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**THE EFFECTS OF DIET AND FEEDING FREQUENCY ON PERIPHERAL  
NUTRIENT SUPPLY AND GROWTH TRAITS OF THE LAMB**

by

**Judith A. Evans, BSc (Hons.)**

**Thesis submitted to the University of Nottingham for the degree of  
Doctor of Philosophy, September 1998**

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## ABSTRACT

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Tissue protein synthesis requires a simultaneous supply of energy and amino acids. The effect of altering diet and frequency of feeding on patterns of amino acid and acetate supply to peripheral tissues and the consequent effects on growth and carcass composition was investigated in growing lambs.

A rumen 'asynchronous' diet with respect to the ratio of the rate of release of organic matter (OM) and nitrogen (N) was fed once daily (AD) or hourly (AH) and a more 'synchronous' diet was fed once daily (SD) to individually penned growing lambs (n=30) for 16 weeks. Plasma concentrations of acetate, amino acids and insulin were more constant throughout the day in animals fed AH compared to those fed AD. Those fed SD or AD had similar, large pulses in plasma acetate concentration following feeding. Animals fed AH had increased growth rates and greater amounts of carcass crude protein compared to those fed AD ( $P<0.001$ ). Animals fed SD had faster growth rates with a higher amount of carcass crude protein and fat deposition than those fed AD ( $P>0.1$ ).

Synchronous diets formulated to have either slow (SS) or fast (FF) release of OM and N into the rumen were fed hourly (SSH or FFH) or once daily (SSD or FFD) to individually penned growing lambs (n=28) for 10 weeks. Plasma concentrations of acetate and insulin in groups fed once daily, increased after feeding, remaining elevated for longer in animals fed SSD. Amino acid concentrations decreased following feeding. Once daily fed animals tended to have greater amounts of crude protein in the carcass and generally heavier muscles than those fed hourly, particularly those fed SS.

Animals fed AH had significantly higher feed intakes in the first experiment compared to those fed the diets once daily ( $P<0.001$ ) which may be why these animals grew better and deposited more carcass protein than those fed once daily. It was thought that a pulse in acetate following feeding may contribute to an enhanced growth and carcass protein in ruminants. When sodium acetate was infused into growing lambs, intravenously, to raise the concentrations of plasma acetate similar to the pulse in acetate concentrations observed in the plasma of sheep following once daily feeding, no increase in whole-body protein synthesis was observed. The effect of temporal changes in the peripheral nutrient supply on protein synthesis in the ruminant requires further investigation.

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# CONTENTS

---

	Page numbers
ABSTRACT	i
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	ix
LIST OF FIGURES	xiv
LIST OF ABBREVIATIONS	xix
<u>CHAPTER 1 - INTRODUCTION</u>	
1.1 General Introduction	1
1.2 Energy Metabolism in the rumen	2
1.2.1 Acetate	5
1.2.1.1 Acetate supply	6
1.2.1.2 Acetate Utilisation	7
1.2.2 Propionate	12
1.2.3 Butyrate	12
1.3 Nitrogen Metabolism in the rumen	13
1.3.1 Degradable fraction of dietary protein	15
1.3.2 Non-protein nitrogen fraction of dietary protein	16
1.3.3 Endogenous nitrogen supply	16
1.4 Efficiency of Microbial Protein Synthesis	18
1.4.1 Nitrogen supply	18
1.4.2 Dietary carbohydrate supply	18
1.4.3 Rate of passage	19
1.4.4 Level of feed intake	20
1.4.5 Frequency of feeding	20
1.4.6 Synchrony of the nitrogen and energy supply	20
1.5 Absorption of amino acids and peptides from the small intestine	24
1.6 Amino acid and protein metabolism in the tissues	25
1.6.1 Synthesis of non-protein compounds	26
1.6.2 Amino acid oxidation	27
1.6.3 Nitrogen excretion	27

1.6.4 Protein turnover	27
1.7 Nutritional influences on fat and protein accretion	28
1.7.1 The effects of frequency of feeding on fat and protein accretion	29
1.8 Overall objectives of this thesis	33

## CHAPTER 2 - MATERIALS AND METHODS

2.1 Plasma Sample Collection	35
2.2 Analysis of plasma samples	35
2.2.1 Determination of plasma acetate concentration	35
(i) Deproteinisation of plasma	35
(ii) Analysis of acetate concentration	36
(iii) Calculation of the acetate concentration	36
2.2.2 Determination of the plasma amino acid concentration	37
(i) Deproteinisation of plasma	37
(ii) Analysis of amino acids	37
(iii) Calculation of plasma amino acid concentrations	38
2.2.3 Determination of plasma insulin concentration	38
2.2.4 Determination of <sup>13</sup> C-Leucine enrichment in plasma	39
(i) Deproteinisation of plasma for the isolation of leucine and ketoacids	39
(ii) Preparation of the ion exchange resin column	40
(iii) Isolation of Ketoacids	40
(iv) Isolation of Leucine	40
(v) Derivatisation of Leucine	41
(vi) Derivatisation of ketoacids	41
(vii) GC/MS analysis of leucine and ketoacids derivatives	41
2.3 Breath Sample Collection	42
2.4 Analysis of <sup>13</sup> CO <sub>2</sub> enrichment in breath samples	43
2.5 Analysis of isotopic enrichment ( <sup>13</sup> C/ <sup>12</sup> C) of sodium acetate	43
2.6 Determination of Animal Liveweight.	44
2.7 Dissection of carcasses at slaughter	44

2.8 Carcass and Muscle Preparation for chemical composition analysis	45
2.8.1 Muscle preparation	45
2.8.2 Carcass preparation	46
2.9 Analysis of the carcass and muscle samples	46
2.9.1 Dry Matter and Ash Determination of carcass and muscle samples	46
2.9.1.1 Freeze Dry Matter	46
2.9.1.2 Oven Dry Matter	46
2.9.1.3 Organic Matter	47
2.9.2 Determination of the lipid content in carcass and muscle samples	47
(i) Lipid extraction	47
(ii) Calculations of total fat content of the samples	48
2.9.3 Determination of the nitrogen content of carcass and muscle samples	48
(i) Digestion	48
(ii) Distillation	48
(iii) Calculation of Nitrogen present in the sample	49
2.10 Analysis of feed samples	49
2.10.1 Preparation of feed samples for chemical composition analyses	49
2.10.2 Dry Matter and Ash determination of feed samples	50
2.10.2.1 Dry Matter	50
2.10.2.2 Organic Matter	50
2.10.3 Determination of Gross Energy of feed samples	50
2.10.4 Determination of the nitrogen content of feed samples	51

**CHAPTER 3 – THE EFFECTS OF ALTERING SYNCHRONY OF RELEASE OF NITROGEN AND ENERGY COMPONENTS FROM THE DIET INTO THE RUMEN ON PERIPHERAL PLASMA METABOLITES AND GROWTH AND CARCASS COMPOSITION TRAITS IN GROWING LAMBS.**

3.1 Introduction	52
3.2 Experimental design	53
3.3 Methodology	55
3.3.1 Animals, diets and treatments	55
3.3.2 Experimental procedures	56
3.3.3 Slaughter Procedures	56

3.3.4 Chemical Analysis	57
3.3.5 Calculations to estimate the initial muscle weights and muscle growth rates	57
3.4 Statistical Analysis	58
3.4.1 Animal performance data	58
3.4.2 Blood metabolite data	59
3.5 Results	60
3.5.1 Feed Analysis	60
3.5.2 Animal Performance	60
3.5.3 Blood Metabolite Data	72
3.6 Preliminary discussion	87

**CHAPTER 4 – THE EFFECTS OF ALTERING THE TIMING OF RELEASE OF ORGANIC MATTER AND NITROGEN INTO THE RUMEN, ON PLASMA METABOLITES AND THE GROWTH AND CARCASS COMPOSITION TRAITS IN GROWING LAMBS**

4.1 Introduction	99
4.2 Methodology	103
4.2.1 Animals, diets and treatments	103
4.2.2 Experimental Procedure	104
4.2.3 Slaughter Procedures	105
4.2.4 Chemical analysis	105
4.2.5 Calculations	106
4.3 Statistical Analysis	107
4.3.1 Animal performance data	107
4.3.2 Blood metabolite data	107
4.4 Results	108
4.4.1 Feed Analysis	108
4.4.2 Animal Performance	109
4.4.3 Plasma metabolites	120
4.5 Preliminary discussion	137



CHAPTER 5- THE EFFECTS OF INCREASING THE PLASMA ACETATE CONCENTRATION ON  
LEUCINE METABOLISM IN THE GROWING LAMB

5.1 Introduction	144
5.2 Estimating amino acid oxidation and whole-body protein synthesis	144
5.2.1 Choice of Isotope	148
5.3 General Methodology	150
5.3.1 Method of infusion	150
5.3.2 Continuous infusion of [1- <sup>13</sup> C]-Leucine	151
5.3.3 Continuous infusion of NaH <sup>13</sup> CO <sub>3</sub>	152
5.3.4 Sodium acetate and sodium chloride infusates	153
5.4 Calculations	154
5.4.1 Isotopic enrichment	154
5.4.2 Plasma entry rates	155
5.4.3 Leucine oxidation Rate	156
5.4.4 Whole Body Protein Synthesis Rate	156
5.5 Preliminary Studies	157
5.5.1 Methodology	157
5.5.2 Results	159
5.5.3 Further work in the preliminary study	162
5.6 The effect of infusing sodium acetate intravenously, on plasma leucine metabolism parameters in sheep	165
5.6.1 Experimental procedure	165
5.6.2 Calculations and statistical analysis	167
5.6.3 Results	169
5.7 Preliminary Discussion	172

## CHAPTER 6 – GENERAL DISCUSSION

6.1 The effect of diet and feeding frequency on predicted rumen metabolism	179
6.2 The effect of diet and feeding frequency on amino acid supply to peripheral tissues	182
6.3 The effect of diet and feeding frequency on acetate supply to peripheral tissues	185
6.4 The effect of diet and feeding frequency on insulin in peripheral plasma	188
6.5 Further work	193
6.6 Summary	196
BIBLIOGRAPHY	198

---

## LIST OF TABLES

---

	Page numbers
<b>CHAPTER 3</b>	
Table 3.1 Composition of the synchronous and asynchronous diets and the SIRE predictions of nutrient content and rate of nutrient release into the rumen.	54
Table 3.2 Dietary composition of the experimental diets.	60
Table 3.3 The effect of feeding the three dietary regimes; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the DLWG, carcass weight, final liveweight, killing out proportion and feed conversion efficiency (FCE).	62
Table 3.4 The effect of feeding the three dietary regimes; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of three fat depots and the subcutaneous backfat thickness.	64
Table 3.5 The effect of feeding the three dietary regimes, asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SM) to growing lambs, on the weight of three muscles.	65
Table 3.6 The effect of feeding the three dietary regimes, asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of chemical components of the carcass.	66
Table 3.7 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of chemical components of the <i>L.Dorsi</i> .	68

- Table 3.8 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the estimated growth of the *L.Dorsi*, *V.Lateralis*, and *Semitendinosus*. 70
- Table 3.9 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD), to growing lambs, on the size of the liver, spleen, heart and the different parts of the ruminant digestive tract. In addition the dietary effects on the total gut-fill are shown . 71
- Table 3.10 The significant correlations observed between plasma acetate concentrations and concentrations of individual and total plasma amino acid at hourly intervals throughout the day, in two sheep fed each diet. 81
- Table 3.11 The significant correlations observed between plasma insulin concentrations and concentrations of individual and total plasma amino acid at hourly intervals throughout the day, in two sheep fed each diet. 83
- Table 3.12 The effects of the two different diets (A or S) fed either hourly or once daily (-H or -D) on the plasma amino acid concentrations measured over a 24 hour period. 86
- Table 3.13 The Effects of the two different diets (A or S) fed either hourly or once daily (-H or -D) on the plasma acetate and insulin concentrations measured over a 24 hour period. 86
- Table 3.14 The amount of OM and N in both the asynchronous and synchronous diets, that is degraded in the rumen in the first hour following feeding, as estimated by the SIRE programme (Sinclair *et al.*, 1993) 93

## CHAPTER 4

- Table 4.1 Composition of the “fast/fast” and the “slow/slow” diets and SIRE predictions of nutrient content and rate of nutrient release into the rumen. 102
- Table 4.2 Dietary composition of the experimental diets. 108
- Table 4.3 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the daily liveweight gain (kg), final liveweight (kg), carcass weight, killing out proportion (g/kg), daily food intake (MJ ME/ kg LW) and feed conversion efficiency (FCE). 111
- Table 4.4 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the mesenteric fat, omental fat and KKCF depots and the subcutaneous backfat thickness. 112
- Table 4.5 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weight of the *L.dorsi*, *V.lateralis* and the *Semitendinosus*. 113
- Table 4.6 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weight and chemical composition of the carcass 115
- Table 4.7 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the estimated growth of the *L.dorsi*, *V.lateralis* and *Semitendinosus* muscles. 116
- Table 4.8 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the estimated growth of the mesenteric fat, omental fat and the KKCF depots. 118

Table 4.9 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weights of the liver, spleen and heart and the weights of the individual components of the digestive tract. 119

Table 4.10 The Effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the mean daily plasma acetate, insulin and amino acid concentrations measured over a 24 hour period. 131

Table 4.11 The Effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the significance of the correlations between the hourly plasma acetate concentrations and their respective hourly plasma amino acid concentrations in three animals from each dietary treatment group. 133

Table 4.12 The Effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the significance of the correlations between the hourly plasma insulin concentrations and their respective hourly plasma amino acid concentrations measured in three animals from each dietary treatment group. 134

## CHAPTER 5

Table 5.1 The natural  $^{13}\text{CO}_2$  of breath in a sheep and the isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of four sodium acetate sources. 162

Table 5.2 The effects of a 3h infusion of sodium acetate, at a rate 3mmoles/min, on measurements of entry rates of leucine ( $\text{Ra}_{\text{KIC}}$ ), the entry rate of total body  $\text{CO}_2$  ( $\text{Ra}_{\text{CO}_2}$ ), and estimates of leucine oxidation and whole-body protein synthesis (WBPS) during selected time periods before, during and after the infusion of sodium acetate. 170

Table 5.3 The effects infusing sodium acetate for 3h (3mmoles/min) on the  $^{13}\text{CO}_2$  enrichment (APE) of the breath of sheep (n=2) 171

Table 5.4 A comparison of the parameters of leucine metabolism shown in the current experiment with those in previously published data 174

## CHAPTER 6

Table 6.1 The effects of insulin treatment on diabetic animals on the arterio-venous differences of acetate and glucose concentrations when expressed as  $\mu\text{moles/g}$  plasma across the sheep hindlimb (Results from Jarrett *et al.*, 1974). 190

---

## LIST OF FIGURES

---

	Page numbers
<u>CHAPTER 1</u>	
Figure 1.1 Carbohydrate fermentation pathways (Source : Van Houtert, 1993)	3
Figure 1.2 Three pentose phosphate reactions (i, ii, iii) that convert pentose monosaccharides (C5) to hexose (C6) and triose (C3) monosaccharides for use in pyruvate producing reactions.	4
Figure 1.3 The predominant metabolic pathways for the production and utilisation of acetate	7
Figure 1.4 Pathways of fatty acid synthesis in ruminant tissues (source : Van Soest, 1994).	10
Figure 1.5 Digestion and metabolism of nitrogenous compounds in the rumen (Source : McDonald <i>et al</i> , (1995)	14
Figure 1.6. Theoretical availability of the carbohydrate and nitrogen fractions in the rumen, over time, when the animal is fed a single meal of ensiled forage (source : Beever, 1993).	21
Figure 1.7 Amino acid flow into and removal from the plasma free amino acid pool. (modified from NRC, 1985)	26
Figure 1.8 The effect of feeding frequency (14 meals per day versus 2 meals per day) on the pH of the rumen throughout the day (Source : Kaufmann., 1976)	30
Figure 1.9 Diurnal cycling of nitrogen-balance at low (a) and high (b) feed intakes (source : Millward, 1985).	33



### CHAPTER 3

Figure 3.1 Mean liveweights of animals in the three dietary treatment groups, Asynchronous fed once daily (AD), synchronous fed once daily (SD) and the asynchronous diet fed hourly (AH), throughout the experimental period 61

Figure 3.2 (a) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the asynchronous diet once daily (AD). 73

(b) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the asynchronous diet hourly (AH). 73

(c) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the synchronous diet once daily (SD). 74

Figure 3.3 (a) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the asynchronous diet once daily (AD). 77

(b) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the asynchronous diet hourly (AH). 77

(c) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the synchronous diet once daily (SD). 78

Figure 3.4 (a) The mean hourly insulin concentration in the peripheral plasma of sheep (n=10) fed the asynchronous diet once daily (AD) shown against the mean hourly acetate concentration in the plasma of sheep (n=3) fed the same diet. 79

(b) The mean hourly insulin concentration in the peripheral plasma of sheep (n=10) fed the asynchronous diet hourly (AH) shown against the mean hourly acetate concentration in the plasma of sheep (n=3) fed the same diet. 79

(c) The mean hourly insulin concentration measured in the peripheral plasma of sheep (n=10) fed the synchronous diet once daily (SD) shown against the mean hourly acetate concentration in the plasma of sheep (n=3) fed the same diet. 80

Figure 3.5 The hourly acetate concentrations in the peripheral plasma of growing lambs fed the asynchronous (A) or synchronous (S) diet once daily 90

Figure 3.6 (a) N and OM release from a theoretical diet (A) following feeding with a rapid release of OM and a slow release of N (asynchronous)	95
(b) N : OM ratio following feeding of theoretical diet A	95
Figure 3.7 (a) N and OM release from a theoretical diet (B) following feeding with a rapid release of N and a slow release of OM (asynchronous)	96
(b) N : OM ratio following feeding of theoretical diet B	96
Figure 3.8 (a) N and OM release from a theoretical diet (C) following feeding with both a rapid release of N and OM (synchronous)	97
(b) N : OM ratio following feeding of theoretical diet C	97
Figure 3.9 (a) N and OM release from a theoretical diet (D) following feeding with both a slow release of N and OM (synchronous)	98
(b) N : OM ratio following feeding of theoretical diet D	98

#### CHAPTER 4

Figure 4.1 (a) The proportion of total dietary N estimated to be released each hour, following once daily feeding of either the Slow/Slow diet (SS) or the Fast/Fast (FF) diet, as predicted by SIRE (Sinclair <i>et al.</i> , 1993).	101
(b) The proportion of total dietary OM estimated to be released each hour, following once daily feeding of either the Slow/Slow diet (SS) or the Fast/Fast (FF) diet, as predicted by SIRE (Sinclair <i>et al.</i> , 1993).	101
Figure 4.2 (a) Hourly plasma acetate and leucine concentrations in the plasma of growing lambs (n=3) fed the Fast/Fast diet once daily (FFD).	121
(b) Hourly plasma acetate and leucine concentrations in the plasma of growing lambs (n=3) fed the Fast/Fast diet hourly (FFH).	121
Figure 4.3 (a) Hourly acetate and leucine concentrations in the plasma of sheep (n=3) fed the slow/slow diet once daily (SSD).	123

- (b) Hourly acetate and leucine concentrations in the plasma of sheep (n=3) fed the slow/slow diet hourly (SSH). 123
- Figure 4.4 (a) The hourly acetate and total amino acid concentration in the plasma of sheep (n=3) fed the fast/fast diet once daily (FFD). 125  
 (b) The acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the fast/fast diet hourly (FFH). 125
- Figure 4.5 (a) The hourly acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the slow/slow diet once daily (SSD). 126  
 (b) The hourly acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the slow/slow diet hourly (SSH). 126
- Figure 4.6 (a) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the fast/fast diet once daily (FFD). 128  
 (b) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the fast/fast diet hourly (FFH). 128
- Figure 4.7 (a) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the slow/slow diet once daily (SSD). 130  
 (b) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the slow/slow diet hourly (SSH). 130
- Figure 4.8 Hourly acetate concentration over a 24 h period in the jugular plasma of sheep fed the fast/fast (n=3) or slow/slow (n=3) diet once daily. 139

## CHAPTER 5

- Figure 5.1 A multi tissue model of whole-body leucine metabolism, showing leucine uptake by tissues and its intracellular transamination to KIC (Matthews, 1982). 148
- Figure 5.2 The effects of infusing sodium acetate, for 2h, at a rate of 3mmoles/min on the plasma acetate concentration throughout the infusion periods 159

- Figure 5.3 The effects of infusing sodium acetate (3mmoles/min) on the  $^{13}\text{CO}_2$  enrichment (APE) in breath samples during a 10h continuous infusion of L-[1- $^{13}\text{C}$ ]-leucine 160
- Figure 5.4 The effects of infusing sodium acetate (3mmoles/min) on the  $^{13}\text{CO}_2$  enrichment (APE) in breath samples during a 10h continuous infusion of  $\text{NaH}^{13}\text{CO}_3$  160
- Figure 5.5 The effects of infusing sodium acetate, at a rate of 3mmoles/min, on the isotopic enrichment (APE) of the natural  $\text{CO}_2$  in the breath of a sheep, fed hourly. 161
- Figure 5.6 The isotopic enrichment (atom percent) of varying dilutions of the [1,2- $^{13}\text{C}$ ]-sodium acetate 164
- Figure 5.7 An example of the effects of a 3h sodium acetate infusion (3mmoles/min) on the APE of expired  $\text{CO}_2$  throughout the time period of an infusion of L-[1- $^{13}\text{C}$ ]-leucine, in one animal, randomly selected from the experimental group 168
- Figure 5.8 An example of the effects of a 3h sodium acetate infusion (3mmoles/min) the APE of expired  $\text{CO}_2$  throughout the time period of an infusion of  $\text{NaH}^{13}\text{CO}_3$ , in one animal, randomly selected from the experimental group 168
- Figure 5.9 The effects of infusing sodium acetate (3mmoles/min) for 3h on plasma concentrations of acetate during the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$ . 169

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## List of Abbreviations

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3-mv - 3-methyl-n-valerate

'A' – period after sodium acetate treatment

AD - treatment group where the asynchronous diet was fed once daily

AFRC - Agricultural and Food Research Council

AH - treatment group where the asynchronous diet was fed hourly

ANCA - automated <sup>15</sup>N <sup>13</sup>C analyser

ANOVA – analysis of variance

AP – atom percent

APE – atom percent excess

ATP – adenosine 5'-triphosphate

'B' – period before sodium acetate treatment

BCAA - branched chain amino acids

'D' – period during sodium acetate treatment

DLWG - daily liveweight gain

DM - dry matter

DMI - dry matter intake

DUP – digestible undegraded protein

FAS - fatty acid synthetase

FCE - feed conversion efficiency

FFD - treatment group where the fast/fast diet was fed once daily

FFH - treatment group where the fast/fast diet was fed hourly

FID - flame ionisation detector

FSG – final slaughter group

GC - Gas chromatograph

GC/MS - gas chromatograph/mass spectrometer

GE – gross energy

GTP – guanosine 5'triphosphate

ID – internal diameter

IGF - insulin-like growth factor  
ILR – irreversible loss rate  
IRMS - isotope ratio mass spectrometer  
ISG – initial slaughter group  
ISTD - internal standard  
KC - ketocaproic acid  
KIC - ketoisocaproic acid  
KKCF - kidney knob and channel fat  
*L.dorsi* - *Longissimus dorsi*  
LW - liveweight  
ME -metabolisable energy  
min - minute(s)  
MP - metabolisable protein  
mRNA - messenger ribonucleic acid  
MTBSTFA : CH<sub>3</sub>CN - N-methyl-N-t-butyl-dimethylsilyltrifluoroacetamide in  
acetonitrile  
N - nitrogen  
NEFA - non-esterified fatty acids  
NPN - non-protein nitrogen  
OM - organic matter  
OMADR - organic matter apparently degraded in the rumen  
PDV - portal drained viscera  
PPP - pentose phosphate pathway  
Q – substrate flux  
Ra – rate of appearance of substrate  
Rd – rate of disappearance of substrate  
RF - response factor  
SD - treatment group where the synchronous diet was fed once daily  
SI - synchrony index  
SIM - selective ion monitoring  
SIRE - synchrony in a rumen environment  
SSD - treatment group where the slow/slow diet was fed once daily  
SSH - treatment group where the slow/slow diet was fed once daily

TAG – triacylglycerol

t-BDMS – tertiary-butyl dimethylsilyl

tRNA - transfer ribonucleic acid

*V.lateralis* - *Vastus lateralis*

v/v - volume to volume

VFA - volatile fatty acid

w/v - weight to volume

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# CHAPTER 1

## INTRODUCTION

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### 1.1 General Introduction

Starch-producing plants, such as cereal grains, roots and tubers and sugar cane, are the most efficient producers of energy for human consumption (Asplund, 1994). The disadvantage of these crops, however, is that they require excellent growing conditions that are not always available. In addition, these crops are deficient in protein that is required in the human diet.

Ruminant animals produced for human consumption can provide a diet that is rich in protein, energy, minerals, trace elements and vitamins. Owing to the unique digestive tract of the ruminant, production of meat does not need to compete for premium land available. Instead ruminants, unlike any monogastric animal, can be produced on non-arable land with crops that are high in fibrous materials, for example cellulose. This results in it being of extreme economic importance across the world, particularly in areas where land is infertile and where both climate and topography are inconducive to growing arable crops.

Manipulating fat and lean deposition in ruminant animals is increasingly under demand from the consumer and producer of meat products and the environmentalist. A leaner product is required by consumer to satisfy health concerns (Reiser & Shorland, 1990), although a certain degree of fat is considered important to maintain the meat tenderness and juiciness quality (Wood & Warriss, 1992). Ruminant systems are renowned for their inefficiency in the deposition of animal protein when compared to similar monogastric systems (Asplund, 1994). Van Soest (1994) reviewed the efficiency of feed utilisation showing that data suggests that the efficiency with which gross energy of a feed is utilised in pigs is almost double that in dairy cows. In order to compete with monogastric systems, supplementing the basic forage diet is necessary by the ruminant producer. Different forms and increased amounts of nitrogen and energy are fed in more intense systems to optimise production. However, inefficiency in utilisation of nutrients in peripheral tissues means that much of the supplementation is lost as heat energy, methane and nitrogen



waste in faeces and urine. These excretory products, in particular methane and the nitrogen waste, are of increasing concern to the environmentalist.

Research is vital in furthering the understanding of factors influencing fat and lean deposition in ruminant animals and the efficiency of utilisation of dietary nutrients. Understanding such mechanisms will help to enhance the efficiency of ruminant production systems and so meet the requirements of the consumer, producer and environmentalist.

This chapter aims to provide an overview of current knowledge of energy and nitrogen metabolism in the rumen, of nutrient metabolism in peripheral tissues and the subsequent fat and protein deposition in the ruminant tissues. Chapter 2 describes the general experimental techniques that were necessary to complete the research. Chapters 3 to 5 provide information of aims, procedures, results and conclusions of experiments that were carried out to fulfill the overall aim of this thesis. Chapter 6 brings together and draws conclusions on the findings of the research discussed.

## **1.2 Energy Metabolism in the Rumen**

Utilisation of dietary carbohydrates by ruminants is different from that of monogastrics because of the anaerobic microbial activity in the rumen. Carbohydrates cannot be completely degraded into carbon dioxide and water in the rumen, as with aerobic metabolism, and instead are metabolised by anaerobic fermentation to a variety of different end-products. Dietary carbohydrates, such as cellulose, hemicellulose, pectin, starch and soluble sugars, are the main substrates of such fermentation. These complex carbohydrates are degraded into monosaccharides (hexoses and pentoses) before being fermented to form volatile fatty acids (VFAs; Blaxter, 1961) and various gases (Figure 1.1).

The majority of plant carbohydrates are broken down to hexose sugars. These are metabolised to pyruvate predominantly by the Embden-Meyerhof glycolytic pathway. Pentose sugars can also be produced and are used in transketolase and transaldolase enzymatic reactions (Figure 1.2) of the pentose phosphate pathway. The net result of these three reactions is the formation of two hexoses (Figure 1.2, in steps (ii) and (iii)) and one triose (in step (iii)), from three pentoses, which can then be converted to glycolytic intermediates and consequently pyruvate.

Figure 1.1 Carbohydrate fermentation pathways (Source : Van Houtert, 1993)

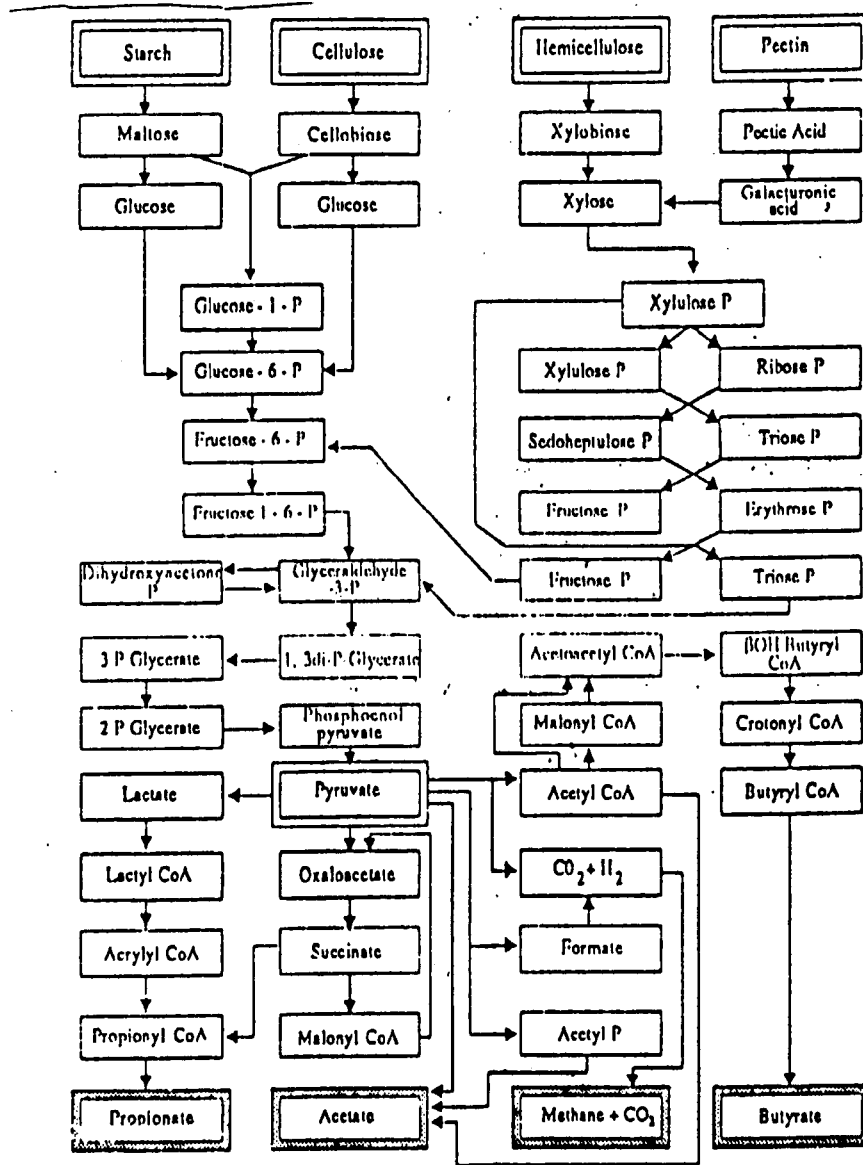
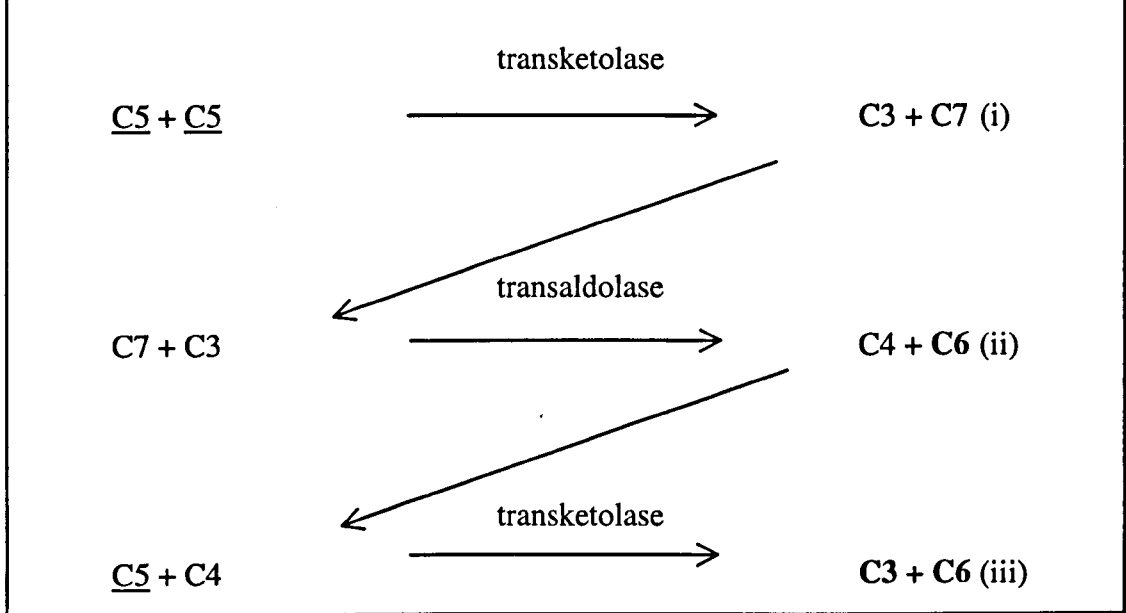


Figure 1.2 Three pentose phosphate reactions (i, ii, iii) that convert pentose monosaccharides (C5) to hexose (C6) and triose (C3) monosaccharides for use in pyruvate producing reactions. Monosaccharides that are produced and consequently used within these three reactions are neither highlighted nor underlined. Adapted from Stryer (1988)



Rumen fermentation continues with the conversion of pyruvate to VFAs via a number of different pathways (Figure 1.1). Some microbial species are able to produce non-VFA end-products, such as ethanol, succinate, lactate and hydrogen. These can be used as substrates by other bacterial species for conversion to VFAs, methane and carbon dioxide (CO<sub>2</sub>) (see van Houtert, 1993).

Acetate, propionate and butyrate are the principle VFAs produced during anaerobic fermentation of dietary carbohydrate in the rumen, although other short, straight- and branched-chain fatty acids (e.g. isovalerate, valerate, isobutyrate) may also be produced in smaller quantities. Overall, VFA production represents nearly 75% of the energy content of the carbohydrate in the diet, the remaining 25% is used by the microbes for growth or lost in other compounds (Bergman, 1990). VFAs that are produced are absorbed across the rumen wall for utilisation by the animal. The VFAs produced are waste products to the microbes, but to the host ruminant are important sources of energy. An estimated 70-80% of the energy requirements of the ruminant animal are met by the VFAs (van Houtert, 1993).

The fermentation process generates energy (ATP) which is used for microbial metabolism or lost as heat. The driving force in microbial metabolism is the availability of ATP for maintenance and growth and hence the rates of nitrogen

utilisation in the rumen are highly dependent on the rate of fermentation of the carbohydrates (Baldwin & Denham, 1979).

The proportions of volatile fatty acids produced and absorbed from the rumen can vary considerably and are ultimately determined by the diet and the time after feeding (Bergman, 1990). The relative concentrations of individual VFAs are commonly known as the fermentation pattern and this is determined by the microbial population. The basal diet of the ruminant dictates the predominant microbial species that are present in the rumen. For example high-fibre forage-based diets maintain a population of acetate-producing cellulolytic bacteria and starch-rich concentrate diets encourage the growth of propionate-producing amylolytic bacteria. It is suggested that forages that induce a fermentation with preference to acetate production are most efficient with regard to ATP production and VFA yield, they do also however, produce more methane (see Beever, 1993).

The importance of glucose utilisation and gluconeogenesis, are quite different in the ruminant compared to the monogastric, as will be described throughout this literature review. As the amount of glucose that is available for absorption from the gastrointestinal tract is limited in ruminants, VFAs provide a considerable amount of energy, instead of glucose. The adult ruminant, appears to strive for glucose conservation and has developed mechanisms to limit glucose utilisation, so that the small amount that is available can be utilised for essential functions such as supplying energy to the brain tissues.

### 1.2.1 Acetate

In the ruminant, acetate is a major source of energy for the peripheral tissues and is rapidly utilised by the body tissues (Annison & Lindsay, 1961). The liver, having removed the majority of propionate and butyrate from the blood, leaves acetate present in the largest quantities in the peripheral circulation. Consequently acetate represents 90-98% of the VFAs in the peripheral blood (see Van Soest, 1994 or Bergman, 1990).

For tissue metabolism, acetate must be converted to acetyl CoA, a reaction that is enzymatically catalysed by acetyl CoA synthetase (Ballard, 1972). The acetyl CoA molecule is then generally either completely oxidized in the citric acid cycle to

CO<sub>2</sub> and H<sub>2</sub>O, producing ATP (Stryer, 1988) or incorporated into fat in the adipose tissue (Bauman & Davis, 1975).

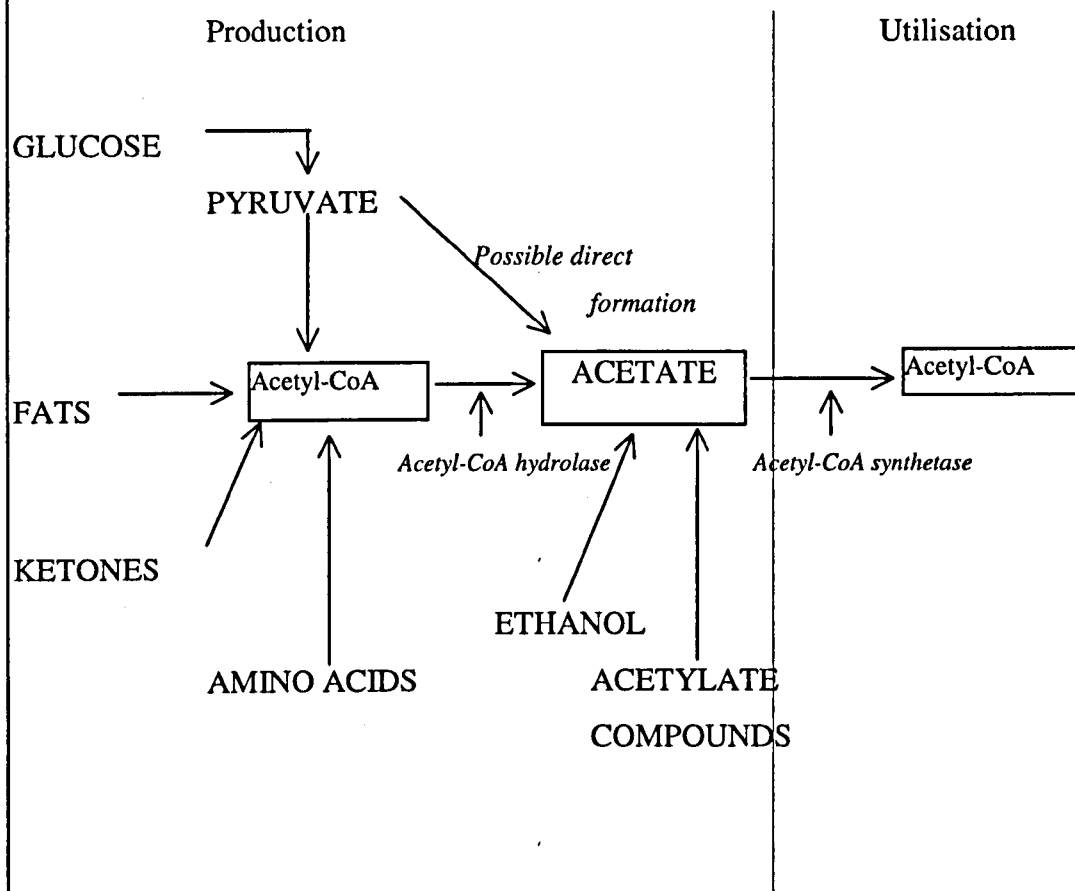
#### 1.2.1.1 Acetate supply

Acetate in the blood originates from two sources. The endogenous source which is derived predominantly from tissue metabolism, and the exogenous source which is of dietary origin.

The endogenous supply of acetate in the blood forms approximately 30% of the total acetate metabolised by sheep when fed *ad libitum* (Bergman, 1975). Endogenous acetate is produced in the liver, adipose tissue, heart, kidney and skeletal muscle (Knowles *et al.*, 1974). It has been proposed that endogenous acetate is produced from acetyl-CoA that is formed from a number of body compounds such as long-chain fatty acids, carbohydrates and amino acids (Ballard, 1972). Figure 1.3 suggests the likely metabolic pathways involved in the production and utilisation of acetate.

A study by Pethick *et al* (1981) showed that the rate of endogenous acetate production in sheep muscle remains constant whether the animal is fed or made diabetic and fasted, whereas the exogenous supply of acetate is more variable and is largely determined by food intake (Pethick *et al.*, 1981). In contrast other studies have shown that endogenous plasma acetate supply also varies depending on the nutritional state (see Van Soest, 1994). Fasting sheep showed an increase in the production of endogenous acetate and this decreased when sheep were fed or supplied with ruminal acetate (Annison & White, 1962).

Figure 1.3 The predominant metabolic pathways for the production and utilisation of acetate



Source : Modified from Ballard (1972)

### 1.2.1.2 Acetate utilisation

It has been observed that acetate utilisation rates can alter with changes in carbohydrate metabolism (Annison & Lindsay, 1961). Reid (1950) suggested that rate of metabolism of acetate is largely dependent upon the rate of production in, and the absorption from, the rumen. In other words, utilisation rate of acetate in the sheep is proportional to the blood concentration. Annison & Lindsay (1961) showed that by infusing acetate, the mean acetate utilisation rate increased by almost exactly the same amount as was infused, until steady state was reached. This study also showed that by infusing glucose with acetate, the mean utilisation rate of acetate was further enhanced.

Acetate acts as a significant energetic fuel for the ruminant muscle and adipose tissue (Bergman, 1990; Pethick *et al.*, 1981) and can be oxidised in the mitochondria generating ATP and GTP. Acetate is also a significant contributor of

carbon for the synthesis of fatty acids in ruminant adipose tissue. The fatty acids that comprise body fat are from two sources, either those that are absorbed from the gut or those synthesised in the body (*de novo* synthesis). A typical forage diet contains less than 3% lipid (Pethick & Dunshea, 1993) and consequently *de novo* synthesis is the major source of body fat. Long-chain fatty acids are predominant in the body as either triacylglycerol (TAG) or as non-esterified fatty acids (NEFA). Esterification is the process involved in the production of TAG molecules and involves the combination of glycerol 3-phosphate to fatty acids to form TAG. The glycerol 3-phosphate is synthesised from glucose and combines with three fatty acyl CoA molecules (formed from fatty acids) through a sequence known as the glycerol 3-phosphate pathway. Owing to the low concentrations of glycerol kinase in adipocytes the glycerol fragment of TAG cannot be re-used following lipolysis and exits the fat cells and enters the peripheral circulation. The fatty acids are either reutilised in TAG formation or are taken into the peripheral circulation where they are bound to albumin for transportation as NEFA.

Droplets of TAG combine to form lipid droplets in the adipocyte. Lipase enzymes catalyse the breakdown of TAG to fatty acids and glycerol. Due to the constant turnover by the hydrolytic cleavage of TAG (lipolysis), the rate at which TAG is deposited is not the same as the rate with which fatty acids are esterified (Vernon, 1981). The relative rates of lipolysis and esterification determine whether there is a net loss or accumulation of lipid within the adipose tissues. Fatty acids that are formed by lipolysis of the TAG molecule appear to comprise the bulk of fatty acids esterified (~75%) i.e. the fatty acids are recycled. The remaining fatty acids are from *de novo* synthesis (Vernon, 1981).

In non-lactating ruminants, the majority of *de novo* fatty acid synthesis (lipogenesis) occurs in the adipose tissue (Vernon, 1981). Lipogenesis requires acetyl CoA as the carbon precursor, NADPH as a reducing agent and a system of enzymes to catalyse the series of reactions that occur in fatty acid synthesis.

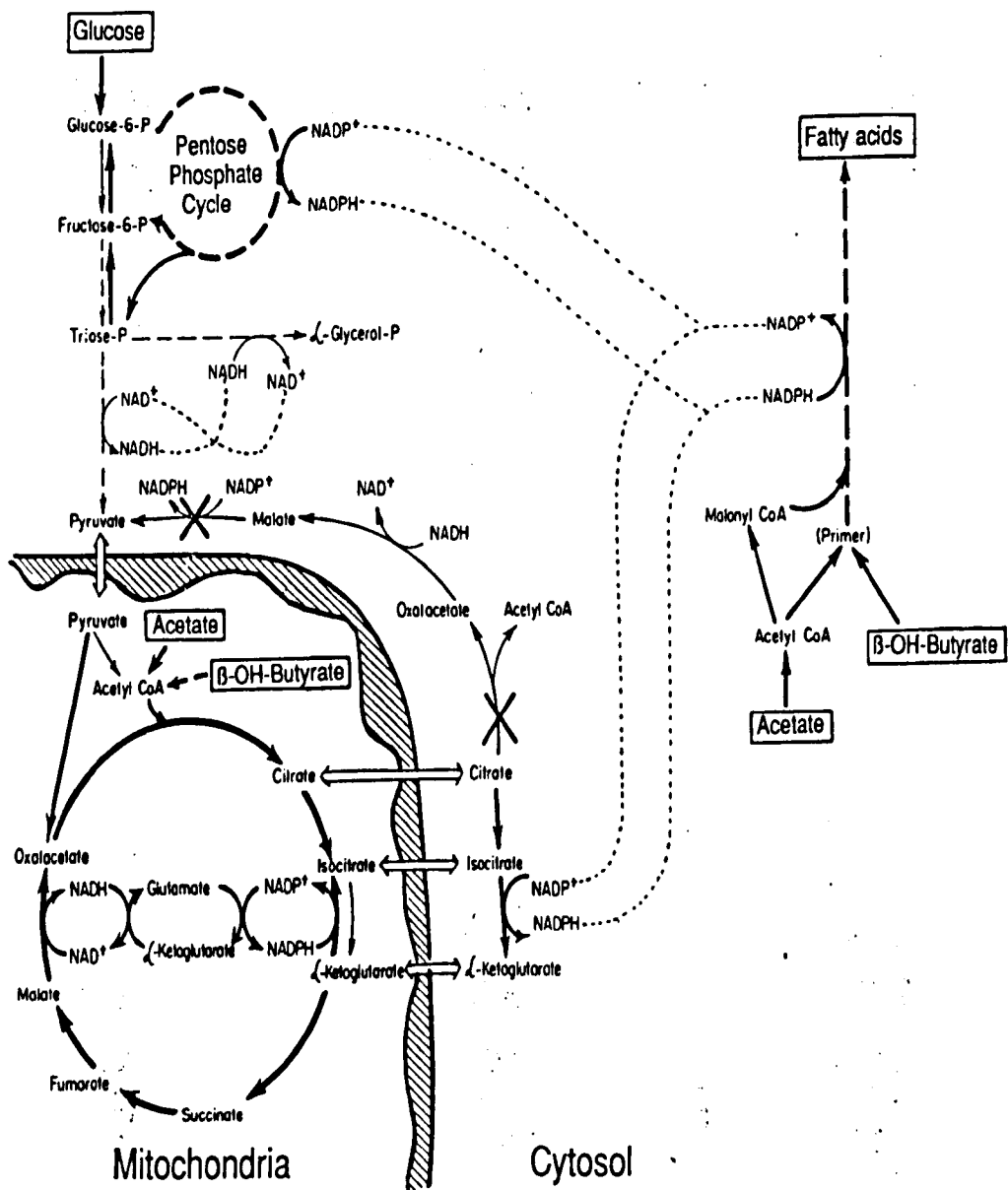
In contrast to monogastrics, ruminant animals utilise mainly acetate instead of glucose in the production of acetyl CoA for fatty acid synthesis, which is a useful method of conserving glucose. This is thought to be due to the low activity of the cytosolic enzymes ATP-citrate lyase and NADP-malate dehydrogenase in ruminant tissues. In monogastric animals these enzymes are involved in the production of

acetyl CoA in the cytoplasm of the cell from pyruvate that has been produced from glucose. The pyruvate is converted to citrate and transferred from the mitochondria where it is cleaved to acetyl CoA and oxaloacetate by ATP-citrate lyase (Figure 1.4). This process uses glucose and so it is thought that a low activity of these enzymes in ruminants is the result of low glucose availability (Robertson *et al.*, 1982). In the ruminant, there is a considerable amount of acetate already in the cytosol and for utilisation of this source of acetate in fatty acid synthesis, the mitochondrial pathway shown in Figure 1.4 is not necessary.

Synthesis of fatty acids requires considerable amounts of the reducing agent NADPH. In ruminants this is derived mainly from the pentose phosphate pathway (PPP; glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and also from the oxidation of isocitrate, supplied by the mitochondrial citric acid cycle, in the cytosol (NADP-isocitrate dehydrogenase). Isocitrate is also used in a non-ATP yielding pathway in the mitochondria known as the isocitrate-oxaloacetate shunt, which is also involved in the formation of NADPH (Bauman & Davis, 1975; Ingle *et al.*, 1972; Vernon, 1981). In the lactating ruminant mammary gland, like that of the monogastric (Flatt, 1970) the relative importance of the NADPH producing pathways is thought to be dependent on the ATP requirement of the tissues (Bauman & Davis, 1975). The isocitrate cycle generates 3 moles of ATP per mole of acetate incorporated into fatty acids. The PPP produces triose phosphate which is extensively recycled and thus results in a process with a net ATP deficit rather than an ATP yield. It has been suggested that the isocitrate cycle is used in preference to the PPP, until ATP requirements of the mammary tissues are met. Once these are met, the reducing equivalents must arise from the PPP (see Bauman & Davis, 1975). The mitochondrial Isocitrate-oxaloacetate shunt may also be involved in the balance of the production of NADPH without yielding excess ATP (see Van Soest, 1994).



Figure 1.4 Pathways of fatty acid synthesis in ruminant tissues. The lack of ATP-citrate lyase and NADP-malate dehydrogenase is denoted by X (source : Van Soest, 1994).



Cronje *et al.* (1991) and Jarrett & Filsell (1961) showed that by increasing the ratio of the glucose precursor, propionate, to acetate in the rumen, the rate with which acetate was cleared from blood was increased. *In vitro* studies also suggest that increasing the concentration of glucose in the media of ovine adipocytes can increase the incorporation of acetate into lipid and the oxidation of acetate to CO<sub>2</sub> (Scollan & Jessop, 1995). These studies suggest therefore that by increasing the glucose concentrations either more glucose will be passed through the PPP pathway for the

production of NADPH or glycerol was limiting and so more glycerol is produced enhancing esterification and incorporation of acetate into lipid.

Acetyl CoA carboxylase is the enzyme which catalyses the first reaction in lipogenesis to form malonyl CoA from acetyl CoA,  $\text{HCO}_3^-$  and ATP. The malonyl CoA is converted into fatty acids by a number of reactions that are catalysed by the fatty acid synthetase (FAS) complex. The principal fatty acids that are formed in ruminants are palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids (Vernon, 1981). Palmitic acid is the main product of the FAS reactions and further elongation reactions occur on the endoplasmic reticulum by the addition of 2 carbon units (Stryer, 1988).

Of the three major VFAs, acetate is the least metabolised by the liver (Leng & Annison, 1963; Bergman & Wolff, 1971). This is thought to be due to low levels of acetyl-CoA synthetase within the hepatic tissue (Ash & Baird, 1973) and inhibition of its utilisation by propionate and butyrate (Bell, 1981). Only small amounts of acetate are oxidised in the liver (Pethick *et al.*, 1981). Acetate that is taken up by the liver is thought to be used for lipogenesis (Bell, 1981).

Acetate has been shown to be utilised quite significantly in the portal-drained viscera (PDV) of fed sheep but not to the same extent in fasted sheep (Bergman & Wolff, 1971). Removal of acetate from the blood into the PDV could be for lipogenesis in mesenteric fat or perhaps oxidation by the smooth muscle (Bergman & Wolff, 1971).

Acetate is also oxidised to produce energy for other synthetic reactions. Synthesis of tissue protein requires hydrolysis of ATP and GTP (section 1.6.4). The rate of generation of these energy-rich phosphate molecules is therefore likely to be a limiting factor in the rate of protein synthesis (see Madsen, 1983a). Initially acetate is converted to acetyl-CoA by acetyl-CoA synthetase which is then transported into the mitochondria by means of carnitine as acetylcarnitine (Madsen, 1983a). Mitochondrial acetyl-CoA is oxidised in the citric acid cycle. Acetate uptake measured across the hindlimb muscle can account for at least 20% of the oxygen uptake of the hindlimb in fed, resting sheep (Jarrett *et al.*, 1976) indicating that it plays a significant part in generating energy within the muscle.

### 1.2.2 Propionate

Approximately two thirds of portal blood propionate is taken up by the liver (Bergman & Wolff, 1971). As previously mentioned, propionate acts as a significant glucose precursor in the ruminant to maintain a high hepatic glucose turnover (Demigne *et al.*, 1991; Leng & Annison, 1963). Reports suggest that up to 95% of propionate is converted into glucose (Steinhour & Bauman, 1986). Propionate is converted to glucose via oxaloacetate. The process is activated by the conversion of propionate to propionyl-CoA and then proceeds via methylmalonyl-CoA into succinyl-CoA which enters the citric acid cycle to form oxaloacetate and consequently pyruvate. The hepatic capacity for gluconeogenesis may be limited by the activities of the enzymes controlling the main pathways. These enzymes (fructose 1,6-bisphosphatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase and glucose 6-phosphatase) respond to variations in nutritional supply (Madsen, 1983b; Ballard *et al.* 1969).

In addition to glucose, amino acids can also be produced from the oxaloacetate that is formed by the conversion of propionate. The respective ketoacids are formed and reductively aminated to form aspartate, glutamate and alanine (Bergman & Pell, 1985).

### 1.2.3 Butyrate

The main function of butyrate is as a substrate for the production of ketone bodies, principally acetoacetate and  $\beta$ -hydroxybutyrate. Ketogenesis occurs in both the rumen epithelium and in the liver. The proportion of butyrate absorbed into the blood is low in relation to the amount produced in the rumen, however of the butyrate that is absorbed, approximately 80% is taken up by the liver (Bergman & Wolff, 1971). Ketone bodies occur in substantial quantities in the peripheral circulation and can be used via acetyl CoA in metabolic processes of the peripheral tissues. Excess levels, however, are toxic to the animal and these are reached when the animal has been subject to a nutritional stress, causing ketoacidosis. In these conditions the animal has been required to mobilise fatty acids and in doing so has increased the concentration of ketone bodies, while glucose, which is required for their utilisation is insufficient.

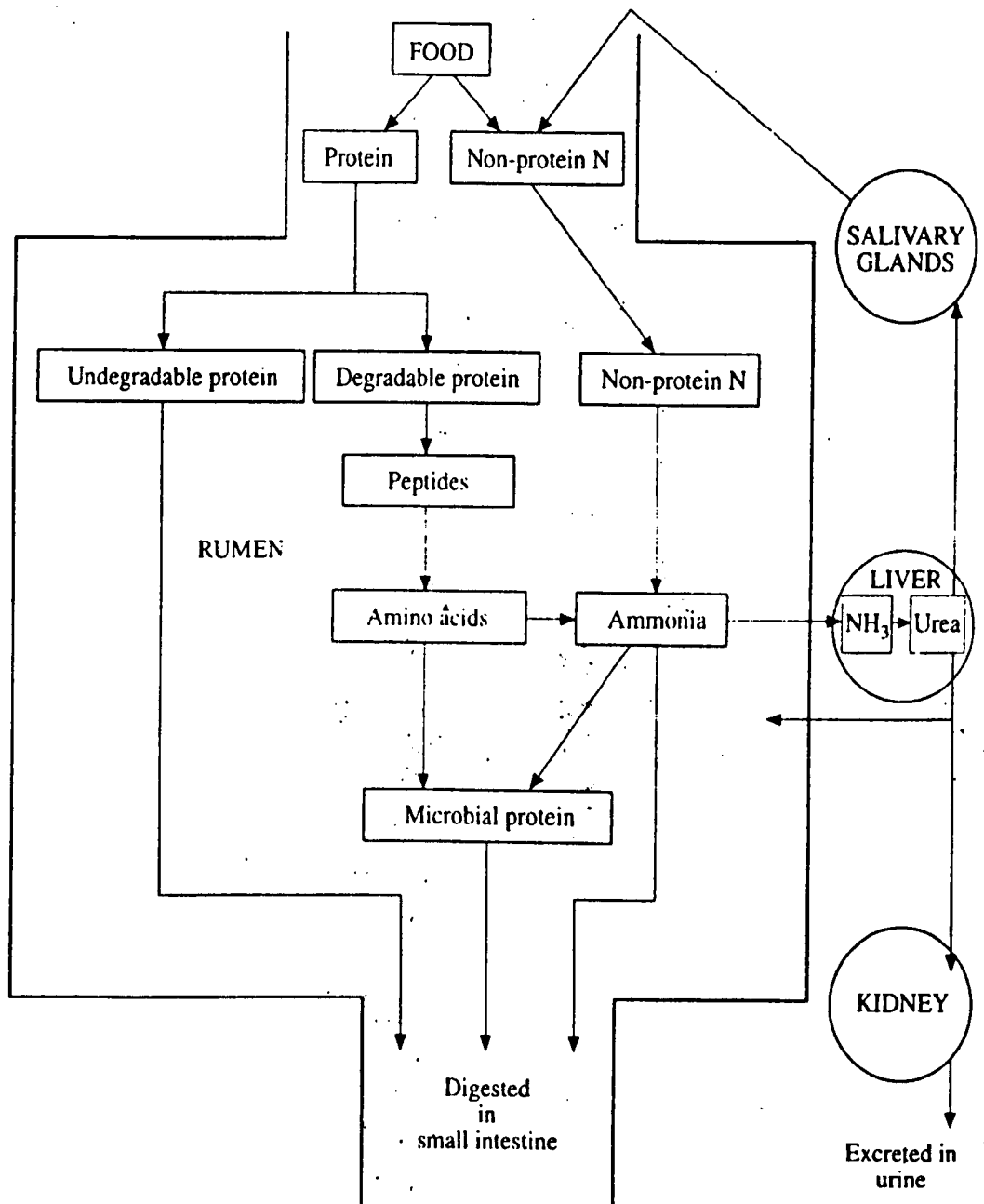
### **1.3 Nitrogen Metabolism in the rumen**

Protein requirements of the ruminant animal are largely met by the synthesis of microbial protein within the rumen and its subsequent digestion in the small intestine. Protein that escapes degradation in the rumen may be degraded in the lower gastro-intestinal tract (predominantly the duodenum) and used as a nitrogen (N) source for the host ruminant. In addition, endogenous N sources can supply the rumen microbes and the duodenum with nitrogenous compounds. Microbial protein, however, is the main component of duodenal protein with most diets (Beever, 1993). Figure 1.5 shows a diagram of the digestion, metabolism and passage of nitrogenous compounds in the rumen.

Synthesis of useful protein by the rumen microbes, benefits the host animal nutritionally as it enables the efficient utilisation of plant proteins and other nitrogenous sources that could otherwise not be readily utilised by the animal (Czerkawski, 1986). Microbial growth rates (protein yield) can vary considerably. Reports in dairy cows have shown a variation in microbial protein yield of up to 44% (Nocek & Russell, 1988).

For the microbes to synthesise protein in the rumen, availability of a N source and an energy source is required. The energy source is ATP and is derived from the anaerobic fermentation process (section 1.2) converting hexoses and pentoses to volatile fatty acids and other microbial waste products. The two main sources of N from the diet, for microbial use, are from either the degradable fraction of dietary protein or non-protein nitrogen fractions present in the rumen. In addition to the dietary sources, there is a substantial endogenous supply of nitrogen within the rumen environment. These nitrogen sources will be discussed.

Figure 1.5 Digestion and metabolism of nitrogenous compounds in the rumen (Source : McDonald *et al.*, (1995)



### 1.3.1 Rumen degradable fraction of dietary protein

The proportion of dietary protein that is degraded to amino acids, peptides and ammonia in the rumen is dependent on a number of factors (see Nolan, 1993; NRC., 1985; Buttery & Lewis, 1982). These factors include the proteolytic capacity of the microbes in the rumen, the microbial access to the protein and the retention time of the protein in the rumen. Protein solubility and rumen pH also determine the extent to which a protein is degraded (see Stern *et al.*, 1994; Nolan, 1993; NRC., 1985; Buttery & Lewis, 1982). Chemical and physical properties of the protein alter the accessibility of the protein to the microbes. Structural characteristics will also affect the accessibility of the protein for enzymatic action (Leng & Nolan, 1984).

The extent of degradability is largely affected by the time the digesta resides in the rumen. Proteins retained for a shorter time in the rumen are degraded to a lesser extent than those that are retained for a longer time. The rate of passage from the rumen is different for the soluble and the particulate fractions of the digesta (Czerkawski, 1986). The soluble fractions are more susceptible to degradation than the particulate fraction (Thomas & Rook, 1981).

Proteolysis and deamination of proteins in the rumen, as enzymatically controlled reactions, are likely to be affected by pH. The optimum pH for these reactions is generally between 6.0 and 7.0. Normal rumen pH is generally between 5.5 and 7.0 (see NRC, 1985).

There is generally a strong correlation between the solubility of a protein and the rate with which it is degraded. A reduction in solubility can be achieved by heat or chemical treatment e.g. formaldehyde treatment (see Buttery & Lewis, 1982) and reduces the degradation of the protein in the rumen. Changes in the population of microbes may change the amounts that favour solid or soluble substrate and so influence the rate of passage (Czerkawski, 1986).

The intake and bulk, physical state and reactivity of the food are all components that have a direct effect on the physical passage of digesta and an indirect effect by influencing fermentation. The structure of a protein is a contributing factor to the degradation characteristics of the protein as was shown by Mahadevan *et al.* (1980). This study showed that there were differences in the rate of degradation of certain protein feed sources and deduced that to a certain extent the disulphide bonds were the cause of this difference. Soya bean meal, when reacted

with mercaptoethanol or performic acid, had an increase in its rate of degradation. The degradation rate of casein, a rapidly degraded feed source, was unchanged by the addition of mercaptoethanol or performic acid. Both mercaptoethanol and performic acid break down disulphide bonds and as casein has no disulfide bonds, it was deduced that the bonding structure strongly affected the rate of degradation of the proteins.

### 1.3.2 Non-protein nitrogen (NPN) fraction of the diet

One of the main advantages in ruminant animal production over monogastric animal production is the ability of the rumen microbes to use dietary NPN components to make quality protein. Most of these NPN fractions can be readily degraded in the rumen to ammonia.

The dietary NPN fraction includes nucleic acids, amides, amines, amino acids and nitrate and these contribute to the supply of nitrogen for microbial protein synthesis (Nolan, 1993).

### 1.3.3 Endogenous nitrogen supply

The endogenous nitrogen supply entering the rumen can come from a number of sources including :

1. urea entering through the rumen wall, recycled from ammonia produced following previous N metabolism in the ruminant
2. nitrogenous compounds within the saliva, e.g. mucoproteins, purine metabolites and urea
3. sloughed epithelial cells and lysed microbes
4. ammonia, which can come from a number of endogenous sources such as lysed cell fragments, the breakdown product of urea, nucleic acids and other endogenous N components and protozoal excretion (Nolan, 1993).

Ammonia is an essential source of N for microbial protein synthesis in the rumen. More than 80% of culturable bacteria are capable of using ammonia as a sole nitrogen source, whereas the protozoa and fungi use amino acids and peptides to meet their requirements (Morrison & Mackie, 1996). The ammonia in the rumen is derived from many sources which include the dietary peptides, amino acids,

protozoal excretion of ammonia, sloughed cells from the epithelia, gaseous nitrogen taken in with the feed and fixed as ammonia by microbes (e.g. *Methanobacterium ruminantium*), lysed rumen microbes and salivary non protein nitrogen including soluble N compounds which are rapidly converted to ammonia (such as endogenous urea, nitrate)(Leng & Nolan, 1984; Nolan, 1993).

Ammonia can leave the rumen in several ways. Either by incorporation into the microbial protein that then passes to the small intestine, flowing out of the rumen in ruminal fluids to the lower gut or by absorption of predominantly unionized ammonia across the rumen wall for conversion to urea in the liver (Leng & Nolan, 1984). Urea can then either be recycled back into the rumen via salivary transfer or excreted from the host animal in the urine (Nolan, 1993).

Salivary transfer of urea contributes to the NPN supply to the rumen. If dietary protein degradation proceeds more rapidly than protein synthesis, there was an accumulation of ammonia in the rumen and the optimum concentration will be exceeded. Ammonia that is not utilised in the rumen is absorbed across the rumen wall, where it is carried to the liver and converted to urea. This urea can be transported back to the rumen via the salivary glands or directly through the rumen wall. Urea that does not get recycled back to the rumen is excreted in the urine.

The concentration of plasma ammonia and rumen ammonia will affect the trans-epithelial urea concentration gradient, which consequently affects the amount of urea that is transported straight across the rumen wall by simple diffusion. A lowered pH promotes ammonium ion formation and decreases the total ammonia concentrations in the rumen which will increase the transfer of urea from the plasma. A decrease in this transfer (adverse gradient) is observed when blood urea concentrations are low or when there is an ammonia build up near the rumen epithelia. Urease activity in the microbes that are adhering to or near the rumen wall, is thought to partially modulate the recycling of urea across the rumen wall. The urease enzyme converts urea to ammonia, but higher concentrations of rumen ammonia may repress urease synthesis in bacteria near the rumen wall and so reduce urea transfer into the rumen from the plasma (see Cheng & Costerton, 1980).



## **1.4 Efficiency of microbial protein synthesis**

Rumen microbes play a vital part in the ruminants supply of amino acids (Armstrong & Hutton, 1975). However, there is a huge variation in the reported efficiency with which microbial protein is synthesised. Some of the main factors that affect microbial efficiency are discussed below.

### **1.4.1 Nitrogen supply**

As N is one of the building blocks for protein synthesis, an adequate supply is necessary for efficient microbial protein synthesis. Microbial growth is likely to be influenced by the actual N source for example, amylolytic rumen bacteria are stimulated by amino acids as a source of N (Maeng & Baldwin, 1976) whereas cellulolytic bacteria require ammonia rather than amino acids for microbial production (Bryant & Robinson, 1963). Both reports suggest that when there is a more readily available energy source, maximal growth will require soluble organic N. Sources of N to the rumen microbes both in the diet and from endogenous sources have been discussed previously in section 1.3

### **1.4.2 Dietary carbohydrate supply**

Dietary carbohydrates are generally the main energy yielding components that are available to the rumen. There are important relationships between the rate of fermentation of a carbohydrate and the microbial efficiencies when expressed as the production of microbial protein per unit of feed ingested e.g. g bacteria per kg organic matter digested in the rumen (see Van Soest, 1994). Rate of fermentation of organic matter (OM) sets the amount of feed energy per unit time for bacteria i.e. the faster the digestion rate of carbohydrates, the more energy is made available for growth. As with animals, growth of bacterial cells cannot occur until the maintenance energy requirements are met. As the growth of rumen bacteria compared to other bacteria is relatively low, the maintenance energy requirement forms a significant part of energy utilisation in the rumen (Nocek & Russell, 1988). This maintenance requirement appears to be variable for different microbial species (Pirt, 1965).

The efficiency with which energy in the rumen is used for synthesis of microbial protein can be measured as the yield of bacterial cells in grams per mole ATP (g cells / mol ATP) used and the expression given is  $Y_{ATP}$ . The  $Y_{ATP}$  value is

greatly dependent on the energy source available within the rumen (Sniffen & Robinson, 1987). A range of values have been measured from 8.3 to about 20 g cells / mol ATP (Russell & Hespell, 1981; Nocek & Russell, 1988; Febel & Fekete, 1996). Theoretical values suggested by Hespell & Bryant (1979) state that the rumen bacterial population can potentially grow very efficiently, more so than has been measured *in vivo*. This potential, it was suggested, is limited by nutrient availability in the rumen, particularly after a significant period of time post-feeding when nutrient availability is diminished.

#### 1.4.3 Rate of Passage

Differences in the rates of passage of the particulate and liquid phases in rumen, appear to affect the efficiency with which microbial protein is synthesised. *In vitro* studies have shown that by increasing the dilution of a culture, the efficiency with which microbial protein is produced is increased (see Buttery & Lewis, 1982). *In vivo* studies report similar conclusions, where strong positive correlations have been shown between passage rate and microbial efficiency (Feng *et al.*, 1993; Piwonka *et al.*, 1994; Firkins *et al.*, 1986). The extensive adhesion of microbes to feed particles in the particulate phase (McAllister, 1994) and the passage of small feed particles in the fluid phase is likely to mean that the efficiency of microbial protein synthesis is influenced by the rates of flow of both phases in the digesta (Firkins, 1996). Differences in efficiency are thought to be due to the fact that by increasing passage rate, the proportion of energy required for maintenance by the microbes relative to microbial growth, is decreased (Clark & Davis, 1983). The basic principle being that with an increased passage rate the mean age of the microbial population is decreased. Older cells are likely to die and be retained in the rumen and the nutrients be recycled or washed out, which will decrease the efficiency with which the nutrients are used for yielding microbial protein (see Van Soest, 1994). Another suggestion is that when rumen contents are diluted by an increasing outflow rate, products that inhibit growth decline, increasing the microbial efficiency (Bergen *et al.*, 1980).

#### 1.4.4 Level of feed intake

Some studies suggest that there is no relationship between feed intake and microbial efficiency (Chamberlain *et al.*, 1976; Tamminga *et al.*, 1979). Others have shown that there are higher bacterial yields at higher feed intakes, particularly in dairy cattle where the intakes vary greatly throughout the lactation cycle (NRC, 1985; Sniffen & Robinson, 1987). Higher intakes are associated with an increase in the outflow of digesta from the rumen (Kaufmann *et al.*, 1979). Such an increase in outflow rate, will increase the efficiency of microbial production, as described above (section 1.4.3). Sheep and beef cattle tend to have a narrower range of feed intake relative to the liveweight of the animal and as result correlations between intake and microbial efficiency have been more difficult to detect (Sniffen & Robinson, 1987).

#### 1.4.5 Frequency of feeding

The effects of feeding frequency on the efficiency of microbial protein synthesis are discussed in section 1.7.2.

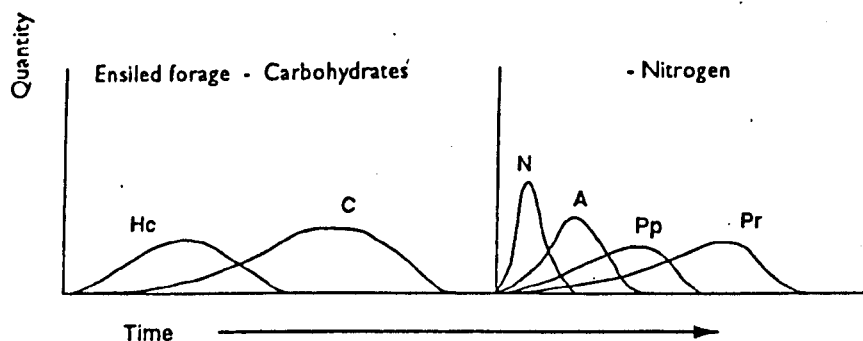
#### 1.4.6 Synchrony of nitrogen and energy supply

The principle focus of much attention in recent years has been manipulating the supply of N and energy in the rumen to improve the efficiency of microbial protein production (Huber & Herrera-Saldana, 1994). Synchronising the release of N and energy from the dietary components, into the rumen, is believed to enhance the efficiency with which the rumen degradable dietary components are utilised for microbial protein synthesis (Huber & Herrera-Saldana, 1994; Sniffen *et al.*, 1983; Sinclair *et al.*, 1993). Energy (ATP) produced from carbohydrate fermentation cannot be stored and consequently needs to be available at the time N is available from dietary protein degradation and other sources, in order to maximise nitrogen capture. An example of a diet with an asynchronous supply of dietary N and energy yielding fraction of the diet to the rumen, is the extensively-fed grass silage diet where ensiling has generally increased the rapidly soluble nitrogen component (i.e. ammonia and amino acids) by partially degrading the forage protein components with plant proteases. The soluble carbohydrate content of the plant material are reduced substantially during ensiling fermentation as they are anaerobically fermented to organic acids (Wilkinson, 1985; McDonald *et al.*, 1991). Figure 1.6 shows the

theoretical difference in availability, with respect to time, of carbohydrate and N components in a silage diet. Microbial protein synthesis in sheep fed grass silage diets was found to be approximately 21 g microbial N / kg organic matter apparently disappearing in the rumen (OMADR) and was generally lower than animals fed on hay diets produced from the same material which yielded approximately 26 g microbial N / kg OMADR (Siddons *et al.*, 1985).

Initial reports by Blackburn (1965) suggested that synchronising the supply of OM and nitrogen in the diet would affect N metabolism of the rumen by decreasing the rate at which rumen ammonia nitrogen ( $\text{NH}_3$  - N) is absorbed across the rumen wall. This would imply that a greater proportion of ammonia would be incorporated into microbial protein, as is the predominant fate of rumen ammonia if it is not absorbed across the rumen wall (Nolan, 1975). Rooke *et al.* (1987) demonstrated that by continuously infusing glucose syrup into the rumen, of cattle fed grass silage, microbial protein synthesis was stimulated and the rumen  $\text{NH}_3$  - N concentration decreased. The improvement in synthesis was attributed to a better synchronisation of available energy with the silage N.

Figure 1.6. Theoretical availability of the carbohydrate and nitrogen fractions in the rumen, over time, when the animal is fed a single meal of ensiled forage (where Hc = hemicellulose; C= cellulose; N= non-amino nitrogen; A = amino acids; Pp = peptides; Pr = proteins) (Beever, 1993).



Differences between dietary carbohydrate sources and their effects on nitrogen metabolism in the rumen have been reported (Chamberlain *et al.*, 1993; Chamberlain *et al.*, 1985; Syrjala, 1972). Chamberlain *et al.* (1985) reported that when a silage diet was fed to sheep with either a glucose or sucrose supplement, the rumen microbes were more effective in using  $\text{NH}_3$  - N for the synthesis of microbial protein, than when silage was supplemented with starch. Starch is a complex

polysaccharide molecule and is consequently more slowly degradable than the smaller di- and mono- saccharides such as sucrose and glucose and is less likely to match the release of the rapidly available soluble N component of the silage diet. The confounding factor with the work of Chamberlain *et al.*(1985) is that they assumed that a decreasing rumen ammonia concentration implies that more  $\text{NH}_3$  - N is incorporated into microbial protein. In fact, often with a dietary change there is a change in rumen pH and this may affect the absorption of the ammonia across the rumen wall, a deceptive route that may cause a decline in rumen  $\text{NH}_3$  - N concentration. A further study by Chamberlain *et al.* (1993), overcame this confounding factor, however, when they carried out an experiment using measurements of both rumen ammonia concentration and the urinary excretion of purine-derivatives as an index of microbial protein supply to the animal (Chen *et al*, 1992) and showed that rumen pH was unchanged between diets.

In contrast, Salter *et al.* (1983) showed that starch was a better carbohydrate supplement than glucose, for maximal capture of the  $\text{NH}_3$  - N for microbial synthesis in the rumen of steers. However, these animals were not fed a basal silage diet as was the case in the other studies reported, but instead were fed straw supplemented with concentrates of which the N component was urea. This would imply that the variability observed in the effects that the carbohydrate component has on  $\text{NH}_3$  - N capture is also affected by the N component. This is clearly demonstrated in work by Rooke and Armstrong (1989), who investigated the effects of supplementing grass silage with intra-ruminal infusions of either urea, casein or a twice daily feed of soya-bean meal on microbial protein synthesis, when sucrose was continuously infused into the rumen in all treatments. Casein, but not urea, enhanced the amount of protein synthesised by the microbes, whilst the animals fed soya-bean meals twice daily tended to have only a slight and non-significant increase in microbial protein synthesis. The purpose of feeding the soya-bean as a treatment was to investigate whether feeding a rumen degradable protein source twice daily achieved similar results in microbial protein metabolism to the continuous infusion of N. The explanation that was offered for these results apart from the fact that different amino acids were being supplied from the different protein sources was that the rates of release of N from the soya-bean and of energy from the sucrose was less well synchronised than when casein was infused.

In the above studies the rate of energy and N release into the rumen was not measured. The work is therefore confounded by the lack of knowledge of the actual quantity or rate of release of the N and energy yielding components into the rumen. This factor is particularly important in studies involving silage supplementation, as the variability in silage quality is likely to have a significant effect on the energy and nitrogen release and consequently the actual synchronisation of the energy and N components with which the experimental hypotheses are based on.

Sinclair *et al.* (1993) overcame this confounding factor by looking at the effects of a quantified N and energy release, on the efficiency of microbial protein synthesis. The release of N and energy from particular dietary ingredients were quantified using the degradation characteristics measured *in situ*. Diets in this study were designed using SIRE (Synchrony Index for the Rumen Environment), a diet formulation computer program that was written to calculate the dietary N and OM supply to the rumen. The program collates the rumen degradability coefficients of a number of different raw feed ingredients and applies them to the first-order model of Ørskov and McDonald (1979) to measure the cumulative amount of N and OM components degraded with time,  $t$  (Equation 1.1).

Equation 1.1 (Source : Ørskov and McDonald (1979))

$$p = a + b(1 - e^{-ct})$$

where :  $p$  is the cumulative amount degraded at time  $t$ ;  $a$  is the readily soluble fraction;  $b$  is the second protein fraction that disappears at a constant fractional rate,  $c$ , per unit time and  $t$  is time (h).

Ørskov and McDonald (1979) in proposing this model assumed that the total dietary protein is made up of only two fractions, namely the very rapidly degradable fraction,  $a$ , and the more slowly degradable fraction,  $b$ . Another assumption made was that the rapidly degradable protein fraction,  $a$ , is completely degraded in the rumen which seems a fairly valid approximation as the early degraded proteins are predominantly water soluble and accessibility of the proteins to the proteases is much greater if the protein is in solution (NRC, 1985).

The SIRE program contains the database of the degradation characteristics of a large number of raw ingredients and can consequently be used to calculate the hourly release of N and OM from a compound diet, when the proportion of each ingredient in the diet, the total dry matter intake (DMI), rumen outflow rate of the solids and the feeding times and feeding frequency throughout the day are included in the computer model.

The degree of OM and N synchrony in a diet can be quantified using a “Synchrony Index” (SI). SI is a measure of the sum of the hourly deviation of the N : OM ratio from an optimum N : OM ratio for efficient metabolism of the rumen microbes throughout the day. This optimum ratio was taken to be 25 :1 gN / kgOM using data reported by Czerkawski (1986). SI is expressed as a factor between 0 and 1, the closer the diet is to 1, the more synchronous the overall daily release of nitrogen and OM is to the recommended optimum. The calculation of SI can be seen in Equation 1.2.

Equation 1.2 (Source : Sinclair *et al.*, 1993)

$$\text{Synchrony Index, SI} = \frac{25 - \left( \frac{\sum (25 - hN:OM)}{1-24} \right)}{24}$$

where :

hN:OM = the hourly N:OM release in the rumen from a particular diet

25 = the optimal release of dietary N:OM degraded in the rumen of sheep (gN/kgOM)

24 = hours measured.

### **1.5 Absorption of amino acids and peptides from the small intestine**

Amino acids are carried from the rumen into the small intestine as undegraded dietary protein, endogenous protein or microbial protein. Digestion of proteins occurs by pancreatic proteases in the lumen of the small intestine. The pH in the lumen is a factor that affects protein degradation and amino acid and peptide absorption. Intestinal proteases function most efficiently between pH 7 and 8. In

contrast to monogastrics, in ruminants only one to two thirds of the intestine are believed to increase to the optimum pH of the proteases (Ben-Ghedalia *et al.*, 1974).

Absorption of a number of the degraded proteins occurs across the brush border membrane of the small intestine. Different sites in the small intestine have different affinities for the absorption of specific amino acids (Philips, 1979), although the ileum is the major site for amino acid absorption in the ruminant small intestine (Walker *et al.*, 1979; Webb & Matthews, 1994). There is evidence that different transport systems exist along the intestine for the absorption of particular amino acids (Webb & Bergman, 1991; Wilson & Webb, 1990). Some of these systems require energy and sodium in order to function effectively, while others function by simple diffusion. For example, diffusion has been shown to be the prevailing form of methionine transport, although active transport and facilitated diffusion have been shown to contribute (Moe *et al.*, 1987). The concentration of the amino acid substrate influences the passive uptake by the intestine of both methionine and lysine (Wilson & Webb, 1990).

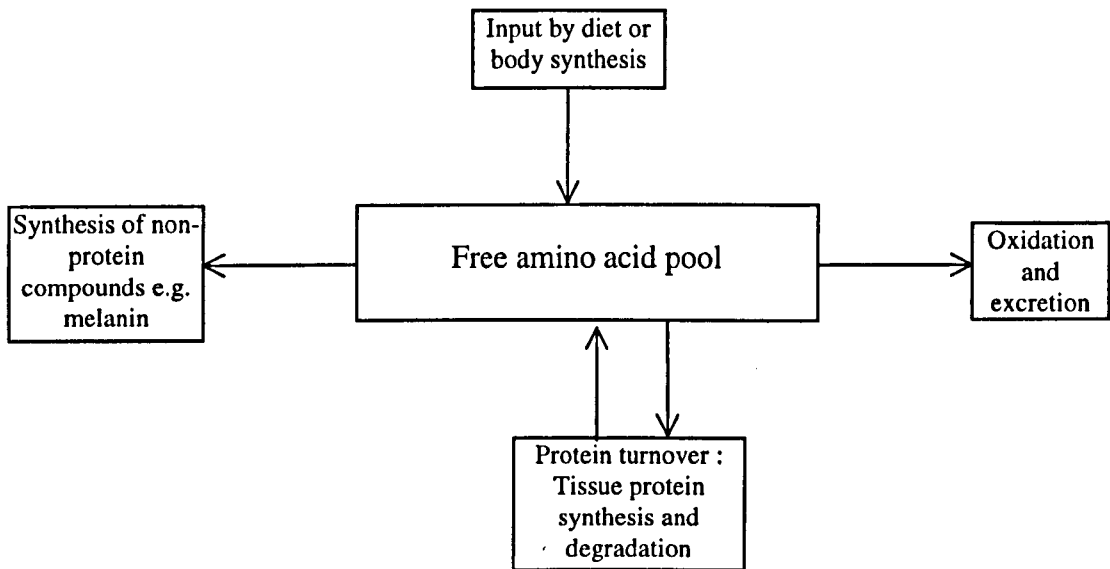
## **1.6 Amino acid metabolism in the tissues**

The amino acids absorbed from the gastrointestinal tract are transported in the blood via the portal vein to the liver, before being distributed to other tissues. The majority of amino acids are transported in the plasma, but there is also evidence that amino acids are transported as serum proteins (McCormick & Webb, 1987) and as peptides in the peripheral plasma (Webb, 1986; Backwell, 1994; Webb *et al.*, 1992; Seal & Parker, 1991; Webb & Bergman, 1991). Differences in the rate of disappearance of amino acids from the small intestine and their rate of appearance in the portal blood, reported by Tagari & Bergman (1977) and Hume *et al.* (1972) indicate utilisation of the amino acids in the portal-drained viscera (PDV). However, as the presence of plasma peptides were not measured in these studies, it may be that the form with which the amino acids are transported to the peripheral blood is different i.e. they may be transported as peptides. Certainly a difference in utilisation of amino acids in the PDV and liver appeared to be the case in steers with greater feed intakes being shown to increase both the removal of amino nitrogen and the utilisation of oxygen by the PDV and liver (Reynolds *et al.*, 1992).



A simple method of describing the amino acids in the circulation that are available for metabolism by the tissues, is to use the concept of plasma free amino acid pools. A broad overview of the flow of amino acids into and removal from such pools is shown in Figure 1.7.

Figure 1.7 Amino acid flow into and removal from the plasma free amino acid pool. (modified from NRC, 1985)



Free amino acid pools are complex and vary in size, the ratios of different amino acids in the pools and the rate and efficiency with which the amino acids are transported from the plasma pools. As a result, the simple concept presented in Figure 1.7 needs to be treated with caution, however, the general concepts of movement of plasma amino acids are necessary to understand the utilisation of amino acids in the tissues.

### 1.6.1 Synthesis of non-protein compounds

Free amino acids are used for the synthesis of a number of non-protein compounds e.g. creatine, melanin, thyroid hormones and purines. The loss from the amino acid pool for these purposes, however, probably account for less than 1% of the total amino acids absorbed from the digestive tract (NRC, 1985).

### 1.6.2 Amino acid oxidation

Amino acid oxidation represents a significant pathway in the irreversible loss of amino acids from the body. Generally surplus amino acids that are not used for protein synthesis become a metabolic fuel. The amino groups are removed and generally converted to urea in the liver. The carbon skeletons are converted into pyruvate, acetyl CoA, acetoacetyl CoA,  $\alpha$ -ketoglutarate, succinyl CoA, fumarate or oxaloacetate. These substrates can either be completely oxidised in the citric acid cycle to CO<sub>2</sub> and water with the generation of energy (ATP), or be used for gluconeogenesis, fatty acid or ketone body synthesis.

The majority of amino acids from the plasma pool, appear to be oxidized in the liver (NRC, 1985), particularly the glucogenic amino acids alanine and glycine (Bergman, 1986). The peripheral muscle tissues are also capable of amino acid oxidation, particularly the branched-chain amino acids (BCAA) valine, isoleucine and leucine (Harris & Loblely, 1991)

### 1.6.3 Nitrogen excretion

In ruminant animals, waste nitrogen from the deamination of amino acids or from ammonia is converted to urea and either excreted from the body in milk or in urine, or alternatively is recycled back into the rumen for microbial usage (section 1.3.3).

### 1.6.4 Protein Turnover

Protein turnover is the term used to express the dynamic substrate cycle of protein synthesis and degradation. The continual breakdown and resynthesis of protein means that the flow of amino acids in the body greatly exceed the amount of amino acids that are absorbed from the gastrointestinal tract (Loblely, 1994). Deposition of protein in the animal is dependent on the difference in the relative amounts of protein synthesis and degradation per unit time. Consequently protein accretion occurs when synthesis of protein exceeds degradation.

Amino acids are transported into cells by a variety of transport mechanisms (Christensen, 1990). In the cytoplasm of the cells, amino acids become activated by attaching to specific tRNA catalysed by aminoacyl-tRNA synthetases. Peptide bond formation takes place on the ribosome, dictated by the base sequence of the mRNA.

Translation of the mRNA for protein synthesis is a three stage process; initiation, elongation and termination. Two high-energy phosphate bonds are used from ATP as an energy source for the formation of each molecule of aminoacyl-tRNA and high-energy phosphate bonds are used when two GTP molecules are hydrolysed during elongation of the peptide bond (Waterlow *et al.*, 1978). Consequently at least four high energy phosphate bonds are necessary for the formation of one peptide bond (Lobley, 1993; Waterlow *et al.*, 1978; Campbell, 1977), making the synthesis of protein a very energetic process.

Considerably less is known about the degradation of proteins than about protein synthesis. A number of distinct proteolysis pathways exist and the enzymes which control these processes appear to be structurally compartmentalised with separate proteolytic systems within, for example, the plasma membrane and different organelles within the cytoplasm (Reeds & Davis, 1992).

The constant turnover of the protein pools enables the animal to cope with submaintenance conditions and maintain a degree of homeostasis, by mobilising amino acids to use their carbon structures for the synthesis of compounds (e.g. glucose). The exothermic reactions involved in the protein synthesis reactions also aid the maintenance of a stable body temperature.

Protein turnover not only contributes to a stable environment, but allows the animal to be flexible in coping with a changing environment e.g. growth, pregnancy, wound healing and fighting infection. The constant mobilisation of amino acids ensures availability of substrates for changes in structure and function within the animal e.g. muscle accretion, hormone, enzymatic and immune response changes.

The control of protein degradation and synthesis, is thought to be strongly influenced by the workload, hormonal balance and physiological and nutritional state of the tissue (Reeds and Davis, 1992)

## **1.7 Nutritional influences on fat and protein accretion**

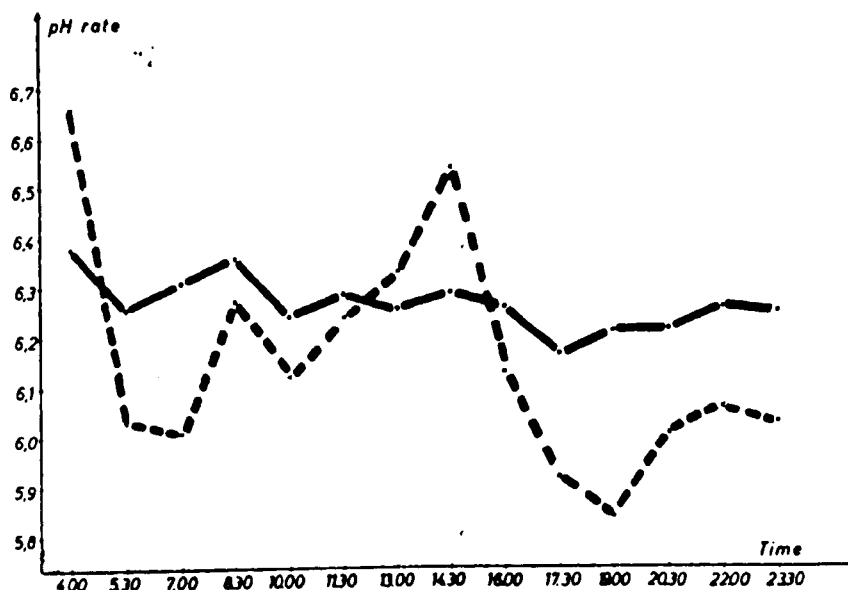
Growth of an animal is dependent on lipid and protein metabolism. Net accretion of muscle and fat tissues is the result of synthesis exceeding degradation. Regulation of metabolism occurs mainly by altering the kinetics of the biochemical reactions involved in tissue metabolism and also by altering the availability of the substrates involved in the biochemical reactions (McDowell & Annison, 1991). The

interaction of hormones and growth factors with cellular receptors causes changes in the metabolic response of tissues. The effects of hormones (e.g. insulin, growth hormone, IGFs and glucagon) appear to be strongly influenced by nutritional parameters. Their secretion and the tissue responsiveness to the hormones can affect net lipid and protein accretion (Reeds & Davis, 1992). The frequency with which an animal is fed can influence hormonal control and the patterns of peripheral nutrient supply. The effects that this has on fat and protein accretion is discussed here.

#### 1.7.1 The effects of frequency of feeding on fat and protein accretion

There are a number of factors that can affect the fermentation pattern of the rumen. Cellulolytic bacteria are pH sensitive, a decrease in pH decreases the activity of the acetate producing cellulolytic bacteria, and the rumen environment favours propionate production by amylolytic bacteria (Orskov, 1975; Kaufmann, 1976). A more frequent feeding results in a more constant pH, with minimum pH throughout the day being higher than if the same feed is fed less frequently (Aronen, 1991; Kaufmann, 1976; Faichney, 1968). With the smaller decrease in pH throughout the day, in more frequently fed animals, it has been shown that the acetate to propionate ratio in the rumen is higher throughout the day (Figure 1.8; Kaufmann, 1976). Similar results were observed by French & Kennelly (1984).

Figure 1.8 The effect of feeding frequency (14 meals per day (solid line) versus 2 meals per day (dotted line)) on the pH of the rumen throughout the day. Twice daily meals were fed at 07:00h and 19:00h and more frequently fed animals were fed at frequent meals between 04:00h and 23:00h (Source : Kaufmann., 1976)



Diurnal fluctuations in rumen VFA concentrations have been reported, with a peak in the total VFAs following meal feeding (Aronen, 1991; Robinson *et al.*, 1986; Phillip *et al.*, 1980). This change in VFA production in the rumen is likely to alter the amount of VFAs absorbed across the rumen wall for use by the ruminant. Absorption of VFAs across the rumen wall is by simple diffusion of the undissociated form of VFAs and is a concentration dependent process (see France & Siddons, 1993). The pKa of the acids is lower than the pH of the rumen contents and therefore the VFAs generally exist in the anionic form in the rumen. A fall in pH is associated with an increase in the undissociated form of VFAs and consequently a decline in rumen pH is likely to enhance absorption of VFAs into the circulation (see France & Siddons, 1993). Peaks in both plasma acetate and plasma propionate concentrations of dairy cows have been observed following meals in a twice daily feeding regime (Sutton *et al.*, 1988). Plasma acetate and propionate concentrations were more constant during a six times daily feeding regime (Sutton *et al.*, 1988).

Feeding infrequently leads to a greater variation throughout the day in several rumen characteristics (Sniffen & Robinson, 1984; Aronen, 1991). Diurnal responses to once daily feeding in both sheep and cattle have been demonstrated in

concentrations of rumen ammonia N (Robinson *et al.*, 1986, Phillip *et al.*, 1980). Low concentrations of ammonia N are thought to limit microbial growth (Satter & Slyter, 1974). Theoretically a more frequent feeding regime should give a steady supply of nutrients and increase microbial efficiency (Robinson, 1989; Johnson, 1976). However, Chen *et al.* (1992) reported that when measuring purine derivatives in the urine, as an estimate of microbial protein synthesis, there was little difference in the variation throughout the day with increasing the frequency of feeding.

The efficiency of microbial yield with variation in the frequency of feeding, has given contrasting results between experiments. Results reported by Bunting *et al.*, (1987) and Hungate *et al.* (1971) support the hypothesis that when feeding frequency is increased there is less diurnal fluctuation in the metabolites derived from fermentation and so with a more stable rumen fermentation, microbial yield is increased and there is an increase in the efficiency of feed utilisation. Hungate *et al.* (1971) showed that the total microbial nitrogen flow from the rumen was nearly doubled (an 89% increase) when sheep were fed every 2h instead of twice daily. This was presumably due to an improved synchrony of nutrient availability to the microbes. Others, however, have found either no difference in the microbial flow to the duodenum (Robinson and Sniffen, 1985) or have observed a lower protozoal yield with a more frequent feeding pattern (John & Ulyatt, 1984). Greater microbial yields with a less frequent feeding pattern was likely to be due to an increase in passage rate due to the rapid feed intake associated with meal feeding. The subsequent escape of feed particles from the rumen is likely to have had a large number of bacteria attached. The protozoa entering the liquid phase following the feeding of soluble dietary fractions may also be washed out of the rumen and so reduce microbial recycling and enhance efficiency (Sniffen & Robinson, 1987).

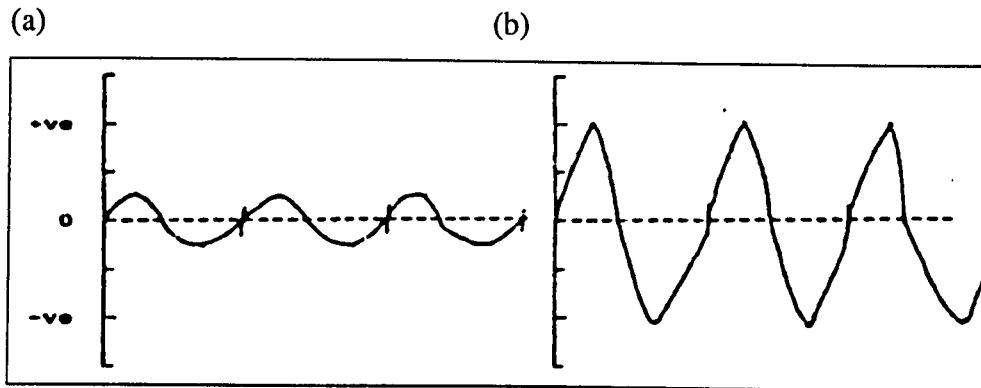
A number of studies on the effect of feeding frequency on body fat and protein metabolism have been reported in both ruminants and non-ruminants. An early review by Cohn *et al.* (1965) stated that the periodicity with which the daily amount of nutrients was ingested was a significant factor influencing intermediary metabolism. Much of their data was based on rat studies, where they demonstrated that when the same daily amount of diet was fed less frequently (twice daily, force-fed versus *ad libitum* nibbling) fat deposition increased and protein deposition was diminished. It was suggested that by meal-feeding rats, the amino acids absorbed into

the peripheral metabolism were in excess of that which could be utilised in protein anabolism and so the nitrogen moiety was excreted as urea and the carbon moiety used in fat metabolism. Similarly, when sheep fed once daily, excreted significantly more N in the urine than those fed three times daily (Raleigh & Wallace, 1965). Several studies have shown that infrequent feeding increases lipogenesis and fat deposition in chickens (Leveille & Hanson, 1965) and in rats (Hollifield & Parson, 1962) although more recent studies have shown no effect on lipogenesis (Jones, 1995).

Rates of ATP generation and the utilisation of oxygen in the hindleg muscles of young steers appeared to decrease with time after feeding when comparing measurements 3h and 20h after feeding (Bell *et al.*, 1975) indicating that there may be a lower protein deposition when animals are fed less frequently. In rats (Garlick *et al.*, 1973) it has also been shown that the fractional rate of protein synthesis was diminished in muscle 24 h after feeding when the animals were acclimatised to meal feeding. Consequently, meal fed animals have shown variation in protein metabolism throughout the day.

Frequency of feeding does not appear to alter nitrogen retention in humans (Irwin & Feeley, 1967), despite there being continuous diurnal changes in body protein, throughout the day (Millward *et al.*, 1974). Protein synthesis in the quadricep muscle of humans was shown to increase on feeding, following a post-absorptive state (McNurlan *et al.*, 1993). Diurnal changes reflect the meal-eating pattern and so in order to maintain nitrogen balance it is necessary to deposit sufficient protein in muscle after feeding to balance losses in the post-absorptive state (Millward, 1985). Millward (1985) proposed that if the intake at each meal is low, then the losses in the post-absorptive state must be regulated and kept low, to maintain nitrogen balance. Price *et al.* (1994) showed that by increasing protein intake that the amplitude of diurnal cycling in human adults increased. This is demonstrated in Figure 1.9 where in the case of low and high intakes there are low and high amplitude diurnal changes to achieve N balance. Changing the size and frequency of the meal, therefore, may change the pattern of peripheral protein synthesis and may influence overall protein turnover and consequently deposition.

Figure 1.9 Diurnal cycling of nitrogen-balance at low (a) and high (b) feed intakes (source : Millward, 1985).



Investigations carried out on ruminants have given variable results. Some studies have concluded that there is no response in the liveweight gain of bulls (Aronen, 1991), steers (Goonewardene *et al.*, 1995) or pigs (Burt & Dunton, 1967) when frequency of feeding is altered. Gibson (1981), however, reviewed a series of literature on the effects of feeding frequency on cattle growth and showed that when increasing the frequency of feeding increased daily weight gain on average by 16% and increased food utilisation efficiency for growth by 19%. In addition, Gordon & Tribe (1952) fed sheep (n=22) either 8 times daily or once daily in a cross over design. The liveweight gain of animals fed 8 times daily was greater than when animals were fed once daily. Ruiz & Mowat (1987) showed that under a restricted feeding regime nitrogen retention was enhanced when weaned calves were fed more frequently. This latter study, however, did not weigh food refusals to measure eating patterns, until five hours after the animals were offered the food, and so time of feeding throughout the day is unclear.

## **1.8 Overall Objectives**

As previously discussed, a synchronous supply of energy and nitrogen in the rumen enhances the production efficiency of microbial protein (section 1.4.6). Knowledge of the effect that the synchrony of nutrients in the rumen has on the patterns of nutrient supply and the effects that these have on efficiency of nutrient utilisation in the tissues of ruminants is limited. The overall aim of this thesis was to investigate the effects of altering the patterns of supply of dietary nitrogen (N) and organic matter (OM) in the rumen on the supply of metabolites to the peripheral



tissues and their subsequent effects on fat and lean deposition. In particular, the effect that altering the pattern of metabolite supply on protein metabolism of the animal, was to be investigated.

The objectives of this thesis were :

- a) to study the effect when the predicted patterns and rates of supply of dietary nitrogen and OM to the rumen was altered throughout the day on the ruminant energy supply (acetate) and nitrogen supply (amino acids) in the peripheral plasma.
- b) to investigate the effects on carcass composition and growth when the predicted patterns and rates of supply of nitrogen and OM to the rumen was altered throughout the day and
- c) to investigate the effects of the acetate supply on amino acid utilisation, in particular estimates of whole-body protein synthesis.

The plasma nutrient supply in this thesis has not been assessed directly, but has been inferred from measurements of plasma concentration.

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## CHAPTER 2

### MATERIALS AND METHODS

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#### **2.1 Plasma Sample Collection**

Blood samples were taken from an indwelling cannula placed in the left jugular vein at least 24 hours before sampling. Cannulae were inserted approximately 10cm into the vein. Cannulae were fitted with a flow switch (16G Universal FloSwitch; Ohmeda, Swindon, Wiltshire). After each sample was taken the cannula was flushed with 1ml heparinised saline (37u/ml, Multiparin heparin injection B.P; CP Pharmaceuticals Ltd., Wrexham), to maintain patency.

Blood taken was placed in ice-chilled tubes containing 25u heparin/ ml of whole blood sample. The samples were gently mixed by inversion and centrifuged at 1000×g for 15 min (MSE-Centaur 2, Fisons Instrumentation, Sussex). The plasma supernatant was pipetted off and stored at -20<sup>0</sup>C until analysis.

After slow thawing at 4<sup>0</sup>C, all plasma samples were centrifuged at 1000×g for 10 min (MSE-Centaur 2, Fisons Instrumentation, Sussex), prior to analysis.

#### **2.2 Analysis of plasma samples**

##### **2.2.1 Determination of plasma acetate concentration**

###### **(i) Deproteinisation of plasma**

Plasma (200µl) was deproteinised, by the addition of an equal volume of ice-cold perchloric acid (0.6M; Fisher Scientific, Loughborough), containing 0.2µmoles of 3-methyl-n-valeric acid (Sigma Chemical Company, St.Louis, USA) as an internal standard (ISTD). After 10 min on ice, the deproteinised solution was microfuged for 10 min at 4<sup>0</sup>C (3600×g, Biofuge A; Heraeus Septatec, West Germany) and the supernatant pipetted off. Excess perchlorate in the supernatant was neutralised by the addition of 30µl of 2M potassium carbonate (Fisher Scientific, Loughborough) and the solution then reacidified with 60µl orthophosphoric acid (10% (w/v); Fisher

Scientific, Loughborough). The precipitate was allowed to settle and the resulting supernatant was placed in 100 $\mu$ l vial inserts and analysed.

#### (ii) Analysis of acetate concentration

Samples were analysed using a gas chromatograph (Perkin Elmer Autosystem; Norwalk, Connecticut, USA). The deproteinised plasma sample was injected, using an autosampler, into a split/splitless injection port (240 °C) that was set at a split ratio of 16:1. The volatile fatty acids (VFA) were separated on a 0.32mm  $\times$  25 m polyethylene glycol polar capillary column (BP21, bonded FFAP, 0.25 $\mu$  film thickness; SGE, Milton Keynes). Helium (Air Products, Surrey) was used as carrier gas through the column at a flow rate of 0.9 ml/min. The oven temperature was programmed at 85°C to increase at a rate of 6°C/min to 180°C. Flame ionisation detection (FID; 290°C) monitored the separated VFA ions that were released from the column, and integration of the detected peaks was carried out using Turbochrom 4 Software (Perkin Elmer-Nelson; San Jose, California) to obtain peak areas of the VFAs. The linearity of the column was checked with standards of increasing concentrations of both 3-methyl-n-valerate (3-mv; 0.2-5mM;  $r^2=1.00$ ) and acetate (0.2-15mM;  $r^2=0.98$ ).

#### (iii) Calculation of the acetate concentration

A calibration curve and internal standard were used to calculate the concentration of acetate. A calibration curve of the ratio of 3-mv concentration (internal standard) to acetate concentration (calibration standard) against the ratio of the 3-mv peak area to the acetate peak area (both measured by Gas Chromatography (GC)), was drawn. The slope of this curve was the response factor (RF).

The calibration curve was linear ( $r^2=0.99$ ), therefore, the RF, the known internal standard concentration (3-mv) and the peak areas of both the acetate and the 3-mv could be used to calculate the unknown acetate concentration in the sample.

The concentration of acetate was calculated using equation 2.1 where 3-mv PA and acetate PA are the respective peak areas measured on the GC, for 3-mv and acetate. [3-mv] is the concentration (mM) of the internal standard (3-mv).

$$\text{Acetate concentration in sample (mM)} = \frac{3 - mv \text{ PA}}{\text{acetate PA}} \times [3 - mv] \times \frac{1}{RF}$$

Equation 2.1

The interassay variation was 4.55% (n=10). Recovery of acetate was calculated by measuring the acetate concentration in a plasma sample containing 200µl of 2mM acetate (n=10) and measuring the same plasma sample but substituting the 2mM acetate with 200µl water. The recovery of acetate was calculated as the actual amount of additional acetate measured in the spiked sample as a percentage of the 2mM acetate solution that was added. Acetate recovery was approximately 87.6% (n=6).

### 2.2.2 Determination of the plasma amino acid concentration

#### (i) Deproteinisation of plasma

To 0.2ml plasma was added 0.07ml 5-sulphosalicylic acid (15% w/v; Fisons Ltd, Loughborough, Leics) containing 40nmoles nor-leucine (Sigma Chemical Co., St.Louis, USA) as an ISTD. Following agitation, the mixture was left to stand at 4°C for one hour and then microfuged for 7 min at 18000×g (A13, Jouan; Saint Nazaire, France). The clear supernatant was decanted and the pH adjusted to between 2.0 and 2.5 (4M lithium hydroxide) and tested using pH indicator paper (pH 1-4; Whatmann). The samples were then frozen at -20°C until analysed.

#### (ii) Analysis of amino acid concentration

Samples were thawed and injected (100µl) into capsules and loaded, with a lithium citrate loading buffer (0.2M, pH 2.2), onto a cation exchange amino acid analyser (Pharmacia Biochrom 20; St Albans, Herts). A molarity change was created in the column (packed resin column, 25cm x 4.6mm) with five lithium citrate cation buffers (from 0.2M-1.65M), each with their own specific pH (2.80-3.55) resulting in an increasing pH gradient. The change in pH together with a change in the temperature in the column (30°C to 85°C) enabled separation of the amino acids by cation exchange. A buffered ninhydrin solution (0.06M ninhydrin and 0.0025M hydrindantin in 70% (v/v) ethanediol and 30% (v/v) sodium acetate buffer (1.0M

anhydrous sodium acetate, 3.0M potassium hydroxide, 0.01M citric acid, 4.8M glacial acetic acid), pH 5.5; Sigma Chemical Co., St.Louis, USA) reacted with the amino group to form a coloured amino acid complex on incubation in a reaction coil (135°C). All amino acid complexes were quantified in a colorimeter at wavelengths of 570nm except for the proline complex which was measured at a wavelength at 440nm. Peaks of the individual amino acids were recorded and the area integrated. The concentration of the individual amino acids in each sample was calculated by reference to the ISTD peak area.

### (iii) Calculation of plasma amino acid concentrations

Calibration standards of each amino acid and the ISTD (norleucine) were used to calculate the concentration of amino acids in the plasma. The ratio of the peak areas (norleucine : amino acid) against the ratio of the respective concentrations were used to obtain a response factor. The unknown concentration of each amino acid could be calculated from the known concentration of internal standard, the peak areas obtained for the amino acids and norleucine and the response factor calculated (see section 2.2.1(iii)). Interassay variation for the total amino acids measured was 4.35% (n=5).

### 2.2.3 Determination of plasma insulin concentration

Plasma insulin concentrations were determined in samples from the experiment that is reported in chapter 3, using a radioimmunoassay kit (Ins-RIA-100; Lifescreen Ltd., Watford, Hertfordshire), produced for the determination of human plasma insulin concentrations. The assay is based on the principle of competition between a labeled antigen (Ag\*; <sup>125</sup>I-insulin) and an unlabelled antigen (Ag; plasma insulin) for specific antibodies (Ab; anti-insulin antiserum). As the presence of the unlabelled antigen increases, a greater proportion of AgAb is formed and a smaller proportion of the Ag\*Ab can be monitored. A standard curve was constructed by adding increasing known quantities of Ag (0-500µU/ml) to constant amounts of Ag\* and Ab. Following incubation of Ag with Ab and Ag\*, the Ag\*Ab was separated from the other reactants and the radioactivity measured. The curve constructed of known quantities of AgAb against the Ag\*Ab measured was used to calculate

quantities of Ag in plasma samples by interpolating the quantity of Ag\*Ab measured in the sample to the standard curve.

Modifications to the original method were necessary to increase the sensitivity of the assay for measurements of insulin concentrations in ruminant plasma. The modifications used, were recommended by the kit manufacturer.

Insulin antiserum (100 $\mu$ l) was added to the sample or standard (200 $\mu$ l), mixed, covered and incubated overnight at 4°C. The iodinated insulin (100 $\mu$ l) was added to the samples and incubated at room temperature for 6 h. A second antiinsulin antiserum solution (1ml), suspended in polyethylene glycol solution was added to the iodinated samples, agitated and incubated for 20 minutes at room temperature. The <sup>125</sup>I-insulin and antibody complex that was produced, formed a precipitate and was sedimented by centrifugation for 30 min at 1500 $\times$ g (Jouan CT422; Saint Nazaire, France). The supernatant was decanted and the LP4 tubes containing the pellet were inverted for 30 min to drain any remaining liquid. The radioactivity in the pellet formed, was counted using a gamma counter (Cobra AutoGamma; Packard Instrument Co., Meridian, U.S.A.). The insulin concentrations, for these samples were measured as  $\mu$ U/ml.

All insulin concentrations measured in the plasma from experiments reported in Chapters 4 and 5 were determined using a radioimmunoassay that had been developed by Dr. Chris Reynolds, who kindly analysed these samples using this assay at Reading University. The insulin concentrations, for these samples were measured as ng/ml.

#### 2.2.4 Determination of <sup>13</sup>C-Leucine enrichment in plasma

##### (i) Deproteinisation of plasma for the isolation of leucine and ketoacids

To 0.5ml of plasma was added 100 $\mu$ l of ISTD solution containing 150nmoles nor-leucine and 10nmoles ketocaproic acid (KC)(Sigma Chemical Company, USA). Samples were deproteinised by adding 0.5ml of 15% (w/v) sulphosalicylic acid to the plasma and internal standard, mixing well and storing on ice for 10 min. The protein precipitate was separated by centrifuging the samples in a microfuge at 18000 $\times$ g (Jouan A13; Saint Nazaire, France) for 5 min. The resulting supernatant (1ml) was

pipetted off and passed through an ion-exchange resin column, to separate the amino acids and ketoacids from the sample.

#### (ii) Preparation of the ion exchange resin column

The Dowex 50W-X8 resin ( $H^+$  form; cross linkage 8%, 100-200 dry mesh; Sigma Chemical Co., USA) was prepared by washing the resin in a sintered funnel containing a filter paper (No.1, Whatman) with HCl (approximately 5ml 1M HCl/ g resin) until the effluent from the funnel had a pH < 2. The resin was then rinsed with deionised water until the effluent reached a pH > 4.8. The prepared resin was stored in an excess of deionised water at room temperature until needed.

The columns were prepared by placing a small plug of glass wool into a disposable 2 ml syringe (plunger removed). A flow switch (16G UniversalFloSwitch; Ohmeda, Swindon, Wiltshire) was attached to the luer nozzle of the syringe, and the columns then placed in a clamp. The resin/water mix was stirred gently to suspend the resin evenly through the water before 1.5ml of resin was pipetted into the syringe. Deionised water was added to the column above the resin and allowed to run through followed by two further head volumes of water. Care was taken not to allow the column to run dry at any point. The column was used for isolation of amino acids and ketoacids.

#### (iii) Isolation of Ketoacids

The deproteinised plasma was applied to the top of the column, allowed to soak into the resin and then immediately eluted with 1ml of deionised water. The eluate was collected in 8ml pyrex culture tubes and contained the ketoacids.

#### (iv) Isolation of Leucine

Following collection of the ketoacids, deionised water (2 x 2 ml) was run through the column and the eluate discarded. The leucine was then eluted from the column with 4M ammonia solution (2ml) followed by deionised water (1ml). The eluate was collected in 8ml pyrex culture tubes and the column allowed to run dry. Amino acid eluates were frozen at  $-80^{\circ}C$  prior to being freeze dried, and then stored at  $-80^{\circ}C$  until analysis.

#### (v) Derivatisation of Leucine

The freeze-dried leucine samples were derivatised by adding 350 $\mu$ l of HCl (0.1M), agitating and drying at 90°C under nitrogen (Pierce Reacti-Therm III heating block; Pierce, Rockford, Illinois). After cooling, 100 $\mu$ l of N-methyl-N-t-butyl-dimethylsilyltrifluoroacetamide in acetonitrile (MTBSTFA:CH<sub>3</sub>CN; 1:1 v/v) was added to the dried sample and vortex mixed. The tube was tightly capped and heated in an oven at 80 °C for 20 min.

After cooling, the t-BDMS leucine derivative was transferred to a GC vial containing a 250 $\mu$ l insert and analysed by Gas Chromatography/Mass spectrometry (GC/MS) using selected ion monitoring for ions m/z 302.2 and 303.2 (see details in section vii)

#### (vi) Derivatisation of ketoacids

To the eluate containing the keto acids, 0.5ml of acidified phenylenediamine (1% (w/v) in 4M HCl) was added. The tubes were purged with nitrogen, tightly capped, vortex mixed and heated in an oven at 90 °C for 1 h. Following cooling, the solution was extracted twice with 2ml ethyl acetate by vigorous shaking after each addition of ethyl acetate. The solvent fractions (the upper layer) were pooled following each extraction and any water removed by adding anhydrous sodium sulphate (approximately 20 crystals) for 15 min. The water-free solvent extract was transferred to a clean 8ml screw-capped Pyrex culture tube and dried under nitrogen on a heating block (Pierce Reacti-Therm III; Pierce, Rockford, Illinois) at 60°C.

After cooling 100 $\mu$ l of MTBSTFA: CH<sub>3</sub>CN (1:1 v/v) was added and the tubes tightly capped, vortex mixed and placed in an oven at 80 °C for 20 min. After cooling the t-BDMS ketoacid derivatives were transferred to a GC vial containing a 250 $\mu$ l insert and analysed by GC/MS for KIC ions m/z 259.1 and 260.1, using selected ion monitoring (see section vii).

#### (vii) GC/MS analysis of leucine and ketoacids derivatives

The t-BDMS amino acid and ketoacid derivatives were analysed using selective ion monitoring (SIM), on a Hewlett Packard 5890 Series II gas chromatograph (Stockport, Cheshire) linked to a electron impact (70 eV) ionisation quadrupole mass spectrometer (Hewlett Packard 5970 Series Mass Selective



Detector; Stockport, Cheshire). Both the amino acid and ketoacid derivatives were analysed on the same column (25m × 0.2mm, HP5 cross-linked capillary column; Hewlett Packard, Stockport, Cheshire). Derivatised samples were automatically injected (Hewlett Packard 7673A Autosampler; Stockport, Cheshire) onto the column (1µl t-BDMS leucine derivative, 3µl t-BDMS ketoacid derivative) into a split/splitless injection port (250°C) at a split ratio of 100:1. The carrier gas used was helium (>99.9995%, Grade 5.5; Air Products, Surrey) set at a column flow rate of 0.7 ml/min. For analysis of the amino acid derivatives the GC oven temperature was set at 150°C for 8 min and then increasing 35°C/min to 290°C and held for 3 min. For analysis of the ketoacid derivatives, the GC oven temperature was set at 150°C for 2 min and then increased by 35°C/min to 200°C and held for 3.32 min and then increased again at 35°C/min to 250°C and held for 3.82 min.

The GC/MS interface temperature was 280°C. The electron impact ionisation ionises the separated t-BDMS molecules to ion fragment peaks in the mass spectrum of M-57 and M-57+1. The ions were monitored by SIM with a dwell time of 50 msec/ion for ions at  $m/z$  302.2 and 303.2 for both t-BDMS leucine and t-BDMS norleucine and ions at  $m/z$  259.1 and 260.1 for both t-BDMS ketoisocaproic acid (KIC) and t-BDMS ketocaproic acid (KC). The  $m/z$  notation represents the ratio of mass to charge of the molecular fragment.

The interassay variation was 1.64% (n=12). Recovery of leucine was calculated in the same way as described for the measurement of acetate recovery (section 2.2.1 (iii)), but instead of acetate, plasma sample (0.3ml) was spiked with 0.2ml of leucine solution (500µM). Leucine recovery was approximately 100% (n=3). The calibration curve was linear ( $r^2=0.91$ ; leucine concentration 0.25µM-300µM).

### **2.3 Breath Sample Collection**

Breath samples were taken to measure the enrichment of  $^{13}\text{C}/^{12}\text{C}$  in the expired  $\text{CO}_2$ . Breath samples were collected into a 2 litre air tight urinary catheter bags (known here as breath bags) fitted with a septum and one-way air valves (DHD Medical Products; Canastota, New York) and face masks. The face mask was held over the animals nostril, holding the other nostril and mouth shut, until the bag was filled (approximately 2 min). The exact time when the face mask was removed was

the time recorded for each sample. Triplicate sub-samples of breath were taken from the bag directly into 13ml evacuated tubes (Exetainers; Labco, High Wycombe, Bucks) through a needle contained in the septum of the breath bag. The needle of the breath bag was held in the lid septum of the evacuated tube for 10 seconds to ensure a constant volume of gas was taken for each sample. Breath samples were stored at room temperature until analysed.

## **2.4 Analysis of $^{13}\text{CO}_2$ enrichment in breath samples**

The  $^{13}\text{CO}_2$  enrichment of the breath was analysed by isotope ratio mass spectrometry (IRMS). Evacuated tubes containing breath samples were loaded onto the IRMS via a Roboprep-G gas purification system (Europa Scientific; Crewe, Cheshire) An automatic needle sampler (Gilson Model 222 Sample Changer; Villiers, France) extracted the sample from the exetainers with helium gas (40ml/min, >99.9995%, Grade 5; Air Products, Surrey) and the breath was passed through a column of magnesium perchlorate to remove water. The  $\text{CO}_2$  was separated from the nitrogen in the breath using a gas chromatograph (oven temperature  $75^\circ\text{C}$ ), before being passed into the IRMS (20-20 Analyser; Europa Scientific).

$^{13}\text{CO}_2$  enrichment of breath samples was determined by comparison with similar volumes of a reference gas (5%  $\text{CO}_2$  in nitrogen; Air Products, Surrey) of known  $^{13}\text{C}$ -enrichment, run frequently throughout the analysis. The  $^{13}\text{C}$ -enrichment of the breath samples was measured as both  $\delta^{13}\text{C}_{\text{PDB}}$  and atom %.

## **2.5 Analysis of isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of sodium acetate**

The isotopic enrichment of sodium acetate samples was determined using an automated  $^{15}\text{N}$   $^{13}\text{C}$  analyser (ANCA). The samples (1mg) were accurately weighed into tin capsules (6x4mm; Elemental Microanalysis Ltd, Devon), and sealed. Reference samples of cane sugar of known  $^{13}\text{C}$  enrichment were also accurately weighed (1mg) into tin capsules. Where a liquid sample was analysed, a small amount of absorbent material (Chromosorb W NAW, mesh size 30-60; PhaseSep, Deeside, Clwyd) was used in the bottom of the tin capsules to avoid leakage from the capsules.

The samples were loaded into the autosampler tray and each sample in turn was dropped into the combustion tube (1000°C), through which, passed a helium carrier gas (60ml/min, >99.9995%, Grade 5; Air Products, Surrey). A pulse of oxygen was injected into the helium carrier gas and flash combustion of the tin capsules occurred which raised the temperature to approximately 1700°C, ensuring a complete reaction. The carbon products (CO<sub>2</sub>) were passed through a copper reduction tube (600°C) where the carbon products were reduced. Water was removed from the sample by passing it through anhydrous MgClO<sub>4</sub> (Europa Scientific, Crewe). The C products were separated on a gas chromatograph (oven temperature 100°C), before being passed into the IRMS (20-20 Analyser; Europa Scientific).

<sup>13</sup>C content of the samples was determined by comparison with similar amounts of the reference cane sugar (of known <sup>13</sup>C-enrichment), which were run at regular intervals throughout the analysis. The <sup>13</sup>C-enrichment of the samples was measured in atom %.

## **2.6 Determination of Animal Liveweight.**

Animals were weighed using a weigh crate connected to a digital balance (Trutest AG500, Tru-Test Distributors Ltd; Auckland, New Zealand). The weighing apparatus was calibrated using a standard weight of 20kg.

## **2.7 Dissection of carcasses at slaughter**

Animals were humanely slaughtered by conventional slaughterhouse techniques (stunned and exsanguinated).

The liver, heart and spleen were removed from the warm carcass and weighed. The digestive tract (excluding the pancreas and oesophagus) was also removed from the carcass and weighed. The reticulo-rumen, omasum and abomasum (all known as the fore-gut) were separated from the intestines, by tying and cutting as close to the abomasal valve as possible. The full foregut and intestines were weighed separately.

All omental fat surrounding the foregut was removed and weighed. A tie was attached to the end of the reticulo-rumen and a cut made between the rumen and the omasum. A weight for the full reticulo-rumen and the full omasum and abomasum

was recorded. The omasum was cut from the abomasum and the full omasum weighed. The reticulo-rumen, abomasum and omasum were emptied, cleaned with water, drained and reweighed.

The full intestine plus fat were weighed. Mesenteric fat was removed from the small and large intestine, the intestinal fill was emptied and the empty gut was cleaned with water. Any fat remaining on the intestines was removed by cutting the gut longitudinally and scraping the fat from the intestine using a scalpel. All mesenteric fat removed from the intestine was weighed and a weight for the empty intestine recorded.

Reticulo-rumen fill, omasal fill, abomasal fill and intestinal fill were all calculated as the difference between the full and empty organ weights. The full abomasum weight was calculated from the weight of the full omasum subtracted from the weight of the full abomasum and omasum.

Dissection of the *Longissimus dorsi* (*L.dorsi*), *Vastus lateralis* (*V.lateralis*) and *Semitendinosus* muscles from the right side of the carcass was carried out within one hour of slaughter. The hung, dressed carcass was weighed, when cold, sawn in half.

The dressed half carcasses were hung and stored at 4°C overnight. The following day, the left half of the carcass was cut between the 11th and 12th rib-bone against the 12<sup>th</sup> rib-bone. Subcutaneous back fat depth was measured against the 12<sup>th</sup> rib using digital calipers. Kidney knob and channel fat (KKCF) was also dissected and weighed.

The right half of the carcass together with dissected muscles were frozen at -20°C until minced and analysed for chemical composition.

## **2.8 Carcass and Muscle Preparation for chemical composition analysis**

### **2.8.1 Muscle preparation**

Prior to mincing the *Longissimus dorsi* for composition analysis, each muscle was partially thawed (room temperature, 1 hour) and cut into pieces. The muscle was passed three times through a Hobart mincer (Hobart MFG. Co. Ltd.) using a 5mm mincing plate with thorough mixing between each sample. A sub-sample

(approximately 25g) was taken from the final mince, weighed into a pot of known weight and frozen at  $-20^{\circ}\text{C}$ . The remaining mince was frozen with the rest of the carcass. The minced sample was freeze-dried to constant weight and finely ground with a coffee grinder. Samples were stored in a dessicator until analysed.

### 2.8.2 Carcass preparation

Frozen half carcasses together with the dissected *V.lateralis* and *Semitendinosus* muscles and the minced *L.dorsi* were removed from the  $-20^{\circ}\text{C}$  freezer and partially thawed (room temperature, 4 hours). Each half carcass was cut into several pieces before mincing through a Wolfking mincer (C160-UN1; Wolfking U.K. Ltd., Southgate, London). The carcass was passed once through a 13mm mincer plate and then twice through a 4mm mincer plate. The mince was collected in a large container and mixed well between each mincing. The final minced half carcass was well mixed and a subsample of approximately 300g placed in an aluminium food container of known weight and frozen ( $-20^{\circ}\text{C}$ ). The sample was freeze-dried to a constant weight and finely ground with a coffee grinder. Ground samples were stored in a dessicator until analysed.

## 2.9 Analysis of carcass and muscle samples

### 2.9.1 Dry Matter and Ash Determination of carcass and muscle samples

#### 2.9.1.1 Freeze Dry Matter

All carcass and tissue samples were freeze-dried to constant weight and the freeze-dry matter calculated as the resulting dry tissue weight as a percentage of the fresh tissue weight.

#### 2.9.1.2 Oven Dry Matter

Sub-samples of the freeze-dried samples (approximately 2g) were oven-dried to ensure any residual water not removed by freeze-drying or reabsorbed during the grinding and storage of the samples was accounted for. Samples were accurately weighed into pre-weighed dry crucibles and placed in a vacuum oven (Gallenkamp, size 2) at  $70^{\circ}\text{C}$  for 16 h. Samples were cooled in a dessicator before weighing to calculate the oven dry matter, as the weight of the oven-dried sub-sample as a proportion of the weight of the sub-sample before oven-drying. The product of the oven dry matter and the freeze-dry matter is the true dry matter of the sample.

### 2.9.1.3 Organic Matter/Ash

The percentage ash content of the sample was determined by placing the crucible containing the oven-dried sample into a muffle furnace (Stuart Scientific Co. Ltd., Redhill, Surrey) at 550 °C for 16 h. The resulting ashed sample was cooled in a dessicator before weighing. Organic matter of the sample was calculated as the weight loss of the oven-dried sample by ashing as a proportion of the weight of the sample prior to ashing. The ash content of the sample was the percentage of sample that was not organic matter.

### 2.9.2 Determination of the lipid content in carcass and muscle samples

#### (i) Lipid extraction

The Weibul method (Osbourne & Voegt, 1978) was used to determine the lipid content of the carcass and muscle samples. The principle of the method was to free the bound and occluded lipid fractions within the sample by boiling in dilute hydrochloric acid before solvent-extract of the lipid from the sample using petroleum ether.

Approximately 2.5g of the freeze-dried sample was accurately weighed into a pyrex beaker (600ml) and the weight recorded. HCl (100ml; 3M; Fisher Scientific, Loughborough, Leicestershire) was added and the beaker placed on a heater bank (Gallenkamp Kehldahl apparatus) and fitted with an overhead condenser. The solution was allowed to boil gently for one hour, before cooling slightly and adding 1-2g of white Kieselguhr filtration aid (Fisons Scientific, Loughborough, Leicestershire). The solution was filtered, while still warm, through moistened, hardened ashless double filter paper (Whatman 541, diameter 15cm; Whatman International Ltd., Maidstone, Kent). The filter paper, containing the bound and occluded lipid fractions of the sample, was carefully folded and placed in a cellulose extraction thimble (28mm × 100mm, single thickness; Whatman International Ltd., Maidstone, Kent) and dried at 100 °C overnight (16h). A plug of absorbent cotton wool was placed above the sample in the dried thimble and the thimble placed in a soxhlet extractor flask (Quick-fit glass). The flask was attached to a Graham condenser and the lipid extracted with petroleum ether (SLR, 40-60°C boiling point;

Fisher Scientific, Loughborough, Leicestershire) for 6 h. The petroleum-ether collected in a pre-weighed flask was then evaporated from the sample on a steam bath. The extracted lipid that had been collected in a pre-weighed flask (Quick-fit glass), was dried in an oven at 100 °C for 90 min and weighed after cooling in a dessicator to determine lipid weight.

#### (ii) Calculations of total fat content of the samples

The total fat in the sample was calculated as the weight of the final dry lipid residue collected from the extraction procedure as a percentage of the weight of the sample before the lipid was extracted (expressed as a percentage of the freeze dry matter).

#### 2.9.3 Determination of the nitrogen content of carcass and muscle samples

Crude protein was calculated by multiplying the nitrogen content of each sample by 6.25. Nitrogen content was determined by Kjeldahl analysis.

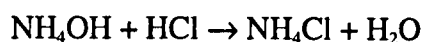
##### (i) Digestion

Freeze-dried sample (0.5g) was weighed into filter paper (Whatman No. 1, 90mm), which was folded to prevent loss of sample, and the weight recorded. The filter paper and the sample were placed in a kjeldahl tube with 1 Kjeltab CX (Thompson & Capper Ltd, Runcorn, Cheshire) and 10ml concentrated H<sub>2</sub>SO<sub>4</sub>. All kjeldahl tubes were placed in a digestion rack on a pre-heated Kjeldatherm heating block (400°C; Gerhardt, Brackley, Northants) to digest the sample. The heating block was situated in a fume cupboard with a manifold connected to a scrubber unit (Gerhardt Turbosog, Brackley, Northants) to remove fumes that developed during the digestion process. When the samples had turned green all were digested for a further 20 minutes. The samples were left to cool.

##### (ii) Distillation

The kjeldahl tubes were placed individually on a Vapodest 5 (Gerhardt UK Ltd., Brackley, Northants) and the sample mixed with 100ml sodium hydroxide (20%(w/v); Fisons; Loughborough, Leicestershire). The sample was steam distilled for 4 min into cooled, saturated boric acid (5% w/v). The NH<sub>4</sub>OH formed was

converted to  $\text{NH}_4\text{Cl}$  by titration with dilute hydrochloric acid (0.2M; Fisons, Loughborough, Leicestershire), the end point of which was determined by pH. The reaction that occurred is shown in Equation 2.2.



Equation 2.2

The amount of HCl required to complete this reaction was recorded and used to calculate the amount of nitrogen present in the sample.

### (iii) Calculation of Nitrogen present in the sample

For every 1ml of 0.2M HCl titrated,  $2.8 \times 10^{-3}$  g of nitrogen was reacted to form  $\text{NH}_4\text{Cl}$ . Consequently the total amount of nitrogen present in the sample was calculated as a product of the volume of HCl titrated multiplied  $2.8 \times 10^{-3}$ . The weight of nitrogen measured in the sample was calculated as a percentage of the total freeze dried sample weight.

## 2.10 Analysis of feed samples

### 2.10.1 Preparation of feed samples for chemical analyses

Representative samples of the feed offered to the animals were prepared for chemical analysis (Gross energy (GE), nitrogen, DM, OM and ash content). A small sample (approximately 10g) was collected from each 25kg sack of feed and pooled every 2 weeks. The pooled sample was ground through a 1mm die using a sample mill (Cyclotec 1093; Tecator, Sweden). Samples were stored in a dessicator until required for analysis.



## 2.10.2 Dry Matter and Ash determination of feed samples

### 2.10.2.1 Dry Matter

All feed samples were accurately weighed into pre-weighed crucibles and placed in an oven at 100°C for 16 h. Samples were cooled in a dessicator before weighing to calculate the dry matter, as the final weight of the sub-sample as a percentage of the fresh sample weight.

### 2.10.2.2 Organic Matter

The organic matter of the feed sample was measured as in section 2.9.1.3.

## 2.10.3 Determination of Gross Energy of feed samples

The gross energy of feed was determined using an adiabatic bomb calorimeter (Parr Instrument Company-Moline, USA). A crucible containing 1g of feed sample, was placed into the crucible holder of the bomb cap. Nickel-chrome ignition wire, approximately 10cm in length, was connected at each end to electrodes in the bomb cap and the wire placed just above the sample. The bomb cap was fitted onto the bomb and the apparatus charged with 30 atmospheres of air.

The calorimeter bucket was filled with 2 litres of water, at a temperature between 24.5°C and 25°C, and the bomb placed into the bucket of water. After connecting the ignition leads to the electrodes, the cover was placed over the bucket and the thermometer lowered into the bucket. Hot water was allowed into the jacket of the calorimeter until the jacket temperature and the bucket temperature were equal. The controller on the calorimeter was switched to maintain temperature equilibrium between the bucket and the jacket for 4 min, before the bucket thermometer was vibrated and an initial temperature read to three decimal places. The bomb was then ignited and after exactly 9 min the thermometer was again vibrated and a final bucket temperature reading taken and recorded.

A bomb constant was determined each day to monitor the consistency of the bomb this was carried out by combusting benzoic acid. The temperature difference obtained with benzoic acid was used to obtain a bomb constant as shown in Equation 2.3. Gross energy was calculated using Equation 2.4, where  $c$  is the bomb constant,  $\Delta$

T is the change in water temperature ( $^{\circ}\text{C}$ ) resulting from the ignition of the sample and W is the weight of the sample (g).

$$\text{Bomb constant, } c = \frac{26.435 \times \text{weight of benzoic acid (g)}}{\text{temperature difference } (^{\circ}\text{C})}$$

Equation 2.3

$$\text{Gross Energy (MJ/kg)} = \frac{c \times \Delta T}{W}$$

Equation 2.4

#### 2.10.4 Determination of the nitrogen content of feed samples

The nitrogen content of the feed was analysed by Kjeldahl analysis (section 2.9.3)

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## CHAPTER 3

# THE EFFECTS OF ALTERING SYNCHRONY OF RELEASE OF NITROGEN AND ENERGY COMPONENTS FROM THE DIET INTO THE RUMEN ON PERIPHERAL PLASMA METABOLITES AND GROWTH AND CARCASS COMPOSITION TRAITS IN GROWING LAMBS

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### 3.1 Introduction

As described in chapter 1, the rate of degradation of dietary carbohydrates and nitrogen components in the rumen is dependent on a number of factors. Consequently, the concentration and pattern of release of the VFAs produced and the efficiency of microbial protein synthesis can vary considerably. Feeding diets where the release of nitrogen and energy from the dietary components into the rumen for utilisation by the microbes is synchronised, is believed to enhance the efficiency of microbial protein synthesis (Huber & Herrera-Saldana, 1994; Sniffen *et al*, 1983; Sinclair *et al*, 1993). Sinclair *et al*. (1993) designed diets which were synchronous or asynchronous, with respect to the hourly release of nitrogen and OM into the rumen throughout the day. Sheep fed these diets twice daily, showed an increase in microbial protein flow to the duodenum and an improvement in the efficiency of microbial protein synthesis when fed the synchronous diet.

Witt *et al*. (1997) subsequently showed that by feeding similar diets, formulated to be synchronous or asynchronous with respect to the rates of nitrogen and energy release into the rumen, the growth rate of lambs was enhanced by 23% in animals fed the synchronous diet. This was presumably due to an improvement in the efficiency of capture of nitrogen in the rumen by microbes which is likely to have increased protein supply to the duodenum of the host ruminant. The effect of feeding such diets on the supply of the metabolites to the peripheral tissues and detailed evaluation of carcass composition were not reported.

Feeding an asynchronous diet more frequently should synchronise the release of nitrogen and energy into the rumen from the dietary components. Diurnal patterns in the plasma metabolites such as acetate, propionate and insulin have been observed following feeding and are less erratic when the animal is fed more frequently (Sutton *et al.*, 1988; Bassett, 1975).

The work reported in this chapter aimed to investigate the effect of improving rumen synchrony on the growth and carcass composition of growing lambs and the temporal nutrient supply in the host animal's peripheral circulation. Three questions were addressed :

1. Does formulating a diet to be more synchronous with respect to the release of energy and nitrogen in the rumen increase liveweight gain and alter the carcass composition, when fed once daily to growing lambs, compared to an asynchronous diet?
2. If an asynchronous diet is fed hourly rather than once daily, which should improve the synchrony of release of N and energy in the rumen, does the liveweight gain increase or the carcass composition differ?
3. Does rumen synchrony or asynchrony affect the temporal supply of nutrients (energy-yielding acetate or amino acids) to the tissues of the host and is this influenced by pattern of feeding?

### **3.2 Experimental design**

Diets used in this experiment were designed using the SIRE program (Sinclair *et al.*, 1993) to be asynchronous (A) or synchronous (S), with respect to the rate of release of OM and N components into the rumen, when fed once daily. The objective of this experiment was to investigate the effects of feeding growing lambs diet A, either hourly or once daily, or diet S once daily, on the temporal supply of plasma amino acids and the predominant energy yielding nutrient (plasma acetate) to the tissues and whether possible changes in the pattern of nutrient supply to the tissues affected growth rates or carcass composition.

Animals were fed diets A or S once daily at 09.30h or were fed the same amount of diet A in 24 equal hourly portions. It was predicted that feeding diet A hourly rather than once daily would result in a more frequent release of the N and

OM components of the diet into the rumen and therefore bring the ratio of release nearer to the optimum ratio of N:OM, resulting in a more synchronous dietary regime. This was considered to be a novel way of synchronising nutrient release in the rumen from an asynchronous diet.

Dietary ingredients and the SIRE predictions of nutrient content and rate of nutrient release are shown in Table 3.1. The asynchronous and synchronous diets have similar metabolisable energy (ME) and metabolisable protein (MP) levels in the diet. However the synchrony index (SI) of the two diets were different with the asynchronous diet having an SI of 0.83 and the synchronous diet an SI of 0.95 (see section 1.4.6, equation 1.2 for calculation of SI).

Table 3.1 Composition of the synchronous and asynchronous diets and the SIRE predictions of nutrient content and rate of nutrient release into the rumen.

INGREDIENT	COMPOSITION (g/kg)	
	ASYNCHRONOUS DIET	SYNCHRONOUS DIET
WHEAT	150	150
BARLEY	150	150
SUGAR BEET PULP	80	80
NUTRITIONALLY IMPROVED STRAW	400	400
SOYPASS	50	-
FISHMEAL	40	-
SOYA	-	70
RAPE	-	15
UREA		5
MOLASSES	100	100
MINERALS	30	30
<b>SIRE PREDICTIONS :</b>		
	ASYNCHRONOUS DIET	SYNCHRONOUS DIET
Synchrony Index	0.83	0.95
Total N release into rumen (g/ kg fresh)	9.66	12.96
* Total OM release into rumen (g/ kg fresh)	482.3	494.6
ME (MJ/ kg fresh)	8.99	8.89
MP (g/ MJ ME)	7.47	7.27
DUP (g/ MJ ME)	2.93	1.56

ME = metabolisable energy; MP = metabolisable protein; DUP = digestible undegraded protein

### **3.3 Methodology**

#### **3.3.1 Animals, diets and treatments**

Thirty six Charolais-cross wether lambs, weighing  $24.0 \pm 0.5$  kg were obtained from 2 farm sources. On arrival animals from both sources were wormed (Parafend Oxfendazole 2.265%, Norbrook Laboratories, Worcs.) and evenly allocated between two buildings in individual pens. Buildings were continually illuminated and animals had free access to water and mineral licks at all times. Prior to weaning onto the experimental diets, animals were fed dried grass and a standard lamb creep feed.

Twelve animals were randomly allocated to each of the three feeding groups, 2 animals from each feed group were subsequently selected for the initial slaughter group (ISG).

The feeding groups were as follows :

- AD - “Asynchronous” diet fed once daily at 09:30h and offered for one hour only
- AH - “Asynchronous” diet fed in 24 hourly portions
- SD - “Synchronous” diet fed once daily at 09:30h and offered for one hour only.

Animals were allowed 10 days to be weaned onto the experimental diets, during which time all were trained to eat the meal within one hour of feeding, by gradually reducing the time during which feed was offered. Animals allocated to the hourly feeding group, AH, were introduced to an hourly feeding regime with automatic feeders, 3 days prior to the beginning of the experiment.

The metabolisable energy (ME) requirements for maintenance and a liveweight gain of 150g/day for housed castrate lambs was calculated in accordance with requirements reported in Agricultural and Food Research Council (AFRC; 1993) (Equation 3.1)

$$\text{ME (MJ/d)} = 0.296 (\text{LW}) + 4.09$$

where:

ME = Metabolisable energy requirement per day (MJ)

LW = Liveweight of the animal (kg)

(AFRC, 1993)(Equation 3.1)

The ME content (MJ / kg) of each experimental diet was estimated using the SIRE program (Sinclair *et al.*, 1993). The “asynchronous” diet was estimated to provide 9.0 MJ ME/ kg fresh weight and the “synchronous” diet 8.9 MJ ME/ kg fresh weight. Using these figures the total daily rations of either the “asynchronous” or the “synchronous” diets were calculated to meet the ME requirements of the individual animals on a liveweight basis. The total daily ME requirements for each individual were calculated each time the animal was weighed and the feed requirements adjusted accordingly.

### 3.3.2 Experimental procedures

Animals fed once daily, were offered the total daily ration of experimental diet at 09:30h every day, for one hour. Animals fed hourly were fed using automatic feeders, which dispensed 1/24th of the total daily ration every hour for 24 hours.

Refusals were removed from the hourly fed animals at 09:30h and from the once daily fed animals at 10:30h each day and weighed. Animals were weighed (section 2.6) three times per week, every Monday, Wednesday and Friday between 11:30h and 12:30h throughout the experiment, to monitor animal growth and to calculate the total daily ration to offer each individual.

After 12 weeks of feeding the diets, jugular cannulae were fitted in all animals 48 hours prior to being blood sampled (section 2.1). Hourly blood samples were taken from all the animals over 24 h, from 05:30h to 04:30h. This ensured 4 samples had been taken prior to the once daily feed being offered.

All animals were fed according to the dietary regime for a 16 week period and then humanely slaughtered using conventional slaughterhouse techniques.

### 3.3.3 Slaughter Procedures

On the first day of the experiment two animals randomly selected from each feed group were humanely slaughtered (stunned and exsanguinated). Animals were weighed the day before (at 09:00h) and on the day of slaughter (at 09:00h) and the final liveweight was defined as the mean of these two weights. All animals were fed according to their allocated dietary regime the day prior to slaughter, but received no feed from 08:30h on the day of slaughter. Details of the slaughter and carcass dissection are described in section 2.7. The *Longissimus dorsi* (*L.dorsi*),

*Semitendinosus* and *Vastus lateralis* (*V.Lateralis*) muscles were dissected from the right side of the carcass and weighed.

After 16 weeks, the remaining experimental animals were slaughtered over a period of 10 days, with equal numbers from each dietary group being slaughtered on each slaughter day. The final liveweight of the animals was defined as the mean weight of the animals on the day before and the day of slaughter. Full details of the slaughter and carcass dissection are described in section 2.7. Briefly, the mesenteric fat, omental fat and KKCF depots were all dissected and weighed. *L.dorsi*, *V.lateralis* and *Semitendinosus* muscles were also dissected from the right side of the carcass. Liver, spleen and heart weights and the gut fill and size of digestive tract organs were measured to investigate any dietary effects that may have occurred. The carcasses were hung overnight and the following day the subcutaneous backfat thickness were determined on the left side of the carcass, against the 12th rib bone. The right side of the dressed carcass (excluding the KKCF but including the 3 dissected muscles) were stored at -20°C until minced (see section 2.8.2) and analysed for chemical composition. The whole *L.dorsi* muscle was minced (see section 2.8.1) and a small subsample taken (approximately 25g). The remaining *L.dorsi* was included in the carcass when mincing.

#### 3.3.4 Chemical Analysis

Chemical composition (water, fat, protein and ash content) of carcasses and the *L.dorsi* muscles was determined, as described in section 2.9.

Samples (approximately 10 g) of the feed were taken from each 25 kg bag of feed and pooled every 2 weeks. Feed analysis was carried out on each pooled sample for Gross Energy (GE), Nitrogen, DM, OM and ash content (see section 2.10).

#### 3.3.5 Calculations to estimate the initial muscle weights and muscle growth rates

Regressions equations of liveweight (LW) against dissected muscle weights were obtained for each muscle from the animals in the initial slaughter group (see equations 3.5, 3.6, 3.7 below). These regressions were used to estimate initial muscle weights of the experimental animals at the start of the experiment. Muscle growth rates (g/d) were estimated by calculating the difference between the final and



estimated initial muscle weights for individual animals throughout the experimental period.

Equation 3.5 Regression equation of LW against *L.Dorsi* weight for the initial slaughter group

$$Y = (9.5698 \times LW) + 60.891$$

where Y = *L.Dorsi* weight (g)

Equation.3.6 Regression equation of LW against *V.Lateralis* weight for the initial slaughter group

$$Y = (2.2278 \times LW) + 34.286$$

where Y = *V.Lateralis* weight (g)

Equation 3.7 Regression equation of LW against *Semitendinosus* weight for the initial slaughter group

$$Y = (0.4545 \times LW) + 49.739$$

where Y = *Semitendinosus* weight (g)

### **3.4 Statistical Analysis**

#### **3.4.1 Animal performance data**

The effect of feeding different diets (S or A) or the same diet in different feeding patterns (hourly (H) or once daily (D)) on growth and carcass composition was tested for difference by analysis of variance. The experiment was analysed as a completely randomised design with dietary treatment (n=3) as the main effect. Analysis of variance was calculated using the Genstat 5 statistical package (Release 3.22; Lawes Agricultural Trust, Rothamsted). Animals used in the experiment were obtained from two different farms and during the experiment were housed in two different buildings. These two factors were accounted for in the analysis by distributing the animals evenly between dietary treatment groups at the start of the trial, and testing for dietary difference by analysis of variance with source and building as factors. Building and source interactions with dietary treatment where detected are indicated in the legends to the appropriate tables.

Initial liveweight of the animals was used as a covariate in the analysis of variance for examining animal performance when the residual mean square was reduced by including the covariance. The design of the experiment gave 17 degrees of freedom for error, when covariance was included in the analysis. When 18 degrees

of freedom for error were used in the analysis, this is indicated at the foot of the appropriate table.

When analysis of variance showed a significant difference between dietary treatment, t-tests were carried out between pairs of dietary treatments using the pooled standard error of difference obtained from the analysis of variance. Differences between groups were assumed not to be significantly different at  $P > 0.1$ .

Liveweight gain was determined by linear regression of the individual liveweights of the animals over the experimental period. The slopes of the individual animal growth rates in each of the 3 dietary groups were tested for difference by analysis of variance. Any difference in initial liveweight between dietary groups was tested using ANOVA with source and building as factors.

#### 3.4.2 Blood metabolite data

Plasma concentrations of acetate and individual amino acids were measured in the hourly samples taken from 2 animals in each of the dietary treatment groups; one from each building. Plasma concentrations of insulin were measured in all 10 animals in each dietary treatment group. Although not all the blood samples taken were analysed for plasma concentrations of acetate and amino acid all animals were blood sampled to ensure all were treated equally throughout the monitoring of the growth rates. The effect of feeding different diets (S or A) or the same diet in different feeding patterns (H or D) on the daily plasma concentrations of amino acids, acetate and insulin was examined by analysis of variance (Genstat 5, Release 3.22; Lawes Agricultural Trust, Rothamsted). Using mean daily plasma amino acid and acetate concentrations analysed in samples obtained from the same 2 animals from each dietary treatment (AD, AH and SD), there were 3 degrees of freedom for error. Differences were assumed not to be significantly different at  $P > 0.1$ .

Analysis of the correlation between the hourly plasma acetate concentration and the concentration of each of the individual or the total amino acids measured was carried out by directly correlating the acetate and amino acid concentration at each of the 24 hourly time points within each animal. The statistical design gave 23 degrees of freedom for error, for each animal analysed. The statistical significance of the correlation coefficient was calculated using statistical tables (Lindley and Scott,

1984). Pairs of variables were assumed to be not statistically significantly correlated at  $P>0.05$ .

## **3.5 Results**

### **3.5.1 Feed Analysis**

The results of the chemical feed analysis are shown in Table 3.2. The two diets were approximately isoenergetic and isonitrogenous. Likewise, the dry-matter (DM) and organic matter (OM) content of the diets were very similar between diets.

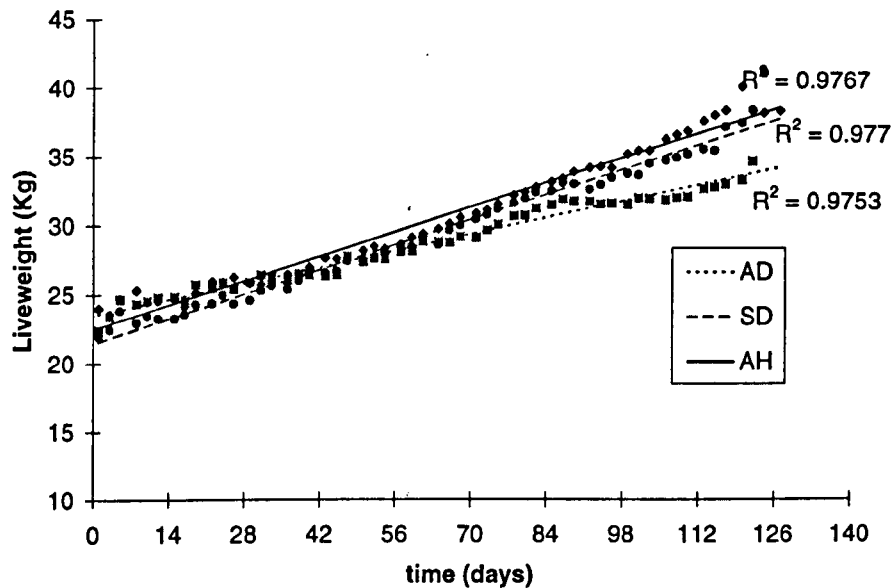
Table 3.2 Dietary composition of the experimental diets.

	DIETS	
	“ASYNCHRONOUS”	“SYNCHRONOUS”
FEED ANALYSIS :		
DM (g/kg fresh)	926.7	939.7
OM (g/kg DM)	897.7	901.3
N ( g/kg DM)	18.46	20.62
GE (MJ/kg DM)	17.37	17.32

### **3.5.2 Animal Performance**

Figure 3.1 shows that the mean growth rates, in the three treatment groups, were linear across the experimental period with  $r^2$  values of 0.98 for each treatment group. Animals fed the asynchronous diet once daily (AD) grew the slowest and those that were fed the asynchronous diet hourly (AH) grew the fastest, with animals being fed the synchronous diet once daily (SD) being in-between. The average daily liveweight gain (DLWG) of AD animals was 82.3g/d, whilst those fed AH was 136.0g/d and those fed SD was 125.6g/d (Table 3.3). No significant differences ( $P>0.1$ ) were seen in the DLWG between animals fed AH and SD (Table 3.3).

Figure 3.1 Mean liveweights of animals in the three dietary treatment groups, Asynchronous fed once daily (AD), synchronous fed once daily (SD) and the asynchronous diet fed hourly (AH), throughout the experimental period



There was no difference ( $P>0.1$ ) in the initial liveweight between dietary treatment groups at the start of the experiment, as can be seen in Table 3.3. The final liveweight, carcass weight, killing out proportion, feed intake (MJ ME/kgLW/d) and feed conversion efficiency (FCE; food consumed/kg liveweight gain) data of the animals in each of the three treatment groups can also be seen in Table 3.3. Significant differences were observed in the DLWG, carcass weight and final liveweight of the animals fed different dietary treatments ( $P<0.01$ ). As expected from the DLWG data, animals fed SD and AH had significantly greater final liveweights and carcass weights compared to those animals fed AD ( $P<0.01$ ). The final liveweights and carcass weights of animals fed AH were significantly higher than those fed SD ( $P<0.1$ ).

Table 3.3 The effect of feeding the three dietary regimes; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the DLWG, carcass weight, final liveweight, killing out proportion and feed conversion efficiency (FCE). Also shown are the liveweights at the start of the trial in each dietary treatment group

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)	Probability of significant differences between dietary groups using t-test	AD×AH	AH×SD	AD×SD
Initial Liveweight (kg)	24.83	24.75	23.25	1.459	0.488				
Daily live-weight gain (g)	82.3	136.0	125.6	13.2 <sup>4</sup>	<0.01	***			**
Carcass weight (kg)	15.15	18.82	17.39	0.869 <sup>1</sup>	0.002	***	+	*	*
Final Liveweight (kg)	30.98	38.69	35.59	1.382	<0.001	***	*	*	**
Killing-out (g carcass/kg LW)	486.9	487.9	484.4	19.25 <sup>4</sup>	0.982				
Food Intake (MJ ME/kg LW/d)	0.29	0.37	0.32	0.010 <sup>4,2</sup>	<0.001	***	***	***	**
FCE (g food consumed / kg live-weight gain)	11.13	8.00	7.90	0.660 <sup>3</sup>	<0.001	***			***

<sup>1</sup> diet × building interaction P<0.1

<sup>2</sup> diet × source interaction P<0.1

<sup>3</sup> diet × building interaction P<0.001

<sup>4</sup>18 degrees of freedom for error

\*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = standard error of difference

Killing out was calculated as the proportion of the LW that was carcass weight. No significant differences ( $P>0.1$ ) were seen in the killing out proportion of the final carcass between dietary treatments.

Feed conversion efficiency was calculated as the total amount of feed consumed throughout the experimental period divided by the total liveweight gain over the same period. The conversion of the total feed consumed to liveweight gain in groups AH and SD was considerably more efficient than that of the animals fed in group AD ( $P<0.001$ ).

There was a significant difference ( $P<0.01$ ) in the feed intake (MJ ME/kg LW/d) between the 3 dietary groups. Animals offered AH consumed the greatest amount, those offered SD ate significantly less and those offered AD ate the least (0.37, 0.32 and 0.29 MJ/kgLW/d respectively;  $P<0.01$ ).

The effects of treatment on the weights of the three dissected internal fat depots and the subcutaneous backfat thickness are shown in Table 3.4. Due to the differences in carcass weight at slaughter between animals fed the 3 dietary regimes, fat depots were expressed as both absolute weights (g) and as a proportion of the carcass weight (g/kg carcass weight). Feeding the asynchronous diet hourly (AH) rather than once daily (AD) resulted in greater deposition of mesenteric fat, KKCF and omental fat ( $P<0.01$ ). Animals fed AD tended to have smaller fat depots compared to those fed SD, both in terms of absolute weight (g) and, with the exception of mesenteric fat, when expressed as a proportion of carcass weight (g/kg), although these differences were not statistically significant ( $P>0.1$ ). The KKCF and omental fat depots were heavier in the AH fed animals compared to the SD fed animals ( $P<0.05$ ). The weight of the omental fat depots expressed as a proportion of carcass weight was significantly increased when animals were fed AH compared to those fed AD or SD ( $P<0.1$ ). The mesenteric fat and KKCF depots expressed as a proportion of the carcass weight showed similar trends but were not significantly different ( $P>0.1$ ). Dietary treatment did not significantly affect the subcutaneous fat thickness ( $P>0.1$ ), although it tended to be thicker in animals fed AH (3.92mm) compared to animals fed AD (2.90mm) or SD (3.23mm).

Table 3.4 The effect of feeding the three dietary regimes; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of three fat depots and the subcutaneous backfat thickness.

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)	Probability of significant differences between dietary groups using t-test <sup>2</sup>	AD × AH	AH × SD
Mesenteric fat depot (g)	398	513	452	43.9	0.049	**	**	
(g / kg carcass wt)	25.93	27.47	25.59	1.909 <sup>1</sup>	0.599			
Omental fat depot (g)	297	476	360	49.4	0.006	**	**	*
(g / kg carcass wt)	18.08	24.75	20.79	2.361	0.032	**	**	+
KKCF depot (g)	281	414	330	46	0.027	**	**	*
(g / kg carcass wt)	17.94	22.05	18.18	2.428 <sup>1</sup>	0.193			
Subcutaneous back fat thickness (mm)	2.90	3.92	3.23	0.938	0.551			

<sup>1</sup> 18 degrees of freedom for error

<sup>2</sup> No significant differences between SD and AD

\*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = standard error of difference

Table 3.5 The effect of feeding the three dietary regimes, asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SM) to growing lambs, on the weight of three muscles.

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)
<i>L.Dorsi</i> (g)	481	518	488	52.2	0.763
(g / kg carcass wt)	33.1	26.9	29.5	4.16 <sup>1</sup>	0.341
<i>V.Lateralis</i> (g)	130.3	142.2	139.2	12.62	0.626
(g / kg carcass wt)	9.11	7.40	8.45	1.227 <sup>1</sup>	0.394
<i>Semitendinosus</i> (g)	93.9	104.9	104.4	8.79	0.39
(g / kg carcass wt)	6.55	5.50	6.30	0.921 <sup>1</sup>	0.505

<sup>1</sup> 18 degrees of freedom for error

SED = standard error of difference

There were no significant dietary effects on the weights of the three muscle depots dissected at slaughter, as shown in Table 3.5 either as absolute weight or when expressed relative to the carcass weight of the animal. Animals fed AH and SD tended to have larger muscles than group AD, although the differences were not statistically significant ( $P > 0.1$ ). However, the *L.dorsi*, *V.lateralis* and *Semitendinosus* muscles comprised a greater proportion of the carcass weight in group AD than group AH or SD, although, this was not statistically significant ( $P > 0.1$ ).



Table 3.6 The effect of feeding the three dietary regimes, asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of chemical components of the carcass.

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)	Probability of significant differences between dietary groups using t-test	AD × AH	SD × AH	SD × AD
Weight of carcass chemical components :									
Crude protein (kg) <sup>3</sup>	2.78	3.34	3.09	0.147 <sup>1</sup>	0.004	***	***	+	*
Fat (kg)	3.05	4.26	3.52	0.353	0.009	**	**	*	*
Ash (kg)	0.74	0.92	0.79	0.072	0.066	*	*	*	*
Water (kg)	8.67	10.43	10.10	0.493	0.005	**	**	**	**
Chemical composition of carcass :									
% Crude protein	18.49	17.68	18.05	0.684 <sup>2</sup>	0.506				
% Fat	18.97	22.33	20.33	1.503	0.097	*	*		
% Ash	4.97	4.86	4.53	0.329 <sup>4</sup>	0.413				
% Water	57.74	55.41	58.58	1.579 <sup>4</sup>	0.144				
Fat : Protein	1.044	1.264	1.141	0.0932	0.079	*	*		

<sup>1</sup> Diet × Building interaction effects (P<0.05)

<sup>2</sup> Diet × Building interaction effects (P<0.1)

<sup>3</sup> Crude protein = % Nitrogen x 6.25

<sup>4</sup> 18 degrees of freedom for error

\*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = standard error of difference

The effects of dietary treatment on the weight of chemical components of the carcass and the *L.dorsi* muscle can be seen in Tables 3.6 and 3.7, respectively. The chemical composition expressed as a percentage of the carcass and the *L.dorsi* are also shown in Tables 3.6 and 3.7 respectively. Percentage protein in the carcass was calculated as the percentage of nitrogen measured in the minced carcass multiplied by 6.25. Carcass protein was significantly greater in animals fed dietary treatment AH compared to animals fed dietary treatment AD and SD ( $P < 0.1$ ). However, when expressed as a percentage of the carcass, the crude protein content showed no differences between the dietary treatments. The weight of fat in the carcass was not significantly different between animals fed the diets once daily (AD or SD), but was significantly greater in animals fed the AH treatment. When fat was expressed as a percentage of the carcass, the carcasses of animals fed AH had a significantly higher proportion of fat than those fed AD ( $P < 0.05$ ). The proportion of the carcass that was fat was slightly higher in animals fed AH than that of those fed SD although this was not statistically significant ( $P > 0.1$ ). As a result the carcass fat : protein ratio (calculated from the percentage of fat and protein in the carcass) was significantly higher in the animals fed AH compared to AD ( $P < 0.05$ ). The fat : protein ratio of the carcass also tended to be higher in AH compared to SD although this was not statistically significant ( $P > 0.1$ ).

There tended to be slightly more crude protein and fat laid down in the *L.dorsi* of animals fed AH compared to those fed SD or AD, however these differences were not statistically significant ( $P > 0.1$ ). When expressed as a percentage of the *L.dorsi* weight, there was significantly more crude protein in animals fed AH compared to animals fed AD or SD. There tended to be a higher percentage of fat in the *L.dorsi* muscle of animals fed AH compared to the other dietary treatments, particularly animals fed AD, but these differences between groups were not statistically different ( $P > 0.1$ ). The fat : protein ratios in the *L.dorsi* muscle were similar between groups.

Table 3.7 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the weight of chemical components of the *L.Dorsi*.

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)	Probability of significant differences between dietary groups using t-test <sup>2</sup>
				AD x AH SD x AH SD x AD		
<b>Weight of <i>L.dorsi</i> chemical components :</b>						
Crude protein (kg) <sup>1</sup>	97.4	110.4	102.9	11.63	0.534	
Fat (kg)	15.9	19.9	19.1	3.36	0.477	
Ash (kg)	4.97	5.43	6.36	0.837	0.332	
Water (kg)	356	376	373	37.6	0.839	
<b>Chemical composition of <i>L.dorsi</i> :</b>						
% Crude protein <sup>1</sup>	20.55	21.44	20.49	0.311	0.011	** **
% Fat	3.36	4.06	3.59	0.613 <sup>2</sup>	0.517	
% Ash	1.07	1.06	1.25	0.135	0.321	
% Water	75.12	73.63	74.48	0.555	0.042	** +
Fat : Protein	0.16	0.19	0.18	0.030	0.660	

<sup>1</sup> % Crude protein = % Nitrogen x 6.25      <sup>2</sup> 18 degrees of freedom for error      \*\* = P<0.01, + = P<0.1

SED = standard error of difference

The growth rate of the *L.dorsi*, *V. lateralis* and the *Semitendinosus* muscles was estimated by subtracting the estimated initial muscle weight from the measured final muscle weight and dividing by the days the animals were on trial. Table 3.8 shows the effects of the dietary treatments on the estimated growth of the *L.dorsi*, *V.lateralis* and the *Semitendinosus* expressed as grams per day (g/d). As expected, with no differences between dietary treatments in the absolute final weights (Table 3.5), there were also no statistically significant dietary differences in the growth of the three muscles between dietary regimes when expressed as g/d, although those animals fed AH tended to have a higher growth in the *L.dorsi* and the *Semitendinosus*.

Table 3.9 shows the effect that the three dietary treatments had on the internal organs that were dissected at slaughter and the weights of the components of the digestive tract. No differences were observed in the spleen and heart weights between diets. However, the liver weight was significantly higher in animals fed AH and SD compared to the group fed AD ( $P < 0.01$ ).

There were no differences in the empty reticulo-rumen, omasum and small intestine weights, between animals in the 3 dietary groups. The abomasum, however, was significantly different between all three dietary groups ( $P < 0.01$ ) being largest in animals fed AH and smallest in those fed AD ( $P < 0.5$ ).

Table 3.8 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD) to growing lambs, on the estimated growth of the *L.Dorsi*, *V.Lateralis*, and *Semitendinosus*.

	DIETARY TREATMENT			Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)
	AD	AH	SD		
Estimated growth of the <i>L.Dorsi</i> (g / day)	1.68	2.04	1.89	0.457	0.73
Estimated growth of the <i>V.Lateralis</i> (g / day)	0.046	0.050	0.058	0.007	0.33
Estimated growth of the <i>Semitendinosus</i> (g / day)	0.015	0.019	0.017	0.004	0.71

<sup>1</sup> 18 degrees of freedom for error

\* = P<0.05

SED = standard error of difference

Table 3.9 The effect of feeding the three dietary treatments; asynchronous diet fed once daily (AD), asynchronous diet fed hourly (AH), and synchronous diet fed once daily (SD), to growing lambs, on the size of the liver, spleen, heart and the different parts of the ruminant digestive tract. In addition the dietary effects on the total gut-fill are shown.

	AD	AH	SD	Pooled SED (17 degrees of freedom for error)	Dietary treatment effect (P Value)	Probability of significant differences between dietary groups using t-test		
						AD × AH	AH × SD	SD × AD
Liver weight (g)	422	519	545	36.6	0.009	**		**
Spleen weight (g)	54.2	64.4	53.1	7.57 <sup>1</sup>	0.281			
Heart weight (g)	143.6	156.7	163.7	9.23				
empty reticulo- rumen weight (g)	485	562	535	42.8 <sup>1</sup>	0.216			
Empty abomasum weight(g)	113.3	155.6	135.9	10.47 <sup>1</sup>	0.003	***	*	*
empty omasum weight (g)	59.6	67.1	59.4	4.42 <sup>1</sup>	0.172			
empty intestine weight (g)	714	778	752	50.1 <sup>1</sup>	0.447			
total gut fill (g)	4419	5124	4661	412.9 <sup>1</sup>	0.248			

<sup>1</sup> 18 degrees of freedom for error      \*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05

SED = standard error of difference

### 3.5.3 Blood Metabolite Data

Profiles of the hourly plasma concentrations of acetate and leucine or the total amino acids were plotted to compare the patterns of nutrients in the plasma throughout the day, when the animals were fed the three different dietary treatments (n=2 per group). Hourly insulin concentration was also plotted as a 24 h profile, for each dietary treatment group, using insulin results for all animals used in the experiment (n=30). Arrows on the profiles denote feeding

Figure 3.2 (a) shows the effects of feeding the asynchronous diet once daily (AD) on hourly plasma concentrations of acetate and total amino acids in the peripheral plasma supplying the tissues. Plasma acetate concentration increased following feeding and this rise continued for 6 h after the 9.30am meal was offered. After the acetate concentration peaked at just under 5mM, there was a gradual decline in concentration for 6 h, at which point the plasma acetate concentration was back to the basal level it had been before the animal was fed (approximately 1mM). This would indicate that in response to once daily feeding, plasma acetate concentration fluctuated over approximately 12 h in the day and for the remaining 12 h was at a relatively constant low level. The total amino acid concentration in the plasma of sheep fed the AD dietary treatment, seemed to fluctuate over a greater length of time, compared to the corresponding plasma acetate concentration. The error bars would suggest that there was only a small variation in concentrations of acetate and amino acids between the two animals measured. Following feeding, the concentration of the total amino acids started to decrease from approximately 2500 $\mu$ M. At the point at which the acetate concentration peaked (approximately 6 h following feeding) the total amino acid concentration appeared to reach a nadir of approximately 1800 $\mu$ M. When the plasma acetate concentration had returned to the original basal level of 1mM (approximately 12 h following feeding) the total amino acid concentration started to rise again and continued to increase until the end of the sampling period.

Figure 3.2 (a) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the asynchronous diet once daily (AD) Error bars represent the standard error of the mean.

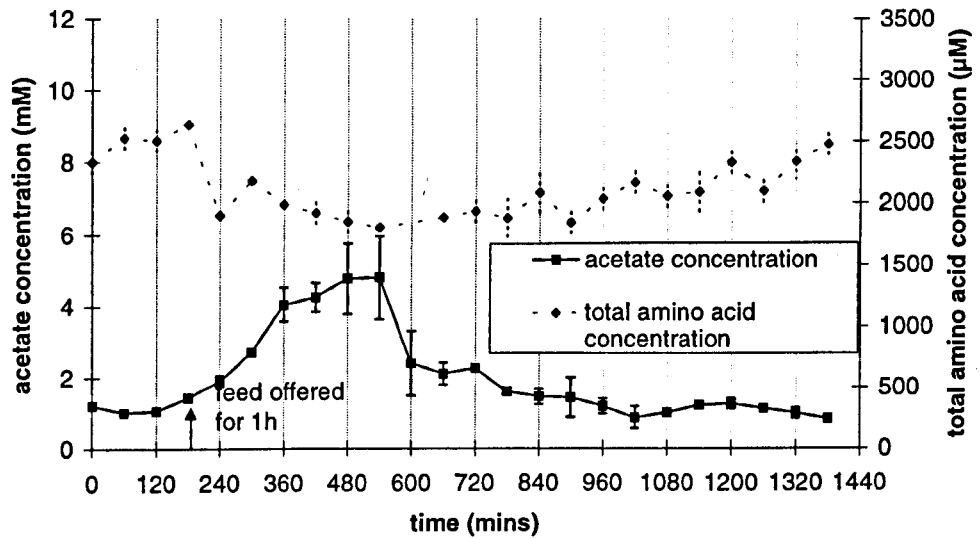
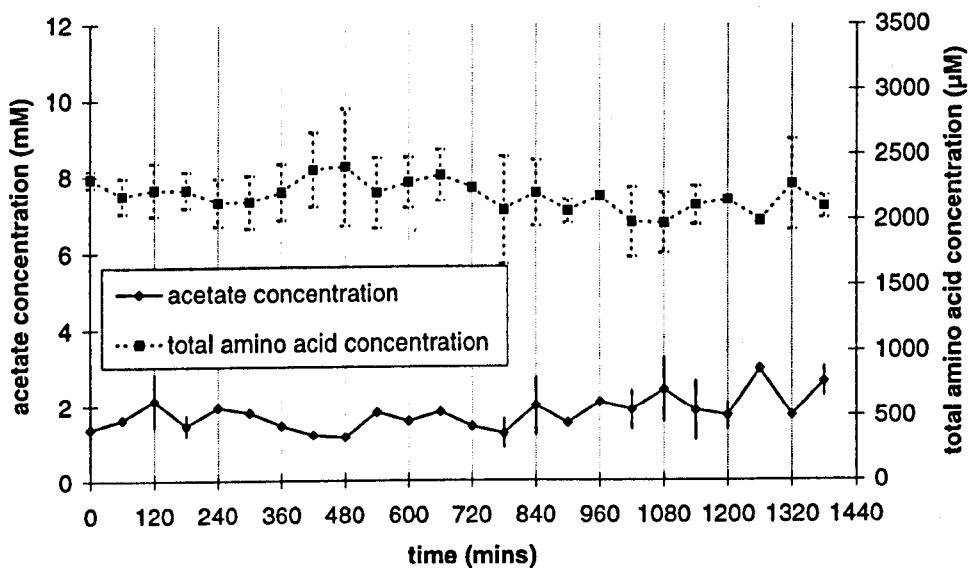


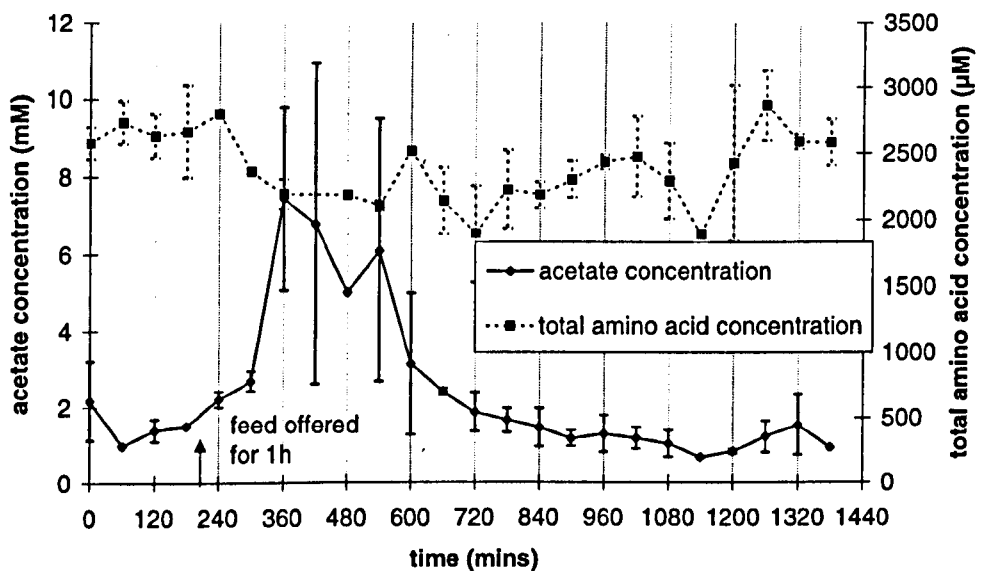
Figure 3.2 (b) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the asynchronous diet hourly (AH). Error bars represent the standard error of the mean.





When the animals were fed the asynchronous diet hourly a different pattern of acetate and total amino acid concentration was observed (Figure 3.2 (b)). The profiles of both hourly plasma acetate and total amino acid concentrations were considerably more constant than those observed in animals fed the same diet once daily. The acetate concentration remained at a level of between approximately 1 and 2 mM and the total amino acid concentration between approximately 2000 and 2400 $\mu$ M over the 24 h period. There were no clear fluctuations throughout the day in concentrations of either of the two metabolites.

Figure 3.2 (c) Hourly acetate and total amino acid concentrations in the peripheral plasma of sheep (n=2) fed the synchronous diet once daily (SD). Error bars represent the standard error of the mean.



The patterns of the hourly plasma concentrations of acetate and total amino acids in the peripheral plasma of animals fed the synchronous diet once daily (SD) can be seen in Figure 3.2(c). The change in plasma acetate concentration was similar to that observed in the animals fed the asynchronous diet once daily (AD) and ranged between approximately 1 and 7.5 mM. The acetate concentration fluctuated from what appeared to be a basal concentration of approximately 1mM for approximately

12 h after feeding, reaching a peak approximately 3h after feeding. The error bars, shown in Figure 3.2 (c) however, show that the variation between the two animals fed SD was large.

The total amino acid concentration started to decrease approximately 1h following feeding for approximately 7h, but following this, the pattern was fairly erratic. The total amino acid concentrations was at a plateau for the four hours leading up to the meal feeding time. The variation in the total amino acid concentration between the 2 animals was relatively small, as indicated by the error bars.

Leucine was selected as a representative essential amino acid, to compare the concentration profiles of an individual amino acid with plasma acetate over time. The concentration profiles of the other amino acids with acetate are not shown, however the correlations between plasma acetate and each individual amino acid are presented in Table 3.11 and show the extent to which the change in the plasma concentrations of the two nutrients correlated over time, in each dietary treatment.

Figure 3.3 (a) shows the plasma leucine concentration in the animals fed the asynchronous diet once daily (AD), was at a plateau in the 4 h prior to feeding (approximately  $100\mu\text{M}$ ) and began to decrease after the meal had been offered. One hour before the acetate concentration peaked, at 6 h following feeding, the leucine concentration rose immediately back to the original plateau concentration ( $100\mu\text{M}$ ) that it had been at prior to feeding, and stayed at that level throughout the day.

In the animals fed the asynchronous diet hourly (AH), the plasma leucine concentration followed a similar pattern to that of total amino acid concentrations with concentration remaining relatively constant throughout the day, as can be seen in Figure 3.3 (b).

In Figure 3.3 (c) it can be seen that the leucine concentration of those animals fed the synchronous diet once daily (SD) had reached a plateau at approximately  $140\mu\text{M}$  prior to the meal being offered. The plasma leucine concentration started to decline 1 h after the meal was offered and continued for approximately 9h until the plasma leucine concentration was approximately  $100\mu\text{M}$ . The plasma leucine

concentration was quite erratic after this point. The standard error bars indicate that there was a large amount of variation between the animals throughout the day.

Plasma insulin concentrations of animals fed treatment AD, increased from approximately  $7.5\mu\text{U/ml}$  to  $13.5\mu\text{U/ml}$  following feeding (Figure 3.4 (a)). The insulin concentration did not increase any further after the first hour but decreased over the following 3 h back to the level that it was prior to feeding (approximately  $7\mu\text{U/ml}$ ). The plasma insulin concentration peaked before the plasma acetate concentration which reached a peak approximately 5h later.

As with the plasma acetate concentrations, plasma insulin concentrations appeared to be relatively constant throughout the day (between approximately 8 and  $10\mu\text{U/ml}$ ; Figure 3.4 (b)) in animals fed the asynchronous diet hourly (AH), although there was a small rise in insulin concentration earlier in the day, around the point at which the once daily fed animals were offered their meal (to approximately  $12\mu\text{U/ml}$ ).

Figure 3.4 (c) shows the insulin concentration in the plasma of animals fed the synchronous diet once daily (SD) rose slightly following feeding to approximately  $13\mu\text{U/ml}$  and then decreased over 3 h back to a basal level concentration of approximately  $7\mu\text{U/ml}$ . In a similar way to animals fed the AD dietary treatment, the plasma acetate concentration in animals fed the SD dietary treatment peaked after the plasma insulin concentration (approximately 2h after).

Figure 3.3 (a) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the asynchronous diet once daily (AD). Error bars represent the standard error of the mean.

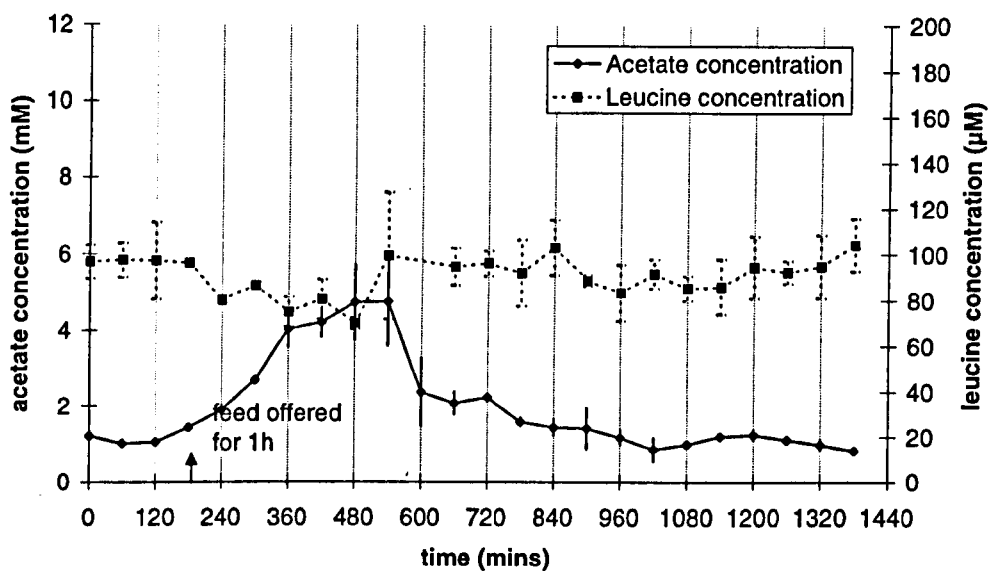


Figure 3.3 (b) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the asynchronous diet hourly (AH). Error bars represent the standard error of the mean.

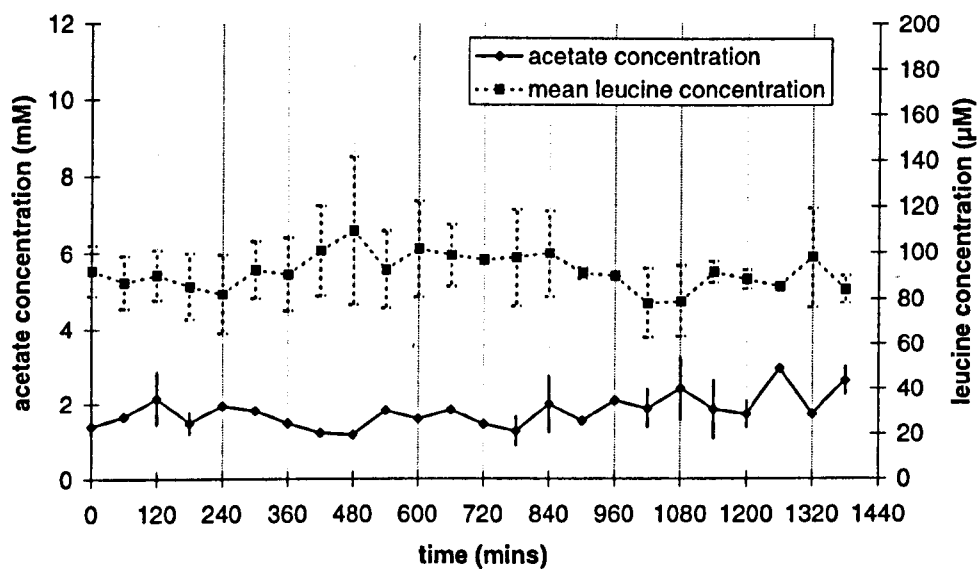


Figure 3.3 (c) The hourly acetate and leucine concentration in the peripheral plasma of sheep (n=2) fed the synchronous diet once daily (SD). Error bars represent the standard error of the mean.

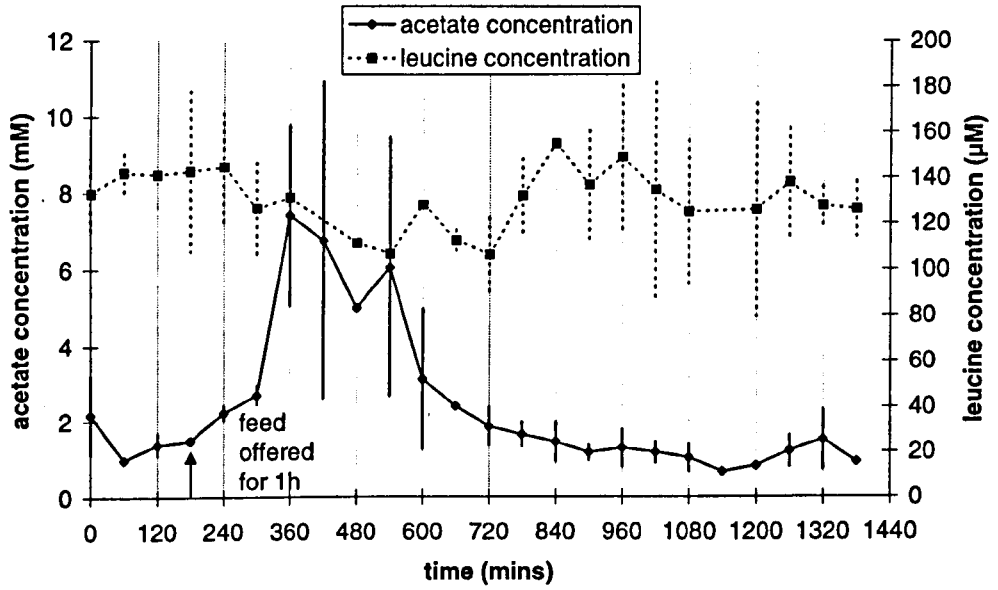


Figure 3.4 (a) The mean hourly insulin concentration in the peripheral plasma of sheep (n=10) fed the asynchronous diet once daily (AD) shown against the mean hourly acetate concentration in the plasma of sheep (n=2) fed the same diet. Error bars represent the standard error of the mean.

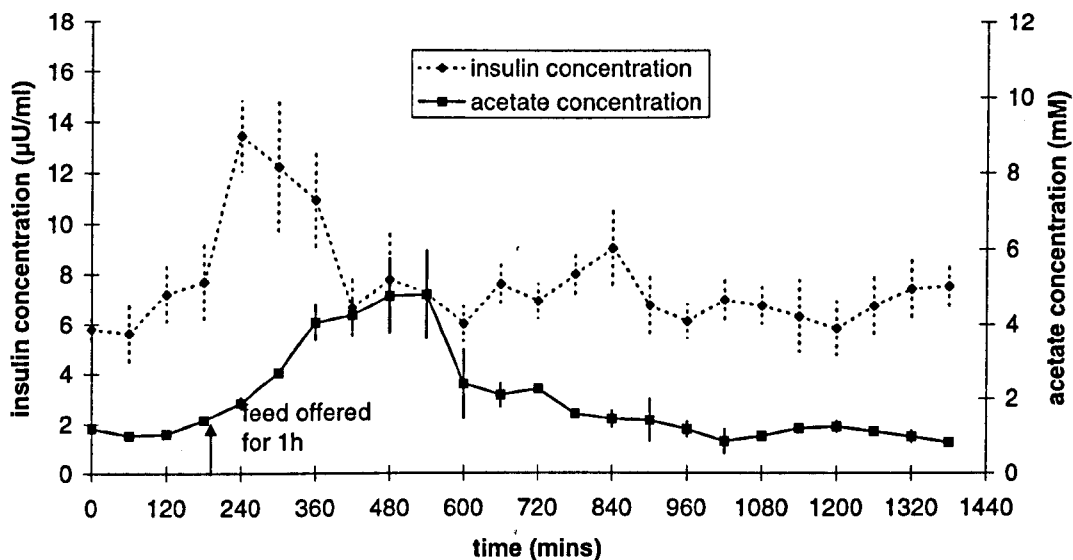


Figure 3.4 (b) The mean hourly insulin concentration in the peripheral plasma of sheep (n=10) fed the asynchronous diet hourly (AH) shown against the mean hourly acetate concentration in the plasma of sheep (n=2) fed the same diet. Error bars represent the standard error of the mean.

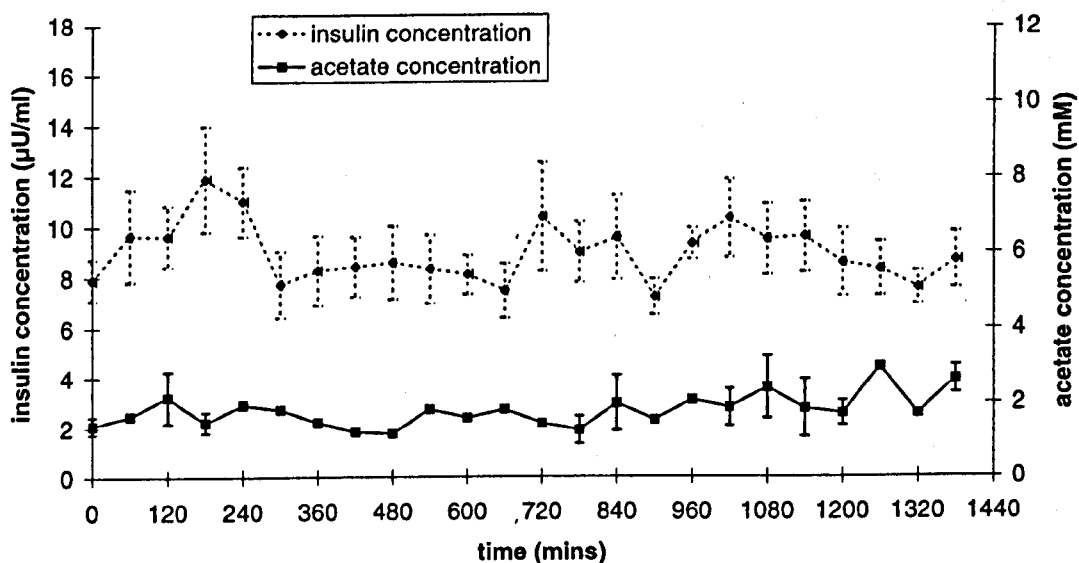


Figure 3.4 (c) The mean hourly insulin concentration measured in the peripheral plasma of sheep (n=10) fed the synchronous diet once daily (SD) shown against the mean hourly acetate concentration in the plasma of sheep (n=2) fed the same diet. Error bars represent the standard error of the mean.

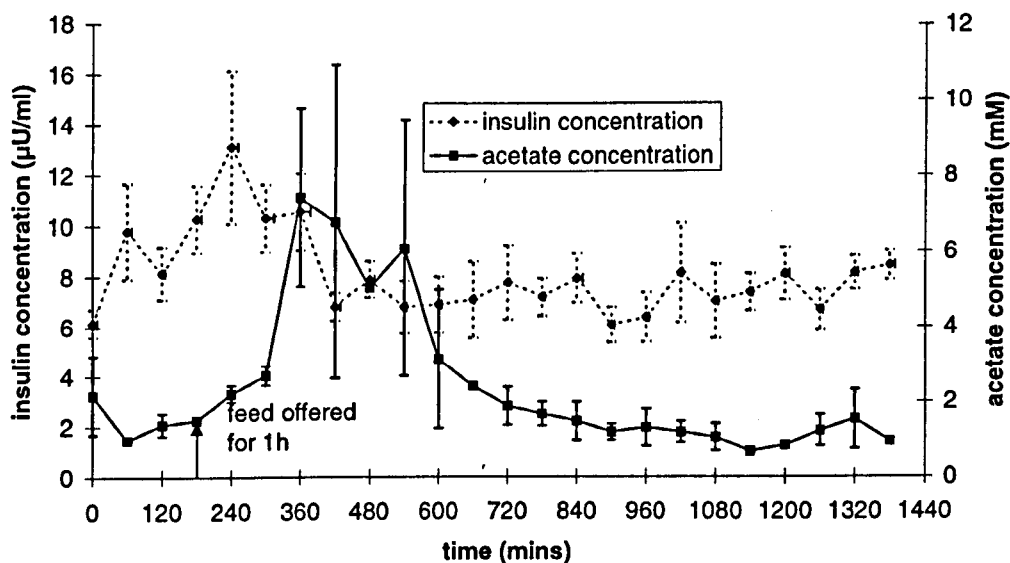


Table 3.10 shows that there were a number of significant correlations between the hourly concentrations of plasma acetate and individual amino acids measured over a 24 h period, for all the dietary treatment groups. The majority of the correlations are negative indicating an inverse relationship in the concentrations of the two nutrients, i.e. when one increases the other decreases, although there is considerable variation between animals within the same dietary treatment group.

In animals fed AD the plasma acetate concentration was significantly correlated with that of alanine, serine, glycine, phenylalanine, valine, arginine, lysine and glutamate ( $P < 0.05$ ) in the two animals measured. Some of these correlations are highly significant ( $P < 0.001$ ).

In those animals fed AH, one animal showed a negative correlation between the plasma acetate concentration and the plasma aspartate concentration ( $P < 0.05$ ), but with no other amino acid. The other animal fed this diet, showed no significant correlation between plasma aspartate concentration and plasma acetate concentration,

but had a number of significant correlations between other plasma amino acid concentrations and the plasma acetate concentration ( $P < 0.01$ ).

Table 3.10 The significant correlations observed between plasma acetate concentrations and concentrations of individual and total plasma amino acid at hourly intervals throughout the day in two sheep fed each diet.

<i>Animal number</i>	DIETARY TREATMENT					
	AD		AH		SD	
	1	2	3	4	5	6
Alanine	***(-)	**			***(-)	
Serine	***(-)	*	**(-)		**	* (-)
Leucine	***(-)		**		**(-)	
Glycine	**(-)	***(-)	**(-)		***(-)	**(-)
Phenylalanine	***(-)	***(-)	*(-)		**(-)	
Aspartate				* (-)		
Valine	***(-)	***(-)	*(-)		**(-)	
Tyrosine		**(-)	*(-)			
Arginine	*(-)	***(-)	**(-)		**(-)	
Lysine	**(-)	***(-)	*(-)		**(-)	
Methionine		**			**(-)	*
Threonine			**(-)			
Isoleucine					*	**
Glutamine					**(-)	*
Glutamate	***	***			*	***
Proline			**(-)		* (-)	
Asparagine					* (-)	
Citrulline						*
Histidine		***(-)	**(-)		**(-)	
Ornithine		*(-)			**(-)	
Total amino acids		***(-)	**(-)		***(-)	

\*\*\* =  $P < 0.001$ , \*\* =  $P < 0.01$ , \* =  $P < 0.05$

(-) = negative correlation



In the dietary group fed SD, there were significant correlations between the plasma acetate concentration and serine, glycine, methionine, isoleucine, glutamine and glutamate concentrations for both animals analysed. One of the two animals fed dietary treatment SD had 16 significant correlations (13 of which were negative) between the acetate and the individual amino acids ( $P < 0.05$ ) which was the largest number of significant correlations observed amongst all the dietary groups. The other animal in this treatment group had only seven significant correlations.

Hourly plasma glutamate concentrations were consistently positively correlated with hourly plasma acetate concentrations in all animals measured from the once daily fed dietary groups (AD and SD;  $P < 0.05$ ). Plasma isoleucine concentrations were significantly positively correlated with plasma acetate concentrations in both animals fed SD, but no significant correlations with acetate, in animals in either of the other two dietary treatment groups were observed.

Hourly plasma serine, glycine and glutamate concentrations were statistically significantly correlated with hourly plasma acetate concentrations in both animals from both once daily fed dietary treatment groups (AD and SD).

There was one animal in each dietary treatment group, where the acetate concentration was correlated with a larger number of amino acids than the other animal in the group. Of the animals with the larger number of correlations, the total amino acid concentrations measured was significantly correlated with the plasma acetate concentration ( $P < 0.01$ ).

Hourly plasma acetate concentrations were not significantly correlated with hourly plasma insulin concentrations in any of the animals measured except one in the dietary treatment AD group ( $P < 0.05$ ).

Plasma insulin concentrations were significantly correlated with the plasma concentrations of several of the individual amino acids in all three dietary treatment groups, as can be seen in Table 3.11. Unlike the correlations between acetate and amino acids, the majority of significant correlations between insulin and amino acids were positive.

Table 3.11 The significant correlations observed between plasma insulin concentrations and concentrations of individual and total plasma amino acid at hourly intervals throughout the day in 2 sheep fed each diet.

<i>Animal number</i>	DIETARY TREATMENT					
	AD		AH		SD	
	1	2	3	4	5	6
Acetate		*(-)				
Alanine						
Serine		**	*(-)	*(-)	***	
Leucine			*(-)		*	
Glycine	*(-)	*	*(-)	*	**	
Phenylalanine	*(-)	***			**	
Aspartate		***		**(-)	*	
Valine	*(-)	**			**	
Tyrosine		**	*(-)			
Arginine		**			*	
Lysine		*	*(-)		***	*
Methionine		**			**	
Threonine			*(-)		***	
Isoleucine						
Glutamine				*		
Glutamate		*(-)		*	***(-)	*(-)
Proline					*	
Asparagine						
Citrulline	*(-)		*(-)	*	***	
Histidine			*(-)			
Ornithine		***			**	
Total amino acids		*			**	

\*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05

(-) = negative correlation

In dietary treatment group AD, plasma concentrations of glycine, phenylalanine and valine were significantly correlated with plasma acetate concentrations in both animals measured ( $P < 0.05$ ), although in one animal the correlations are negative and in the other animal the correlations are predominantly positive. This suggested that there were very few similarities in the correlations observed between hourly plasma amino acids and hourly plasma insulin concentrations of animals within this dietary group.

In dietary treatment group AH, only plasma serine, glycine and citrulline concentrations were significantly correlated with plasma insulin concentrations in both animals measured ( $P < 0.05$ ). The plasma insulin concentration of one animal was positively correlated with the plasma glycine and citrulline concentrations while in the other animal the plasma insulin concentration was negatively correlated with plasma glycine and citrulline.

In the animals fed the synchronous diet once daily, the correlations that were statistically significant between amino acids and insulin, were all positive except for the correlations between glutamic acid and insulin and these were negatively correlated for both animals analysed.

There were 14 individual plasma amino acids concentrations that were significantly correlated with hourly plasma insulin concentrations in one animal fed the SD dietary treatment. In the other animal, fed the same treatment, there were only 2 significant correlations. Similarly in one animal from the dietary treatment group, AD, there were 12 individual plasma amino acids concentrations that were significantly correlated with hourly plasma insulin concentrations. The other animal in the same treatment group had only 4 individual plasma amino acids concentrations that were significantly correlated with plasma insulin concentrations.

It should be noted here that the individual animals with the greatest number of correlations between plasma insulin and individual plasma amino acids, in the groups fed all three dietary treatments, also had the greatest number of significant correlations between individual plasma amino acids and plasma acetate concentrations. Of the animals with the greatest number of correlations within each of the three dietary groups, the same individual amino acids concentrations were shown to be significantly correlated with both acetate and insulin, with the exception of aspartate, threonine and citrulline.

Of the animals in treatment group AH, with the least number of individual amino acid concentrations correlated with plasma acetate or plasma insulin concentrations, only aspartate concentration was correlated with both insulin and acetate concentrations. Similarly, of the animals within the treatment groups SD or AD, with the least number of individual amino acid concentrations correlated with hourly plasma acetate or insulin concentration, glutamate concentration was the only hourly amino acid concentration that was correlated with both acetate and insulin concentrations in group SD and citrulline in group AD.

The animals that had a large number of significant correlations between insulin and the individual amino acids, in the treatment groups where the diets were fed once daily, also had a significant correlation between insulin and the total individual amino acids ( $P < 0.05$ ). Those in treatment group AH showed that insulin was not correlated with the mean of the individual amino acids in either animal measured.

The daily means of the individual amino acid concentrations and the total amino acid concentrations that were measured can be seen in Table 3.12. There were no significant differences between dietary treatments in the daily mean concentrations of the individual amino acids with the exception of the histidine which was significantly higher in animals fed the dietary treatment AH, compared to those fed SD or AD ( $P < 0.05$ ).

The daily mean concentrations of acetate and insulin are shown in Table 3.13. There were no significant differences between dietary treatment, although the mean daily acetate concentration appeared to be higher in animals fed once daily, especially in dietary treatment SD, compared to those fed AH. The mean daily insulin concentration showed the opposite trend, tending to be higher in animals fed the AH dietary treatment ( $P > 0.1$ ).

Table 3.12 The effects of the two different diets (A or S) fed either hourly or once daily (-H or -D) on the plasma amino acid concentrations measured over a 24 hour period.

	DIETARY TREATMENT			SED	P Value	Probability of significant differences between dietary groups using t-test <sup>1</sup>	
	AD	AH	SD			AD x AH	AH x SD
Aspartate (μM)	15.1	18.0	17.4	3.325	0.689		
Threonine (μM)	94.0	102.5	101.2	24.22	0.933		
Serine (μM)	76.9	66.9	96.7	14.94	0.273		
Asparagine (μM)	13.8	18.4	16.9	4.430	0.624		
Glutamate (μM)	152.3	173.9	218.8	49.85	0.486		
Glutamine (μM)	198.1	213.3	163.9	35.65	0.462		
Glycine (μM)	451.9	366.9	463.1	61.20	0.358		
Alanine (μM)	132.8	177.6	145.8	15.48	0.127		
Citrulline (μM)	111.1	109.9	128.1	11.04	0.321		
Valine (μM)	151.9	158.9	219.5	40.57	0.324		
Methionine (μM)	10.5	9.2	12.5	1.747	0.306		
Isoleucine (μM)	69.0	79.9	90.0	12.878	0.386		
Leucine (μM)	90.3	91.9	128.1	19.96	0.249		
Tyrosine (μM)	49.3	53.0	51.1	7.367	0.889		
Phenylalanine (μM)	50.2	50.2	51.6	4.767	0.943		
Ornithine (μM)	84.0	79.9	99.8	21.616	0.662		
Lysine (μM)	93.8	108.8	96.9	18.09	0.710		
Histidine (μM)	53.8	64.7	51.2	4.016	0.083	*	*
Arginine (μM)	125.6	145.9	108.8	19.30	0.299		
Proline (μM)	111.6	110.4	137.5	23.10	0.499		
Total amino acids (μM)	2125	2186	2392	211.4	0.503		

\* = P<0.05 SED = standard error of difference<sup>1</sup> no significant differences between AD and SD

Table 3.13 The effects of the two different diets (A or S) fed either hourly or once daily (-H or -D) on the plasma acetate and insulin concentrations measured over a 24 hour period.

	DIETARY TREATMENT			SED (3 degrees of freedom for error) <sup>1</sup>	P Value
	AD	AH	SD		
Acetate (mM)	1.94	1.79	2.36	0.360	0.386
Insulin (μU/ml)	7.50	8.97	8.21	0.999	0.357

SED = standard error of difference<sup>1</sup> 27 degrees of freedom for error for insulin

### **3.6 Preliminary discussion**

Previous reports have shown that improving the synchrony of a diet, with respect to the release of dietary N and OM into the rumen, enhances the production and efficiency of microbial protein synthesis (Sinclair *et al.*, 1993). It has also been suggested that feeding a more synchronous diet improves liveweight gain presumably through the increase in microbial protein flow to the duodenum (Witt *et al.*, 1997). The problem of feeding different rumen synchronous and asynchronous diets is that the differences are confounded by the fact that different ingredients are used to make the diets. The experiment reported in this chapter overcomes this problem by feeding an asynchronous diet hourly, rather than once daily, to increase the rumen synchrony. A synchronous diet was also fed once daily for comparison. In this experiment, feeding the “asynchronous” diet hourly aimed to make the ratio of dietary N:OM release in the rumen more synchronous throughout the day, than when the same diet was fed once daily. The SIRE program (Sinclair *et al.*, 1993) used to formulate the asynchronous and synchronous diets, predicted that the two diets were isoenergetic and when fed once daily had similar levels of metabolisable protein. The effects that these dietary treatments, with differing rates of supply of the N and energy components to the rumen had on the growth and carcass parameters of growing lambs, was investigated. In addition, the effect that feeding these diets had on patterns of nutrients that are available for utilisation by the peripheral tissues (i.e. the concentrations of plasma acetate and amino acids in the jugular plasma) was investigated, to see if variation in the synchrony of nutrient supply to the tissues could be detected which may have contributed to changes in the carcass composition, particularly the efficiency of muscle protein deposition .

The growth rate was 52% higher in animals fed SD compared to those fed AD. The effect of a synchronous diet increasing the growth rate when compared to and asynchronous diet was also observed by Witt *et al* (1997) who showed that animals fed synchronous diets twice daily grew 23% more each day compared to animals fed an asynchronous diet twice daily. The increase in carcass weight of animals fed SD compared to those of animals fed AD is possibly due to significantly greater amounts of protein deposited in the carcass ( $P<0.05$ ) and perhaps also slightly higher amounts of fat deposited in the carcass although the differences in the amount

of carcass fat were not significant ( $P>0.1$ ). Similarly animals fed SD had higher final liveweights compared to those fed AD which may in addition to the carcass measurements, have been due to a tendency to lay down more fat in the depots that were measured (Table 3.4), although differences between these groups were not significant ( $P>0.1$ ).

Differences observed between dietary treatments AD and SD could be due to dietary formulation differences as different ingredients were used, which could lead to a difference in the microbial metabolism and consequently a difference in efficiency of feed utilisation. The FCE was more efficient in the animals fed SD compared to those fed AD indicating a better utilisation of the nutrients that were supplied in the diet for animal growth. The SIRE program uses data from AFRC (1993), which does not account for an improvement in the rumen synchrony contributing to an enhanced efficiency in microbial protein synthesis. In this experiment, despite DUP levels being lower in the synchronous diet, a more synchronous release of N and OM into the rumen each hour, may have enhanced the capture of N as microbial protein, above that predicted (see section 6.1 for further discussion). If the amino acid N arriving at the duodenum was greater, when the synchronous diet was fed, this may have enhanced growth rate which may account for the improved efficiency of feed utilisation, measured as FCE ( $P<0.001$ ).

The problem with confounding a difference in the patterns of supply of nutrients to the rumen with feeding different ingredients was overcome by feeding an additional group the same diet hourly (AH) instead of once daily (AD), causing a more synchronous supply of hourly OM and N to the rumen, but feeding exactly the same ingredients throughout the day. By feeding the asynchronous diet hourly (AH), making it more rumen synchronous, the animals grew at a faster rate and showed the greatest differences in carcass composition compared to animals fed the same diet once daily (AD). Animals fed AH had heavier carcass weights which would appear to be due to a tendency to lay down more fat and crude protein in the carcass and also to their tendency to have heavier *L.Dorsi* and *V.Lateralis* muscles, although these differences were not significant between the two groups ( $P>0.1$ ). The fat depots were significantly heavier (omental fat, mesenteric fat and KKCF) in animals fed AH compared to AD which would account for the final liveweight being significantly heavier in these animals. Gut-fill also tended to be heavier in animals fed AH which

would enhance final liveweight in these animals although gut-fill differences were not significant. A tendency for a higher gut-fill may imply that feeding hourly slowed down the passage rate of nutrients in the gut. This may enhance the rate of protein degradation in the rumen (see section 1.3.1), and change microbial protein synthesis which may alter the supply of nitrogen to the duodenum and consequently the nitrogenous supply to the host ruminant. It is likely that feeding the asynchronous diet hourly enhances microbial protein synthesis, as was shown by Bunting *et al.* (1987) perhaps because the synchrony of the supply of N and OM are made more synchronous throughout the day. In addition there is a greater DUP in the asynchronous diet compared to the synchronous diet and consequently the N supply to the duodenum may be higher in the animals fed AH which could be another reason that a greater growth rate was observed in these animals.

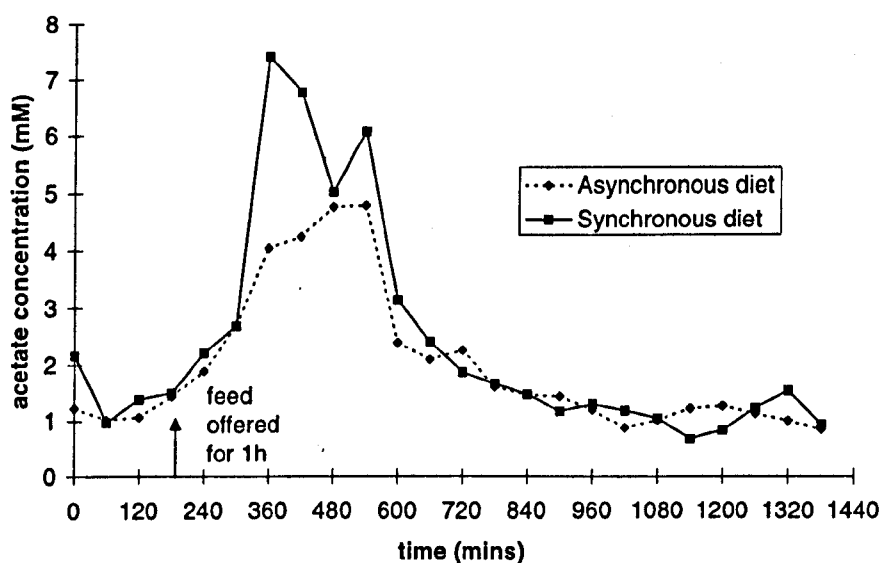
A limitation of this experiment was that voluntary feed intakes were higher when animals were fed SD and higher still when animals were fed AH resulting in a higher daily intake of energy. The differences in growth rate could primarily be caused by the differences in feed intake. A number of reasons for differences in voluntary intake have been reported and are discussed in chapter 6 in more detail. Those fed the diets once daily may have been limited by the amount that they could consume with metabolic and physical factors (see Forbes, 1996). Another reason why hourly-fed animals in the current work and in other experiments (Leveille, 1970; Cohn, 1965) eat more than those fed once daily is likely to be due to boredom and eating every hour is an activity to break this boredom.

On investigating the patterns of metabolite supply when feeding the different dietary regimes, it was interesting to note that the energy-yielding acetate in the peripheral plasma varied in temporal concentrations throughout the day between dietary feeding groups. Animals fed the diets once daily (AD or SD) showed that there was no effect on the pattern of plasma acetate concentration in the jugular plasma over a 24 hour period. When animals were fed both the diets once daily the acetate concentration in the jugular plasma fluctuated over a 12 hour period, following feeding such that there was a peak in the acetate concentration at approximately the same time of day following the meal being offered. This can be seen when the mean hourly plasma acetate concentrations are superimposed on the



same graph (Figure 3.5). Despite the N : OM ratio in the SD diet being far more synchronous throughout the day than the AD diet, when feeding once daily, (i.e. different synchrony indexes), the rates of degradation and the total amount of the OM released into the rumen, in the AD and SD diets were estimated using the SIRE program to be very similar to each other (53% and 50% of the total OM in the diets was degraded in the first hour, respectively). The similar peaks in acetate concentration following feeding were, therefore, likely to be the end-product of the rapidly fermented dietary OM component that was absorbed across the rumen wall relatively quickly and so raised the concentration of plasma acetate relatively soon after the animals were offered their meal.

Figure 3.5 The hourly acetate concentrations in the peripheral plasma of growing lambs fed the asynchronous (A) or synchronous (S) diet once daily



The peak in acetate concentration following feeding was slightly higher in the plasma of animals fed the synchronous diet compared with those fed the asynchronous diet which may be due to the voluntary intake of the animals fed SD being higher than animals fed AD. In addition, the overall daily acetate concentration also tended to be higher in animals fed SD compared to those fed AD, although this was not significant. However, the daily acetate concentration tended to be higher ( $P>0.1$ ) in animals fed SD compared to those fed AH. Those fed AH ate significantly

more than animals fed SD, so there are likely to be factors other than voluntary feed intake that increase the daily acetate concentration. A tendency for a higher acetate concentration in animals fed SD may be because the ingredients in the diets differed. Consequently, the amount of acetate producing bacteria in the rumen may have been different producing slightly more acetate in animals fed SD resulting in a slightly higher quantity of acetate being absorbed across the rumen wall. As there is also possibly an enhanced nitrogen supply to the duodenum when feeding this more synchronous diet, a better daily supply of amino acids and energy could be supplying the peripheral tissues enhancing the overall growth of the animals fed SD. A fourth treatment group where the synchronous diet was fed hourly was not included in the experiment, due to space requirements being greater than availability, as large groups of animals are required in each treatment group to allow for animal variation. Had this been fed to the animals, the growth rate may have been greater than the growth that we observed in animals fed SD, as perhaps the nitrogen supply to the duodenum was enhanced further and the energy in the form acetate supplying the tissues may have been more constant. If this had been carried out, it may have been possible to establish whether the efficiency of nutrient utilisation throughout the day would be enhanced if energy supply is at a constant level.

Plasma acetate concentrations in the animals fed AH was relatively constant throughout the day which is likely to be due to the OM being continuously degraded during the day with the animals being offered small portions of the same diet at hourly intervals. The resulting fermentation end-product, acetate, was then likely to be absorbed continuously across the rumen. This pattern of acetate supply was considerably different from the once daily-fed animals, although the daily concentration between treatment groups were very similar. This difference in the pattern of supply of energy-yielding acetate to the peripheral tissues may be a reason for the differences that were observed in the growth and carcass composition of the animals. The patterns of plasma amino acids were also more constant throughout the day, when animals were fed AH compared to those fed SD or AD. This would indicate that the amino acids are being supplied and utilised more continuously. A more constant concentration of acetate and amino acids throughout the day, may cause a more efficient synthesis of protein in the carcass which resulted in the higher level of crude protein that was observed in the carcass. A constant concentration of

acetate throughout the day may also contribute to the increase in fat deposition that was observed, not only in the overall carcass measurements but also in the fat depots.

Correlations between plasma concentrations of acetate or insulin and the amino acids throughout the day were carried out to see if there was any relationship between their concentrations, during the fluctuations that were observed throughout the day. There were a considerable number of negative correlations between the acetate concentrations and the amino acids which might suggest that as the energy-yielding acetate concentration goes up, the amino acid concentrations decrease, and vice versa. This is possibly because the amino acids are being utilised in metabolic processes (e.g. protein synthesis, gluconeogenesis), as the availability of the energy increases, and so the amino acid concentration declined. The correlations between the metabolites, however, were not consistent within groups which may be because there was considerable standard error of the means within dietary treatment groups, as shown by the substantial error bars in the plasma acetate profiles, particularly in those fed SD. The analysis of plasma from more animals that were sampled in each group may have given a more consistent picture of the significant correlations.

These diets were designed based on the synchrony index (SI). This is calculated as hourly deviation of the N : OM ratio in the rumen, from the optimum N : OM ratio (i.e. 25 : 1 gN/kgOM) (see Equation 1.2, section 1.4.6 for more details on calculating SI). The disadvantage with basing design of experimental diets on the rumen SI is that the patterns of release of the OM and N into the rumen throughout the day is not taken into account. The daily pattern of the nitrogen and organic matter ratio can deviate from the optimum at different times of the day, but theoretically the overall SI can be the same. This is demonstrated in Figures 3.6 and 3.7 where the N and OM release of theoretical asynchronous diets are shown. In Figure 3.6 (theoretical diet A) a rapid release of OM and a slow release of N is seen, so consequently a gradual increase in the N:OM will be observed across the day. In contrast theoretical diet B (Figure 3.7) shows a fast release of N and a slower release of OM, causing an decrease in the N:OM throughout the day. The deviation of the N : OM ratio from the optimum N : OM for both diets is the same and it is therefore possible that despite the release of the N and OM occurring at completely different

times of the day, the synchrony index is the same i.e. Synchrony index of diet A = Synchrony index of diet B.

Figures 3.8 and 3.9 show the N and OM release of theoretical synchronous diets, where the N and OM release into the rumen occur at the same time within the diets, so maintaining a constant N:OM ratio, but in diet C the OM and N are rapidly released and in diet D the OM and N are more slowly released. Despite the release of the nutrients being at completely different times of the day, the SI of diets C and D could be identical.

The results of this experiment would imply that the predicted rate with which OM was degraded into the rumen was related to the pattern of plasma acetate concentration. Despite the overall daily release of the OM and N in the rumen being different, as was estimated by the SI (Sinclair *et al.*, 1993) of the once daily fed dietary regimes (AD and SD), the proportions of OM released into the rumen in the first hour of feeding A and S diets once daily, relative to the total daily OM released from the diet, were fairly constant (see Table 3.14). A similarity in the pattern of energy supply may be the reason that the patterns of plasma acetate, insulin and the amino acids were generally very similar. For this reason therefore, the rate of OM degradation appears to be a significant contributing factor to the similarities between the plasma acetate concentrations in the animals fed the two diets, A and S, with the same pattern of feeding. The concentration of plasma amino acids also showed a very similar pattern throughout the day, between animals fed the two different diets with contrasting SI.

Table 3.14 The amount of OM and N in both the asynchronous and synchronous diets, that is degraded in the rumen in the first hour following feeding, as estimated by the SIRE programme (Sinclair *et al.*, 1993)

DIET	DEGRADATION IN FIRST HOUR	
	% N	% OM
ASYNCHRONOUS	52	53
SYNCHRONOUS	52	50

In contrast, the animals fed more frequently (AH) had very constant supply of acetate to the tissues throughout the day. This indicates that perhaps the pattern of

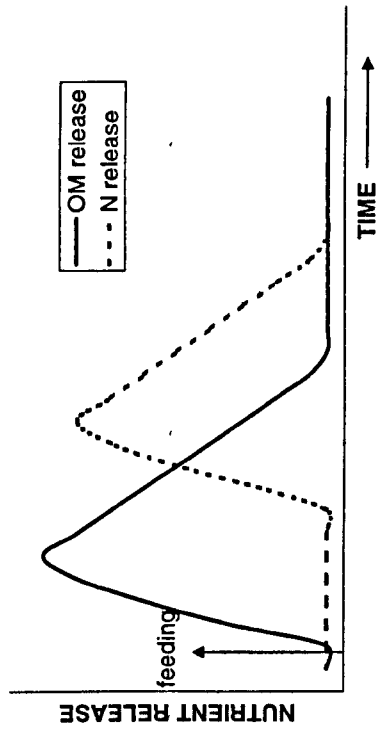
feeding and consequently the pattern of release of dietary OM and N into the rumen throughout the day is more significant with respect to the daily patterns of plasma metabolites than the ratio of release of the hourly N : OM throughout the day, represented here as the SI.

In these particular diets, feeding a diet with a relatively rapid degradation of OM frequently (i.e. every hour) gave a more constant supply of nutrients to the tissues than when fed just once daily, which tended to be conducive to an increased carcass weight and deposition of crude protein and fat in the carcass. Whether slowing down the rate of OM degradation in the rumen, by diet formulation, will make the supply of acetate to the tissues more constant, when the animal is fed once daily, needs to be investigated. In addition, the effects of the pattern of feeding i.e. feeding frequency, when diets with differing OM degradation are fed also requires investigation. If energy-yielding acetate and amino acids can be supplied more constantly throughout the day this may increase the deposition of crude protein and / or fat in the carcass and also perhaps the growth of the animal, as was indicated by the experiment reported in this chapter.

Figure 3.6 (a) N and OM release from a theoretical diet (A) following feeding with a rapid release of OM and a slow release of N (asynchronous)

(b) N : OM ratio following feeding of theoretical diet A

(a)



(b)

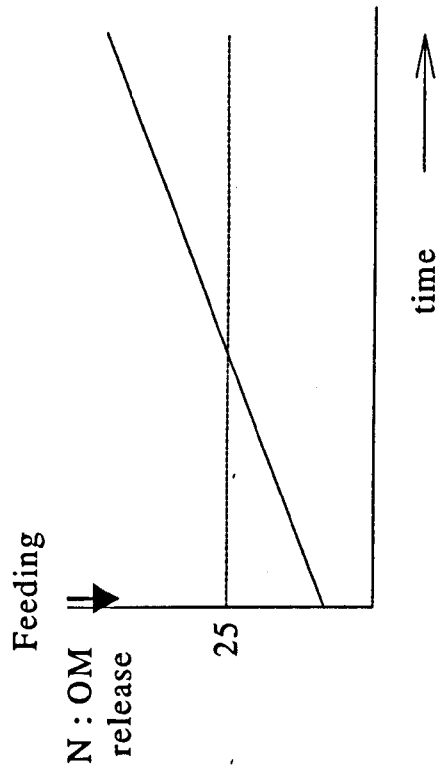
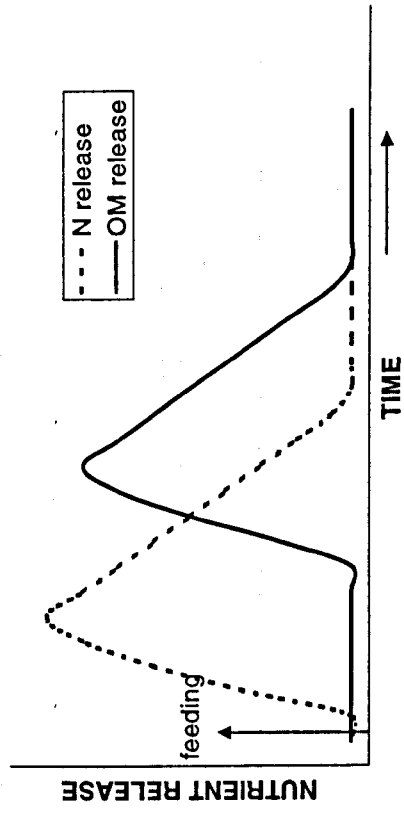


Figure 3.7 (a) N and OM release from a theoretical diet (B) following feeding with a rapid release of N and a slow release of OM (asynchronous)

(b) N : OM ratio following feeding of theoretical diet B

(a)



(b)

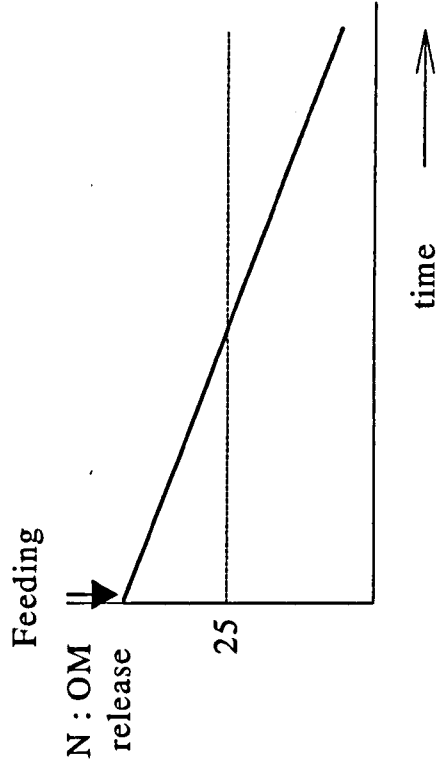
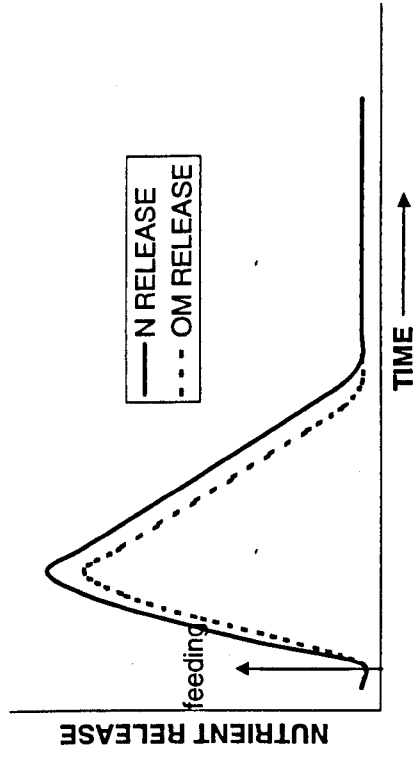


Figure 3.8 (a) N and OM release from a theoretical diet (C) following feeding with both a rapid release of N and OM (synchronous)

(b) N : OM ratio following feeding of theoretical diet C

(a)



(b)

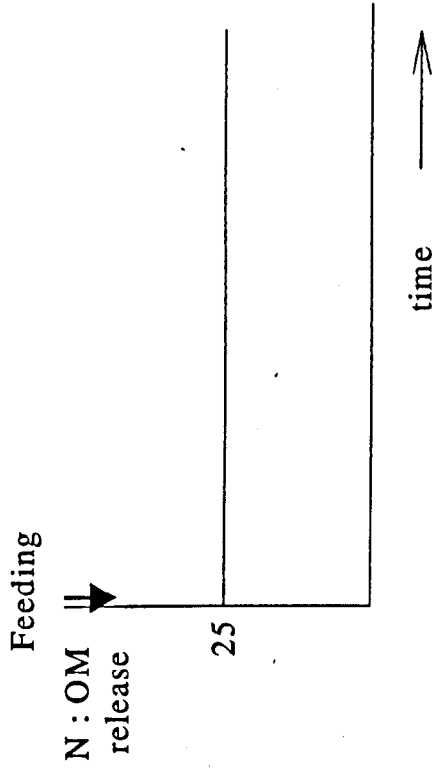
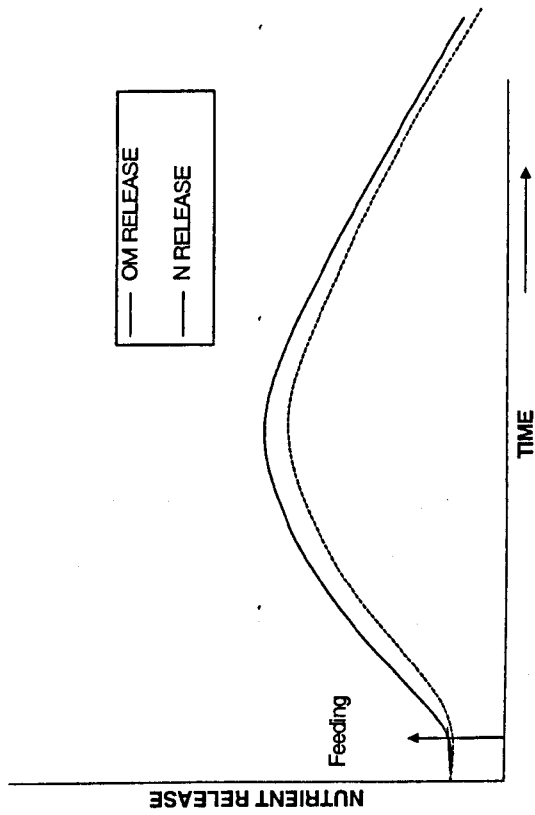


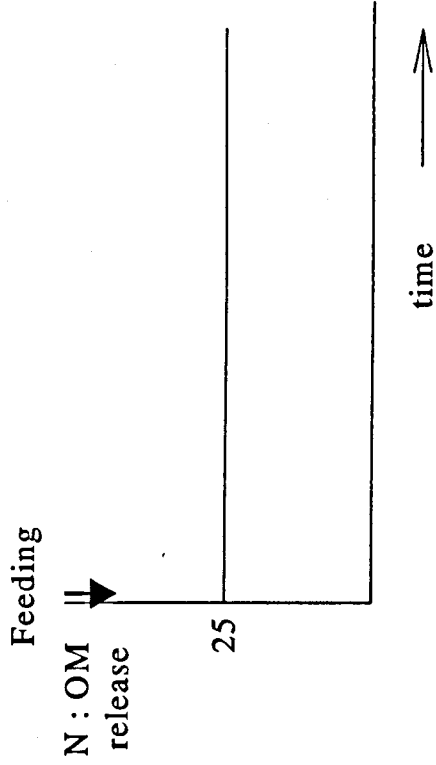


Figure 3.9 (a) N and OM release from a theoretical diet (D) following feeding with both a slow release of N and OM (synchronous)  
 (b) N : OM ratio following feeding of theoretical diet D

(a)



(b)



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## CHAPTER 4

# THE EFFECTS OF ALTERING THE TIMING OF RELEASE OF ORGANIC MATTER AND NITROGEN INTO THE RUMEN, ON PLASMA METABOLITES AND THE GROWTH AND CARCASS COMPOSITION TRAITS IN GROWING LAMBS

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### 4.1 Introduction

In the previous trial, sheep were fed two diets each with a differing synchrony index (SI) based on the ratio of OM : N release into the rumen over 24 h. However, although one diet was more synchronous than the other, the proportion of the total OM that was degraded in the rumen in the first hour following feeding was similar in both diets (approximately 50%). This probably accounted for the similar large rise in plasma acetate detected following once-daily feeding, when either the “asynchronous” or the “synchronous” diet was fed. The concentration of energy-yielding acetate in the peripheral circulation throughout the day is likely to be related to the rate with which the OM was degraded, rather than the ratio of N : OM in the rumen. A trial was therefore designed to investigate whether the concentration of plasma metabolites at different times of the day could be manipulated by formulating diets to have different rates of OM degradation, and how would this affect growth and carcass composition. When the lambs in the previous trial were fed the asynchronous diet hourly rather than once daily, it was predicted that the degradation of OM would be more constant, and indeed the concentrations of acetate in the plasma of hourly fed animals were observed to be considerably more constant throughout the day. Growth of lambs in the previous trial appeared to be increased by feeding the asynchronous diet hourly rather than once daily. This current experiment therefore examined the effects of feeding 2 diets, predicted to have OM contents with different rates of degradation in the rumen, to animals hourly or once daily to observe whether the plasma acetate concentration could be further manipulated and to investigate whether this could influence the growth or carcass composition of growing lambs.

The diets used in the present experiment, were designed using the SIRE program (Sinclair *et al.*, 1993), which enabled the rate of degradation of the dietary OM and N in the rumen, following feeding, to be predicted. Two diets were formulated which differed as much as possible in their rate of release of OM and N following feeding but were very similar in total metabolisable protein (MP) and metabolisable energy (ME) content. One diet (designated fast/fast, FF) contained ingredients that were estimated, when fed once daily, to have a greater proportion of the total OM (62%) and N (72%) degraded in the rumen in the first hour following feeding than was degraded for the remaining 23h of the day. The other diet (designated slow/slow, SS) contained ingredients that were estimated to have a smaller proportion of the total OM (31%) and N (32%) degraded in the rumen in the first hour following feeding than was degraded for the remaining 23h of the day. Figure 4.1 shows the predicted proportions of (a) the total N and (b) the total OM released each hour following a once daily feed of the FF or SS diets. It is clear from the predictions that the initial release of OM and N in the first hour after feeding the FF diet, should be larger than when the SS diet was fed, but in each subsequent hour the release of OM and N should be greater from the SS diet than from the FF diet. It was hypothesised therefore, that feeding the SS diet once daily would give a more constant release of OM into the rumen throughout the day than feeding the FF diet once daily and that this may give a more constant supply of acetate to the tissues which may possibly enhance synthesis of protein at the tissues, by making a continuous supply of energy available throughout the day for this highly energetic process. The ingredients used in the FF or SS diets and the predictions made by the SIRE program of total N and OM release into the rumen and the MP, ME and DUP content can be seen in Table 4.1. The ME and MP of the diets were predicted to be relatively similar and therefore, the main difference in the diets was the rate with which the N and OM components were degraded throughout the day.

When the FF or SS diets were fed hourly it was assumed that the overall release of OM and N into the rumen would be far more constant throughout the day.

Figure 4.1 (a) The proportion of total dietary N estimated to be released each hour, following once daily feeding of either the Slow/Slow diet (SS) or the Fast/Fast diet (FF) diet, as predicted by SIRE (Sinclair *et al.*, 1993).

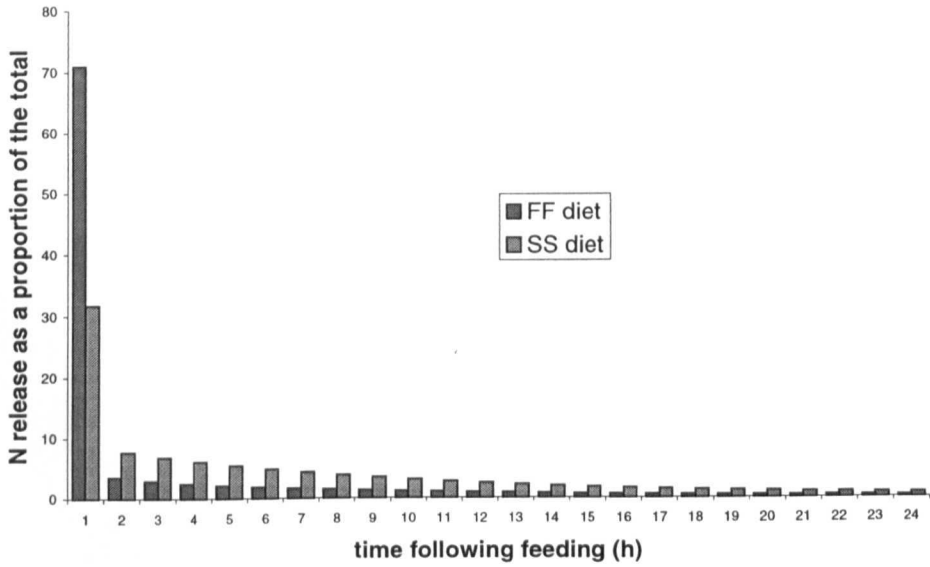


Figure 4.1 (b) The proportion of total dietary OM estimated to be released each hour, following once daily feeding of either the Slow/Slow diet (SS) or the Fast/Fast (FF) diet, as predicted by SIRE (Sinclair *et al.*, 1993).

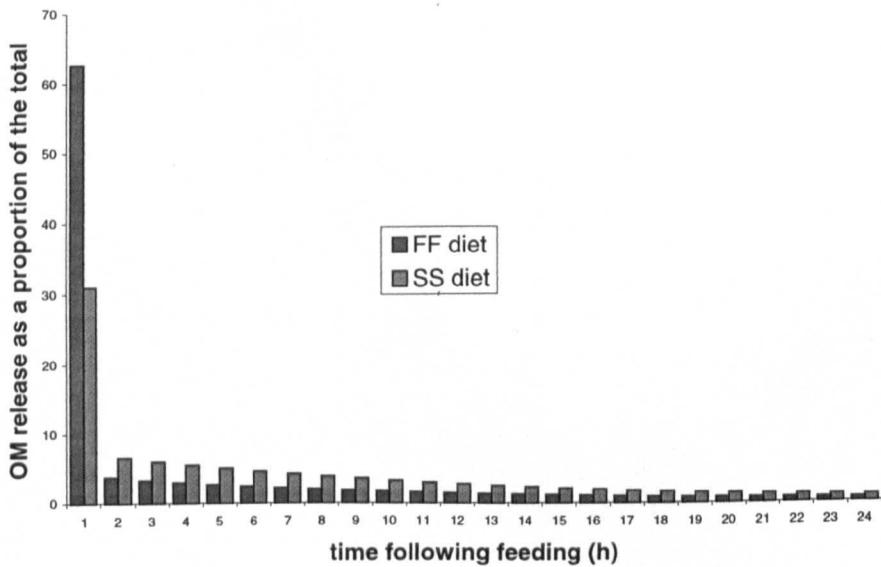


Table 4.1 Composition of the “fast/fast” and the “slow/slow” diets and SIRE predictions of nutrient content and rate of nutrient release into the rumen.

INGREDIENT	COMPOSITION (g/kg)	
	“SLOW/SLOW” DIET	“FAST/FAST” DIET
WHEAT	-	50
BARLEY	-	50
SUGAR BEET PULP	-	40
NUTRITIONALLY IMPROVED STRAW	400	370
OATS	-	260
MAIZE	380	-
MAIZE GLUTEN FEED	-	50
SOYA	90	40
DRIED GRASS	100	-
UREA	-	10
MOLASSES*	-	100
MINERALS	30	30
<b>SIRE PREDICTIONS :</b>		
	“SLOW/SLOW” DIET	“FAST/FAST” DIET
Synchrony Index	0.90	0.85
Total N release into rumen (g/kg fresh)	12.171	14.25
Total OM release into rumen (g/kg fresh)	453.6	445.8
ME (MJ/kg fresh)	9.203	8.905
MP (g/MJ ME)	7.78	7.40
DUP (g/MJ ME)	2.16	1.51

\* Rumenco - 48% sugar sucrose sugar cane molasses.

ME = metabolisable energy; MP = metabolisable protein; DUP = digestible undegraded protein

This experiment therefore, addressed two questions that arose from the previous experiment reported in Chapter 3 :

1. Can altering the rate of release of N and OM into the rumen, by diet formulation or frequency of feeding, affect growth and carcass composition of the animals?
2. If differences are seen, can this be due to the temporal pattern of peripheral plasma metabolites supplying the tissues (acetate and amino acids).

## **4.2 Methodology**

### **4.2.1 Animals, diets and treatments**

Thirty-four Charolais-cross wether lambs, initially weighing  $28.5 \pm 0.6$  kg, were wormed (Parafend Oxfendazole 2.265%, Norbrook Laboratories, Worcs.) and individually penned in one building, prior to the experiment. All animals had been born within one week and all were obtained from the same farm source. The building was continually illuminated and animals had free access to water and mineral licks at all times. Prior to weaning onto the experimental diets, animals were fed dried grass and a standard lamb creep feed.

The animals were initially randomly allocated to the two experimental diets. Seventeen were weaned to the FF diet and seventeen to the SS diet. Animals were allowed 10 days to acclimatise to the experimental diets, during which time all were trained to eat the feed offered within one hour of feeding, by gradually reducing the time the feed was offered.

The lambs, within each dietary group, were then randomly allocated to a feeding regime (hourly or once daily) or to an initial slaughter group (ISG). From each dietary group, seven were allocated to an hourly feeding regime, seven to a once-daily feeding regime and three to an ISG.

The feeding groups were as follows :

- FFH : “Fast/Fast” (FF) diet fed in 24 hourly portions
- FFD : FF diet fed once daily at 09:00h and offered for one hour only.
- SSH : “Slow/Slow” (SS) diet fed in 24 hourly portions
- SSD : SS diet fed once daily at 09:00h and offered for one hour only.

Animals allocated to the hourly feeding groups, SSH or FFH, were introduced to an hourly feeding regime using automatic feeders, 3 days prior to the beginning of the experiment. At the start of the experiment, animals allocated to the ISG were slaughtered.

The metabolisable energy (ME) requirements for maintenance and a liveweight gain of 150g/day for housed castrate lambs, was calculated in accordance with requirements reported in AFRC, 1993 (Equation 4.1).

Equation 4.1 Maintenance requirement for maintenance and liveweight gain of 150g/day for housed castrate lambs fed the Slow/Slow (SS) or Fast/Fast (FF) experimental diets.

$$\text{ME (MJ/d)} = 0.224 (\text{LW}) + 2.65$$

where :

ME = Metabolisable energy requirement per day (MJ)

LW = Liveweight of the animal (kg)

Source : AFRC (1993)

The ME content (MJ / kg) of each experimental diet was estimated using the SIRE program. The FF diet was estimated to provide 8.9 MJ ME/ kg fresh food and the SS diet 9.2 MJ ME/ kg fresh food and using these figures the total daily rations of either the FF or the SS diets were calculated to meet the ME requirements of the individual animals on a liveweight basis.

The total daily ME requirements for each individual was updated each time the animal was weighed and the feed requirements adjusted accordingly.

#### 4.2.2 Experimental Procedure

Animals fed once daily were offered their total daily ration of experimental diet at 09:00h each day for one hour. Animals fed hourly were fed using automatic feeders, which dispensed 1/24th of the total daily ration every hour for 24 hours. Refusals were removed from the hourly-fed animals, at 09:00h and from the once-daily fed animals at 10:00h. Weights of refusals were recorded daily. Animals were weighed (see section 2.6) three times per week, every Monday, Wednesday and Friday between 11:30h and 12:30h, throughout the experiment to monitor growth of the animals within each dietary group and also to calculate the total daily ration of feed to offer each individual.

After 7 weeks of feeding the diets, jugular cannulae were fitted in all animals 48 h prior to a blood sampling period (section 2.1). Hourly blood samples were taken from all the animals over a 25 hour period, from 05:00h to 05:00h. This ensured four samples had been taken prior to the once daily feed.

### 4.2.3 Slaughter Procedures

On the first day of the experiment the 6 initial slaughter group (ISG) animals were slaughtered by conventional slaughterhouse techniques (stunned and exsanguinated) and the carcasses dressed. Animals were weighed at 08:30h on the day before and on the day of slaughter. All were starved on the day of slaughter.

After 10 weeks of feeding the experimental dietary regimes, all the remaining animals were also humanely slaughtered by conventional slaughterhouse techniques over a period of 4 days, with equal numbers from each dietary group on each day. All animals were fed according to their allocated dietary regime the day prior to slaughter, but not from 08:00h on the day of slaughter. The final liveweight of the animals was defined as the mean weight of the animals on the day before (at 08.30h) and the day of slaughter (at 08:30h).

Dissection of the carcasses in both the ISG and the final slaughter group (FSG) was carried out as described in section 2.7. Briefly, the *Longissimus dorsi*, *Semitendinosus* and *Vastus lateralis* muscles were dissected from the right side of the carcass and weighed. The omental fat, mesenteric fat and kidney knob and channel fat from both sides of the carcass (KKCF) were dissected and weighed. Liver, spleen and heart weights and the gut fill and size of the digestive tract organs were also dissected and weighed on the day of slaughter of the FSG. The carcasses of the FSG were hung overnight and the following day, sawn in half and a measurement of the subcutaneous backfat thickness was taken against the 12th rib bone, using digital calipers.

The right side of the dressed carcass together with the dissected muscles were stored at -20°C until minced together (section 2.8.2) and analysed for chemical composition.

### 4.2.4 Chemical analysis

Chemical composition analysis (water, fat, protein and ash content) of the carcasses was determined as described in section 2.9.

Samples (approximately 10 g) of the feed were taken from each 25 kg bag of feed and pooled every 2 weeks. Chemical analysis of the feed was carried out to determine Gross Energy (GE), Nitrogen, DM, OM and ash content (see section 2.10).



Equation 4.2 Regression equation of LW against the *L.Dorsi* weight for the initial slaughter group

$$Y = (16.84 \times LW) - 100.13$$

Where Y = *L.Dorsi* weight (g)

Equation.4.3 Regression equation of LW against the *V.Lateralis* weight for the initial slaughter group

$$Y = (4.2893 \times LW) - 1.7624$$

Where Y = *V.Lateralis* weight (g)

Equation 4.4 Regression equation of LW against the *semitendinosus* weight for the initial slaughter group

$$Y = (2.4921 \times LW) + 5.3295$$

Where Y = *Semitendinosus* weight (g)

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Equation 4.5 Regression equation of LW against the mesenteric fat depot weight for the initial slaughter group

$$Y = (4.1629 \times LW) + 124.79$$

Where Y = mesenteric fat depot weight (g)

Equation. 4.6 Regression equation of LW against the omental fat depot weight for the initial slaughter group

$$Y = (5.1778 \times LW) + 51.85$$

Where Y = omental fat depot weight (g)

Equation 4.7 Regression equation of LW against the Kidney knob and channel fat (KKCF) depot weight for the initial slaughter group

$$Y = (9.1855 \times ILW) - 98.283$$

Where Y = KKCF depot weight (g)

#### 4.2.5 Calculations

Regression equations for liveweight (LW; kg) against individual dissected muscle and fat depot weights obtained from the initial slaughter animals, were calculated (see Equations 4.2-4.7 above). Using these regression equations an initial muscle or fat weight for each of the animals in the treatment groups could be estimated, based on their individual liveweights at the start of the experiment.

Growth rates (g/d) of individual muscles and fat depots were estimated by calculating the difference between the final and the estimated initial tissue weights for individual animals over the experimental period.

### **4.3 Statistical Analysis**

#### **4.3.1 Animal performance data**

The effect of feeding different diets (FF or SS) or the same diet in different feeding patterns (hourly or once daily) on growth and carcass composition was examined by analysis of variance, using Genstat 5 statistical package (Release 3.22; Lawes Agricultural Trust, Rothamsted). The experiment was analysed as a completely randomised design in a split plot ANOVA with diet and frequency of feeding as the main plots.

Initial liveweight of the animals obtained at the start of the experiment was used as a covariate in the analysis of variance for examining animal performance when the residual mean square was reduced by including the covariance. The design of the analysis gave 23 degrees of freedom for error, when covariance was included in the analysis. When 24 degrees of freedom for error were used in the analysis, it has been shown at the foot of the appropriate results table.

When analysis of variance showed a significant treatment effect, t-tests were carried out between pairs of treatments (diets or feeding frequency) using the pooled standard error of difference. Differences between groups were assumed not to be significantly different at  $P > 0.1$ .

Daily liveweight gain (DLWG) was determined by regression of the individual liveweights of each animal over time. The slopes of the individual animal growth rates were tested for difference by analysis of variance.

#### **4.3.2 Blood metabolite data**

Plasma concentrations of acetate, amino acids and insulin were determined in individual hourly samples from 3 animals in each of the 4 dietary treatment groups. The first sample taken in the 25h sampling period (05:00h) was discarded and was not analysed. The data is presented graphically as profiles of mean (n=3) hourly plasma acetate, insulin and amino acid concentrations throughout the day with error

bars representing the hourly standard error of the mean (SEM). Arrows on the profiles denote feeding.

The effect of feeding two different diets (FF or SS) with two different feeding patterns (hourly or once daily) on the hourly plasma concentrations of acetate and the individual amino acids throughout the day, in 3 animals from each treatment group, were examined by analysis of variance. Analysis of variance was calculated using the Genstat 5 statistical package (Release 3.22; Lawes Agricultural Trust, Rothamsted). There were 8 degrees of freedom for error when the experiment was analysed as a completely randomised design with diet and frequency as the main plots. Differences between groups were assumed not to be significantly different at  $P>0.1$ .

Direct correlations between the hourly plasma concentrations of acetate or insulin with each individual amino acid measured and the total amino acids measured were obtained. The statistical design gave 23 degrees of freedom for error, for each animal analysed. Pairs of variables were assumed not to be significantly correlated at  $P>0.05$ .

## **4.4 Results**

### **4.4.1 Feed Analysis**

The results of the feed analysis (Table 4.2) show that the two diets were approximately isoenergetic and isonitrogenous, as was predicted by the SIRE program. Likewise, the DM and OM content of the diets were very similar between diets. This indicates that any differences observed in animal performance are likely to be due to the pattern of dietary nutrient supply and their utilisation by the microbes and the host ruminant.

Table 4.2 Dietary composition of the experimental diets.

	DIETS	
	"FAST/FAST" DIET	"SLOW/SLOW" DIET
<b>FEED ANALYSIS :</b>		
DM (g/kg fresh)	892.5	893.8
OM (g/kg DM)	916.8	924.7
N ( g/kg DM)	21.4	18.3
GE (MJ/kg DM)	15.9	15.9

#### 4.4.2 Animal Performance

The initial liveweight, DLWG, final liveweight, carcass weight, killing-out proportion of the carcass, food intake and feed conversion efficiency (FCE) of the animals in each of the dietary treatment groups can be seen in Table 4.3. The initial liveweight, measured at the beginning of the experiment, followed a 10 d acclimatisation period to the diets before which the animals were allocated to their dietary groups. Initial liveweight was significantly different between diets (SS or FF), with animals in the group fed the SS diet being slightly heavier than animals fed the FF diet.

Animals fed the diets once daily (SSD or FFD) tended to have a higher DLWG compared to those animals fed the diets hourly (SSH or FFH). However, the difference between hourly and once-daily feeding was significantly greater for animals fed the SS diet (0.203 and 0.094 kg/d for SSD and SSH respectively;  $P < 0.001$ ) compared to those fed the FF diet (0.137 and 0.132 kg/d for FFD and FFH respectively;  $P > 0.1$ ). This resulted in a significant diet\*frequency interaction ( $P < 0.01$ ). Similarly diet\*frequency interactions were detected, in the final liveweight and carcass weights. Animals fed SSD tended to have the highest final liveweight of all the dietary treatments while animals fed SSH tended to have the lowest ( $P < 0.01$ ). Feeding animals hourly instead of once daily increased the carcass weight of animals fed the FF diets, but decreased the carcass weight of animals fed the SS ( $P < 0.001$ ). Despite the growth rates and consequently the final liveweights of animals fed FFH and FFD being similar, the carcasses of animals fed FFD were significantly smaller than those of animals fed FFH ( $P < 0.1$ ). This contributed to the significant diet\*frequency interaction on killing out proportion ( $P < 0.05$ ), where animals fed FFD tended to be considerably lower (474g carcass/kg LW) than the animals in the other groups (500.1, 501.6 and 501.3g carcass/kg LW for animals FFH, SSD and SSH respectively).

A significant diet\*frequency interaction was also detected in feed intakes when expressed as MJ ME consumed per kg LW per day. Animals fed hourly (SSH or FFH) tended to consume more food, but as the DLWG was not significantly higher in these animals, the feed conversion efficiency (FCE) when calculated as g food per kg liveweight gain, was significantly ( $P < 0.001$ ) higher in animals fed either diet hourly (FFH or SSH) compared to those fed once daily.

The effects of dietary treatment on the weights of three fat depots dissected at slaughter and the subcutaneous backfat thickness are shown in Table 4.4. Significant diet\*frequency interactions were detected in all three fat depots measured and in the subcutaneous backfat thickness ( $P < 0.1$ ). When feeding the animals once daily instead of hourly, fat depots tended to be slightly bigger in animals fed the SS diet ( $P > 0.1$ ) but significantly smaller in animals fed the FF diet ( $P < 0.05$ ).

When the fat depots measured at slaughter, were expressed as a proportion of the carcass weight, there was a significant effect of feeding frequency on the mesenteric fat, omental fat and KKCF depots ( $P < 0.1$ ). In all cases, animals fed the diets once daily, had significantly smaller fat depots, than those animals fed the diets hourly, when expressed as a proportion of the carcass weight ( $P < 0.05$ ) particularly when fed the FF diet.

Table 4.5 shows the effects of feeding the four dietary treatment groups on the mass of three muscles dissected at slaughter. There was a significant effect of feeding frequency on the weight of the *Semitendinosus* muscles ( $P < 0.1$ ) which were bigger in animals fed the diets once daily compared to those fed the diets hourly. There was a significant diet\*frequency interaction on the weight of the *L.dorsi* muscle which was due to the once daily fed animals having a larger *L.dorsi* when fed the SS diet, but a smaller *L.dorsi* when fed the FF diet, when compared to the hourly fed animals. When the muscles were expressed relative to carcass weight there were no significant differences between dietary treatment groups ( $P > 0.1$ ) with the exception of a diet effect on the *V.lateralis* which was greater in animals fed the FF diet ( $P < 0.1$ ).

Table 4.3 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the daily liveweight gain (kg), final liveweight (kg), carcass weight, killing out proportion (g/kg), food intake (MJ ME/kg LW) and feed conversion efficiency (FCE). Also shown are the initial liveweight in each dietary treatment group at the start of the experiment.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Initial Liveweight (kg)	27.17	27.09	29.89	29.83	1.494 <sup>1</sup>	*		
Daily liveweight gain (kg)	0.137	0.132	0.203	0.094	0.0251		**	**
Carcass weight (kg)	16.96	18.14	19.64	17.05	0.715			***
Final Liveweight (kg)	35.87	36.41	39.02	33.93	1.457		*	**
Killing-out (g carcass/kg LW)	473.7	500.1	501.6	501.3	8.61 <sup>1</sup>	*	*	*
Food Intake (MJ ME/kg LW/ d)	0.26	0.32	0.29	0.31	0.009 <sup>1</sup>	+	***	***
FCE(g food/kg liveweight gain)	8.44	12.1	5.81	15.25	2.507 <sup>1</sup>		**	

<sup>1</sup> 24 degrees of freedom

\*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED =Standard error of difference

Table 4.4 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the mesenteric fat, omental fat and KKCF depots and the subcutaneous backfat thickness.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Mesenteric fat depot (g)	381.4	478.3	479.4	450.2	26.86		+	**
(g / kg carcass wt)	22.19	26.58	24.45	26.34	1.350 <sup>1</sup>		**	
Omental fat depot (g)	312	486	506	461	80			+
(g / kg carcass wt)	17.4	26.4	26.2	26.5	3.71 <sup>1</sup>		+	
KKCF depot (g)	232	378	362	343	50		+	*
(g / kg carcass wt)	13.17	21.05	18.33	19.42	2.509		*	+
Subcutaneous Back fat thickness (mm)	1.40	3.03	3.65	2.37	1.027 <sup>1</sup>			+

<sup>1</sup> 24 degrees of freedom for error      \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = Standard error of difference

Table 4.5 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weight of the *L.dorsi*, *V.lateralis* and the *Semitendinosus*.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
<i>L.Dorsi</i> (g)	467.6	482.3	547.7	454.8	26.28		*	**
(g / kg carcass wt)	27.87	26.86	27.72	26.58	1.149 <sup>1</sup>			
<i>V.Lateralis</i> (g)	184	146	171	149	36.50 <sup>1</sup>			
(g / kg carcass wt)	11.60	9.07	7.77	7.82	1.779	+		
<i>Semitendinosus</i> (g)	109.4	102.1	123.5	104.4	10.7 <sup>1</sup>			+
(g / kg carcass wt)	6.76	6.00	5.97	5.86	0.422 <sup>1</sup>			

<sup>1</sup> 24 degrees of freedom

\*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = standard error of difference



The effect of dietary treatment on the chemical composition of the carcass is shown in Table 4.6. There were significant diet\*frequency interactions ( $P < 0.05$ ) in the amount of the carcass that was crude protein and that which was fat. This was due to the animals fed the SS diet once daily having a greater amount of fat and protein in the carcass compared to those fed hourly while animals fed the FF diet once daily had a smaller amount of fat and protein in the carcass compared to those fed the same diet hourly.

Animals fed FFD tended to have a higher percentage of protein in the carcass than animals in the other three dietary groups, although this was not statistically significant ( $P > 0.1$ ). Animals fed the diets hourly tended to have a higher percentage of fat in the carcass than animals fed once daily, although this was not statistically significant ( $P > 0.10$ ) as there was a diet\*frequency interaction. This was due to there being a significantly larger difference in the percentage fat ( $P < 0.01$ ) between animals fed the FF diet (16.99 and 21.22% fat for animals fed FFD and FFH respectively) than those fed the SS diet (21.08 and 21.39 % fat for animals fed SSD and SSH respectively;  $P > 0.1$ ). The fat : protein ratio was significantly affected by frequency of feeding ( $P < 0.1$ ), where the animals fed the diets once daily have a significantly lower fat : protein ratio ( $P < 0.1$ ) than those fed hourly, although the difference between the fat : protein ratio in animals fed SSD and SSH was small.

The effects of the four dietary treatments on the estimated growth rate of the three muscles over the experimental period are presented in Table 4.7. As expected, the differences between treatments in the estimated growth of the muscles were similar to the differences observed between treatments in final weight of the muscles dissected at slaughter. The frequency of feeding significantly affected the *Semitendinosus* ( $P < 0.05$ ), with the animals fed the diets once daily having significantly faster growth rates compared to those fed hourly. No significant differences were observed in the *V.lateralis* between treatments ( $P > 0.1$ ). There was a significant diet\*frequency interaction ( $P < 0.01$ ) in the estimated growth rate of the *L.dorsi* muscle, which was due to the *L.dorsi* of animals fed once daily, growing faster in animals fed the SS diet and slower in animals fed the FF diet, than when the animals were fed hourly.

Table 4.6 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weight and chemical composition of the carcass

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Weight of carcass chemical components :								
Crude protein (kg) <sup>2</sup>	2.75	2.85	3.12	2.68	0.135		+	**
Fat (kg)	2.94	3.86	4.17	3.72	0.374	+		*
Ash (kg)	0.74	0.72	0.82	0.60	0.069		*	*
Water (kg)	10.53	10.72	11.72	10.06	0.441		*	**
Carcass Composition :								
% Crude protein	16.29	15.77	15.81	15.73	0.375 <sup>1</sup>			
% fat	16.99	21.22	21.08	21.39	1.666		+	+
% ash	4.30	3.90	4.25	3.57	0.381 <sup>1</sup>		+	+
% water	62.43	59.10	59.80	59.28	1.176		*	+
Fat : Protein	1.051	1.358	1.347	1.360	0.1367		+	

<sup>1</sup> 24 degrees of freedom

<sup>2</sup> Crude protein = Nitrogen content x 6.25    \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED =Standard error of difference

Table 4.7 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the estimated growth rate of the *L.dorsi*, *V.lateralis* and *Semitendinosus* muscles.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Estimated growth of the								
<i>L.dorsi</i>								
(g / day)	1.19	1.40	2.33	1.04	0.340 <sup>1</sup>		*	**
Estimated growth of the								
<i>V.lateralis</i>								
(g / day)	0.32	0.44	0.61	0.31	0.454			
Estimated growth of the								
<i>Semitendinosus</i>								
(g / day)	0.498	0.402	0.601	0.339	0.1190 <sup>1</sup>		*	

<sup>1</sup> 24 degrees of freedom

\*\* = P<0.01, \* = P<0.05

SED = Standard error of difference

The effects of the four dietary treatments on the estimated growth rate of the three fat depots over the experimental period are presented in Table 4.8. There were significant diet\*frequency interactions on the growth rate of all three fat depots. In each case, this was due to fat depots of the animals fed the diet once daily having a faster growth rate when fed the SS diet and a slower growth rate when fed the FF diet, compared to the fat depots of the animals fed the same diets hourly. The rate of fat depot growth was significantly less in animals fed FFD compared to the other 3 dietary groups ( $P<0.05$ ).

Table 4.9 shows the effect of feeding the four dietary treatment groups on the weights of the liver, spleen and heart and the weights of the different empty gut components of the sheep digestive tract. Also shown in Table 4.9 is the effect of diet on total gut fill. Feeding the diets hourly significantly reduced the liver and heart weights compared to feeding the diets once daily ( $P<0.1$ ). There were diet\*frequency interaction on the empty reticulo-rumen and abomasum, as both gut components were significantly heavier ( $P<0.01$ ) in animals fed SSD compared to those fed SSH but there was no significant difference between animals fed the FF diet hourly or once daily. There were significant effects of both diet and feeding frequency on total gut fill, with no diet\*frequency interaction. Animals fed diets once daily had significantly larger gutfills than those fed hourly ( $P<0.1$ ), despite the animals being starved prior to slaughter. Those fed the FF diet had a significantly heavier gutfill than those fed the SS diet ( $P<0.05$ ). These differences may have accounted for the differences observed between diets in the killing out proportion.

Table 4.8 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the estimated growth rate of the mesenteric fat, omental fat and the KKCF depots.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Estimated growth of the mesenteric fat depot (g / day)	1.88	3.21	3.25	2.84	0.365	+	+	**
Estimated growth of the omental fat depot (g / day)	1.53	3.93	4.20	3.57	1.083			+
Estimated growth of the KKCF depot (g / day)	0.94	2.94	2.72	2.44	0.676		+	*

<sup>1</sup> 24 degrees of freedom

\*\*\* = P<0.001, \*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = Standard error of the mean

Table 4.9 The effect of feeding either the fast/fast diet once daily (FFD) or hourly (FFH) or the slow/slow diet once daily (SSD) or hourly (SSH), on the weights of the liver, spleen and heart and the weights of the individual components of the digestive tract.

	DIETARY TREATMENT				Pooled SED (23 degrees of freedom)	Probability of significant differences		
	FFD	FFH	SSD	SSH		DIET	FREQUENCY	DIET*FREQUENCY
Liver weight (g)	531	497	524	437	32.9		*	
Spleen weight (g)	52.2	57.0	53.6	49.0	5.89			
Heart weight (g)	168.2	158.9	166.8	156.4	7.99		+	
Empty reticulo-rumen weight (g)	584	578	634	480	35.8		**	**
Empty abomasum weight(g)	132.3	130.7	132.5	106.6	7.84		*	*
Empty omasum weight (g)	70.4	71.0	74.1	67.1	6.61			
Empty intestine weight (g)	925	926	1041	888	73.5			
Total gut fill (g)	5686	5059	4824	3841	650.4		*	+

<sup>1</sup> 24 degrees of freedom      \*\* = P<0.01, \* = P<0.05, + = P<0.1      SED = Standard error of difference

#### 4.4.3 Plasma metabolites

Leucine was selected as a representative essential amino acid with which to compare the concentration profiles of plasma amino acid and acetate in animals fed the different dietary treatments, over time. The remaining amino acid profiles are not shown, however the correlations of hourly concentrations of each amino acid with acetate can be seen in Table 4.11 and show the extent to which plasma acetate was correlated with the individual amino acids, throughout the day, for each dietary treatment. The change in plasma concentrations of leucine and acetate over a 24 h period in sheep (n=3) fed the different dietary regimes, can be seen in Figures 4.2 and 4.3.

Figure 4.2 (a) shows the mean hourly plasma leucine and acetate concentration of sheep fed FFD, over a 24 h period. There was a dramatic increase in the plasma acetate concentration, immediately after the animals were offered the fast/fast diet. The concentration continued to rise for 4 h following feeding at which point the acetate concentration peaked at approximately 8.5mM. Following the peak concentration there was a decline in the acetate concentration over approximately 7 h at which point the acetate concentration in the plasma returned to the basal concentration, that it was at prior to feeding, approximately 0.8mM. Consequently, in animals fed the FF diet once daily, the plasma acetate concentration fluctuated for 11 hours of the day and was constant at approximately 0.8mM for the remaining 13 hours. The hourly concentration of plasma leucine was highly negatively correlated ( $P<0.001$ ) with the hourly acetate concentration in all animals fed the FF diet once daily (see Table 4.13). This occurred because the plasma leucine concentration began to decline in the hour following feeding, almost at the same time that the plasma acetate concentration started to increase, and reached the lowest concentration as the plasma acetate concentration reached its peak. At that point (approximately 3 h after feeding) the plasma leucine concentration started to increase gradually. The leucine concentration appeared to take approximately 2-3 hours longer to plateau than the acetate concentration. The error bars shown for the leucine concentration would suggest that the change in leucine concentration throughout the day was significant.

Figure 4.2(a) Hourly plasma acetate and leucine concentrations in the plasma of growing lambs (n=3) fed the Fast/Fast diet once daily (FFD). Error bars represent the standard error of the mean.

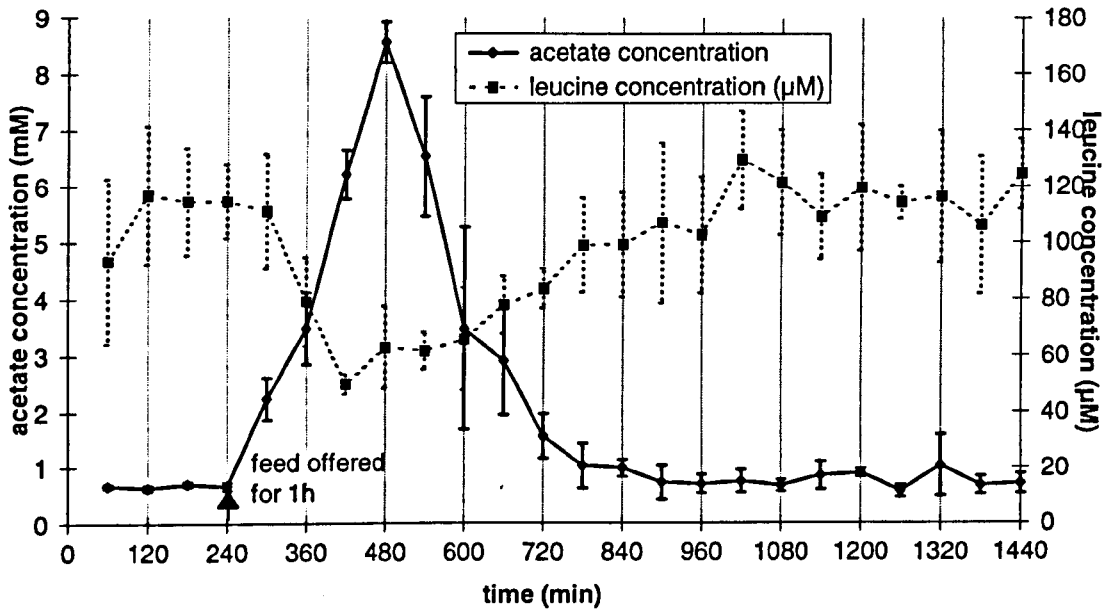
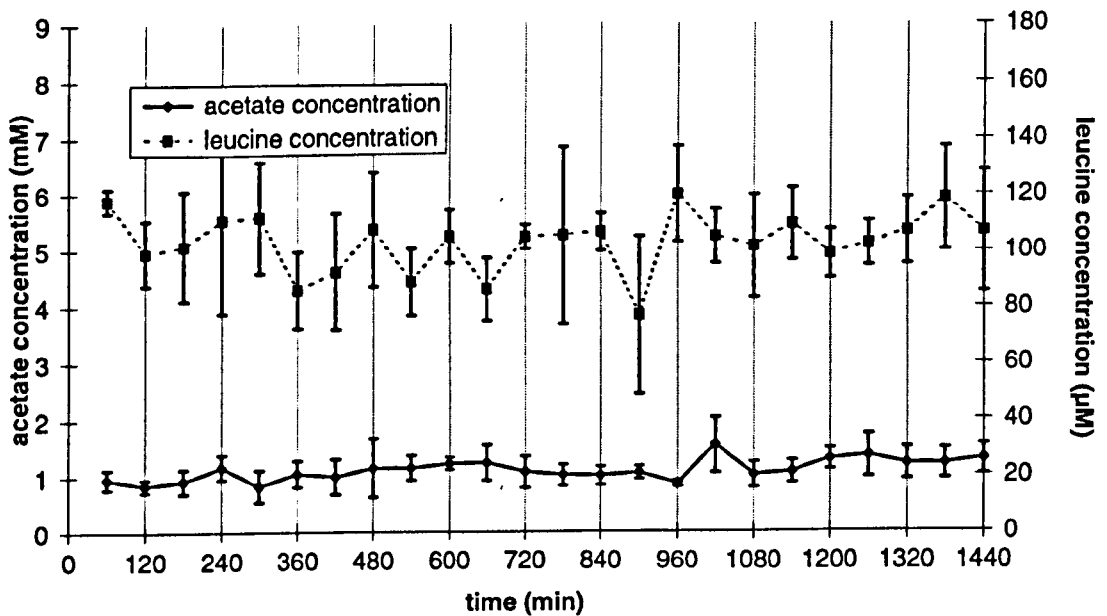


Figure 4.2(b) Hourly plasma acetate and leucine concentrations in the plasma of growing lambs (n=3) fed the Fast/Fast diet hourly (FFH). Error bars represent the standard error of the mean.





In contrast, when the same FF diet was fed to animals hourly, a completely different metabolite profile was observed as shown in Figure 4.2 (b). Plasma acetate concentrations were constant at approximately 1mM throughout the day and similarly the leucine concentration remained at a constant level at approximately 100  $\mu$ M.

A similar pattern was observed in the metabolite concentrations measured when the SS diet was fed (Figure 4.3), although the magnitude of the fluctuations in acetate and leucine concentration in the once daily fed animals were less than those observed with the FF diet. As can be seen in Figure 4.3 (a) the hourly plasma acetate concentration in animals fed SSD rose over approximately 5 h following feeding to approximately 2.5mM. This elevation, was considerably less (approximately 2.5 times the basal concentration prior to feeding) than the elevation observed in plasma acetate concentration following feeding of the FFD dietary regime (approximately 8 times the basal concentration). Although not as large, the increase in acetate concentration was slightly more prolonged (approximately 1 h) than was seen in the animals fed FFD and the decline in acetate concentration following the peak, appeared to be more gradual in animals fed SSD, not decreasing to the basal concentration that was observed prior to feeding (approximately 1mM) for the remaining part of the sampling period. The overall mean daily acetate concentrations (Table 4.10) tended to be less in animals fed dietary treatment SSD compared to those fed FFD (1.93mM), although this was not significant ( $P>0.1$ ).

Figure 4.3 (a) Hourly acetate and leucine concentrations in the plasma of sheep (n=3) fed the slow/slow diet once daily (SSD). Error bars represent the standard error of the mean.

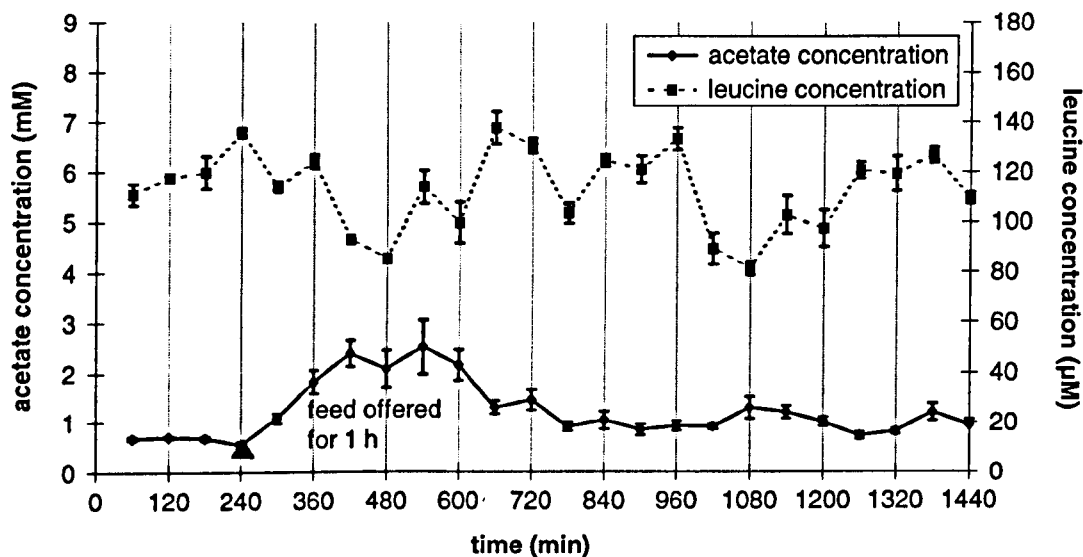
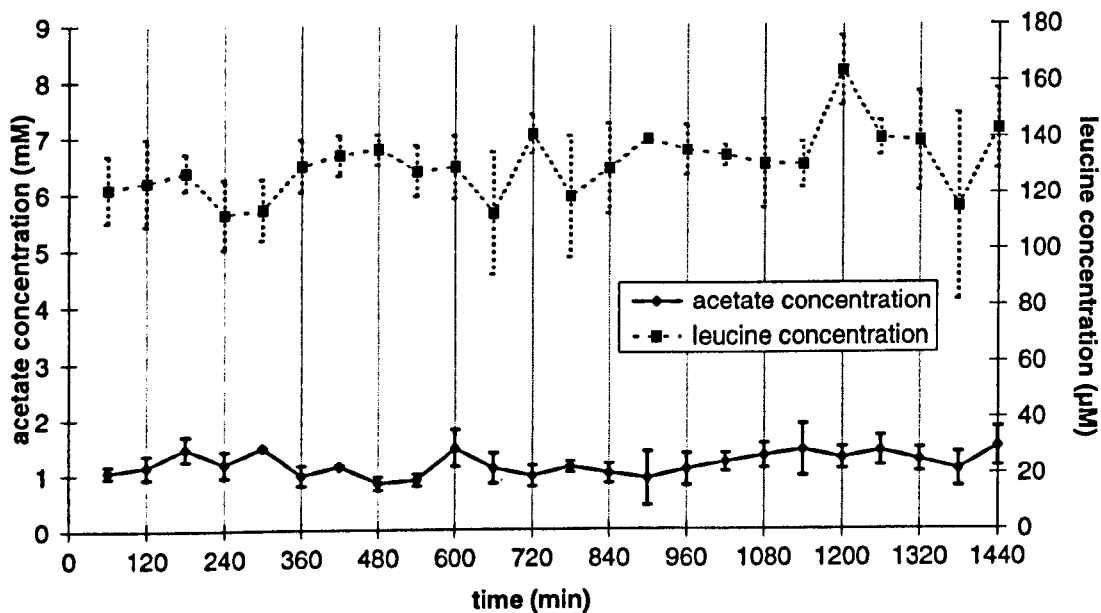


Figure 4.3 (b) Hourly acetate and leucine concentrations in the plasma of sheep (n=3) fed the slow/slow diet hourly (SSH). Error bars represent the standard error of the mean.



Plasma leucine concentrations in animals fed treatment group SSD showed a small decline following feeding, similar, but of lower magnitude to that observed in the plasma of animals fed the FFH dietary treatment group. The leucine concentration started to increase again, approximately 4 h following feeding. The increase in the leucine concentration was fairly erratic, but approximately 12 h following feeding there appeared to be another drop in the leucine concentration, down to the level of leucine concentration that the original decrease had been at, 4 h post-feeding (approximately 80 $\mu$ M). Following this second decrease, there was a gradual increase in the leucine concentration through the remaining part of the sampling period. It should be noted that there appeared to be an increase in the leucine concentration during the 4 h prior to feeding, at the start of the sampling period. Consequently, it would appear that the concentration of leucine in the plasma was continually fluctuating throughout the day in animals fed the SSD dietary treatment. The error bars on the profiles suggest that these changes in the leucine and acetate concentrations that were observed for the animals fed SSD, were significant.

In animals fed the SSH dietary treatment, both the plasma acetate and the plasma leucine concentrations were relatively constant throughout the day, however, the overall acetate concentration appeared to be higher for animals fed the SSH treatment (slightly higher than 1mM), compared to those fed the FFH dietary treatment (slightly lower than 1mM) (see Figures 4.2 (b) and 4.3 (b)). This corresponded to the trends observed for the mean daily acetate concentration (Table 4.10), with the mean for the SSH treated animals being higher than for the FFH treated animals, although these differences were not significant ( $P>0.1$ ).

Figure 4.4 (a) The hourly acetate and total amino acid concentration in the plasma of sheep (n=3) fed the fast/fast diet once daily (FFD). Error bars represent the standard error of the mean.

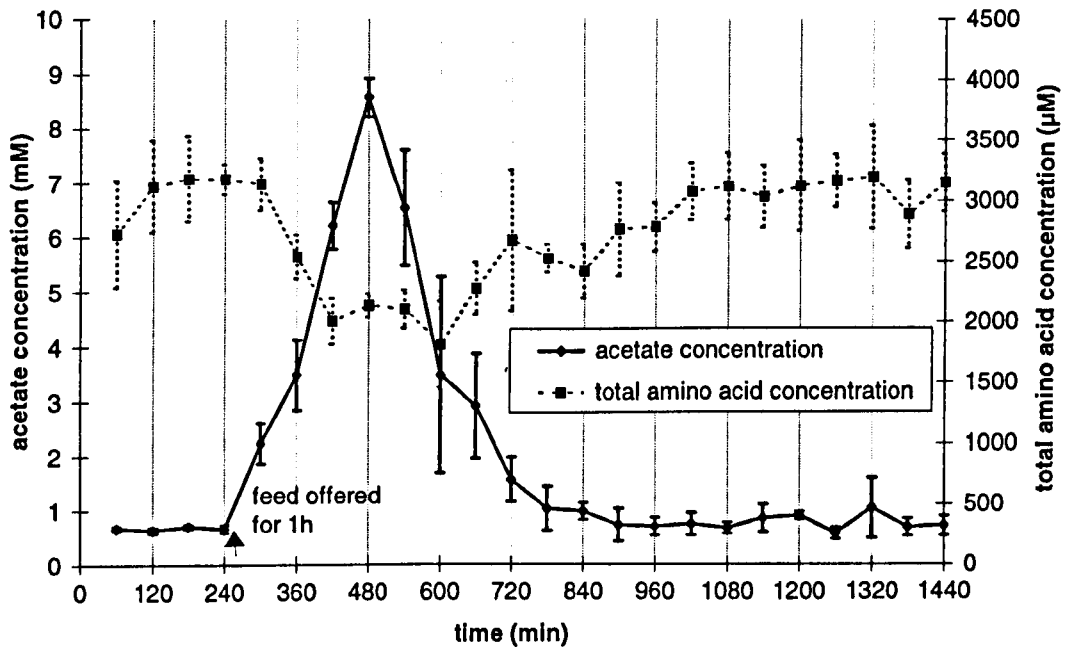


Figure 4.4 (b) The acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the fast/fast diet hourly (FFH). Error bars represent the standard error of the mean.

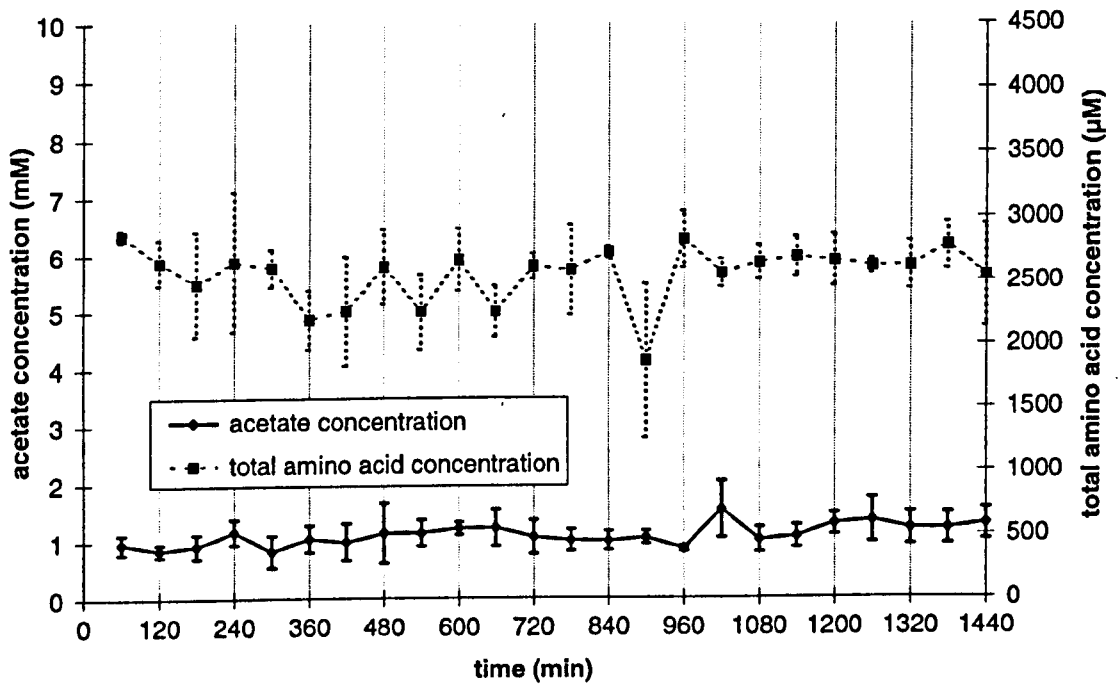


Figure 4.5 (a) The hourly acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the slow/slow diet once daily (SSD). Error bars represent the standard error of the mean.

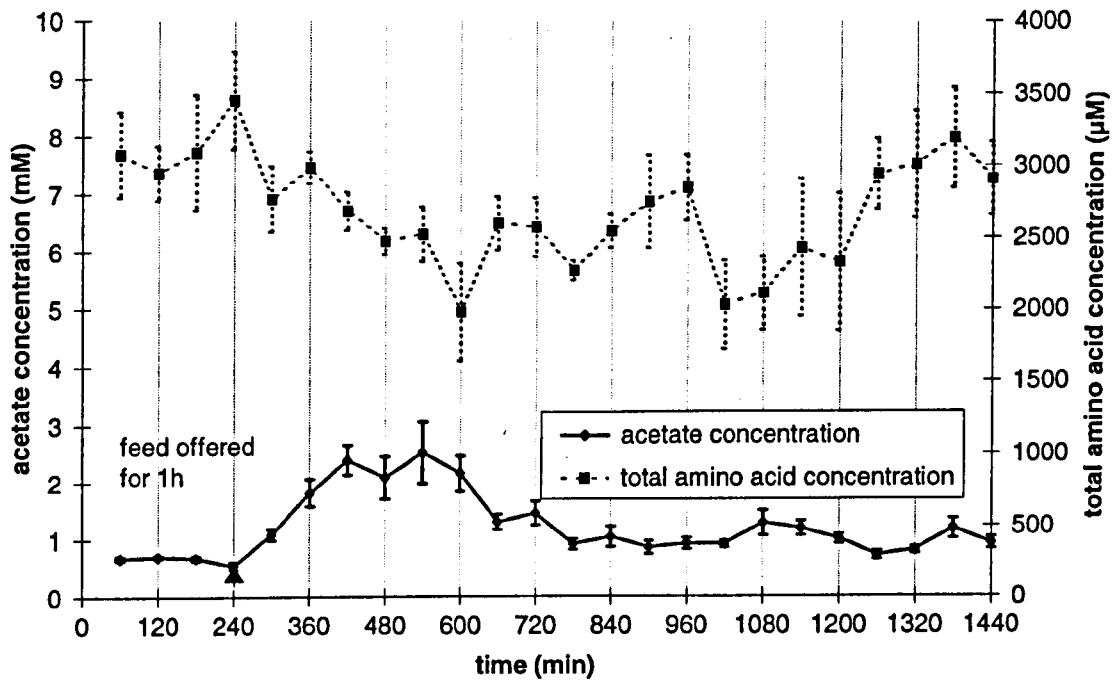
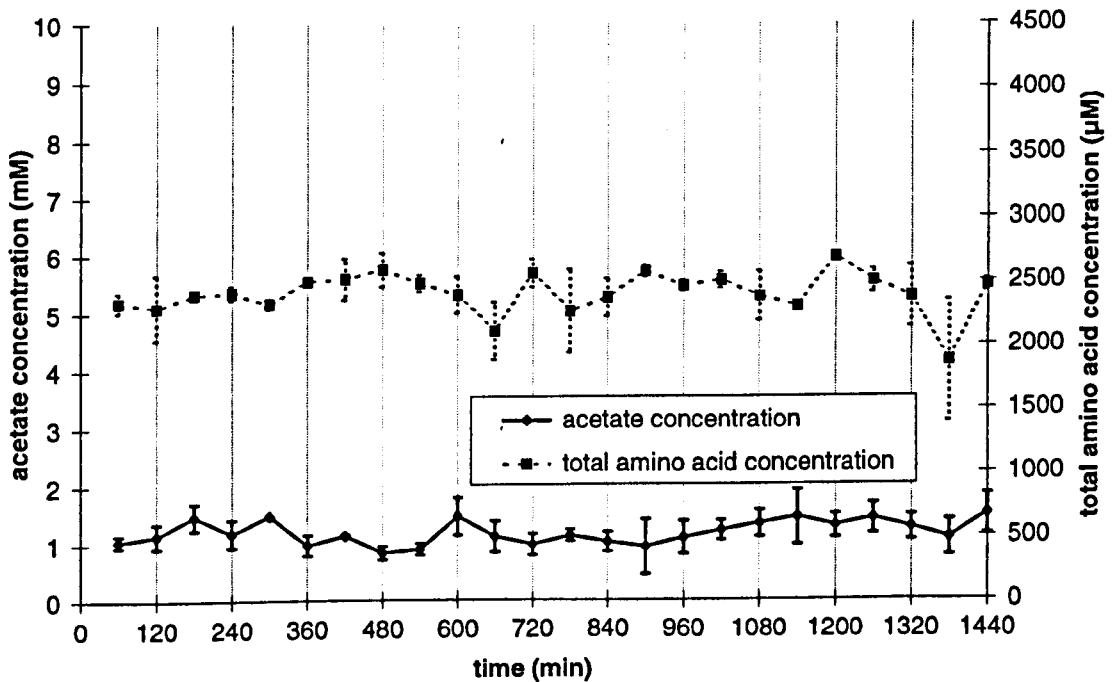


Figure 4.5 (b) The hourly acetate and total amino acid concentration in the plasma of growing lambs (n=3) fed the slow/slow diet hourly (SSH). Error bars represent the standard error of the mean.



The profiles of the hourly plasma concentrations of the total amino acid and acetate over a 24 h period in the plasma of the sheep fed the different dietary regimes can be seen in Figures 4.4 and 4.5. The total amino acid concentration profiles, follow a similar pattern to those observed with the leucine profiles. The animals fed either the SS or FF diets hourly have similar concentrations at constant levels throughout the day (See Figure 4.4 (b) and 4.5 (b)).

The animals fed dietary treatment FFD showed a decrease in the concentration of total plasma amino acids approximately 1h after feeding, which continued for a further 5h (Figure 4.4(a)). The total amino acid concentration increased again and returned to the original concentration that it was at, at the point of feeding, approximately 14h following feeding.

In contrast, the total amino acid concentration of animals fed the SSD dietary treatment appeared to fluctuate for the majority of the day (see Figure 4.5 (a)). There was a drop in the leucine concentration following the once daily feed which didn't appear to rise until after approximately 16h after feeding. Following this, there was a gradual rise in total amino acid concentration for the remaining time in the sampling period.

Figure 4.6(a) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the fast/fast diet once daily (FFD). Error bars represent the standard error of the mean.

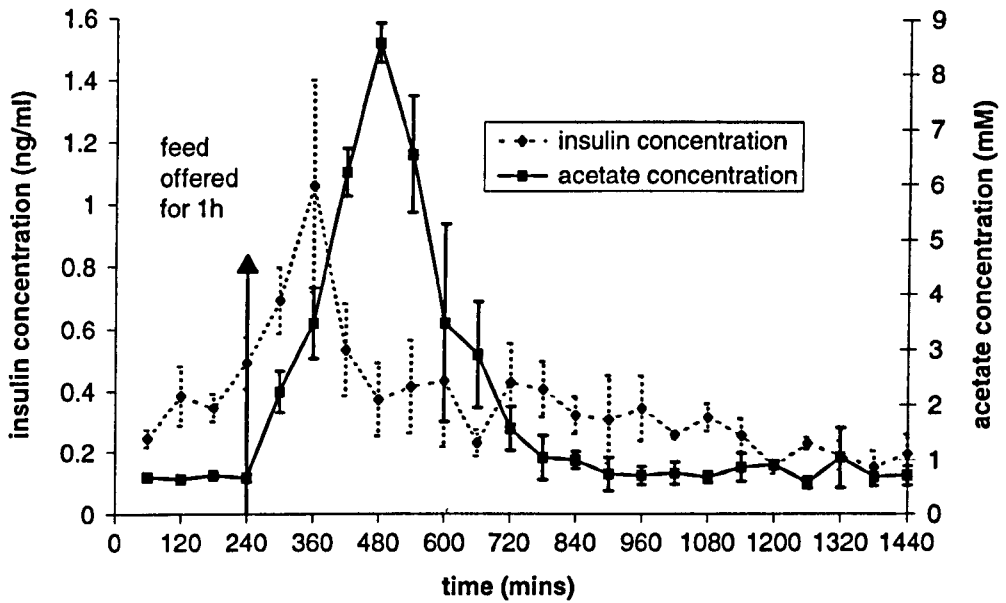
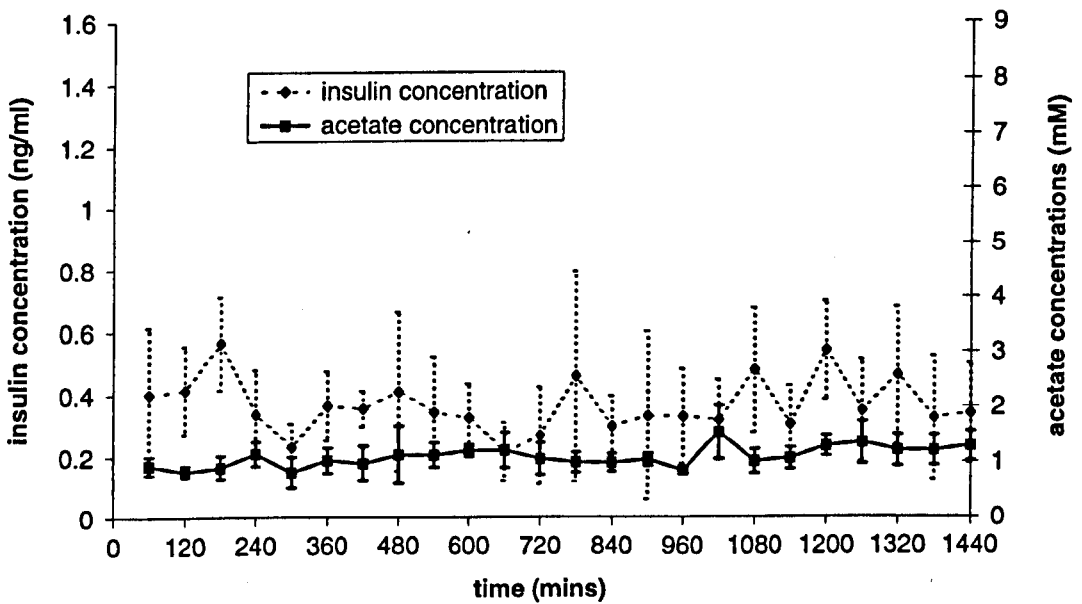


Figure 4.6 (b) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the fast/fast diet hourly (FFH). Error bars represent the standard error of the mean.



The profiles of the hourly concentrations of plasma insulin and plasma acetate can be seen in Figures 4.6 and 4.7. Figure 4.6 (a) shows the pattern of plasma insulin concentration, throughout a 24 h period when animals were fed FFD. There was a peak in the insulin concentration (approximately 1.1ng/ml) 2 h post-feeding, followed by a rapid decrease, back to the concentration observed prior to feeding (between approximately 0.2 and 0.4 ng/ml), for the rest of the day. The rise in insulin concentration occurred prior to the rise in acetate concentration, and the decline in total amino acids (not shown in Figure 4.6 (a)).

Figure 4.6 (b) shows the pattern in the insulin concentration that was observed in the plasma when animals were fed FFH. These animals had relatively steady insulin concentration throughout the 24 h sampling period, with concentrations ranging between approximately 0.2ng/ml and 0.6ng/ml.

The pattern in the insulin concentration in the plasma of sheep fed SSD are shown in Figure 4.7 (a). As observed in animals fed the FF diet once daily, those animals fed the SS diet once daily also had a rapid increase in the concentration of insulin following feeding. However, the insulin concentration appeared to remain elevated for longer following feeding in animals fed SSD compared to those fed FFD. It took approximately 9 h following feeding for the insulin concentration to decrease to approximately 0.4ng/ml, which was a level similar to that observed prior to the animal being offered the feed in animals fed SSD. In contrast in those fed FFD, it took only approximately 4 h following feeding for the insulin concentration to decline back to the level observed prior to feeding (0.4ng/ml) .

The pattern of the insulin concentration in the peripheral plasma of sheep fed the SSH dietary regime is shown in Figure 4.7 (b). This appeared to be very constant throughout the day at a level between approximately 0.3 and 0.5ng/ml. Table 4.10 shows that the mean daily plasma insulin concentrations tended to be higher in animals fed SSD (0.51ng/ml) compared to those fed the SSH (0.39ng/ml) and FFD (0.37ng/ml), although these differences were not statistically significant ( $P>0.1$ ).



Figure 4.7 (a) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the slow/slow diet once daily (SSD). Error bars represent the standard error of the mean.

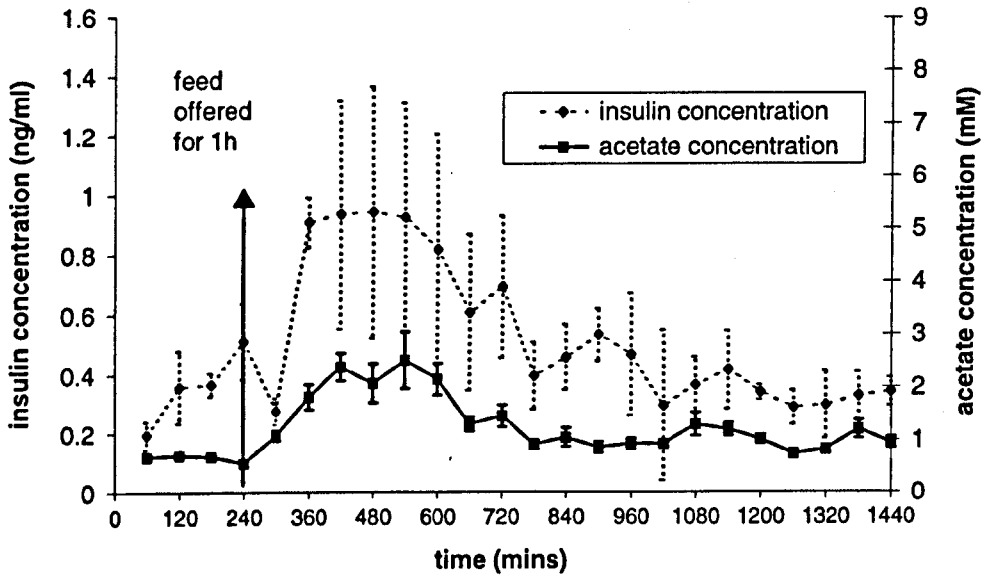


Figure 4.7 (b) The hourly insulin and acetate concentration in the peripheral plasma of sheep (n=3) fed the slow/slow diet hourly (SSH). Error bars represent the standard error of the mean.

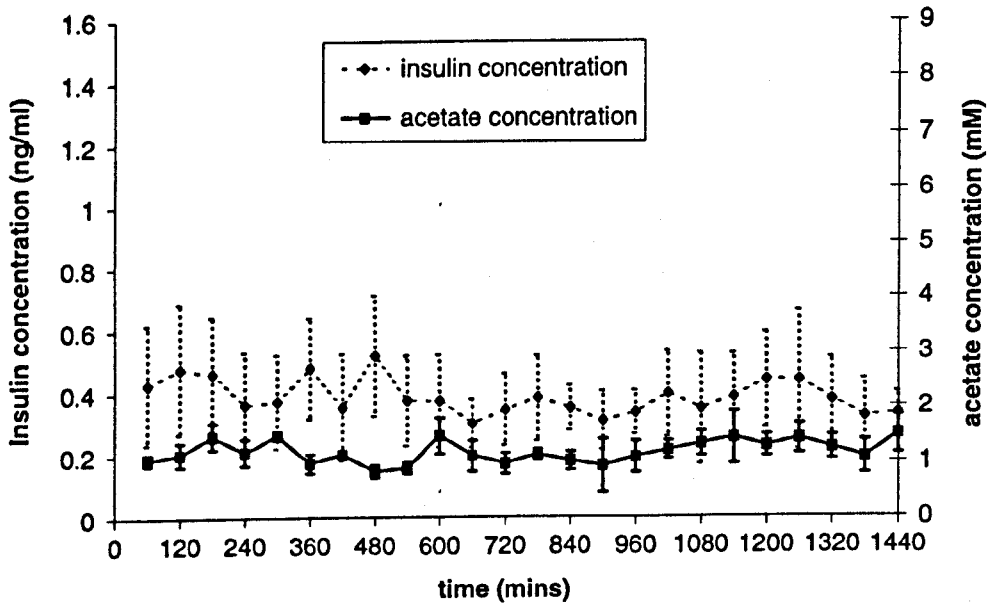


Table 4.10 The effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the mean daily plasma acetate, insulin and amino acid concentrations measured over a 24 hour period (\*\*=P<0.001, \*\*\*=P<0.01, +=P<0.1).

	DIETARY TREATMENT							SED (8 degrees of freedom for error)	Probability of significant differences frequency
	FFD	FFH	SSD	SSH	FFD	FFH	SSD		
Acetate (mM)	1.93	1.09	1.19	1.18	0.374				
Insulin (ng/ml)	0.37	0.36	0.51	0.39	0.177				
Aspartate (µM)	14.5	14.9	16.4	12.1	4.20				
Threonine(µM)	135.8	134.0	142.4	116.6	29.34			**	
Serine(µM)	170.3	92.4	122.7	76.0	20.90			**	
Asparagine(µM)	30.18	32.64	31.86	29.13	7.147				
Glutamic acid (µM)	174.2	151.9	133.8	148.3	65.11				
Glutamine(µM)	284.8	291.2	352.9	291.2	68.91				
Glycine(µM)	497.3	408.2	500.7	351.2	30.84			***	
Alanine(µM)	189.0	194.9	225.5	186.9	25.93				
Citrulline(µM)	206.4	176.8	178.6	163.4	24.78				
Valine(µM)	187.4	203.1	159.2	184.2	26.29				
Methionine(µM)	13.5	13.0	12.9	12.2	2.16				
Isoleucine(µM)	80.6	93.6	72.0	87.0	8.71			+	
Leucine(µM)	98.6	103.1	113.0	131.1	14.95			+	
Tryptophan(µM)	14.7	15.5	17.0	12.6	4.93				
Tyrosine(µM)	64.8	70.3	83.1	82.1	13.97				
Phenylalanine (µM)	49.6	57.2	59.2	61.4	6.49				
Ornithine(µM)	106.4	82.0	78.1	74.3	18.36				
Lysine(µM)	127.2	127.4	95.6	119.9	23.29				
Histidine(µM)	61.1	62.9	70.8	68.8	6.40				
Arginine(µM)	160.3	170.6	147.8	132.1	23.78				
Proline(µM)	83.0	70.1	83.9	72.5	10.94				
Total amino acid (µM)	2740	2560	2694	2410	207.3				

<sup>1</sup> No significant effects of diet or diet\*frequency on the daily means SED = standard error of difference

There were no diet\*frequency interactions between the daily means of the concentrations of plasma acetate, insulin or amino acids (Table 4.10). Animals fed the diets hourly had significantly higher daily plasma leucine and isoleucine concentrations than those fed the diets once daily ( $P < 0.1$ ). Daily concentrations of plasma glycine and serine were significantly higher in animals fed the diets once daily than those fed the diets hourly ( $P < 0.01$ ). The difference in the mean daily plasma acetate concentration between the animals fed the SS diet hourly and once daily tended to be less (0.01mM) than the difference between the two groups of animals fed the FF diet (0.84mM). The plasma acetate concentration tended to generally be higher in animals fed the FF diet once daily (1.93mM) compared to those fed the other three dietary treatment groups (1.09-1.19mM), although this was not significant ( $P > 0.1$ ).

Significant correlations between the hourly concentrations of acetate and each of the individual amino acids measured in 3 animals from each of the 4 dietary treatment groups are shown in Table 4.11. The majority of the correlations are negative indicating an inverse relationship in the relative concentrations of the two nutrients, i.e. when one increased the other decreased. It is clear from this table that when the FF diet was fed once daily (FFD) there were highly significant correlations between the hourly concentrations of acetate and most of the individual amino acids in all three animals measured, the majority of which were negative correlations. The hourly concentrations of plasma acetate were also significantly negatively correlated with the hourly total amino acid concentration, in two out of three of the animals fed FFD. Plasma acetate was significantly positively correlated with plasma glutamate concentration in dietary treatment group FFD, in two of the three animals measured ( $P < 0.001$ ).

In contrast, animals fed the FF diet hourly (FFH) showed virtually no significant correlations between the hourly concentrations of acetate and individual or total amino acids measured.

Hourly plasma acetate concentrations were significantly correlated with a number of the individual amino acid concentrations measured in the animals fed the SS diet both hourly and once daily (SSH or SSD). However where correlations between hourly concentrations of plasma acetate and individual or total amino acids

Table 4.11 The effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the significance of the correlations between the hourly plasma acetate concentrations and their respective hourly plasma amino acid concentrations in 3 animals from each dietary group.

Animal Number	DIETARY TREATMENT											
	FFD			FFH			SSD			SSH		
	1	2	3	4	5	6	7	8	9	10	11	12
Aspartate	*(-)	*				**		**(-)				
Threonine	***(-)	***(-)	***(-)					**(-)			*(-)	
Serine	***(-)	***(-)	***(-)					**(-)	*(-)			
Asparagine	***(-)	***(-)	***									*
Glutamate		***									*(-)	*(-)
Glutamine	*(-)				*							
Glycine	***(-)	***(-)	*(-)				*(-)	*(-)	*(-)	**(-)		
Alanine	***(-)	***(-)								*(-)	*(-)	
Citrulline	*(-)	***(-)						**(-)	**(-)	*(-)		
Valine	***(-)	***(-)	*(-)					**(-)				
Methionine	***(-)	***(-)	*(-)					**(-)	*(-)			*(-)
Isoleucine	***(-)	***(-)	***(-)					*(-)			*(-)	**(-)
Leucine	***(-)	***(-)	***(-)					**(-)			*(-)	**(-)
Tryptophan		**(-)										
Tyrosine	**(-)	**(-)						*(-)				**(-)
Phenylalanine	***(-)	***(-)	**(-)					*(-)			*(-)	
Ornithine	***(-)	***(-)						*(-)				
Lysine	***(-)	***(-)	*(-)					*(-)	*(-)			
Histidine	***(-)	***(-)						*(-)	**(-)			
Arginine	***(-)	***(-)	**(-)					*(-)	*(-)		*(-)	*(-)
Proline	***(-)	***(-)						*(-)				
total amino acid	***(-)	***(-)						*(-)				

\*\*\* = P<0.001 \*\* = P<0.01 \* = P<0.05 (-) = negative correlation

Table 4.12 The effects of the two different diets (FF or SS) fed either hourly (H) or once daily (D) on the significance of the correlations between the hourly plasma insulin concentrations and their respective hourly plasma amino acid concentrations measured in 3 animals from each dietary group.

Animal Number	DIETARY TREATMENT											
	FFD			FFH			SSD			SSH		
	1	2	3	4	5	6	7	8	9	10	11	12
Acetate	*	*					*	***	*			
Aspartate	*(-)							*(-)			*(-)	
Threonine	*(-)				**		*	**(-)				
Serine		*(-)			***			**(-)	*(-)			
Asparagine	*(-)	**(-)			***			**(-)				
Glutamate					**						*	
Glutamine												
Glycine								**(-)	*(-)			
Alanine	*(-)				**							
Citrulline								***(-)			*	
Valine					***		*	***(-)				
Methionine			*		***			***(-)				*
Isoleucine	*(-)				***		*	*(-)				
Leucine	*(-)				***		*	*(-)				
Tryptophan					***			**(-)				
Tyrosine			*		**			*(-)				
Phenylalanine					***			*(-)				
Ornithine					**		**	*(-)				
Lysine		*(-)			**		*	**(-)				*
Histidine	*(-)	*(-)			***		*	**(-)				*
Arginine			*		**		*	*(-)				*
Proline	*(-)			*			*	**(-)				
Total amino acid	*(-)				***			**(-)				

\*\*\* = P<0.001 \*\* = P<0.01 \* = P<0.05

(-) = negative correlation

were observed the significance was generally not as high as when the FF diet was fed once daily (FFD).

The hourly concentrations of plasma insulin, measured in 3 sheep from each dietary treatment group, that were significantly correlated with concentrations of plasma acetate, the individual amino acids or the total amino acids is shown in Table 4.12. Between diets there appeared to be no consistent patterns in the significance of the correlations. No amino acid was significantly correlated to insulin in all three animals measured in any of the dietary treatment groups.

One of the three animals measured, in the FFH treated, had a considerable number ( $n=15$ ) of the individual amino acids that were significantly correlated ( $P<0.01$ ) with plasma insulin concentration. All of these correlations were positive. No significant correlations between the plasma insulin and the amino acids in the other two animals in the FFH group (with the exception of one amino acid in one animal) were detected.

There were few significant correlations observed between the plasma concentrations of amino acids and insulin, measured throughout the day in the animals fed dietary treatment SSH. One animal in this group had insulin concentrations that were not correlated with any of the concentrations of amino acids that were measured. The significant correlations of the concentrations of insulin with individual amino acids were not with the same amino acids between animals fed SSH.

Similarly in dietary treatment group FFD there were few significant correlations between the hourly plasma concentrations of insulin and the individual amino acids. No amino acid was consistently significantly correlated in all three animals in the dietary treatment group, FFD.

In treatment group SSD, one animal had plasma insulin concentrations that were significantly correlated with a number of amino acids. All of these correlations were negative. Compared to the other groups, the animals in treatment group SSD had more plasma amino acid concentrations that were correlated with the plasma insulin concentration in more than one animal. These amino acids were threonine, serine, glycine, valine, isoleucine, lysine, proline and arginine.

Plasma acetate concentration was significantly positively correlated with insulin concentration in all three animals in dietary treatment group SSD ( $P<0.05$ )

and in two of the three animals in treatment group FFD ( $P < 0.05$ ). Plasma insulin concentration did not appear to be correlated with plasma acetate concentration in any of the animals in either of the hourly fed treatment groups ( $P > 0.05$ ).

## **4.5 Preliminary discussion**

The current experiment used diets that were formulated to contain similar amounts of OM and N, but which contained ingredients that had different degradation characteristics in the rumen so that OM and N was either released rapidly (approximately 65% released in the first hour) or more constantly throughout the day (approximately 35% released in the first hour). Both diets were fed to growing lambs either once daily or hourly. The aim of the experiment was to investigate the effects of altering the timing of OM degradation in the rumen by diet formulation and by frequency of feeding, on the growth and carcass composition of growing lambs and to find out if the effects that were observed could be accounted for by the pattern of supply of acetate and other metabolites in the blood supplying the peripheral tissues.

The growth rate and carcass weight of animals fed SSD were significantly larger ( $P < 0.05$ ) than those of the animals fed the other dietary treatments. This was probably because the animals fed SSD had a significantly greater deposition of crude protein in the carcass ( $P < 0.05$ ). In addition animals fed SSD tended to have an increased deposition of carcass fat, although this was only significantly greater when compared to that of animals fed FFD ( $P < 0.01$ ). Muscle weights also tended to be heavier in the animals fed SSD, which will have contributed to heavier final liveweights, although this was only significant in the *L.dorsi*. Fat depot weights, however, were not greater in the animals fed SSD. These results would imply therefore, that the increase in final liveweight in animals fed SSD was due to an improvement in the efficiency with which protein rather than fat is synthesised. This was a little surprising as feeding hourly was originally thought to provide a more constant supply of nutrients throughout the day and therefore improve the efficiency with which nutrients are utilised. This apparently does not occur when animals were fed the SS diet as there is a significant increase in the feed conversion efficiency and the growth rate when these animals were fed once daily compared to hourly ( $P < 0.1$ ).

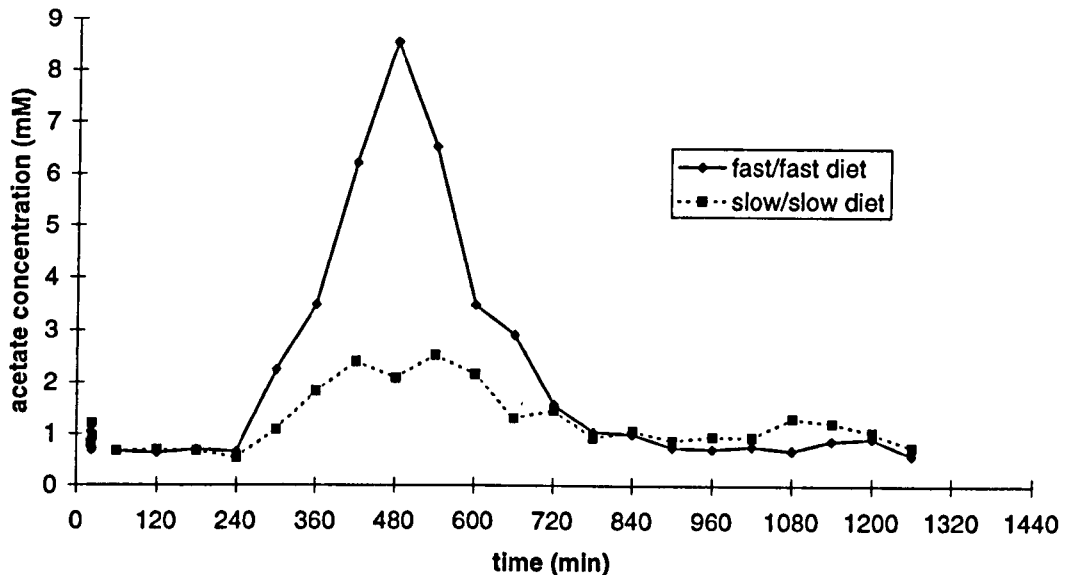
The carcass weights of the animals fed FFD were significantly smaller than those of animals fed FFH and SSD ( $P < 0.1$ ). The weight of the carcasses relative to the final liveweight was significantly smaller in animals fed FFD ( $P < 0.01$ ) compared to those in the other dietary treatment groups, as was shown by the killing out proportion. This was probably due to the animals fed FFD tending to have a higher



gutfill compared to those in the other dietary treatments and so showing an increased final liveweight without an increased carcass weight. The decrease in the carcass weight of animals fed FFD may be accounted for by these animals having significantly less fat deposited in their carcasses compared to those fed FFH or SSD. This would imply therefore that those fed FFD did not utilise the nutrients supplied in the diet as efficiently for growth or deposit fat as efficiently as those fed FFH or SSD. However, there were no apparent differences in the crude protein deposition in the carcasses of animals fed the FF diet either hourly or once daily. In fact, the *V.Lateralis* and the *Semitendinosus* muscles tended to be bigger in the animals fed FFD compared to those fed FFH. This would suggest that the pattern of nutrient supply when animals are fed once daily was more conducive to muscle growth than when exactly the same diet was fed small portions every hour.

Following a meal, degradation of OM in the rumen is likely to occur which is likely to have caused a drop in the pH of the rumen, favouring the fermentation of dietary compounds by acetate producing, cellulolytic bacteria (Kaufmann, 1976). Following the decrease in the pH and an increase in the production of acetate, there is likely to be an enhanced absorption of acetate across the rumen wall due to an increase in the anionic form of acetate (see France & Siddons, 1993) and so one would expect to see an enhanced plasma acetate concentration following once daily feeding. This was seen in the experiment reported in chapter 3 and is also seen in the current experiment. In this experiment the magnitude of the pulse appeared to have been altered by feeding the two different diets (SS or FF) once daily. There was a smaller pulse in the plasma acetate concentration immediately following a single feeding of the SS diet, than the pulse observed in the plasma acetate concentration of animals fed the FF diet once daily. This is highlighted in Figure 4.8, where the hourly acetate concentrations of animals fed either SSD or FFD, are plotted against time.

Figure 4.8 Hourly acetate concentration over a 24 h period in the jugular plasma of sheep fed the fast/fast (n=3) or slow/slow (n=3) diet once daily.



The differences in the elevation of plasma acetate concentration following feeding were likely to be because the OM degraded in the first hour was predicted to be far less (31% of the total OM was predicted to be degraded in the first hour) than when animals were fed the FF diet once daily (62% of the total OM was predicted to be degraded in the first hour). It can be concluded from this experiment that when the rate of degradation of OM in the rumen was more constant throughout the day, as in the hourly fed and the SSD fed animals, there was a more constant hourly concentration of plasma acetate throughout the day compared to when FFD was fed. As protein synthesis requires a large amount of energy, a more constant supply of energy-yielding acetate would be expected to improve the rate of protein synthesis for longer during the day and therefore increase the overall amount of muscle deposited. The muscles of animals fed the SS diet once daily tended to be bigger than those fed the FFD, although this was only significant in the *L.dorsi* muscle ( $P < 0.01$ ). The acetate concentration was more constant in the animals fed SSH compared to that in animals fed SSD, however, the muscles of animals fed SSH tended to be smaller than those fed SSD. This was also seen in the animals fed the FF diet, where those fed once daily with an erratic acetate concentration throughout the day, tended to have the same size or larger muscle depots than those fed hourly. Feeding the FF diet hourly

did not enhance the crude protein content of the carcass either. This implies that those fed once daily utilised the nutrients more efficiently for protein synthesis than those fed the same diets hourly. Nutrients appeared to be utilised more efficiently for growth as was shown by an improved FCE in animals fed once daily compared to those fed hourly ( $P < 0.01$ ). The most efficient group in converting feed to liveweight gain were the animals fed SSD and the least efficient, the animals fed SSH. This suggests that a more constant level of acetate was not solely responsible for a higher rate of muscle deposition, although it may have been when comparing the animals that were fed once daily.

The hourly total concentrations of plasma amino acids appeared to decrease for a longer period of the day following feeding when the SS diet was fed once daily. The leucine concentration appeared to be at a lower level, for a longer period throughout the day in animals fed the diets daily compared to those fed the diets hourly. The daily concentrations both leucine and isoleucine were significantly lower in the animals fed the diets once daily compared to animals fed the same diets every hour. Both leucine and isoleucine are branched chain amino acids which are known to play a significant role in regulating protein metabolism (see Haymond *et al.*, 1980). This may mean that more amino acids are being taken up by the tissues for protein synthesis, with an increase in the availability of acetate, as an energy source. This may consequently result in a tendency for a greater amount of protein deposited in the carcasses and in the muscles in the once daily fed animals compared to those fed the same diets hourly.

In addition, the daily insulin concentration tended to be higher in animals fed SSD compared to those fed the other dietary regimes, although this was not significant ( $P > 0.1$ ). This appears to have been caused by the elevation in the concentration of insulin lasting longer in the animals fed SSD. The effects of insulin on conserving protein within the muscle and amino acid utilisation are to be discussed more fully in chapter 6 (see section 6.4), however it is possible that longer elevation in insulin concentration observed in the animals fed SSD was related to a longer decrease in the concentrations of amino acids. This may perhaps be due to insulin inhibiting protein degradation, resulting in a protein turnover with a greater synthesis than degradation and consequently more amino acids being taken up by the animal than released, resulting in a decrease in amino acid concentration. The correlations

between hourly concentrations of plasma amino acids with insulin were negative and highly significant in one particular animal fed the SSD diet. This relationship indicates that as the insulin increased following feeding there was a drop in the amino acid concentration, which may coincide with less muscle protein being degraded. This would correspond with data from Grizard *et al.* (1987) who showed that following an insulin injection, essential plasma amino acids decrease and that there was a close correlation with the drop in amino acids and the incorporation of the same amino acids in muscle tissue. Consequently this may contribute to the higher growth rate and final liveweight, and a generally higher muscle deposition in the *L.dorsi* and *Semitendinosus*, observed in the animals fed SSD. Standard error of the means were large, particularly with insulin concentrations of the animals fed SSD, as were shown by the large error bars on the concentration profiles.

The fat depots of the animals with a more constant supply of acetate in the peripheral plasma throughout the day (i.e. SSD, SSH, FFH) tended to be relatively similar, but the group of animals with a very sudden pulse of acetate following feeding (i.e. the animals fed FFD) had the smallest fat depots of all the groups ( $P < 0.1$ ). This may be due to an insufficient amount of glucose being produced from the liver to produce NADPH which was necessary to synthesise fatty acids and utilise the acetate. Glucogenic amino acids were significantly decreased with the increase in plasma acetate concentrations following feeding (as were shown by the significant correlations of alanine and glycine to acetate in 2 of the three animals measured  $P < 0.001$ ) This indicates that there is possibly an attempt by the animal to produce glucose from the liver for the production of NADPH. Consequently the rapid pulse in acetate following feeding was likely to be utilised for other purposes other than fat synthesis. This is discussed further in Chapter 6.

There were no statistically significant differences in the mean daily plasma concentrations of acetate or individual amino acids between diets, which suggests that over 24 h the overall concentrations in these metabolites were unaffected by the predicted differences in temporal release of N and OM components from the diet into the rumen. This is perhaps not surprising as the OM and N content of the diets were similar, although diet did have an effect on food intake ( $P < 0.1$ ) and therefore energy

and OM intakes were not exactly the same between diet groups. Although not significant ( $P>0.1$ ), the mean daily acetate concentration tended to be higher in animals fed the FF diet once daily than in the other dietary treatments. This was probably increased due to the large surge in acetate in the jugular plasma, following feeding. Considering the acetate concentration in the FFD treated animals was increased approximately 8 times above the original basal acetate concentration, the daily mean was not greatly elevated when compared to the SSD, SSH or FFH fed animals. This may have been due to the voluntary food intake in the animals fed FFD being lower than that of animals in the other dietary treatment groups. Had the food intakes been the same between treatment groups, the daily mean concentrations of plasma acetate may have been increased in animals fed FFD. The growth rate of the animals may also have been improved, with an increasing amount of ME and MP consumed, which would have implied that those animals fed once daily grew better than those fed hourly. The feed conversion efficiency showed that animals fed the diets once daily utilised the nutrients more efficiently for growth than those fed hourly. Irrespective of feed intakes, the pattern of acetate concentration throughout the day was clearly affected by the diet and pattern of feeding whilst the daily mean of the acetate concentration was similar between dietary treatment groups.

The daily acetate concentration did not differ between dietary treatment groups, but the muscles and the deposition of crude protein in the carcass tended to be greater in animals fed the diets once daily. This would suggest that the differences in the patterns of the amino acids and acetate may contribute to the efficiency with which protein is synthesised. Millward (1985) suggested that there was an increase in the synthesis of protein following feeding which balanced out degradation and losses in the post-absorptive state. This is discussed further in chapter 6. Acetate as an energy source may be influencing the efficiency with which protein is synthesised throughout the day at the tissues, which as a result, is decreasing the concentrations of amino acids in the plasma. If the amino acids are diminished for longer, the efficiency with which protein is synthesised may be increased.

To summarise, it appears that a large pulse in acetate following feeding may contribute to a decrease in fat deposited in the animal. However, surprisingly a less constant pattern of acetate concentration (i.e. a pulse in the concentration of acetate

following feeding) may be related to a more enhanced deposition of crude protein deposition and muscle growth.

This may mean that during a pulse in acetate following feeding, there was an increase in the energy available to the tissues, which increased the synthesis of protein following feeding and as a result enhanced the overall deposition of muscle in these animals. In animals fed SSD, there appeared to be a more constant, but elevated pulse in acetate throughout the day, which have led to an increase in protein synthesis for a longer period during the day. Acetate is a significant energy source to the ruminant for metabolic processes. Amino acids are essential for protein synthesis. It would appear from the studies shown in chapters 2 and 3 that the changes in patterns of the supply of plasma acetate and amino acids to the tissues may be a reason for differences in the efficiency of protein synthesis in the peripheral tissues of the growing ruminant. It is necessary to investigate further the effects of changing the plasma acetate concentration on protein synthesis. The remaining experimental work in this thesis aimed to study the effects of increasing the plasma acetate concentration by infusing sodium acetate intravenously, on estimates of leucine oxidation and whole-body protein synthesis.

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## CHAPTER 5

# THE EFFECTS OF INCREASING THE PLASMA ACETATE CONCENTRATION ON LEUCINE METABOLISM IN THE GROWING LAMB

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### 5.1 Introduction

Tissue protein synthesis is a highly energetic process dependent on a nitrogen source in the form of amino acids. Acetate is a major energy source to the ruminant. The experiments reported in chapters 3 and 4 show that when differences in the hourly plasma acetate and amino acid concentration were observed throughout the day, differences were also seen in the growth of the animals, the fat : protein ratio and the crude protein content of the muscle and carcass. It is speculated that changing patterns in metabolite concentrations may contribute to the changes observed in growth and carcass composition. The following experiment described in this chapter aimed to examine the effect of raising, plasma acetate, an energy source to the rumen, by intravenously infusing sodium acetate, on estimates of whole body protein synthesis.

### 5.2 Estimating amino acid oxidation and whole-body protein synthesis

The isotope dilution technique, uses isotopic tracers to label metabolic pools so that metabolic events can be monitored and quantified *in vivo*. A basic general assumption of most tracer methodology is that there is no recycling of tracer and it is necessary to assume, for the period of the experiment, that whatever leaves the metabolic pool is irreversibly lost. The irreversible loss rate procedure is a technique that has been adopted to quantify whole-body metabolic processes, such as amino acid oxidation and protein synthesis, using the isotope dilution technique. Exogenous application of tracer, therefore, is considered the only route of entry of the tracer into the metabolic pool and consequently calculations to quantify the passage of tracer leaving the pool for use in metabolic processes can be made.

The principle of isotope dilution for estimating whole-body protein synthesis and amino acid oxidation originates from the idea that the animal body contains a

series of pools or compartments which each contain identical molecules or substrates. Plasma, intracellular- and extracellular- fluid are examples of such body pools.

Molecules can enter and leave these pools, but when the pool is of a constant concentration, the input of molecules into a pool equals the output of the molecules from the pool. This dynamic equilibrium is known as 'steady state' (Shipley & Clarke, 1972). Using this principle, when an isotopic tracer is continuously infused into an animal that is in metabolic steady state, the tracer will leave the pool that it is being infused into at a rate proportional to the abundance of tracer, relative to tracee in the body pool. When the infusion of a tracer begins, the relative abundance of the tracer in the pool will be lower than the abundance in the infusate. Consequently the proportion of tracer relative to the tracee leaving the pool will be at a lower rate than the rate with which it appears in the pool, as initially, the abundance of the tracer relative to tracee in the pool is low. As isotope is infused, the abundance in the body pool increases and so the relative concentration of tracer in the pool increases. Eventually, at some point in the infusion, the rate with which the tracer is lost from the pool (rate of disappearance,  $R_d$ ) will be at the same rate that it enters the pool (entry rate,  $R_a$ ), a rate which is also known as the substrate flux ( $Q$ ) or turnover rate (see Equation 5.1). When this is achieved the enrichment of the body pool reaches a plateau and this state is known as isotopic equilibrium (Wolfe, 1992). To state that the isotope exit rate is equal to the entry rate it must be assumed that the isotope is irreversibly lost and is not recycled back into the pool. Using this irreversible loss procedure, the  $R_d$  is equal to the rate of entry of the tracer in the tracee pool as a proportion of the relative amounts of tracer to tracee in the body pool.

The  $R_d$  of the metabolite is, in the case of amino acids, assumed to be the rate at which the metabolites are either incorporated into the tissues by protein synthesis or lost from the pool by oxidation (Waterlow *et al*, 1978). The  $R_d$  of a pool can be measured when there is no change in the pool size, i.e. the pool is in steady state. The  $R_a$  of the amino acid pool is the rate of degradation of body proteins and the rate of amino acid entry by dietary intake (see Equation 5.1).

Metabolic steady state of the animal is assumed to be achieved when a healthy animal is fed continuously. In experimental procedures animals are usually fed small amounts hourly which is generally considered satisfactory to make estimates of  $R_d$ .



An estimate of protein synthesis can be made as the proportion of the measured amino acid  $R_d$  that is not leaving the pool by oxidation (Equation 5.1).

Equation 5.1 adapted from Loble (1993)

Under steady state conditions :

$$R_d = R_a = \text{flux rate (Q)}$$

$$R_d = \text{protein synthesis} + \text{amino acid oxidation}$$

$$R_a = \text{protein degradation} + \text{dietary intake}$$

$$= \frac{I}{S}$$

where :  $I$  = rate of infusion ( $C_i$  / h or atom % excess / h)

$S$  = plateau isotopic activity ( $C_i$  / mmol or atom % excess / mmol)

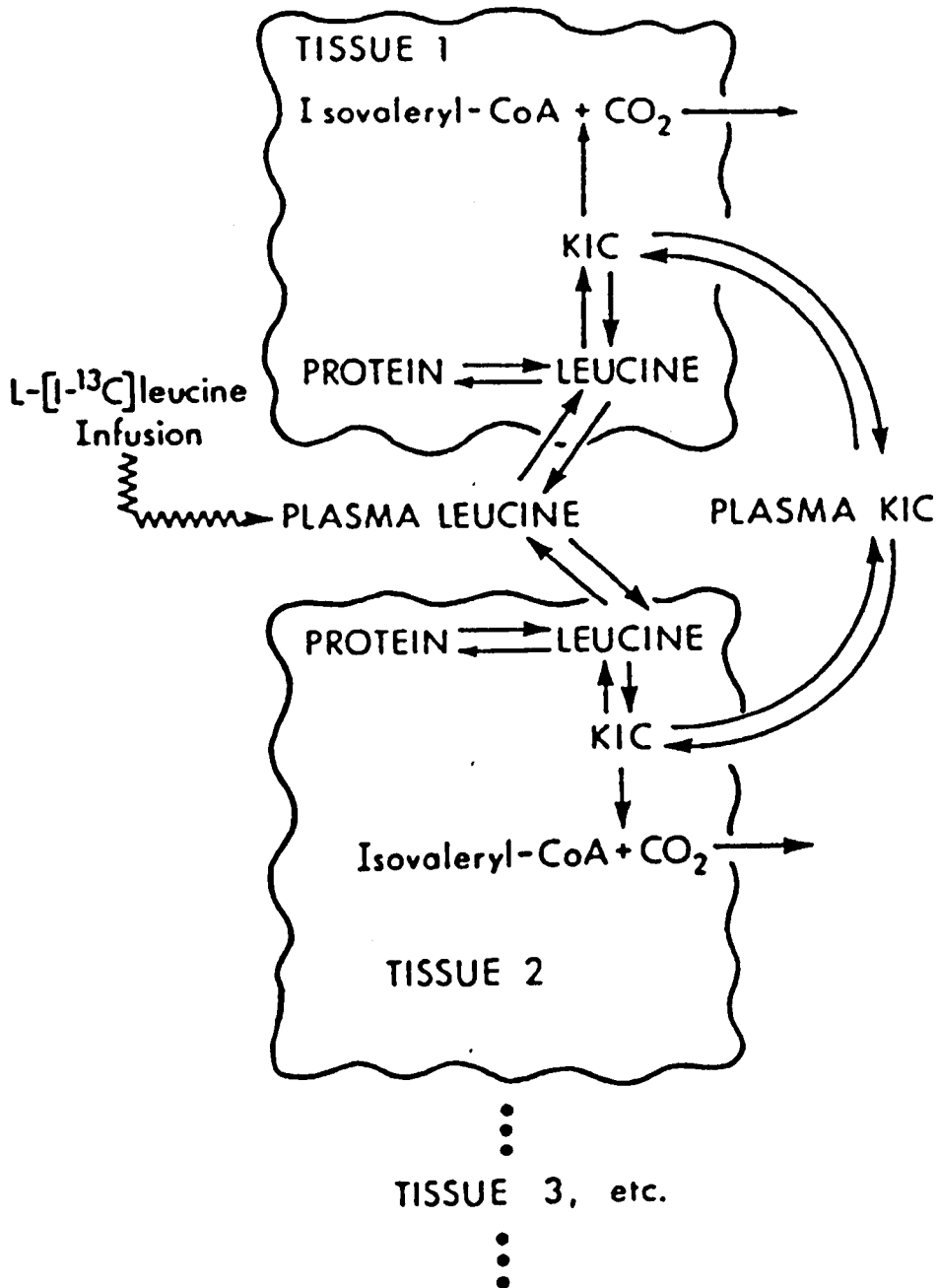
In order to calculate the rate of amino acid oxidation in the whole body, it must be assumed that the animal is in steady state. The calculation of absolute rate of substrate oxidation also requires the determination of total  $CO_2$  expired by oxidation of all substrates. Total body  $CO_2$  production in an animal can be estimated by isotopically labeling the  $CO_2$  pool of an animal assumed to be in steady state, with  $NaH^{13}CO_3$ . The  $R_a$  of the  $CO_2$  can then be calculated in the same way as for calculating the  $R_a$  of any substrate using the ILR procedure, as described previously in this chapter (Equation 5.1). When in isotopic equilibrium, the entry rate of the  $CO_2$  is equal to the exit rate from the pool and consequently this represents the total body  $CO_2$  production in an animal.

The proportion of a substrate that is oxidised can be determined from the isotopic enrichment of the  $CO_2$  expired in breath during the continuous infusion of an isotope that labels the substrate pool, as a proportion of the isotopic enrichment of the substrate in the pool. The proportion of substrate oxidised can then be used to calculate the overall oxidation rate of the substrate by multiplying it by the calculated total  $CO_2$  production.

To calculate the  $R_d$  of an amino acid pool in the plasma, the isotopic enrichment of the amino acid in the plasma pool can be measured, following intravenous infusion of a tracer until isotopic equilibrium is reached, and the  $R_d$  calculated using the ILR procedure (Equation 5.1). The disadvantage with this method is that the plasma amino acid pool is not the true precursor amino acid pool for protein synthesis. The aminoacyl-tRNA in the intracellular pool are the true precursors. As the tracer is infused into the plasma pool and unlabelled protein is degraded into the intracellular pool, the isotopic activity in the plasma pool will generally be higher than in the diluted secondary intracellular pool. As a result the irreversible loss rate, of the pool will be less when measuring the plasma pool than when measuring the intracellular pool (Equation 5.6) and consequently protein synthesis will be underestimated (see Loble, 1993).

If the amino acids are transaminated, the  $R_d$  of the amino acids in the true precursor pool can be estimated indirectly, by measuring the  $R_d$  of labeled  $\alpha$ -ketoacids in the plasma pool. All labeled branched-chain amino acids e.g. leucine in the intracellular pool are either passed back into the plasma pool or are transaminated to  $\alpha$ -ketoacids. The  $\alpha$ -ketoacids pass into the plasma pool or are oxidised (See Figure 5.1)

Figure 5.1 A multi tissue model of whole-body leucine metabolism, showing leucine uptake by tissues and its intracellular transamination to KIC (Matthews *et al.*, 1982).



### 5.2.1 Choice of Isotope

Tracers are molecules that contain at least one atom that is isotopically labeled. An atom that is isotopically labeled contains the same number of protons as an unlabelled atom and so the atomic number is the same. The difference occurs in the number of neutrons that exist within the atom, and so the atomic weight of the isotope differs, but the overall charge of the atom is unchanged. Chemical properties

of atoms are generally determined by the atomic number and so isotopes of the same atomic number i.e. the same element, but with different atomic weights react, similarly.

The basic elements that comprise the majority of metabolites and tissues are carbon, nitrogen, oxygen and hydrogen and all exist as different isotopes in the natural environment e.g. living organisms and atmospheric gases. The isotopes can either be stable or unstable as radioactive atoms depending on the neutron to proton ratio of the nuclei of the atoms. If the nuclei is unstable the neutron to proton ratio is outside the limits of stability and there is a mutual proton repulsion which causes a nuclear reaction and an emission of radioactivity. The larger the atomic number, the more significant this reaction is and consequently the faster the radioactivity is emitted. Stable isotopes, in contrast, have nuclei which contain neutrons and protons within a narrow limit and so the nucleus remains stable (Thornburn, 1972).

In metabolic studies the choice of isotopically labeled molecules is important as it must reflect the passage of particular metabolites through pools and so the molecule chosen must be representative of the pathways of metabolism.

Isotopically labeled branched-chain amino acids (BCAA) leucine, isoleucine and valine are considered suitable for protein metabolism studies (Haymond *et al.*, 1980), although any essential amino acid can theoretically be used. The ruminant is not able to synthesise BCAA and consequently the only source with which these can move into the plasma pool is either by dietary intake or catabolism of endogenous proteins. Another advantage in using BCAA for protein metabolism tracer studies is that they are thought to play a direct role in the regulation of protein metabolism and they play a significant role in supplying nitrogen to other non-essential amino acids (Haymond *et al.*, 1980).

Stable isotopes have the advantage over radio-isotopes in that they are less hazardous to the user. In addition, they avoid the need for specialised disposal of radioactive wastes, including faeces, urine and carcasses. This is a particular advantage with large animal work, where the amount and the containment of waste can present significant difficulties.

In the work described in this thesis, L-[1-<sup>13</sup>C]-leucine, a stable isotopically labeled, BCAA, was used to measure the flux and oxidation of the leucine pool. It is

important that the first carbon in the leucine chain is labelled, for quantifying leucine oxidation as this is the first carbon to be released as CO<sub>2</sub> during oxidation.

In the experiment reported in this chapter, L-[1-<sup>13</sup>C]-leucine was continuously infused, intravenously, to label the plasma leucine pool enabling a measurement of leucine flux through this pool. Infusion of NaH<sup>13</sup>CO<sub>3</sub> was carried out to label the CO<sub>2</sub> pool which enabled total body CO<sub>2</sub> to be estimated and consequently an estimate of leucine oxidation, to be made. The proportion of leucine disappearing from the plasma pool which was not oxidised, was assumed to be incorporated into protein. Thus an estimate of whole-body protein synthesis was obtained. This chapter describes general methodology for infusate preparation and infusions and the calculations that were used to measure leucine oxidation and to estimate whole-body protein synthesis, indirectly by using the isotopic enrichment of plasma KIC. Methodology and results from preliminary studies are shown prior to the report of the experiment that was carried out to study the effects of sodium acetate infusion on measurements of leucine oxidation and estimates of whole-body protein synthesis.

## **5.3 General Methodology**

### **5.3.1 Method of infusion**

Two catheters were inserted in the jugular vein either side of the neck. One was used for entry of the infusate, the other was used for blood sampling. Flow switches at the end of each catheter enabled the site to be opened or closed during infusion or sampling. The infusion catheter was placed on the right side of the neck, 5 cm deeper into the vein than the sampling catheter, in the left side of the neck. Catheters were kept patent as described in Section 2.1 prior to and during the experiment.

Infusates were administered through fine bore polythene tubing (0.86mm ID, Portex; Smiths Industries, Kent) with the aid of a peristaltic infusion pump (Minipuls 2; Gilson, France). Peristaltic tubing (1.14mm ID, red tag; Anachem, Luton, Bedfordshire) was placed in the infusion pump and connected to the polythene tubing with cut down 19G x 1 inch needles and connector pieces (male luer-male luer double cone; Vygon, Laboratoires Pharmaceutiques, Ecoen, France). The infusion line was then attached to the flow switch (16G UniversalFloSwitch; Ohmeda, Swindon,

Wiltshire) of the infusion catheter. Where two infusates were simultaneously administered to the animal, two infusion lines were run through the same infusion pump and a Y-piece attached at the end of the lines to connect to the flow switch of the infusion catheter.

Before connecting the infusion line to the catheter the infusate was run through the lines to the point of exit to obtain the exact time of entry into the cannula and also to prevent air bubbles being pumped into the blood stream.

Infusates in all experiments reported in this chapter were infused at approximately 0.7ml/min. Infusates were weighed, prior to and after the infusion, to establish the exact infusion rate for each animal.

### 5.3.2 Continuous infusion of [1-<sup>13</sup>C]-Leucine

L-[1-<sup>13</sup>C]-leucine (MassTrace Ltd., Massachusetts, USA.; 99 atom %) was continuously intravenously infused to measure the rate of appearance of leucine in the plasma pool and to calculate the rate of leucine oxidation in the animal.

[1-<sup>13</sup>C]-leucine was continuously infused into the animal at a rate of approximately  $7\mu\text{mol/kg}^{0.75}$  LW /h. The L-[1-<sup>13</sup>C]-leucine infusate was prepared in sterile, pyrogen-free saline bags (0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). The solid L-[1-<sup>13</sup>C]-leucine was initially dissolved in a small volume of saline solution (50ml) that had been removed from the bag and the resulting solution was returned to the bag through a sterilising syringe filter (0.2 $\mu\text{m}$  Minisart syringe filter; SartoriusGmbH, Gottingen, Germany).

It was necessary to prime the leucine pool immediately prior to the continuous infusion of L-[1-<sup>13</sup>C]-leucine, to ensure that isotopic equilibrium of the leucine pool was reached rapidly. Previous work within these laboratories (Greathead, 1997) has shown that the L-[1-<sup>13</sup>C]-leucine priming dose should be equal to the amount of L-[1-<sup>13</sup>C]-leucine that is infused in one hour during the continuous infusion. Consequently the L-[1-<sup>13</sup>C]-leucine priming dose was prepared by dissolving the solid L-[1-<sup>13</sup>C]-leucine ( $7\mu\text{mol/kg}^{0.75}$  LW) in approximately 20ml saline solution (0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). The priming dose was injected over a period of 20 seconds directly into the right jugular catheter through a sterilising syringe filter (0.2 $\mu\text{m}$  Minisart syringe filter; SartoriusGmbH, Gottingen, Germany)

immediately before a priming dose of  $\text{NaH}^{13}\text{CO}_3$  (see below) and the continuous infusion of L-[1- $^{13}\text{C}$ ]-leucine commenced.

The bicarbonate pool is large and consequently has a slow turnover. In order to ensure that the expired  $^{13}\text{CO}_2$  in the breath reaches a rapid and stable plateau it was necessary to prime the bicarbonate pool in addition to the leucine pool. The bicarbonate pool was primed prior to the continuous infusion of L-[1- $^{13}\text{C}$ ]-leucine, immediately after the leucine pool has been primed. The priming dose was calculated using equation 5.2

$$\text{NaH}^{13}\text{CO}_3 \text{ priming dose prior to continuous leucine infusion } (\mu\text{g}/\text{kg}^{0.75} \text{ LW}) \\ = I \times 85 \times 0.2$$

where - I is the infusion rate of leucine ( $\mu\text{g}/\text{kg}^{0.75} \text{ LW}$ ) in one minute,

- 85 is the optimum ratio of the priming dose as a proportion of the infusion rate and

- 0.2 is the proportion of leucine uptake that is oxidised.

Equation 5.2 (source : Wolfe, 1992)

The  $\text{NaH}^{13}\text{CO}_3$  priming dose was prepared by dissolving the solid  $\text{NaH}^{13}\text{CO}_3$  in approximately 20ml saline solution (0.9% (w/v) NaCl). The priming dose was injected over a period of 20 seconds into the right jugular catheter through a sterilising syringe filter (0.2 $\mu\text{m}$  Minisart syringe filter) immediately before the continuous infusion of L-[1- $^{13}\text{C}$ ]-leucine.

### 5.3.3 Continuous infusion of $\text{NaH}^{13}\text{CO}_3$

$\text{NaH}^{13}\text{CO}_3$  (MassTrace Ltd., Massachusetts, USA.; 99 atom %) was continuously infused to measure the total-body  $\text{CO}_2$  entry rates. These infusions were carried out using the same animals following measurements made on leucine flux. It was essential to ensure that the isotopic enrichment had returned to background levels, so that the enrichment of the previous infusion did not interfere with the enrichment of this infusion. Consequently total-body  $\text{CO}_2$  measurements were made two days after the  $^{13}\text{C}$  labeling of the leucine pool. The  $\text{NaH}^{13}\text{CO}_3$  was infused at a rate of  $13\mu\text{mol}/\text{kg}^{0.75} \text{ LW} / \text{h}$  and was prepared in sterile, pyrogen-free saline bags (0.9%

(w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). The solid  $\text{NaH}^{13}\text{CO}_3$  was initially dissolved in a small volume of the saline solution (50ml) that had been removed from the saline bag. The resulting solution was returned to the bag through a sterilising syringe filter (0.2 $\mu\text{m}$  Minisart syringe filter).

To ensure that the bicarbonate pool reached isotopic equilibrium in as short a time as is possible, it was necessary to prime the pool immediately before the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$ . The priming dose for the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$  was calculated as shown in Equation 5.3

$$\text{NaH}^{13}\text{CO}_3 \text{ priming dose prior to continuous NaH}^{13}\text{CO}_3 \text{ infusion } (\mu\text{g}/\text{kg}^{0.75} \text{ LW}) \\ = I \times 85$$

where - I is the infusion rate of  $\text{NaH}^{13}\text{CO}_3$  ( $\mu\text{g}/\text{kg}^{0.75} \text{ LW}$ ) in one minute,  
- 85 is the ideal ratio of the priming dose as a proportion of the infusion rate.

Equation 5.3 (Source : Wolfe, 1992)

The  $\text{NaH}^{13}\text{CO}_3$  priming dose was prepared by dissolving the solid  $\text{NaH}^{13}\text{CO}_3$  in approximately 20ml saline solution (0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). The priming dose was injected over a period of 20 seconds directly into the right jugular catheter through a sterilising syringe filter (0.2 $\mu\text{m}$  Minisart syringe filter) immediately before the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$ .

#### 5.3.4 Sodium acetate and sodium chloride infusates

Sufficient sodium acetate (4.4M) was prepared on the day of the first infusion for use during the whole experiment. This ensured exactly the same concentration of sodium acetate was used as the experimental treatment. Sodium acetate was dissolved in sterile saline solution (0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). Sufficient solution for each animal used, for each infusion period, was measured out and the pH corrected to 7.4 with HCl (1M). The solution was filter sterilised for intravenous infusion by passing through a bottle top filter (Falcon 7111 0.22 $\mu\text{m}$ ; Becton Dickenson, New Jersey) and collecting the filtrate into a sterile bottle by vacuum. Sodium acetate solution that had been prepared in advance for infusion



periods that were to be carried out within 7d, was sterilised by passing through a bottle top filter (Falcon 7111 0.22 $\mu$ m) and stored at 4°C. On the day of infusion the solution was brought to room temperature, the pH corrected to 7.4 with 1M HCl, and the solution re-sterilised by filtering through a bottle top filter into a sterile infusate bottle.

Sodium chloride infusate was freshly prepared on the day of infusion. Sodium chloride (4.4moles/ litre saline) was dissolved in sterile saline solution (0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk). The solution was pH corrected to 7.4 using potassium hydroxide (5M) and made sterile by passing through a sterile filter (0.2 $\mu$ m membrane filter; Gelman Sciences Inc., USA) by peristaltic pump (H.R. Flow Inducer; Watson Marlow Ltd, Falmouth, England)

## **5.4 Calculations**

The calculations shown here were used in both the initial studies and the experiment described later in this chapter.

### **5.4.1 Isotopic enrichment**

Measurement notation for the isotope enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of the breath samples analysed on an isotope ratio mass spectrometer (Europa Scientific, Crewe; see section 2.4 for methodology) was atom percent (AP). Atom percent excess (APE) is a measure of the isotope enrichment in excess of natural abundance. This was calculated by subtracting the background atom percent value, measured in samples taken before the plasma pool was isotopically enriched, from the enriched atom percent value.

Measurement notation for the isotopic enrichment of the plasma samples analysed on the GC/MS (Hewlett Packard, Stockport Cheshire; see section 2.2.4 for methodology) using SIM was also AP. APE was calculated using Equation 5.4, where  $R_e$  and  $R_n$  are the ratios of heavy to light isotope ( $^{13}\text{C}/^{12}\text{C}$ ) for plasma samples that had been enriched and plasma samples of natural enrichment (background), respectively.

$$APE = \left( \frac{(R_e - R_b)}{((R_e - R_b) + 1)} \right) \times 100$$

Equation 5.4 (Wolff, 1992)

#### 5.4.2 Plasma entry rates

All substrate rate calculations were dependent on the substrate pool being in isotopic equilibrium ('steady state'). Isotopic equilibrium of the total CO<sub>2</sub> and the leucine pools was achieved in both within 180 min, as was demonstrated in the initial studies carried out prior to the experiment. At this point it was assumed that the entry rate of the substrate was equal to the exit rate (rate of disappearance, Rd ; Ra = Rd).

The entry rate (Ra, rate of appearance in the body pool) of the total body CO<sub>2</sub>, measured during the primed continuous infusion of NaH<sup>13</sup>CO<sub>3</sub>, was calculated as shown in Equation 5.5, where Ra<sub>CO2</sub> is the rate of appearance of total body CO<sub>2</sub>, *F* is the infusion rate (μmol/min/kg<sup>0.75</sup> LW) of the tracer (NaH<sup>13</sup>CO<sub>3</sub>) and APE<sub>CO2</sub> is the enrichment of the <sup>13</sup>CO<sub>2</sub> in the breath during the primed continuous infusion of NaH<sup>13</sup>CO<sub>3</sub> at isotopic equilibrium.

$$Ra_{CO2} (\mu\text{mol}/\text{min}/\text{kg}^{0.75} \text{ LW}) = \frac{F}{(APE_{CO2}/100)}$$

Equation 5.5 (source : Wolfe, 1992)

The Ra of leucine (Ra<sub>KIC</sub>) was calculated from the primed continuous infusion of [1-<sup>13</sup>C]-leucine as an indirect measure using the measured isotopic enrichment (<sup>13</sup>C/<sup>12</sup>C) of KIC (see section 5.2). The entry rate of the plasma KIC pool was assumed to represent the flux of leucine from the true precursor pool (intracellular pool) and was calculated using Equation 5.6; where *F* is the infusion rate (μmol/min/kg<sup>0.75</sup> LW) of the tracer ([1-<sup>13</sup>C]-leucine) and APE<sub>KIC</sub> is the isotopic enrichment (APE) of the plasma KIC pool at isotopic equilibrium (Wolfe, 1981) and APE<sub>i</sub> is the isotopic enrichment (APE) of the tracer infused, which was assumed to be 99%.

$$Ra_{KIC} (\mu\text{mol}/\text{min}/\text{kg}^{0.75} \text{ LW}) = F \times \left( \left( \frac{APE_i}{APE_{KIC}} \right) - 1 \right)$$

Equation 5.6 (Source : Wolfe 1981)

#### 5.4.3 Leucine oxidation Rate

The rate of leucine oxidation was calculated indirectly by estimating oxidation of the intracellular leucine precursor (KIC) pool. Equation 5.7 was used where  $APE_{KIC}$  is the isotopic enrichment of the plasma KIC pool at isotopic equilibrium. The excretion of  $^{13}\text{CO}_2$  is a measure of the rate of production of  $\text{CO}_2$  as a result of leucine oxidation and is calculated as shown in Equation 5.8 where  $APE_{\text{CO}_2}$  is the isotopic enrichment of the expired  $\text{CO}_2$  at isotopic equilibrium, measured during the primed continuous infusion of leucine and  $Ra_{\text{CO}_2}$  is the rate of appearance of total body  $\text{CO}_2$  (Wolfe, 1992).

$$\text{Oxidation rate } (\mu\text{mol}/\text{min}/\text{kg}^{0.75} \text{ LW}) = \frac{\text{CO}_2 \text{ excretion}}{APE_{KIC}}$$

Equation 5.7 (Wolfe, 1992)

$$\text{CO}_2 \text{ excretion } (\mu\text{mol}/\text{min}/\text{kg}^{0.75} \text{ LW}) = APE_{\text{CO}_2} \times Ra_{\text{CO}_2}$$

Equation 5.8

#### 5.4.4 Whole Body Protein Synthesis Rate

The rate of whole-body protein synthesis ( $\text{g}/\text{kg}^{0.75} \text{ LW}/\text{d}$ ) was calculated using Equation 5.9, where  $Ra_{KIC}$  is the Ra of the leucine precursor pool (KIC), Leu Ox is the estimated rate of leucine oxidation and 0.068 is the proportion of leucine in the carcass protein of sheep (Reeds & Loble, 1980). It was assumed that with the animal in 'steady state' the  $Ra_{KIC}$  represents the rate of disappearance of leucine from the true precursor intracellular leucine pool. Multiplication by  $131.2 \times 10^{-6}$  converts the

μmoles of leucine into grams and by 1440 converts the rate from minutes to days (Krishnamurti & Janssens, 1988).

$$\text{Whole-body protein synthesis (g/kg}^{0.75}\text{LW/d)} = \left( \frac{(\text{Ra}_{\text{KIC}} - \text{Leu Ox})}{0.068} \right) \times (131.2 \times 10^{-6}) \times 1440$$

Equation 5.9 (Krishnamurti & Janssens, 1988)

## **5.5 Preliminary Studies**

Pilot studies were carried out prior to the main experiment to check that the concentrations of the priming doses and of the leucine,  $\text{NaH}^{13}\text{CO}_3$  and sodium acetate infusates were correct. Problems encountered with the experimentation during the preliminary studies are described and the solutions that overcame these problems were used for the experiment to measure the effects of sodium acetate infusion on estimates of amino acid utilisation.

### **5.5.1 Methodology**

One Charolais-cross wether weighing 30kg was acclimatised to hourly feeding, using an automatic feeder, for 10 days prior to an initial pilot study. The animal was fed the 'asynchronous' diet, as described in chapter 3 (see Table 3.1) and the total daily ration calculated on a liveweight gain basis, to meet the ME requirements for a liveweight gain of 150g/day (see section 3.3.1; AFRC, 1993). The animal was held in a metabolism crate 5 days prior to the pilot study. Catheters were inserted into the jugular vein of both the left and right side of the neck 48 hours prior to infusing (section 5.3.1). The building that held the animal was continually illuminated and animal had free access to water and mineral licks at all times.

On the first day of the experiment, the animal was continuously intravenously infused with L-[1- $^{13}\text{C}$ ]-leucine at a rate of 0.7ml/ min for 10 h to determine the plasma leucine entry rate and the rate of leucine oxidation (see section 5.3 for details of leucine infusate and the infusion). Saline (0.9% (w/v) NaCl) was simultaneously infused, with the L-[1- $^{13}\text{C}$ ]-leucine, at a rate of 0.7ml/ min (i.e. 0.15mmoles/min), via a separate infusion line for the first 6 h of the infusion (see section 5.3.2 for method of

infusion). Immediately before the infusion commenced, the leucine and the bicarbonate pools were primed with L-[1-<sup>13</sup>C]-leucine and NaH<sup>13</sup>CO<sub>3</sub> to increase the rate at which the expired <sup>13</sup>CO<sub>2</sub> reached equilibrium (see section 5.3.2 for details of priming doses). After 6 h of infusion, the L-[1-<sup>13</sup>C]-Leucine infusion continued the saline infusion was replaced by an infusion of sodium acetate at a rate of 3mmol/min for 2 h. After 2h, the infusion of the sodium acetate terminated, and the saline (0.9% (w/v) NaCl) infusate was restarted and continued for a further 2 h. After a total of 10h of infusion, the pump infusing the L-[1-<sup>13</sup>C]-leucine and saline was stopped. Saline was infused, so that when the treatment infusate of sodium acetate commenced, there was no change in the volume of fluid that was entering the animal body. This therefore, excluded volume as a confounding factor in treatment effects that were observed.

Two days later, the Ra of the total body CO<sub>2</sub> was measured by labeling the CO<sub>2</sub> pool by continuous intravenous infusion of NaH<sup>13</sup>CO<sub>3</sub> for 10 h (see section 5.3.3 for details of the infusate and the method of infusion). Simultaneous continuous infusions of NaH<sup>13</sup>CO<sub>3</sub> and saline (0.9%; 0.7 ml/min) were started immediately following the priming of the bicarbonate pool with NaH<sup>13</sup>CO<sub>3</sub> (see section 5.3.3 for details). In the same manner as for the L-[1-<sup>13</sup>C]-leucine infusion, the NaH<sup>13</sup>CO<sub>3</sub> continued to be infused but the saline infusate was exchanged for sodium acetate infusate 6h after the start of the infusion. The sodium acetate was infused at a rate of 3mmol/min, for 2 h, before stopping and the saline infusion recommencing for a further 2 h.

Breath and blood samples (for details of collection see sections 2.3 and 2.1 respectively) were taken from the animals before the infusion commenced, as background samples. Breath samples were taken every ten minutes throughout both 10 hour infusion periods. Blood samples (3ml) were taken at 20 min intervals prior to the sodium acetate infusion and every 10 minutes thereafter. The whole blood samples were heparinised (37u heparin /ml whole blood) and centrifuged to separate the plasma (see section 2.1 for collection of plasma samples). Plasma was frozen at -20°C until required for analysis.

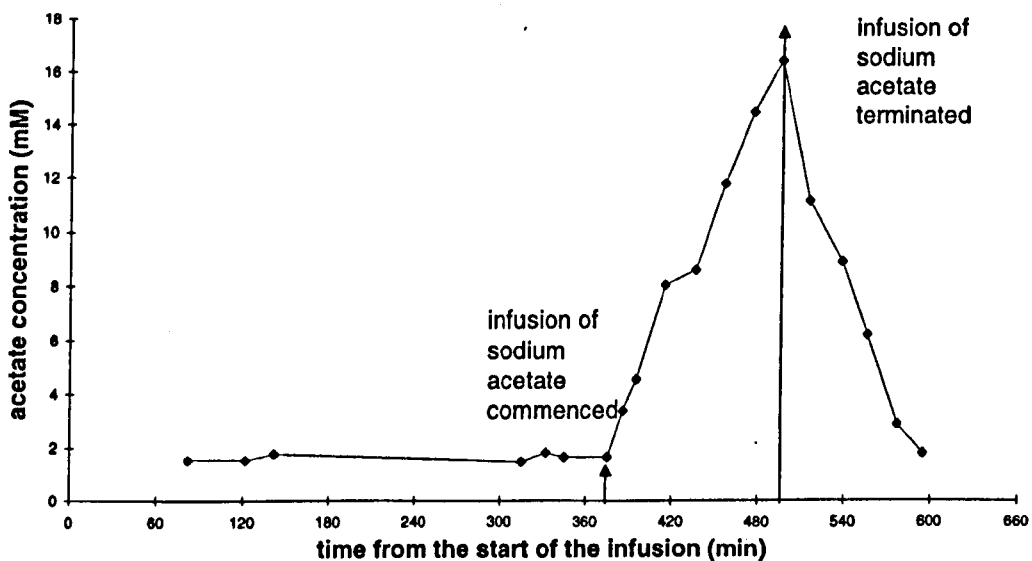
The enrichment (<sup>13</sup>C/<sup>12</sup>C) of leucine and KIC in the blood plasma and the enrichment of CO<sub>2</sub> (<sup>13</sup>C/<sup>12</sup>C) in the breath were measured and the irreversible loss rate (Rd) of the leucine pool and the total body CO<sub>2</sub> then calculated (see section 2.2.4 and

2.4 respectively for methods of analysis). In addition, plasma acetate concentrations were measured throughout the two infusion periods (see section 2.2.1 for method of analysis).

### 5.5.2 Results

Plasma acetate concentrations throughout the infusion period can be seen in Figure 5.2. There was a large rise in plasma acetate concentration during the sodium acetate infusion and a rapid decline when the sodium acetate infusion was terminated.

Figure 5.2 The effects of infusing sodium acetate, for 2h, at a rate of 3mmoles/min on the plasma acetate concentration throughout the infusion periods



The effects that sodium acetate infusion had on the isotopic enrichment (APE) of the expired  $^{13}\text{CO}_2$  during the continuous infusions of L-[1- $^{13}\text{C}$ ]-leucine and  $\text{NaH}^{13}\text{CO}_3$  are shown in Figures 5.3 and 5.4 respectively. The isotopic enrichment of the expired  $^{13}\text{CO}_2$  reached a plateau within 180 min from the start of both continuous infusions.

Figure 5.3 The effects of infusing sodium acetate (3mmoles/min) on the  $^{13}\text{CO}_2$  enrichment (APE) in breath samples during a 10h continuous infusion of L-[1- $^{13}\text{C}$ ]-leucine

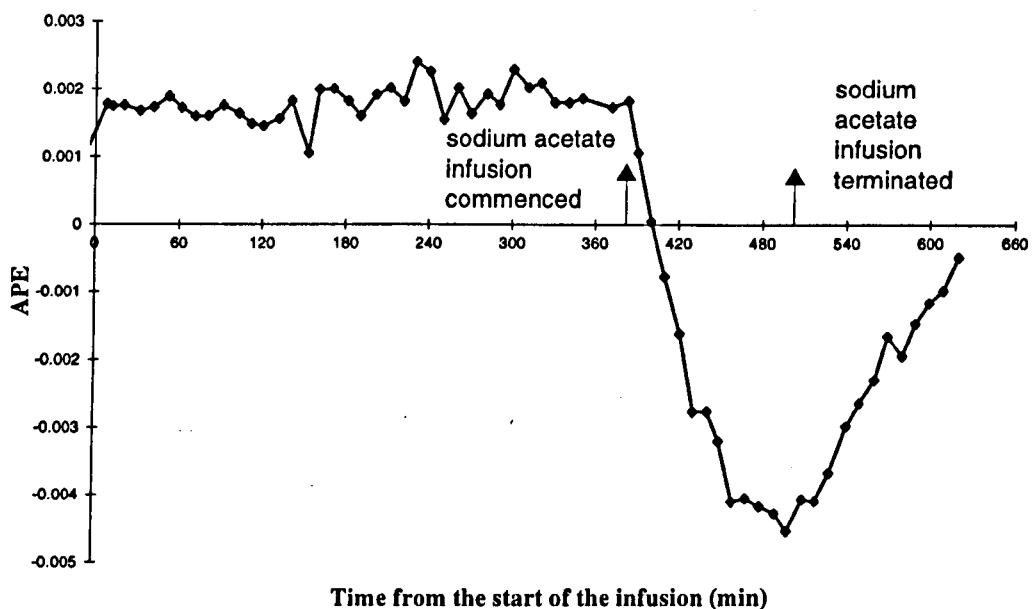
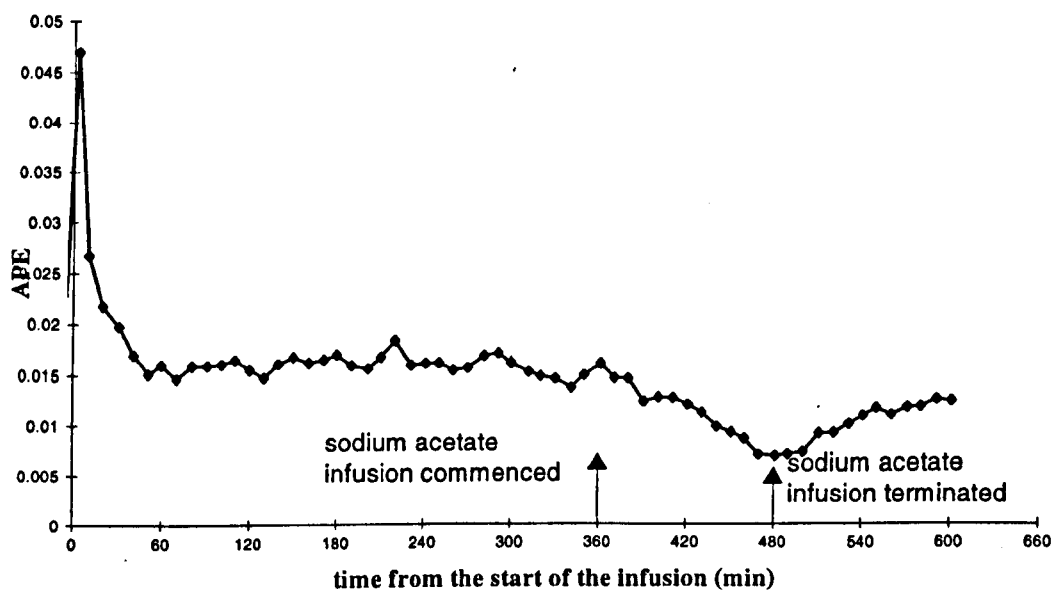
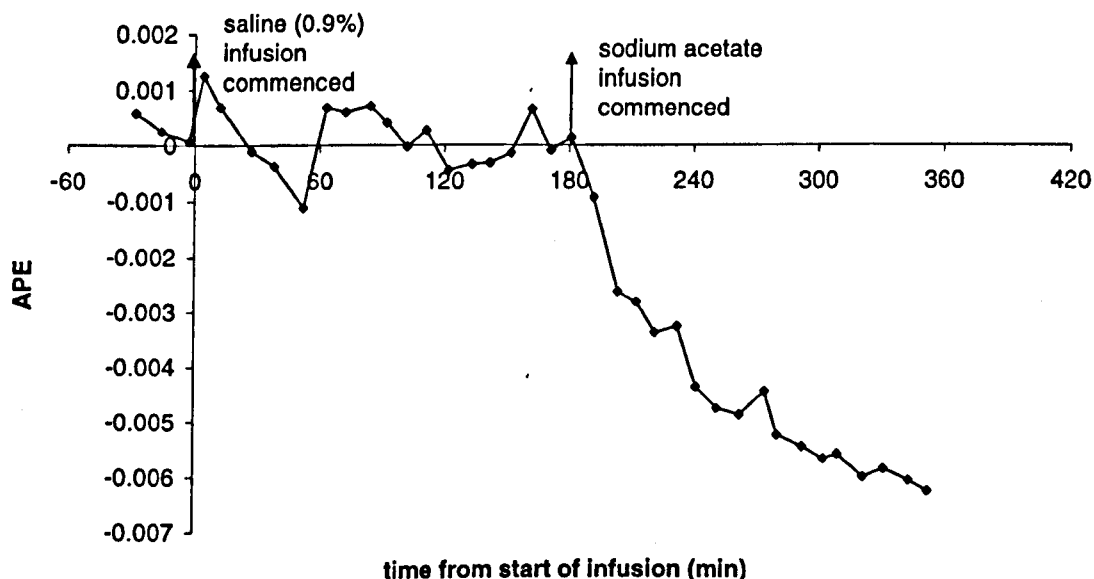


Figure 5.4 The effects of infusing sodium acetate (3mmoles/min) on the  $^{13}\text{CO}_2$  enrichment (APE) in breath samples during a 10h continuous infusion of  $\text{NaH}^{13}\text{CO}_3$



These results show that there was a dramatic reduction in the APE of the expired  $^{13}\text{CO}_2$  of the sheep on infusing sodium acetate. During the infusion of sodium acetate, in both infusion periods, there was a decrease in the enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of the  $\text{CO}_2$  in the breath. Following the termination of the sodium acetate infusion there was a rapid increase in the  $^{13}\text{CO}_2$  enrichment of the breath samples taken during the continuous infusions of both L-[1- $^{13}\text{C}$ ]-leucine and  $\text{NaH}^{13}\text{CO}_3$ . This pattern was particularly apparent in the samples taken during the continuous infusions of L-[1- $^{13}\text{C}$ ]-leucine where the  $^{13}\text{CO}_2$  enrichment of the breath (APE) became negative during the sodium acetate infusion. This meant that the isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of the breath during the sodium acetate infusion fell below that of the background samples measured prior to the infusion. This was a surprising result. The only explanation that could be offered was that the natural isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of the carbon in the sodium acetate infused was lower than the enrichment of the  $\text{CO}_2$  in the breath. This was confirmed when a sheep was infused with no isotope, but continuously infused with saline for 3 h, which was then exchanged to a continuous infusion of sodium acetate (3mmoles/min) for 3h and then terminated. The isotopic enrichment of the  $\text{CO}_2$  in the breath can be seen in Figure 5.5.

Figure 5.5 The effects of infusing sodium acetate, at a rate of 3mmoles/min, on the isotopic enrichment (APE) of the natural  $\text{CO}_2$  in the breath of a sheep, fed hourly.





The APE of the breath was approximately zero during the saline infusion alone (i.e. was maintained at background level), but during the infusion of sodium acetate, the isotopic enrichment of the breath decreased rapidly below that of the natural isotopic enrichment of the animal.

In order to measure the effects of sodium acetate on leucine flux and oxidation it was necessary to find a sodium acetate source of the same isotopic enrichment as that of the CO<sub>2</sub> in breath, to be able to assume that any change in the isotopic enrichment of the CO<sub>2</sub> in breath was due to the effects of sodium acetate on the metabolism of leucine and not due to the isotopic enrichment of the sodium acetate infusate. It was concluded, therefore, that before looking at the effects of infusing sodium acetate on leucine metabolism, a sodium acetate source that had no effect on the natural <sup>13</sup>CO<sub>2</sub> enrichment in the breath needed to be studied.

### 5.5.3 Further work in the preliminary study

Sodium acetate was obtained from a number of sources and the isotopic enrichment of each source analysed using an automated <sup>15</sup>N <sup>13</sup>C analyser (ANCA) (Europa Scientific, Crewe; see section 2.5 For details of methods). The natural <sup>13</sup>CO<sub>2</sub> of the breath (n=3) (see section 2.4 for the method of analysis) was measured in one sheep fed the same diet as was to be fed in the experiment ('asynchronous' diet; section 3.1). The results of these analyses are shown in Table 5.2.

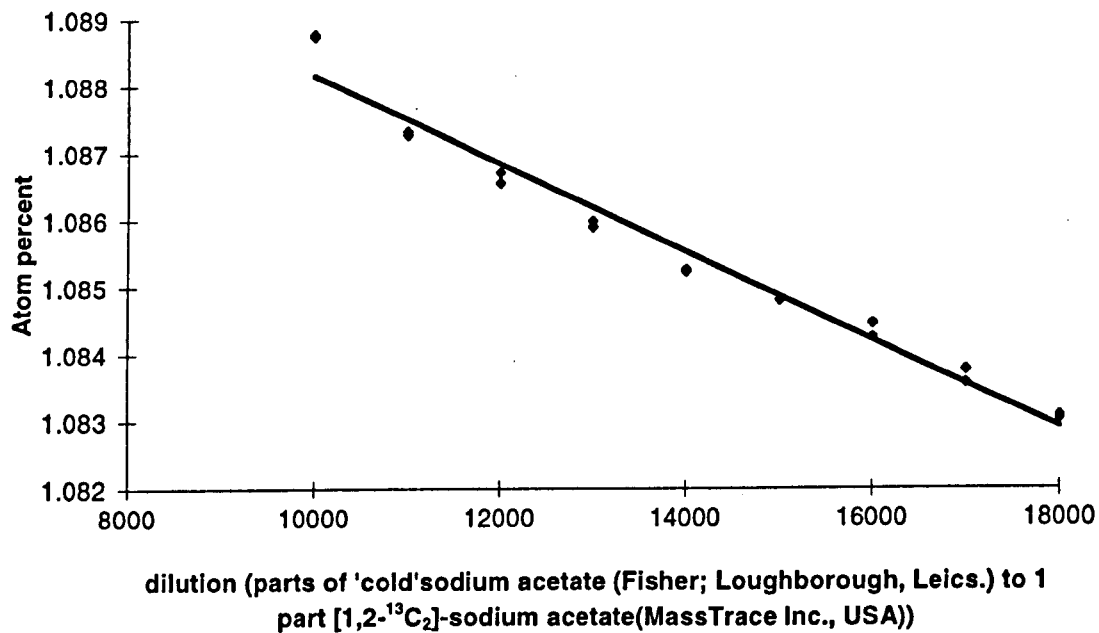
Table 5.1 The natural <sup>13</sup>CO<sub>2</sub> of breath in a sheep and the isotopic enrichment (<sup>13</sup>C/<sup>12</sup>C) of four sodium acetate sources.

Carbon source	Mean isotopic enrichment of carbon source (Atom Percent) ± standard error
Sheep breath CO <sub>2</sub>	<b>1.084 ± 0.001</b>
Sodium acetate (Fisher)	1.071 ± 4.39×10 <sup>-5</sup>
Sodium acetate (BDH)	1.078 ± 3.85×10 <sup>-5</sup>
Sodium acetate (Sigma)	1.076 ± 9.89×10 <sup>-5</sup>
tri-sodium acetate (Fisher)	1.058 ± 1.59×10 <sup>-4</sup>

The isotopic enrichment of the C in the different sources of sodium acetate measured was variable and were lower than the natural  $^{13}\text{C}$  isotopic enrichment of the animal's breath.

As previously indicated in this chapter, in order to examine the effects of the infusion of sodium acetate on leucine metabolism using the isotope dilution technique it was essential that the isotopic enrichment of C in the sodium acetate was of the same isotopic enrichment as that of natural  $^{13}\text{C}$  in sheep breath. As no source of sodium acetate could be found with a  $^{13}\text{C}/^{12}\text{C}$  enrichment similar to the natural  $^{13}\text{C}$  of sheep breath, a solution of appropriate enrichment (1.084 Atom Percent) had to be prepared by mixing of [1,2- $^{13}\text{C}$ ]-sodium acetate (99 atom %; MassTrace, Inc., USA) with 'cold' sodium acetate. A series of solutions were prepared by diluting a solution of known concentration (4.4M) of [1,2- $^{13}\text{C}$ ]-sodium acetate (99 atom %; MassTrace, Inc., USA) solution with a source of 'cold' sodium acetate (Fisher Chemicals, Loughborough, Leics.) solution of the same concentration (4.4M). This ensured the resulting solution was of constant sodium acetate concentration but had a different enrichment of  $^{13}\text{C}/^{12}\text{C}$  to the 'cold' sodium acetate. A range of dilutions of [1,2- $^{13}\text{C}$ ]-sodium acetate in 'cold' sodium acetate (Fisher Chemicals, Loughborough, Leics.) were analysed by ANCA (for details see section 2.5). The results can be seen in Figure 5.6 and showed that to match the isotopic enrichment of C in natural abundance in the sheep, the [1,2- $^{13}\text{C}$ ]-sodium acetate had to be diluted 1 : 16,000 with 'cold' sodium acetate.

Figure 5.6 The isotopic enrichment (atom percent) of varying dilutions of the [1,2-<sup>13</sup>C]-sodium acetate



## **5.6 The effect of infusing sodium acetate intravenously, on plasma leucine metabolism parameters in sheep**

### **5.6.1 Experimental procedure**

The effects of infusing a sodium acetate solution with the same  $^{13}\text{C}$  enrichment as the natural sheep breath  $\text{CO}_2$  on selected parameters of leucine metabolism in sheep were investigated.

Four 8 month old Charolais-cross wether lambs, initially weighing  $47.1 \pm 2.32$  kg, were acclimatised to hourly feeding, using automatic feeders, for 10 d prior to the experiment. Animals were fed the 'asynchronous' diet, as used in chapter 3 (see section 3.1) and the total daily ration was calculated on a liveweight basis, to meet the ME requirements for a liveweight gain of 150g/d (AFRC, 1993; see equation 4.1). All animals had been fed this diet for approximately 6 months prior to the experiment. Animals were placed in metabolism crates 5 days before the start of the experiment and the jugular veins of both the left and right side of the neck were cannulated 48 h before infusing (section 5.3.1). The building was continually illuminated and animals had free access to water and mineral licks at all times.

Entry rates ( $R_a$ ) of leucine and the rate of leucine oxidation were determined after each animal was continuously intravenously infused with L-[1- $^{13}\text{C}$ ]-leucine for 11 h (section 5.3.2). Sodium chloride was simultaneously infused through a separate infusion line at a rate of 3mmol/min for the first 5 h of the infusion. Immediately before the infusion, the leucine and bicarbonate pools were primed with L-[1- $^{13}\text{C}$ ]-leucine and  $\text{NaH}^{13}\text{CO}_3$  to increase the rate at which the  $^{13}\text{CO}_2$  in the breath reached isotopic equilibrium (section 5.3.2). In the initial studies it took less than 180 min for the isotopic enrichment of the  $\text{CO}_2$  in the breath to reach a plateau, where dynamic equilibrium was assumed. On this basis, when L-[1- $^{13}\text{C}$ ]-leucine had been continuously infused for 5 h, it was assumed that the enrichment of the  $\text{CO}_2$  in the sheep breath had reached a plateau. While the infusion of L-[1- $^{13}\text{C}$ ]-leucine continued, the sodium chloride infusion was stopped and an infusion of sodium acetate commenced at a rate of 3mmol/min for 3 h. Sodium chloride was infused at the same rate and concentration as the sodium acetate, so that when the sodium acetate infusion

commenced, there was no change in either the volume of fluid or the sodium level that was entering the animal. This excluded sodium concentration and volume of fluid entering the animals as confounding factors affecting the treatment. After 3 h the sodium acetate infusion was terminated and the sodium chloride infusate recommenced (3mmol/min) and continued for a further 3 h. After 11 h of infusion, both L-[1-<sup>13</sup>C]-leucine and sodium chloride infusions were terminated.

Two days after the infusion of L-[1-<sup>13</sup>C]-leucine, measurements of the entry rate of the total body CO<sub>2</sub> were made. To estimate total body CO<sub>2</sub> entry rate, the bicarbonate pool of each animal was labeled by continuous intravenous infusion of NaH<sup>13</sup>CO<sub>3</sub> for 11 h (see section 5.2.3). Simultaneous continuous infusions of NaH<sup>13</sup>CO<sub>3</sub> and sodium chloride (3mmol/min) were started immediately after the priming of the bicarbonate pool with NaH<sup>13</sup>CO<sub>3</sub> (see section 5.3.3 for details of infusates, priming doses and methods of infusion). While the NaH<sup>13</sup>CO<sub>3</sub> continued to be infused, sodium chloride infusate was exchanged for sodium acetate infusate 6h after the start of the infusion and continued for 3h, at rate of 3 mmoles/min, before being changed back to the sodium chloride infusion for a further 3h.

To ensure that the sodium acetate solution infused throughout the experiment had no effect on the natural <sup>13</sup>CO<sub>2</sub> enrichment of the breath of the sheep, as previously seen in the preliminary study, infusions were subsequently repeated on two of the four animals used for the experiment with sodium chloride (4.4M) and the same sodium acetate (4.4M) solution that had been infused throughout the experiment. Infusates were infused at the same rate and for the same duration, using identical methodology, as used when measuring the rate of plasma leucine entry and leucine oxidation. No other isotopically labeled material was infused into the animals.

Breath and blood samples (for details of sampling see sections 2.3 and 2.1, respectively) were taken before the start of the experiment (n=3), as background samples. Breath samples were taken every ten minutes throughout 11 h infusion periods. In addition, blood samples (5ml) were taken every 20 minutes prior to the sodium acetate infusion, every 10 minutes for 1h following the start of the sodium acetate infusion and every 15 minutes thereafter. The whole blood samples were heparinised (37u heparin/ ml of whole blood) and centrifuged to separate the plasma

(see section 2.1 for details of plasma sampling). The plasma was frozen at  $-20^{\circ}\text{C}$  until required for analysis.

Of the plasma samples taken, samples taken approximately every 30min, were analysed. All the breath samples taken were analysed. The enrichment of leucine and KIC in the blood plasma and the  $^{13}\text{CO}_2$  enrichment of the breath were measured and the flux rate of the leucine intracellular pool and the total body  $\text{CO}_2$  for each animal was calculated. Estimates of leucine oxidation and whole-body protein synthesis were also calculated (see section 5.4 for calculations). Concentrations of plasma acetate and plasma insulin were determined throughout the infusion periods (see Chapter 2 for methods of all analyses).

### 5.6.2 Calculations and statistical analysis

A plateau in the isotopic enrichment of the breath was reached within 3h from the start of the infusion of both L-[1- $^{13}\text{C}$ ]-leucine and  $\text{NaH}^{13}\text{CO}_3$  as can be seen in Figures 5.7 and 5.8 respectively. Measurements of plasma leucine and KIC concentration and the estimates of leucine metabolism parameters were statistically analysed using the Genstat 5 statistical package (Release 3.22; Lawes Agricultural Trust, Rothamsted) to determine the effects of sodium acetate infusion. Three time periods were defined for comparison of measurements within the two infusions. A mean of all the samples that were analysed within the time periods 'before' ('B'; 180-300 min from the start of the infusion;  $n=4$ ), 'during' ('D'; 360-480 min from the start of the infusion;  $n=4$ ) and 'after' ('A'; 540-660 min from the start of the infusion;  $n=4$ ) the sodium acetate infusion commenced, were compared. A repeated measure split-plot ANOVA was used, with animals as the main plot and the time period within animals as the sub-plot factor, resulting in 6 degrees of freedom for error. Where only 2 animals were used for confirming that the sodium acetate did not alter the natural APE in the breath, there were 2 degrees of freedom. Degrees of freedom for error for the between-time F tests were adjusted to account for the repeat measures taken on the same animal, using the Greenhouse-Geisser epsilon (Winer *et al.*, 1991). This method of analysis was used for all parameters that were statistically analysed.

Figure 5.7 An example of the effects of a 3h sodium acetate infusion (3mmoles/min) on the APE of expired CO<sub>2</sub> throughout the time period of an infusion of L-[1-<sup>13</sup>C]-leucine, in one animal, randomly selected from the experimental group

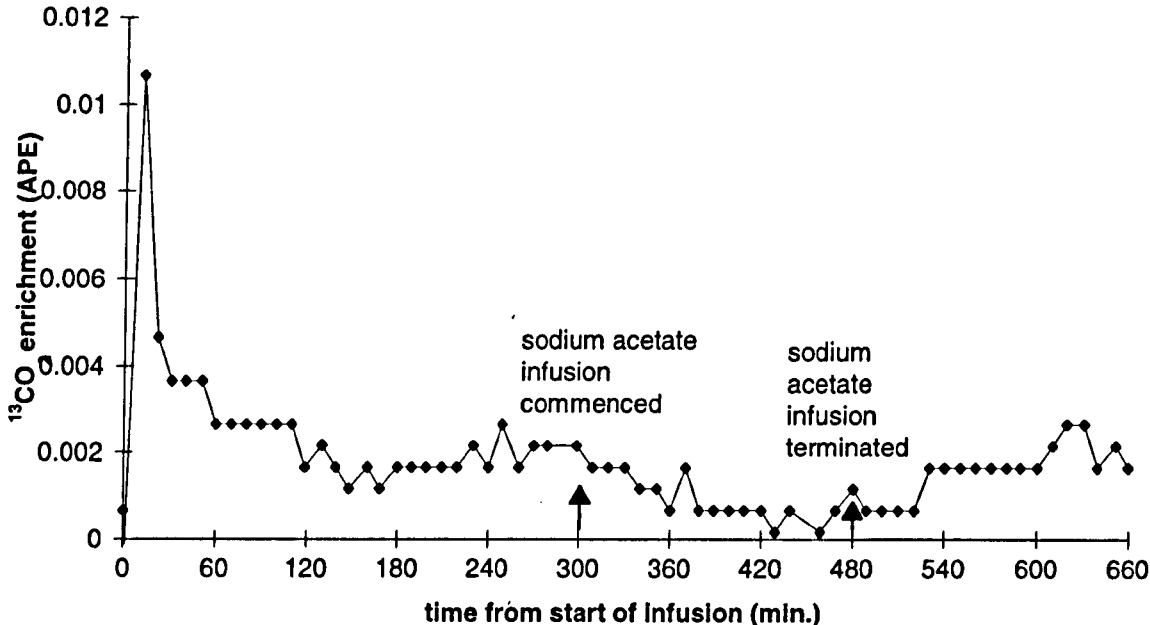
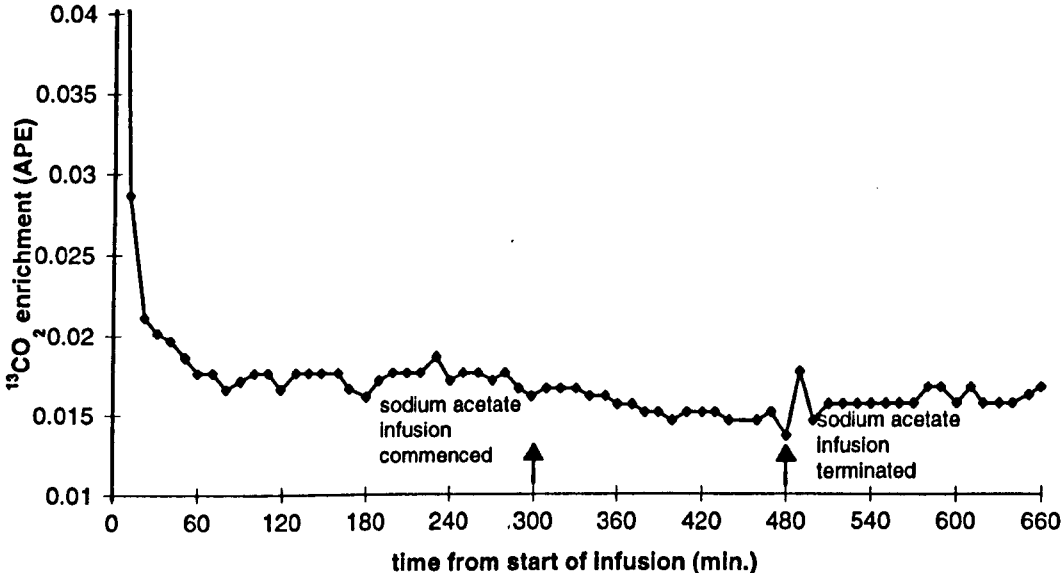


Figure 5.8 An example of the effects of a 3h sodium acetate infusion (3mmoles/min) the APE of expired CO<sub>2</sub> throughout the time period of an infusion of NaH<sup>13</sup>CO<sub>3</sub>, in one animal, randomly selected from the experimental group



### 5.6.3 Results

The effects of infusing sodium acetate (3mmoles/min) intravenously for 3h on plasma concentrations of acetate during the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$  are shown in Figure 5.9. All animals have a similar rise and fall in the plasma acetate concentration from the start of the sodium acetate infusion.

Figure 5.9 The effects of infusing sodium acetate (3mmoles/min) for 3h on plasma concentrations of acetate during the continuous infusion of  $\text{NaH}^{13}\text{CO}_3$ .

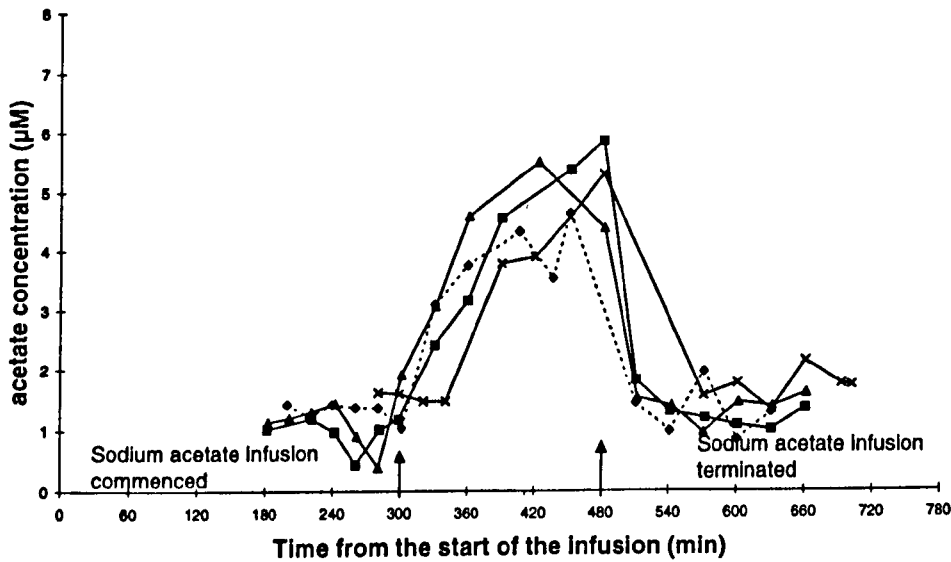




Table 5.2 The effects of a 3h infusion of sodium acetate, at a rate 3mmoles/min, on measurements of entry rates of leucine (Ra<sub>KIC</sub>), the entry rate of total body CO<sub>2</sub> (Ra<sub>CO2</sub>), and estimates of leucine oxidation and whole-body protein synthesis (WBPS) during selected time periods before (B; 180-300min from the start of the overall infusion), during (D; 360-480min from the start of the overall infusion) and after (A; 540-660min from the start of the overall infusion) the infusion of sodium acetate.

Time from start of infusion	'B' (180-300min)	'D' (360-480min)	'A' (540-660min)	SED (6 degrees of freedom for error)	P Value	Probability of significant differences between treatment groups using t-test
Leucine flux (Ra <sub>KIC</sub> ) ( $\mu\text{mol}/\text{min}/\text{kg}^{0.75}\text{LW}$ )	9.98	8.29	9.01	0.700	NS	B × D D × A A × B
Total CO <sub>2</sub> Flux (Ra <sub>CO2</sub> ) ( $\mu\text{mol}/\text{min}/\text{kg}^{0.75}\text{LW}$ )	1156	1283	1190	29.9	<0.05	** *
Leucine oxidation ( $\mu\text{mol}/\text{min}/\text{kg}^{0.75}\text{LW}$ )	2.00	1.06	2.22	0.243	<0.05	* **
WBPS ( $\text{g}/\text{kg}^{0.75}\text{LW}/\text{d}$ )	22.5	20.0	19.5	0.98	<0.1	* *
Leucine concentration (mM)	151.3	135.5	138.9	8.40	NS	
KIC concentration (mM)	4.023	5.999	3.950	0.1786	<0.01	*** **
Insulin concentration (ng/ml)	0.748	0.863	0.777	0.1041	NS	

\*\* = P<0.01, \* = P<0.05, + = P<0.1

SED = Standard error of difference

NS = not significant (P>0.1)

Table 5.2 show that the Ra of leucine ( $Ra_{KIC}$ ) was unaffected by the infusion of sodium acetate. Also shown in this table is the Ra of the total body  $CO_2$  ( $Ra_{CO_2}$ ) calculated from measurements taken at the three timepoints during the continuous infusion of  $NaH^{13}CO_3$ . An increase in the Ra of the total body  $CO_2$  ( $Ra_{CO_2}$ ) was observed during the infusion of sodium acetate ( $P<0.01$ ), but this returned to the original level that it was at prior to the sodium acetate infusion, after the sodium acetate treatment had been terminated.

Estimates of leucine oxidation and whole-body protein synthesis, in each of the three time periods are also shown in Table 5.2. The rate of leucine oxidation decreased during the infusion of sodium acetate and increased again after the sodium acetate infusion was terminated ( $P<0.05$ ). The rate of whole-body protein synthesis in the periods 'during' ('D') and 'after' ('A') the sodium acetate infusion was lower than the rate before the infusion of sodium acetate commenced ( $P<0.05$ ).

Table 5.2 also shows the effects of sodium acetate infusion on the plasma concentrations of KIC, leucine and insulin. Leucine concentration in the plasma remained unchanged before, during and after the sodium acetate was infused. The concentration of plasma KIC was the same before and after the sodium acetate treatment, but was significantly increased during the infusion of sodium acetate ( $P<0.001$ ). There was a slight increase in the insulin concentration during the sodium acetate infusion period, although this was not statistically significant ( $P>0.1$ ).

Table 5.3 shows that the infusion of enrichment-corrected sodium acetate had no effect on the natural  $^{13}CO_2$  enrichment of the breath of the sheep.

Table 5.3 The effects infusing sodium acetate for 3h (3mmoles/min) on the  $^{13}CO_2$  enrichment (APE) of the breath of sheep (n=2)

	'B' (280-300min)	'D' (360-480min)	'A' (540-660min)	SED (2 degrees of freedom for error)	P Value
APE of breath	-0.0009	-0.0014	-0.0006	0.00023	NS

NS = not significant ( $P>0.1$ )

## **5.7 Preliminary Discussion**

This experiment aimed to examine the effect of increasing concentrations of energy yielding plasma acetate by infusing sodium acetate, on estimates of leucine oxidation and whole-body protein synthesis. To investigate the effect of infusing sodium acetate on leucine metabolism it was necessary to correct the  $^{13}\text{C}$  isotopic enrichment of sodium acetate so that it was the same as that of  $^{13}\text{CO}_2$  in natural abundance in the breath of sheep. Once corrected, the sodium acetate was infused into the sheep to raise the plasma acetate concentration. Concentrations of plasma acetate peaked at levels between 5mM and 7mM, which were similar to levels observed when animals were fed the same diet once daily (AD; Chapter 3). The asynchronous diet was fed hourly in this current study, so that the animals could be assumed to be in metabolic 'steady state'. In addition, the animals fed the diet hourly were thought to have a very constant plasma acetate concentration as was shown in Chapter 3, when the same diet was fed in this way. By infusing sodium acetate, it was thought that the increase in plasma acetate concentration would be similar to that observed following a once daily feeding of the asynchronous diet (see Chapter 3). The effects of raising the plasma acetate concentration in this way, on estimates of leucine metabolism parameters were studied. This approach assumed that the pool size remained stable and unchanged between treatment periods.

The results presented in this chapter show estimates of leucine metabolism (leucine entry rate, leucine oxidation and whole body protein synthesis) calculated using the isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of plasma KIC. As intracellular leucine is the precursor of plasma KIC, plasma KIC enrichment is assumed to be representative of its former intracellular leucine environment. Intracellular KIC and leucine are true precursors of protein synthesis. Consequently the isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of plasma KIC can be used as an index of the intracellular  $[1-^{13}\text{C}]\text{-KIC}$  and the  $[1-^{13}\text{C}]\text{-leucine}$  enrichments (see Figure 5.1). By measuring the isotopic enrichment ( $^{13}\text{C}/^{12}\text{C}$ ) of plasma KIC, whole-body leucine entry rate and leucine oxidation rates can be estimated. Indirectly, the rate of leucine incorporation into protein via whole body protein synthesis can also be estimated (Matthews, 1982).

The values obtained for the measurements obtained for leucine metabolism are similar to the values that are recorded in published literature. Table 5.4 shows comparisons of the values obtained in the current experiment and other values

reported. Small differences in the leucine metabolism values obtained may be due to the physiological state, sex, species, breed and feeding level differences between experiments. In addition, the method of measuring leucine flux may alter the value obtained. In this experiment, the isotopic enrichment of plasma KIC was measured as an indirect measure of the isotopic enrichment of the true intracellular leucine pool. The [1-<sup>13</sup>C]-KIC enrichment in the plasma is reported to be lower than that of plasma [1-<sup>13</sup>C]-leucine enrichment due to the release of leucine from protein breakdown (Matthews, 1982; Nissen & Haymond, 1981). As a result, the flux measured is higher. This may explain why the leucine flux measured in this experiment had higher values than those recorded in the growing steers of Lappierre *et al* (1996) who measured leucine flux using plasma [1-<sup>13</sup>C]-leucine enrichment.

Table 5.4 A comparison of the parameters of leucine metabolism shown in the current experiment with those in previously published data

Leucine flux rate ( $\mu\text{mol}/\text{min}/\text{kg}^{0.75}$ )	Leucine Oxidation rate ( $\mu\text{mol}/\text{min}/\text{kg}^{0.75}$ )	Whole body protein synthesis rate ( $\text{g}/\text{kg}^{0.75}/\text{d}$ )	Experimental animals and their mean liveweight	Reference
9.98	2.00	22.5	Growing wether lambs - 47kg	Current experiment
4.0-6.6	0.7-1.1	-	Dairy heifers - 158kg	Lappierre <i>et al.</i> (1996)
5.2-8.2	0.5-1.5	14.7-21.0	Growing beef steers - 315kg	Lappierre <i>et al.</i> (1996)
5.2	0.8	8.9-12.2	Ewes - 58kg	Pell <i>et al.</i> (1986)
4.8-6.0	0.4-0.7	12.0-15.2	Ewes - 46kg	Krishnamurti & Janssens (1988)
7.1-9.8	2.2-3.7	14.2-17.0	Growing beef steers - 119kg	Greathead (1997)

The measurements of leucine oxidation decreased during the infusion of sodium acetate ( $P < 0.05$ ). The total body  $\text{CO}_2$  during the infusion of sodium acetate increased, however, which indicated that although oxidation of leucine decreased, that of a particular substrate was increased. It would appear from these results, therefore, that there was a switch in the substrate oxidation from leucine, to another substrate and as there was an abundance of acetate in the plasma during the infusion, it is possible that the acetate was oxidised instead of leucine, during the infusion of sodium acetate. The rate of disappearance of leucine ( $R_{a_{\text{KIC}}}$ ) was not significantly different before, during or after the infusion of sodium acetate ( $P > 0.1$ ), although there was a tendency for it to decrease during the infusion of sodium acetate and increase when the infusion of sodium acetate was terminated. If  $R_{a_{\text{KIC}}}$  stayed the same and the oxidation of the KIC was decreased, this might indicate an increase in the estimates of whole-body protein synthesis. This was not seen. Whole body protein synthesis significantly decreased following the start of the sodium acetate infusion and this decrease continued for the remainder of the infusion period ( $P < 0.05$ ). Consequently, the decrease in the estimates in whole-body protein synthesis when calculated is likely to be because there was a tendency for both the KIC that was oxidised and for the rate of disappearance of the KIC to decrease.

Although the concentration of plasma leucine was unchanged before, during and after the infusion of sodium acetate, the KIC concentration in the plasma was significantly increased. This indicates that the increase in plasma concentrations of KIC is coming from one or more of three places. Either less of the intracellular KIC is being oxidised to  $\text{CO}_2$  or less is incorporated into the muscle as leucine or more is available following degradation of the intracellular muscle proteins (see Figure 5.1). From the estimates made, it would appear that during the sodium acetate infusion, the rate of disappearance tended to decrease, and as the rate of oxidation and whole-body protein synthesis also decreased, the plasma KIC collected in the plasma pool, increased in concentration during the infusion of sodium acetate. Metabolic clearance can be calculated by dividing the plasma flux by the plasma concentration (Nissen & Haymond, 1981). With the results obtained in this experiment it would appear that the metabolic clearance rate is decreased by approximately 44%, during the infusion of sodium acetate, but increases again when the sodium acetate infusion is terminated.

The method used in this chapter to estimate protein synthesis has its advantages in that it uses stable isotopes, rather than radioactive isotopes. The benefits of stable isotopes, particularly in large animal work, are that they reduce the problems that exist with safe waste disposal of radioactively labeled products. The use of stable isotopes also reduces health hazards to the user. Another advantage with using this method is that the method is relatively non-invasive. This is better for the animal, as stress induced changes in metabolism can be minimised. A reduction in stress, when taking measurements of protein metabolism, is highly beneficial as any changes in blood hormones and metabolites that may occur in a stressed situation will give inaccurate results. In addition this method of estimating whole-body protein synthesis means that the animals do not need to be slaughtered at the end of the procedure, but can instead be used again.

There are, however, a number of disadvantages, with this method, which must be considered when drawing conclusions from the results obtained. A number of assumptions have to be made when using the isotope dilution technique. It is assumed that entry of tracer into the metabolite pool, is only from the isotopic infusion. In practice this may be incorrect, as with protein turnover comes protein degradation and so over time there will be recycling of the isotope back into the amino acid pool. Recycling, therefore, can lead to an underestimation of entry rate and consequently an underestimation in protein synthesis. If the duration of a protein metabolism experiment is kept as short as possible, as was done in this experiment, the proportion of tracer that is recycled back into the amino acid pool will be minimised. The surprising decrease in whole-body protein synthesis, that was observed from the start of the sodium acetate infusion onwards, however, could have been due to the labelled amino acid being recycled and so reducing the estimates of protein synthesis. After the sodium acetate infusion had been terminated, there was a tendency for the rate of disappearance of the KIC from the plasma pool, not to rise back to the levels that it had been at prior to the treatment, indicating that perhaps there may have been some label recycled from the muscle tissues, raising the isotopic enrichment of the plasma and so reducing the entry rate of the KIC slightly, as the infusion progressed. However, as there tended to be an increase in the plasma concentration of KIC, together with an apparent slight decrease in the Ra of the

leucine and a significant decrease in leucine oxidation, it could be concluded from these results that even if there were discrepancies in the estimates, there are unlikely to be large increases in whole-body protein synthesis, as these would probably have been detected.

Another assumption made is that the pool is in steady state, and unchanged in size throughout the duration of the experiment. Factors that affect the pool size, in addition to the possibility of recycling are growth and starvation of the animal. Starvation can be avoided by feeding the animal continuously, e.g. hourly, throughout the experiment, as was done in this experiment. The error is minimised with a change in growth of the animal, if the duration of the experiment is kept short and consequently the overall change in growth is kept to a minimum throughout the sampling period (Waterlow *et al.*, 1978). These potential problems were endeavored to be minimised.

It must also be assumed that tracer is mixed completely and instantaneously in the metabolite pool. It is very difficult to measure the extent of error introduced with this assumption without sampling the whole pool (Waterlow *et al.*, 1978). Also, the isotope dilution theory assumes that there is no preference in the metabolism for labelled or unlabelled metabolite. This is likely to be a fairly valid assumption, as the atomic number of the labelled and unlabelled molecules are the same and the chemical reactivity of the two is therefore considered almost identical in their affinity for reactions.

The technique estimates whole body protein synthesis and it is necessary to recognise that the contribution of individual tissues to the whole body protein metabolism are not measured. Muscle protein synthesis has been estimated to be approximately 20% of whole-body protein synthesis (Lobley *et al.*, 1980), and therefore if a difference in muscle protein synthesis was to occur, there would need to be very large differences in whole-body protein synthesis, with treatment. These were not seen.

In conclusion, increasing the acetate concentration in the plasma using intravenous sodium acetate does not increase estimates of whole-body protein synthesis, as might be expected with an increase in the availability of energy source



(i.e. acetate). It would appear that an increase in the concentration of plasma acetate, however, reduces leucine oxidation, possibly by an inhibition in the oxidation pathway. Although this method is very useful for obtaining results when there are large differences in the metabolism during a treatment, it is relatively crude when small differences occur, as have been discussed with regard to the assumptions that must be made. As no increase in whole-body protein synthesis was observed during the infusion of sodium acetate, either an increase in protein synthesis in the whole-body does not occur, during an increase in the plasma concentrations of acetate or the conditions of the experiment reported here are quite different from those that occurred in the animal following a once daily feed in the experiment reported in Chapter 3. The latter is more feasible as there are a number of factors (discussed in Chapter 6) that are likely to affect protein synthesis that have not been accounted for in this experiment. One example is a deficiency in available amino acids for protein deposition. Insulin is another example that was measured in Chapters 3 and 4, and altered in concentration following feeding, but remained constant during the infusion experiment, possibly resulting in different metabolic environments between experiments.

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## CHAPTER 6

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### GENERAL DISCUSSION

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The aim of the work presented in the thesis was to investigate the effects of altering the temporal patterns of supply of dietary nitrogen (N) and organic matter (OM) to the rumen on the supply of metabolites to the peripheral tissues and the subsequent effects on fat and lean deposition. Two experiments were carried out to investigate the effects that changing the predicted rates of OM and N degradation into the rumen by diet formulation or feeding frequency had on the temporal supply of acetate and amino acids and the growth and carcass composition of growing lambs. A separate study was also undertaken which investigated the effects of increasing the plasma concentration of the energy-yielding metabolite, acetate, on amino acid utilisation.

#### **6.1 The effect of diet and feeding frequency on predicted rumen metabolism**

Predicted rates of OM and N degradation were altered either by formulating diets, using the SIRE diet formulation computer programme (see section 1.4.6 for details), to contain different ingredients with different rates of OM and N degradation, or by altering the frequency of feeding. Diets fed in the two growth experiments (reported in Chapters 3 and 4) were formulated to have similar predicted metabolisable protein (MP) and metabolisable energy (ME) contents, so that differences observed in energy and nitrogen components supplying the peripheral tissues were not due to differences in the overall amount of dietary energy and nitrogen supplied to the ruminant. In Chapter 3, two diets were fed which differed in synchrony index, an index indicating the synchrony of N and OM availability to the rumen microbes by estimating the hourly release of N : OM (Sinclair *et al.*, 1993). It has been reported that synchronising the rates of N and OM degradation in the rumen enhances nitrogen capture for microbial protein production (Sinclair *et al.*, 1993; Huber & Herrera-Saldana, 1994). If this is the case then more microbial nitrogen, will be transported to the duodenum and made available for absorption and utilisation

by animals fed the synchronous compared to animals fed the asynchronous diets. The SIRE (Sinclair *et al.*, 1993) programme for this experiment was updated to predict the MP content of the diet on the basis of the estimating the microbial crude protein production using fermentable metabolisable energy (AFRC, 1993). Differences in the microbial protein synthesis to the duodenum, with differences in the synchrony of release of OM and N, however, was not accounted for in the programme. The SIRE program, predicted that the asynchronous diet would have a much higher digestible undegradable protein (DUP) component than the synchronous diet (see Table 3.1). When these diets were fed once daily to growing lambs, those fed the synchronous diet grew significantly more each day ( $P < 0.01$ ) and as result had a significantly higher final liveweight and carcass weight ( $P < 0.05$ ), than those fed the asynchronous diet. This agreed with data from Witt *et al.* (1997) who showed that feeding a synchronous diet increased the growth rate of lambs. This would indicate that the dietary nutrients in the synchronous diet, were more readily available to the animals and were conducive to enhanced growth rates. Synchrony of the energy and nitrogen components in the synchronous diet may have supplied the animal with more microbial protein than was estimated by SIRE. Consequently the SIRE formulation programme may have underestimated microbial protein resulting in an underestimate of MP in the synchronous diet.

Comparing animal performance with diets of differing rumen synchrony is confounded by the diets containing different ingredients. Consequently, a novel approach was adopted to improve the synchrony of the nutrients in the rumen, by feeding the same dietary ingredients. To improve the synchrony of an asynchronous diet, the same diet was fed to lambs as small portions every hour instead of one large meal. This should therefore have made the release of OM and N into the rumen more synchronous throughout the day, compared to feeding the asynchronous diet once daily. By increasing the frequency of feeding of the asynchronous diet (i.e. hourly), therefore, microbial protein production and consequently the amount of microbial nitrogen to the duodenum may be enhanced, as was shown by Bunting *et al.* (1987). Animals fed the asynchronous diet hourly, with improved synchrony of OM and N to the rumen and containing more DUP than the synchronous diet, were likely to have the most N available in the duodenum for utilisation by the ruminant of all three dietary treatment groups. Animals fed the asynchronous diet hourly had significantly

greater growth rates than animals fed either the asynchronous or synchronous diet once daily ( $P < 0.05$ ). This was attributed to a better availability of N for utilisation, than the animals fed either diet once daily.

In Chapter 4 animals were fed two diets formulated to contain OM and N with different rates of degradation in the rumen, but with similar SIs. It was estimated that the “slow/slow” (SS) diet had a higher DUP content than the “fast/fast” (FF) diet. If feeding hourly increases microbial protein entering the duodenum, in the second experiment (Chapter 4), the animals fed the SS diet hourly (SSH), with a higher DUP content than the FF, would be expected to grow the fastest and those fed the FF diet once daily, would be expected to grow the slowest of all the animals in the experimental group. With both diets, if nitrogen capture was enhanced by increasing the frequency of feeding, a better growth rate would be expected in the hourly fed animals. There was very little difference in the growth of animals fed the FF diet either once daily (FFD) or hourly (FFH). However, animals fed the SSH grew considerably slower, resulting in significantly lighter final liveweights and carcass weights ( $P < 0.001$ ) than animals fed SSD. The results obtained from the first experiment reported in Chapter 3, where the hourly fed animals had greater growth rates and a significantly higher amount of crude protein laid down in the carcass may be due to the fact that the animals fed AH consumed 22% more MP per kg LW per day than those fed the same diet once daily. MP intakes in the animals fed the FF diet hourly were 25% higher per kg LW per day than those fed the same diet once daily. Had animals fed the FF diet eaten the same between the hourly and once daily feed groups, there may have been higher growth rates in those fed the diets once daily compared to those fed the diets hourly. There was only a 4% increase in MP intake (MP/kg LW/d) in animals fed the SS diet hourly compared to those fed the same diet once daily. However, in animals fed the SS diet once daily there was a 15% increase in the final liveweight and a 16% increase in the amount of crude protein in the carcass compared to those fed SS hourly.

This highlights the fact that the growth rate and carcass composition is dependent on a number of other factors, other than total duodenal nitrogen supply, which influence nitrogen and energy metabolism at the peripheral tissues in the ruminant. The temporal supply of the nutrients to the tissues and the effect on nutrient partitioning in the tissues is to be discussed.

## **6.2 The effect of diet and feeding frequency on amino acid supply to peripheral tissues**

Frequency of feeding appeared to affect the pattern of plasma amino acid concentrations throughout the day. The plasma concentrations of most of the amino acids decreased following feeding. Amino acid concentration in the plasma is dictated by the release and uptake of amino acids from tissues (gut, liver and peripheral tissues). The appearance of essential amino acids in the portal plasma can vary as much as between 30-80% of the intestinal disappearance of the amino acids (Tagari & Bergman, 1978). This is thought to be due to a large amount of the amino acids that are absorbed from the intestine being utilised by the gut mucosa for synthesis of plasma proteins (Tagari & Bergman, 1978). The gastro-intestinal tract was shown to contribute a significant proportion (32-46%) of whole-body protein synthesis (Lobley *et al.*, 1980). This suggests that the gut mucosa is a strong regulator of the amino acids that are present in the peripheral plasma. The liver is also considered to play a central role in regulating nitrogen metabolism in the ruminant (Van der Walt, 1993) and removes amino acids from the blood in varying amounts depending on the individual amino acid (see Huntingdon, 1990). For example glycine and alanine are removed by the liver in greater quantities than are absorbed from the gastrointestinal tract, resulting in the requirement for a significant continuous release of these amino acids from the muscle tissue and to a certain extent from the kidneys (Bergman & Pell, 1984). In contrast the liver removes only 40% of the branched chain amino acids, with the remaining 60% being taken up by the muscle brain and other tissues (Bergman & Pell, 1982). In the muscle, they are deaminated to their respective ketoacids which are either oxidised or used in synthesis. The amino groups from these amino acids are used for the formation of alanine, glycine, or arginine and are removed by the liver (Bergman & Pell, 1984). Wolff *et al.* (1972) and Bergman & Heitmann (1978) found that the liver removed a greater amount of amino acid nitrogen from the portal vein than was absorbed across the gut tissues, from the intestine, suggesting that inter-organ tissue exchange plays an important role in amino acid transfer and protein turnover. During fasting, the concentrations of plasma alanine and glycine and the branched chain amino acids increase in the plasma to balance out the uptake of amino acids by the liver to meet the hepatic demands that appear to continue irrespective of feeding (Bergman & Pell, 1984).

Hormonal secretions, however, appear to alter the release and uptake of the liver and peripheral tissues. For example, increasing the amino acid requirements for protein synthesis in peripheral tissues using growth hormone releasing factor injections in growing cattle decreased the removal of amino acids by the liver (Reynolds *et al.*, 1992). Consequently, the gut tissues and liver utilise a significant amount of the available tissue nutrients and whilst doing so, work in concert with other body tissues to co-ordinate nutrient partitioning (Reynolds & Maltby, 1994).

It is likely, therefore, that energy and nitrogen levels and secretion of hormones are important factors dictating amino acid utilisation by different tissues and consequently amino acid concentration in the peripheral plasma. The temporal supply of energy, amino acids and the hormone, insulin, observed between diets fed with a different frequency of feeding were quite different. In chapter 3, no differences were observed in the patterns or daily concentrations of amino acids between the different diets (asynchronous or synchronous) fed once daily. Despite both diets having different SI, both were isoenergetic and the pattern of acetate and insulin concentrations following once daily feeding was similar. The temporal demands of the gut tissues and the liver may consequently be similar, resulting in the liver and gut tissues utilising plasma amino acid concentration to a similar extent throughout the day. In the second experiment reported (Chapter 4) significant differences were observed with frequency of feeding but not between diet groups in the daily plasma concentrations of serine, glycine, leucine and isoleucine. The serine and glycine concentrations were significantly lower and leucine and isoleucine concentration were significantly higher in animals fed hourly compared to those fed once daily. This would indicate that either there was a difference in the amino acid supply from the splanchnic tissues when the animals were fed hourly compared to once daily or the peripheral tissues and liver were taking up and releasing the amino acids at different rates and consequently altering the concentrations of amino acids between frequency of feeding groups. These differences may have been due to a huge difference in the pattern of acetate supply (energy) and/or insulin supply that was observed between treatment groups, which may have influenced the efficiency with which amino acids were used for protein synthesis at the muscles and also the efficiency with which amino acids were used as glucogenic precursors, consequently altering the amino acid concentration in the peripheral blood. If this is the case, the

frequency of feeding, with differing rates of release of OM into the rumen (SS and FF), may theoretically have influenced the efficiency of energy metabolism at the peripheral tissues and consequently altered the uptake and release of amino acids by the liver and peripheral tissues. This may account for the differences observed in the amount of crude protein in the carcasses and the differences in muscle sizes that were observed between frequency of feeding groups in the second experiment that was reported (Chapter 4).

The liver removes large quantities of different amino acids, particularly the glucogenic amino acids alanine and glycine, from the blood of fed sheep (Bergman, 1986). The exception to this hepatic removal of amino acids is glutamate and, to a large extent, the urea cycle intermediates, ornithine and citrulline, which are instead released from the liver (Bergman, 1986; Wolff *et al*, 1972). Glutamate concentration, in contrast to many of the amino acid concentrations was shown, in both growth experiments to be positively correlated with plasma acetate concentration in animals fed the diets once daily. This implies that while many of the amino acid concentrations decreased with increasing acetate concentration, the glutamate concentrations increased, following a once daily feed. Therefore, the positive correlation with acetate and glutamate that is observed in all the once daily fed animals in the first experiment (Chapter 3) and the 2 out of three of the animals fed FFD in the second experiment reported (Chapter 4), may be due to an increase in the release of glutamate from the liver following feeding. The negative correlations observed in many of the other amino acids with acetate could be due to increased uptake by either the liver or the peripheral tissues, following feeding. Therefore, temporal changes in the concentration of amino acids in the peripheral blood may indicate temporal changes in their uptake and release by the liver and peripheral tissues. The requirements of the tissues for the amino acids are likely to be dependent on the energy that is available for their utilisation (i.e. protein synthesis or oxidation) or the influence of circulating hormones.

### 6.3 The effect of diet and feeding frequency on acetate supply to peripheral tissues

The pattern of the concentration of the energy-yielding acetate in the plasma, differed considerably, throughout the day, between the groups with a different frequency of feeding. As was shown by Sutton *et al.* (1988) in dairy cows, the sheep in this experiment, fed hourly had a very constant plasma acetate concentration throughout the day and those that were fed once daily had large peaks in the acetate concentration following feeding. Fat deposition appeared to be enhanced in animals fed the diets hourly compared to those fed the same diets once daily, in both experiments with the exception being animals fed the SS diet. In ruminants acetate is a significant source of energy and carbon (as acetyl CoA) for *de novo* synthesis of fatty acids which are used to form TAG and consequently lipid molecules, in adipose tissue. Acetate incorporation into adipose tissue, in ruminants, is thought to be utilise a supply of glucose. *In vitro* studies have shown that increasing the glucose present in the media of ovine adipocytes, increases the incorporation of acetate into lipid and the oxidation of acetate to CO<sub>2</sub> (Scollan & Jessop, 1995). Other studies have shown that increasing the proportion of the glucose precursor, propionate relative to acetate in the rumen, increases the rate with which acetate is removed from the blood (Cronje *et al.*, 1991; Jarrett & Filsell, 1961). Synthesis of fatty acids requires considerable amounts of NADPH which can be produced from either the pentose phosphate pathway (PPP) or from the oxidation of isocitrate in the cytosol. The substrate for the PPP is glucose (see Figure 1.4). Glucose is used to produce NADPH which is required for triglyceride formation. Glucose is also required for the synthesis of sufficient glycerol to form the TAG molecules. It is possible therefore that in the animals fed once daily (with the exception of animals fed SSD), sufficient glucose could not be produced from the propionate rapidly enough in the liver, following feeding, to utilise the sudden surge of acetate that was available in the plasma, for fatty acid synthesis. The acetate in the plasma of the animals fed hourly, therefore, may have been utilised more efficiently for TAG formation, as the demand for glucose was not as erratic and could therefore be met. This would result in more fat deposited in the hourly fed animals, as was seen in both experiments. Animals fed the SSD not only had constant acetate concentration throughout the day, but the acetate concentration was also elevated for longer than those fed FFD. The relatively



constant elevated acetate concentration may have enhanced fatty acids synthesis. In animals fed the diets once daily, in both experiments, the glucogenic amino acids (glycine and alanine) generally rapidly decreased following feeding with an increasing acetate concentration, as was seen with these amino acids having significantly strong negative correlations with the acetate concentration in most animals fed the diets once daily. This decrease in concentration may mean that there is a greater amino acid uptake by the liver, from the plasma, following feeding which would indicate that there is an attempt by the animal to increase the production of glucose in the liver with the increasing acetate concentration. In the animals fed the "slow/slow" diet once daily (SSD), the correlations of these glucogenic amino acids with acetate were only significant when correlating glycine and these were not as highly significant as when the same correlations were made in animals fed FFD. The total amino acids appeared to decrease less suddenly and more gradually throughout the day, indicating that the demands on the liver for synthesising glucose may not have been as great, immediately following feeding, as when the animals were fed FFD.

Animals fed the diets once daily in the second experiment reported (Chapter 4), tended to have greater amounts of crude protein in the carcass and generally heavier muscles, compared to the animals fed the same diet, hourly. This difference between hourly and once daily fed animals was particularly noted in animals fed the SS diet, and may have been greater in the animals fed the FF diet, had the animals fed this diet once daily, eaten all that they were offered. As acetate is considered a source of energy for protein synthesis (see Hocquette *et al.*, 1998; Madsen, 1983), a large surge in acetate concentration following feeding, as was seen in once daily fed animals, could contribute to a surge in energy for the anabolism of tissue proteins. Utilisation of the amino acids for tissue synthesis may account for the decrease in plasma amino acid concentrations. A decrease in the concentration of total amino acids and leucine was observed in all animals fed once daily, in the experiments reported in Chapters 3 and 4, following the meal. Grizard *et al.* (1987) showed in growing sheep, that after an insulin injection, essential plasma amino acids decreased and that there was a close correlation between the decrease in amino acids in the plasma and an increase in the same amino acids in the muscle tissue indicating that

there may be an increase in the incorporation of amino acids into muscle following feeding. In rats Garlick *et al.* (1973) showed, following a meal, there was a cyclic pattern in protein synthesis with an increase for up to 18h after the meal, followed by a decrease. Millward (1985), on the basis of similar results in humans, proposed that in adult humans there were continuous diurnal changes in the body protein, reflecting the meal pattern. He stated that overall N balance is achieved by depositing sufficient protein after a meal to balance the post-absorptive losses that will occur. With small feed intakes there are small post-absorptive losses and with large feed intakes there are greater losses in body protein, but a balance is achieved, probably by hormonal control. The animals used in the experiments reported in this thesis were growing and consequently in a different physiological state to that of Millward's human subjects. However, this indicates that size of the meal is likely to affect the rate at which protein is deposited following feeding. In these experiments those fed once daily ate one large meal and those fed hourly ate regular small meals. As the animals in the experiment reported in Chapter 4 tended to have bigger *Semitendinosus* and *V.lateralis* in animals fed once daily compared to those fed hourly, particularly in those fed the diet with an elevated but more constant plasma acetate (energy) and insulin supply following feeding (i.e. animals fed SSD). The animals fed SSD also had significantly larger amounts of crude protein deposited in the carcass ( $P < 0.01$ ). This suggests that the anabolism of proteins exceeded catabolism (post-absorptive losses) to a greater extent in these animals fed once daily which would cause a greater net protein deposition. The difference in the pattern of acetate and insulin that were observed, may have altered the pattern and rate of protein anabolism and catabolism, which possibly determined the extent to which protein was deposited.

One factor that should be considered from the two growth experiments performed here, is the difference in feed intake between dietary groups. Those fed the diets hourly in both experiments, reported in chapters 3 and 4, consumed more energy than when the diets were fed once daily. This could account for some of the differences in growth in the first experiment (Chapter 3) and also in the animals fed the FF diet in the second experiment (Chapter 4), but not the differences observed in the animals fed the SS diet (Chapter 4), as these animals consumed the same amount. Studies on the frequency of feeding have shown that when rats are fed less frequently the feed intake is significantly reduced (Leveille, 1970; Cohn, *et al.*, 1965). The

animals, being individually penned, were seen, it was thought, to suffer from boredom, and this may be the reason that animals fed hourly ate more, simply for want of something to do. The differences in feed intake could also be due to the difference in the pattern of supply of nutrients and consequently a difference in the efficiency of utilisation of the nutrients by the animal. For example, reports have suggested that without adequate glucose, blood acetate concentration rises which results in metabolic feedback which may reduce feed intake (Illius & Jessop, 1996). As previously suggested, following feeding there was such a large and rapid rise in the concentration of acetate in animals fed the diets once daily, that glucose may be insufficient or cannot be metabolised rapidly enough, resulting in a feedback response which reduced feed intake in animals fed diets once daily.

Voluntary food intake, is thought to be controlled by a number of different integrated factors, which together have been shown to have an additive effect (Forbes, 1996). An imbalance in nutrients is one factor, an example being discussed above with glucose and acetate, but distension of the rumen (Allen, 1996) and the efficiency with which oxygen is used to yield energy for maintenance and growth of the animal (Ketelaars & Tolkamp, 1996) may be other factors that together send signals to the central nervous system and restrict intake in the animals fed the diets once daily. Feeding once daily is likely to be affected by the factors described above and so it is maybe not surprising the animals ate less within the hour than the hourly fed animals.

#### **6.4 The effect of diet and feeding frequency on insulin in peripheral plasma**

A peak in the insulin concentration was observed following feeding when the animals were fed once daily. This coincides with data on the pattern of insulin concentrations with respect to feeding that was reported by both Sutton *et al.*, (1988) and Bassett (1974). In contrast those fed hourly had a more constant supply of insulin throughout the day. These differences may contribute to the rate of protein anabolism and catabolism, which may in turn affect the extent to which protein was deposited in the carcass and at the muscle. As previously mentioned, in the second experiment (Chapter 4) animals fed the diet once daily tended to have heavier *V.lateralis* and *Semitendinosus* than those fed the same diet hourly. In addition, in animals fed the

SS diet, the amount of carcass protein deposited was significantly greater than those fed the same diet hourly. No difference was seen in the carcass crude protein between animals fed the FF diet once daily (FFH) or hourly (FFH), however the feed intake was significantly less in animals fed FFD which could suggest that had the animals had same level of feed intake, there may have been a greater deposition of carcass protein in animals fed FFD compared to those fed FFH.

Insulin is believed to Previous studies in ruminants, have shown that muscle protein synthesis was unaffected by insulin when infused into lactating goats (Tauveron *et al.*, 1994) and dry goats (Tesseraud *et al.*, 1993). Both studies, however, showed that insulin inhibited protein degradation, especially during lactation (Tesseraud *et al.*, 1993) which implies that insulin plays a significant mechanistic role in conserving protein within the muscle (see Grizard *et al.*, 1995 or Wolff *et al.*, 1989). Whether similar responses of protein synthesis to insulin occur in rapidly growing animals has not been reported (Weekes, 1996), but perhaps another hormone or factor contributed to an increase in protein synthesis. In growing animals for protein deposition and consequently growth to occur, rates of protein synthesis must exceed rates of degradation. The concentrations of plasma insulin appeared to be elevated for a longer period of time throughout the day and although, not statistically significant, animals fed SSD tended to have a higher mean daily insulin concentration than those fed the other dietary treatments in the experiment reported in chapter 4. If the insulin reduced protein catabolism within the muscle for a longer period of time, causing a shorter period of postabsorptive loss, this may be why the animals fed SSD tended to have larger muscles. With an elevated energy supply for a period in the day than the hourly fed animals and more gradually throughout the day, compared to the FFD fed animals, there would appear to be an enhanced acetate concentration for a longer period and therefore a more continuous elevated energy source for synthetic processes.

Previous studies have shown that acetate uptake by the sheep hindlimb, when measured by arterio-venous (A-V) difference was significantly impaired in diabetic animals and the uptake restored back to the level of normal animals when insulin was infused (Table 6.1, Jarrett *et al.*, 1974).

Table 6.1 The effects of insulin treatment on diabetic animals on the arterio-venous differences of acetate and glucose concentrations when expressed as  $\mu\text{moles/g}$  plasma across the sheep hindlimb (Results from Jarrett *et al.*, 1974).

Metabolite	Normal (untreated animals)	Diabetic animals	Diabetic animals treated with insulin
Glucose	0.164**	0.077	0.086*
Acetate	0.41**	0.12	0.31**

Values that are statistically greater than zero are indicated as \*\* =  $P < 0.01$ , \* =  $P < 0.05$ .

The uptake of glucose by the sheep hindlimb, in the study by Jarrett *et al.*, (1974), followed a similar pattern to that of acetate in response to insulin, although not to the same extent (Table 6.1). The uptake of glucose by skeletal muscle appears to be less responsive to insulin in ruminants than in rats (Prior *et al.*, 1984). This would suggest that insulin plays some part in increasing energy supply to the tissues that are synthesising protein, although it appears that these effects are not as obvious as in the monogastric. The effects of increasing the acetate concentration in the peripheral plasma, by intravenous infusion of sodium acetate, on estimates of amino acid utilisation (including whole-body protein synthesis) were investigated in the final experiment (reported in chapter 5). No increase in protein synthesis was seen when sodium acetate was intravenously infused, although measurements of leucine oxidation decreased during the sodium acetate infusion. The increase in plasma acetate concentration, following an infusion of sodium acetate, did not appear from this study (Chapter 5) to correspond with an increase in the secretion of insulin into the peripheral circulation. This is not surprising as several studies would suggest that acetate does not stimulate the secretion of insulin (Mineo *et al.*, 1994 and 1990; De Jong, 1982). Mineo *et al.* (1994) showed that by injecting short-chain fatty acids intravenously into sheep, butyrate was the most effective at increasing insulin secretion and acetate was very ineffective. Propionate, the precursor to gluconeogenesis in the liver, also appeared to stimulate insulin secretion (Mineo *et al.*, 1994; Sano *et al.*, 1993; Sutton *et al.*, 1988, Bines & Hart, 1984; De Jong, 1982). Following feeding in the once daily animals, when acetate increased, insulin also appeared to increase.

However, by infusing sodium acetate alone into the peripheral blood, as was done in Chapter 5, no increase in plasma concentrations of insulin was seen. With no

increase in plasma insulin concentration, the sensitivity of peripheral tissue to acetate may not have increased with increasing acetate concentration and so energy was not made available for an increase in protein synthesis as might have been expected by an increasing concentrations of plasma acetate in the peripheral plasma. Garlick & Grant (1988) reported that in rats the sensitivity of muscle to insulin increased with increasing plasma amino acid concentrations, particularly the branched-chain amino acids, which was thought to increase the incorporation of amino acids into the muscle tissue. Reports would suggest, therefore, that the effects of insulin, on protein synthesis are limited, but that other factors (e.g. amino acids) and hormones may influence the use of acetate in protein synthesis in the ruminant. Consequently, with an increase in just plasma acetate concentrations in the final experiment reported (Chapter 5), the effects that are seen are unlikely to be the same as those following a once daily feeding, where insulin certainly increased with feeding and probably a multitude of hormones and factors may be influencing the effect on protein deposition.

The excess acetate, following infusion, may have been oxidised which may be why in the experiment reported in Chapter 5 there was an increase in total CO<sub>2</sub> entry rate. A large pulse in acetate may have caused the decrease in the oxidation of leucine that was observed, due to a switch in the substrate that was oxidised. Leucine, as a ketogenic amino acid, is oxidised to isovaleryl CoA (Wolfe, 1992) and eventually to acetyl CoA (Stryer, 1988). It is possible that the pathway oxidising the ketoacid intracellularly (Figure 5.1) is inhibited by a surge in acetate concentration, with a preference for the oxidation of acetate to occur. This may also account for the highly significant increase in plasma concentrations of KIC, as if the leucine is not oxidised directly an alternative pathway is necessary. It is possible, therefore, that more leucine was transaminated in the cells to KIC, but due to a decrease in the oxidation of KIC (see Figure 5.1) the concentration of KIC increased.

If excess acetate was oxidised, as the energy generated does not appear to have been used for whole-body protein synthesis, some of the energy generated from this oxidation may have been used by fat synthesis. There is, however, strong evidence that insulin plays a regulatory role in acetate utilisation particularly by adipose tissue in the presence of glucose (Yang & Baldwin, 1973; Skarda & Bartos,

1969) as adipose tissue is well supplied with insulin receptors (Vernon, 1992). In addition, insulin is thought to activate acetyl CoA carboxylase and so increase lipogenic flux (Etherton & Evcock, 1986; Vernon & Finlay, 1988), although the actual mechanism with which this occurs remains to be elucidated (Vernon, 1992). Insulin has been shown to stimulate glucose and acetate utilisation (Vernon & Taylor, 1988). Insulin administration to ruminants resulted in a rapid decrease in plasma fatty acids (Bauman, 1976) and glycerol levels (Bergman, 1968), indicating that perhaps acetate is necessary to replace these. Insulin can also reduce the rate of fatty acid release from the adipose tissue by stimulating glucose uptake and so enhancing fatty acid re-esterification (Vernon, 1981). It is therefore likely that as there were no changes in insulin during the sodium acetate infusion, fat synthesis may also have been unaffected by the increase of an infusion of just sodium acetate. The growth trials reported in Chapters 3 and 4 showed that the groups of animals with increased fat deposition also tended to have an increase in the daily insulin concentration (i.e. AH in the first experiment and SSD in the second), although these increases were not significant.

If excess acetate is oxidised, and the energy not utilised for synthetic functions (e.g. protein synthesis or fat synthesis) then the energy must be lost as heat. This may have been what occurred in the experiment performed in Chapter 5. The excess acetate may have been oxidised, inhibiting the leucine oxidation pathway, but the energy generated from acetate oxidation apparently was not used to fuel an increase in whole-body protein synthesis possibly because other metabolites and hormones were limiting. Consequently the energy formed may have been lost as heat. If the experiment reported in Chapter 5 was to be repeated it would be useful to measure the heat produced before, during and after the acetate infusion, to predict whether the possible increase in acetate oxidation did coincide with an increase in heat production. Studies of intravenously infused acetate (2 moles per day) have given conflicting results. Studies by Crabtree *et al.* (1987) showed that the heat produced by animals was not significantly increased when acetate was infused. MacRae & Lobley (1985), however, showed that there was an increase in heat production when acetate was infused into animals fed a poor quality forage diet, but not when a high quality forage diet was fed. It is possible in their experiment that when the good quality diet was fed, sufficient nutrients were being supplied to utilise the energy

generated by acetate oxidation efficiently for synthetic processes. Feeding different diets apparently alters the efficiency with which an infusion of acetate is utilised (Macrae & Lobley, 1985), which may account for the conflicting results that have been obtained with regard to infusing acetate. In the experiment reported in Chapter 4, conditions may have been such that the energy that may have been produced during oxidation of the surge in acetate concentration following feeding, was utilised for protein synthesis. In the experiment reported in Chapter 5, no increase in whole-body protein synthesis was observed which may be due to an absence of other metabolites and hormones, which consequently may prevent the energy produced by oxidation to be used efficiently for increasing whole-body protein synthesis. This suggests that there are a number of interactive factors between the type of nutrients absorbed, the energy supplied and the nitrogen metabolism in the rumen, that may affect the efficiency of feed utilisation.

Another possible reason for the apparent differences in the utilisation of a surge in acetate concentration between the experiments reported in Chapter 4 and 5 may be that the animals were not accustomed to the sudden change in acetate concentration and therefore did not respond in the same way as an animal acclimatised to a regular pulse in acetate concentration following a regular daily feeding. Perhaps if these animals had been infused each day with sodium acetate for approximately one week prior to the measurements of leucine oxidation and estimates of whole-body protein synthesis, a different result may have been obtained.

Elevated concentrations of plasma insulin and perhaps patterns of other hormones may be factors that work together with the energy source acetate to enhance the synthetic processes discussed here. The timing of nutrient supply to the peripheral tissues and the influence that hormonal balance has on the deposition of fat and lean needs to be investigated further.

## **6.5 Future work**

A more constant, but elevated concentration of both plasma acetate and insulin may be the reason that the animals fed SSD had a greater growth rate and muscle deposition than those fed SSH and FFD. Acetate and insulin concentrations could perhaps be made more constant and maintain the elevation in concentrations,



for longer throughout the day, particularly in the second half of the day, by feeding the SS diet 2 -4 times per day. The effects on growth and carcass composition and the blood metabolites can be measured and compared to another group of animals fed the same SS diet once daily. If an animal is fed a diet with a slowly degraded OM and N component, such that there is a constant and elevated level of acetate and insulin throughout the 24 hours in the day and as a result produces a fast growing and leaner carcass because of enhanced protein synthesis or inhibited protein degradation at the muscles, this information could be of practical use to the animal producer, who particularly in the sheep industry is striving for a leaner and faster growing carcass.

Growth trials performed in the future should pair-feed animals so that feed intake is not a confounding factor in drawing conclusions from the results, as was the case in work reported here in Chapters 3 and 4. Although the efficiency with which the feed consumed is utilised for growth can be calculated, it is very difficult to draw conclusions on feed utilisation for fat and muscle deposition as the nutrients available between feed groups in both growth trials performed, were not the same.

As was carried out in the experimental work in this thesis, it is vital that sufficient animals are used in each treatment group. This is because the variation between animals can be fairly considerable, as was demonstrated in the results in chapters 3 and 4, especially regarding the metabolite concentrations when correlated with each other (Table 3.10-11 and 4.11-12) . As with these experiments, animals will need to be individually penned. In addition, although very time consuming, as many animals from each dietary groups, as would be possible should be analysed for concentrations of plasma metabolites from each treatment group to reduce the error in the statistical analyses. These requirements are likely to limit the size of the experiment with regard to number of groups and number of animals, because of cost, time and space allowances.

Measurements of the energetic efficiency of utilisation of the diets fed in this experiment could be measured in the future. It would be useful to investigate the effects of feeding diets with a slow or fast rate of OM degradation and also hourly or once daily on the production of heat. This would further knowledge with regard to the efficiency with which these diets and the patterns of feeding, with different temporal supplies of energy-yielding acetate (as were seen in the work carried out in this thesis), are used for growth. As previously mentioned (section 6.4) it would also

be useful to measure the production of heat, when infusing sodium acetate to measure leucine metabolism parameters, to attempt to conclude how a pulse in plasma acetate concentration is being utilised.

Amino acid concentrations in this work, were measured in the plasma. Amino acids are also transported in the erythrocyte. It has been shown, however, that for a number of the amino acids there are no differences between erythrocyte and plasma concentrations (see Heitmann & Bergman, 1980; Lobley *et al.*, 1996). In their experiments no temporal patterns of amino acid concentrations were investigated. Further work needs to be carried out to investigate the importance of possible differences in trends between the plasma and the whole blood for particular amino acids with respect to feeding throughout the day.

Future work could look at the levels of hormones other than insulin, when lambs are fed such that the rumen is supplied with different rates of OM degradation. Growth hormone (GH) and IGF-1 are all thought to play a role in muscle protein metabolism. GH is considered to have indirect effects on cell division and growth (Harper & Buttery, 1992). Reports suggest that the growth promoting effects on skeletal muscle seen by GH are mediated by IGF-1 (McDowell & Annison, 1991; Davis *et al.*, 1988; Etherton & Kensinger, 1984). IGF-1 is a polypeptide growth factor, which has been shown to stimulate protein synthesis, amino acid and glucose uptake and to inhibit protein degradation in skeletal muscle cells (Harper *et al.*, 1987). The capacity of the high affinity hepatic GH receptor is particularly sensitive to nutritional status (Breier *et al.*, 1988) It is thought that this receptor could be responsible for the mediation between GH, IGF-1 and growth. When nutritional level is high, the plasma GH is low and the IGF-1 levels are raised. Production studies in relation to the patterns of feeding and the pattern of peripheral nutrient supply and the patterns and plasma concentrations of GH and IGF-1 would be interesting with regard to the extent of fat and protein deposition in the carcass. It should be noted that GH can fluctuate considerably within an hour, resulting in a need for very frequent blood sampling (every 5-10 min). If very regular samples needed to be taken, a larger ruminant model would be necessary e.g. the steer, so that the blood volume is not decreased more than 5%.

As a follow on study from the experiment reported in Chapter 5, the effects of increasing plasma acetate concentration on fat and protein metabolism should be

further investigated. Results previously have indicated that insulin is responsible for the conservation of body protein, as previously discussed. To confirm whether insulin and/ or other hormones and/ or amino acids were the limiting factors preventing a detection of an effect on protein synthesis when sodium acetate was infused into the peripheral blood a further series of experiments should be carried out. A similar experiment could be performed to that reported in Chapter 5, but in addition to infusing sodium acetate a series of metabolite and hormone mixes could be infused where the effects of sodium acetate with or without amino acids and /or insulin and /or other hormones, on estimates of whole-body protein synthesis or amino acid incorporation could be measured. It is proposed that metabolite mixes (hormone, amino acids and/ or acetate) be dissolved in saline to make an infusate. If each infusate mix was infused into animals, 5 hours after the start of a 11h continuous infusion of [1-<sup>13</sup>C]-leucine, for 3 hours (using the same method as discussed in Chapter 5), it would be possible to find out whether acetate, insulin or amino acids or a mixture of any or all of the three, will alter estimates of whole-body protein synthesis. The method for estimating whole-body protein synthesis (described in Chapter 5), however, is fairly crude and therefore large differences are likely to be required to have a significant effect in the measurements, as were discussed. An alternative method of measuring incorporation of amino acids into muscle would be to take biopsies of a representative muscle before, during and after the infusate was infused. If animals were continuously infused with radioactively labeled amino acid (e.g. [1-<sup>14</sup>C]- phenylalanine) and isotopic equilibrium of the label was reached, the infusion of the metabolite mixes could begin and muscle biopsies taken to measure for the incorporation of <sup>14</sup>C-phenylalanine . The influence of the metabolite mixes on the incorporation of amino acids into the muscles will then be established. This would enable further knowledge on other factors that effect the use of acetate as an energy source in muscle protein synthesis

## **6.6 Summary**

Feeding a diet hourly gave a more constant supply of nutrients to the tissues throughout the day compared to when the same diet was fed once daily. From the first experiment (Chapter 3), a more constant supply of nutrients to the tissues was thought to enhance growth and deposition of crude protein in the carcass. Supply of

nutrients was not assessed directly, but was inferred from measurements of the nutrient concentrations. The pulse in acetate concentration following feeding appeared to be strongly related to the rate with which the OM was degraded in the rumen. In the second experiment (Chapter 4), animals were fed two diets, predicted to have differing OM release into the rumen following feeding. The two diets in this experiment were fed either hourly or once daily. The once daily fed animals tended to lay down more crude protein in the carcass and have larger muscles than those fed the same diets once daily. Animals fed either diet once daily had a pulse in acetate and insulin concentration following feeding. In contrast, those fed hourly had a more constant supply of peripheral nutrients (amino acids and acetate) to the tissues.

It was thought that the reason that the hourly fed animals grew faster and had significantly more crude protein deposited in the carcass, in the first experiment, was due to a significantly greater feed intake in the hourly fed animals compared to those fed the same diet once daily ( $P < 0.001$ ). The pulse in acetate concentration, therefore, may contribute to an increase in the synthesis of protein in tissues throughout the day and consequently enhance the deposition of protein in the whole animal, as was seen in animals fed the SS diet in the second experiment.

However, when sodium acetate was infused into the peripheral blood of lambs (the third experiment; Chapter 5) no increase in the estimates made of whole-body protein synthesis were observed. The acetate alone appeared unlikely to affect the measurements of whole-body protein synthesis and it is therefore thought that the amino acid supply, the plasma insulin concentrations and /or other plasma hormone concentrations, in addition to the temporal changes in acetate concentration may be responsible for the differences in carcass composition, when animals are fed different diets and at different feeding frequencies.

The rate of OM degradation may affect the magnitude and length of time that the pulse in acetate in the blood is elevated throughout the day. This pulse in acetate concentration could consequently affect the rate of protein synthesis and deposition. Further work needs to be carried out to investigate the effect of a pulse in acetate concentration on protein synthesis and deposition.

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