). Ammonia is also produced in the lower GIT in a similar manner.

Deamination of amino acids provides the bulk of the N for ureagenesis, as both ammonia and aspartate (Krebs *et al.* 1976). The major sites of amino acid deamination are as follows;

1) One of the major end products of muscle metabolism is glutamine, which is used by the GIT mucosa as a respiratory fuel (Haussinger & Gerok, 1986), in addition to being a major contributor of N to the urea cycle (Cooper *et al.* 1988). Deamination of glutamine provides both an ammonium ion and glutamate. Similarly asparagine deamination provides both an ammonium ion and aspartate.

2) The liver is a major site of deamination for most amino acids, with the exception of branched chained amino acids (Nishikawa *et al.* 1994).

For ureagenesis, the amine group of glutamate is sourced from the transamination of most amino acids (Frieden, 1976; Rennie *et al.* 1990). Within the hepatocytes, glutamate is transported into the mitochondria by either the glutamate hydroxyl carrier (Krebs, 1972), or it is exchanged for aspartate *via* the glutamate-aspartate shuttle (LaNoue & Schoolwerth, 1979). Thus the mitochondrial and cytosolic aspartate-glutamate pools are thought to be in equilibrium (Cooper *et al.* 1987). A proportion of mitochondrial glutamate is oxidatively deaminated in the presence of glutamate dehydrogenase (EC 1.4.1.2; GLDH), producing α-ketoglutarate and an ammonium ion for use by CPS*I* and ultimately, incorporation into urea (Wanders *et al.* 1983). The available data suggests that rat and bovine GLDH have similar properties (Wanders *et al.* 1983).

The reaction catalysed by GLDH was initially considered to be freely bi-directional (Frieden, 1976), however more recent findings have demonstrated that the direction of this reaction depends on the redox state of the NAD(P) couple and the availability of the substrates and products of both the forward and reverse reactions (Wanders *et al.* 1983). The forward reaction requires $NAD(P)^+$, GTP and ADP as allosteric regulators and is inhibited by α-ketoglutarate production (Engel & Chen, 1975; Wanders *et al.* 1983). Thus under normal metabolic conditions, the forward reaction predominates (Wanders *et al.* 1983).

The reversal of the reaction catalysed by GLDH allows the N from both ammonia and glutamate, and therefore most amino acids, to contribute both of the N atoms to the urea molecule (Meijer *et al.* 1978; Parker *et al.* 1995). This suggestion was investigated using ovine hepatocytes cultured with $^{15}NH_4Cl$ as the sole N source, with the result that predominantly [$^{15}N^{15}N$]urea was produced (Luo *et al.* 1995). However, when the incubation medium was supplemented with a physiological amino acid mixture, both [$^{14}N^{15}N$]urea and [$^{15}N^{15}N$]urea were produced (Luo *et al.* 1995). When the ammonia concentration in the incubation medium was increased, the proportion of urea synthesised as [$^{15}N^{15}N$]urea also increased (Luo *et al.* 1995). However, in sheep fed lucerne pellets and infused with $^{15}NH_4Cl$, predominantly [$^{14}N^{15}N$]urea was produced (Lobley *et al.* 1995; Lobley *et al.* 1996a; Lobley & Milano, 1997). Therefore, whilst the *in vitro* ovine data (Luo *et al.* 1995), in conjunction with theoretical considerations (Brosnan *et al.* 1996; Weijs *et al.* 1996), indicate that reversal of the GLDH complex is possible, the available *in vivo* ovine data suggests that this does not normally occur (Lobley *et al.* 1995; Lobley *et al.* 1996a; Lobley & Milano, 1997).

The accepted doctrine of CPS*I* utilising GLDH generated ammonia (Frieden, 1959; Lowenstein, 1972) was challenged by the suggestion that GLDH functions primarily in the direction of glutamate formation *via* reductive amination of α-keto-glutarate, with ammonia derived from the deamination of AMP in the purine nucleotide cycle (McGivan & Chappell, 1975; Mendes-

Mourao *et al.* 1975; Moss & McGivan, 1975; Skilleter, 1975). Under this scenario, both of the N's of the urea molecule would be sourced from aspartate. However, further work suggested that the hepatic GLDH pathway supplies most of the required ammonia to carbamoyl-phosphate (Rognstad, 1977; Krebs *et al.* 1978; Wanders *et al.* 1983) and that the contribution of the purine nucleotide cycle to urea-N was insignificant (Nissim *et al.* 1992). This latter theory has subsequently been confirmed and refined, showing that the first N of the urea molecule was sourced primarily from the 5-N of glutamine *via* glutaminase (Cooper *et al.* 1988).

Mitochondrial glutaminase activity, which is restricted to periportal hepatocytes (Haussinger & Sies, 1984), produces ammonia-N which is directly channelled to CPS*I* (Meijer, 1985) and therefore ureagenesis (Katz, 1992). Using isolated rat hepatocytes incubated in ^{15}N-labelled glutamine, glutamate or aspartate, Nissim *et al.* (1992) demonstrated that glutamine-N contributed up to 70% of urea-N, confirming previous reports indicating that glutamine is the major amino acid-N contributor to urea-N (Haussinger & Gerok, 1983; Ochs, 1984; Meijer, 1985; Zaleski *et al.* 1986; Cooper *et al.* 1988; Haussinger *et al.* 1989; Haussinger, 1990; Meijer *et al.* 1990).

2.8.6.5.2 The second N of the urea molecule

The aspartate used in ureagenesis receives its amine group from glutamate (Rennie *et al.* 1990), *via* transamination with oxaloacetate in the presence of aspartate aminotransferase (Krebs *et al.* 1976). Glutamate receives the amine group from most amino acids (*via* transamination with α-keto-glutarate), but predominantly from alanine, glutamine, proline and histidine (Meijer *et al.* 1990). Contrary to earlier suggestions (McGivan & Chappell, 1975), the purine nucleotide cycle is not a major N contributor to ureagenesis (Krebs *et al.* 1978).

2.8.6.6 Regulation of ureagenesis

Ureagenesis is regulated to maintain non-toxic peripheral ammonia concentrations (less than 50 μmol/l) rather than complete removal of portal

ammonia (Haussinger & Gerok, 1986). Ammonia is used in the synthesis of glutamate and through glutamate, for the synthesis of all non-essential amino acids. It is also a key metabolite in the mitochondrial redox state (Haussinger & Gerok, 1986).

The rate of ureagenesis may be regulated by the availability of substrates, the rate of product removal or changes in ureagenic enzyme activity, assuming that both energy and cofactors are non-limiting (Meijer *et al.* 1990).

The major substrates used to synthesis urea are ammonia, bicarbonate, aspartate and water (Krebs & Henseleit, 1932). The near equilibrium of aspartate aminotransferase and the GLDH reaction ensures that aspartate and ammonia are always in equilibrium and that aspartate is available for ureagenesis (Haussinger & Gerok, 1986). Aspartate is produced by the transamination of glutamate (Ratner, 1973), therefore it is unlikely that the supply of this amino acid, nor any of the other substrates, would limit the rate of ureagenesis (Ratner, 1976).

Ureagenesis requires HCO_3^-, not CO_2, however HCO_3^- cannot penetrate the mitochondrial membrane. Therefore HCO_3^- must be produced within the mitochondria by carbonic anhydrase in addition to the HCO_3^- produced from the hydration of CO_2 (Dodgson *et al.* 1983). There is the possibility that this mechanism affects the mitochondrial and cellular *p*H, however it has been suggested that ureagenesis has no role in the maintenance of cellular *p*H (Boon *et al.* 1994).

The products of ureagenesis are urea and fumarate. Excess fumarate can be used in the citric acid cycle (Kelly *et al.* 1993) and urea is excreted in the urine *via* the kidneys (Gans & Mercer, 1984).

The long term (hours or days) control of ureagenesis is achieved by changes in enzyme mass (Saheki *et al.* 1977; Mori *et al.* 1981) *via* modifications to the rate(s) of enzyme synthesis or degradation (Schimke, 1964; Tsuda *et al.* 1979).

Short term (seconds or minutes) control is achieved *via* the activation or inhibition of the existing enzyme molecules (Meijer e*t al.* 1990). The enzymes have half lives of 3-9 days (Schimke, 1964; Nicoletti e*t al.* 1977; Tsuda e*t al.* 1979; Haggerty e*t al.* 1982; Wallace e*t al.* 1986), whereas the mRNA's coding for the synthesis of these enzymes have half lives of several hours (Morris, 1992).

The activities of the five enzymes involved in ureagenesis are all increased under conditions of increased amino acid catabolism, such as high protein intakes (Schimke, 1962; Schimke, 1963; Morris, 1992). The abundance of the mRNA's for the ureagenic enzymes correlates well with the activities of these enzymes and the level of dietary protein intake (Mori e*t al.* 1981; Ryall e*t al.* 1984).

Two of the enzymes of ureagenesis are potentially rate limiting, that is argininosuccinate synthetase and CPS*I* (Haussinger & Gerok, 1986). Argininosuccinate synthetase has been shown to be limiting when the substrates are available at saturation concentrations, both *in vitro* and in isolated perfusion preparations (Saheki & Katunuma, 1975; Morris, 1992). However *in vivo*, with non-saturating substrate concentrations, CPS*I* is the rate limiting enzyme of ureagenesis (Duda & Handler, 1958; Meijer & Hensgens, 1982; Cohen e*t al.* 1985; Meijer e*t al.* 1985; Morris, 1992). This is more biologically economical, considering CPS*I* catalyses the first step of ureagenesis (Newsholme & Leech, 1983b).

The activity of CPS*I* is largely dependent on the concentration of its allosteric regulator, N-acetylglutamate (NAG) (Shigesada & Tatibana, 1971; Lusty, 1981), as confirmed by the high correlation between intra-mitochondrial NAG concentration and the rate of citrulline synthesis (McGivan e*t al.* 1976). The mitochondrial synthesis of NAG is catalysed by NAG synthetase (Kawamoto e*t al.* 1982; Kawamoto e*t al.* 1985), which is activated by arginine (Shigesada e*t al.* 1978) and increased protein intake (McGivan e*t al.* 1976). With a mitochondrial half life of 20 minutes (Shigesada e*t al.* 1978), NAG provides

effective short term regulation of ammonia metabolism *via* ureagenesis (Kawamoto *et al.* 1982; Kawamoto e*t al.* 1985).

The rate of ureagenesis is affected by the portal ornithine concentration (Chamalaun & Tager, 1970). The availability of ornithine within the mitochondria may limit the rate of ureagenesis (Lund & Wiggins, 1986). There is, to date, insufficient evidence to include ornithine transport into the mitochondria as *a* rate limiting step in ureagenesis (De Bandt e*t al.* 1995).

The rate of ureagenesis is increased by hormones including glucagon, catecholamines, angiotensin II, vasopressin, and thyroid hormone, due to the influence these hormones have on substrate supply (Haussinger & Gerok, 1986; Morris, 1992).

2.8.6.7 The energetic cost of ureagenesis

Hepatic ureagenesis is energetically expensive, as indicated by the percentage of hepatic and whole body oxygen consumption attributed to ureagenesis *per se*; 7.1% and 1.4% respectively (McBride & Kelly, 1990).

Ureagenesis consumes four moles of ATP per mole of urea synthesised (Martin & Blaxter, 1965; Parker *et al.* 1995). The hydrolysis of ATP yields 30.5 kJ/mole (Lehninger *et al.* 1993), therefore the energy cost of urea synthesis is 122 kJ/mole.

It has been suggested that the energy cost of ureagenesis should be discounted by the energy produced from the oxidation of fumarate, which is produced in equi-molar quantities to urea during ureagenesis, *via* the citric acid cycle (Newsholme & Leech, 1983a; Reynolds *et al.* 1991; Kelly *et al.* 1993). This hypothesis would suggest that the cost of ureagenesis would be one mole of ATP per mole of urea synthesised. This theory has not received significant support (Parker e*t al.* 1995), and subsequent *in vivo* work has confirmed earlier calculations of 4 moles of ATP per mole of urea (Lobley *et al.* 1995; Lobley e*t al.* 1998).

2.8.6.8 Variations in hepatic ammonia load

The ammonia produced in the PDV is almost entirely removed from the portal blood by the liver (Reynolds & Huntington, 1988; Huntington, 1989; Burrin *et al.* 1991; van der Walt, 1993). The rate of PDV ammonia production is predominantly influenced by dietary factors such as the level of protein intake, the type and form of dietary protein, the site of protein digestion, the rate of digesta transit through the GIT, and the composition of the feed.

There is a positive correlation (R^2 = 0.74-0.91) between N intake and the rate of hepatic ammonia absorption (van der Walt, 1993; Parker *et al.* 1995). Changes in the intake level of rumen degradable protein dramatically influence the level of PDV ammonia production (Huntington, 1990). In beef steers fed iso-energetic diets containing either 12 or 17% crude protein, there was a two fold increase in PDV ammonia production, liver ammonia absorption, and hepatic urea production and a three fold increase in α-amino-N removal for the animals on the high compared to low protein intakes (Reynolds *et al.* 1991; Reynolds, 1992). Similar results have also been demonstrated in other species (Brosnan, 1976; Cooper *et al.* 1987).

Hepatic ureagenesis has a finite capacity of approximately 1.5-2.0 μmol urea-N/g liver/min (Symonds *et al.* 1981; Orzechowski *et al.* 1987; Lobley & Milano, 1997). Therefore, hepatic ammonia absorption at greater than the capacity of ureagenesis may result in either increased glutamine synthesis, expansion of the intracellular free ammonia pools, increases in the proportion of NH_3-N contributing to urea-N or hyperammonaemia (Lobley & Milano, 1997).

Estimates of the contribution of NH_3-N to urea-N range from 27 to 110% (Huntington, 1989; Maltby *et al.* 1991; Reynolds *et al.* 1991; Parker *et al.* 1995). When hepatic ammonia loads were increased by acute 3 h (Wilton & Gill, 1988) or chronic 5 day (Lobley *et al.* 1995) NH_4Cl infusions into the mesenteric vein of sheep, the increase in hepatic urea production was approximately twice that needed to detoxify the additional NH_3-N load. However, when the

experiment was repeated using NH_4HCO_3, there was no affect on arterial *p*H and the additional urea-N produced was equivalent to 1.2 times the NH_3-N infused (Lobley *et al.* 1996b), confirming *in vitro* work (Mutsvangwa *et al.* 1996). When the hepatic ammonia detoxification load exceeds the ureagenic capacity it is theoretically possible for both of the N atoms of the urea molecule to be provided by NH_3-N *via* the reversal of the normal direction of the GLDH complex (Meijer *et al.* 1978; Parker *et al.* 1995). However, the low, close to detection limits, atoms percent enrichment (APE) of [$^{15}N^{15}N$]urea following the chronic 5 day infusion of $^{15}NH_4Cl$ into the mesenteric vein of sheep (Lobley *et al.* 1995), suggests that reversal of the GLDH complex is not a medium to long term mechanism to avert hepatic ammonia overload. This finding was confirmed by a comparable study in humans fed $^{15}NH_4Cl$ (Weijs *et al.* 1996). More recent acute (20 min) infusions in the ovine of 1.1 mmol/min NH_4HCO_3 into the mesenteric vein, which induced peripheral hyperammonaemia, however indicates that the contribution of NH_3-N to urea-N increases from 46% to 89%, suggesting that the reversal of the GLDH complex is a possible short term (30 min) mechanism which may be used to prevent peripheral hyperammonaemia (Lobley & Milano, 1997).

2.8.6.9 Variations in hepatic amino acid supply

The specific amino acids required for ureagenesis are aspartate, producing fumarate, and glutamine, producing glutamate *via* glutaminase, and in turn glutamate can provide NH_3-N to ureagenesis (Krebs *et al.* 1976). The hepatic aspartate concentrations are kept relatively constant *via* amino-transferases (Krebs *et al.* 1976).

Ureagenesis is continuous, even during fasting (Heitmann & Bergman, 1980a; Miller, 1982; Jackson, 1995), as a result of ammonia production by non-GIT tissues (van der Walt, 1993). Hepatic amino acid extraction still occurs during fasting (Bergman & Pell, 1984), with the amino acid requirement for ureagenesis (and other hepatic functions) being supplied by muscle protein degradation which yields predominantly glutamine and alanine (Elwyn *et al.* 1968; Bergman & Heitmann, 1978).

The proportion of the PDV amino acid output that is absorbed by the liver ranges from 20% (Guerino *et al.* 1991) to greater than 100% (Burrin *et al.* 1991). The level of hepatic amino acid uptake is highly correlated (R^2 = 0.88) with protein intake (van der Walt, 1993). When the liver is supplied with amino acids in excess of hepatic requirements, the rate of hepatic amino acid absorption increases (Lobley & Milano, 1997). One of the major demands on hepatic amino acid supply is hepatic protein synthesis. When hepatic amino acid supply is in excess of requirements the rate of protein synthesis is maintained, whilst the rate of protein degradation decreases, resulting in a net increase in protein synthesis (Natori, 1995). The rate of hepatic ammonia absorption for ureagenesis does not decrease when hepatic amino acid absorption increases, which indicates that peripheral hyperaminoacidaemia is preferred to hyperammonaemia (Lobley & Milano, 1997).

2.8.6.10 Comparison with in vitro work

It is often convenient to draw comparisons between *in vivo* and *in vitro* work, however such comparisons should be treated with caution. *In vitro* methodology typically utilises isolated cell preparations, which effectively disrupts the cellular organisation present *in vivo* and therefore affects the channelling of metabolites between enzymes and from the cytosol to the mitochondria (Watford, 1989). Cellular enzymes are typically considered to be soluble and free, however a number of the enzymes of ureagenesis are either membrane bound or show a degree of compartmentalisation (Powers-Lee *et al.* 1987; Srere, 1987; Cheung *et al.* 1989), as such *in vitro* work may destroy these relationships. However, with the above considerations in mind, the use of *in vitro* analysis provides indicative findings with which to direct further *in vivo* work.

The isolated perfusion of an organ is a technique which has also been used extensively for metabolic studies (Haussinger, 1990). This technique effectively translates an internal organ into an isolated perfused tissue, simply by altering the vascular supply and drainage (Mortimore & Surmacz, 1984).

The liver, particularly of small laboratory species, is particularly amenable to this technique and has been studied extensively (Linzell *et al.* 1971; Haussinger, 1990). By reversing the direction of the blood flow through such liver preparations, differential hepatocyte functionality was first demonstrated (Lueck & Miller, 1970; Haussinger *et al.* 1975; Haussinger *et al.* 1986).

2.8.6.11 Ureagenesis versus glutamine synthetase activity

Ureagenic capacity is restricted to the periportal hepatocytes with glutamine synthetase activity reciprocally restricted to the perivenous hepatocytes (Gebhardt & Mecke, 1983; Gaasbeekjanzen *et al.* 1984; Gebhardt & Mecke, 1984). Ureagenesis is a low affinity-high capacity ammonia removal regimen whereas glutamine synthetase activity has a high affinity-high capacity for ammonia removal (Deuel *et al.* 1978). Under normal metabolic conditions, the bulk of the hepatic ammonia load is detoxified *via* ureagenesis. Glutamine synthetase is used as a backup, scavenger system for ammonia detoxification (Haussinger, 1990). A proportion of the hepatic urea production undergoes renal hydrolysis and subsequent excretion (Haussinger, 1990).

Hepatic glutamine synthetase activity effectively prevents peripheral hyperammonaemia by removing excess ammonia from the hepatic venous blood (before it enters the *vena cava*) and synthesising glutamine from the glutamate that the perivenous hepatocytes absorb (Haussinger *et al.* 1989).

The bulk (70-100%) of hepatic glutamate uptake is *via* the perivenous hepatocytes *per se* (Haussinger & Gerok, 1983; Taylor & Rennie, 1987; Cooper *et al.* 1988; Haussinger *et al.* 1989) where it is predominantly used for glutamine synthesis (Haussinger & Gerok, 1983; Haussinger *et al.* 1989; Stoll & Haussinger, 1989).

The liver has both periportal glutaminase and perivenous glutamine synthetase activity operating concurrently (Haussinger & Sies, 1979; Haussinger, 1983; Haussinger *et al.* 1983; Sies & Haussinger, 1984; Haussinger & Gerok, 1986; Haussinger *et al.* 1986). This represents an intercellular cycling of glutamine.

Under normal metabolic conditions (particularly blood *p*H, portal ammonia and HCO_3^- concentrations) the rate of this glutamine cycle is approximately 0.1 μmol/min/g liver (Haussinger & Sies, 1979).

The flux through glutaminase is *p*H sensitive, due to the direct effect of *p*H on the glutamine transporter systems (Lueck & Miller, 1970; Haussinger e*t al.* 1983; McGivan & Bradford, 1983; Verhoeven e*t al.* 1983; Haussinger e*t al.* 1984; Lenzen e*t al.* 1987). The activity of glutaminase is not product inhibited (Krebs, 1935), however this enzyme does require ammonia as an essential activator (Haussinger e*t al.* 1975; Haussinger & Sies, 1979; Haussinger e*t al.* 1983; McGivan & Bradford, 1983; Verhoeven e*t al.* 1983). Fluctuations in portal ammonia concentrations are paralleled by changes in glutaminase activity (Haussinger & Sies, 1979). There is evidence to suggest that the products of glutaminase, glutamate and ammonium, are channelled directly to CPS*I* (Lenzen e*t al.* 1987), and that both enzymes require NAG as an activator (Meijer & Verhoeven, 1986). Glutaminase activity provides up to 30% of the hepatic ammonia detoxificaiton capacity (Haussinger, 1983; Haussinger e*t al.* 1984).

Ureagenesis effectively removes equimolar quantities of HCO_3^- and NH_4^+, (a strong base and weak acid respectively), therefore the relative rate of ureagenesis can influence whole body *p*H (Atkinson & Bourke, 1984; Haussinger e*t al.* 1984). For example, under acidotic conditions, the liver can decrease the rate of ureagenesis, effectively retaining HCO_3^- and thereby neutralising the acidosis. With the decreased rate of ureagenesis there is a potential for peripheral hyperammonaemia. However, under this scenario, the perivenous glutamine synthetase activity effectively removes the ammonia, thereby preventing peripheral hyperammonaemia (Haussinger e*t al.* 1984). The role of ureagenesis in *p*H homeostasis is regulated by the rate of HCO_3^- and NH_4^+ delivery to CPS*I* and possibly *via* NAG activation of CPS*I* (Haussinger, 1990).

2.8.6.12 The inter-relationship between the urea and citric acid cycles

Most dietary carbohydrates are fermented to VFA's within the rumen (Reynolds *et al.* 1988a; Seal & Parker, 1993; Goetsch *et al.* 1994), consequently only small quantities of glucose are available to be absorbed from the GIT (Bergman & Heitmann, 1978; Reynolds, 1992). Therefore ruminants are reliant on *de novo* gluconeogenesis to meet the bodys' glucose requirements (Weighart *et al.* 1986; Mutsvangwa *et al.* 1996). Gluconeogenesis occurs in both the liver (85%) and kidneys (15%) (Lindsay & Armstrong, 1982). Propionate, produced from rumen carbohydrate fermentation, is the major gluconeogenic precursor in fed ruminants (Armstrong, 1965), however other precursors, such as amino acids, are also utilised (Bergman & Heitmann, 1978).

The relative contribution of amino acids to hepatic gluconeogenesis is still to be ascertained (Reynolds, 1992; Seal & Reynolds, 1993).

It has been estimated that up to 30% of the hepatic glucose production can be attributed directly to hepatic amino acid metabolism (Wolff & Bergman, 1972a). However, data from various isotopic studies suggest that this percentage is approximately half (15% of glucose synthesised by the liver) the earlier figure (Bergman & Heitmann, 1978). Approximately 50% of the amino acid contribution to hepatic gluconeogenesis is from just two amino acids; alanine and glutamate (Wolff & Bergman, 1972a; Bergman & Heitmann, 1978; Lindsay & Armstrong, 1982). Accordingly these glucogenic amino acids generally have a high rate of hepatic extraction (Haussinger & Gerok, 1986).

The processes of gluconeogenesis and ureagenesis are integrated *via* a common requirement for energy and shared intermediaries, such as aspartate, glutamate and α-keto-glutarate (Krebs *et al.* 1976).

Maximal rates of gluconeogenesis and ureagenesis do not appear to be limited by energy (ATP) supply (Krebs *et al.* 1976).

Ureagenesis requires lactate as a precursor of the carbon skeletons of glutamate and aspartate (*via* α-keto-glutarate and oxaloacetate), however, lactate can also be replaced with glutamine, asparagine, proline, alanine or pyruvate (Krebs e*t al.* 1976). The rate of gluconeogenesis is decreased in the presence of a high ammonium concentration, not elevated rates of ureagenesis *per se*, due to the reduced supply of α-keto-glutarate under such conditions (Krebs e*t al*. 1976).

2.9 CONCLUSION

Digestion of dietary protein in the rumen produces peptides, amino acids and ammonia (Ulyatt & MacRae, 1974). Within the rumen, highly soluble proteins are rapidly degraded, predominantly to ammonia (Mangan, 1982). Therefore, the high soluble protein content of fresh forage results in considerable ammonia production in the GIT, which is subsequently detoxified to urea by the liver. This requires stoichiometric increases in the utilisation of aspartate-N, supplied from either glutamate *via* amino-transferases or NH_3-N *via* the reversal of the GLDH complex (Meijer e*t al.* 1990).

If the GLDH complex were to function in the reverse direction, urea would be produced with both N atoms originating from ammonia (Meijer e*t al.* 1990), however, the *in vivo* data suggest that this does not normally occur (Lobley e*t al.* 1995; Lobley e*t al.* 1996a; Lobley & Milano, 1997).

Ureagenesis utilises the amine group of aspartate, which is initially provided by glutamate and ultimately sourced from the transamination of most amino acids (Lobley e*t al.* 1996b). Hence ureagenesis has an obligatory requirement for amino acids. Thus, amino acid utilisation for ureagenesis effectively restricts the availability of amino acids for other purposes such as protein synthesis (van der Walt, 1993), therefore high rates of ureagenesis can potentially impact on the growth rate of the animal (Reynolds, 1992). The amino acid metabolism of ruminants grazing fresh forages may be subjected to a double negative penalty; firstly in terms of the large loss of ingested dietary proteins as NH_3-N

from the rumen and secondly, amino acid catabolism to detoxify ammonia *via* ureagenesis (Reynolds, 1992; Lobley & Milano, 1997).

2.9.1 Objectives

The objectives of the present study were given in section 1.2. Briefly, the study was designed to test the hypothesis that increased rates of hepatic ammonia detoxification require an increase in the rate of hepatic amino acid catabolism. To test this hypothesis, the rate of hepatic ammonia absorption, urea production and concomitant hepatic amino acid metabolism have been determined in sheep fed different feeding regimens affecting soluble protein intakes. Three separate experiments were conducted in which sheep were fed: 1) lucerne pellets or fresh white clover, 2) different intake levels of fresh white clover, and 3) fresh white clover offered during two 2 hour periods per day. Finally, the activities of some of the key enzymes involved with controlling the rate of ammonia detoxification have been investigated.

3 CHAPTER 3. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) OR LUCERNE (*MEDICAGO SATIVA*) PELLETS

3.1 ABSTRACT

The effect of feeding two high protein diets (fresh white clover or lucerne pellets), differing in protein degradability in the rumen-reticulo (rumen), on splanchnic ammonia and urea production, oxygen consumption, amino acid utilisation and whole body and hepatic protein synthesis was studied in four sheep. A cross-over design was used so that the same four sheep were used for each diet. The animals were offered 900 g DM/d for 20 d prior to continuous (10 h) infusions of $^{15}NH_4Cl$, [^{35}S]-methionine and *para*-aminohippurate *via* the mesenteric vein and [^{3}H]-phenylalanine *via* the jugular vein. Blood samples were collected to determine ammonia, urea, oxygen, and amino acid concentrations in the mesenteric, portal and hepatic vein and aorta. Rumen ammonia production was significantly higher in the animals fed the fresh white clover diet (746 v 185 μmol/min; SED 51.1; $P < 0.001$), however this difference was not reflected in hepatic urea production (1.64 v 0.57 mmol/min; SED 1.63; $P = 0.534$). There was no difference in hepatic amino acid utilisation between the two diets (12.0 v 9.8 g N/d; SED 1.99; $P = 0.305$) and hence no consistent difference in protein synthesis rates. Both mass transfer and ^{15}N kinetics confirmed that hepatic ammonia extraction accounted for no more than 0.50 of the urea produced by the liver. The absence of [$^{15}N^{15}N$]-urea suggested that ammonia contributed only one N atom to urea.

There was no apparent difference in the energetic cost of ureagenesis as a proportion of metabolisable energy intake (0.031 v 0.009; SED 0.0962; $P = 0.505$). This work demonstrated that the hepatic ammonia detoxification rate of these sheep (fed near maintenance intake levels) approached known metabolic maximum levels. In grazing sheep with higher than maintenance intakes of fresh forages, it may be expected that hepatic ammonia overload will occur (at least at a sub-clinical level) and this may impair animal productivity.

3.2 INTRODUCTION

Ruminant animal production in temperate countries such as New Zealand is reliant on the utilisation of fresh forages (Ulyatt, 1997) which are characterised by containing highly soluble protein (0.75-0.80 solubility; Mangan, 1982). In ruminants fed fresh forage diets, substantial rapid degradation of dietary protein to ammonia occurs in the reticulo-rumen (rumen), with a proportion of this ammonia subsequently incorporated into microbial protein (Parker *et al.* 1995). A substantial proportion of the ammonia produced in the rumen is utilised for microbial protein synthesis (0.30-0.50 N intake; Huntington, 1982; Parker *et al.* 1995). Of the remainder, most is passively absorbed across the wall of the rumen into the venous blood for transport to the liver and a smaller amount is passed with digesta to the lower digestive tract (Nolan & Leng, 1972). Ammonia is removed from the venous blood by the liver, where it is metabolised into urea to maintain systemic ammonia concentrations below those toxic to the central nervous system (Remond *et al.* 1993).

Previous studies, reviewed by Parker *et al.* (1995), have shown that the rate of hepatic ureagenesis typically exceeds the rate of hepatic ammonia extraction. From these findings it has been suggested that a significant quantity of amino acid-N is diverted from anabolic processes to provide additional N for the formation of urea under conditions of high rates of hepatic ammonia extraction (Lobley *et al.* 1995). This would certainly be the case in ruminants fed fresh forage, particularly during spring and early summer.

In the present study sheep were fed two high N content diets, differing markedly in the degradability of the protein in the rumen. The aim was to determine whether the metabolic cost of ammonia detoxification was higher in animals fed a fresh forage (fresh white clover {*Trifolium repens*}; high rumen protein degradability) compared to lucerne pellets (*Medicago sativa*; low rumen protein degradability; Lobley *et al.* 1995). Grinding, pelleting and drying feeds increases the proportion of dietary protein that escapes degradation in the rumen, thus effectively reducing the rumen ammonia production (Preston & Leng, 1987b).

To test this hypothesis, four sheep were first subjected to a N balance then rumen ammonia production, hepatic ammonia and amino acid metabolism were quantified to determine the metabolic cost of ammonia detoxification in these sheep. The animals were infused with $^{15}NH_4Cl$, to trace the hepatic detoxification of ammonia to urea, and both [^{3}H]-phenylalanine and [^{35}S]-methionine to investigate whole-body and hepatic protein synthesis rates.

3.3 MATERIALS AND METHODS

These experimental procedures and protocols were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Palmerston North, New Zealand according to the Animals Protection Act (1960), Animals Protection Regulations (1987) and amendments.

3.3.1 Animals and surgical procedures

Four Romney-cross wether lambs with body weights between 35 and 42 kg (39.8 SE 1.6) and aged 6-9 months were habituated to metabolism crates and automatic hourly feeding (900 g DM/d lucerne pellets) for ten days prior to surgery.

General anaesthesia was maintained with halothane whilst indwelling catheters were inserted into the posterior aorta *via* the femoral artery, and into the mesenteric (x2), portal, and hepatic veins following the procedure originally described by Katz & Bergman (1969a) and modified by Lobley *et al.* (1995). For further details on the surgical procedure see section 8.2.1. The catheters comprised silicone rubber for that portion residing inside the vessel, with polyvinyl chloride (PVC) being used for the remainder of the catheter (Lobley *et al.* 1995). For further details on the catheter construction see section 8.2.2. Temporary catheters (PVC only) were inserted into the jugular vein on the day prior to each experimental period using the procedures described by Harris *et al.* (1992). The animals were allowed a four week post surgical recovery period before the experiment commenced.

3.3.2 Diets and design

The sheep were fed a diet of either fresh white clover or lucerne pellets. The white clover was harvested daily from a pure sward with a minimum of 4-6 weeks growth. The lucerne pellets were sourced from a commercial company and were made from a single harvest of lucerne.

The animals were offered 900 g DM/d for 10 days before entering a 10 day N balance. Immediately following the N balance, the sheep were infused with $^{15}NH_4Cl$ into the mesenteric vein to quantify the rate of ureagenesis and to identify the sources of N for ureagenesis. The rate of protein synthesis (both whole body and hepatic) was estimated following infusions of [^{3}H]-phenylalanine and [^{35}S]-methionine into the jugular and mesenteric veins, respectively.

Using a cross-over design, each sheep was then placed on the alternate diet and given a minimum adjustment period of two weeks prior to repeating the experimental procedure.

3.3.3 Infusion protocol

Each sheep was continuously infused (24.6 g/h) into the mesenteric vein with either 146 mmol/l (animals fed fresh white clover) or 73 mmol/l (animals fed lucerne pellets) of $^{15}NH_4Cl$ (99.8 atoms percent enrichment, APE; Icon, Mt Marion, USA) for 9.5 hours. The duration and infusion rate of the $^{15}NH_4Cl$ was based on earlier work with lucerne pellet fed sheep (Lobley *et al.* 1995) and assumed a two fold increase in portal ammonia flux for the fresh white clover fed animals. After infusing $^{15}NH_4Cl$ for 3.5 hours, 259 MBq/l L-[ring 2,6 ^{3}H]-phenylalanine (NEN Research products, Wilmington, USA) containing 60 μmol/l of unlabelled phenylalanine (Sigma Chemicals, St Louis, USA) as a carrier was infused (24.6 g/h) into the jugular vein for 6 hours. After 5.75 hours of $^{15}NH_4Cl$ infusion, the $^{15}NH_4Cl$ infusate was supplemented with sodium *para*-aminohippurate (*p*AH; Sigma Chemicals, St Louis, USA; 203 mmol/l), 444

MBq/l [^{35}S]-methionine (NEN Research products, Wilmington, USA) and 60 μmol/l of unlabelled methionine (Sigma Chemicals, St Louis, USA) as a carrier. The infusion was continued for a further 3.75 hours. Thus the $^{15}NH_4Cl$, [^{3}H]-phenylalanine, [^{35}S]-methionine and the *p*AH were infused for a total of 9.5, 6, 3.75 and 3.75 hours respectively. For further details on the infusion of $^{15}NH_4Cl$ see section 8.2.3.

Prior to commencing blood sampling, the sheep were manually infused with a bolus of 5,000 IU heparin (Ovine origin; New Zealand Pharmaceuticals, Palmerston North, New Zealand). Blood samples were withdrawn (at 24.6 g/h) with a peristaltic pump (302F, Watson-Marlow Limited, Cornwall, England) from the mesenteric, portal, and hepatic vein, and arterial catheters for the first 30 minutes of each of the last four hours of the $^{15}NH_4Cl$ infusion. The collection lines were passed through ice to reduce the risk of coagulation and degradation of the blood constituents, with the blood collected into 15 ml polypropylene vials containing 50 μl sodium ethylenediaminetetra-acetic acid (EDTA; 0.15 w:v in water) as an anti-coagulant.

3.3.4 Analytical measurements

The packed cell volume of whole blood was determined for each sample by haematocrit. Blood oxygen concentrations were measured immediately after the blood was collected using a galvanic oxygen cell (Grubb & Mills, 1981). All subsequent procedures were performed gravimetrically.

To 0.5 g blood, 5 g 12% (w:w) trichloroacetic acid (TCA) was added and the sample was centrifuged (at 3270 G for 15 minutes at 4 °C). The *p*AH concentration was determined in the resultant supernatant using the method described by Harris *et al.* (1992) following acid deacetylation of the supernatant at 90 °C for 65 minutes (Lobley *et al.* 1995). For further information on the estimation of the blood flow see section 8.2.4.

Whole blood (2 g) was haemolysed with an equal weight of chilled 200 μmol/l L-nor-leucine (BDH Chemicals Ltd., Poole, England) to which 200 mg of 80 mmol/l dithiothreitol (DTT) in 0.2 mol/l phosphate buffer (*p*H 8.0) was added.

Haemolysed blood (1 g) was ultra-filtered through a 10,000 molecular weight cut-off membrane (Centrisart®; Sartorius, Gottingen, Germany). Then, 100 mg of the resultant supernatant was used for the determination of amino acid concentration by picotag analysis and reverse phase high performance liquid chromatography (HPLC) according to the method described by Cohen *et al.* (1986). All values were corrected to the initial gravimetric addition of the internal L-nor-leucine standard. For further details on the HPLC methodology see section 8.2.5.

The enrichment of [^{15}N]-amino acids and [^{15}N]-urea in haemolysed blood was determined by gas chromatography and mass spectrometry (GCMS) using the methods described by Nieto *et al.* (1996). The remaining blood was then centrifuged and the plasma removed. For further details on the GCMS methodology see section 8.2.6.

The ammonia concentration (conducted non-gravimetrically) was determined in plasma (100 μl) using a commercial kit (#171; Sigma Chemicals, St Louis, USA), based on the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase. Plasma samples were stored on ice for up to six hours prior to analysis. For further details on the assay method used to quanitate the ammonia concentration see section 8.2.7.

The ammonia present in plasma (1 g; stored on ice for up to six hours) was converted to nor-valine to allow the subsequent determination of the [^{15}N] enrichment of ammonium using a GCMS (VG Trio-1, VG Masslab Ltd, Manchester, Cheshire, UK) according to the method described by Nieto *et al.* (1996). For further details on the GCMS methodology see section 8.2.6.

Urea concentrations were determined in plasma (100 mg) using a commercial kit (#535; Sigma Chemicals, St Louis, USA), utilising the conversion of urea to hydroxyl-amine in the presence of diacetylmonoxime when heated with sulphuric acid. The assay was repeated until the coefficient of variation between duplicate samples was less than 0.1. For further details on the method used to quantitate the urea concentration see section 8.2.8.

Plasma cysteine concentrations were determined in whole blood (1 g) following haemolysis using a spectro-photometric method after reaction with acid ninhydrin as described by Gaitonde (1967) and modified by Lee *et al.* (1993).

The radioactivities associated with [^{3}H] and [^{35}S] in each of the haemolysed blood and plasma samples were measured simultaneously using a liquid scintillation counter (Model 1500 Tricarb, Packard, Zurich, Switzerland) within one month of the infusion to avoid significant [^{35}S] decay. The relative proportion of the total radioactivity attributable to each amino acid and oxidation products were determined by inline flow through a liquid scintillation counter (Model 2, β-ram, IN/US systems Inc., New Jersey, US) using an HPLC (LC4A, Shimadzu, Kyoto, Japan) and the methodology described by Lee *et al.* (1993).

3.3.5 Calculations and statistics

Blood flow (BF) was calculated using the equations described by Katz & Bergman (1969b). Hepatic arterial blood flows were calculated by difference between the hepatic and portal venous flows, with negative values omitted from subsequent calculations (White e*t al.* 1967; Bergman, 1975).

Mass transfers of metabolites, Z, across the mesenteric drained viscera (MDV) were calculated using equation 1 as described by Huntington & Reynolds (1987) and Lobley *et al.* (1995);

$$MDV = (Z_m - Z_a) \times BF_m \qquad \text{Equation 1}$$

where the subscripts *m* and *a* represent the mesenteric vein and artery, respectively. Net metabolite appearance or production was indicated by positive values whilst negative values indicated net utilisation by the tissue(s).

Similarly transfers of metabolites across the portal drained viscera (PDV) were calculated from equation 1 after substituting the portal concentration (Z_p) and portal blood flow (BF_p) for those of the mesenteric vessel.

The mass transfer of metabolites across the rumen was calculated as the difference between the PDV and MDV transfers (Reynolds & Huntington, 1988).

Hepatic mass transfer of metabolites (Z) were calculated using equation 2 as described by Huntington & Reynolds (1987) and Lobley *et al.* (1995);

$$HMT = (Z_h \times BF_h) - (Z_p \times BF_p) - (Z_a \times BF_{ha}) \qquad \text{Equation 2}$$

where the subscript *h* denotes the hepatic vein and *ha*, the hepatic artery.

The irreversible loss rate (ILR) of phenylalanine and methionine were calculated using equation 3 as described by Shipley & Clark (1972);

$$ILR = IR_a / SRA_a \qquad \text{Equation 3}$$

where IR_a was the infusion rate (MBq/h) of either [^{3}H]-phenylalanine or [^{35}S]-methionine and SRA_a (MBq/mmol) was the specific radioactivity associated with either phenylalanine or methionine in arterial (posterior aorta) blood, at plateau. There was no measurable SRA in the amino acid oxidation products (tyrosine and phenylpyruvate; cysteine, methionine-sulphoxide, and sulphate, respectively), therefore it was assumed that the contribution of oxidation (for the two infused amino acids) to the ILR was negligible (Waterlow *et al.* 1978a). Therefore, the rate of whole body protein synthesis was calculated from the

phenylalanine and methionine ILR (mmol/h) using the procedure described by Harris *et al.* (1992). It was assumed that ovine whole-body protein comprised 0.035 and 0.015 phenylalanine and methionine, respectively (MacRae *et al.* 1993). It was also assumed that the ILR of phenylalanine and methionine to whole-body protein synthesis were proportional to the fractional contribution of each amino acid to whole-body protein content (Cronje *et al.* 1992).

The fractional synthesis rate (FSR) of whole body protein was calculated using equation 4 as described by Waterlow *et al.* (1978b);

FSR = rate of protein synthesis / whole body protein content Equation 4

where the rate of protein synthesis was based on the ILR of either phenylalanine or methionine. It was assumed that protein comprised 155 g/kg body weight (MacRae *et al.* 1993).

The net ^{15}N-ammonia and ^{15}N-urea transfers across the liver were calculated using equation 5;

$$H^{15}NT = (E_h \times Z_h \times BF_h) - (E_p \times Z_p \times BF_p) - (E_a \times Z_a \times BF_{ha}) \quad \text{Equation 5}$$

where $H^{15}NT$ was the hepatic ^{15}N transfer and E_h, E_p and E_a were the respective ammonia and urea enrichments (as APE above natural abundance), whilst *Z* and BF were the ammonia or urea concentrations and blood flows respectively, in the hepatic, portal and posterior aorta.

The fractional hepatic extraction (FHE) rates for [^{3}H]-phenylalanine and [^{35}S]-methionine were calculated using equations 6-9 as described by Waterlow *et al.* (1978b);

$$Q_p = (SRA_p \times Z_p \times BF_p) \quad \text{Equation 6}$$

$$Q_h = (SRA_h \times Z_h \times BF_h) \quad \text{Equation 7}$$

$$Q_a = (SRA_a \times Z_a \times BF_{ha}) \quad \text{Equation 8}$$

$$FHE = [(Q_a + Q_p) - Q_h] / (Q_a + Q_p) \quad \text{Equation 9}$$

where Q was the flux (MBq/h) of either [^{3}H]-phenylalanine or [^{35}S]-methionine for each of the portal and hepatic veins and the posterior aorta. Negative FHE rates for individual time points were omitted from the calculations.

The hepatic extraction rates of labelled phenylalanine and methionine were calculated from the FHE of either arterial or venous specific radioactivity using equations 10 and 11;

$$\text{Hepatic extraction rate}_{ha} = FHE \times [(Z_p \times BF_p) + (Z_a \times BF_{ha})] \quad \text{Equation 10}$$

$$\text{Hepatic extraction rate}_{h} = FHE \times (Z_h \times BF_h) \quad \text{Equation 11}$$

Estimates of hepatic protein synthesis rates were then calculated assuming that hepatic protein comprised 0.055 and 0.032 phenylalanine and methionine, respectively (MacRae *et al.* 1993). It was also assumed that the fractional hepatic extraction rates for [^{3}H]-phenylalanine and [^{35}S]-methionine were proportional to the fractional contribution of each amino acid to hepatic protein (Cronje *et al.* 1992).

The data were subjected to analysis of variance using the generalised linear model procedure (SAS, 1985) with animals treated as blocks for the diet effect.

3.4 RESULTS

All catheters remained patent for the duration of the experimental period (6 months) with the exception of one mesenteric sampling catheter which failed within one week following surgery.

3.4.1 Feed intake and body weight gains

The dry matter (DM; 164 v 896 g/kg; SED 0.0212), organic matter (OM; 136 v 828 g/kg; SED 0.0153) and N (43.0 v 26.8 g N/kg DM; SED 1.12) contents of the two diets (fresh white clover v lucerne pellets, respectively) were all significantly different ($P < 0.001$). The intakes of DM (870 v 855 g/d; SED 57.0; $P = 0.804$) and OM (765 v 735 g/d; SED 72.0; $P = 0.691$) were similar, whereas N intake (37.9 v 22.9 g N/d; SED 1.99; $P < 0.001$) was significantly higher in the animals fed fresh white clover, predominantly due to the higher N content of that diet. The apparent digestibility of DM (0.729 v 0.593; SED 0.0447; $P = 0.022$), OM (0.808 v 0.616; SED 0.0160; $P < 0.001$) and N (0.813 v 0.660; SED 0.0234; $P < 0.001$) were all significantly higher in the animals fed fresh white clover. However, the net N retention in the animals fed fresh white clover tended to be lower when compared to the animals fed lucerne pellets (-1.8 vs 1.7 g N/d; SED 1.59; $P = 0.070$).

3.4.2 Blood flows and oxygen exchange

The average blood flow in the mesenteric, portal and hepatic veins were similar for the animals fed fresh white clover and lucerne pellets with portal flows of 52.5 and 46.8 g/min per kg LW or 134.4 and 119.8 g/min per kg $LW^{0.75}$, respectively. The contribution of the hepatic artery to hepatic venous flow (0.061 v 0.066; SED 0.0624; $P = 0.847$) was similar for the animals fed fresh white clover and lucerne pellets respectively (Table 3.1).

Hepatic oxygen consumption tended ($P = 0.058$) to be higher in the animals fed fresh white clover compared to those fed lucerne pellets (Table 3.1).

Table 3.1. Blood flow (g/min) and oxygen consumption (mmol/min) across the mesenteric drained viscera (MDV), portal drained viscera (PDV), rumen and the liver of sheep fed lucerne pellets (L) or fresh white clover (WC).

(Mean value and standard error of the difference {SED} between means of four sheep)

	WC	L	SED	*P*
Blood flow				
Mesenteric vein†	1425	1164	436.3	0.582
Portal vein	2249	2004	169.5	0.199
Hepatic vein	2395	2197	326.5	0.566
Hepatic artery*	147	146	160.8	0.998
Oxygen transfers				
MDV†	-2.83	-2.22	1.081	0.598
PDV	-3.98	-3.45	0.627	0.424
Rumen†	-1.52	-0.99	0.731	0.508
Liver	-3.05	-1.55	0.643	0.058

* By difference (H - P).

† Four sheep fed fresh white clover and three sheep fed lucerne pellets.

Positive and negative values indicate net production and net extraction of oxygen by the relevant tissue respectively.

3.4.3 Ammonia and urea transfers

Rumen ammonia production in the fresh white clover fed animals was approximately four times that of the animals fed lucerne pellet (746 v 185; SED 51.1; $P < 0.001$; Table 3.2). The portal ammonia concentration was significantly ($P = 0.002$) higher and consequently the PDV ammonia production and hepatic ammonia extraction also tended ($P = 0.084$ and 0.072, respectively) to be higher in the animals fed fresh white clover compared to those fed lucerne pellets.

Urea concentrations in the three veins and artery were significantly ($P < 0.05$) higher for the animals fed fresh white clover compared to lucerne pellets (Table 3.2). However urea transfers were similar between treatment groups.

Hepatic N (as g N/d) transfers as ammonia, amino acids or urea were similar in the animals fed fresh white clover and lucerne pellets (Table 3.3). The portal ammonia-N flux was significantly higher for the animals fed fresh white clover

compared to lucerne pellets, consequently both the portal and hepatic urea fluxes were also significantly higher for the fresh white clover fed animals.

Table 3.2. Ammonia and urea concentrations and transfers across the mesenteric drained viscera (MDV), portal drained viscera (PDV), rumen and the liver of sheep fed fresh white clover (WC) or lucerne pellets (L).

(Mean value and standard error of the difference {SED} between means of four sheep)

	WC	L	SED	*P*
Ammonia concentrations (μmol/l)				
Mesenteric vein*	467	325	77.2	0.141
Portal vein	646	320	61.7	0.002
Hepatic vein*	72	48	39.6	0.574
Posterior aorta	132	56	59.5	0.249
Urea concentrations (mmol/l)				
Mesenteric vein*	10.85	6.03	0.961	0.001
Portal vein	10.38	6.23	1.311	0.020
Hepatic vein	11.04	6.18	1.816	0.037
Posterior aorta	10.50	6.13	1.704	0.043
Ammonia transfer (μmol/min)				
MDV*	571	382	339.6	0.607
PDV	1169	567	290.2	0.084
Rumen*	746	185	51.1	<0.001
Liver*	-1319	-664	287.2	0.072
Urea transfer (mmol/min)				
MDV*	-0.72	-0.34	1.977	0.857
PDV	-0.25	0.40	1.316	0.638
Rumen*	0.04	0.49	0.692	0.555
Liver	1.64	0.57	1.627	0.534

* Four sheep fed fresh white clover and three sheep fed lucerne pellets.
Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.

Table 3.3. Summary of nitrogen (N) fluxes in sheep fed fresh white clover (WC) or lucerne pellets (L).

(Mean value and standard error of the difference {SED} between means of four sheep)

g N/d	WC	L	SED	*P*
N Intake	37.9	22.9	1.12	<0.001
NH_3-N flux				
Mesenteric vein	13.5	8.5	5.30	0.397
Portal vein	29.7	13.2	2.91	0.001
Hepatic vein	3.4	2.5	1.89	0.461
Posterior aorta	0.4	0.2	0.23	0.496
AA-N flux				
Mesenteric vein	107.6	92.9	35.53	0.701
Portal	164.9	157.1	30.77	0.808
Hepatic	161.4	169.4	39.38	0.845
Posterior aorta	10.6	20.6	9.81	0.161
Urea-N flux				
Mesenteric vein	586.7	272.3	186.97	0.168
Portal	941.9	512.0	149.36	0.028
Hepatic	1080.2	575.5	241.91	0.082
Posterior aorta	72.1	46.8	51.9	0.644
Hepatic N transfers (g N/d)				
NH_3-N*	-26.6	-13.4	0.70	0.072
AA-N	-14.1	-8.3	0.81	0.604
Urea-N	66.2	23.0	13.07	0.534
Balance*	-25.4	3.0	12.47	0.668

* Four sheep fed fresh white clover and three sheep fed lucerne pellets.
Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Flux = metabolite concentration x blood flow in the respective vessels.

3.4.4 Amino acid transfers

Arterial amino acid concentrations were similar for the animals fed fresh white clover and lucerne pellets (results presented for phenylalanine and methionine only; Table 3.5). Net absorption of amino acids across the PDV were similar for the animals fed fresh white clover and lucerne pellets (Table 3.4). The PDV of the fresh white clover fed animals utilised glutamine, glutamate and

ornithine, whereas only glutamine was utilised across the PDV of the animals fed lucerne pellets. Amino acid-N appearance in the portal vein was similar for the animals fed fresh white clover and lucerne pellets, and was equivalent to 11.9 and 12.5 g N/d (SED 3.36; P = 0.869), or 0.314 and 0.554 (SED 0.1201; P = 0.102) of N intake, respectively. The hepatic extraction of amino acids was not significantly different in the animals fed fresh white clover compared to those fed lucerne pellets. The fractional extraction rate of amino acids absorbed by the PDV was unaffected by diet but the branched chain amino acids had the lowest fractional extraction rates. There was almost complete removal of essential amino acids from the portal blood by the liver.

Table 3.4. Amino acid transfers (μmol/min) across the portal drained viscera (PDV) and liver and fractional removal of amino acids by the liver in sheep fed fresh white clover (WC) or lucerne pellets (L).

(Mean value and standard error of the difference between means {SED} of four sheep)

		Thr	Val	Met	Ile	Leu	Phe	Lys	His	Trp	Arg	Tyr
PDV	WC	21.3	11.3	10.7	22.0	31.7	22.8	27.7	4.5	9.0	13.2	14.2
	L	24.6	30.4	7.2	26.4	40.3	25.1	27.5	7.3	5.3	19.4	19.8
	SED*	8.78	12.44	3.82	7.81	11.02	5.76	11.27	6.64	2.43	6.01	4.81
Liver	WC	-22.9	-2.9	-7.0	-3.8	-10.6	-20.4	-12.4	-8.3	-4.4	-17.7	-18.4
	L	-12.1	-1.0	-3.4	-2.2	-3.9	-21.0	-10.8	-5.9	-3.9	-17.3	-15.3
	SED*	9.11	13.44	2.67	5.74	9.46	4.51	14.87	3.00	2.31	5.36	4.71
Fractional removals§	WC	1.1	0.3	0.7	0.2	0.3	0.9	0.4	1.9	0.5	1.3	1.3
	L	0.5	0.0	0.5	0.1	0.1	0.8	0.4	0.8	0.7	0.9	0.8
	SED*	0.69	0.77	0.88	0.23	0.58	0.26	0.54	2.22	0.38	1.12	0.50

		Asp	Gln	Glu	Asn	Ser	Gly	Ala	Pro	Cit	Om
PDV	WC	2.7	-35.9	-8.1	30.9	34.3	34.7	26.1	6.2	13.7	-2.6
	L	3.2	-20.0	13.0	29.2	36.4	60.0	40.3	20.1	16.1	1.0
	SED†	1.22	28.01	5.64	8.22	11.45	31.15	13.39	10.96	5.59	4.21
Liver	WC	1.0	-15.8	40.8	-17.9	-23.9	-52.1	-18.2	-36.9	4.8	13.4
	L	-0.7	-20.6	21.2	-18.0	-22.6	-69.6	-40.2	-6.8	-0.5	15.3
	SED*	1.32	20.80	31.65	6.39	5.19	18.26	25.71	27.31	7.85	7.54
Fractional removals§	WC	0.4	0.4	5.0	0.6	0.7	1.5	0.7	5.9	0.3	5.1
	L	0.2	1.0	1.6	0.6	0.6	1.2	1.0	0.3	0.0	15.3
	SED*	0.82	1.91	10.22	0.19	0.22	0.74	6.91	1.87	0.60	7.32

Note; Citrate and ornithine included because of involvement with ureagenesis.

* No significant differences between diets.

† Only glutamate significantly ($P = 0.009$) different between diets.

§ (Net hepatic removal)/(net PDV appearance) ratio.

3.4.5 Phenylalanine and methionine kinetics

Whole body phenylalanine and methionine fluxes were similar in the animals fed fresh white clover and lucerne pellets. On the basis of phenylalanine kinetics, the estimate of ILR, protein synthesis and fractional whole body protein synthesis rate in the animals fed fresh white clover were significantly ($P < 0.05$) higher than in those animals fed lucerne pellets (Table 3.5). However, there were no significant differences between these parameters when the ILR was calculated from the SRA of methionine. The absolute rates of hepatic protein synthesis calculated from the net disappearance of phenylalanine or methionine from either the venous or arterial pool were similar for the animals fed either fresh white clover or lucerne pellets.

3.4.6 ^{15}N transfers

The ^{15}N-urea enrichment had not reached a plateau value after 10 h of $^{15}NH_4Cl$ infusion (Figure 3.1). The flux of [^{15}N]-urea at the four sampling sites exceeded the rate of $^{15}NH_4Cl$ infusion, indicative of [^{15}N] recycling during the blood sampling period. The hepatic ^{15}N-ammonia transfers reflect the relative infusion rates for each diet, with most (0.88 v 1.04, respectively; SED 0.054; $P = 0.055$, Table 3.6) of the infused $^{15}NH_4Cl$ removed by the liver as $^{15}NH_3$ and recovered as hepatic ^{15}N-urea (1.16 v 0.93, respectively; SED 1.832; $P = 0.904$). The majority (0.97) of the ^{15}N-urea molecules synthesised were in the single labelled form, [$^{14}N^{15}N$], with the proportion of double labelled [$^{15}N^{15}N$]-urea present below the detection limits (0.2 APE) of the GCMS.

Table 3.5. Methionine and phenylalanine whole body and liver kinetics and protein metabolism in sheep fed fresh white clover (WC) or lucerne pellets (L).

(Mean value and standard error of the difference {SED} between means of four sheep)

	WC	L	SED	*P*
Whole-body kinetics				
Arterial phenylalanine (mmol/l)	43.1	52.6	6.80	0.211
Arterial methionine (mmol/l)	18.8	21.5	3.11	0.411
ILR phenylalanine (mmol/h)*	2.2	2.6	0.15	0.036
Protein synthesis (g/d)*	249.2	296.0	16.39	0.036
Fractional synthesis rate*	0.037	0.047	0.0029	0.022
ILR methionine (mmol/h)*	0.9	1.2	0.46	0.465
Protein synthesis (g/d)*	207.6	294.1	109.30	0.465
Fractional synthesis rate*	0.031	0.047	0.0171	0.419
Hepatic kinetics				
Fractional extraction rates				
^{3}H-phenylalanine*	0.06	0.05	0.007	0.446
^{35}S-methionine†	0.05	0.06	0.011	0.333
Net extraction rates (mmol/h)				
^{3}H-phenylalanine$_a$*	0.40	0.49	0.133	0.509
^{3}H-phenylalanine$_v$*	0.35	0.43	0.107	0.526
^{35}S-methionine$_a$†	0.18	0.12	0.054	0.360
^{35}S-methionine$_v$†	0.15	0.14	0.064	0.902
Net uptake rates (mmol/h)				
Phenylalanine	1.26	1.22	0.160	0.233
Methionine	0.21	0.42	0.271	0.893
Protein synthesis (g/d)				
^{3}H-phenylalanine$_a$*	28.50	35.31	10.230	0.509
^{3}H-phenylalanine$_v$*	25.56	30.80	7.676	0.526
^{35}S-methionine$_a$†	20.44	13.17	6.023	0.360
^{35}S-methionine$_v$†	16.66	15.79	5.795	0.902

Note; Calculations use the SRA of whole blood. Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.

The subscripts a and v indicate the use of arterial and venous blood estimates of the precursor pool SRA. No detectable activity in oxidation products of either amino acid.

* Four sheep fed fresh white clover and three sheep fed lucerne pellets.

† Three sheep fed fresh white clover and two sheep fed lucerne pellets.

The hepatic ^{15}N-ammonia transfer was significantly (P = 0.011) higher in the animals fed fresh white clover compared to lucerne pellets (Table 3.6). However, the resultant ^{15}N-urea transfers were similar for the animals fed either fresh white clover or lucerne pellets. There was no detectable ^{15}N incorporated into free amino acid-N.

Table 3.6. Hepatic ^{15}N transfers in sheep fed fresh white clover (WC) or lucerne pellets (L).

(Mean value and standard error of the difference {SED} between means of four sheep)

	WC	L	SED	*P*
Hepatic ^{15}N transfers (μmol/min)				
Ammonia*	-52.0	-33.2	3.38	0.011
Urea	68.0	33.0	73.50	0.651

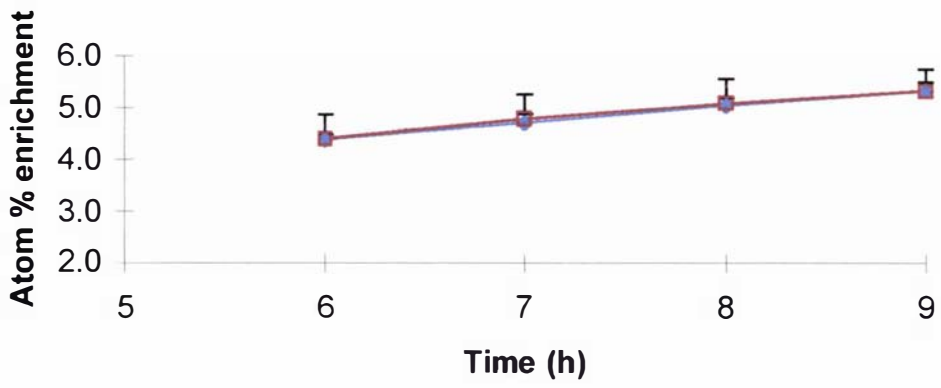
* Three sheep fed fresh white clover and two sheep fed lucerne pellets.

^{15}N was not detected in any amino acid.

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.

Figure 3.1 Changes in arterial urea atoms % excess during infusion of $^{15}NH_4Cl$ into the mesenteric vein of sheep fed fresh white clover (□) or lucerne pellets (♦).

(Values are means of four sheep, with standard errors indicated by vertical bars)



3.5 DISCUSSION

The objective of the current experiment was to estimate the metabolic cost of hepatic ammonia detoxification in sheep fed two high N content diets (fresh white clover and lucerne pellets) with marked differences in rumen protein degradability. Changes in the rate or extent of dietary protein degradation in the rumen are characterised by changes in rumen ammonia-N production (Huntington, 1987). In the current experiment, rumen ammonia production increased when the sheep were fed fresh white clover compared to when the same sheep were fed lucerne pellets. This was probably due to the protein in the fresh white clover being more degradable in the rumen than the protein in the lucerne pellets (Ulyatt & Egan, 1979). The increased rate of rumen ammonia production was associated with a coincident increase in the rate of hepatic ammonia detoxification and ureagenesis (to maintain basal systemic ammonia concentrations). It has been suggested that increased ureagenesis may require additional input of amino acid-N (Reynolds, 1992). This could result in both the N atoms of the urea molecule being sourced from ammonia *via* amino acids, predominantly glutamine (Lobley *et al.* 1995).

Although the DM intakes were similar for the animals fed fresh white clover and lucerne pellets, the higher N intake of the animals fed fresh white clover was predominantly due to the significantly higher N content of that diet, however the net N retention was similar for both diets. Rumen ammonia production for the animals fed fresh white clover was approximately twice that recorded for the animals fed lucerne pellets (Table 3.2). Ammonia production in the animals fed lucerne pellets was similar to previous reports with lucerne pellet fed sheep (Lobley *et al*. 1995; Goetsch *et al.* 1997).

As a proportion of N intake the PDV ammonia-N production in the animals fed fresh white clover was comparable to that in the animals fed lucerne pellets (0.62 v 0.50; SED 0.121; P = 0.564). The contribution of the rumen to PDV ammonia-N production was significantly higher in the animals fed fresh white clover than in those fed lucerne pellets (0.40 v 0.16; SED 0.042; P = 0.003). This indicates that the animals fed fresh white clover approached the known

metabolic maximum levels for PDV ammonia-N production (0.65 of N intake; Seal & Reynolds, 1993).

3.5.1 Hepatic ammonia metabolism

The hepatic ammonia extraction rate in the animals fed fresh white clover was approximately twice (P = 0.072) that recorded in the same animals fed lucerne pellets (Table 3.2). Assuming that liver mass represented between 0.016 (Lobley *et al.* 1995) and 0.025 (Orzechowski *et al.* 1987) of body weight, the hepatic ammonia clearance rate for the animals fed fresh white clover was between 1.2 and 1.9 μmol/min/g wet liver tissue. This is similar to the metabolic maximum rate recorded in sheep (1.5 μmol/min/g; Orzechowski *et al.* 1987) and cattle (1.9 μmol/min/g; Symonds *et al.* 1981). The ammonia concentration in the hepatic vein (72 μmol/l) was well below the critical ammonia overload concentration of 700 μmol/l (Symonds *et al.* 1981), therefore hepatic ammonia overload did not occur. Whilst the intake of fresh white clover (870 g DM/d) was relatively high compared to a maintenance intake for this diet (550 g DM/d; Bird *et al.* 1981; Beever *et al.* 1985), it was considerably lower than that of sheep grazing high quality pasture (Ulyatt, 1997). Therefore it may be expected that with higher intakes of fresh forages the possibility of sub-clinical hepatic ammonia toxicity will increase and may be sufficient to impair animal productivity (Ulyatt, 1997).

3.5.2 Urea metabolism

Although urea concentration was significantly different in the mesenteric, portal and hepatic veins and posterior aorta, there were no significant differences in urea transfers across the MDV, PDV, rumen or liver of the animals fed fresh white clover compared to the animals fed lucerne pellets (Table 3.2).

In general, the urea produced by the liver is either recycled to the gastro-intestinal tract (GIT) or excreted in the urine (Obara *et al.* 1991). The total N excreted in the urine was higher in the animals fed fresh white clover than in those fed lucerne pellets (33 v 14 g N/d, respectively; SED 2.4; $P < 0.001$) due

to a higher urine output (3.2 v 1.0 kg/d, respectively; SED 0.23; $P < 0.001$), rather than a higher urine N concentration *per se* (11 v 14 g N/kg, respectively; SED 2.1; $P = 0.103$).

3.5.3 ^{15}N metabolism

If it is assumed that one mole of ammonia generates one mole of urea, then the hepatic ammonia extraction rates, as a proportion of urea production were similar for the animals fed fresh white clover and lucerne pellets (0.80 v 1.17; SED 0.894; $P = 0.809$). However, comparing the level of ammonia-N (g N/d) extraction with urea-N production suggested that the hepatic ammonia extraction only contributed 0.40 of the urea produced (0.40 v 0.37; SED 0.443; $P = 0.809$). This observation was confirmed by the ^{15}N data which suggested that ^{15}N-ammonia contributed less than half of the ^{15}N-urea (0.38 v 0.50, respectively).

The absence of detectable ^{15}N-amino acid enrichment was confirmed by the conservation of infused ^{15}N as ^{15}N-urea. Lobley *et al.* (1995) reported ^{15}N-amino acid enrichment levels of 0.15-3.05 APE for the six amino acids analysed, whereas there was no detectable ^{15}N-amino acid enrichment in the current study. This suggests a fundamental difference between the metabolic channelling of the ^{15}N-ammonia to urea and amino acids in the animals used by Lobley *et al.* (1995) compared to those used in the current study. The animals fed fresh white clover may have adapted to the need to increase their rate of hepatic ammonia detoxification by either increasing the rate of glutamine synthesis (by increasing the glutamine synthetase {EC 6.3.1.2} activity) or increasing the rate of ureagenesis or a combination of the two (Meijer *et al.* 1990). Increased glutamine synthetase activity would have resulted in an increase in hepatic glutamine production and enrichment of glutamine by ^{15}N, neither of which were evident in this work. Therefore the findings of the current study do not present any evidence to support a significant role for any amino acid in the hepatic detoxification of excess ammonia.

The absence of detectable levels of [$^{15}N^{15}N$]-urea in this work suggests that these animals were not under severe hepatic ammonia overload. The spatial distribution of the urea cycle within the liver may offer an explanation as to why [$^{15}N^{15}N$]-urea was not produced. The peri-portal hepatocytes are the site of ureagenesis and contain glutaminase and release, but do not extract, glutamate (Katz, 1992). The perivenous cells of the rat liver do not synthesise urea, but extract glutamate and aspartate (Haussinger, 1983). These cells can also synthesise glutamate. If a similar situation is present in the ovine liver, then the site of glutamate uptake and synthesis is cranial to the ureagenic cells, thus preventing the formation of [$^{15}N^{15}N$]-urea in at least the first pass metabolism.

3.5.4 Amino acid metabolism

In general, the amino acids absorbed by the GIT (as estimated by PDV production) were removed by the liver, except for the branched chain amino acids (Harper *et al.* 1984). The PDV amino acid-N production and liver extraction (as g N/d) were similar for the two dietary treatments and are comparable to previous reports (Wolff *et al.* 1972; Lobley *et al.* 1995).

Glutamate was utilised by the PDV in the animals fed fresh white clover whereas it was produced by the PDV of the animals fed lucerne pellets (Table 3.4). The level of hepatic glutamate production in the animals fed fresh white clover was comparable to that recorded by Lobley *et al* (1996a), but was approximately twice that recorded for the lucerne fed animals, which was comparable to other work (Wolff *et al.* 1972; Lobley *et al.* 1995). This is indicative of the large variation associated with hepatic metabolism of this amino acid.

There was a trend for decreased hepatic glutamine extraction in the animals fed fresh white clover compared to those fed lucerne pellets, confirming previous findings of decreased glutamine extraction with increased hepatic ammonia load (Lobley *et al.* 1995). This may be directly attributed to the increase in hepatic glutamine synthetase activity generally associated with

increased hepatic ammonia detoxification rates (Deuel *et al.* 1978; Meister, 1985).

Although the calculated net hepatic N balance (ammonia-N plus amino acid-N input minus urea-N output; Table 3.3) for the fresh white clover fed animals was more than twice that recorded for the lucerne fed animals, this difference was not significant. These calculations assume that all of the hepatic amino acid-N uptake was utilised for urea production, which is clearly an over estimation and does not account for such hepatic N transactions as hepatic protein synthesis (such as albumin, which in humans is 200-400 mg/kg bwt/d; Ganong, 1983), metabolite formation (such as hippurate and glutathione) or oxidation *via* the citric acid cycle (Lobley *et al.* 1996b). The ovine liver cannot consistently generate N deficits, thus we are left to speculate the cause of such a deficit or to reconsider the validity of such measurements and calculations (with the necessary assumptions) generated from arterio-venous differences across a defined tissue (Parker *et al.* 1995). Alternatively, there may be an hepatic N source not considered in the above calculations such as peptide-N (Webb, 1986; Webb, 1990) which could be used to provide the additional N required for ureagenesis in the sheep fed the fresh white clover.

3.5.5 Whole-body and hepatic protein metabolism

The estimates of whole body ILR, protein synthesis and fractional synthesis rates were lower in the animals fed fresh white clover compared to those fed lucerne pellets, however this difference was only significant when the estimates were based on the SRA of [^{3}H]-phenylalanine (Table 3.5).

The estimates of whole body protein synthesis are comparable to previous work based on plasma or whole blood (Davis *et al.* 1981; Harris *et al.* 1992; Lobley *et al.* 1995). However, using the SRA of tissue homogenates, Davis *et al.* (1981) reported estimates of whole body protein synthesis almost twice those generated (from whole blood) in the current work, indicative of the potential variation associated with the choice of amino acid pool used to estimate the SRA of the true precursor pool (Airhart *et al.* 1974).

The absolute rates of hepatic protein synthesis and the percentage of whole body protein synthesis attributable to hepatic protein synthesis were similar to previous work (Davis *et al.* 1981; Lindsay, 1982; Reynolds, 1992; Lobley *et al.* 1994; Lobley *et al.* 1995).

Methionine is an essential amino acid which is first limiting for both skin growth and wool production (Lee *et al.* 1995). Methionine undergoes trans-sulphuration to cysteine in the liver (Cobon *et al.* 1988), therefore the absence of a detectable SRA for cysteine in the hepatic venous blood indicates that the rate of trans-sulphuration is indeed rapid and that cysteine is rarely the end product of that metabolism.

3.5.6 Energy metabolism

The trend toward a higher rate of hepatic oxygen consumption in the animals fed fresh white clover was a result of an increased rate of hepatic ammonia detoxification relative to that generated when the sheep were fed lucerne pellets. From empirical calculations (Martin & Blaxter, 1965) it was estimated that 0.13 of liver oxygen consumption is normally used for ureagenesis (Parker *et al.* 1995). In the current study, the process of ureagenesis consumed 0.36 of the hepatic oxygen consumption in the animals fed fresh white clover and only 0.25 in the animals fed lucerne pellets. Both of these values are greater than previous reports (0.11-0.16; Reynolds *et al.* 1991; Lobley *et al.* 1995).

The energetic cost of urea synthesis was 288 kJ/d (assuming 122 kJ/mole of urea synthesised; Martin & Blaxter, 1965; Waghorn & Wolff, 1984) for the animals fed the fresh white clover compared to 100 kJ/d (SED 284.3; P = 0.534) for the animals fed lucerne pellets. This represents 0.031 of metabolisable energy (ME) intake for the animals fed fresh white clover and 0.009 (SED 0.0962; P = 0.505) for the lucerne pellet fed animals, assuming an ME content of 12.0 and 10.5 MJ/kg DM, respectively (Waghorn & Wolff, 1984; Beever *et al.* 1985; Lobley *et al.* 1995). Any increase in the proportion of ME used for ureagenesis would decrease the energy available for whole body

metabolism. For example, the energetic cost of ureagenesis in the animals fed fresh white clover is equivalent to approximately 14 g/d live weight gain (Waghorn & Wolff, 1984).

3.6 CONCLUSION

The current study clearly demonstrated the magnitude of hepatic ammonia detoxification in sheep fed near maintenance levels of two high N content diets (fresh white clover or lucerne pellets) differing in rumen protein degradability. The observed differences in the metabolic parameters of the sheep fed the two diets were probably due to the higher rumen protein degradability associated with the fresh white clover diet. However, there was no incremental increase in the utilisation of amino acids associated with the increased hepatic ammonia detoxification that resulted from feeding fresh white clover compared to lucerne pellets. There was no evidence to suggest that ammonia provided both of the N atoms for the synthesis of urea, even under the elevated ammonia metabolism conditions generated when the animals were fed fresh white clover. There was a trend toward increased metabolic costs (hepatic oxygen consumption) associated with the increased ureagenesis that occurred in the sheep fed fresh white clover.

In practice, the intake of grazing lambs would normally exceed the 900 g DM/d fed in this trial, consequently the hepatic ammonia detoxification load could be expected to exceed that recorded in this study. For this reason the metabolic cost of ureagenesis in grazing lambs may exceed the 0.031 of ME intake recorded in this work. Further work should investigate the impact of higher intakes of fresh white clover on hepatic ammonia load with the possibility of inducing a measurable increase in hepatic amino acid catabolism associated with the resultant ureagenesis.

4 CHAPTER 4. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) AT TWO LEVELS OF INTAKE

4.1 ABSTRACT

The effect of offering fresh white clover (*Trifolium repens*) at low and high (800 v 1400 g DM/d offered) intake levels on splanchnic ammonia and urea production, hepatic oxygen consumption and amino acid utilisation, and estimates of whole body and hepatic protein synthesis were studied in six sheep. The animals were infused (9.5 h) with $^{15}NH_4Cl$, [^{35}S]-cysteine and *para*-aminohippurate *via* the mesenteric vein. Blood samples were collected to determine ammonia, urea, oxygen, and amino acid concentrations. Although there were significant differences in both dry matter (DM; 807 v 1118 g DM/d; SED 50.2; $P < 0.001$) and nitrogen (N; 31.6 v 43.9 g N/d; SED 1.96; $P < 0.001$) intakes between the low and high intake groups, there were no significant differences in DM and N digestibility, ammonia, urea nor amino acid concentrations and transfers across the portal drained viscera and liver. However the hepatic ^{15}N-ammonia transfer was significantly lower in the low intake group (39.8 v 67.8 μmol/min, respectively; SED 10.58; $P = 0.033$) compared to the high intake group. The absence of [^{15}N^{15}N]-urea confirmed that ammonia contributed only one N atom to urea. It was estimated that ureagenesis accounted for 0.017 and 0.019, respectively (SED 0.0059; $P = 0.789$) of metabolisable energy intake in the low compared to high intake groups. There were no treatment effects on the estimates of both whole body and hepatic protein synthesis rates. Although the N intake of the high intake group was 0.39 above that of the low intake group, there was no difference in the rate of hepatic ammonia detoxification, urea production nor amino acid catabolism, suggesting that the additional N intake did not increase the cost of ammonia detoxification.

4.2 INTRODUCTION

Fresh forages, such as white clover, are characterised by containing protein which is readily soluble (0.75-0.80 solubility; Mangan, 1982). During degradation of dietary protein in the reticulo-rumen (rumen), large quantities of ammonia are produced. The resident rumen microbes can utilise this ammonia directly (Parker *et al.*, 1995), however the rate of utilisation is often below the rate of ammonia production, resulting in the passive absorption of excess ammonia across the rumen wall into the blood for transport to the liver (Huntington, 1982). Ammonia is converted to urea, which incurs an amino acid cost (Reynolds, 1992; Lobley *et al.*, 1995; Lobley *et al.*, 1996b), effectively reducing the availability of amino acids for other purposes, such as productive gain.

Previous work in this laboratory with sheep fed either fresh white clover or lucerne pellets (both with high protein content but the latter having a lower rumen protein degradability) failed to demonstrate an incremental amino acid cost for the increase in rumen ammonia production associated with fresh white clover (Chapter Three). However, in that study the intake of fresh white clover by the sheep was moderate (900 g DM/d) and about 1.4-1.6 x maintenance (M), if the maintenance requirements for these animals is 550-650 g DM/d (Bird *et al.* 1981, Ulyatt, 1997). In the present study sheep were fed fresh white clover at a level similar to the previous study (800 g DM/d, 1.2-1.5xM) and at a higher level (1400 g DM/d, 2.2-2.5xM). The higher level of intake would be required to achieve high growth rates in sheep grazing fresh pasture (Ulyatt, 1997). The objective was to determine whether the resultant increased rate of hepatic ammonia detoxification would also increase the hepatic amino acid requirements for ureagenesis disproportionately.

Using blood samples taken from surgically implanted catheters within the splanchnic vasculature, the portal drained viscera (PDV) ammonia production was quantified and the resultant effects on hepatic ammonia detoxification, nitrogen (N) balance and amino acid metabolism were determined. The

animals were infused with $^{15}NH_4Cl$, to trace the hepatic detoxification of ammonia to urea and the incorporation of the label into amino acids. Additionally, [^{35}S]-cysteine was infused to measure whole-body and hepatic protein synthesis.

4.3 MATERIALS AND METHODS

These experimental procedures and protocols were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Palmerston North, New Zealand according to the Animals Protection Act (1960), Animals Protection Regulations (1987) and amendments.

4.3.1 Animals and surgical procedures

Six Romney-cross wether lambs (mean initial body weight 33.4, SE 0.6; 6-9 months old) were prepared with silicone based catheters in the posterior aorta, mesenteric (x2), portal and hepatic veins as described previously (Chapter Three). The animals were fed fresh white clover (*Trifolium repens*) hourly from automatic feeders and allowed a minimum of 4 weeks recovery following surgery prior to allocation to a treatment group. All animals were offered 800 g DM/d during this period.

4.3.2 Diets and design

The sheep were offered either 800 (low intake) or 1400 g DM/d (high intake) of fresh white clover harvested daily from a pure sward with a minimum of 4-6 weeks growth. The animals were accustomed to each intake level for 10 days before entering a 10 day N balance.

To assist with the quantification of the rate of hepatic ammonia detoxification, ureagenesis and amino acid catabolism, the sheep received a continuous infusion of $^{15}NH_4Cl$ *via* the mesenteric vein for 9.5 h. The rate of protein synthesis (both whole body and hepatic) was estimated from [^{35}S]-cysteine kinetics (which was also infused *via* the mesenteric vein for 3.75 h). Each sheep was then placed on the alternate intake level and allowed a minimum

adjustment period of two weeks prior to repeating the experimental procedure in a cross over design.

The methodology employed during this trial was similar to previous work in this laboratory (Chapter Three).

4.3.3 Infusion protocol

Each sheep was continuously infused using a peristaltic pump (302F, Watson-Marlow Limited, Cornwall, England) with 146 mmol/l (low intake) or 248 mmol/l (high intake; SED 14.8; $P < 0.001$) $^{15}NH_4Cl$ (99.8 atoms percent enrichment, APE; Icon, Mt Marion, USA) for 9.5 hours at a rate of 22.7 g/h. Following 5.75 hours of $^{15}NH_4Cl$ infusion, the $^{15}NH_4Cl$ infusate was supplemented with sodium *para*-aminohippurate (*p*AH; Sigma Chemicals, St Louis, USA; 203 mmol/l), 925 MBq/l [^{35}S]-cysteine (ICN Biomedicals Inc., California, USA) and 60 μmol/l of unlabelled cysteine (Sigma Chemicals, St Louis, USA) as a carrier and the infusion continued for a further 3.75 hours.

The sheep were manually infused with a bolus of 5,000 IU heparin (Ovine origin; New Zealand Pharmaceuticals, Palmerston North, New Zealand) prior to the commencement of blood sampling. Mesenteric, portal, hepatic and arterial blood samples were withdrawn (at 22.7 g/h) for the first 30 minutes of each of the last four hours of isotope infusion. The blood collection lines were passed through ice to minimise the risk of coagulation and degradation of the blood constituents with the blood collected into 15 ml polypropylene vials (on ice) containing 50 μl sodium ethylenediaminetetra-acetic acid (EDTA; 0.15 w:v in water) as an anticoagulant.

4.3.4 Analytical measurements

The packed cell volume of whole blood was determined by haematocrit for each sample taken. Blood oxygen concentrations were evaluated immediately following collection using a galvanic oxygen cell (Grubb & Mills, 1981). All subsequent procedures were determined gravimetrically.

Following the addition of 5 g 12% (w:w) trichloroacetic acid (TCA) to 0.5 g whole blood, the sample was centrifuged (at 3270 G) for 15 minutes at 4 °C. The *p*AH concentration in whole blood was determined using the methods described by Harris e*t al*. (1992) with an additional acid deacetylation step at 90 °C for 65 minutes (Lobley e*t al*. 1995).

Whole blood (2 g) was haemolysed by the addition of an equal weight of chilled 200 μmol/l L-nor-leucine (BDH Chemicals Ltd., Poole England) to which 200 mg of 80 mmol/l dithiothreitol in 0.2 mol/l phosphate buffer (*p*H 8.0) was added.

The remaining blood was then centrifuged and the plasma removed.

Haemolysed blood (1 g) was ultra-filtered through a 10,000 molecular weight cut-off membrane (Centrisart®; Sartorius, Gottingen, Germany), with amino acid concentrations determined in 100 mg of the resultant supernatant by picotag analysis and reverse phase high performance liquid chromatography (Cohen e*t al*., 1986).

The enrichments of both [^{15}N]-amino acids and [^{15}N]-urea in haemolysed blood were determined by gas chromatography and mass spectrometry (GCMS; VG Trio-1, VG Masslab Ltd, Manchester, Cheshire, UK) using the methods described by Nieto e*t al.* (1996).

Plasma ammonia concentrations were measured (conducted non-gravimetrically) with a commercial kit (#171; Sigma Chemical, St Louis, USA) that utilised the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase.

The ammonia present in 1 g of plasma was converted to nor-valine to allow the subsequent determination of [^{15}N]-ammonium enrichment by GCMS using the methods described by Nieto e*t al*. (1996).

Urea concentrations were determined in plasma using a commercial kit (#535; Sigma Chemical, St Louis, USA), that was based on the conversion of urea to hydroxyl-amine in the presence of diacetylmonoxime when heated with sulphuric acid.

The radioactivity from [^{35}S]-cysteine was determined in both haemolysed blood and plasma using a liquid scintillation counter (Model 1500 Tricarb, Packard, Zurich, Switzerland) within one month of the infusion using the methods described by Lee *et al.* (1993). The proportions of total radioactivity attributable to each amino acid and oxidation products were determined by inline flow through a liquid scintillation counter (Model 2, β-ram, IN/US systems Inc., New Jersey, US) using an HPLC (LC4A, Shimadzu, Kyoto, Japan) using the methodology described by Lee *et al.* (1993).

4.3.5 Calculations and statistics

The mass and isotope transfers were calculated as outlined previously (Chapter Three).

The ^{35}S-cysteine SRA was corrected for SRA in the oxidation products, tyrosine and sulphate. Estimates of whole body protein and hepatic protein synthesis rates were calculated using the assumption that whole body and hepatic protein comprised 0.015 and 0.025 cysteine, respectively (MacRae *et al.*, 1993).

The data were subjected to analysis of variance using the generalised linear model procedure (SAS, 1985) with animals treated as blocks for the effect of intake.

4.4 RESULTS

All catheters remained patent for the duration of the experimental period (6 months).

4.4.1 Feed intake and body weight gains

The dry matter (DM) and nitrogen (N) content of the fresh white clover was similar for the two levels of intake (Table 4.1). The DM and N intakes for the high intake groups were 0.38 (807 v 1118 g/d; SED 50.2, P < 0.001) and 0.39 (31.6 v 43.9 g N/d DM; SED 1.96, P < 0.001) above those of the low intake group. The DM and N digestibility, and N retention were not significantly different for the low and high groups.

Table 4.1 The dry matter (DM) and nitrogen (N) intake and digestibility of fresh white clover offered at low or high intakes.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

	Low	High	SED	*P*
Intake (g/d)				
DM	807	1118	50.2	< 0.001
N	31.6	43.9	1.96	< 0.001
Digestibility				
DM	0.812	0.826	0.0095	0.199
N	0.811	0.793	0.0194	0.373
N retention (g/d)	0.3	2.7	3.15	0.477

4.4.2 Blood flows and oxygen exchange

Estimates of the blood flow in the mesenteric vein were highly variable (473 – 6667 g/min), therefore data from this catheter (and subsequently the mesenteric drained viscera *per se*) were omitted from further consideration. The average portal, hepatic vein and hepatic arterial blood flows were similar for the two intake groups ($P > 0.05$; Table 4.2). The contribution of the hepatic artery to hepatic venous flow was not affected by the intake level (0.075 v 0.168; SED 0.0771; $P = 0.283$).

Although portal oxygen concentrations approached the 10% level of significance (4.50 v 3.53 mmol/l; SED 0.470; P = 0.101), the oxygen transfer values for the portal drained viscera (PDV) and the liver were similar (P > 0.05) for the two intake groups.

Table 4.2. Blood flow (g/min) and oxygen consumption (mmol/min) across the portal drained viscera (PDV) and the liver of sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

	Low	High	SED	P
Blood flow				
Portal vein	1746	1399	186.6	0.110
Hepatic vein	1893	1776	293.9	0.712
Hepatic artery*	146	376	187.2	0.272
Oxygen transfers				
PDV†	-2.71	-4.33	0.949	0.165
Liver††	-3.04	-2.74	0.586	0.660

* By difference (H - P).
† Six sheep in the low intake group and four sheep in the high intake group.
†† Four sheep in the low intake group and four sheep in the high intake group.
Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.

4.4.3 Ammonia and urea transfers

There was no significant (P > 0.05) difference in portal and hepatic vein ammonia or urea concentrations, consequently the mass transfers (across the PDV and liver) of these two metabolites were also similar for the two intake levels (Table 4.3).

Considered as g N/d, the portal, hepatic and arterial fluxes, and consequently the hepatic transfers, of ammonia, urea and amino acids, and the resultant net N balance across the liver, were all similar for the high and low intake groups (Table 4.4).

Table 4.3. Ammonia and urea transfers across the portal drained viscera (PDV) and the liver of sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

	Low	High	SED	P
Ammonia concentrations (μmol/l)				
Portal vein	706	666	73.0	0.617
Hepatic vein	97	74	47.8	0.632
Posterior aorta	96	81	29.7	0.644
Urea concentrations (mmol/l)				
Portal vein	11.28	10.12	1.550	0.495
Hepatic vein	11.65	10.82	1.632	0.639
Posterior aorta	11.51	10.43	1.611	0.539
Ammonia transfer (μmol/min)				
PDV	1089	823	235.0	0.309
Liver	-1090	-827	254.1	0.352
Urea transfer (mmol/min)				
PDV	-0.44	-0.50	0.521	0.910
Liver	0.69	1.23	0.359	0.174

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.

Table 4.4. Summary of nitrogen (N) fluxes in sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

g N/d	Low	High	SED	*P*
N Intake	31.6	43.9	1.96	< 0.001
NH_3-N flux				
Portal	25.2	19.4	3.35	0.217
Hepatic	3.5	3.0	1.45	0.768
Posterior aorta	0.4	0.8	0.51	0.510
AA-N flux				
Portal	128.3	111.0	17.35	0.495
Hepatic	133.4	127.2	18.04	0.813
Posterior aorta	9.3	21.8	7.64	0.274
Urea-N flux				
Portal	813.1	601.5	131.32	0.241
Hepatic	907.1	768.2	155.91	0.506
Posterior aorta	66.3	123.9	52.83	0.418
Hepatic N transfers (g N/d)				
NH_3-N	-22.0	-16.7	5.13	0.352
AA-N	-7.5	-7.9	18.33	0.847
Urea-N	27.7	49.6	14.10	0.174
Balance	31.6	17.6	13.43	0.347

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.

Flux = metabolite concentration x blood flow in the respective vessels.

Table 4.4. Summary of nitrogen (N) fluxes in sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

g N/d	WC	L	SED	*P*
N Intake	31.6	43.9	1.96	< 0.001
NH_3-N flux				
Portal	25.2	19.4	3.35	0.217
Hepatic	3.5	3.0	1.45	0.768
Posterior aorta	72.1	46.8	51.9	0.644
AA-N flux				
Portal	128.3	111.0	17.35	0.495
Hepatic	133.4	127.2	18.04	0.813
Posterior aorta	72.1	46.8	51.9	0.644
Urea-N flux				
Portal	813.1	601.5	131.32	0.241
Hepatic	907.1	768.2	155.91	0.506
Posterior aorta	72.1	46.8	51.9	0.644
Hepatic N transfers (g N/d)				
NH_3-N	-22.0	-16.7	5.13	0.352
AA-N	-7.5	-7.9	18.33	0.847
Urea-N	27.7	49.6	14.10	0.174
Balance	31.6	17.6	13.43	0.347

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Flux = metabolite concentration x blood flow in the respective vessels.

4.4.4 Amino acid transfers

Amino acid concentrations were similar for the low and high intake groups, with the exception of glycine in the hepatic vein (386 v 324 μmol/l; SED 21.4; P = 0.022). Concentration results are presented for cysteine only (Table 4.6). Net PDV amino acid production was similar for the two intake levels (Table 4.5). Net hepatic amino acid extraction (7.5 and 7.9 g N/d) was also not significantly affected by intake level (Table 4.4). The branched chain amino acids had the lowest hepatic fractional extraction rate, with the essential amino acids nearing total hepatic absorption (Table 4.5).

Table 4.5. Amino acid transfers (μmol/min) across the portal drained viscera (PDV) and liver, and fractional removal of amino acids by the liver in sheep fed fresh white clover at either low or high intake levels.
(Mean value and standard error of the difference between means {SED} of six sheep in the low intake group and five sheep in the high intake group)

		Thr	Val	Met	Ile	Leu	Phe	Lys	His	Trp	Arg	Tyr
PDV	Low	18.6	23.6	9.7	19.9	27.6	17.3	20.9	7.4	16.9	14.1	5.0
	High	18.3	21.6	5.9	18.4	25.5	16.1	22.7	7.6	12.6	14.0	0.9
	SED*	8.23	13.05	4.61	8.82	12.47	7.66	9.91	3.80	6.94	8.71	3.37
Liver	Low	-5.7	-6.2	-4.8	-2.3	-4.8	-13.8	-8.9	-4.1	-11.8	-13.1	-5.5
	High	-6.7	2.2	1.5	2.4	1.5	-14.4	-5.3	-4.6	-12.9	-12.8	-3.9
	SED*	6.01	11.05	3.89	5.02	6.23	5.16	5.28	2.64	2.33	7.27	4.01
Fractional removals†	Low	0.8	0.4	-0.3	0.1	-0.1	1.0	0.8	0.6	0.8	1.0	1.0
	High	0.3	-0.2	-0.7	-0.2	-0.1	0.9	0.2	0.6	0.7	0.7	1.0
	SED*	6.95	0.51	1.41	0.20	0.21	0.39	0.33	0.98	2.22	0.62	0.27

		Asp	Gln	Glu	Asn	Ser	Gly	Ala	Pro	Cit	Om
PDV	Low	5.8	-20.0	26.3	18.9	22.7	55.2	32.4	14.9	14.1	2.9
	High	5.7	-15.8	9.2	15.5	22.3	32.4	31.2	15.8	15.7	4.6
	SED*	2.35	12.27	25.89	7.72	7.60	27.75	14.32	7.19	5.32	4.07
Liver	Low	0.6	-12.8	34.0	-12.0	-14.0	-31.6	-27.7	-5.6	-0.4	5.2
	High	3.1	-23.2	18.1	-10.0	-19.7	-26.8	-35.3	-7.5	2.0	8.8
	SED*	1.44	14.46	35.28	4.01	5.73	18.24	9.60	2.94	3.45	3.53
Fractional removals†	Low	-0.5	-0.6	-0.4	0.9	0.7	1.0	1.1	0.8	0.1	3.9
	High	-0.6	-1.3	-4.7	0.7	0.9	0.5	1.2	0.5	-0.3	2.2
	SED*	0.49	0.90	2.02	0.70	0.36	0.93	0.33	0.34	0.39	3.57

Note; Citrate and ornithine included because of involvement with ureagenesis.
* No significant differences in amino acid transfers across the PDV and liver or in fractional removal of amino acids by the liver probability values are presented.
† (Net hepatic removal)/(net PDV appearance) ratio.

4.4.5 Cysteine kinetics

There were no significant ($P > 0.05$) differences between the measurements of cysteine SRA in whole blood and plasma, consequently there were no significant differences in the estimates of either whole body or hepatic protein synthesis rates. Therefore, only the whole blood data is presented in Table 4.6.

The estimates of whole body protein synthesis based on the SRA of cysteine in whole blood were similar for the two intake levels. Hepatic protein synthesis rates were similar, for both groups (Table 4.6).

Table 4.6. Cysteine whole body and liver kinetics, and protein metabolism in sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of three sheep in the low intake group and three sheep in the high intake group)

	Low	High	SED	*P*
Cysteine concentration				
Arterial cysteine (mmol/l)	47.5	40.8	7.12	0.509
Whole body kinetics				
ILR cysteine (mmol/h)	1.1	1.5	0.30	0.375
Protein synthesis (g/d)	227	287	59.8	0.375
Fractional synthesis rate	0.04	0.05	0.012	0.430
Hepatic kinetics				
Fractional extraction rates	0.05	0.09	0.038	0.308
Net extraction rates (mmol/h)				
Based on arterial ^{35}S-cysteine activity	0.15	0.20	0.076	0.543
Based on venous ^{35}S-cysteine activity	0.15	0.21	0.080	0.499
Net uptake rates (mmol/h)				
Cysteine	-0.11	0.02	0.151	0.255
Protein synthesis (g/d)				
Based on arterial ^{35}S-cysteine activity	17.7	23.6	8.82	0.543
Based on venous ^{35}S-cysteine activity	17.3	24.1	9.23	0.499

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.

4.4.6 ^{15}N transfers

The ^{15}N-urea (arterial) enrichment had reached 5.9 APE (non-plateau) after ten hours of $^{15}NH_4Cl$ infusion, comparable to the non-plateau value achieved in previous work in this laboratory (Chapter Three). Label recycling was evident because of ^{15}N-urea at the three sampling sites, exceeding the rate of $^{15}NH_4Cl$ infusion. Of the labelled urea ($[^{14}N^{15}N]$ or $[^{15}N^{15}N]$) recovered across the liver, the single labelled form, $[^{14}N^{15}N]$, predominated. Double labelled $[^{15}N^{15}N]$ urea was not detected in any of the blood samples. The detection limit of the GCMS was 0.2 APE.

The hepatic ^{15}N-ammonia transfers were significantly higher (0.70 above the low intake group) for the high intake group compared to the low intake group (Table 4.7). However the ^{15}N-urea transfers were not significantly different between the two intake groups. There was no detectable ^{15}N incorporation into free amino acid-N.

Table 4.7. Hepatic ^{15}N transfers in sheep fed fresh white clover at either low or high intake levels.

(Mean value and standard error of the difference {SED} between means of six sheep in the low intake group and five sheep in the high intake group)

	Low	High	SED	*P*
Liver ^{15}N transfers (μmol/min)				
Ammonia	-39.8	-67.8	10.58	0.033
Urea*	117.3	147.7	151.60	0.864

* Six sheep fed low intake and four sheep fed the high intake.
^{15}N was not detected in any amino acid.
Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.

4.5 DISCUSSION

The objective of this study was to estimate the metabolic cost of hepatic ammonia detoxification in sheep fed fresh white clover at either near maintenance (M) intake (comparable to previous work; Chapter Three) or at an elevated intake (1.75xM). Previous reports have suggested that increased rates of ureagenesis may incur a coincident metabolic cost in terms of elevated amino acid catabolism (Reynolds, 1992). This work tested the hypothesis that the hepatic ammonia load will increase in response to an increased intake of fresh white clover. Accordingly, hepatic ureagenesis may be expected to increase, requiring an increased rate of hepatic amino acid catabolism.

The significant results of the current study were that although the DM and N intakes were significantly different between the two treatment groups, there was no significant difference in ammonia production from the PDV and therefore the hepatic ammonia extraction and urea production rates were also similar. However, the ^{15}N-ammonia transfers across the liver increased significantly with intake. There was no detectable increase in hepatic amino acid catabolism.

4.5.1 Intake parameters

The DM intake as a proportion of DM offered was significantly lower in the high intake group (0.988 v 0.835 for the low and high intake groups respectively; SED 0.0297; $P < 0.001$), suggesting that the higher intake level of fresh white clover (1118 g DM/d) approached metabolic maximum intake levels under these feeding conditions. Previous work with fresh white clover offered to mature sheep in metabolism crates recorded maximal intake levels below those recorded here (MacRae & Ulyatt, 1974; Ulyatt & MacRae, 1974; Ulyatt & Egan, 1979). Intake levels of mature sheep consuming pure fresh white clover swards under most grazing conditions in New Zealand are also similar to the high intake group (Ulyatt, 1971). Therefore it may be surmised that the sheep on the high intake treatment ate the maximum quantity of fresh white clover possible.

As DM and N intake increases, it would be expected that the rumen digesta pool size would increase (Waghorn *et al.*, 1986), whilst the digesta retention time in the rumen would decrease (Reid *et al.*, 1977). It has been shown that the N digestibility of fresh white clover decreases as N intake increases, and that the proportion of the N intake digested in the rumen also decreases as N intake increases (Beever *et al.*, 1985). Therefore in the present experiment, whilst the N digestibility remained constant for increased N intakes, it may be assumed that the rumen digesta retention time was decreased.

4.5.2 Blood flows

The blood flow in the portal and hepatic veins were comparable to previous reports (Barnes *et al.*, 1983a; Lush & Gooden, 1988; Burrin *et al.*, 1989; Huntington, 1990; Harris *et al.*, 1992; Lobley *et al.*, 1995). There was no elevation in blood flow with increased intake (Table 4.2), confirming previous work (Webster, 1974). However there was a trend towards lower venous blood flows for the higher intake that may have contributed to reducing the difference between the treatment groups. The large variation in the hepatic arterial contribution to hepatic venous blood flow is indicative of both the variation quoted in the literature (Pell *et al.*, 1986; Ortigues *et al.*, 1994) and the variation in blood flow between and within the two treatment groups. The variability associated with the estimates of the mesenteric blood flow is discussed in section 6.2.3.

4.5.3 Hepatic ammonia metabolism

Although the nitrogen intakes were significantly different, there were no significant differences in ammonia concentrations (portal and hepatic veins, and the posterior aorta) and therefore the PDV and liver ammonia transfers were also similar for the two intake groups (Table 4.3). Hepatic ammonia overload did not occur, as evidenced by the arterial ammonia concentrations (96 v 81 μmol/l) remaining well below metabolic overload concentrations of 700 μmol/l (Symonds *et al.*, 1981).

The significant difference in hepatic ^{15}N-ammonia transfer between treatment groups was contrary to the mass transfer data (no significant difference) and suggests that there was in fact a difference in the hepatic ammonia detoxification rate between the two intake levels (Table 4.7). It is expected that the ^{15}N-ammonia data would be a more concise representation of the actual ammonia status of these animals (Weijs *et al.*, 1996) and therefore this difference truly reflects the difference in N intake between treatment groups.

Nitrogen digestion in the rumen ultimately produces either ammonia-N or amino acid-N, therefore the increase in N intake should have resulted in an increase in either PDV ammonia or amino acid production. The mass transfer data for both ammonia and the amino acids do not support this theory, however the more sensitive ^{15}N-ammonia data are consistent with this theory. This suggests that, for the difference in N intake recorded in this experiment, either the mass transfer method of quantifying N transfers was not sufficiently sensitive or that the inter-animal variation inherent in such experiments obscured any treatment differences.

4.5.4 Amino acid metabolism

The PDV amino acid production was generally extracted by the liver, with the exception of the branched chain amino acids (Table 4.5). There was no increase in hepatic amino acid catabolism associated with the increase in nitrogen intake by the high intake group.

Although the PDV utilised glutamine, there was also a net utilisation of glutamine across the liver for both intake levels. The net hepatic glutamate production (for both intake levels), in addition to the glutamate production from the PDV also suggests that the glutamine extracted by the liver was providing ammonium for ureagenesis as previously reported by Krebs *et al.* (1978). The net transfer of the amino acid couplet asparagine / aspartate failed to demonstrate net hepatic ammonium donation as previously reported by Krebs *et al.* (1976) and Bergman & Pell (1984).

The absence of detectable quantities of ^{15}N in any amino acid is in contrast to previous findings (Lobley *et al.*, 1995) and suggests that the animals used in this study detoxified ammonia utilising a different mechanism to that used by the animals assessed by Lobley *et al.* (1995). For example, Lobley *et al.* (1995) recorded significant quantities of ^{15}N-glutamine (APE 3.05), suggesting that these animals increased the activity of hepatic glutamine synthetase to remove the additional hepatic ammonia load. The absence of ^{15}N-glutamine enrichment in the current study suggests that the capacity of ureagenesis was sufficient to remove the ammonia load and that the alternative ammonia detoxification regimen (of increased glutamine synthetase activity) was not required. This also suggests that there could be a period of (hepatic ammonia load) adaptation involved, with the animals in the present study given at least twenty days on an intake level prior to infusion and assessment of ^{15}N-amino acid enrichment, during which the capacity of ureagenesis could have adapted and therefore increased. This compares to a maximum of only five days in which the liver could have adapted to the dietary regimen in the work of Lobley *et al.* (1995).

The net hepatic nitrogen transactions (ammonia-N plus amino acid-N input minus urea-N output) were similar for the two intake levels (Table 4.4). Inherent in these calculations was the assumption that all of the absorbed amino acid-N was utilised for ureagenesis, which is clearly an over estimation and does not account for other hepatic N transactions such as hepatic protein synthesis (Ganong, 1983) and metabolite formation or oxidation *via* the citric acid cycle (Lobley *et al.*, 1996a). The ovine liver cannot sustain continual N deficits, therefore we are left to speculate the cause of such a deficit or to reconsider the validity of such analyses and calculations (with their inherent assumptions) which generate these values from arterio-venous differences across defined tissues. However, there may also be an alternative N source which has not been included in the hepatic N balance calculations such as peptide-N (Webb, 1986; Webb, 1990).

4.5.5 Urea metabolism

The urea concentrations in the blood vessels sampled were similar for the two intake groups, therefore the urea transfer values for the PDV and liver were also similar (Table 4.3). Considering that the mass transfer data recorded similar ammonia production levels for the two intake levels, this result was expected. The urea production, expressed as g N/d, was comparable to N intake for both intake groups (Table 4.4).

The ^{15}N-urea metabolism also confirms similar mass transfer values for the two intake groups, although the relative difference between the two treatments was much less than for the urea mass transfer values. The absence of double labelled urea (^{15}N^{15}N) confirms earlier reports suggesting that the contribution of ammonia to urea-N is restricted to the single N atom (Lobley *et al.*, 1995; Lobley *et al.*, 1996b).

4.5.6 Energy metabolism

Using the assumption that urea synthesis requires four high energy phosphate bonds per molecule synthesised and that there are six of these bonds per oxygen molecule consumed (Martin & Blaxter, 1965), ureagenesis accounted for 0.156 and 0.317 (SED 0.071; P = 0.078) of hepatic oxygen consumption in the low and high intake groups, respectively. These values are greater than those reported previously (0.11-0.16; Reynolds *et al.*, 1991; Lobley *et al.*, 1995). In terms of direct energetic cost, ureagenesis used 147 and 216 kJ/d (SED 58.2; P = 0.311; assuming 122 kJ/mole urea synthesised; Martin & Blaxter, 1965; Waghorn & Wolff, 1984), which was 0.017 and 0.019 (SED 0.0059; P = 0.789) of metabolisable energy (ME) intake (ME content of fresh white clover 10.5 MJ/kg DM; Waghorn & Wolff, 1984; Beever *et al.*, 1985) in the low and high intake groups, respectively. This represents 0.029 v 0.042 of the energetic requirements of an average daily gain of 200 g/d (Waghorn & Wolff, 1984).

4.5.7 Whole body and hepatic protein metabolism

The estimates of whole body ILR, protein synthesis and fractional synthesis rates were similar when based on the SRA of either whole blood or plasma (data not shown). The estimates of protein synthesis were comparable to previous work using the SRA of either whole blood or plasma with sheep fed in a similar manner (Davis e*t al.*, 1981; Harris e*t al.*, 1992; Lobley e*t al.*, 1995).

There were no significant differences in the rate of hepatic protein synthesis between the sheep fed the two intake levels (Table 4.6).

Cysteine is a non-essential amino acid which is the oxidation product of methionine, an essential amino acid which is known to be first limiting in both skin growth and wool production (Lee e*t al.*, 1995). There was detectable SRA in the oxidation products of cysteine, taurine and sulphate, with the latter indicative of the terminal cysteine oxidation.

4.6 CONCLUSION

The current study clearly demonstrated that significant differences in both DM and N intake did not induce significant differences in ammonia, urea and amino acid transfers across the PDV and liver, with the sole exception of ^{15}N-ammonia hepatic transfers. There was no evidence to suggest that elevated rates of hepatic ammonia detoxification require a disproportionate increase in amino acid-N for ureagenesis. The difference between the ammonia mass transfer and ^{15}N-ammmonia transfer data highlight the potential limitations of the former technique for quantifying small metabolite concentration and hence transfer differences. It is possible that differences in ammonia transfers between treatments could be generated by increasing the intake for the high intake group, however to the authors knowledge there are no reports of sheep eating pure fresh white clover (in metabolism crates) at greater than the intake levels recorded here. These data confirm that the energetic cost of ureagenesis accounts for a sizeable proportion of ME intake, hence any increment in the energetic requirements for ureagenesis can restrict the rate of productive gain.

Future work could evaluate other techniques of inducing hepatic ammonia overload and hence a measurable increase in hepatic amino acid metabolism whilst feeding fresh white clover, such as ammonia infusions or to manipulate the feeding regimens.

5 CHAPTER 5. THE METABOLIC COST OF HEPATIC AMMONIA DETOXIFICATION IN SHEEP FED FRESH WHITE CLOVER (*TRIFOLIUM REPENS*) IN TWO 2 HOUR PERIODS PER DAY

5.1 ABSTRACT

The effect of rapidly consuming fresh white clover (*Trifolium repens*) in two 2 hour feeding periods per day on splanchnic ammonia and urea production, hepatic oxygen consumption and amino acid utilisation were studied in six sheep. The animals were infused (16.5 h) with $^{15}NH_4Cl$ and *para*-aminohippurate *via* the mesenteric vein. Blood samples were collected to determine ammonia, urea, oxygen, and amino acid concentrations. During the two 2 hour feeding periods, the sheep ate a total of 560 g DM/d (0.69 of the DM offered) and 21.9 g N/d. Ammonia production from the portal drained viscera and extraction by the liver tended to increase following feeding, both of which had returned to pre-feeding levels within seven hours following the conclusion of feeding. There was no resultant increase in hepatic amino acid metabolism, nor hepatic urea production. There was a trend for increased hepatic glutamine production during feeding, followed by an increase in glutamine extraction when the ammonia concentration returned to pre-feeding levels. Hepatic glutamine synthetase activity followed an inverse trend to that of hepatic glutamine metabolism. This suggests that hepatic glutamine synthetase activity was used to supplement the ammonia detoxification capacity of ureagenesis. Although there was a trend for increased ammonia production following the rapid consumption of fresh white clover, there was no elevation in urea production nor amino acid catabolism.

5.2 INTRODUCTION

Fresh forages such as white clover, are characterised by containing protein which is highly soluble (solubility 0.75-0.80; Mangan, 1982). The degradation of dietary protein in the reticulo-rumen (rumen) produces large quantities of ammonia. The rumen microbes can utilise this ammonia directly (Parker *et al.* 1995). However, the rate of ammonia utilisation is often below the rate of production, with the result that any excess of ammonia is passively absorbed across the rumen wall and transported to the liver (Huntington, 1982). The liver converts ammonia to urea, potentially incurring an amino acid cost (Reynolds, 1992; Lobley *et al.* 1995; Lobley *et al.* 1996b), which may reduce the quantity of amino acids available for other purposes, such as productive gain.

Previous work in this laboratory with sheep fed at two intake levels (800 and 1400 g DM/d) of fresh white clover failed to demonstrate any incremental cost in amino acid utilisation associated with the increase in ammonia production incurred with the higher feeding level (Chapter Four). In the current study sheep were trained to consume fresh white clover rapidly by allowing the animals access to their daily ration for only two 2 hour periods per day. This pulse feeding approach was adopted as opposed to a mesenteric ammonia infusion to more closely simulate the physiological conditions associated with increased intake of a whole diet which would occur under grazing conditions. Previous attempts to quantify the increase in hepatic amino acid catabolism in response to mesenteric ammonia infusions have not been successful (Lobley *et al.* 1995).

Using blood samples taken from surgically implanted catheters within the splanchnic vasculature, the portal drained viscera (PDV) ammonia production was quantified and the resultant effects on hepatic ammonia detoxification, nitrogen (N) balance and amino acid metabolism were determined. The animals were infused with $^{15}NH_4Cl$ to trace the hepatic detoxification of ammonia to urea and ^{15}N incorporation into amino acids.

5.3 MATERIALS AND METHODS

These experimental procedures and protocols were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Palmerston North, New Zealand according to the Animals Protection Act (1960), Animals Protection Regulations (1987) and amendments.

5.3.1 Animals and surgical procedures

Six Romney-cross wether lambs, aged 6-9 months with a mean initial body weight of 33.4 kg (SE 0.6), were prepared with silicone based catheters in the posterior aorta, mesenteric (x2), portal and hepatic veins as described previously (Chapter Three). These animals were adjusted to hourly feeding, from automatic feeders of fresh white clover (*Trifolium repens*). A four week recovery period followed surgery during which the animals were offered 800 g DM/d.

5.3.2 Diets and design

During the experimental period the sheep were offered fresh white clover harvested daily from a pure sward with a minimum of 4-6 weeks growth. Rapid consumption of feed (offered 800 g DM/d) was achieved by restricting the animals access to feed to two 2 hour periods per day. The two feeding intervals began at 0800 and 1600, respectively. The uneaten feed at the conclusion of each 2 hour period was removed. The animals were accustomed to this feeding regimen for ten days prior to entering a 10 day N balance.

The sheep were infused with $^{15}NH_4Cl$ *via* the mesenteric vein for 16.5 h to assist with the quantification of the rate of hepatic ammonia detoxification, ureagenesis and amino acid catabolism.

The methodology employed during this trial was identical to previous work in this laboratory (Chapter Three).

5.3.3 Infusion protocol

The sheep were continuously infused with 146 mmol/l $^{15}NH_4Cl$ (99.8 atoms percent enrichment, APE; Icon, Mt Marion, USA) for 16.5 hours at a rate of 22.7 g/h using a peristaltic pump (302F, Watson-Marlow Limited, Cornwall, England). After 5.75 hours the $^{15}NH_4Cl$ infusate was supplemented with sodium *para*-aminohippurate (*p*AH; Sigma Chemicals, St Louis, USA; 203 mmol/l) and the infusion continued for a further 10.75 hours.

The sheep were manually infused with a bolus of 5,000 IU heparin (Ovine origin; New Zealand Pharmaceuticals, Palmerston North, New Zealand) before blood sampling began. Blood samples were withdrawn (at 22.7 g/h) from the mesenteric, portal, hepatic and arterial catheters into 15 ml polypropylene vials (on ice) containing 50 μl sodium ethylenediaminetetra-acetic acid (EDTA; 0.15 w:v in water, as an anticoagulant). Blood was collected for the first 30 minutes of each of nine sampling hours, that is, the 6th (the first sampling hour), 7th, 8th, 9th, 10th, 11th, 12th, 14th and 16th (11th sampling hour) hours of $^{15}NH_4Cl$ infusion. Each infusion was scheduled such that a two hour feeding period occurred from the beginning of the 8th hour (0800) to the end of the 9th hour of infusion, that is, the beginning of the third to the end of the fourth sampling hour.

5.3.4 Analytical measurements

A galvanic oxygen cell was used to evaluate blood oxygen concentrations immediately following collection (Grubb & Mills, 1981).

The *p*AH concentration in whole blood was determined using the methods described by Harris *et al.* (1992) with an additional acid deacetylation step at 90 °C for 65 minutes (Lobley *et al.* 1995).

Whole blood (2 g) was haemolysed with an equal weight of chilled 200 μmol/l L-nor-leucine (BDH Chemicals Ltd., Poole England) to which 200 mg of 80 mmol/l dithiothreitol in 0.2 mol/l phosphate buffer (*p*H 8.0) was added. The remaining blood was centrifuged and the plasma removed.

Haemolysed blood (1 g) was ultra-filtered through a 10,000 molecular weight cut-off membrane (Centrisart®; Sartorius, Gottingen, Germany). Amino acid concentrations were determined in 100 mg of the resultant supernatant *via* picotag analysis and reverse phase high performance liquid chromatography (Cohen *et al.* 1986).

The enrichment of both [^{15}N]-amino acids and [^{15}N]-urea in the haemolysed blood were determined by gas chromatography and mass spectrometry (GCMS; VG Trio-1, VG Masslab Ltd, Manchester, Cheshire, UK) using the methods described by Nieto *et al.* (1996).

Plasma ammonia concentrations were determined (non-gravimetrically) with a commercial kit (#171, Sigma Chemical, St Louis, USA) that was based on the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase.

Plasma urea concentrations were measured with a commercial kit (#535, Sigma Chemical, St Louis, USA), based on the conversion of urea to hydroxyl-amine in the presence of diacetylmonoxime when heated with sulphuric acid.

The ammonia present in 1 g of plasma was converted to nor-valine for the subsequent determination of [^{15}N]-ammonium enrichment *via* GCMS using the methods described by Nieto *et al.* (1996).

5.3.5 Terminal procedures

Following a two week recovery period the animals were again infused with *p*AH and two blood samples taken to ascertain blood flow rates and ammonia concentrations only, using the methodology outlined previously. The animals were then euthanased at various times relative to the feeding period, that is corresponding to the sampling hours of 1, 3, 4, 6, 9, 11. Liver tissue samples were taken and frozen at –85 °C for the subsequent determination of glutamine synthetase (EC 6.3.1.2) activity. The rate of formation of glutamylhyrodxamate following hydroxylamine substitution for ammonia, was used to estimate the

glutamine synthetase activity, using the procedures outlined by Rowe *et al.* (1970).

5.3.6 Calculations and statistics

The mass and isotope transfers were calculated as outlined previously (Chapter Three).

The data were subjected to analysis of variance using the generalised linear model procedure (SAS, 1985) with animals treated as blocks for the effect of time relative to feeding. Where appropriate (a significant effect of time from the above analysis) the significance of the difference between means was assessed by t-test using the 0.05 level of significance. The first two, pre-ingestion, time points, were individually compared against each of the last seven sampling time points.

5.4 RESULTS

All catheters remained patent for the duration of the experimental period (6 months).

5.4.1 Feed intakes

The dry matter (DM) and N content of the feed were 138 g/kg and 39.2 g N/kg DM respectively. The sheep ate 0.69 of the DM offered within the two 2 hour feeding periods per day, with a total intake of 560 g DM/d and 21.9 g N/d. The DM and N digestibilities were 0.818 and 0.848 respectively. The net N retention was 1.5 g N/d.

5.4.2 Blood flows and oxygen exchange

The estimates of blood flow in the mesenteric vein were highly variable (351-40,451 g/min), therefore data from this catheter (and subsequently the mesenteric drained viscera *per se*) were omitted from further consideration. The portal and hepatic vein and hepatic arterial blood flows were similar for the nine time points, therefore the measured blood flow rates were not significantly affected by the onset of feeding (Figure 5.1 and appendix Table 8.1). The contribution of the hepatic arterial flow to hepatic venous flow ranged from 0.08 to 0.27 and was not significantly affected by time.

There were no significant differences in PDV and liver oxygen transfers for the nine time points (Figure 5.2 and appendix Table 8.1).

5.4.3 Ammonia and urea transfers

The ammonia and urea concentrations in the portal and hepatic vein, and the posterior aorta were not significantly affected by time (Table 5.1 and appendix Table 8.2). The ammonia transfers across the PDV and liver were significantly affected by the time after the onset of ingestion (Figure 5.3, Table 5.1 and appendix Table 8.2). Urea transfers across the PDV and liver were not affected by the time after the onset of ingestion (Figure 5.4 and appendix Table 8.3).

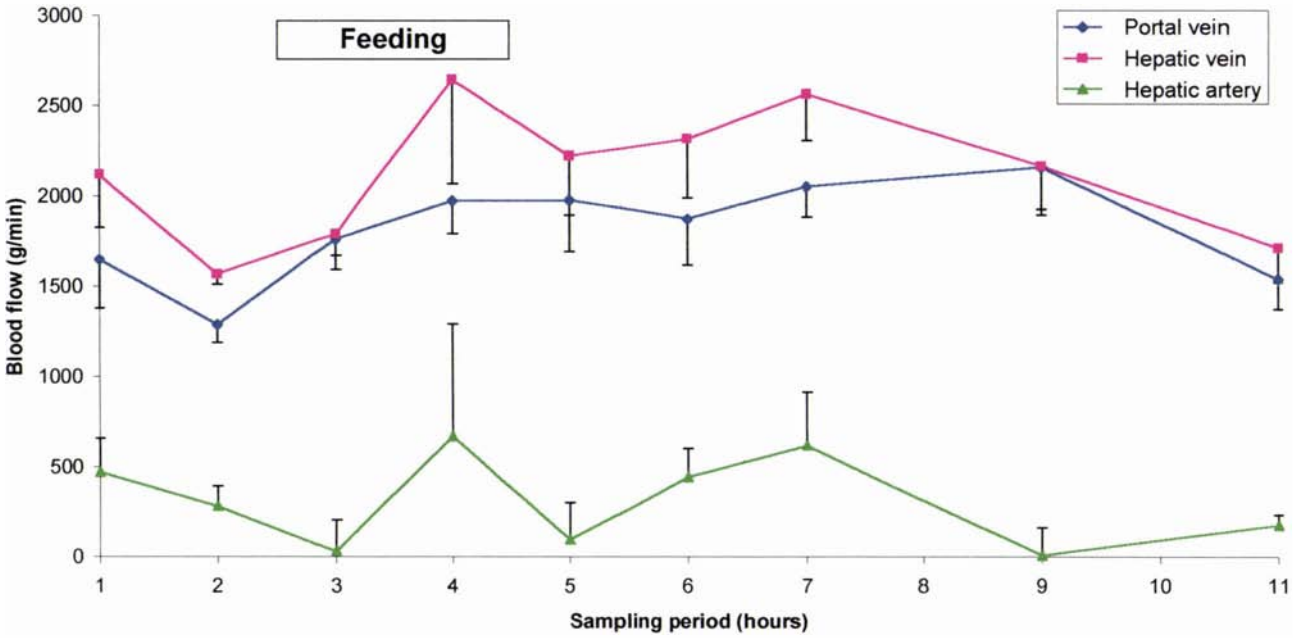


Figure 5.1 Blood flow in the portal and hepatic veins and hepatic artery in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

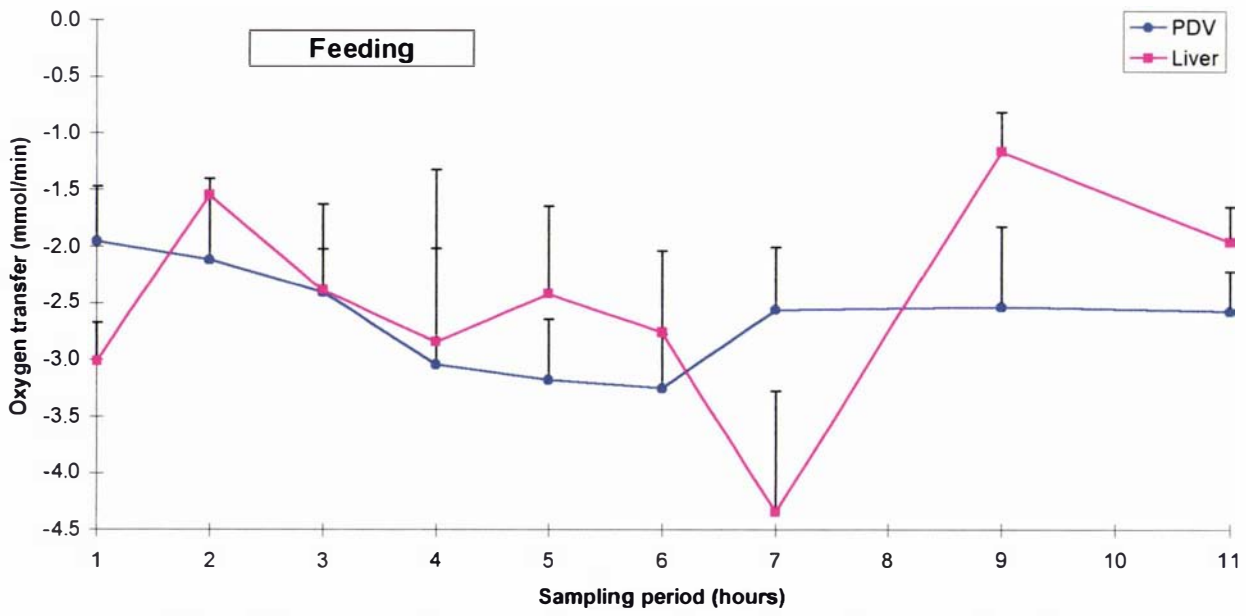


Figure 5.2 Oxygen transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

Considered as g N/d, the estimates of hepatic ammonia transfer ranged (across the nine time points) from 9.0 to 22.7 g N/d, with hepatic urea production ranging from 0.9 to 44.1 g N/d (Table 5.2).

The portal and hepatic fluxes of ammonia-N were significantly affected by the time after the onset of ingestion (Table 5.2). Urea fluxes in the portal and hepatic veins and the posterior aorta were not significantly affected by the time after the onset of ingestion (Table 5.2).

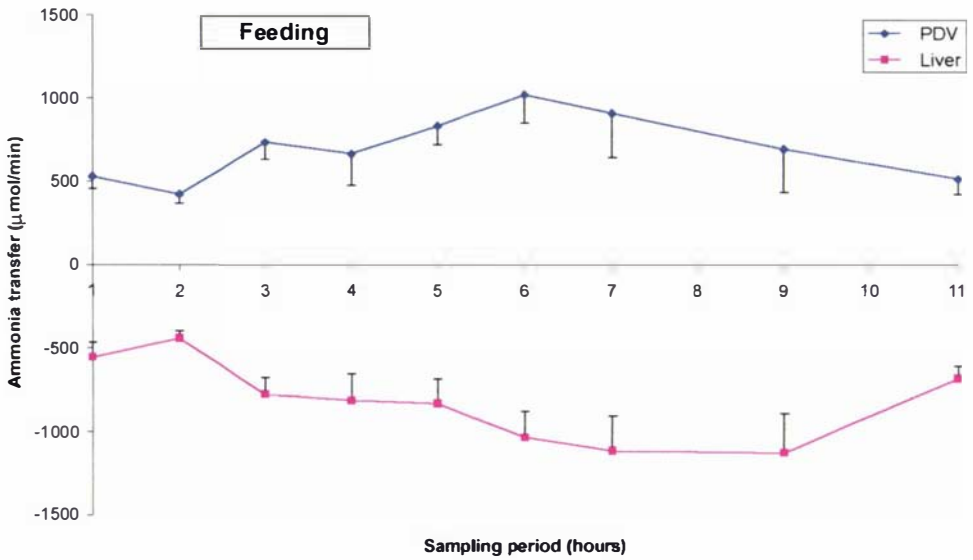


Figure 5.3 Ammonia transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

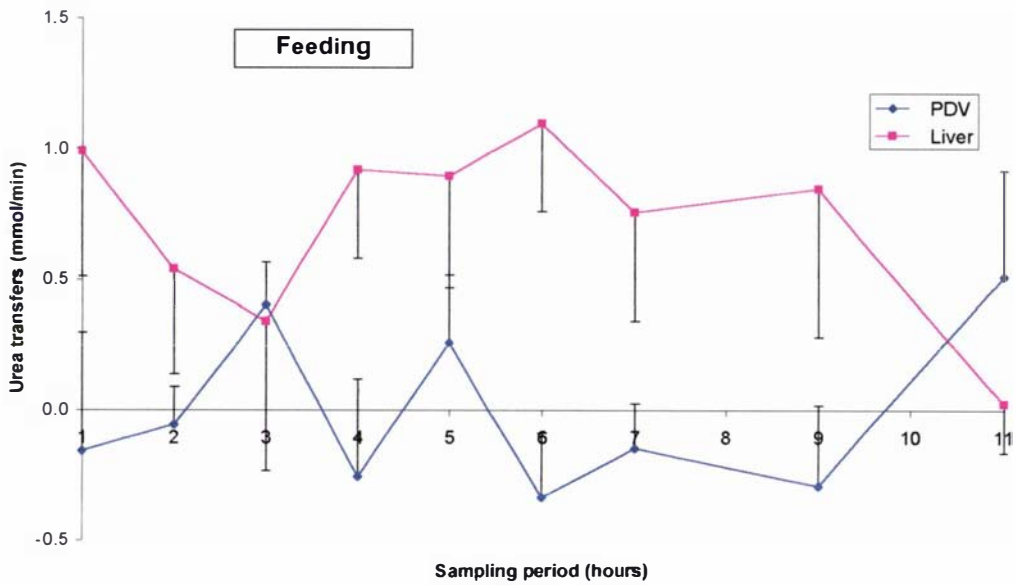


Figure 5.4 Urea transfers across the portal drained viscera (PDV) and liver in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

Table 5.1. Ammonia concentrations and transfers in sheep offered white clover in two 2 hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

Ammonia (μmol/l)	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Portal vein	467	393	397	501	490	474	538	554	464	392	89.7	0.494
Hepatic vein	63	51	52	64	83	71	83	86	47	33	24.9	0.462
Hepatic artery	81	66	68	92	91	69	88	98	84	73	25.5	0.890
Ammonia transfers (μmol/min)												
PDV	700	^{da}529	^{dc}421	^{a}733	^{b}663	^{b}830	^{b}1019	^{b}904	^{a}690	^{ac}511	199.0	0.033
Liver	-821	^{da}-557	^{db}-446	^{a}-780	^{a}-818	^{c}-836	^{a}-1035	^{a}-1113	^{a}-1125	^{a}-682	179.1	0.009

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.
Means with the same super-script are not significantly different at 0.05.

Table 5.2. Summary of nitrogen (N) fluxes in sheep offered white clover in two 2 hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

gN/d	Average	Sampling period (hours)									SED	*P*
N intake	21.9	1	2	3	4	5	6	7	9	11		
NH_3-N flux												
Portal	18.9	^{ad}12.9	^{cd}10.3	^{a}18.0	^{a}19.9	^{b}19.8	^{b}24.2	^{b}25.8	^{a}25.0	^{a}14.6	3.94	0.045
Hepatic	2.9	^{ad}2.1	^{cd}1.6	^{ac}2.4	^{bc}4.9	^{ac}2.8	^{b}4.2	^{b}4.4	^{ac}2.5	^{ac}1.2	1.13	0.048
Post. aorta	0.5	0.5	0.3	0.1	1.5	-0.1	0.9	1.1	0.2	0.3	0.62	0.379
AA-N flux												
Portal	103.2	^{a}87.4	^{a}86.0	^{b}104.5	^{a}101.3	^{b}151.1	118.1^{b}	^{a}89.9	^{b}101.0	^{a}89.8	14.21	0.008
Hepatic	201.6	^{ad}151.7	^{cd}147.1	^{b}190.7	^{b}217.1	^{b}264.5	247.6^{b}	^{bc}185.9	^{b}254.3	^{ac}155.8	18.37	<0.001
Post. aorta	27.0	^{a}18.2	^{c}7.8	^{b}14.5	^{b}28.5	^{a}26.0	64.6^{b}	^{b}29.9	^{b}36.2	^{a}17.5	3.08	<0.001
Urea-N flux												
Portal	694.0	618.2	470.0	653.2	734.8	767.5	727.2	823.4	847.5	604.5	116.73	0.076
Hepatic	842.1	827.8	589.2	683.3	1023.4	902.8	946.5	1053.2	880.3	672.4	162.22	0.080
Post. aorta	118.1	169.8	97.5	16.4	251.6	35.7	175.2	251.2	-1.3	67.0	128.50	0.459
Hepatic N transfers												
NH_3-N	-16.6	^{ad}-11.2	^{bd}-9.0	^{a}-15.7	^{a}-16.5	^{c}-16.9	^{a}-20.9	^{a}-22.5	^{a}-22.7	^{a}-13.8	4.26	0.009
AA-N	-2.1	-2.5	0.7	-0.6	-1.1	-11.8	12.1	-6.0	-0.2	-9.1	7.00	0.684
Urea-N	28.6	39.8	21.7	13.7	37.0	36.0	44.1	30.4	34.1	0.9	9.27	0.689
Balance	-48.9	-53.5	-30.0	-30.1	-54.6	-61.7	-60.3	-61.3	-70.4	-18.6	14.09	0.916

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Flux = metabolite concentration x blood flow in the respective vessels.
Note; AA-N arterial points 1 & 2 significantly different. Post. aorta = posterior aorta.
Feeding between sampling hours 3 and 4, beginning at 0800.
Means with the same super-script are not significantly different at 0.05.

5.4.4 Amino acid transfers

Amino acid concentrations were similar across all time points (data not presented). Net PDV and liver amino acid metabolism was not affected by time (Table 5.3 and 5.4). The amino acid fractional removal rates were highly variable but not significantly affected by time (Table 5.5).

The amino acid-N fluxes in the portal and hepatic veins and the posterior aorta were significantly affected by the time after the onset of ingestion (Table 5.2).

Although the hepatic glutamine synthetase activity data was based on only one animal per time point, there was a trend for the activity to respond inversely to the level of hepatic glutamine transfer (Figure 5.5).

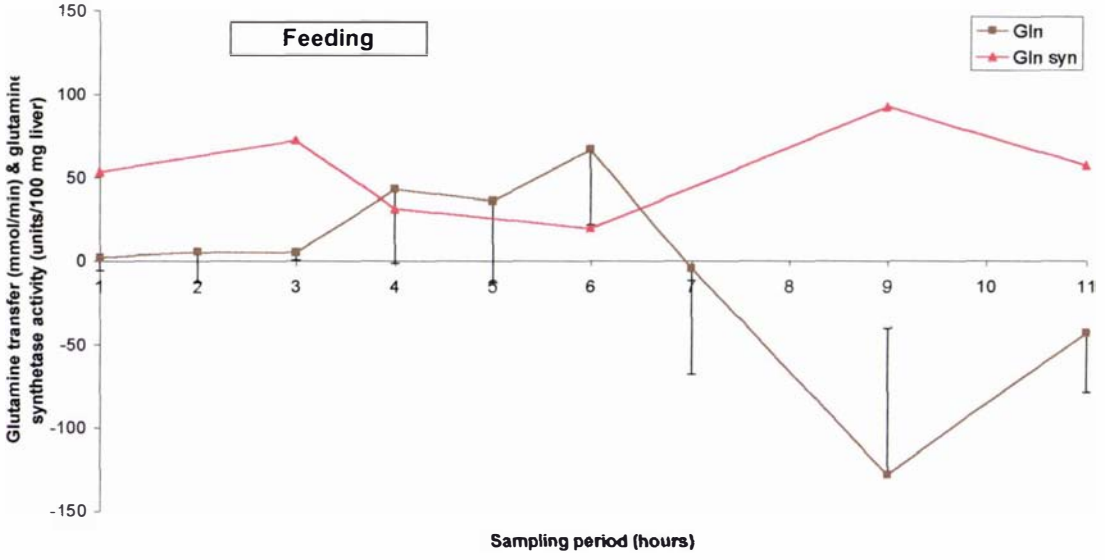


Figure 5.5 Hepatic glutamine transfers & glutamine synthetase activity in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

Table 5.3. PDV amino acid transfers (μmol/min) in sheep offered fresh white clover in two 2 hour periods per day.
(Mean and standard error of the difference between means {SED} for six sheep)

AA	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Thr	11.6	-9.3	11.9	2.8	6.1	36.3	9.1	13.6	23.5	10.5	10.80	0.100
Val	14.4	-11.4	13.8	1.3	16.2	37.8	-32.1	10.7	82.8	10.3	31.21	0.313
Met	3.4	6.0	2.4	0.0	0.4	10.2	0.7	1.7	4.6	4.8	4.99	0.799
Ile	14.3	5.1	11.1	5.4	11.9	35.7	1.3	13.7	32.4	11.7	10.68	0.172
Leu	17.4	7.0	16.0	6.2	18.1	42.1	-6.0	17.5	43.8	12.3	15.62	0.269
Phe	10.8	7.2	10.3	4.9	10.2	22.8	2.9	10.9	20.0	7.8	5.29	0.084
Lys	17.9	5.4	18.1	5.9	15.6	40.4	5.7	21.0	36.0	12.8	11.45	0.164
His	-18.2	-62.6	-55.6	-13.6	-22.3	53.6	-79.5	-9.0	29.9	-4.6	8.81	0.601
Trp	1.7	-1.1	0.6	0.1	0.8	10.1	1.3	-0.6	1.0	3.0	2.42	0.095
Arg	11.8	30.3	4.4	-4.7	8.8	20.5	-10.1	4.9	46.2	5.9	24.00	0.742
Tyr	8.3	0.9	7.1	1.6	7.5	19.6	-1.2	8.1	25.2	5.4	6.88	0.082
Asp	1.5	0.4	8.4	-1.8	5.0	-3.3	-1.6	1.8	4.5	0.3	4.52	0.526
Gln	-27.0	-43.5	-26.3	-29.0	-37.1	-12.4	-51.0	-32.5	15.1	-26.0	32.57	0.771
Glu	-11.5	14.9	-19.7	-17.0	-20.5	-33.6	-89.3	-1.8	52.7	10.8	46.62	0.607
Asn	14.1	7.4	12.5	6.2	13.3	32.3	5.1	12.8	25.4	12.0	7.09	0.062
Ser	16.7	-4.3	16.6	8.3	20.2	38.5	6.3	14.1	33.7	17.1	9.84	0.057
Gly	18.5	-0.5	30.4	-19.5	61.6	34.0	-97.4	18.3	118.4	20.9	51.15	0.194
Ala	13.7	8.9	15.4	1.6	21.8	6.5	3.1	16.0	35.8	13.8	20.19	0.954
Pro	7.1	4.3	8.7	-0.8	8.0	24.5	-12.4	2.6	24.2	4.9	11.51	0.320
Cit	9.9	1.9	10.1	3.0	10.6	20.2	-2.6	5.8	30.9	9.6	10.13	0.335
Orn	5.9	-0.2	8.1	0.9	7.4	13.4	-1.4	5.7	16.0	3.4	6.93	0.521

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.

Table 5.4. Hepatic amino acid transfers (μmol/min) in sheep offered fresh white clover in two 2 hour periods per day.
(Mean and standard error of the difference between means {SED} for six sheep)

AA	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Thr	3.3	3.8	2.4	5.4	3.4	-13.9	18.3	2.0	8.4	-0.1	16.04	0.935
Val	-3.3	-11.5	-2.8	0.4	-1.6	-35.3	49.9	2.2	-25.5	-4.9	42.28	0.910
Met	0.8	-3.5	1.1	0.1	2.5	-1.0	5.8	1.9	3.3	-2.9	6.13	0.958
Ile	-1.2	-4.1	0.2	-0.3	-5.1	-20.4	16.2	0.5	3.9	-1.6	15.65	0.858
Leu	-10.5	-7.7	-0.2	-42.6	-10.9	-34.1	16.8	-0.3	-4.5	-10.9	26.08	0.750
Phe	-3.6	-8.5	-6.0	-3.6	-2.4	-8.7	12.5	4.3	-7.3	-12.8	10.03	0.635
Lys	-8.8	-6.5	-3.0	0.6	-4.0	-31.3	10.4	-13.7	-17.3	-14.4	13.57	0.435
His	2.2	20.3	80.4	54.0	127.7	24.6	-151.2	41.6	87.5	-265.3	10.92	0.497
Trp	-2.1	-6.9	-2.3	-2.9	-1.1	-8.2	3.7	-1.0	1.2	-1.5	5.52	0.874
Arg	-26.3	-6.6	-7.3	-1.4	-33.2	-50.8	-5.6	-33.0	-69.2	-29.6	25.47	0.430
Tyr	-2.6	-7.3	-4.8	-1.6	1.3	-8.0	17.3	-0.7	-10.3	-9.0	11.99	0.732
Asp	2.1	-1.8	5.3	8.8	-0.6	-1.7	8.4	0.5	-2.1	1.9	6.65	0.809
Gln	-2.1	1.9	5.2	4.9	42.8	35.7	66.7	-4.6	-128.0	-43.4	46.83	0.524
Glu	37.6	1.7	29.6	-7.2	91.2	74.0	103.3	2.3	14.7	28.8	53.66	0.648
Asn	-4.5	-4.8	-3.3	-2.0	0.1	-16.1	11.0	-0.3	-15.6	-9.6	10.08	0.530
Ser	0.4	4.7	4.4	-0.2	2.4	-16.1	13.8	-6.2	0.6	0.5	16.88	0.959
Gly	-81.3	-25.9	7.7	3.1	-123.4	-188.1	-30.3	-49.4	-229.8	-95.8	71.59	0.121
Ala	-15.8	-16.8	-13.3	-7.0	-23.9	-5.3	4.2	-15.2	-34.5	-30.8	21.69	0.907
Pro	-8.7	-9.4	-7.5	-4.8	-4.3	-21.6	12.4	-11.1	-16.8	-15.1	14.74	0.795
Cit	-0.7	-0.2	4.0	3.0	6.2	-8.3	12.2	-3.7	-11.1	-8.2	14.47	0.935
Orn	8.5	2.1	7.5	4.1	11.9	1.8	18.8	11.9	13.0	5.3	13.68	0.983

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.

Table 5.5. Fractional hepatic amino acid transfers in sheep offered fresh white clover in two 2 hour periods per day.
(Mean and standard error of the difference between means {SED} for six sheep)

AA	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Thr	0.28	-0.41	0.20	1.95	0.56	-0.38	2.00	0.15	0.36	-0.01	7.192	0.439
Val	-0.23	1.01	-0.20	0.28	-0.10	-0.93	-1.55	0.20	-0.31	-0.47	4.837	0.417
Met	0.24	-0.58	0.48	5.00	7.01	-0.10	8.93	1.10	0.73	-0.61	1.171	0.195
Ile	-0.08	-0.81	0.02	-0.05	-0.43	-0.57	12.47	0.04	0.12	-0.14	4.674	0.270
Leu	-0.60	-1.09	-0.01	-6.92	-0.60	-0.81	-2.81	-0.02	-0.10	-0.89	2.647	0.320
Phe	-0.34	-1.18	-0.58	-0.74	-0.24	-0.38	4.34	0.39	-0.37	-1.64	7.132	0.594
Lys	-0.49	-1.20	-0.16	0.10	-0.26	-0.77	1.81	-0.65	-0.48	-1.12	3.098	0.821
His	-0.12	-0.32	-1.45	-3.97	-5.72	0.46	1.90	-4.64	2.93	57.77	3.651	0.247
Trp	-1.25	6.45	-3.68	-24.67	-1.41	-0.82	2.89	1.86	1.20	-0.50	5.272	0.385
Arg	-2.23	-0.22	-1.63	0.30	-3.76	-2.48	0.55	-6.72	-1.50	-5.00	3.495	0.311
Tyr	-0.31	-7.72	-0.68	-0.99	0.17	-0.41	-14.91	-0.09	-0.41	-1.65	7.138	0.434
Asp	1.38	-4.35	0.64	-4.77	-0.12	0.52	-5.09	0.30	-0.46	6.67	2.912	0.111
Gln	0.08	-0.04	-0.20	-0.17	-1.15	-2.88	-1.31	0.14	-8.45	1.67	7.601	0.369
Glu	-3.27	0.11	-1.50	0.43	-4.45	-2.20	-1.16	-1.27	0.28	2.66	1.325	0.141
Asn	-0.32	-0.65	-0.26	-0.32	0.01	-0.50	2.18	-0.03	-0.61	-0.81	12.362	0.676
Ser	0.03	-1.11	0.27	-0.03	0.12	-0.42	2.18	-0.44	0.02	0.03	1.595	0.851
Gly	-4.40	52.67	0.25	-0.16	-2.00	-5.54	0.31	-2.70	-1.94	-4.57	7.640	0.233
Ala	-1.16	-1.88	-0.86	-4.28	-1.09	-0.80	1.36	-0.95	-0.97	-2.23	3.517	0.986
Pro	-1.22	-2.22	-0.86	5.91	-0.54	-0.88	-1.00	-4.30	-0.69	-3.07	2.672	0.060
Cit	-0.07	-0.09	0.40	1.00	0.58	-0.41	-4.74	-0.64	-0.36	-0.86	8.654	0.405
Orn	1.43	-9.31	0.92	4.36	1.61	0.13	-13.40	2.10	0.81	1.55	1.953	0.266

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.

5.4.5 ^{15}N transfers

The ^{15}N-urea (arterial) enrichment had reached 7.6 APE after sixteen hours of $^{15}NH_4Cl$ infusion (Figure 5.6). Label recycling was evidenced by the flux of ^{15}N-urea at the three sampling sites exceeding the rate of $^{15}NH_4Cl$ infusion. Of the labelled urea ($[^{14}N^{15}N]$ or $[^{15}N^{15}N]$) recovered across the liver, the single labelled form $[^{14}N^{15}N]$ predominated. Double labelled $[^{15}N^{15}N]$ urea was not detected. The detection limit of the GCMS was 0.2 APE.

The hepatic ^{15}N-ammonia transfers were not significantly affected by time (Table 5.6). The level of ^{15}N incorporation in the free amino acids was below the detection limits of the GCMS.

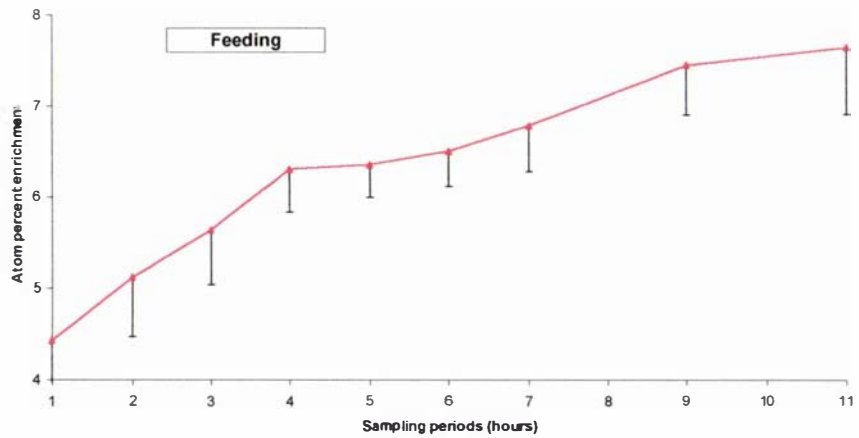


Figure 5.6 Arterial ^{15}N-urea enrichment in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n = 6).

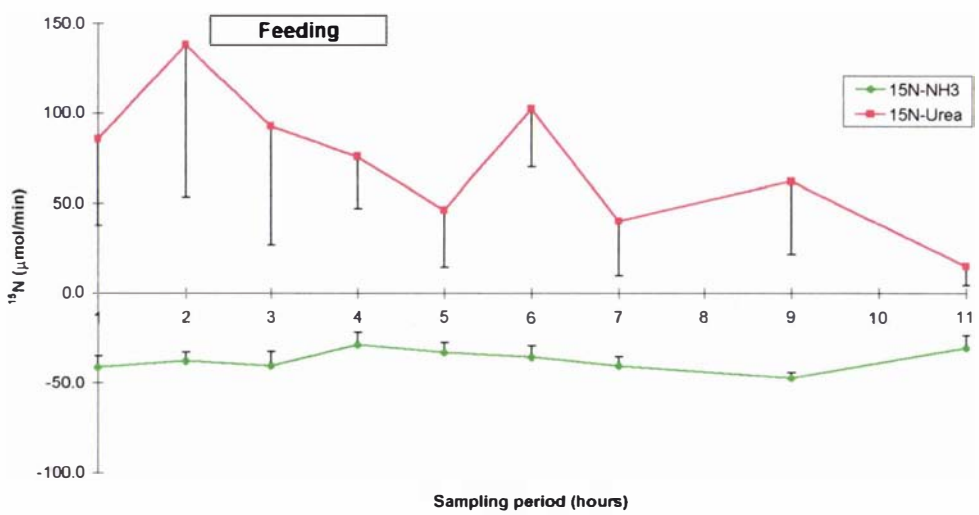


Figure 5.7 Hepatic ^{15}N-ammonia and ^{15}N-urea transfers in 6 sheep fed fresh white clover for two 2 hour periods per day (mean +/- SE; n= 6).

Table 5.6. Hepatic ^{15}N fluxes in sheep offered white clover in 2 two hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

		Sampling period (hours)								SED	P
	Average	1	2	3	4	5	6	7	9	11		
Liver ^{15}N transfers (mmol/min)												
^{15}N-NH_3	-37.3	-41.3	-37.8	-40.4	-28.9	-33.2	-35.7	-40.4	-47.4	-30.6	6.76	0.625
^{15}N-Urea	72.7	85.2	137.9	92.4	75.6	45.8	101.9	39.9	61.7	14.5	50.94	0.660

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.
No significant difference between sampling hours.
Means with the same super-script are not significantly different at 0.05.

5.5 DISCUSSION

The objective of this study was to estimate the metabolic cost of hepatic ammonia detoxification in sheep rapidly consuming fresh white clover for two 2 hour periods per day. Previous reports have suggested that increased rates of ureagenesis may incur a coincident metabolic cost in terms of elevated amino acid catabolism (Reynolds, 1992). However attempts to induce such increases in amino acid catabolism with *ad libitum* feeding of fresh white clover were unsuccessful (Chapter Four). This work investigated the hypothesis that the hepatic ammonia load will increase (at least briefly) above those previously recorded in this laboratory (Chapter Three and Four) in response to "pulse" (two by 2 hour) feeding of fresh white clover. Further, hepatic ureagenesis may increase, requiring an increase in hepatic amino acid catabolism.

The significant results of the current study were that although there was a significant increase in both PDV ammonia production and hepatic ammonia extraction following feeding, there was no significant increase in hepatic urea production nor amino acid catabolism. There was a suggestion of increased hepatic glutamine production during feeding followed by an increased rate of extraction when the ammonia detoxification rate returned to pre-feeding levels. The opposite trend was observed for glutamine synthetase activity. This suggests that hepatic glutamine synthesis was used to temporarily remove ammonia that could not be detoxified immediately *via* ureagenesis.

5.5.1 Intake parameters

The DM intake as a proportion of DM offered (0.69) was lower than previous work in this laboratory (0.84; Chapter Four), as a direct result of the restricted feeding period these animals were permitted.

The feeding regimen employed in this experiment conditioned the sheep to rapidly consume the feed when available, a phenomenon which is typical of the regimen often employed to feed dairy cows in New Zealand. That is, following a period of fasting (during milking), the cow is returned to the pasture and eats

rapidly. To the author's knowledge, there are few reports of metabolic evaluations following such feeding regimens under experimental conditions (Waghorn, 1986; Waghorn *et al.* 1989), and none with sheep. Compared to previous work in this laboratory using continuous (24 hour) feeding from automated feeders, where the animals consumed 816 g DM/d {34 g/h} (Chapter Four), the intake of 560 g DM/d {140 g/h} achieved by these animals in a total of four hours feeding per day, indicates that four hours feeding per day is sufficient for maintenance purposes. Both the DM and N digestibilities were also comparable to previous work in this laboratory (Chapter Four), indicative of a normal digestive process following the abnormal ingestion pattern.

5.5.2 Blood flows

The average blood flow in the portal and hepatic veins were comparable to previous reports (Barnes *et al.* 1983a; Lush & Gooden, 1988; Burrin *et al.* 1989; Huntington, 1990; Harris *et al.* 1992; Lobley *et al.* 1995). Although there was no significant difference in blood flow (in any vessel measured) across the nine time points, there was a trend for increased blood flow during ingestion which extended for three hours following the cessation of ingestion (appendix Table 8.1) confirming previous reports (Whitt *et al.* 1996). The variability associated with the estimates of the mesenteric blood flow is discussed in section 6.2.3.

5.5.3 Hepatic ammonia metabolism

Although there was a trend for increased portal ammonia concentration starting one hour after feeding and extending for seven hours, the difference was not significant (Table 5.1). This trend was not evident in either the hepatic vein or posterior aorta, with the latter ammonia concentration maintained below 100 μmol/l, well below the recognised metabolic ammonia overload concentration of 700 μmol/l (Symonds *et al.* 1981). There were significant increases in PDV ammonia production and hepatic extraction following feeding. The increase in ammonia production from the PDV had receded within seven hours of the conclusion of ingestion. This finding was also demonstrated in cows fed fresh ryegrass during two 2 hour periods per day, with a peak in rumen ammonia

concentration recorded two hours after the initiation of feeding, returning to pre-feeding concentrations within two hours following the cessation of ingestion (Waghorn *et al.* 1989).

There was no discernible change in hepatic ^{15}N-ammonia extraction over time (Table 5.6), further confirming that these animals were able to metabolise the ammonia produced during the digestive process without major changes to the ureagenic pathway or increases to the various ammonia storage pools.

5.5.4 Amino acid metabolism

The PDV amino acid production was generally extracted by the liver, with the exception of the branched chain amino acids (Table 5.3 and 5.5). There was no measurable increase in hepatic amino acid catabolism associated the onset of feeding (Table 5.4).

The large variation associated with these amino acid data potentially obscures any true biological findings. However, there was a trend toward increased hepatic glutamine production following the onset of feeding which changed to net glutamine extraction for the last three time points (5, 7, 9 hours after the onset of ingestion, respectively). The level of hepatic glutamine synthetase activity measured in the six sheep at various times relative to the feeding period was comparable to that recorded for the rat liver (Meister, 1985). Together, these two trends suggest that hepatic glutamine synthetase activity was used to detoxify the hepatic ammonia load, in addition to ureagenesis during the period of elevated ammonia load, but when this load decreased, the glutamine produced was recycled to the liver. That is, hepatic glutamine synthetase activity was used as a backup, short-term ammonia detoxification regimen to support ureagenesis.

The net hepatic nitrogen transactions (ammonia-N plus amino acid-N minus urea-N) clearly demonstrated the variability associated with such measurements (Table 5.2; average –48.9 g N/d). However, if the difference between the averages were taken (ammonia-N + amino acid-N – urea-N) for

each time point rather than the average of each of the six animals' net liver N transactions, values close to net balance were recorded (Table 5.2; 9.9 g N/d). These re-calculated values may offer a closer representation of the actual situation that occurred in these experimental animals, as the liver could not sustain the large N loss suggested with the previous, more accepted method of calculating net hepatic N balance.

5.5.5 Urea metabolism

The urea concentrations in the blood vessels sampled were similar to previous reports (Chapter Four) and did not demonstrate any effect of time after feeding (appendix Table 8.3). Urea transfers for the PDV and liver were highly variable, as is often the case with this metabolite (Parker *et al.* 1995), and were not affected by the time after feeding. Hepatic ^{15}N-urea metabolism was also not affected by the time after feeding. The absence of detectable quantities of double labelled urea ($^{15}N^{15}N$) confirms earlier reports suggesting that the contribution of ammonia-N to urea-N is restricted to the single N atom (Lobley *et al.* 1995; Lobley *et al.* 1996b; Chapter Three and Four). The hourly hepatic urea production values (Table 5.2) may be used to provide estimates of the daily urea production, as g N/d, which were of a similar magnitude to the daily N intake. This urea data clearly shows that the liver detoxified the pulse of ammonia generated by the pulse feeding regimen, without significantly increasing the rate of urea production.

5.5.6 Energy metabolism

From the assumption that urea synthesis requires four high energy phosphate bonds per molecule synthesised and that there are six of these bonds per oxygen molecule consumed (Martin & Blaxter, 1965), ureagenesis accounted for 0.211 (SED 0.127; P = 0.744) of hepatic oxygen consumption. This value (the average of nine time points) is comparable to previous reports (0.11-0.16; Reynolds *et al.* 1991; Lobley *et al.* 1995). In terms of direct energetic cost, ureagenesis used 113 kJ/d (SED 76; P = 0.634; assuming 122 kJ/mole urea synthesised; Martin & Blaxter, 1965; Waghorn & Wolff, 1984), which was 0.05 of metabolisable energy (ME) intake (ME content of fresh white clover 10.5

MJ/kg DM; Waghorn & Wolff, 1984; Beever *et al.* 1985). This represents 0.022 of the energetic requirements of an average daily gain for 200 g/d (Waghorn & Wolff, 1984).

5.6 CONCLUSION

The results from this study indicate that animals accustomed to two 2 hour feeding periods per day exhibited a trend toward elevated ammonia production following feeding, resulting in a similar trend in hepatic ammonia extraction. However, urea production did not follow this trend. There was no discernible increase in hepatic energy consumption. Hepatic glutamine production tended to increase following feeding, then change to net extraction five hours after the onset of feeding. Conversely hepatic glutamine synthetase activity increased during and immediately after feeding, with a subsequent decrease in activity, suggesting that hepatic glutamine synthetase activity was used to supplement the ammonia detoxification capacity of ureagenesis. This study did not record any elevation in hepatic amino acid catabolism as a result of elevated levels of hepatic ammonia detoxification. Future work could utilise the same feeding regimen employed in the current work, with more than the six animals used here to reduce the measured variation.

6 CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 INTRODUCTION

The New Zealand agricultural system is based on the efficient utilisation of fresh temperate pastures. The climate in New Zealand is sufficiently mild to allow grass growth throughout the year. A typical New Zealand pasture is dominated by ryegrass (*Lolium perenne*) and white clover (*Trifolium repens)*. Ruminants generally graze pastures year round, although forage conservation, as both silage and hay, is widely practised.

Fresh forage is characterised by a crude protein content (20-35%) that is rapidly degraded in the rumen with a resultant high loss of N as ammonia (MacRae, 1975). The carbohydrate content of fresh forage comprises predominantly structural carbohydrates that are digested relatively slowly in the rumen, hence the availability of energy (supplied from carbohydrate) and nitrogen for microbial protein synthesis in the rumen may be asynchronous. Additionally, seasonal climatic variations induce significant changes in the relative proportions of protein and carbohydrate in fresh forages, further exacerbating these problems. The asynchrony of energy and nitrogen supply within the rumen is one of the contributing factors to the reduction in productivity associated with ruminants fed fresh forages as compared to iso-energetic concentrate diets (Reynolds *et al.* 1991).

The high protein content of Lucerne (*Medicago sativa*) makes it an important protein source for ruminants in many countries. Lucerne has a higher protein content than ryegrass, for example, therefore digestion of lucerne within the rumen is rapid and yields a higher concentration of volatile fatty acids, consequently lucerne has a higher nutritive value than ryegrass (Waghorn *et al.* 1989). The efficiency of lucerne digestion can be increased by protecting the protein from digestion within the rumen, that is, increasing the proportion of by-pass protein. This maybe achieved by a number of different techniques, however a common method is to dry, then grind and finally pelletise the lucerne (Preston & Leng, 1987b).

In New Zealand fresh white clover is rarely used as a pure sward, predominantly due to the prevalence of bloat following grazing such a sward. Instead, white clover is the minority species, comprising 0 to 30% of the ryegrass/white clover combination that predominates in New Zealand's pastures. The protein content of fresh white clover is characteristically high, 20-35%, with significant seasonal variation (Rattray & Joyce, 1975; Ulyatt & Egan, 1979; Beever *et al.*, 1985). Once ingested, the protein content of fresh forage is highly soluble (75-80%), with a large proportion of this protein rapidly degraded (90% within one hour) in the rumen (Ulyatt, 1997). The soluble carbohydrate content of fresh white clover is also relatively high and seasonally variable (5-15%), therefore the total energy content of this forage is also high (10-20 MJ/kg DM).

Ruminants grazing a typical New Zealand pasture are presented with a diet consisting of a high crude protein content, which is readily soluble in the rumen and is rapidly fermented producing large quantities of ammonia (Ulyatt & MacRae, 1974). The resident rumen microbial population utilises the available ammonia for protein synthesis, however the energy supply for this process is quickly exhausted (Ulyatt *et al.*, 1984). The excess rumen ammonia is passively absorbed into the venous blood system for subsequent detoxification to urea in the liver. The rate of hepatic urea-N production generally exceeds the rate of hepatic ammonia-N extraction (Parker *et al.*, 1995). Such findings have lead to the suggestion that hepatic ureagenesis requires an amino acid input, which may reduce amino acid availability for productive processes (Reynolds, 1992; Ulyatt, 1997).

This thesis investigated the hypothesis that increased hepatic ammonia detoxification would require an increase in hepatic amino acid catabolism for ureagenesis. The animals productive capability may be impaired as a result of the decreased hepatic amino acid output.

6.2 METHODOLOGICAL CONSIDERATIONS

6.2.1 Surgical methodology

The surgical approach adopted in this study was first published the same year this work began, therefore the techniques applied were contemporary. Whilst the surgical technique *per se* has remained predominantly unchanged, the catheter construction has improved with simplified construction methods using materials which are less prone to thrombus formation or blocking, potentially increasing the term of catheter patency (Milano *et al.*, 2000). Additionally these catheters are easier to insert into the appropriate veins / arteries during surgery, which reduces surgery times and consequently the stress on the animal.

6.2.2 The arterio-venous difference technique

The arterio-venous difference technique is dependent upon accurate measurements of the primary determinants; blood flow and metabolite concentration (Boisclair *et al.*, 1993). Any errors in the primary measurements are compounding and have considerable influence on the final estimate of metabolite transfer. Clear definition of the tissue across which the arterio-venous differences are taken is fundamental to obtaining accurate measurements.

Comparison of the nitrogen flux data with net hepatic nitrogen (g N/d) transfer data presented in Tables 3.3, 4.4 and 5.2 illustrates an interesting problem with the presentation of arterio-venous difference data such as these. Theoretically the net hepatic N transfer could be compiled from the summation of the appropriate fluxes in each of the above tables (hepatic - portal – arterial), however the difference in the number of time points that contributed to the mean for each animal did not allow such a summation. That is, the fluxes (as g N/d) of the various metabolites within each of the vessels considered are presented, with data from the hepatic transfers (as g N/d) presented at the bottom of Tables 3.3, 4.4 and 5.2. To calculate the flux data presented requires only the data from the specific vessel under consideration to generate

a mean, that is, the blood flow and the metabolite concentration, and for the data presented in Chapter Three, this comprised four time points or samples per animal. However, to calculate the hepatic transfer data, blood flow from the portal and hepatic veins and the estimation of the hepatic arterial blood flow, plus the concentration data for each of these vessels was required. If one of these pieces of data were missing (for example, the portal vein blood flow estimation was not available) then the entire hepatic transfer result for that time point would not contribute to the mean for that animal. The net result is that there may be a different number of time points contributing to the individual animal mean from which the average of the treatment groups is presented in these tables, however the number of animals per mean are the same.

6.2.3 Estimation of blood flow

Dye dilution of *p*AH was used to estimate the blood flow rates in this work because *p*AH is not significantly metabolised by the liver, with the exception of an acetylation step. An acid deacetylation step was added to the standard *p*AH analysis (Harris *et al.* 1992) to remove the acetyl group accumulated following hepatic metabolism. The *p*AH is predominantly removed from the blood by the kidneys, almost eliminating the recycling of *p*AH to the mesenteric vein. The probe technique (Tepperman & Jacobson, 1982), with its numerous variants, was not considered for these experiments because the hepatic vein is inaccessible for probe placement and it is desirable to utilise the same method of blood flow determination for all of the catheterised vessels to allow concise quantitation of sequential blood flows.

The estimates of blood flow presented in Chapters Four and Five for the mesenteric vein were highly variable (351-40,451 g/min), therefore the results were not presented. Although the surgical technique was identical to that used in Chapter Three, there was no obvious explanation for this finding. The distance between the mesenteric infusion and sampling catheters were the same between experiments, however the estimates of blood flow presented in Chapters Four and Five are suggestive of *p*AH streaming within that vein. At autopsy the mesenteric catheters appeared normal.

The hepatic arterial blood flow was estimated by difference between the portal and hepatic vein flows, therefore any variation in either estimate would distort the estimate of arterial flow, further increasing the variability associated with the blood flow estimate in the artery. This was confirmed by the estimates of hepatic arterial blood flow in the current work, which had a higher level of variation (SED) than that recorded for the other vessels considered.

6.2.4 Tracer methodology

The infusion of $^{15}NH_4Cl$ provided quantitative information on the hepatic metabolism of ^{15}N, particularly the rate of formation of single and double labelled ^{15}N-urea. Conservation of label is indicative of accurate quantitation and concise measurements. The absence of detectable levels of ^{15}N in any amino acid was unexpected and contrasts with the findings of Lobley *et al.* (1995) using similar analytical methodology and in the case of the work described in Chapter Three, the analysis was conducted in Dr Lobleys laboratory. There were no other concise direct comparisons of ^{15}N metabolism between the current report and that of Lobley *et al.* (1995), therefore this apparent difference is difficult to explain. However in the work of Lobley *et al.* (1995), it is possible that the acidosis caused by the relatively high level of $^{15}NH_4Cl$ infusion, which is in contrast to the present experiments, may have altered the normal amino acid metabolism, and therefore may be the cause of the observed differences.

6.3 AMMONIA DETOXIFICATION

Rumen ammonia production was increased when the animals were fed fresh white clover compared with lucerne pellets. However, the significance of this difference was reduced to that of a trend (P = 0.084) when considered across the PDV. There was no significant difference in PDV ammonia production recorded for the low and high intake groups in Chapter Four, despite a 39% increase in N intake. Whilst both the blood flow and ammonia concentrations were not significantly different, the values recorded for both parameters were

lower for the high intake group, combining to ensure no significant differences in hepatic transfer values. In the work described in Chapter Five, there was a trend towards elevated portal blood flow and ammonia concentrations and consequently the rate of hepatic ammonia extraction, for a period of 4-6 hours following the onset of ingestion. These data cumulatively demonstrate that the experimental animals removed the elevated levels of ammonia production without increasing the hepatic vein or arterial ammonia concentrations. This was achieved by equimolar increases in the level of urea production. The ratio of hepatic ammonia extraction to urea production was within the range 0.6-1.6 for all of the comparisons with the exception of the first and last time points following the initiation of ingestion in the pulse fed trial (Chapter Five). The former exception is perhaps indicative of the lag phase required for ureagenesis to respond to a rapid increase in hepatic ammonia extraction (with the next time point within the expected ratio), whereas the latter was due to a very low level of ureagenesis for that time point.

6.4 SOURCES OF N FOR UREAGENESIS

The urea molecule contains two N atoms which are sourced from ammonia *via* carbamoyl-phosphate synthesis and from cytoplasmic aspartate. The mitochondrial and cytoplasmic aspartate / glutamate transamination pools are in equilibrium (Cooper *et al.* 1988), therefore it is possible for the N of ammonia (or amino acids) to contribute both of the N atoms of the urea molecule by reversing the action of glutamate dehydrogenase (GLDH). If the mitochondrial supply of ammonia is not able to meet the elevated demand for ureagenesis, the amino acid-N is the only other source of the ammonium ion, effectively inducing an amino acid deamination cost for ureagenesis. In this work, there was no evidence to suggest that ammonia contributed both of the N atoms of the urea molecule, nor was there an increase in hepatic amino acid catabolism as the rate of hepatic ammonia detoxification to urea increased.

The spatial arrangement of the liver ensures that any excess ammonia escaping periportal ureagenesis is incorporated into glutamine in the perivenous cells (Parker *et al.* 1995). Therefore elevated levels of glutamate

extraction and glutamine production are indicative of periportal ammonia overload. Increased levels of glutamate extraction were noted in the comparison between lucerne pellets and fresh white clover but not in the intake level comparison nor with the pulse fed animals. Elevated levels of hepatic glutamine production were recorded in the comparison with lucerne pellets and following the onset of feeding in the pulse fed animals, but not in the intake level comparison. The level of glutamine synthetase activity was not significantly affected by the onset of ingestion, nor was there a significant change in activity during the period of investigation (pulse fed data only). Therefore, this study does not support a clear role for the glutamate / glutamine shuttle as a means of temporal ammonia detoxification.

The rate of ureagenesis may be increased in response to high protein diets by increasing the capacity of the urea cycle enzymes, predominantly by an increase in enzyme mass (Morris, 1992). Carbamoyl phosphate synthetase (CPS*I*) has been shown to be the rate limiting enzyme of ureagenesis (Cohen *et al*. 1985; Meijer e*t al*. 1985; Morris, 1992). Short term (minutes) regulation of CPS*I* is achieved by changes in substrate concentrations, whereas longer term (hours / days) regulation is achieved by changes in CPS*I* mass (Cohen e*t al*. 1985). It has been shown that the activity of CPS*I* is highly dependent on the concentration of NAG as an allosteric regulator (Shigesada & Tatibana, 1971; Lusty, 1981). Therefore the concentration of NAG was evaluated using the methods of Alonso & Rubio (1985) and the activity of CPS*I* was recorded following the methods outlined by Kaseman & Meister (1985). Following many modifications to these methods, it was concluded that the liver tissue samples were not prepared pre-storage nor stored appropriately to allow either the NAG concentration or CPS*I* activity to be determined. It should also be noted that the interpretation of the hepatic enzyme activity was also impaired by the lack of animals available for evaluation. The lack of reportable data for both the NAG concentration and CPS*I* activity from this work was disappointing, as increasing the throughput of this enzyme and hence ureagenesis was definitely the mechanism these animals utilised to effectively respond to the various ammonia challenges presented to them. This method of ammonia

detoxification is the most economical (metabolically) available to these animals and appears to be the first choice method of dealing with such sub-clinical ammonia over-load situations as those presented by this work.

6.5 THE ENERGETIC COST OF UREAGENESIS

If it is assumed that the energetic cost of ureagenesis is 122 kJ/mole (Martin & Blaxter, 1965; Waghorn & Wolff, 1984) and that the energetic cost of body weight gain is 3.4-4.1 MJ/g (Waghorn & Wolff, 1984), then the energetic equivalent of the urea produced by the liver in these trials equates to 4-14 g of live weight gain per day. Obviously the urea produced by the liver is not entirely lost to the body, with a variable proportion returned to the GIT. The pulse fed animals used in the experiment reported in Chapter Five required the highest proportion of ME intake for ureagenesis, which was associated with a relatively low proportion of hepatic oxygen consumption, indicative of the ease with which the liver dealt with the pulse of ammonia generated by this dietary regimen.

6.6 THE INFLUENCE OF ACIDOSIS ON HEPATIC N METABOLISM

It has been suggested that mild acidosis may be induced by the infusion of NH_4Cl (Lobley *et al.,* 1996; Milano *et al.*, 2000), following earlier reports which demonstrated that amino acid oxidation was stimulated by NH_4Cl administration in humans (Reaich *et al.*, 1992), providing a surplus of amino acid-N available for ureagenesis. Unfortunately the blood *p*H was not recorded during the current work, therefore direct comparisons with earlier reports (Lobley *et al.,* 1995, 1996; Milano *et al.*, 2000) cannot be made. Although the levels of NH_4Cl infusion in the present work were relatively low (0.13-0.4 of that used by Lobley *et al.,* 1995) mild acidosis may still have been induced which may have elevated the level of amino acid oxidation providing supplemental amino acid-N for ureagenesis. However there was no evidence from any of the three experiments reported here, to suggest that the rate of hepatic amino acid catabolism increased as the rate of ureagenesis increased.

6.7 HEPATIC ADAPTATION

The liver is able to rapidly adapt to changes in dietary intake, therefore the changes to intake levels used in this trial may not have been severe enough to induce changes in hepatic ammonia metabolism and hence ureagenesis. In Chapter Four, the experimental animals were fed *ad-libitum* prior to the initiation of the N balance and infusion period. The animals were able to sustain an intake level of 1118 g DM/d (43.9 g N/d), a level which is below that achievable by animals grazing a similar pure sward of fresh white clover *ad-libitum* (Ulyatt, 1997). This apparent depression of intake may be partially explained by an apathetic approach from these animals to the same diet. Alternatively an excess of a micro-nutrient or toxin, such as cyanide (Gurnsey *et al.*, 1977), may have been partially responsible.

6.8 CONCLUSIONS

Despite a significant increase in rumen ammonia production from the animals fed fresh white clover, there was only a trend toward higher hepatic ammonia extraction rates in the sheep fed fresh white clover compared to the animals fed lucerne pellets. Consequently there was no significant difference in amino acid catabolism between the two diets. There was no evidence to suggest that ammonia provided both of the N atoms required for urea synthesis.

The experiment reported in Chapter Four showed that despite significant differences in both DM and N intake there was no significant difference in ammonia, urea and amino acid transfers across the PDV and liver, with the exception of ^{15}N-ammonia transfer across the liver. This finding suggests that the differences (if any) between treatments in this experiment were too small to be detected with the mass transfer technique, particularly with only six animals being used in this experiment.

Restricting feed access to only two 2 hour periods per day resulted in a trend toward elevated ammonia production following feeding, which generated a similar trend in hepatic ammonia extraction. Urea production did not follow this

trend, nor was there any evidence to suggest an increase in hepatic amino acid catabolism following the onset of ingestion.

This set of experiments has utilised various different dietary treatments to try to increase the rate of hepatic ammonia detoxification required to maintain basal systemic ammonia concentrations. Hepatic urea production has been shown to rise without any apparent increase in hepatic amino acid catabolism. There was no evidence to suggest that ammonia contributed both of the N atoms of the urea molecule. It has been assumed that the ureagenic capacity of the liver has been modified by changes to enzyme mass, specifically the enzymes CPS*I* and GLDH, without the need to reverse the reaction of the latter.

From this set of experiments it may be concluded that the level of amino acid catabolism associated with hepatic ureagenesis was similar across the range of diets (and therefore protein solubility) and intake levels presented to the experimental animals. It is well documented that the liver is able to adapt to changes in dietary intake (Reynolds, 1992), as such the dietary and intake challenges presented to the sheep used in these experiments may not have been sufficiently severe to induce measurable changes in hepatic ammonia metabolism. It is suggested that changes in the activity of the primary regulator of the rate of ureagenesis, CPS*I*, allowed these experimental animals to effectively detoxify the ammonia challenges presented to them without incurring any additional amino acid catabolism. The variation associated with the primary determinants of metabolite transfer, blood flow and metabolite concentration, were relatively large in this set of experiments, suggesting that future work should seek to reduce both the intra- and inter-animal variation to increase the probability of quantifying the true differences, if any, between treatments.

6.9 FUTURE WORK

Future work should address the causes of the high inter- and intra-animal variability inherent with experiments of this nature. Sample pooling within a day of infusion could be utilised to reduce the intra-animal variation, as utilised

to good effect in the amino acid analysis reported by Milano *et al.* (2000). The experimental period could be extended to cover multiple days of infusion with samples (say four) taken over an extended period (say six hours) per day. The easiest way to reduce variability is to increase the number of animals per treatment group, however the experimental costs escalate accordingly, which may be prohibitive.

The arterio-venous difference technique is reliant upon accurate determinations of the primary components, namely blood flow and metabolite concentration. A reduction in the variation associated with both of these parameters could be achieved by multiple measurements of the same variable within a time point.

Having improved the empirical measurements and thereby reduced the variation, consideration should be given to methods of taking the experimental animals to the extremes of ammonia metabolism such that the ratio of ammonia extraction to urea production greatly exceeds one-to-one. This may be achieved by adding other physiological demands such as lactation or pregnancy. Alternatively, intensive investigation of the period immediately following a short term starvation period (for example, the third time point in the pulse data, which is the first time point following the onset of ingestion) may yield more conclusive results as to the shorter term mechanisms controlling the rate of ureagenesis. This approach may utilise a more rapid sampling regimen such that blood samples are extracted every fifteen minutes over a period of two hours, for example.

It has been shown that diets containing condensed tannins, such as *Lotus pedunculatus*, effectively reduce rumen ammonia production and increase the absorption of dietary amino acids (Waghorn *et al.*, 1987; McNabb *et al.*, 1993; Waghorn *et al.*, 1994). This diet could be offered to similarly catheterised experimental animals to evaluate whether the rate of rumen ammonia production and hepatic amino acid catabolism would be lower than that recorded in these experiments.

Consideration should be given to developing a working method to evaluate the concentration of NAG and CPS*I* activity in Ovine hepatocytes, which will lead to a clearer understanding of how the Ovine liver metabolises the ammonia challenges presented to it.

Extrapolation of the above findings, using pure fresh white clover, to other diets and feeding regimens, in addition to other physiological conditions (for example pregnancy or lactation) need careful consideration or preferably further work.

In conclusion, these data clearly demonstrate that the Ovine liver is capable of adequately metabolising the ammonia challenges presented by the various feeding regimens applied in this set of experiments. The ratio of hepatic ammonia extraction to urea production didn't significantly exceed one to one, therefore there was no increment in hepatic amino acid catabolism to provide additional N for ureagenesis. Future work should endeavour to reduce the variation associated with the empirical measurements utilised to generate tissue metabolite transfer values. Accurate quantification of the activity of the primary rate regulator of ureagenesis, CPS*I*, is required to confirm that increasing the activity of this enzyme is how the Ovine liver responds to sub-clinical ammonia challenges.

7 CHAPTER 7. REFERENCES

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Table 8.1. Blood flows and oxygen transfers in sheep offered fresh white clover in two 2 hour periods per day.
(Mean and standard error of the difference between means {SED} for six sheep)

Blood flow (g/min)	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Portal vein	1807	1648	1288	1759	1973	1976	1871	2050	2157	1536	290.3	0.126
SE	201	265	100	165	180	283	254	166	233	161		
Hepatic vein	2120	2118	1568	1788	2642	2219	2313	2561	2163	1709	389.0	0.120
SE	267	291	56	117	576	327	325	257	270	187		
Hepatic artery	309	470	280	29	669	92	442	616	6	173	339.2	0.491
SE	220	187	112	176	625	209	159	296	158	60		
Oxygen (mmol/min)												
PDV	-2.6	-2.0	-2.1	-2.4	-3.0	-3.2	-3.3	-2.6	-2.5	-2.6	0.90	0.845
SE	0.61	0.49	0.54	0.78	1.03	0.54	0.48	0.56	0.71	0.35		
Liver	-2.5	-3.0	-1.5	-2.4	-2.8	-2.4	-2.8	-4.3	-1.2	-2.0	0.94	0.130
SE	0.62	0.34	0.14	0.36	1.52	0.77	0.72	1.07	0.35	0.31	0.94	0.130

SE, Standard error of the mean.

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.

Feeding between sampling hours 3 and 4, beginning at 0800.

Table 8.2. Ammonia concentrations and transfers in sheep offered white clover in two 2 hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

Ammonia (μmol/l)	Average	Sampling period (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Portal vein	467	393	397	501	490	474	538	554	464	392	89.7	0.494
SE	57	27	37	22	35	80	84	103	79	46		
Hepatic vein	63	51	52	64	83	71	83	86	47	33	24.9	0.462
SE	17	21	21	24	20	14	7	24	7	11		
Posterior aorta	81	66	68	92	91	69	88	98	84	73	25.5	0.890
SE	18	20	21	20	17	19	7	19	14	21		
(μmol/min)												
PDV	700	^{da}529	^{dc}421	^{a}733	^{b}663	^{b}830	^{b}1019	^{b}904	^{a}690	^{ac}511	199.0	0.033
SE	146	75	54	104	190	111	171	262	260	90		
Liver	-821	^{da}-557	^{db}-446	^{a}-780	^{a}-818	^{c}-836	^{a}-1035	^{a}-1113	^{a}-1125	^{a}-682	179.1	0.009
SE	136	89	47	101	163	150	157	210	235	77		

SE, Standard error of the mean.
Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.
Feeding between sampling hours 3 and 4, beginning at 0800.
Means with the same super-script are not significantly different at 0.05.

Table 8.3. Urea concentrations and transfers in sheep offered fresh white clover in two 2 hour periods per day.

(Mean and standard error of the difference between means {SED} for six sheep)

Urea (mmol/l)	Average	Sampling period (hours)									SED	P
		1	2	3	4	5	6	7	9	11		
Portal vein	9.51	9.35	8.98	9.32	9.16	9.54	9.58	9.99	9.88	9.76	0.438	0.344
SE	0.30	0.28	0.39	0.32	0.24	0.22	0.23	0.33	0.40	0.33		
Hepatic vein	9.76	9.77	9.33	9.39	9.53	9.78	10.05	10.18	10.10	9.70	0.505	0.654
SE	0.35	0.34	0.33	0.45	0.25	0.26	0.27	0.34	0.47	0.41		
Posterior aorta	9.44	9.38	9.00	9.04	9.29	9.41	9.74	9.92	9.81	9.32	0.480	0.503
SE	0.33	0.47	0.38	0.30	0.22	0.20	0.26	0.30	0.38	0.44		
(mmol/min)												
PDV	-0.01	-0.16	-0.05	0.40	-0.25	0.26	-0.33	-0.15	-0.29	0.51	0.423	0.375
SE	0.28	0.45	0.14	0.16	0.37	0.26	0.25	0.17	0.31	0.41		
Liver	0.71	0.99	0.54	0.34	0.92	0.89	1.09	0.75	0.85	0.02	0.578	0.689
SE	0.41	0.48	0.40	0.57	0.34	0.43	0.33	0.41	0.56	0.19		

SE, Standard error of the mean.

Positive and negative values are indicative of net production and net extraction of the metabolite by the relevant organ respectively.

Feeding between sampling hours 3 and 4, beginning at 0800.

Table 8.4. Hepatic nitrogen (N) transfers in sheep offered white clover in two 2 hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

gN/d	Average	Sampling periods (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
Liver N transfers												
NH_3-N	-16.6	[ad]-11.2	[bd]-9.0	[a]-15.7	[a]-16.5	[c]-16.9	[a]-20.9	[a]-22.5	[a]-22.7	[a]-13.8	4.26	0.009
SE	2.8	1.8	1.0	2.0	3.3	3.0	3.2	4.2	4.7	1.6		
AA-N	-2.1	-2.5	0.7	-0.6	-1.1	-11.8	12.1	-6.0	-0.2	-9.1	7.00	0.684
SE	6.0	5.4	7.6	1.3	3.0	4.0	13.3	7.2	4.8	7.2		
Urea-N	28.6	39.8	21.7	13.7	37.0	36.0	44.1	30.4	34.1	0.9	9.27	0.689
SE	5.8	6.5	8.3	1.4	4.2	3.3	13.8	5.9	6.2	2.7		
Liver ^{15}N transfers (mmol/min)												
^{15}N-NH_3	-37.3	-41.3	-37.8	-40.4	-28.9	-33.2	-35.7	-40.4	-47.4	-30.6	6.76	0.625
SE	5.9	6.3	5.0	8.0	7.1	5.6	6.4	4.9	3.1	6.9		
^{15}N-Urea	72.7	85.2	137.9	92.4	75.6	45.8	101.9	39.9	61.7	14.5	50.94	0.660
SE	41.3	47.6	84.6	65.8	28.9	31.7	32.0	30.5	40.4	10.2		

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Flux = metabolite concentration x blood flow in the respective vessels.
Feeding between sampling hours 3 and 4, beginning at 0800.
Means with the same super-script are not significantly different at 0.05.
SE, Standard error of the mean.

Table 8.5. Summary of nitrogen (N) fluxes in sheep offered white clover in two 2 hour periods per day.
(Mean value and standard error of the difference between means {SED} for six sheep)

gN/d	Average	Sampling periods (hours)									SED	*P*
		1	2	3	4	5	6	7	9	11		
N intake	21.9											
NH_3-N flux												
Portal	18.9	^{ad}12.9	^{cd}10.3	^{a}18.0	^{a}19.9	^{b}19.8	^{b}24.2	^{b}25.8	^{a}25.0	^{a}14.6	3.94	0.045
SE	1.8	1.9	1.3	2.1	3.4	3.1	3.7	4.6	5.2	1.2		
Hepatic	2.9	^{ad}2.1	^{cd}1.6	^{ac}2.4	^{bc}4.9	^{ac}2.8	^{b}4.2	^{b}4.4	^{ac}2.5	^{ac}1.2	1.13	0.048
SE	0.4	0.7	0.6	1.0	1.4	0.8	0.6	1.1	0.7	0.4		
Arterial	0.5	0.5	0.3	0.1	1.5	-0.1	0.9	1.1	0.2	0.3	0.62	0.379
SE	0.2	0.2	0.1	0.2	1.2	0.5	0.4	0.6	0.4	0.2		
AA-N flux												
Portal	103.2	^{a}87.4	^{a}86.0	^{b}104.5	^{a}101.3	^{b}151.1	^{b}118.1	^{a}89.9	^{b}101.0	^{a}89.8	14.21	0.008
SE	6.9	8.1	9.5	6.3	7.5	17.7	17.4	12.2	9.4	8.7		
Hepatic	201.6	^{ad}151.7	^{cd}147.1	^{b}190.7	^{b}217.1	^{b}264.5	^{b}247.6	^{bc}185.9	^{b}254.3	^{ac}155.8	18.37	<0.001
SE	15.4	5.8	8.6	14.2	15.0	21.6	21.8	8.5	24.8	10.0		
Arterial	27.0	^{a}18.2	^{c}7.8	^{b}14.5	^{b}28.5	^{a}26.0	^{b}64.6	^{b}29.9	^{b}36.2	^{a}17.5	3.08	<0.001
SE	5.5	1.7	0.6	1.4	2.3	2.0	6.1	2.4	3.3	1.9		
Urea-N flux												
Portal	694.0	618.2	470.0	653.2	734.8	767.5	727.2	823.4	847.5	604.5	116.73	0.076
SE	42.0	91.6	47.3	47.0	58.0	174.2	106.8	70.6	116.7	66.8		
Hepatic	842.1	827.8	589.2	683.3	1023.4	902.8	946.5	1053.2	880.3	672.4	162.22	0.080
SE	57.2	102.3	24.5	70.1	215.4	144.9	145.6	118.8	134.8	84.7		
Arterial	118.1	169.8	97.5	16.4	251.6	35.7	175.2	251.2	-1.3	67.0	128.50	0.459
SE	32.5	64.8	36.6	61.0	235.0	81.5	63.0	121.0	68.2	22.4		

Positive and negative values indicate net production and net extraction of the metabolite by the relevant tissue respectively.
Flux = metabolite concentration x blood flow in the respective vessels. Means with the same super-script are not significantly different at 0.05.
Note; AA-N arterial points 1 & 2 significantly different. Feeding between sampling hours 3 and 4, beginning at 0800.
SE, Standard error of the mean.

8.2 SUPPLEMENTAL MATERIALS AND METHODS

8.2.1 Surgical procedures

The portal and hepatic vein catheters were inserted *via* the liver *per se* rather than the respective veins directly. These vessels were located by palpation, that is, an indentation was felt in the liver surface, indicating a medium sized vein lay beneath. A small incision was made approximately 5 mm deep until blood flowed freely. If this did not occur, another potential site was located. A catheter was inserted blindly into the apparent source of the free flowing blood. This was often difficult, especially considering the catheter tip was constructed of silicone and therefore very flexible. Once the catheter was inserted into a vein up to 50 mm, blood was aspirated to confirm the catheter was correctly placed. A second catheter was then similarly inserted into another vessel. The difference between portal and hepatic veins was established by considering the hydrostatic pressure in the two vessels, that is the height to which the blood rose when the catheters were held vertically alongside one another. The difference in height was 20-30 mm, with the lesser pressure vessel deemed to be the hepatic vein, due to flow restrictions through the liver reducing the blood pressure in the hepatic vein. Relative blood oxygen concentrations were also used as an additional method to determine which vessel the catheter was inserted into, however this was more complex and less reliable than the hydrostatic pressure method.

The posterior aorta catheter was inserted *via* the femoral artery for a length of 300 mm, as such it was assumed that the tip of this catheter was in the posterior aorta. The balance of the surgery was performed as per the references quoted in the relevant chapters.

8.2.2 Catheter construction

Catheter construction followed the procedures outlined by Lobley e*t al.* (1995). Toluene was used to soften the PVC portion of the catheters to allow the silicone sections to be inserted into the dilated PVC, with silicone glue affixing the two sections together. A larger diameter PVC sheath was then placed over

the catheter *per se* up to the junction between the silicone and PVC, providing additional strength. The catheters were sutured into the respective tissues with the aid of a further PVC sheath covering the junction between the silicone and PVC which was punctured with suture material in four places, allowing two horizontal mattresses to be used to secure the catheters to the tissue. The finished catheters were gas sterilised with ethylene oxide following construction.

At post-mortem, the catheters were without damage and thrombi formation, with the exception of the single mesenteric catheter which failed in the first experiment, which had a large thrombus around the tip. There was no obvious sign of trauma to the vessel wall surrounding the catheter tips.

8.2.3 $^{15}NH_4Cl$ infusion

The infusates were prepared in isotonic saline to minimise the osmotic shock to the animal. The choice of ^{15}N infusate was limited to $^{15}NH_4Cl$ to allow direct comparison with the report of Lobley *et al.* (1995). At the time these experiments were conducted (1995 – 1996), the reports suggesting that acidosis resulting from the $^{15}NH_4Cl$ infusion *per se* may alter the hepatic amino acid metabolism were not yet published (Lobley *et al.* 1996b; Lobley & Milano, 1997; Milano *et al.*, 2000). Therefore a change from $^{15}NH_4Cl$ as the source of ^{15}N was not considered as the $^{15}NH_4Cl$ provided a tracer for all of the molecules of interest in these experiments and it was deemed important to keep the methodology comparable between experiments.

The $^{15}NH_4Cl$ infusion rates were varied depending on the expected portal ammonia flow from the various dietary regimens applied. The basal (lucerne pellet fed animals, Chapter Three) $^{15}NH_4Cl$ infusion rate was chosen to achieve a 7% enrichment of the estimated portal ammonia flow (based on that recorded by Lobley *et al.*, 1995). The natural abundance of ^{15}N is 0.364 APE (Sarraseca *et al.*, 1998) and the detection limit of the GCMS was 0.2, therefore any enrichment above 0.6 APE was theoretically detectable.

In order to allow comparison with the report of Lobley *et al.* (1995), which infused $^{15}NH_4Cl$ (at the same APE as the current experiment) at a rate of 1.66 mmol/h, the lucerne pellet fed animals were infused with $^{15}NH_4Cl$ at a rate of 1.80 mmol/h. The fresh white clover fed animals (Chapter Three) were infused at twice the level of the lucerne pellet fed animals in anticipation of a two fold increase in portal ammonia flow. The low intake animals (Chapter Four) were infused at the same rate as the lucerne pellet fed animals, whilst the high intake animals received 70% more $^{15}NH_4Cl$ based on the 75% increase in DM offered to this group. The pulse fed animals of Chapter Five were infused with the same rate as the low intake group of Chapter Four as these animals were offered the same amount of DM per day.

8.2.4 Blood flow estimation

Application of the dye-dilution method for blood flow determination was chosen over other available methods because the dye-dilution technique can be used in all of the splanchnic blood vessels of interest in these experiments. P*ara*-aminohippurate (*p*AH) was used in these experiments because *p*AH is not significantly metabolised by the liver, with the exception of an acetylation step, the effect of which was rectified with the addition of an acid deacetylation step to the standard *p*AH analysis (Harris *et al.* 1992). Additionally the infused *p*AH is predominantly removed from the blood by the kidneys almost eliminating the recycling of *p*AH to the mesenteric vein.

The *p*AH analysis followed the method outlined by Harris *et al.* (1992), with an additional acid deacetylation step at 90 °C for 65 minutes rather than for the two hours as recommended by Lobley *et al.* (1995). Numerous preliminary runs were performed to ensure the methodology was functional, including background analyses to confirm that the *p*AH was predominantly removed by the kidneys in a single pass.

Application of the Fick principle allowed the blood flows to be calculated from the quotient of the infusion rate divided by the difference between the inflow

and outflow dye concentrations (Webster, 1974; Tepperman & Jacobson, 1982). Therefore the blood flows were calculated from the following formula;

$$Flow_p = I_r / (C_p - C_a)$$

$$Flow_h = I_r / (C_h - C_a)$$

where $flow_p$ and $flow_h$ are the blood flows (g/h) in the portal and hepatic veins, I_r is the infusion rate of the *p*AH (μmol/min), C_p, C_h and C_a are the concentrations (μmol/g) of *p*AH in the portal and hepatic veins plus the posterior aorta respectively.

8.2.5 HPLC method

The amino acid concentrations in the ultrafiltrates (produced as for the ^{15}N-amino acid analysis) were determined after reverse phase HPLC separation of phenylisothiocyanate derivatives (Bidlingmeyer *et al.*, 1984) using a Waters Pico-Tag® column (3.9 x 300 mm; Waters Corporation, Milford, MA 01757, USA) and a Shimadzu LC-10/A HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA) with a 90 minute run time between injections.

8.2.6 GCMS method

The level of ^{15}N-ammonium enrichment was determined in plasma. The ammonia present in 1 g of plasma was converted to nor-valine in the presence of glutamate dehydrogenase. The samples were then stored at –85 °C for subsequent determination.

Following the addition of 150 μl of 48% sulphosalicylic acid, the samples were centrifuged at 13680 G for 10 minutes at 4 °C. The supernatant was then loaded on to a cation exchange column (AG 50W-X8, 0.8ml; Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804, USA) and washed with 3 by 1 ml deionised water, then 2 by 2 ml 2 mol/l ammonia solution to elute the amino acids, followed by 1 ml deionised water. The eluate was then loaded on to an anion exchange column (AG 1-X8, 0.8ml, Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804, USA) and washed with 3 by 1 ml deionised water and eluted with 3 by 1 ml 1 mom/l acetic

acid. The resulting solution was freeze dried. The samples were re-hydrated with 200 μl 0.1 mol/l HCL and heated at 90 °C to dryness. After the samples cooled, 200 μl of a 1 : 1 solution of N-*tert*.-butyldimethylsilyl-N-methyl-trifluoroacetamide (Sigma Chemicals, St Louis, USA) and aceto-nitrile was added and the samples heated at 90 °C for one hour, yielding N-*tert*.-butyldimethylsilyl-N-methyl (TBDMS) derivatives.

Isotopic enrichment was determined by gas chromatography-mass spectrometry (GCMS; Gas chromatograph Model 17A and Mass Selective Dectector Model QP5050A, Shimadzu Scientific Instruments Ltd., Columbia, MD, 21046, USA) which was equipped with a capillary column (DB-5MS; 30 m, 0.25 mm ID, 0.25 μm film thickness; J & W Scientific, Folsom, CA, 95630-4714, USA), using helium as the carrier gas. The mass selective detector was operated in electron impact mode and selected ion monitoring was performed for the TBDMS derivative of norvaline at *m/z* 186.2 and 187.2 as outlined by Nieto *et al.* (1996).

The level of ^{15}N enrichment in the amino acids and urea was determined in whole blood. Samples of whole blood (2 g) were haemolysed by the addition of an equal weight of chilled 200 μmol/l L-nor-leucine, as an internal standard (BDH Chemicals Ltd., Poole, England) with a further 200 mg of 80 mmol/l dithiothreitol (DTT) in 0.2 mol/l phosphate buffer (*p*H 8.0) added prior to deproteinisation by ultrafiltration (Centrisart®; molecular weight cut-off 10,000, Sartorius, Gottingen, Germany).

The ultrafiltrate was then loaded on to a cation exchange column (AG 50W-X8, 0.8ml; Bio-Rad Laboratories, 3300 Regatta Boulevard, Richmond, CA 94804, USA) and washed with 3 by 1ml deionised water, then 2 by 2 ml 2 mol/l ammonia solution to elute the urea and amino acids, followed by 1 ml deionised water. The resulting solution was freeze dried, then rehydrated with 200 μl 0.1 mol/l HCL and heated at 90 °C to dryness. The samples were allowed to cool prior to adding 200 μl of a 1 : 1 solution of N-*tert*.-butyldimethylsilyl-N-methyl-trifluoroacetamide (Sigma Chemicals, St Louis, USA) and aceto-nitrile. This

solution was then heated at 90 °C for one hour, yielding N-*tert*.-butyldimethylsilyl-N-methyl (TBDMS) derivatives. Isotopic enrichment was determined on the same GCMS as for the ^{15}N-nor-valine analysis.

For the ^{15}N-urea analysis, the mass selective detector was operated in electron impact mode and selected ion monitoring was performed for the TBDMS derivatives of the single and double labelled forms of urea at *m/z* 231, 232, 233 and 234 according to the procedures outlined by Brosnan e*t al*. (1996). For the ^{15}N-amino acid analysis, the mass selective detector was operated in electron impact mode and scan mode for the TBDMS derivatives. The resultant chromatograms were examined for the presence of additional side peaks on all of the TBDMS-amino acid derivatives.

8.2.7 Ammonia method

The plasma samples were stored on ice for up to six hours, prior to analysis. Ammonia concentrations were determined with a commercial enzymatic kit (#171; Sigma Chemicals, St Louis, USA). The reaction was based on the reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase and NADPH. The decrease in absorbance at 340 nm, due to the oxidation of NADPH, was proportional to the plasma ammonia concentration.

8.2.8 Urea method

Plasma urea concentrations were determined at a later date following the freezing (-85 °C) of the samples on the day of collection. A commercial kit (#535; Sigma Chemicals, St Louis, USA) was used to determine the plasma urea concentrations. The assay was based on the conversion of urea to hydroxyl-amine in the presence of diacetyl monoxime in boiling sulphuric acid. The urea concentration is directly proportional to the intensity of the colour produced, which was measured spectrophotometrically at 520 nm. The assay was repeated until the coefficient of variation between the duplicate samples was less than 0.1.

LIST OF EMENDATIONS

Page ii, paragraph 1, lines 1 & 2 – replace "a characteristic of which is a" with "that characteristically have a".

Page iii, paragraph 1, line 5 – replace "Hepatic amino catabolism" with "Hepatic amino acid catabolism".

Page iii, paragraph 2, line 5 – replace "rate" with "rates".

Page 1, paragraph 3, line 5 – replace "determined" with "will quantify".

Page 4, paragraph 1, line 6 – replace "**N** containing" with "**N**-containing"

Page 9, paragraph 4, line 8 – replace "protein deposition" with "deposited **N**".

Page 10, paragraph 5, line 10 – replace "animal productivity" with "animal's productivity"

Page 24, paragraph 1, line 5 – replace "apoliprotien" with "apoliprotein".

Page 25, paragraph 1, lines 2 and 4 – replace "capillary like" with "capillary-like".

Page 49, paragraph 2, line 13 – replace "detoxificaiton" with "detoxification"

Page 54, paragraph 2, line 6 – replace "would certainly be" with "may be".

Page 55, paragraph 4, line 11 – replace "four week post surgical" with "four-week post-surgical".

Page 69, line 5 – replace "higher" with "lower".

Page 72, paragraph 2, line 7 – replace "lucerne pellet fed" with "lucerne pellet-fed"

Page 75, paragraph 2, line 3 – insert sentence "The results of the current experiment were consistent with those of Harper *et al.* (1984)."

Page 76, paragraph 2, line 2 – replace "fresh white clover fed" with "fresh white clover-fed"

Page 76, paragraph 2, line 3 – replace "lucerne fed" with "lucerne-fed".

Page 77, paragraph 3, line 2 - replace "a result of an" with "approximately in proportion to the".

Page 86, paragraph 1, line 1 – replace "Although portal oxygen concentrations approached the 10% level of significance," with "Although the difference in portal

oxygen concentrations between the two intake levels approached the 10% level of significance,".

Page 88, Table 4.4 - this table is replaced with the table on the facing page.

Page 94, line 5 – replace "concise" with "precise".

Page 94, paragraph 2, line 1 – replace "Nitrogen digestion" with "protein digestion".

Page 121, paragraph 3, line 2 – replace "therefore digestion of lucerne within the rumen is rapid and yields" with "; the rapid digestion of lucerne with the rumen yields"

Page 125, paragraph 2, line 7 – replace Dr Lobleys" with "Dr Lobley's"