

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**AMINO ACID UTILISATION BY THE  
MAMMARY GLAND OF DAIRY COWS  
FED FRESH FORAGE**

A thesis presented in partial fulfilment of the requirements  
for the degree of

**Doctor of Philosophy**

in

Animal Science

at Massey University, Palmerston North,

New Zealand

David Pacheco-Ríos

2000

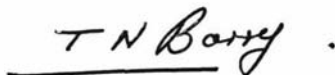
## DECLARATION

The studies presented in this thesis were completed by the author whilst a postgraduate student in the Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand. I hereby affirm that the content of this thesis is original research conducted by the author. All views and conclusions are the sole responsibility of the author. All references to previous work are included in the References section of each chapter. Any assistance received during the preparation of this thesis has been acknowledged.

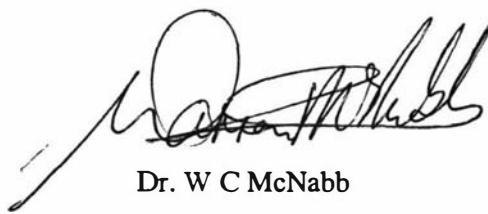
I certify that the content of this thesis has not already been submitted for any degree and is not being currently submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis, and all sources of materials used, have been acknowledged in this thesis



David Pacheco-Rios  
PhD Candidate



Prof. T N Barry  
Chief Supervisor



Dr. W C McNabb  
Co-supervisor



Assoc. Prof. D D S Mackenzie  
Co-supervisor



Dr J P Hill  
Co-supervisor

## ABSTRACT

A series of experiments was conducted to assess the utilisation of amino acids by the mammary gland of lactating dairy cows fed fresh forages. Experiments were conducted at Palmerston North (AgResearch and Massey University) and Hamilton (Dairying Research Corporation), New Zealand with the purpose of identifying the amino acids which may limit milk protein synthesis.

The first two experiments (Chapter 3) were conducted to assess the effect of methionine supplementation on the productive performance of Friesian cows fed fresh cut pasture (ryegrass (*Lolium perenne*) / white clover (*Trifolium repens*) at two different stages of lactation (mid and late lactation). Methionine has been nominated as one of the first limiting amino acids in concentrate-fed cows, and theoretical calculations showed that its supply could also be limiting milk protein synthesis in pasture-fed cows. Supplementation with methionine (15 g d<sup>-1</sup>) did not have significant effects upon the concentrations and yields of milk protein, fat or lactose in either experiment. In late lactation, methionine-supplemented cows produced less  $\beta$ -casein ( $P < 0.05$ ). In mid lactation, intravenous infusion of methionine increased ( $P < 0.05$ ) the efficiency of conversion of pasture nitrogen to milk protein.

Results from the methionine supplementation trials highlighted the lack of reliable information available to explain the responses to extra supply of amino acids in pasture-fed dairy cows. Therefore, a third experiment was conducted to provide information about the utilisation of amino acids by the mammary gland of Friesian cows fed two levels of dry matter intake (*ad libitum* and 75% of *ad libitum*). This experiment was designed to provide information on amino acid metabolism from two different approaches. The first approach consisted in the measurement of amino acid utilisation by the mammary gland using an arterio-venous preparation (Chapter 5). The second approach consisted of the use of isotopic markers to measure the total flux of amino acids in the whole body (Chapter 6). Additionally, an evaluation of two methods for measuring mammary blood flow was conducted as part of this experiment (Chapter 4).

The comparison of methods for blood flow measurement (Chapter 4) showed that the arterio-venous difference and output in milk protein of methionine and phenylalanine+tyrosine yielded similar estimates of mammary blood flow (8.1 and 8.8 litres per minute, respectively) when used as markers with the Fick principle. On the other hand, the use of tritiated water as a marker gave a significantly lower ( $P < 0.05$ ) estimate of mammary blood flow (5.3 litres per minute) than the method using methionine or phenylalanine+tyrosine. Therefore, it was concluded that methionine and/or phenylalanine can be used as an indirect approach for measuring mammary blood flow when direct methods such as flow meters are not available.

Results from the arterio-venous preparation of the mammary gland identified plasma as the main source of free amino acids for milk protein synthesis. Contribution from erythrocytes was limited to isoleucine, leucine, phenylalanine and tyrosine and accounted for 5-14% of the total uptake of these amino acids by the mammary gland. However, uptake of free amino acids from plasma was in some cases not enough to account for their appearance in milk protein. Histidine was the amino acid with the greatest deficit between output and uptake. Sources other than free histidine appear to provide up to  $4.2 \text{ g d}^{-1}$  of histidine to the mammary gland for milk protein synthesis. It is speculated that the high contribution of non-free histidine (most likely small blood peptides) is the result of a limitation in the transfer of free histidine from the blood to the mammary gland.

The whole body fluxes of amino acids were measured by isotopic dilution using continuous infusion of a mixture of universally labelled  $^{13}\text{C}$ - AA. The whole body flux of essential amino acids was reduced by 20% as a result of restricted feed intake, with exception of glutamic acid, for which the whole body flux was up to 8% higher in restricted animals. On average, the mammary utilisation accounted for one third of the whole body flux of essential amino acids. The branched-chain amino acids, plus lysine, are the amino acids with the greatest partitioning towards the mammary gland. It is concluded that the high mammary demand for this group of amino acids may create potentially limiting conditions in terms of their supply for milk protein synthesis.

From this series of studies on amino acid utilisation by lactating dairy cows fed fresh forage, it can be concluded that a) it is unlikely that extra supply of only one amino acid may elicit positive responses in milk protein production; b) potentially limiting amino acids have to be identified using several criteria for different metabolic conditions. Summarising several methods of assessment of limiting amino acids, it can be concluded that histidine, lysine, phenylalanine, threonine and leucine are the main candidates for limiting amino acids in pasture-fed dairy cows.

In the General Discussion, methods for confirming or rejecting that the supply of these amino acids restrict milk protein production in forage-fed dairy cows are proposed, and practical methods for increasing the supply of limiting amino acids in grazing dairy cows are discussed.

**THIS THESIS IS DEDICATED TO MY PARENTS**

**AARON AND MARGARITA PACHECO**

I walk the maze of moments  
but everywhere I turn to  
begins a new beginning  
but never finds a finish

I walk to the horizon  
and there I find another  
it all seems so surprising  
and then I find that I know

Enya. *Anywhere is*



## ACKNOWLEDGMENTS

I express my deepest gratitude to my Supervisors: Prof. Tom Barry, Dr. Jeremy Hill, Assoc. Prof. Duncan Mackenzie, and Dr. Warren McNabb for their helpful advice, support and encouragement given all the way through my PhD studies. I admire and respect their solid scientific knowledge. But being a good supervisor is more than just science... all your advice and support in the "big little things" behind the scenes is most appreciated.

I sincerely thank the support provided by: Ms. Penny Back, Ms. Jennifer Burke, Ms. Sarah Cridland, Dr. Tricia Harris, Dr. Julian Lee, Mr. Jason Peters, Dr. Nicole Roy, Dr. Michael Tavendale, Mr. Bryan Treloar (Nutrition and Behaviour, AgResearch); Dr. Steve Davis (Food Science, AgResearch); Dr. Vicki Carruthers, Dr. Eric Kolver, Ms. Erna Jansen, Mr. David Phipps (Dairying Research Corporation); Mr. Geoff Paterson, Mrs. Ruth Lowe (Food Section, New Zealand Dairy Research Institute); Nicolás López-Villalobos (IVABS, Massey University) and Dr. H elene Lapierre (Agri-Food and Agriculture Canada);. Thanks for your help provided in different stages of this project including skillful technical assistance in the laboratory and experimental phases of this PhD, discussion of results, and constructive criticism of manuscripts.

Thanks to the Direcci n General de Asuntos del Personal Acad mico (DGAPA) of the National Autonomous University of Mexico (UNAM) for providing the scholarship that allowed me to undertake my doctoral studies. Special thanks to the staff in the Subdirecci n de Becas al Extranjero (DGAPA) and the staff of the Secretar a de Superaci n Acad mica and Departamento de Nutrici n Animal y Bioqu mica (Facultad de Medicina Veterinaria y Zootecnia, UNAM).

The experimental phase of this project was supported in part by the Foundation for Research, Science and Technology, New Zealand.

To the staff and postgraduate students of the Institute of Food Nutrition and Human Health, Massey University and the Nutrition and Behaviour Group, AgResearch, thanks for providing a great work environment at both institutions.

To my friends, both in Mexico and New Zealand: thanks for your support, I'm in debt to all of you. A whole extra chapter would be required to name you all and thank you for your help in the good and the bad times... you have been with me throughout this roller coaster ride ... you know this thesis is also for you. Special thanks to Veronica for the wonderful time we spent together in New Zealand.

To Aarón y Margarita, because all what I am is your labour of love: you are a wonderful example to follow. May the Lord bless you forever. To Lupita, Fernando, Aarón, Moisés and Victor, and their partners and children... your support and continuous encouragement gave me the strength required to achieve this goal. A mis padres Aarón y Margarita, porque todo lo que tengo y lo que soy es producto de su labor de amor ustedes son un ejemplo a seguir. A mis hermanos Lupita, Fernando, Aarón, Moisés y Victor, a mis cuñados(as) y sobrinos(as)... su comprensión, apoyo y palabras de aliento han sido manantial de donde he obtenido fortaleza para llegar hasta donde he llegado. Que Dios los bendiga por siempre !

To the Supreme Being... because you listened to me every time I needed someone who would listen to my plans, my fears, my dreams... Everything has taught me that You are there.

# TABLE OF CONTENTS

ABSTRACT .....	I
ACKNOWLEDGMENTS .....	VI
TABLE OF CONTENTS.....	VIII
LIST OF TABLES .....	XVII
LIST OF FIGURES .....	XXI
LIST OF ABBREVIATIONS.....	XXIII
<b>CHAPTER 1. GENERAL INTRODUCTION, OBJECTIVES AND FORMAT OF THE                   <b>THESIS</b> .....</b>	<b>1</b>
1.1. GENERAL INTRODUCTION.....	2
1.2. OBJECTIVES .....	2
1.3. FORMAT OF THE THESIS.....	3
<b>CHAPTER 2. A REVIEW OF LITERATURE .....</b>	<b>5</b>
2.1. MILK .....	6
2.1.1. <i>The evolutionary role of milk</i> .....	6
2.1.1.1. Evolution of lactation.....	6
2.1.1.2. Evolution and functions of milk.....	7
2.1.2. <i>The role of milk in human nutrition</i> .....	8
2.1.3. <i>Milk production in New Zealand</i> .....	10
2.1.3.1. World market of dairy products: the New Zealand perspective.....	10
2.1.3.2. The pastoral system: advantages and limitations .....	12
2.1.3.3. Pasture as a nutrient source for milk protein production.....	13

2.2. MILK PROTEIN .....	16
2.2.1. <i>Proteins present in milk</i> .....	16
2.2.1.1. Caseins (CN) .....	17
2.2.1.1.1. Alpha casein ( $\alpha$ -CN) .....	19
2.2.1.1.2. Beta casein ( $\beta$ -CN) .....	19
2.2.1.1.3. Kappa casein ( $\kappa$ -CN).....	20
2.2.1.2. Whey proteins .....	20
2.2.1.2.1. Alpha lactalbumin ( $\alpha$ -LA).....	21
2.2.1.2.2. Beta lactoglobulin ( $\beta$ -Lg) .....	21
2.2.1.2.3. Serum albumin (SA).....	22
2.2.1.2.4. Immunoglobulins (IG).....	22
2.2.2. <i>Milk protein synthesis in the mammary gland</i> .....	23
2.2.2.1. Factors affecting milk protein production in dairy cows .....	24
2.2.2.1.1. Nutrition .....	25
2.2.2.1.1.1. Dietary energy.....	26
2.2.2.1.1.2. Dietary protein.....	27
2.2.3. <i>Role of amino acids as milk protein precursors</i> .....	27
2.2.3.1. Essential amino acids .....	27
2.2.3.1.1. Group I of EAA .....	28
2.2.3.1.2. Group II of EAA.....	28
2.2.3.2. Non essential amino acids.....	29
2.2.4. <i>Amino acids as limiting nutrients for milk protein synthesis</i> .....	29
2.2.4.1. The concept of limiting amino acid.....	30
2.2.4.2. Pools of amino acids for milk protein synthesis .....	31
2.2.4.2.1. Free amino acids (FAA) .....	32
2.2.4.2.1.1. Plasma.....	32

---

2.2.4.2.1.2. Erythrocytes.....	33
2.2.4.2.1.3. Plasma vs. whole blood.....	34
2.2.4.2.2. Sources other than FAA.....	35
2.2.4.2.2.1. Peptides.....	36
2.2.4.2.2.2. Protein turnover in the epithelial cell.....	36
2.2.4.3. Amino acid supply to the mammary gland as the limiting factor for milk protein synthesis.....	37
2.2.4.3.1. Dietary factors affecting the amino acid supply to the mammary gland..	37
2.2.4.3.1.1. Microbial protein supply.....	39
2.2.4.3.1.2. Nature of the dietary protein: rumen-degradable vs. rumen undegradable.....	40
2.2.4.3.1.3. Protein digestibility.....	41
2.2.4.3.2. Metabolic factors affecting the amino acid supply to the mammary gland..	42
2.2.4.3.2.1 Absorption of amino acids in the small intestine.....	43
2.2.4.3.2.2. Impact of splanchnic organs on amino acid supply to the mammary gland.....	43
2.2.4.3.2.3. Partitioning of amino acids for bodily functions.....	44
2.2.4.3.2.4. Blood flow to the mammary gland.....	45
2.2.4.3.2.5. Importance of the mammary blood flow assessment in the validity of balance studies.....	47
2.2.4.4. Amino acid metabolism in the mammary gland as the limiting factor.....	48
2.2.4.4.1. Amino acid uptake: Transport systems in the mammary gland.....	48
2.2.4.4.2. Amino acid transport as a limiting factor.....	49
2.3. APPROACHES TO DETERMINE LIMITATIONS IN THE AMINO ACID SUPPLY TO THE MAMMARY GLAND.....	50

2.3.1. Amino acid profiles: the concept of ideal supply.....	50
2.3.2. Arterio-venous difference and extraction rates from arterial supply.....	52
2.3.3. Balance studies: Uptake vs. output comparisons .....	52
2.4. CONCLUSIONS AND FUTURE RESEARCH NEEDED .....	53
2.5. REFERENCES.....	55
<b>CHAPTER 3. THE EFFECTS OF METHIONINE SUPPLEMENTATION UPON MILK COMPOSITION AND PRODUCTION OF FORAGE-FED DAIRY COWS</b> .....	<b>76</b>
3.1. ABSTRACT .....	77
3.2. INTRODUCTION .....	78
3.3. MATERIALS AND METHODS .....	79
3.3.1. Experimental Design .....	79
3.3.2. Animals and Diets.....	79
3.3.2.1. Experiment 1.....	80
3.3.2.2. Experiment 2.....	80
3.3.3. Treatments .....	80
3.3.3.1. Experiment 1.....	80
3.3.3.2. Experiment 2.....	81
3.3.4. Measurements.....	81
3.3.5. Analytical Methods.....	82
3.3.6. Statistical Methods .....	83
3.3.7. Animal Care.....	84
3.4. RESULTS .....	84
3.4.1. Feed Intake .....	84
3.4.2. Blood Metabolites.....	86
3.4.3. Milk Production and Composition.....	86

3.5. DISCUSSION .....	89
3.6. CONCLUSIONS .....	92
3.7. ACKNOWLEDGEMENTS .....	92
3.8. REFERENCES .....	93

**CHAPTER 4. COMPARISON OF TWO VARIANTS OF THE FICK PRINCIPLE FOR ESTIMATION OF MAMMARY BLOOD FLOW IN DAIRY COWS FED TWO LEVELS OF DRY MATTER INTAKE..... 98**

4.1. ABSTRACT .....	99
4.2. INTRODUCTION .....	100
4.3. MATERIAL AND METHODS .....	101
4.3.1. <i>Animals</i> .....	101
4.3.2. <i>Diets</i> .....	102
4.3.3. <i>Methods for estimating mammary blood flow</i> .....	102
4.3.3.1. Amino acid arterio-venous differences .....	102
4.3.3.2. Tritiated water .....	103
4.3.3.3. Milk sampling and udder volume .....	103
4.3.4. <i>Laboratory methods</i> .....	103
4.3.4.1. Amino acid(s) A-V .....	103
4.3.4.2. Tritiated water .....	104
4.3.5. <i>Calculations</i> .....	105
4.3.5.1. Amino acid(s) A-V difference .....	105
4.3.5.2. Tritiated water .....	105
4.3.6. <i>Statistical analysis</i> .....	106
4.4. RESULTS .....	106
4.4.1. <i>TOH method: Effect of sample preparation</i> .....	106
4.4.2. <i>Comparison between the amino acid A-V and TOH methods</i> .....	107

---

4.5. DISCUSSION .....	108
4.6. CONCLUSIONS .....	110
4.7. REFERENCES.....	111
 <b>CHAPTER 5. AMINO ACID UTILISATION BY THE MAMMARY GLAND OF DAIRY COWS FED FRESH PASTURE: PRECURSOR POOL AND EFFECT OF DRY MATTER INTAKE RESTRICTION.....</b>	
5.1. ABSTRACT .....	116
5.2. INTRODUCTION.....	117
5.3. MATERIALS AND METHODS .....	118
5.3.1. <i>Animals and diets</i> .....	118
5.3.2. <i>Measurements</i> .....	120
5.3.3. <i>Calculations</i> .....	121
5.3.3.1. AA concentrations in whole blood, plasma and erythrocyte.....	121
5.3.3.2. AA utilisation by the mammary gland .....	122
5.3.4. <i>Statistical analysis</i> .....	123
5.3.4.1. Differences between whole blood and plasma.....	123
5.3.4.2. Dietary treatment effects .....	123
5.4. RESULTS .....	123
5.5. DISCUSSION.....	133
5.6. ACKNOWLEDGEMENTS.....	140
5.7. REFERENCES.....	142
 <b>CHAPTER 6. WHOLE BODY FLUXES AND PARTITIONING OF AMINO ACIDS TO THE MAMMARY GLAND OF COWS FED FRESH PASTURE AT TWO LEVELS OF INTAKE DURING EARLY LACTATION .....</b>	
6.1. ABSTRACT.....	149



---

6.2. INTRODUCTION .....	150
6.3. MATERIALS AND METHODS .....	151
6.3.1. <i>Animals and diets</i> .....	151
6.3.2. <i>Measurements</i> .....	153
6.3.2.1. Determination of whole body irreversible loss rate of AA .....	153
6.3.2.1.1. Preparation of the mixture of <sup>13</sup> C-universally labelled amino acids.....	153
6.3.2.1.2. Infusions .....	154
6.3.3. <i>Laboratory methods</i> .....	155
6.3.3.1. Sample preparation for stable isotope determination.....	155
6.3.3.2. Determination of <sup>13</sup> C concentration in samples.....	155
6.3.4. <i>Calculations</i> .....	156
6.3.5. <i>Statistical methods</i> .....	158
6.3.5.1. Estimation of plateau of enrichment in samples .....	158
6.3.5.2. Treatment and pool effects .....	158
6.3.5.3. Ranking of partitioning .....	159
6.4. RESULTS .....	159
6.5. DISCUSSION.....	164
6.5.1. <i>Methodological considerations</i> .....	164
6.5.2. <i>Irreversible loss rates</i> .....	167
6.5.3. <i>Partitioning</i> .....	168
6.6. ACKNOWLEDGEMENTS.....	172
6.7. REFERENCES.....	174
<b>CHAPTER 7. GENERAL DISCUSSION AND CONCLUSIONS .....</b>	<b>179</b>
7.1. INTRODUCTION.....	180
7.2. METHODOLOGICAL CONSIDERATIONS .....	180
7.3. PRECURSOR POOLS FOR MILK PROTEIN SYNTHESIS.....	181

---

7.4. LIMITING AMINO ACIDS FOR MILK PROTEIN SYNTHESIS .....	183
7.5. CONFIRMING LIMITING EAA AND PRACTICAL IMPLICATIONS .....	189
7.6. CONCLUSIONS.....	193
7.7. REFERENCES .....	195
<b>APPENDIX .....</b>	<b>201</b>
APPENDIX A.1. IMAGE OF THE EXPERIMENTAL CONDITIONS DESCRIBED IN CHAPTER 3 .....	202
APPENDIX A.2. IMAGE OF THE EXPERIMENTAL CONDITIONS DESCRIBED IN CHAPTERS 4, 5 AND 6.....	203
APPENDIX A.3. DIAGRAM OF THE EXPERIMENTAL PROTOCOL USED IN THE METHIONINE SUPPLEMENTATION TRIALS (CHAPTER 3).....	204
APPENDIX A.4. DIAGRAM OF THE EXPERIMENTAL PROTOCOL DESCRIBED IN CHAPTERS 4, 5, AND 6.....	205
APPENDIX A.5. IRREVERSIBLE LOSS RATES OF AMINO ACIDS MEASURED IN DEEP JUGULAR SAMPLE (COW 2235; CHAPTER 6) .....	206
APPENDIX A.6. PRELIMINARY REPORT ON THE EFFECTS OF METHIONINE SUPPLEMENTATION IN MID-LACTATION (CHAPTER 3) PUBLISHED IN THE <i>PROCEEDINGS OF THE NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION</i> 57: 147-150 .....	207
APPENDIX A.7. PRELIMINARY REPORT ON THE EFFECTS OF METHIONINE SUPPLEMENTATION IN LATE LACTATION (CHAPTER 3) PUBLISHED IN THE <i>PROCEEDINGS OF THE NUTRITION SOCIETY OF NEW ZEALAND</i> 22: 184-191 .....	211
APPENDIX A.8. PRELIMINARY REPORT ON THE ARTERIO-VENOUS DIFFERENCES OF ESSENTIAL AMINO ACIDS IN THE PLASMA OF LACTATING DAIRY COWS (CHAPTER 5) PUBLISHED IN THE <i>PROCEEDINGS OF THE NEW ZEALAND SOCIETY OF ANIMAL PRODUCTION</i> 58: 98-101 .....	219
APPENDIX A.9. PRELIMINARY REPORT ON THE DIFFERENCES BETWEEN WHOLE BLOOD AND PLASMA AS POOLS OF FREE AMINO ACIDS FOR MILK PROTEIN SYNTHESIS	

(CHAPTER 5) PUBLISHED IN THE *PROCEEDINGS OF THE NEW ZEALAND SOCIETY  
OF ANIMAL PRODUCTION* 59: 62-65 ..... 223

## LIST OF TABLES

### Chapter 2

Table 2.1. Components present in milk grouped by function. ....	7
Table 2.2. Average composition of bovine milk produced in New Zealand (g/L milk). (Creamer and MacGibbon, 1996). ....	9
Table 2.3. Summary of dairyfarming statistics in New Zealand for the period 1989-99 <sup>a</sup> (Livestock Improvement Corporation, 1999). ....	10
Table 2.4. Summary of advantages and disadvantages of ryegrass/white clover pasture for dairy cow nutrition (Clark <i>et al.</i> , 1997). ....	13
Table 2.5. Main proteins present in cow's milk, known genetic polymorphs and some structural characteristics. ....	17
Table 2.6. Chemical composition, based on primary structure, of the commonly occurring caseins. (Swaigood, 1993). ....	18
Table 2.7. Chemical composition, based on primary structure, of the major whey proteins (Jenness, 1974). ....	21
Table 2.8. Fractional and absolute rates <sup>a</sup> of tissue protein synthesis in dry and lactating goats. ....	30
Table 2.9. Limiting amino acids in dairy cows as proposed by different authors. ....	31
Table 2.10. Average $\pm$ 95% confidence interval (CI) and coefficient of variation (CV) of published <sup>a</sup> amino acid concentrations ( $\mu$ M) in plasma of dairy cows. Each observation represents the average of an experimental treatment. ....	33
Table 2.11. Proportional contribution (% <sup>2</sup> ) of erythrocyte amino acids to whole blood amino acid concentrations in calves fed soybean- or urea-based purified diets. ....	34
Table 2.12. Whole blood (B) and plasma (P) arterio-venous differences (A-V) of essential amino acids across the mammary gland of dairy cows from two different studies. ....	35
Table 2.13. Comparison of the average amino acid composition (% of total N) of duodenal contents of cattle and microbial protein synthesised in the rumen from two types of forages. ....	40

<b>Table 2.14. Digestibility coefficients for protein leaving the rumen according to different protein evaluation systems.....</b>	<b>42</b>
<b>Table 2.15. Summary of measurements of mammary blood flow using different techniques and experimental conditions.....</b>	<b>47</b>
<b>Table 2.16. Relative proportions (g AA/100 g EAA) of essential amino acids in milk protein and the required absorbable according to the Cornell Net Carbohydrate and Protein System. ....</b>	<b>51</b>
<b>Chapter 3</b>	
<b>Table 3.1. Effect of methionine supplementation on feed intakes and blood methionine, cysteine and urea concentrations in mid lactation (Experiment 1).....</b>	<b>85</b>
<b>Table 3.2. Effect of methionine supplementation on feed intakes and blood methionine, cysteine and urea concentrations in late lactation (Experiment 2).....</b>	<b>85</b>
<b>Table 3.3. Effect of methionine supplementation on milk production, milk composition and efficiency of CP utilisation for milk protein synthesis in mid lactation (Experiment 1).....</b>	<b>87</b>
<b>Table 3.4. Effect of methionine supplementation on milk production, milk composition and efficiency of CP utilisation for milk protein synthesis in late lactation (Experiment 2).....</b>	<b>88</b>
<b>Chapter 4</b>	
<b>Table 4.1. Estimated mammary blood flow (litres per minute) using amino acid arterio-venous differences and TOH. ....</b>	<b>107</b>
<b>Chapter 5</b>	
<b>Table 5.1. Chemical composition of the pasture offered during the experimental periods (% of DM, unless otherwise stated). ....</b>	<b>119</b>
<b>Table 5.2. Dry matter intakes and milk yields during the experimental period (n=4). ....</b>	<b>124</b>
<b>Table 5.3. AA concentrations in arterial whole blood, plasma and red blood cells, together with the proportional contribution of erythrocytes to whole blood free AA in lactating dairy cows fed fresh pasture (Means <math>\pm</math> SE).....</b>	<b>125</b>
<b>Table 5.4. Arterio-venous differences (nmol g<sup>-1</sup>) calculated from whole blood or plasma AA concentrations in lactating dairy cows fed fresh pasture (means <math>\pm</math> SE). ....</b>	<b>126</b>

Table 5.5. Least square means of the arterio-venous differences (nmol g <sup>-1</sup> ) measured in blood or plasma of dairy cows offered <i>ad libitum</i> or restricted dry matter intakes.....	127
Table 5.6. Least square means of the extraction percentage of AA from whole blood or plasma of cows offered <i>ad libitum</i> or restricted dry matter intakes. ....	128
Table 5.7. Least square means of the net uptake of AA by the mammary gland from either whole blood or plasma, and their respective output in milk protein of dairy cows offered <i>ad libitum</i> ( <i>Ad lib</i> ) or restricted ( <i>Rest</i> ) dry matter intakes. ....	130
Table 5.8. Regression coefficients (slope ± SE) and <i>R</i> <sup>2</sup> values of the regression <i>y</i> = <i>bx</i> , where <i>y</i> =AA output and <i>x</i> =AA uptake from either whole blood or plasma by the mammary gland.....	133
 Chapter 6	
Table 6.1. Chemical composition of the pasture offered during the experimental periods (% of DM unless otherwise stated). ....	152
Table 6.2. Concentration (mean ± SD) of amino acids (mmol per ml) of infusates.....	154
Table 6.3. Chromatograph retention times of essential amino acids studied and ions monitored using the mass selective detector. ....	156
Table 6.4. Least square means of the different components of the nitrogen balance measurements during the experimental periods (days 6 to 11; <i>n</i> =4).....	159
Table 6.5. Plateau isotopic enrichment ( <sup>13</sup> C MPE) of amino acids in whole blood or plasma as determined from the model <i>IE</i> = <i>A</i> (1- <i>e</i> <sup>-<i>kt</i></sup> ).....	161
Table 6.6. Whole body irreversible loss rates (ILR; mmol h <sup>-1</sup> ) of amino acids, calculated from arterial whole blood or plasma <sup>13</sup> C isotopic enrichments, of lactating dairy cows fed <i>ad libitum</i> or restricted dry matter intakes ( <i>n</i> =3).....	162
Table 6.7. Whole body equivalent protein fluxes ( <i>PF</i> ; g d <sup>-1</sup> ); as calculated from the ILR of essential amino acids of lactating dairy cows fed <i>ad libitum</i> or restricted dry matter intakes ( <i>n</i> =3).....	163
Table 6.8. Partitioning (net uptake/ILR*100) of amino acids from whole blood and plasma to the mammary gland of lactating dairy cows fed fresh pasture at <i>ad libitum</i> and restricted intakes ( <i>n</i> =3).....	164

---

<b>Table 6.9. Comparison of the ratio of isotopic enrichments measured in whole blood and plasma in this study and previously published studies with sheep (Lobley <i>et al.</i>, 1996) and goats (Bequette <i>et al.</i>, 1997).....</b>	<b>166</b>
<b>Table 6.10. Comparison of the ILR (<math>\mu\text{mol h}^{-1}</math> per kg LW<sup>0.75</sup>) of selected EAA measured in lactating dairy cows.....</b>	<b>167</b>
<b>Table 6.11. Estimation of the contribution of EAA absorbed or protein breakdown to the AA flux and the partitioning of this flux between protein synthesis and oxidation in lactating dairy cows fed fresh pasture<sup>3</sup>. ....</b>	<b>169</b>
<b>Chapter 7</b>	
<b>Table 7.1. Percent contribution of plasma peptides to the total plasma concentrations (free amino acid plus peptide-bound amino acid) of selected amino acids in lactating ruminants. ....</b>	<b>182</b>
<b>Table 7.2. Summary of potentially limiting amino acids for dairy cows fed fresh forage or concentrate-based mixed rations, as assessed from the study of different metabolic parameters.....</b>	<b>185</b>
<b>Appendix</b>	
<b>Table A.5.1. Whole body irreversible loss rates (ILR; <math>\text{mmol h}^{-1}</math>) of amino acids in deep jugular whole blood and plasma. Results from animal no. 2235 .....</b>	<b>206</b>

## LIST OF FIGURES

### Chapter 2

- Figure 2.1. Processed milk protein consumption (Valeur, 1997). ..... 11
- Figure 2.2. Main milk protein producers (Valeur, 1997)..... 12
- Figure 2.3. Annual pasture growth curve (solid line) for a dairyfarm in Hamilton, New Zealand, compared with the pasture requirements for three (dashed line) and four (dotted line) cows per hectare (Clark *et al.*, 1997)..... 13
- Figure 2.4. Seasonal changes in the composition of pasture sampled from four dairy farms (two in the Manawatu and two in the Waikato areas). ADF = acid detergent fibre, NDF = neutral detergent fibre, CP= crude protein, DOMD = digestible organic matter per 100 g dry matter, Sol CHO = soluble carbohydrates. Each symbol represents a different farm (Wilson *et al.*, 1995). ..... 15
- Figure 2.5. A schematic outline of the processes for milk protein synthesis and secretion of milk protein and lactose in the secretory cells of the mammary gland (Thomas, 1983). ..... 23
- Figure 2.6. Influence of the energy and protein supply on the milk protein content (Kaufmann, 1980)..... 26
- Figure 2.7. A general scheme depicting the main factors affecting metabolisable protein supply (Oldham, 1996)..... 38
- Figure 2.8. Schematic representation of the main paths of amino acid utilisation in lactating dairy cows..... 42
- Figure 2.9. Classification and characteristics of the amino acid transport systems present in the mammary gland (From Baumrucker, 1985).. ..... 48

### Chapter 4

- Figure 4.1. Curve of tritium activity in arterial and venous plasma samples processed using the different protocols described in the Material and Methods section (Means  $\pm$  SEM;  $n=3$ ). ..... 107

### Chapter 5



---

<b>Figure 5.1. Ratio of the net uptake to output of Group I EAA, Group II EAA and NEAA (see text for explanation) across the mammary gland of dairy cows fed <i>ad libitum</i> or restricted dry matter intakes. Uptake calculated from both whole blood and plasma.....</b>	<b>131</b>
<b>Chapter 6</b>	
<b>Figure 6.1. Examples of the isotopic enrichment curves for His, Ile, Leu, Met, Tyr and Val, as measured in whole blood (○) or plasma (●) in one cow during the course of infusion in Period 1, together with the fitted lines from <math>MPE = A(1 - e^{-kt})</math> to determine the plateau values in whole blood (broken line) and plasma (solid line). .....</b>	<b>160</b>
<b>Figure 6.2. Schematic representation of the two-compartment model used to describe the whole body flux (ILR) of amino acids (modified from Lobley, 1993).....</b>	<b>168</b>
<b>Chapter 7</b>	
<b>Figure 7.1. Diagram summarising the characterisation of the metabolism of amino acids by the lactating dairy cow fed fresh-forages.....</b>	<b>184</b>
<b>Figure 7.2. Flowchart representing the results described in this thesis in relationship with future research needs and their practical implementation in pastoral dairyfarming in New Zealand. ....</b>	<b>189</b>

## LIST OF ABBREVIATIONS

AA	amino acid(s)
AgR	AgResearch
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
A-V	arterio-venous difference
BCAA	branched-chain amino acid(s)
BF	blood flow
CN	casein
CP	crude protein
Cys	cysteine
DCRU	Dairy Cattle Research Unit, Massey University
DIM	days in milk
DM	dry matter
DMI	dry matter intake
dpm	disintegrations per minute
EAA	essential amino acid(s)
EI	electron impact
FAA	free amino acid(s)
GC-MS	gas chromatography-mass spectrometry
GIT	gastrointestinal tract
Gln	glutamine
Glu	glutamic acid
Gly	Glycine
His	histidine
IE	isotopic enrichment
Ig	immunoglobulins
Ile	isoleucine
ILR	irreversible loss rate

---

Leu	leucine
Lys	lysine
MBF	mammary blood flow
ME	metabolisable energy
Met	methionine
MPE	mole percent excess
MPS	milk protein score
N	nitrogen
NCN	non-casein nitrogen
NEAA	non-essential amino acid(s)
NPN	non-protein nitrogen
P+T	phenylalanine plus tyrosine
PBAA	peptide-bound amino acid(s)
PCV	packed cell value (haematocrit)
PF	equivalent protein flux
Phe	phenylalanine
Pro	Proline
RBC	red blood cell
RPAA	ruminally-protected amino acid(s)
RPM	ruminally-protected methionine
SA	serum albumin
Ser	Serine
TBDMS-	N, O- <i>tert.</i> butyldimethylsilyl derivative
Thr	threonine
TN	total nitrogen
TOH	tritiated water
Trp	tryptophan
U <sup>13</sup> C-	universally-labelled with <sup>13</sup> C
Val	valine
α-LA	alpha-lactalbumin
β-Lg	beta-lactoglobulin

**CHAPTER 1.**

**GENERAL INTRODUCTION, OBJECTIVES AND  
FORMAT OF THE THESIS**

## 1.1. GENERAL INTRODUCTION

The New Zealand economy is unique in the developed world due to its large dependence on agricultural production for obtaining revenues from overseas trade. With a total value of NZ\$ 4,005 million, milk powder, butter and cheese are major contributors to the total export earning in New Zealand. While the contribution of other agricultural products to the total export value has been reduced, the increase in export value of dairy products in the last few years is noticeable. The growing contribution of casein and caseinates deserves particular attention with increases of 25% in their export value during the period 1998-99. In 1999 the export value of casein products was almost as big as the total export value of wool (784 vs. 797 million NZ\$) in New Zealand<sup>1</sup>.

Because of this, there is growing interest in the dairy industry to increase the output of milk casein from dairy farms in New Zealand. Research on concentrate-fed animals has emphasised the role of amino acids as important nutrients for milk protein synthesis. In pastoral farming systems, amino acid nutrition appears to have potential for improvement of milk protein production. For instance, Wang *et al.*, (1996)<sup>2</sup> reported that milk protein was increased 14% in sheep grazing *Lotus corniculatus*, presumably due to an increase in the flow of essential amino acids to the small intestine. However, there is no direct information on amino acid requirements of dairy cows fed fresh forages.

## 1.2. OBJECTIVES

Given the lack of published information on the metabolism of amino acids in pasture-fed dairy cows, the current study was conducted to assess several metabolic aspects of the utilisation of amino acids by the mammary gland. This study has the

---

<sup>1</sup> Statistics New Zealand: [www.stats.govt.nz](http://www.stats.govt.nz). Consulted on 11-03-2000.

<sup>2</sup> Wang, Y. et al. 1996. *Journal of Agricultural Science, Cambridge* 126:353-362

ultimate purpose of providing information about the role of these nutrients as limiting factors for milk protein synthesis (particularly casein) in pastoral dairyfarming.

Intermediate objectives of this study include:

- a) the assessment of the productive responses to supplementation of the amino acid(s) that are predicted to be limiting using current schemes available for concentrate-fed cows
- b) to measure different aspects of mammary utilisation of amino acids using the arterio-venous difference procedure to assess the adequacy of the supply of amino acids for milk protein synthesis in pasture-fed cows
- c) to assess the whole body metabolism of amino acids, and to provide information about the magnitude of the partitioning of amino acids towards the mammary gland for milk protein synthesis.

### **1.3. FORMAT OF THE THESIS**

Chapter 2 is a review of literature covering a) general aspects of the importance of milk protein for the dairy industry in New Zealand, b) milk protein synthesis and the role of amino acids in this process and c) methods of assessing limiting amino acid for milk protein synthesis. Results from three different experiments are presented in the self-contained Chapters 3, 4, 5, and 6. Chapter 3 examines the role of methionine as a limiting amino acid for pasture-fed dairy cows at two different stages of lactation. Chapter 4 describes the assessment of two methods for estimation of mammary blood flow, which is required in studies to measure the utilisation of amino acids by the mammary gland. Chapter 5 reports the results obtained characterising the mammary utilisation of the whole array of amino acids in pasture-fed dairy cows. Finally, Chapter 6 describes the utilisation of amino acids by the mammary gland as a proportion of their utilisation in the whole body.

The results presented herein have already been published (Chapter 3) or are currently under review for publication in refereed journals (Chapters 4, 5 and 6). Preliminary results of the experiments described have been published elsewhere (see Appendices A.6- A.9).

**CHAPTER 2.**

**A REVIEW OF LITERATURE**



The recognition of the nutritional, industrial and economic value of milk protein has been reflected in research efforts to increase its production. The New Zealand dairy industry, as a major international supplier of milk products, would benefit from research targeted to, first, understand milk protein synthesis and subsequently, devise strategies to increase its production. The present work represents an attempt towards a better understanding of amino acid metabolism in dairy cows, with the ultimate target of identifying the role of amino acids as limiting nutrients for milk protein synthesis.

## **2.1. MILK**

Milk is the characteristic secretion of the mammary gland. It comprises two phases: a) an aqueous phase in which lactose, mineral ions and water soluble vitamins are present in a simple solution whilst proteins and other ions are present in a colloidal suspension, and b) a lipid phase containing milk fat and fat-soluble compounds such as vitamins, sterols and carotenoids (Mephram, 1987).

### **2.1.1. The evolutionary role of milk**

It is generally thought that the main role of milk in the survival of mammals is the transfer of nutrients to the neonate. However, it may not have been the primary role of mammary secretions during the evolution of the class Mammalia, as milk serves other functions in addition to being strictly nutritional (Fox and Flynn, 1992).

#### **2.1.1.1. *Evolution of lactation***

Lactation is a distinguishing mammalian characteristic; however, the evolutionary development of the mammary gland remains obscure. It is generally accepted that the mammary gland evolved from a modified skin gland over millions of years to attain its structure and function as known today (Blackburn, 1993; Hayssen, 1993). However, all the theories about mammary gland evolution are inconclusive as no fossil record is preserved of the primitive mammary gland, and also because even the most conservative extant (living) mammals (order Monotremata: echidna and platypus)

possess fully functional, complex mammary glands (Blackburn, 1993). Reviews on the origins of lactation and the evolution of the mammary gland and its secretion have been published elsewhere (see Jenness, 1986; Blackburn *et al.*, 1989; Blackburn, 1993; Hayssen, 1993).

### 2.1.1.2. *Evolution and functions of milk*

Milk is the secretion produced by the mammary gland of mammals. It represents the main source of nutrients for the offspring and it is essential for the survival, growth and development of the neonate (Pearlman, 1991). Milk is a source of energy (carbohydrate and fat), protein, minerals, vitamins and water (Jenness, 1986). It also

**Table 2.1. Components present in milk grouped by function.**

Function	Examples	Reference
Nutrients:	Protein Fat Lactose Vitamins Minerals	1,9
Hormones:	Insulin Cortisol Thyroxine Erythropoietin Prolactin Somatotropin Somatostatin Vasoactive intestinal peptide	1,3,5
Defense:	Immunoglobulin G Immunoglobulin A Immunoglobulin M Casein immunoregulatory peptide Lactoferrin	2,6,7
Growth factors:	Epidermal growth factor (EGF) Transforming growth factor (TGF) Nerve growth factor (NGF) Insulin-like growth factors I and II (IGF-I & IGF-II)	1,4,8
Others:	Opioid peptides Bombesin Neurotensin	1,7

1. (Xu, 1996).
2. (Watson, 1980)
3. (Pearlman, 1991)
4. (Odle *et al.*, 1996)
5. (Zinn, 1997)
6. (Schanbacher *et al.*, 1997)
7. (Meisel, 1997)
8. (Weaver, 1997)
9. (Gurr, 1992)

serves as a vehicle for immunoglobulins, antimicrobial agents (i.e. lysozyme, lactoferrin, transferrin), growth factors, bioactive peptides and hormones. Comprehensive reviews on the different roles of the mammary secretion have been published elsewhere and are summarised in Table 2.1.

Milk composition varies greatly among species (for reviews see Jenness, 1974; Jenness, 1986; Mepham, 1987) and it is accepted, given the lack of evidence otherwise, that the nutrient composition of a species has been tailored by natural selection to meet closely (perhaps with exception of vitamin D and iron) the nutrient requirements of the young of that species (Mepham, 1987; O'Connor *et al.*, 1997). The composition of the milk from different species is determined by the amount of nutrients required by the neonate, which has a strong correlation with the timing of birth and the postnatal growth rate (Pearlman, 1991). Milks from different species, nevertheless, share a number of compositional features, such as the presence of caseins and lactose (Jenness, 1974). The diversity of extant mammals with respect to milk composition, together with some common compositional features suggest a potential for manipulation of milk composition in mammals of agricultural importance, given that the controlling mechanisms common for all living species are understood.

### **2.1.2. The role of milk in human nutrition**

Milk collection and use for human consumption is associated with the domestication of cattle, sheep and goats by man as early as 9,000 BC in southwest Asia and Africa. In that early period, milk would spoil rapidly after collection and, therefore, was consumed fresh or transformed into soured dairy products such as yoghurts and cheeses. In Europe, an export market for dairy products was created between 100 and 300 AD by the Roman Empire (Kosikowski, 1981). In the Americas and Oceania, the consumption of dairy products was initiated by European migrants. Nowadays, the use of milk as a food staple is observed practically all over the world, including regions in which foods of animal origin were relatively uncommon, such as the Far East (Kosikowski, 1981). Most of the milk commercially produced for human consumption comes from cows, with small participation of other species such as water buffalo, sheep,

goat, camel, yak, llama and reindeer. The composition of typical cow's milk is presented in Table 2.2.

**Table 2.2. Average composition of bovine milk produced in New Zealand (g/L milk). (Creamer and MacGibbon, 1996).**

Component	
Water	860
Fat	50
Lactose	47
Protein	34
Caseins	27
Whey protein	7

Milk products are often included as a separate group in most basic dietary guides. The nutritional benefits of dairy foods are not confined to young humans, but are important for all ages. Dairy foods are significant sources of several nutrients for humans, particularly calcium, riboflavin, phosphorus, high quality protein, magnesium and water-soluble vitamins (Speckmann *et al.*, 1981).

Cheese, after milk, is the predominating dairy food of the world, followed by butter, evaporated and condensed milks, dried milks and dried whey (Kosikowski, 1981). With the advance in separation techniques, specific components of milk have found multiple applications in a vast range of food products such as nutritional beverages, infant formulae, meal replacement products, sports bars, nutraceuticals and foods for specific physiological functions (Gurr, 1992; Giese, 1994; Mann, 1996a; Mann, 1996b; Dibley, 1997; Meisel, 1997; O'Connor *et al.*, 1997). Particular interest and development has arisen for the protein fractions from both casein and whey. The value of milk proteins as food ingredients resides in their high nutritive value (high digestibility and high levels of essential amino acids), the absence of antinutritional factors, their functionality (surfactants, emulsification, solubility, heat stability), their physiological properties and bland flavour (Speckmann *et al.*, 1981; Gurr, 1992; Wong *et al.*, 1996; Dibley, 1997).

### 2.1.3. Milk production in New Zealand

New Zealand dairyfarming systems are unique because of their reliance on pasture as the main source of nutrients for milk production. The New Zealand dairy industry processed 10,168 million litres of milk in the 1998-99 season, equivalent to 363 million kilograms of milk protein, with a national herd of approximately 3.3 million cows (Livestock Improvement Corporation, 1999). A summary of dairyfarming statistics in the last ten years is presented in Table 2.3.

**Table 2.3. Summary of dairyfarming statistics in New Zealand for the period 1989-99<sup>a</sup> (Livestock Improvement Corporation, 1999).**

Season	Milk processed (million litres)	Milk solids processed (million kgs)	Milk protein processed (million kgs)	Number of herds	Total cows (million)	Average kg protein per cow	\$ per kg of milk solids <sup>b</sup>
1989/90	6 868	572	242	14 595	2.31	na	4.51
1990/91	7 077	599	254	14 685	2.40	na	2.82
1991/92	7 454	637	270	14 452	2.44	na	3.79
1992/93	7 629	651	277	14 458	2.60	111 <sup>c</sup>	4.11
1993/94	8 603	736	313	14 497	2.74	119 <sup>c</sup>	3.68
1994/95	8 633	733	311	14 649	2.83	115 <sup>c</sup>	3.73
1995/96	9 325	788	335	14 736	2.94	120 <sup>c</sup>	4.19
1996/97	10 339	880	375	14 741	3.06	128 <sup>c</sup>	3.73
1997/98	10 651	893	379	14 673	3.22	124 <sup>c</sup>	3.48
1998/99	10 168	850	363	14 362	3.29	109 <sup>c</sup>	3.58

<sup>a</sup> does not include town milk supply

<sup>b</sup> inflation adjusted

<sup>c</sup> includes town milk supply

na: not available

#### 2.1.3.1. *World market of dairy products: the New Zealand perspective*

As 95% of the milk produced in New Zealand is destined to fulfil demand overseas, the world market for dairy products has a strong influence on the decisions made by the New Zealand Dairy Board. It is predicted a reduction in butter production as a result of an increased demand for cheese, whole milk powder and fresh dairy products. The increasing demand for cheese, together with high market prices and improved access to markets are expected to be reflected in increased trade of the this dairy product (Valeur, 1997; Viatte, 1997). Another commodity expected to increase its

share in the world market of dairy products is milk powder, mainly as the result of increased demand in Asia and Latin America.

Depending on the product to be manufactured, higher concentrations of particular components are sought. For example, butter yield relies heavily on the content of milkfat per kilogram of milk. In the same way, milk protein (and particularly casein) is one of the main factors affecting the yield of cheese (Gilles and Lawrence, 1985). The type and concentration of casein has marked effects on rennet coagulation properties, such as curd strength, moisture and fat retention (Gilles and Lawrence, 1985; Grandison *et al.*, 1985). The composition of milk protein also affects the stability of pasteurised milks and milk powder manufacturing (Dalglish, 1992).

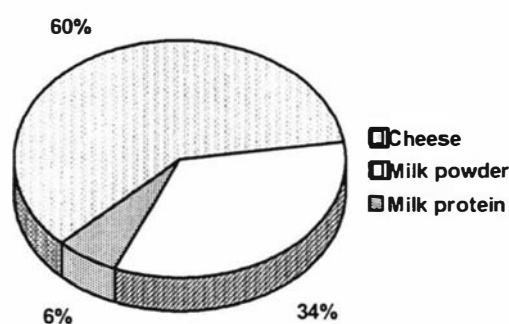


Figure 2.1. Processed milk protein consumption (Valeur, 1997).

Furthermore, milk protein *per se* is one milk component which has increased considerably its share in the world trade of dairy products. Caseinates, whey protein concentrates (WPC) and milk protein concentrates (MPC) are used extensively in the alimentary industry as ingredients for nutritionally enhanced foods (Valeur, 1997). Although milk protein isolates represent at present only a small portion of the international trade in the processed milk protein market (Figure 2.1), it is expected to increase sharply in the future. New Zealand currently occupies an important position in this market as an exporter (Figure 2.2).

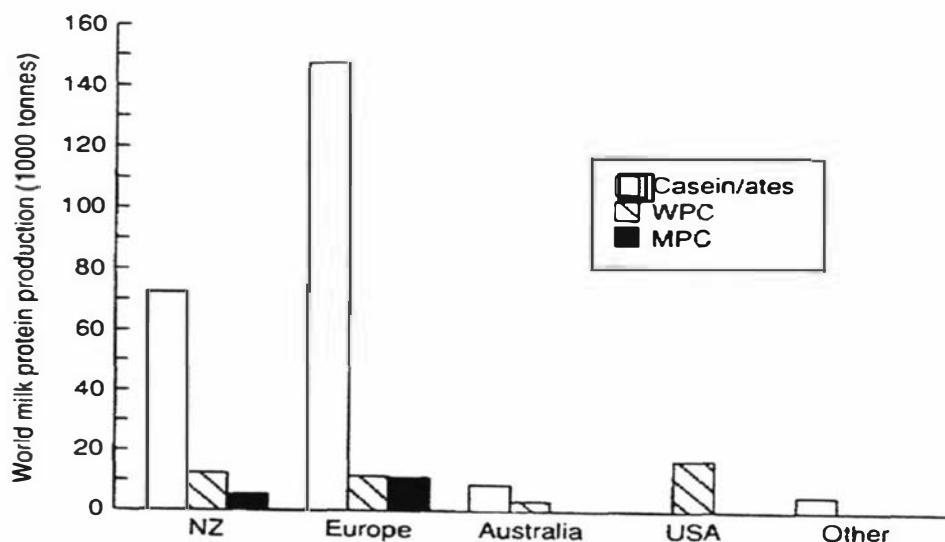


Figure 2.2. Main milk protein producers (Valeur, 1997).

For New Zealand, these forecasts are expected to be translated as an increase in the demand of protein-based dairy products. Therefore, strategies to increase the output of milk protein in the national herd have to be implemented. The interest of the New Zealand dairy industry on milk protein is portrayed in the milk pricing scheme used by dairy companies. Dairy companies pay seasonal supply farmers based upon the “A+B-C” system which incorporates payments for milkfat (A) and protein (B) with penalties for milk volume (C) (Livestock Improvement Corporation, 1999).

### 2.1.3.2. *The pastoral system: advantages and limitations*

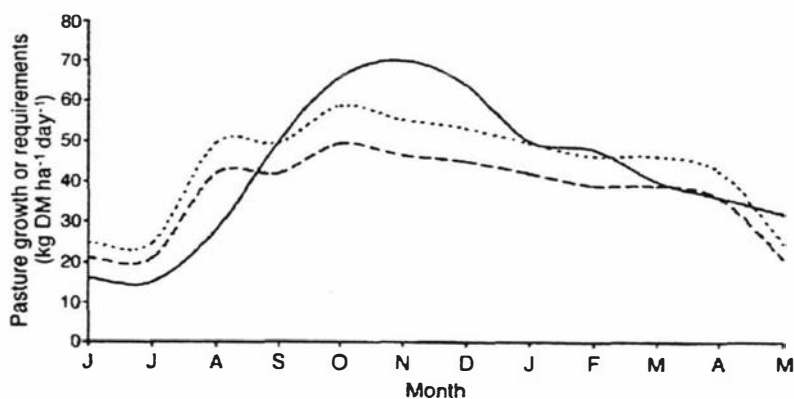
The pastoral system of dairy farming in New Zealand relies on perennial ryegrass (*Lolium perenne*) / white clover (*Trifolium repens*) pastures as the main forage species. A summary of the advantages and disadvantages of this type of pasture for milk production was outlined by Clark *et al.*, (1997) and is presented in Table 2.4.

**Table 2.4. Summary of advantages and disadvantages of ryegrass/white clover pasture for dairy cow nutrition (Clark *et al.*, 1997).**

Advantages:
● low-cost growing
● self-harvesting
● energy efficient
● semi-natural environment
● minimal conservation losses
Disadvantages:
● variable nutritive value
● variable feed supply
● mycotoxic diseases
● environmental stresses difficult to control

### 2.1.3.3. Pasture as a nutrient source for milk protein production

The main advantage of pastoral dairy farming is the low cost of pasture compared with systems based on conserved forages and concentrates. The main disadvantage of the system is the high seasonal variability in pasture supply and seasonal variations in nutritive value. Figure 2.3 shows the annual pasture growth curve compared with the pasture requirements for two different stocking rates. Grazing pasture fulfils the requirement for approximately eight months of the year. Some supplementation to pasture is required for the other four months, particularly during winter, and this is generally met from pasture conserved during late spring/summer as either silage or hay.



**Figure 2.3. Annual pasture growth curve (solid line) for a dairyfarm in Hamilton, New Zealand, compared with the pasture requirements for three (dashed line) and four (dotted line) cows per hectare (Clark *et al.*, 1997).**



Furthermore, the variation in pasture supply is accompanied by fluctuations in the composition of the forage. For example, Wilson *et al.*, (1995), observed that reproductive plant growth and drought stress during summer is associated with increases in fibre content (both acid- and neutral detergent fibres), reductions in crude protein and soluble carbohydrate content and reductions in digestibility (Figure 2.4). Highest digestibility values occur during winter and early spring, when the fibre content is at its lowest and that of the soluble carbohydrates levels is at its highest levels. Crude protein concentration is highest during autumn. In summary, dairy production in pastoral systems may be limited, at certain times of the year, by both the limited supply of pasture and the quality of the ingested feed.

As a brief summary of this section, it can be concluded that, within the group of dairy products, milk protein occupies a relevant position given its nutritional properties and also because of its growing value as a trade commodity, particularly for the New Zealand industry. However, there are shortcomings in the pastoral systems that have to be surmounted in order to increase/optimize the output of milk protein from the New Zealand's herd.

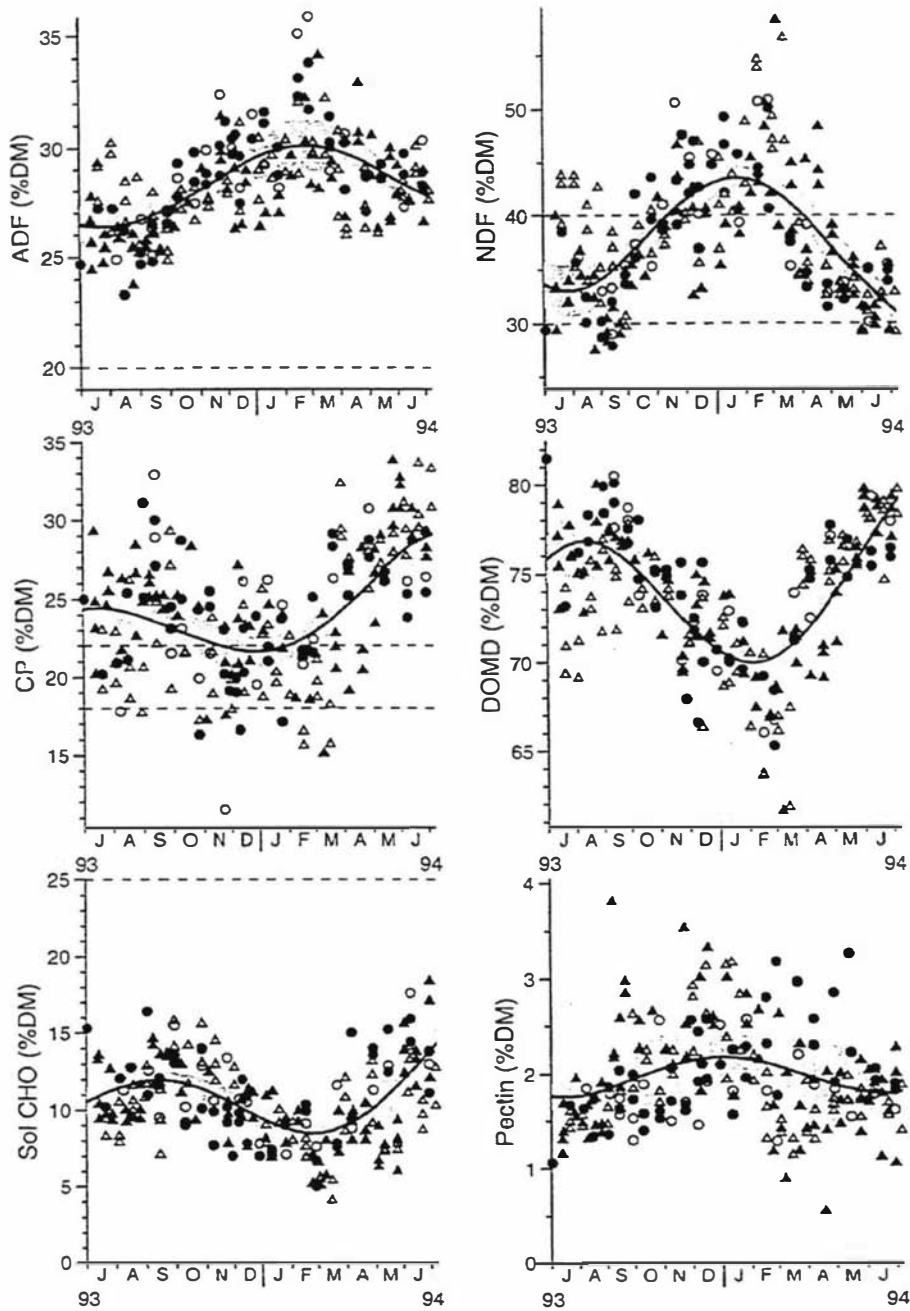


Figure 2.4. Seasonal changes in the composition of pasture sampled from four dairy farms (two in the Manawatu and two in the Waikato areas). ADF = acid detergent fibre, NDF = neutral detergent fibre, CP = crude protein, DOMD = digestible organic matter per 100 g dry matter, Sol CHO = soluble carbohydrates. Each symbol represents a different farm (Wilson *et al.*, 1995).

## 2.2. MILK PROTEIN

The main role of the milk proteins of mammary origin is to provide amino acids for incorporation into protein by the young nursling (Jenness, 1986; Blackburn *et al.*, 1989). Milk protein is also a source of energy for the neonate, providing 15-30% of the total energy content of the milk (Blackburn *et al.*, 1989).

For characterisation purposes, milk “total protein” is estimated as the total nitrogen content of milk multiplied by 6.38 (assuming that milk protein contains 156.8 g N/kg). However, it is well established that not all the milk nitrogen is present as protein. From the “total protein” figure, a small proportion is non-protein nitrogen, but typically more than 94% is true protein (Thomas, 1983). The functions, classification and characteristics of the true protein component of milk are presented in the following sections.

### 2.2.1. Proteins present in milk

Although generally described and analysed as one component, milk protein is far from being a single entity. Milk protein comprises a variety of fractions with particular composition and properties, as shown in Table 2.5. A basic classification of proteins in milk comprises two broad groups: a) caseins and b) whey proteins. The proportion of casein to whey proteins vary greatly among species from about 4:1 in ruminants to 0.8:1 in some primates (Jenness, 1986).

In species of agricultural importance, milk proteins occur as different genetic variants, or polymorphs, differentiated by substitutions in their amino acid sequence. The genetic variants are coded for by two genes (co-dominant alleles) which are independently inherited in a Mendelian way. Detailed reviews on genetic polymorphism of milk protein, including frequency distribution in different species and breeds of cattle and their relation with production traits in dairy cattle have been published elsewhere (Ng-Kwai-Hang and Grosclaude, 1992; FitzGerald, 1997).

**Table 2.5. Main proteins present in cow's milk, known genetic polymorphs and some structural characteristics.**

Protein	Percent of skim milk protein <sup>a</sup>	Origin <sup>b</sup>	Known genetic polymorphs <sup>c</sup>	Approximate molecular mass <sup>d</sup>	Amino acid residues <sup>e</sup>	P per mole <sup>f</sup>	-S-S- per mole <sup>g</sup>	-SH per mole <sup>g</sup>
<b>Casein</b>	75-85							
$\alpha_{s1}$ -Caseins	39-46	MG	A, B, C, D, E	23 600	199	8-9	-	-
$\alpha_{s2}$ -Caseins	8-11	MG	A, B, C, D	25 150	207	10-13	-	2
$\beta$ -Caseins	25-35	MG	A <sup>1</sup> , A <sup>2</sup> , A <sup>3</sup> , B, C, D, E	24 000	209	4-5	-	-
$\kappa$ -Caseins	8-15	MG	A, B	19 000	169	1-2	-	2
$\gamma$ -Caseins	3-7	MG						
<b>Whey protein</b>	15-22							
$\beta$ -Lactoglobulin	7-12	MG	A, B, C, D, E, F, G, (Dr)	18 300	162	-	2	5
$\alpha$ -Lactalbumin	2-5	MG	A, B	14 200	123	-	4	8
Serum albumin	0.7-1.3	Blood	A	66 300	582	-	17	35
Immunoglobulins	1.9-3.3							
IgG1	1.2-3.3	Blood		162 000				
IgG2	0.2-0.7	Blood		152 000				
IgA	0.2-0.7	Blood		400 000				
IgM	0.1-0.7	Blood		950 000				
Proteose-peptone	2-4	MG						

<sup>a, d</sup> (Brunner, 1981)<sup>b</sup> MG: mammary gland<sup>c</sup> (Eigel *et al.*, 1984)<sup>e, f</sup> (Fox and Flynn, 1992)<sup>g</sup> (Dalglish, 1992)

Besides its biological importance, protein is the single most important component in milk from a manufacturing point of view. Higher average milk protein concentrations would benefit a number of important products (i.e. cheese, milk powder, evaporated milk, ultra-high temperature milk) and this benefit would be greater if the casein concentration in milk was selectively increased (Muir *et al.*, 1983; Dalglish, 1992).

### 2.2.1.1. Caseins (CN)

Caseins are phosphoproteins synthesised in the mammary gland, with molecular masses usually in excess of 20 000, which are precipitated at acid pH (pH 4.6 for cow's milk) or by action of the gastric enzyme rennin (Mephram, 1987). Besides their role as

amino acid and energy sources, CN carry calcium and phosphate necessary for the growth of skeletal structures in young mammals (Jenness, 1986).

A unique feature of the structure of CN is the amphipathic nature of their sequences. This suggests tertiary structures organised into polar and hydrophobic domains. The presence of phosphoserine residues in the polar domain is significant because of their interaction with calcium salts and ions, and the formation of micelles. Compared with other proteins, CN are characterised by a large number of proline residues (Swaisgood, 1993; Creamer and MacGibbon, 1996; Wong *et al.*, 1996).

Using electrophoretic separation techniques, CN in cow's milk, is separated into four main types:  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\kappa$ -CN. Additional studies established  $\alpha$ -CN as two different sub-types, namely  $\alpha_{s1}$  and  $\alpha_{s2}$ -CN, whilst  $\gamma$ -CN was found to be a product of the proteolysis of  $\beta$ -CN by plasmin (Brunner, 1981; Eigel *et al.*, 1984; Mephram, 1987). Each of these CN possess a unique amino acid composition (Table 2.6), which in turn is reflected in particular tertiary structures and functional properties.

**Table 2.6. Chemical composition, based on primary structure, of the commonly occurring caseins. (Swaisgood, 1993).**

Amino acid	$\alpha_{s1}$ -Casein	$\alpha_{s2}$ -casein	$\kappa$ -casein	$\beta$ -casein
Aspartic acid	7	4	3	4
Asparagine	8	14	8	5
Threonine	5	15	14	9
Serine	8	6	12	11
Serine-P	8	11	1	5
Glutamic acid	25	24	12	19
Glutamine	14	16	14	20
Proline	17	10	20	35
Glycine	9	2	2	5
Alanine	9	8	15	5
Half Cystine	0	2	2	0
Valine	11	14	11	19
Methionine	5	4	2	6
Isoleucine	11	11	13	10
Leucine	17	13	8	22
Tyrosine	10	12	9	4
Phenylalanine	8	6	4	9
Tryptophan	2	2	1	1
Lysine	14	24	9	11
Histidine	5	3	3	5
Arginine	6	6	5	4
Pyroglutamic acid	0	0	1	0
Total residues	199	207	169	209
Molecular mass	23 623	25 238	19 006	23 988

Caseins are not present in milk as individual molecular structures. They are organised in micelles, which are large protein complexes that also incorporate milk minerals, particularly calcium (Swaisgood, 1992). Caseins in the micelles are present in a 3:1:3:1 ratio for  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$ , respectively. In bovine milk,  $\kappa$ -casein is found mostly on the surface of the micelle, whilst  $\alpha$ - and  $\beta$ -caseins predominate in the interior of the micelle (Dalgleish, 1992).

#### 2.2.1.1.1. *Alpha casein ( $\alpha$ -CN)*

This is represented in bovine milk as two protein families:  $\alpha_{s1}$  and  $\alpha_{s2}$ . The  $\alpha_{s1}$ -CN consist of one major and one minor component, both with the same amino acid sequence (199 residues), varying only in the degree of phosphorylation. Up to 45% of the total protein in bovine milk occurs as  $\alpha_{s1}$ -CN, making it the single most abundant protein in the milk of cattle. There are 5 genetic variants identified to date in the  $\alpha_{s1}$ -CN family (Eigel *et al.*, 1984). Compared with the other caseins,  $\alpha_{s1}$ -CN has a proportionally higher content of serine-P, glutamic acid and glycine in its primary structure (Table 2.6)

The  $\alpha_{s2}$ -CN primary structure consists of 207 amino acid residues, with different degrees of phosphorylation among the members of this group (Eigel *et al.*, 1984). It naturally occurs as a dimer, with disulphide bridges linking the two monomers together (Rasmussen *et al.*, 1994). Similarly to  $\alpha_{s1}$ -CN, the proportion of serine-P and glutamic acid in the molecule of  $\alpha_{s2}$ -CN is higher than in other caseins, and also lysine is abundant. However, its proline content is low.

#### 2.2.1.1.2. *Beta casein ( $\beta$ -CN)*

This group includes  $\beta$ -CN *per se*, plus the products of its proteolysis by plasmin, known as  $\gamma$ -caseins and the proteose-peptone components 5, 8-slow and 8-fast (Eigel *et al.*, 1984). This group is the second most abundant protein in bovine milk, comprising up to 35% of the total protein in skim milk. Compared with the other caseins,  $\beta$ -CN is rich in proline, leucine, valine, methionine and low in tyrosine.

### 2.2.1.1.3. *Kappa casein ( $\kappa$ -CN)*

The  $\kappa$ -CN group consists of a major carbohydrate-free component comprising 169 amino acid residues, and a minimum of six minor components, which are believed to be glycosylated forms of the major component.  $\kappa$ -CN occurs naturally as multimers kept together by disulphide linkages, which are presumed to have an important role stabilising the micelles in milk (Rasmussen *et al.*, 1994). Different polymorphs of  $\kappa$ -casein are associated with variations in the protein content (Ng-Kwai-Hang and Grosclaude, 1992; Coulon *et al.*, 1998) and manufacturing properties, particularly heat stability of the milk (Robitaille, 1995). When compared with the other caseins, its primary structure is characterised by a relative abundance of proline, alanine and isoleucine, while serine-P is present in lower proportion.

### 2.2.1.2. *Whey proteins*

Whey proteins include those milk proteins that remain soluble in the “milk serum” or whey after precipitation of caseins at pH 4.6 and 20°C. This group includes  $\beta$ -lactoglobulin,  $\alpha$ -lactalbumin, serum albumin, immunoglobulins and proteose-peptone fractions. Some of these proteins are synthesised in the mammary gland ( $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin), whilst others (such as immunoglobulins and serum albumin) are preformed and transported from blood and secreted into milk. The term “whey proteins” include a wide range of minor components such as enzymes and growth factors. Some of the proteose-peptone components, that were traditionally classified as “whey proteins”, are part of the  $\beta$ -casein group (*vide supra*; Eigel *et al.*, 1984). Table 2.7 shows the chemical composition of the two major bovine milk whey proteins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin.

**Table 2.7. Chemical composition, based on primary structure, of the major whey proteins (Jenness, 1974).**

Amino acid	$\alpha$ -Lactalbumin	$\beta$ -Lactoglobulin
Aspartic acid	9	16
Asparagine	12	
Threonine	7	8
Serine	7	7
Serine-P		
Glutamic acid	8	25
Glutamate	5	
Proline	2	8
Glycine	6	3
Alanine	3	14
Half Cystine	8	5
Valine	6	10
Methionine	1	4
Isoleucine	8	10
Leucine	13	22
Tyrosine	4	4
Phenylalanine	4	4
Tryptophan	4	2
Lysine	12	15
Histidine	3	2
Arginine	1	3
Total residues	123	162
Molecular mass	24 183	18 363

#### 2.2.1.2.1. *Alpha lactalbumin ( $\alpha$ -LA)*

Considered one of the most primitive milk proteins,  $\alpha$ -LA forms part of the lactose synthetase complex, together with UDP-galactose-*N*-acetylglucosamine galactosyltransferase. Because of its structural similarity to lysozyme, it has been speculated that  $\alpha$ -LA evolved from an antibacterial protein (Blackburn *et al.*, 1989). In bovine milk,  $\alpha$ -LA accounts for 2-5% of the skim milk total protein. It is a calcium-binding protein, formed by 123 amino acids residues, rich in leucine, lysine and cysteine.

#### 2.2.1.2.2. *Beta lactoglobulin ( $\beta$ -Lg)*

This protein is taxonomically restricted, being found in milk from several species (cows, sheep, goats, horse, pig, dog, cat) but absent in the milk of other species (human, guinea pig, mouse; Blackburn *et al.*, 1989; Hambling *et al.*, 1992). Besides its



role as an amino acid source, it is proposed that  $\beta$ -Lg also serves as a carrier of vitamin A and fatty acids in milk (Fox and Flynn, 1992; Hambling *et al.*, 1992; Creamer and MacGibbon, 1996; Wong *et al.*, 1996). In bovine milk,  $\beta$ -Lg is the major whey protein (7-12% of skim milk protein). It is a globular protein formed by 162 amino acid residues with an approximate molecular mass of 18 300. Its primary structure is characterised by a relative abundance of glutamic acid, leucine and lysine. Genetic polymorphism of  $\beta$ -Lg is linked to productive traits such as milkfat concentration and casein:total protein ratio (Ng-Kwai-Hang and Grosclaude, 1992; Hill, 1993; Coulon *et al.*, 1998).

#### 2.2.1.2.3. *Serum albumin (SA)*

A milk protein with the electrophoretic characteristics of blood serum albumin has been found in most of the species analysed to date. In bovine, this protein is an albumin identical in all respects to the albumin found in the blood serum (Jenness, 1974). The presence of SA in milk is mainly the result of “leakage” from blood into the alveolar space through basolateral junctions in the secretory cells in the mammary gland (paracellular transfer). Therefore, its concentration in milk does not depend on the rate of biosynthesis, but rather in the rate of transfer from blood.

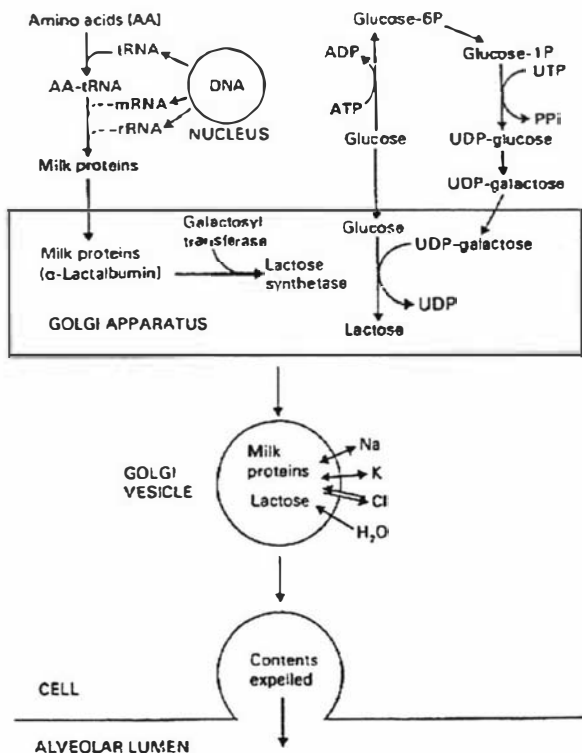
#### 2.2.1.2.4. *Immunoglobulins (Ig)*

A very important role for milk is the transfer of immunoglobulins from the mother to her offspring. The concentration of Ig is specially high in colostrum, which plays a vital role in neonatal survival in those species in which the placental transfer of Ig is absent (Jenness, 1974). Although absorption of Ig occurs for only the first hours of life, detectable concentrations of Ig are found in milk throughout lactation, possibly defending the mammary gland against microbial diseases and providing the young with some protection from enteric diseases. Immunoglobulins are found in milk as a result of both transcellular and paracellular transfer from blood into the alveolar spaces of the mammary gland (Mephram *et al.*, 1982).

### 2.2.2. Milk protein synthesis in the mammary gland

Synthesis and secretion of specific milk proteins are the main functions of mammary epithelial cells during lactation. The process, which is under endocrine and autocrine control, requires the concomitant activation of a) the genes encoding for milk protein and b) the genes encoding for constitutive protein and enzymes required for the synthesis of milk proteins (Mephram *et al.*, 1992).

The composition of individual milk proteins depends on the sequence of nucleotides in messenger RNA transcribed from nuclear DNA. Details about this process are not within the scope of this review, and can be found elsewhere (Mercier and Gaye, 1983; Mephram, 1987; Mephram *et al.*, 1992). A summarised account of events during milk protein synthesis is shown in Figure 2.5 and includes:



**Figure 2.5.** A schematic outline of the processes for milk protein synthesis and secretion of milk protein and lactose in the secretory cells of the mammary gland (Thomas, 1983).

a) Transcription of nuclear information (DNA) to different types of RNA (mRNA, tRNA, rRNA). In the bovine, the genes controlling the synthesis of individual caseins appear to be in the same chromosome and their expression is closely linked. In contrast, there is no indication of a link between casein and whey protein controlling genes, neither between  $\alpha$ -LA and  $\beta$ -LG genes.

b) Assembly of polypeptide chains in the ribosomes. This process involves formation of aminoacyl-tRNA, initiation of the polypeptide chain, elongation cycle and chain termination. Some polypeptide chains suffer co-translational modifications at this stage, for example the transfer of mannose to asparaginyl residues in  $\alpha$ -lactalbumin.

c) Modifications of the polypeptide chain. The polypeptide chain is subject to post-translational modifications, such as phosphorylation (i.e.  $\alpha$ -,  $\beta$ -CN) and glycosylation (i.e.  $\kappa$ -CN), depending on their structure. These post-translational changes occur in the Golgi apparatus, together with the initiation of lactose synthesis. Although it is well established that micelle formation occurs in the Golgi apparatus when caseins are combined with calcium, it is not clear whether caseins and whey protein are together in the same type of vesicle.

d) Vectorial transport to the luminal border of the epithelial cell. Milk proteins and lactose are transported within the mammary epithelial cell in secretory vesicles formed by Golgi apparatus membrane. Because of the osmotic potential of lactose, the vesicles swell with water and ions during their passage through the cytoplasm. Finally, these vesicles discharge their contents into the acinar lumen after fusion-fission of vesicle and apical plasma membranes.

#### **2.2.2.1. *Factors affecting milk protein production in dairy cows***

Two terms are often used to describe milk protein production: milk protein concentration (g protein per kg milk) and milk protein yield (g protein per day). Milk protein concentration is relative to the milk volume produced, which in turn is markedly influenced by lactose synthesis, as stated above (Jenness, 1974). Therefore, higher

protein concentration does not necessarily mean that more protein is synthesised in the mammary gland. However, milk protein concentration is important from a manufacturer's point of view, as the yield of dairy products are expressed per litre of milk (Mackenzie, 1997).

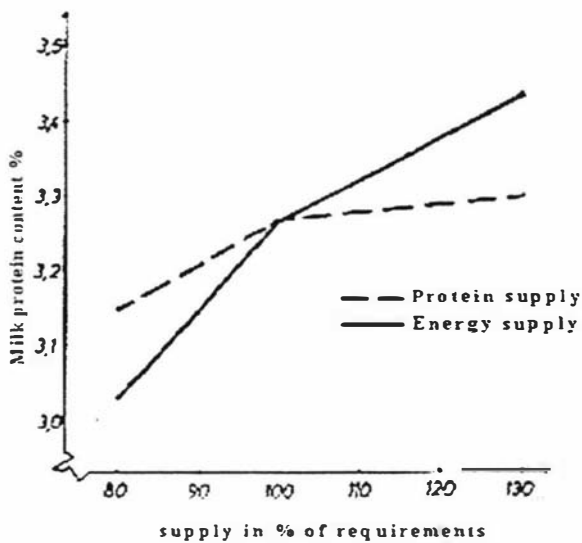
On the other hand, milk protein yield reflects the absolute amount of protein synthesised in the mammary gland (Mackenzie, 1997). This term should be used whenever comparisons on the efficiency of protein synthesis are made.

A multitude of factors may affect both the protein concentration and the protein yield of lactating dairy cows. Some of the major factors have been reviewed elsewhere (DePeters and Cant, 1992) and their detailed description is not within the scope of this review. However, it is important to recognise the effect of these factors when analysing the responses of milk protein concentration and yield to experimental treatments. Among those factors, it is possible to highlight genetic variants (McLean *et al.*, 1984; McLean *et al.*, 1984; FitzGerald, 1997; Hill *et al.*, 1997; Mayer *et al.*, 1997), cattle breed (Gibson, 1989); stage of lactation (DePeters and Cant, 1992; Auldist *et al.*, 1998); parity (DePeters and Cant, 1992; Coulon *et al.*, 1998); disease (Feagan, 1979; Kitchen, 1981; Coulon *et al.*, 1998) and hormonal status (Annison and McDowell, 1980; Falconer, 1980; Mercier and Gaye, 1983; Mepham *et al.*, 1992).

#### 2.2.2.1.1. *Nutrition*

From the major factors affecting the milk protein concentration and yield, nutrition provides the quicker responses (DePeters and Cant, 1992; Fredeen, 1996; Schingoethe, 1996). Several dietary manipulations have been used to modify the concentration and yield of milk proteins in dairy species. For a descriptive purpose, they can be classified in two broad groups: a) responses to changes in dietary energy, and b) responses to changes in dietary protein. However, limiting the effects to one of these two factors is not always easy, as protein can influence diet intake and digestibility and therefore energy intake.

Generally, the responses in milk protein concentration to increases in dietary energy and protein are greater in underfed animals, as seen in Figure 2.6. It can also be seen that responses to extra energy are greater than those observed for extra protein, both below and above of the optimum requirement. A brief account of the effects of dietary energy and protein supply is given here, with the effects of protein being dealt with in more detail later in this review.



**Figure 2.6. Influence of the energy and protein supply on the milk protein content (Kaufmann, 1980).**

#### 2.2.2.1.1.1. Dietary energy

This section includes a variety of diet management strategies which affect the energy intake of animals, such as changes in the forage:concentrate ratios, concentrate supplementation, changes in composition of forages (fibre content, soluble carbohydrate content). In a review of 66 feeding trials, Coulon and Remond (1991) reported that milk protein content is increased linearly with increasing energy supply. However, the protein content of milk is not increased, and may sometimes be reduced when the extra energy is provided as fat (Petersen *et al.*, 1985; Spömdly, 1989; Coulon *et al.*, 1998).

#### 2.2.2.1.1.2. Dietary protein

In most cases it is stated that milk protein concentration is only moderately affected by dietary protein supply. On the other hand, milk protein yield is markedly affected by the type and amount of protein in the diet (Thomas, 1983; DePeters and Cant, 1992; Murphy and O' Mara, 1993; Rajczyk *et al.*, 1994; Schingoethe, 1996).

In concentrate fed animals, the responses in milk protein concentration and yield associated with increasing dietary protein supply are dependant on the type and amount of protein source fed to the animals. In general, protein of low degradability produces larger responses than those of high degradability (Thomas, 1980). It is accepted that responses to dietary protein depend on the supply of amino acids available for absorption in the small intestine (Murphy and O' Mara, 1993; Schingoethe, 1996; Coulon *et al.*, 1998).

### 2.2.3. Role of amino acids as milk protein precursors

Every protein found in nature is assembled from 20 single building units, the genetically coded  $\alpha$ -amino acids. Quantitatively, amino acids in blood are the main precursors available to the mammary gland for milk protein synthesis. Amino acids can be classified in several ways depending on their structural and physiological properties. Common classifications of AA are based on their carbon chain type (aliphatic, aromatic, branched chain), or their electrical charge (acid, basic and neutral). However, in nutritional studies amino acids are commonly classified as essential and non-essential.

#### 2.2.3.1. Essential amino acids

Essential or indispensable amino acids are those which can not be synthesised by animal cells in adequate amounts to meet the requirements for protein synthesis and therefore have to be supplied by the diet (Bender, 1985). It is generally accepted that this group includes methionine (**Met**), threonine (**Thr**), tryptophan (**Trp**), phenylalanine (**Phe**), isoleucine (**Ile**), leucine (**Leu**), lysine (**Lys**), valine (**Val**) and histidine (**His**). However, the list of essential amino acids varies depending on the species and

physiological state of the animals. For example, in young mammals the synthesis of arginine is inadequate to meet the needs for growth; therefore, this amino acid is considered "semi-essential" for mammals (Bender, 1985; Buttery and Foulds, 1988; Buttery and D'Mello, 1994).

Mepham (1982) related uptake and output of essential amino acids in the mammary gland, and this relationship has been used as a classification criterion for this group of amino acids in studies with lactating cows.

#### 2.2.3.1.1. *Group I of EAA*

The amino acids of this group are taken up by the mammary gland in quantities just sufficient to account for their secretion in milk protein. This stoichiometric transfer denotes that their use for any purpose other than direct incorporation into milk protein has to be minimal. This group includes methionine, phenylalanine, histidine and tryptophan. Phenylalanine can be metabolised in the mammary gland to yield tyrosine, which is not metabolised any further before its inclusion in mammary protein (Mepham, 1982). Therefore, tyrosine is also included in this group and then the stoichiometric balance is expressed as phenylalanine plus tyrosine.

Given their high percentage extractions by the mammary gland, it has been speculated that AA in this group might be limiting protein synthesis as a result of restricted transmembrane transport processes (Mepham *et al.*, 1982).

#### 2.2.3.1.2. *Group II of EAA*

Group II, according to Mepham (1982) includes the essential amino acids valine, leucine, isoleucine, arginine, lysine and threonine. The amino acids in this group are taken up by the mammary gland in excess of their output in milk protein. The excess in uptake of these amino acids represents potential sources of  $\alpha$ -amino nitrogen carbon skeletons for the synthesis of non-essential amino acids and other metabolites (Clark *et al.*, 1978). Because of their obligatory participation in catabolic and transamination

reactions, AA in this group might become limiting for milk protein synthesis (Mepham *et al.*, 1982).

### **2.2.3.2. Non essential amino acids**

The non-essential amino acids are synthesised by transamination of intermediates of glycolysis and the carboxylic acid cycle (Buttery and D'Mello, 1994). This group encompasses alanine (**Ala**), asparagine (**Asn**), aspartic acid (**Asp**), cysteine (**Cys**), glutamic acid (**Glu**), glutamine (**Gln**), glycine (**Gly**), proline (**Pro**) and serine (**Ser**). It is important to emphasise that an adequate amount of amino groups must be available for these amino acids to be synthesised in adequate amounts to meet the demand for protein synthesis. It has been suggested that under certain conditions, the demand for some NEAA may be greater than the synthetic capacity, and therefore they can become dietary essentials (Mepham, 1982; Bender, 1985).

Normally, the NEAA are taken up by the mammary gland in amounts that, on average, show a deficit compared with their secretion in milk protein (Clark *et al.*, 1978; Mepham, 1982). Therefore, this deficit should be counterbalanced in the mammary gland mainly by the synthesis from the excess uptake of group II EAA.

### **2.2.4. Amino acids as limiting nutrients for milk protein synthesis**

Lactation represents a dramatic shift in protein metabolism. In goats, it has been demonstrated that the absolute and fractional synthetic rates of tissue protein rise dramatically during lactation (Table 2.8). In the dairy cow, this increase in protein synthesis in the mammary gland represents up to 84% of the total metabolisable protein requirement (AFRC, 1993); cow 450 kg LW, producing 25 kg milk with 3.4% of protein).



**Table 2.8. Fractional and absolute rates<sup>a</sup> of tissue protein synthesis in dry and lactating goats.**

Tissue	Fractional synthetic rate (%/d) <sup>a</sup>			Absolute synthetic rate (mg/d/kg EBW) <sup>b</sup>		
	Dry	Lactating	Lactating:Dry	Dry	Lactating	Lactating:Dry
Mammary gland	5.7	41.6	7.3	12	2242	186.8
Uterus	10.1	5.1	0.5	27	27	1.0
Liver	9.6	10.8	1.1	268	339	1.3
Small intestine	19.0	22.9	1.2	307	408	1.3

<sup>a</sup> Calculated from Champredon *et al.*, (1990; plasma free methionine as precursor pool) and Baracos *et al.*, (1991; plasma free phenylalanine as precursor pool).

<sup>b</sup> From Champredon *et al.*, (1990).

The intracellular availability of amino acids for milk protein synthesis is a function of a) their blood concentration, b) blood flow to the mammary gland, and c) membrane transport by the epithelial cells in the mammary gland (Davis and Collier, 1985). Each of these factors is analysed in the following sections of this chapter.

#### **2.2.4.1. The concept of limiting amino acid**

From any given combination of amino acids, the one supplying the least amount relative to the requirement for protein synthesis will be considered to be the first limiting amino acid (Cole and Van Lunen, 1994). It is possible to rank the amounts of AA available in order to establish the “order of limitation” of amino acids. From these ideas, it emerges that the limiting amino acid will depend both on the supply (absolute amount and composition) and the type of productive process (requirement). When one amino acid is limiting protein synthesis, the rest of the amino acid supply will be metabolised and ultimately excreted. Therefore, the amount of protein synthesised will be determined by the amount of the first limiting amino acid. When the requirement of the first limiting amino acid is met, the second limiting amino acid then becomes the first limiting and successively (Bender, 1985).

There is no general consensus on which AA (if any) is limiting milk protein synthesis in dairy cows. Schwab *et al.*, (1976) stated that only EAA are limiting for dairy cows. However, Meijer *et al.*, (1993) proposed a non-essential AA, glutamine, as a potentially limiting AA for dairy cows, based on the amount of glutamine present in milk protein and its high extraction by the mammary gland. Most studies published to date include methionine in the group of most limiting amino acids, often closely followed by lysine, histidine, phenylalanine and the branched-chain amino acids (leucine, isoleucine and valine). A summary of published research on determining the limiting AA and order of limitation is presented in Table 2.9. However, there are no reports indicating whether this information applies also to the dairy cow fed fresh forages.

**Table 2.9. Limiting amino acids in dairy cows as proposed by different authors.**

Proposed limiting AA	Diet/Protein source	Reference
Phe, Met, Lys	Lucerne hay and maize grain/soybean meal	(Derrig <i>et al.</i> , 1974)
Thr, Phe, Met	Mixed pasture/lucerne hay and barley/casein	(Kellaway <i>et al.</i> , 1974)
Met, Lys, Phe	Lucerne/grass hay and maize grain/soybean meal	(Spires <i>et al.</i> , 1975)
Lys, Met	Lucerne hay and maize grain	(Schwab <i>et al.</i> , 1976)
Met, Phe, Lys	Lucerne/grass hay and maize grain/soybean meal plus infusion sodium caseinate	(Clark <i>et al.</i> , 1977)
Met, Phe, Thr	Maize silage/haylage	(Foldager <i>et al.</i> , 1980)
Lys, Met	Maize hay and maize grain	(Rulquin, 1987)
Met, Lys, Phe	Lucerne hay/Maize silage and maize grain/chickpeas	(Hadsell and Sommerfeldt, 1988)
Met, Lys, Phe	Lucerne hay/Maize silage and maize grain/soybean meal	(Austin <i>et al.</i> , 1991)
Lys, Met, His	Chopped straw and casein	(Fraser <i>et al.</i> , 1991)
Met, Lys, Phe	Maize silage/lucerne hay and maize grain/soybean meal	(Munneke <i>et al.</i> , 1991)

#### 2.2.4.2. Pools of amino acids for milk protein synthesis

Although it has been known from early research (Cary, 1920) that free amino acids in blood are quantitatively the major precursor pool for milk protein synthesis, some researchers have suggested a significant contribution of other precursor pools. However, the proportional participation of each of the suggested pools is still a subject

of debate. In the following sections, each of the precursor pools for milk protein synthesis are described.

#### 2.2.4.2.1. Free amino acids (FAA)

Precursors for tissue protein synthesis are generally assumed to be free amino acids derived either from the diet or from tissue degradation (Backwell *et al.*, 1996). The mammary tissue is not an exception, and it is generally accepted that FAA in blood are quantitatively the major precursor pool for protein synthesis in the mammary gland. Clark *et al.*, (1978) demonstrated the major contribution of the FAA pool to the mammary gland reporting the close balance between the uptake and output of AA carbon and nitrogen (95 and 110%, respectively).

Amino acids in blood are found in two compartments, plasma and erythrocytes. The transfer of FAA from each of these compartments to the mammary gland is not clearly understood, with contrasting reports in the literature.

##### 2.2.4.2.1.1. Plasma

Plasma is generally regarded as the blood compartment from which amino acids are extracted by the mammary gland for protein synthesis. Table 2.10 shows the average of amino acid concentrations in plasma from dairy cows under several dietary conditions. It is noteworthy that all the studies summarised in this Table include only data obtained from concentrate-fed animals. The amino acid profile of blood from pasture-fed dairy cows is still to be characterised.

**Table 2.10. Average  $\pm$  95% confidence interval (CI) and coefficient of variation (CV) of published<sup>a</sup> amino acid concentrations ( $\mu\text{M}$ ) in plasma of dairy cows. Each observation represents the average of an experimental treatment.**

Amino acid	Arterial			Venous		
	mean $\pm$ 95% CI	CV %	<i>n</i>	mean $\pm$ 95% CI	CV %	<i>n</i>
<i>Group I</i>						
Histidine	47.1 $\pm$ 3.6	38.2	98	48.9 $\pm$ 5.4	40.8	55
Methionine	22.9 $\pm$ 1.7	36.0	98	21.5 $\pm$ 2.6	56.0	85
Phenylalanine	42.6 $\pm$ 2.0	23.4	98	34.4 $\pm$ 3.3	43.9	84
Tryptophan	37.4 $\pm$ 4.4	33.8	81	34.6 $\pm$ 3.7	26.5	26
Tyrosine	48.7 $\pm$ 3.7	35.9	90	24.2 $\pm$ 3.0	55.4	78
<i>Group II</i>						
Arginine	80.4 $\pm$ 7.8	48.7	98	73.0 $\pm$ 8.9	42.0	55
Isoleucine	106.6 $\pm$ 5.8	27.3	98	81.6 $\pm$ 5.6	31.0	80
Leucine	133.4 $\pm$ 7.3	23.4	98	126.5 $\pm$ 14.4	52.8	85
Lysine	75.3 $\pm$ 3.0	19.5	94	63.8 $\pm$ 5.4	37.0	77
Threonine	90.8 $\pm$ 4.9	27.0	98	69.7 $\pm$ 5.6	36.0	79
Valine	211.4 $\pm$ 11.3	26.7	98	192.2 $\pm$ 12.0	28.8	85
<i>Non-essential</i>						
Alanine	213.8 $\pm$ 9.0	24.0	94	221.4 $\pm$ 9.8	44.8	78
Aspartic acid	21.8 $\pm$ 6.7	141.8	84	34.7 $\pm$ 5.4	61.0	61
Asparagine	51.3 $\pm$ 6.7	44.7	47	52.6 $\pm$ 9.8	54.4	35
Glutamic acid	92.1 $\pm$ 14.4	76.4	94	122.8 $\pm$ 24.8	89.5	78
Glutamine	224.9 $\pm$ 25.8	32.8	34	181.0 $\pm$ 23.4	37.7	35
Glycine	340.7 $\pm$ 18.1	25.9	94	412.7 $\pm$ 44.1	47.4	78
Proline	87.5 $\pm$ 4.9	24.0	74	84.4 $\pm$ 8.9	42.0	64
Serine	91.4 $\pm$ 4.7	24.9	94	60.1 $\pm$ 10.7	79.3	78

<sup>a</sup> (Fisher, 1972; Bickerstaffe *et al.*, 1974; Clark *et al.*, 1974; Derrig *et al.*, 1974; Spires *et al.*, 1975; Clark *et al.*, 1977; Erfle J.D. and Fisher, 1977; Peeters *et al.*, 1979; Foldager *et al.*, 1980; Rulquin, 1981; Papas *et al.*, 1984a; Papas *et al.*, 1984b; Drackley and Schingoethe, 1986; Yang *et al.*, 1986; Illg *et al.*, 1987; Rogers *et al.*, 1987; Casper and Schingoethe, 1988; Hadsell and Sommerfeldt, 1988; Donkin *et al.*, 1989; Austin *et al.*, 1991; Munneke *et al.*, 1991; Polan *et al.*, 1991; Chamberlain *et al.*, 1992; Choung and Chamberlain, 1992; Lanham *et al.*, 1992; Aldrich *et al.*, 1993; Cant *et al.*, 1993; Guinard J. and Rulquin, 1994; Pösö and Lindberg, 1994; Colin-Schoellen *et al.*, 1995; Meijer *et al.*, 1995; Metcalf *et al.*, 1996; Overton *et al.*, 1996; Pisulewski *et al.*, 1996; Bremmer *et al.*, 1997; Rulquin and Delaby, 1997).

#### 2.2.4.2.1.2. Erythrocytes

As seen in Table 2.11, erythrocyte amino acids account for up to 40% of the total free amino acid content in blood from ruminants (Danilson *et al.*, 1987b) and monogastrics (Proenza *et al.*, 1994). The accumulation of amino acids within the erythrocyte will reflect the permeability of the membranes to free amino acids present in the plasma and the activity of transport mechanisms. Seven different transport systems

**Table 2.11. Proportional contribution (%)<sup>a</sup> of erythrocyte amino acids to whole blood amino acid concentrations in calves fed soybean- or urea-based purified diets.**

Group of amino acids	Soy	Urea
Branched chain amino acids	20.0	17.0
Essential amino acids	26.5	28.5
Non essential amino acids	36.9	41.3
Total amino acids	33.2	37.7

<sup>a</sup> Calculated from Danilson *et al.*, (1987a)

have been described, involving both secondary active transport and facilitated diffusion (Tunnickliff, 1994). The ability of the erythrocyte to take up amino acids has given rise to the suggestion that erythrocytes contribute actively to the AA exchanges between blood and tissues. Studying human blood, Proenza *et al.*, (1994) stated that the contribution of erythrocytes to the whole blood was more important than the plasma for some amino acids (aspartic acid, glutamic acid, asparagine, taurine and ornithine) than for others. The same researchers suggested that amino acids adsorbed to the cell membrane could be readily exchanged with tissue. However, the relative size of this pool (4% of the AA in whole blood) may limit the erythrocyte's net contribution of AA for protein synthesis given the requirement of a high demanding tissue such as the mammary gland.

#### 2.2.4.2.1.3. Plasma vs. whole blood

It has been proposed that using plasma AA concentrations to calculate the uptake by the tissues would result in underestimation, as the contribution from red blood cells is neglected (Heitmann and Bergman, 1980; Danilson *et al.*, 1987b; Hanigan *et al.*, 1991). Measurements of arterio-venous differences in several studies have shown that red blood cells were involved in AA transfer across the portal drained viscera (PDV) of sheep (Heitmann and Bergman, 1980), the hindlimb of calves (Danilson *et al.*, 1987b) and the mammary gland of dairy cows (Hanigan *et al.*, 1991).

In dairy cows, contrasting results have been reported by the same laboratory (Hanigan *et al.*, 1991; Cant *et al.*, 1993), casting doubt on the AA contribution of red blood cells to the mammary tissue. Table 2.12 shows that, according to Hanigan *et al.*, (1991), the use of plasma AA concentrations would underestimate total uptake of all

essential AA except leucine. In contrast, data from Cant *et al.*, (1993) shows that plasma overestimates total uptake of all AA but leucine. Unfortunately, they did not suggest any explanation for such a contrast, which is marked for methionine, lysine and leucine, commonly nominated as limiting AA for milk protein synthesis.

**Table 2.12. Whole blood (B) and plasma (P) arterio-venous differences (A-V) of essential amino acids across the mammary gland of dairy cows from two different studies.**

Amino acid	A-V <sup>a</sup> ( $\mu$ M)			A-V <sup>b</sup> ( $\mu$ M)		
	Plasma	Blood	P/B	Plasma	Blood	P/B
Arginine	26.0	31.1	0.9	24.8	20.0	1.2
Histidine	-33.9	7.8	-4.3	8.3	8.3	1.0
Isoleucine	17.1	81.7	0.2	53.3	22.5	2.4
Leucine	34.6	24.8	1.4	27.5	42.3	0.7
Lysine	22.6	31.3	0.7	27.1	28.9	0.9
Methionine	5.9	7.4	0.8	9.3	5.4	1.7
Phenylalanine	10.8	11.9	0.9	11.9	10.7	1.1
Threonine	15.2	17.6	0.9	16.3	19.3	0.8
Tryptophan	1.1	1.3	0.8	1.4	1.0	1.4
Valine	32.7	44.0	0.7	33.0	46.2	0.7

<sup>a</sup> (Hanigan *et al.*, 1991).

<sup>b</sup> (Cant *et al.*, 1993; average of four treatments). Assuming a packed cell volume of 28%.

On the other hand, Oddy *et al.*, (1988) found that the net uptake of leucine by the mammary gland of lactating goats was  $6.8 \pm 0.8 \mu\text{M}/\text{min}$  when calculated from whole blood, and  $7.6 \pm 0.8 \mu\text{M}/\text{min}$  when calculated from plasma. This reveals little net transfer of red cell leucine to the mammary gland. Furthermore, when isotopically-labelled AA are continuously infused into the blood, the plateaux for label concentration in casein synthesised during the infusion period is closer to the label plateaux for plasma FAA than that of whole blood FAA (Bequette *et al.*, 1994).

#### 2.2.4.2.2. Sources other than FAA

Several studies (Bequette *et al.*, 1994; Backwell *et al.*, 1994; Backwell *et al.*, 1996) have been conducted to investigate the incorporation of AA into milk proteins using isotopic labelling. The contribution of different precursor pools can be assessed by comparing the label concentration of precursors with that of newly synthesised milk protein. In those experiments, the curve of activity/enrichment of milk protein was

significantly lower than the corresponding label in blood amino acids, suggesting that other pools are also involved.

#### 2.2.4.2.2.1. Peptides

Peptides are absorbed from the gastrointestinal tract of ruminants to meet the demand of AA for tissue growth and maintenance (Webb *et al.*, 1992). Bequette *et al.*, (1994) postulated that peptides may contribute to the precursor pool for milk protein synthesis. This suggestion is based on experimental results with goats, using both synthetic (Backwell *et al.*, 1994) and naturally-occurring (Backwell *et al.*, 1996) peptides. The possible contribution of peptide-bound amino acid (PBAA) deserves attention given that approximately one third of the total blood AA occurs as peptides. Earlier estimates (McCormick and Webb, 1982) suggested a greater contribution of PBAA to the AA pool in blood; however, those values are likely artefacts caused by an incomplete deproteinisation of the samples prior to peptide determination (Backwell *et al.*, 1997). Using improved methods, it has been reported that PBAA represent, depending on the AA, between 10-30% of the total blood pool (PBAA + FAA) in the lactating goat (Backwell *et al.*, 1996); between 25-30% in sheep (Backwell *et al.*, 1997) and between 11-36% in dairy cows (Meijer *et al.*, 1997; Tagari *et al.*, 1997). However, there have been failures to prove arterio-venous concentration differences for peptide-bound individual amino acids (Backwell *et al.*, 1997; Meijer *et al.*, 1997; Tagari *et al.*, 1997). Nevertheless, Backwell *et al.*, (1996) reported a significant uptake for the sum of peptide-bound histidine, alanine, leucine, proline and phenylalanine, accounting for 15% of the total extraction across the mammary gland.

#### 2.2.4.2.2.2. Protein turnover in the epithelial cell

It is well known that secretory proteins are first synthesised as higher molecular mass precursors, which undergo cleavage as part of the post translational process (Bienkowski, 1983). Razooki Hasan *et al.*, (1982) reported that secreted casein is only 10% of the casein synthesised by mammary gland explants of rabbits, the remaining 90% being degraded intracellularly. Similarly, it has been demonstrated that milk protein synthesis represents approximately 60% (*in vivo*; Oddy *et al.*, 1988) and 70% (*in vitro*;

Wilde *et al.*, 1989) of the total protein synthesised in the caprine mammary gland. It is likely that the AA liberated from the intracellular casein may be recycled and used for the synthesis of secreted casein (Bequette *et al.*, 1994). Furthermore, not only casein is degraded intracellularly. The constitutive protein in the mammary gland may also contribute to the pool of AA used for milk protein synthesis. This hypothesis has been supported by the high fractional protein synthesis rate (107-130% d<sup>-1</sup>) measured in the mammary tissue of lactating goats (Champredon *et al.*, 1990; Baracos *et al.*, 1991). Mathematical models have predicted that the epithelial cells could become more dependent on AA from constitutive protein turnover when uptake from blood is reduced (Maas *et al.*, 1997).

#### **2.2.4.3. *Amino acid supply to the mammary gland as the limiting factor for milk protein synthesis***

From the previous sections, it may be concluded that milk protein synthesis in the mammary gland is closely dependent upon the supply of AA to the gland. The following sections review the factors that influence the flux of AA to the gland. These factors will be discussed as two groups, those determined by the dietary supply and those related to the metabolism of the individual.

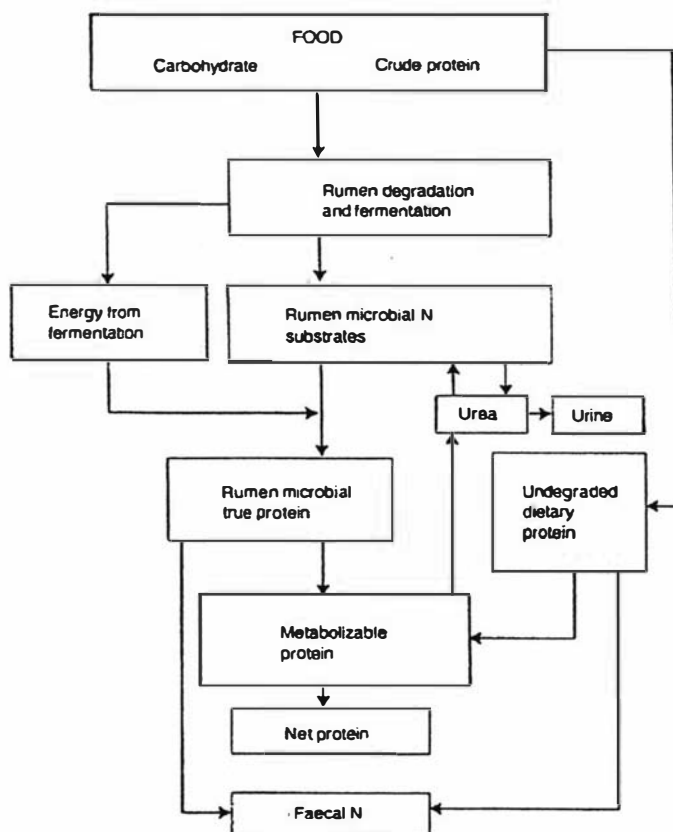
##### **2.2.4.3.1. *Dietary factors affecting the amino acid supply to the mammary gland***

In non-ruminants, the amount and profile of amino acids reaching the small intestine are highly correlated with these components in the diet. However, in ruminants this relationship is not observed because of the complex role of rumen digestion and microbial protein synthesis. Thus, it is necessary to explain briefly how some ruminal events alter the AA supply to the host.

Most of the protein evaluation systems currently in use have coined a term to describe the mixture of AA (of microbial and dietary origin) which is made available to the ruminant for its metabolism (Oldham, 1996). For the AFRC (1993) this term is



metabolisable protein (MP). Metabolisable protein is constituted by microbial true protein synthesised in rumen and undegraded dietary protein. Figure 2.7 shows the main events determining both the quantity and quality of the metabolisable protein reaching the duodenum.



**Figure 2.7. A general scheme depicting the main factors affecting metabolisable protein supply (Oldham, 1996).**

Two major statements can be inferred from Figure 2.7:

a) the amount of AA which becomes available for milk production is markedly influenced by the extensive microbial fermentation of ingested food in the rumen; and

b) the absorption of essential AA from the small intestine is dependent on the amount and AA composition of both dietary undegradable protein and of microbial protein flowing from the rumen and their digestibility in the small intestine.

Non ammonia nitrogen (NAN) is regarded as an indicator of the amount of protein reaching the intestine. In cows fed fresh ryegrass, amino acid N represents 50-70% of the NAN flow. This amount of AA flowing to the duodenum accounts for only 45-55% of the total N intake (Van Vuuren *et al.*, 1992). The same authors reported NAN flow to duodenum as a function of N intake, given by:

$$y = 1.271 - 0.0163(x) \quad (r^2 = 0.69) \quad (\text{Eq. 1.1})$$

where  $y$  is the NAN flow per N intake ( $\text{g g}^{-1}$ ) and  $x$  is grams of N intake per kg of OM intake.

According to Equation 1.1, the predicted NAN flow in lactating cows fed fresh pasture appears to have a ceiling of 270 to 380 g/d within a DM intake range of 12 to 17 kg/d, respectively. Accordingly, the predicted flow of AA N to the duodenum would be in the range of 160 to 270 g/d. As means of comparison, a dairy cow producing moderately (25 kg milk, 3.5% milk protein) secretes approximately 130 g/d of AA N in her milk. From this theoretical calculation it may appear that enough AA N reaches the duodenum to supply the AA N secreted into milk. However, it is not known for certain whether this assumption holds in practical trials. Furthermore, more research is required to quantify the impact of extramammary tissues in the AA economy of dairy cows fed fresh forages.

#### 2.2.4.3.1.1. Microbial protein supply

In animals grazing ryegrass-based pasture, the microbial contribution to the protein reaching the small intestine is expected to be in the range 50-60% (Ulyatt *et al.*, 1975; Van Vuuren *et al.*, 1992; Van Vuuren *et al.*, 1993). Although microbial protein produced in the rumen is one of the highest quality proteins reaching the duodenum, its amino acid profile cannot be considered optimal for milk protein synthesis.

The amino acid composition of rumen microbes remains relatively constant irrespective of the diet and strongly influences the composition of protein in duodenal contents (Table 2.13). Schingoethe (1996) reported that microbial protein is limiting in

histidine, leucine and valine by comparison with milk protein, but methionine and lysine may also be limiting (Storm and Ørskov, 1984). Therefore, it has been stated that ruminal microbes do not provide enough protein for maximum milk production, thus some dietary protein must escape ruminal degradation and pass to the small intestine to supply sufficient amounts of at least these amino acids (Chalupa and Sniffen, 1996).

**Table 2.13. Comparison of the average amino acid composition (% of total N) of duodenal contents of cattle and microbial protein synthesised in the rumen from two types of forages.**

Amino acid	Duodenal contents <sup>a</sup>				Microbial protein <sup>b</sup>	
	Mean	CV	min.	max.	Ryegrass	White clover
<i>Group I</i>						
Histidine	2.21	11.9	1.34	2.89	1.45	1.20
Methionine	1.97	17.6	1.27	2.99	3.60	3.00
Phenylalanine	5.12	7.1	4.13	6.06	4.90	6.00
Tyrosine	4.47	11.9	2.98	5.87	4.75	5.00
<i>Group II</i>						
Arginine	4.96	10.6	3.76	7.07	4.60	4.50
Isoleucine	5.45	8.0	4.44	6.73	5.85	5.70
Leucine	8.87	10.5	6.77	11.90	7.75	7.90
Lysine	6.88	9.7	4.82	8.42	8.45	8.20
Threonine	5.32	7.0	4.36	6.16	5.85	5.90
Valine	6.01	10.8	4.03	7.33	5.65	5.50
<i>Non-essential</i>						
Alanine	6.92	5.6	5.54	7.79	7.35	7.10
Aspartic acid	10.94	6.6	8.90	12.23	12.20	12.40
Glutamic acid	14.52	8.5	11.93	18.08	12.50	12.40
Glycine	6.24	19.2	3.20	10.10	5.15	5.10
Proline	5.01	15.3	3.63	5.87	4.35	4.50
Serine	5.12	8.8	4.01	7.19	4.80	4.90

<sup>a</sup> (Rulquin and Verite, 1996). Cattle. Literature review on 133 diets.

<sup>b</sup> (Ulyatt *et al.*, 1975)

#### 2.2.4.3.1.2. Nature of the dietary protein: rumen-degradable vs. rumen undegradable

When feeding low degradable protein, a close similarity between whole dietary and whole duodenal protein composition can be expected for a range of protein supplements and protection treatments. This close relationship would not hold for highly degradable proteins. In those circumstances, it is expected that the profile of the protein reaching the duodenum will resemble that of microbial protein. (reviewed by Rulquin and Verite, 1996). Furthermore, using multi-variate analysis of a range of diets,

the same authors concluded that the amino acid composition of the intestinal protein is dependant on the nature of the diet from which they originate. For example, high concentrate diets tend to have a higher content of lysine, arginine, methionine, histidine, and a lower content of branched-chain amino acids and phenylalanine than high roughage diets.

Therefore, it seems clear that the amount of protein (and its AA profile) is highly dependent on the type and amounts of ingredients used in dairy rations. In New Zealand, the use of protein supplements in dairyfarming is constrained by economic and management practices. Therefore, in cows fed fresh forage there is little opportunity to modify either the amount or the profile of protein leaving the rumen by means of diet formulation. In general terms, the protein in fresh forages occurs predominantly as a highly degradable fraction (rumen degradable protein; RDP). Ulyatt *et al.*, (1975) reported that RDP of ryegrass and white clover pastures represented 70% of the total N intake. One problem associated with high ruminal degradation of dietary protein, is the wastage of N as ammonia, since the ability of the ruminal bacteria to synthesise AA is surpassed by the ammonia excess. The net loss of dietary N as ammonia has reportedly reached as much as 270 g/kg N intake in lucerne-orchardgrass pasture (Jones-Endsley *et al.*, 1997) and 300 g/kg N intake in ryegrass-clover pasture (Beever and Thorp, 1997). The implications of such losses in the amino acid economy of the grazing cow are not clearly understood.

#### 2.2.4.3.1.3. Protein digestibility

In spite of the variability among dietary ingredients, it is generally accepted that the protein reaching the duodenum possesses a digestibility close to 75% (Table 2.14). However, this may be a simplistic assumption, and the possibility of having different protein digestibilities in duodenal digesta is not discarded. However, the technical difficulties associated with the determination of duodenal digestibility (particularly estimates of the digestibility of the rumen undegradable fraction) are still to be solved (Schingoethe and Blair, 1996).

**Table 2.14. Digestibility coefficients for protein leaving the rumen according to different protein evaluation systems.**

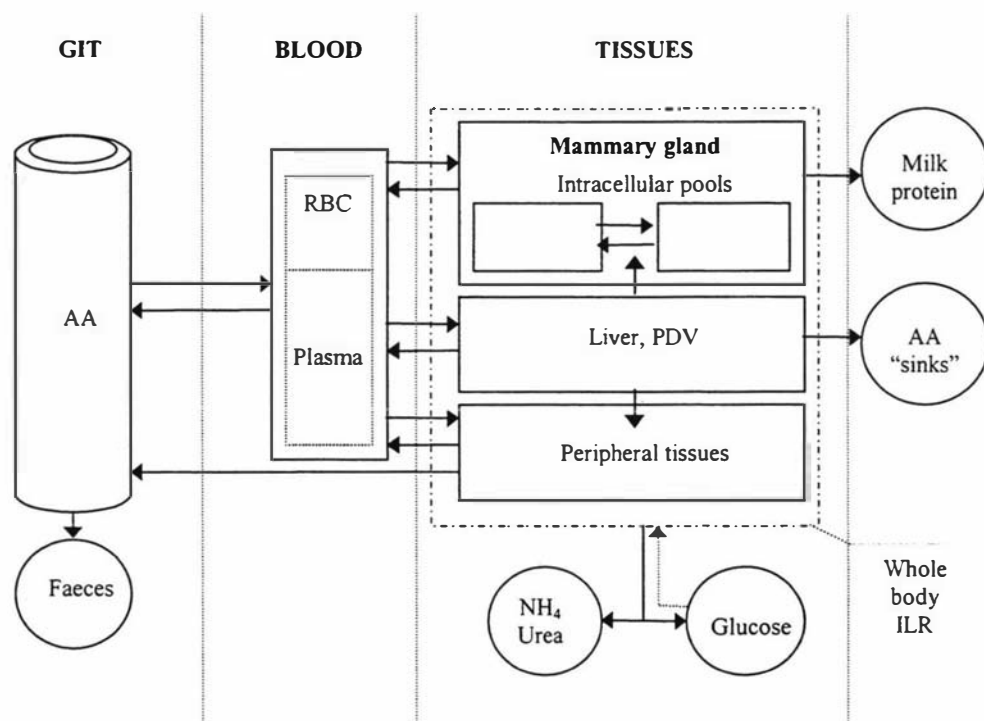
System (Country)	Microbial true protein as % of microbial N	Digestibility of microbial protein	Digestibility of dietary undegraded protein
PDI (France)	80%	80%	Variable <sup>a</sup>
DVE (Netherlands)	75%	85%	Variable <sup>a</sup>
AAT (Scandinavia)	70%	85%	82%
MP (UK)	70%	85%	Variable <sup>a</sup>
AP (USA)	80%	80%	80%

<sup>a</sup>The value depends on the type of protein supplement in the diet.

References: (Verite, 1987; Subnel *et al.*, 1994; Madsen, 1987; Webster, 1987; Owens, 1987).

#### 2.2.4.3.2. Metabolic factors affecting the amino acid supply to the mammary gland

The supply of amino acids to the mammary gland depends not only on the amounts supplied by the diet and flowing from the rumen to the small intestine. Once the AA have reached the duodenum, they are subject to several metabolic steps which influence in a lesser or greater extent, the final mixture reaching the mammary gland.



**Figure 2.8. Schematic representation of the main paths of amino acid utilisation in lactating dairy cows.**

The amount and composition of the amino acid mixture reaching the mammary gland is the result of a complex orchestration of events involving both the mammary gland and non-mammary tissues (Figure 2.8). This orchestration is under two types of control: homeostasis and homeorhesis (Bauman and Currie, 1980). Amino acids are partitioned mainly to one of the following functions: protein synthesis, oxidation and gluconeogenesis (Thomas, 1980). The proportional flux to those pathways depend on physiological state, hormonal controls, and limitations in the supply of one or more AA.

#### 2.2.4.3.2.1. Absorption of amino acids in the small intestine

The main sites for amino acid absorption are the duodenum and jejunum (Tagari and Bergman, 1978). It has been demonstrated that, in general, the net absorption of AA in the small intestine is related to the amounts present in duodenal digesta (Armstrong, 1973; Tagari and Bergman, 1978). However, individual amino acids are absorbed from the small intestine at different rates. In experiments with different dietary treatments, it has been shown that the absorption (% of duodenal flux) in the small intestine varies for individual amino acids in sheep fed grass (25 – 88 %; Armstrong, 1973) and dairy cows fed total mixed rations (51 - 90 %, Stern *et al.*, (1985); 65 – 92 %, Mabjeesh *et al.*, (1996). In ruminants, it has been demonstrated that essential amino acids are absorbed more readily than non-essentials, with methionine being the amino acid with the greatest absorption rate in the small intestine of both sheep and cattle (Webb, 1990).

#### 2.2.4.3.2.2. Impact of splanchnic organs on amino acid supply to the mammary gland

In studies measuring simultaneously AA disappearance from the small intestine and their appearance in portal flow (Tagari and Bergman, 1978; MacRae *et al.*, 1997), it has been shown that the amounts disappearing from the small intestine are usually greater than those appearing in the portal plasma. Using <sup>13</sup>C-labeled AA, MacRae *et al.*, (1997) reported that the recovery of tracer infused at the duodenum ranged from 61% for histidine to 83% for leucine. However, the AA of luminal origin represent less than 20% of the AA used by the gastrointestinal tract. The remaining 80% seems to be extracted from circulating blood. In contrast to previous assumptions, the

gastrointestinal tract actually competes for AA against other peripheral tissues (i.e. muscle, mammary gland).

Once in the portal blood, the absorbed AA undergo metabolic transformations in the liver. These transformations include liver protein synthesis, gluconeogenesis, ketogenesis, synthesis of blood proteins, and amino acid transamination and oxidation (Lescoat *et al.*, 1996). It has been demonstrated that there is an hepatic uptake of all amino acids except glutamate, citrulline and ornithine, for which there is a net production (Bergman and Heitmann, 1978). Unfortunately, these studies involving only measurements of the hepatic uptake and release of amino acids do not provide an insight into the fate of individual amino acids in the liver.

Despite this shortfall, the data available are consistent with the metabolic activity of that organ and with its demands for protein synthesis and gluconeogenesis (Thomas and Chamberlain, 1984). Ruminants derive approximately 15-25% of their glucose requirements from AA. However, the percent contribution of AA to gluconeogenesis can be as high as 36% when other glucogenic substrates are not available (Danfær *et al.*, 1995). Therefore, it seems clear that the availability of glucogenic substrates is an important regulatory factor for the metabolism of AA in the liver. The main glucogenic AA, alanine and glutamine, together with glycine and serine represent, on a molar basis, up to 70% of the total AA uptake by the liver (Bergman and Heitmann, 1978). Hence, the liver has an important role modifying the profile of the amino acids available to the mammary gland for milk protein synthesis.

#### 2.2.4.3.2.3. Partitioning of amino acids for bodily functions

The percent contribution of different tissues to whole body protein synthesis has been reported in non-lactating cattle as follows: muscle 12-16%, carcass (muscle+bone+brain) 32-33%; gastrointestinal tract (GIT) 38-46%, liver 7-8% and skin 14-21% (Lobley *et al.*, 1980). The contributions of liver and GIT deserve particular attention because of their disproportionate contribution considering that they represent only 2 and 6% of the total body protein mass, respectively. Thus, the requirement of individual tissues for AA is markedly affected not only by the protein mass of each

tissue, but also by their metabolic rate (measured as turnover rate or fractional synthesis rate of their protein).

In dry goats, Baracos *et al.*, (1991) reported that the mammary gland contributed only 1% to the whole body Phe flux. During lactation, this contribution increased to 31%. Milk protein alone contributed 18% of the whole body Phe flux. Champredon *et al.*, (1990) reported that the mammary gland protein synthesis contributed less than 1% and 46% of the whole body Met flux in dry and lactating animals, respectively. In the latter experiment, milk protein synthesis accounted for 25% of the whole body Met flux. Similarly, Riis (1988) reported that milk protein synthesis contributed 15-20% of the total protein synthesis as estimated from leucine whole body flux in lactating goats.

The use of universally labeled amino acids obtained from hydrolysed algal (*Spirulina spp.*) has allowed the measurement of whole body fluxes and partitioning of up to 13 AA simultaneously (Bequette *et al.*, 1997). These authors reported that, on average, 25% of the plasma amino acid flux was directed to the mammary gland of dairy goats. In that experiment, the relative pattern on individual AA partitioning was constant among animals: flux to the mammary gland was less than 20% of the plasma flux for His, Ser, Phe and Ala; between 20-30% for Arg, Thr, Tyr and Leu; and between 30-40% for Pro, Ile, Lys and Val.

Therefore, it is noticeable that the protein synthesis in the mammary gland plus the net export of milk represent the largest individual tissue demand for AA. However, from the data of Bequette *et al.* (1997) it appears that the partitioning of AA to the mammary gland varies between individual amino acids. Although the experimental work with dairy goats has provided an insight into the amino acid metabolism in lactating ruminants, there is no comparable information of amino acid partitioning in lactating dairy cows.

#### 2.2.4.3.2.4. Blood flow to the mammary gland

Mammary supply of any particular nutrient generally described as the concentration of the nutrient in the blood multiplied by the rate of mammary blood flow



(MBF). Under normal conditions, approximately 15% of the cardiac output perfuses the mammary gland. However, MBF is highly variable and has been found to be affected by factors such as fasting and milking. The regulation of MBF is exerted by both extramammary and local control. Sympathetic nerves play a role maintaining normal vascular tone in the mammary gland (Davis and Collier, 1985). Local vasoactive compounds are produced by the mammary epithelia, demonstrating the ability of the mammary tissue to regulate its own blood supply, and therefore, nutrient flow for milk synthesis (Prosser *et al.*, 1996).

According to Cant and McBride (1995) the major determinant of blood flow in a particular organ is the number of actively perfused capillaries. Locally produced vasoactive agents, produced in response to the metabolic activity of the organ, are responsible for constriction or dilation of precapillary sphincters in a process of capillary recruitment. This phenomenon was proposed by Davis and Collier (1985) as being responsible for increases in mammary blood flow following udder massage, milking and oxytocin injection.

Different methods have been used to estimate blood flow through the mammary gland, and are reviewed elsewhere (Linzell, 1974; Davis and Collier, 1985). Although milk yield and MBF are highly correlated, it is not completely understood how blood flow influences mammary metabolism and *vice versa*. Table 2.15 shows a summary of MBF measurements using different techniques and under different experimental conditions. In general, measured MBF is in the range 0.3-0.6 ml blood per gram of tissue per minute. From these values, it is generally accepted that the ratio of blood flow to milk yield is between 450-900.

**Table 2.15. Summary of measurements of mammary blood flow using different techniques and experimental conditions.**

Condition	Method <sup>a</sup>	Mammary blood flow		Ratio BF:Milk	Ref.
		l min <sup>-1</sup>	ml g <sup>-1</sup> min <sup>-1</sup>		
Jersey cows (383 kg BW)	TD	5.6	0.53	457	(Bickerstaffe <i>et al.</i> , 1974)
Friesian cows (510 kg BW)	TD	6.7	0.28	458	(Bickerstaffe <i>et al.</i> , 1974)
Red and White East Flemish cows (568 kg BW)	EM	5.5	--	507	(Peeters <i>et al.</i> , 1979)
Holstein cows, different fat intake	FP (Phe+Tyr)	14.9	--	812	(Cant <i>et al.</i> , 1993)
Normal cows, various breeds	AP			681	(Kronfeld <i>et al.</i> , 1968)
Fasted cows, various breeds	AP			900	(Kronfeld <i>et al.</i> , 1968)
Control, Friesian cows (514 kg BW)	FP ( <sup>3</sup> H <sub>2</sub> O)	7.8	0.36	526	(McDowell <i>et al.</i> , 1987)
Somatotropine treated, Friesian cows (510 kg BW)	FP ( <sup>3</sup> H <sub>2</sub> O)	9.7	0.44	576	(McDowell <i>et al.</i> , 1987)

<sup>a</sup> TD: thermodilution; EM: electromagnetic probe; FP: Fick principle; AP: antipyrine absorption.

#### 2.2.4.3.2.5. Importance of the mammary blood flow assessment in the validity of balance studies

Most of the studies on mammary metabolism are based on the analysis of the uptake of substrates by the mammary gland and their appearance in the milk secreted

per unit of time. Therefore, reliable estimates of blood flow are necessary to quantify more accurately the fluxes of amino acids from the blood to the mammary gland and finally into the milk.

#### 2.2.4.4. Amino acid metabolism in the mammary gland as the limiting factor

##### 2.2.4.4.1. Amino acid uptake: Transport systems in the mammary gland

The presence and identification of AA transport systems have been reviewed by (Baumrucker, 1985). According to this author, there are seven transport systems which are likely to be found in the mammary gland (Figure 2.9). The three neutral transport systems (A, L and ASN) and the cationic ( $Y^+$ ) have been positively identified in bovine mammary tissue. There is suggestive evidence of the existence of N and anionic ( $X_{AG}$ ) systems. The presence or absence of a separate system for Gly (Gly system) still is debatable (Baumrucker, 1985).

CLASS	NEUTRAL				N	CATIONIC	ANIONIC	GLY
	A S C	A	L	Y <sup>+</sup>		-		
NAME OF SYSTEM	A S C	A	L	N	Y <sup>+</sup>	-	GLY	
NAMED FOR:	Ala Ser Cys	Ala	Leu	Gln His Asn	Lys Arg	Asp Glu	Gly	
PREFERENCE	Small Sidechains OH or SH	Small Unbranched Sidechains	Large branched or aromatic Sidechains	Nitrogen bearing Sidechains	+ Charged Sidechains	-Charged Sidechains	Gly Sacrosine	
SELECTIVE SUBSTRATE	L-Cys	MeAIB	BCH (c-Leu)	L-His	L-Lys L-Arg L-Orn	L Cysteate	Gly + MeAIB	
NAME OF SYSTEM	A S C	A	L	N	Y <sup>+</sup>	-	GLY	
REGULATION HORMONAL ADAPTIVE	no no	yes yes	no yes?	no yes	no ?	? ?	no no	

Figure 2.9. Classification and characteristics of the amino acid transport systems present in the mammary gland (From Baumrucker, 1985). MeAIB = 2-(methylamino)-isobutyric acid; BCH = 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid; c-Leu = cycloleucine.

The transport systems found in the bovine mammary gland include both Na<sup>+</sup> dependent and Na<sup>+</sup> independent systems. In general terms, the Na<sup>+</sup> dependent systems concentrate amino acids within the cell. In these cases, the rate of transport of amino acids depends on the concentration of Na<sup>+</sup> in the media, while the direction of the transport is given by the orientation of the Na<sup>+</sup> gradient. On the other hand, Na<sup>+</sup> independent systems (L and Y<sup>+</sup>) do not concentrate amino acids to the same extent as Na<sup>+</sup> dependent systems. These systems transport amino acids across the membrane in both directions simultaneously (i.e. the absorption of one mole of amino acid requires the transfer of one mole of amino acid to the extracellular space; Maas *et al.*, 1998).

#### 2.2.4.4.2. *Amino acid transport as a limiting factor*

Amino acid uptake in the mammary gland depends on both the MBF and the exchange across the capillary wall. This exchange, in turn, is a function of the surface area available for exchange and the relative permeability of the capillary wall. Furthermore, the permeability of the capillary wall can differ according to the substrate, depending on the presence and activity of specific transmembrane transporters (Prosser *et al.*, 1996). Therefore, the activity of transporters plays an important role in the amount of nutrient extracted by the mammary gland for synthetic purposes. For example, the mathematical model proposed by Cant and McBride (1995) predicts that, for nutrients with high extraction rate, such as acetate and fatty acids, the increase in mammary blood flow would increase net uptake. For nutrients with low transport rates (glucose and amino acids), net uptake would increase up to an optimal blood flow, decreasing at higher blood flow rates.

The existence of a countertransport system, such as the L system, means that a large proportion of the intracellular AA is released from the cell back to the blood. The L system is responsible for the transport of some AA regarded as limiting for dairy cows (i.e. Met, Phe, Leu). For these amino acids, the net rates of influx into the cell or efflux out of a cell depend on the relative concentration gradients of AA on opposite sides of the membrane (Maas *et al.*, 1998). Indeed, this has led to the hypothesis that a limitation in the net rate of uptake by transport systems in the mammary gland

(particularly non-concentrative systems such as the L system) may be involved in defining which AA are limiting milk protein synthesis in lactating animals.

This hypothesis has been supported by predictions from mathematical models such as the one enunciated by Maas *et al.*, (1997) for Phe and Tyr fluxes in the mammary gland. These authors suggested that intracellular concentrations of Phe and Tyr must be substantially higher than that of arterial blood to accommodate even an moderate level of milk protein synthesis. The same model predicted that only 11% of the Phe+Tyr influx was used for intramammary protein synthesis, and the remaining 89% was recycled back to blood. Although this would appear inefficient, it may be a true reflection of the biology of AA countertransport, such as the L-system. The manipulation of the kinetic parameters in the model indicated that milk protein secretion appears to be more sensitive to the number/activity ( $V_{max}$ ) rather than the affinity ( $K_m$ ) of L-system transporters on the basal membrane side of the alveolar cell. However, data from *in vivo* experiments involving more amino acids are required to prove the predictions from this and other mathematical models.

## **2.3. APPROACHES TO DETERMINE LIMITATIONS IN THE AMINO ACID**

### **SUPPLY TO THE MAMMARY GLAND**

In lactating cows, various techniques have been used to determine which amino acid is limiting milk protein synthesis. These techniques include: comparison of the amino acid pattern of ruminal bacteria with that of milk protein, changes in the amino acid concentrations of blood after protein supplementation and arterio-venous differences across the mammary gland, as well as theoretical calculations (Kaufmann and Lüpping, 1982).

#### **2.3.1. Amino acid profiles: the concept of ideal supply**

An 'ideal' protein has been defined as one in which the composition of the AA absorbed from the small intestine matches closely the AA mixture required by the animal for a particular productive purpose (Fraser *et al.*, 1991). This concept was

developed from experimental work with non-ruminants (Cole and Van Lunen, 1994). However, the results obtained in France (Rulquin *et al.*, 1995) indicate that it is possible to predict productive responses by dairy cows to Met and Lys when the amounts and proportion of absorbable amino acids are known. In similar fashion, and based on their calculations using the Cornell Net Carbohydrate and Protein System, Wu *et al.*, (1997) proposed the proportion of absorbable essential amino acids required by cows consuming total mixed rations of alfalfa hay and maize grain. With exception of arginine, these theoretical values are remarkably similar to the proportions of EAA in milk protein (Table 2.16).

**Table 2.16. Relative proportions (g AA/100 g EAA) of essential amino acids in milk protein and the required absorbable according to the Cornell Net Carbohydrate and Protein System.**

Amino acid	Milk protein <sup>a</sup>	Absorbable required <sup>b</sup>
Arginine	6.3	12.4
Histidine	5.1	5.8
Isoleucine	10.4	11.4
Leucine	17.4	17.2
Lysine	14.8	16.0
Methionine	5.2	5.0
Phenylalanine	8.9	8.8
Threonine	7.8	8.7
Tryptophan	2.6	2.7
Tyrosine	9.8	NA
Valine	11.7	12.0

<sup>a</sup> Calculated from Mackenzie (1997).

<sup>b</sup> From Wu *et al.*, (1997).

Following this idea, Schingoethe and Blair (1996) proposed the term “milk protein score (MPS)” to qualify a diet depending on its content of the most limiting AA for milk protein synthesis. The MPS value is the amino acid content of the most limiting amino acid in a protein supplement or diet relative to that amino acid in milk.

A major drawback of this approach is the underlying assumption that all the amino acids are absorbed in the intestine and utilised by the mammary gland with the same efficiency. Individual amino acids are transferred with different efficiencies in their path to the secretory cells in the mammary gland (Oldham, 1994). Therefore, more research is necessary to increase the knowledge on the efficiency of utilisation of

individual AA within the mammary gland of lactating dairy cows to determine the composition and amount of absorbable AA required to elicit predictable responses in milk protein production.

### **2.3.2. Arterio-venous difference and extraction rates from arterial supply**

Information relevant to the flow of AA in different tissues has been obtained by the technique of arterio-venous (A-V) sampling. Basically, this technique consists in sampling of blood at pre- (arterial sample) and post-organ sites (venous sample, normally the superficial abdominal veins) and determines the changes in concentration of AA. Because of the wide variation between molar concentrations of amino acids in blood, the A-V provides little information on the extent of limitation of individual amino acids for milk protein synthesis. However, when the A-V are expressed as a proportion of the arterial supply (extraction rate or extraction percentage), it is possible to determine the extent to which amino acids are being removed from the blood by the mammary gland. Based on the later, some authors have proposed a ranking of limiting amino acids depending on their extraction percentages. In this way, Davis *et al.*, (1978) proposed that Met, Lys and Leu were limiting, in that order, for milk protein synthesis in forage-fed lactating ewes. However, and as discussed previously, any conclusion derived from this type of study is highly dependent on the choice of precursor pool (whole blood or plasma).

### **2.3.3. Balance studies: Uptake vs. output comparisons**

The measurement of the arterio-venous difference, together with a valid estimate of blood flow permits quantification of the net flux and uptake of AA per unit of time across a certain organ. In the case of the mammary gland, the calculated uptake can be readily compared with the output of metabolites in the milk and then the balance of a specific metabolite may also be calculated (Mephram, 1982). The mammary gland is suitable for this approach because its secretion, milk, is not recycled by the epithelial cells and therefore the measurement of AA output is relatively straightforward.

This technique, *per se*, provides little information about the metabolic transformations occurring in the mammary tissue to the metabolite being studied. However, when tracers are included in this approach, it is possible to obtain an estimate of the relative impact of the mammary tissue in terms of the whole body metabolism of any particular precursor (Bequette *et al.*, 1997). The combination of balance studies and tracer techniques also provides an insight into the type and extent of the metabolic transformations within the mammary tissue (Bequette *et al.*, 1994; Bequette *et al.*, 1996).

#### **2.4. CONCLUSIONS AND FUTURE RESEARCH NEEDED**

From this review, it can be concluded that an adequate AA supply to the mammary gland is of paramount importance for the synthesis of milk proteins. However, the research conducted to date on the AA metabolism and utilisation has not provided enough information to understand and quantify the role of AA as limiting nutrients for lactating dairy cows. Furthermore, the majority of the published information on AA metabolism of dairy cows has been obtained in concentrate-fed animals. There is no information that allows a characterisation of the AA metabolism in animals fed fresh forages as sole diet.

Therefore, in order to devise strategies to increase/modify/optimize protein synthesis in pasture-fed dairy cows, it is necessary to understand and quantify the several metabolic steps involved in the transformation of dietary proteins into AA available to productive purposes and their incorporation into milk proteins.

Some areas of future research are outlined as the first step towards a better understanding of the metabolism of amino acids in lactating dairy cows fed fresh forage:

- To assess the productive responses of lactating dairy cows fed fresh-forages to the supplementation with limiting amino acids. To compare these responses to those obtained in concentrate-fed animals.



- 
- To assess the role of whole blood and plasma as pools of free amino acids for milk protein synthesis in the mammary gland of lactating dairy cows fed fresh forage.
  - To quantify the flux of amino acids through the mammary gland and their usage for milk protein synthesis in relation with whole body amino acid fluxes in lactating dairy cows fed fresh forages.

## 2.5. REFERENCES

AFRC. 1993. Energy and protein requirements of ruminants. An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients. Wallingford, U.K.: CAB International.

Aldrich, J.M.; Muller, L.D.; Varga, G.A. 1993. Effect of somatotropin administration and duodenal infusion of methionine and lysine on lactational performance and nutrient flow to the small intestine. *British Journal of Nutrition* **69**:49-58

Annison, E.F.; McDowell, G.H. 1980. The influence of nutrition on milk yield and composition in ruminants. *Proceedings of the Nutrition Society of Australia* **5**:96-103

Armstrong, D. G. 1973 The uptake of amino acids by the small intestine of the ruminant animal. In Production disease in farm animals (Ed. Payne, J. M., Hibbitt, K. G., and Sansom, B. F.) 43-53. London, U.K.: Baillière Tindall.

Auldist, M.J.; Walsh, B.J.; Thomson, N.A. 1998. Seasonal and lactational influences on bovine milk composition in New Zealand. *Journal of Dairy Research* **65**:401-411

Austin, C.L.; Schingoethe, D.J.; Casper, D.P.; Cleale, R.M. 1991. Influence of bovine somatotropin and nutrition on production and composition of milk from dairy cows. *Journal of Dairy Science* **74**:3920-3932

Backwell, F.R.C.; Bequette, B.J.; Wilson, D.; Calder, A.G.; Metcalf, J.A.; Wray-Cahen, D.; MacRae, J.C.; Beever, D.E.; Lobley, G.E. 1994. Utilization of dipeptides by the caprine mammary gland for milk protein synthesis. *American Journal of Physiology*. **267**:R1-R6

Backwell, F.R.C.; Bequette, B.J.; Wilson, D.; Metcalf, J.A.; Franklin, M.F.; Beever, D.E.; Lobley, G.E.; MacRae, J.C. 1996. Evidence for the utilization of peptides

for milk protein synthesis in the lactating dairy goat *in vivo*. *American Journal of Physiology* **271**:R955-R960

Backwell, F.R.C.; Hipolito-Reis, M.; Wilson, D.; Bruce, L.A.; Buchan, V.; MacRae, J.C. 1997. Quantification of circulating peptides and assessment of peptide uptake across the gastrointestinal tract of sheep. *Journal of Animal Science* **75**:3315-3322

Baracos, V.E.; Brun-Bellut, J.; Marie, M. 1991. Tissue protein synthesis in lactating and dry goats. *British Journal of Nutrition* **66**:451-465

Bauman, D.E.; Currie, W.B. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. *Journal of Dairy Science* **63**:1514-1529

Baumrucker, C.R. 1985. Amino acid transport systems in bovine mammary tissue. *Journal of Dairy Science* **68**:2436-2451

Beever, D. E.; Thorp, C. L. 1997 Supplementation of forage diets. In Milk composition, Production and Biotechnology (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 419-440. Cambridge, UK.: CAB International.

Bender, D. A. 1985. Amino acid metabolism. Chichester, U.K.: John Wiley & Sons.

Bequette, B.J.; Backwell, F.R.C.; Calder, A.G.; Metcalf, J.A.; Beever, D.E.; MacRae, J.C.; Lobley, G.E. 1997. Application of a U-<sup>13</sup>C-labeled amino acid tracer in lactating dairy goats for simultaneous measurements of the flux of amino acids in plasma and the partition of amino acids to the mammary gland. *Journal of Dairy Science* **80**:2842-2853

Bequette, B.J.; Backwell, F.R.C.; Dhanoa, M.S.; Walker, A.; Calder, A.G.; Wray-Cahen, D.; Metcalf, J.A.; Sutton, J.D.; Beever, D.E.; Lobley, G.E.; MacRae, J.C.

1994. Kinetics of blood free and milk casein-amino acid labelling in the dairy goat at two stages of lactation. *British Journal of Nutrition* **72**:211-220

Bequette, B.J.; Backwell, F.R.C.; MacRae, J.C.; Lobley, G.E.; Crompton, L.A.; Metcalf, J.A.; Sutton, J.D. 1996. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. *Journal of Dairy Science* **79**:2217-2224

Bergman, E.N.; Heitmann, R.N. 1978. Metabolism of amino acids by the gut, liver, kidneys, and peripheral tissues. *Federation Proceedings* **37**:1228-1232

Bickerstaffe, R.; Annison, E.F.; Linzell, J.L. 1974. The metabolism of glucose, acetate, lipids and amino acids in lactating dairy cows. *Journal of Agricultural Science, Cambridge*. **82** :71-85

Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatisation. *Journal of Chromatography* **336**:93-104

Bienkowski, R.S. 1983. Intracellular degradation of newly synthesised secretory proteins. *Biochemical Journal* **214**:1-10

Blackburn, D.G. 1993. Lactation: Historical patterns and potential for manipulation. *Journal of Dairy Science* **76**:3195-3212

Blackburn, D.G.; Hayssen, V.; Murphy, C.J. 1989. The origins of lactation and the evolution of milk: a review with new hypotheses. *Mammal Review* **19**:1-26

Bremmer, D.R.; Overton, T.R.; Clark, J.H. 1997. Production and composition of milk from Jersey cows administered bovine somatotropin and fed ruminally protected amino acids. *Journal of Dairy Science* **80**:1374-1380

Brunner, J.R. 1981. Cow milk proteins: Twenty-five years of progress. *Journal of Dairy Science* **64**:1038-1054

Buttery, P. J.; D'Mello, J. P. F. 1994 Amino acid metabolism in farm animals: An overview. In *Amino acids in farm animal nutrition* (Ed. D'Mello, J. P. F.) 1-10. Wallingford, Oxon, U.K.: CAB International.

Buttery, P. J.; Foulds, A. N. 1988 Amino acid requirements of ruminants. In *Recent Developments in Ruminant Nutrition -2* (Ed. Haresign, W. and Cole, D. J. A.) 19-33. London: Butterworths.

Cant, J.P.; DePeters, E.J.; Baldwin, R.L. 1993. Mammary amino acid utilization in dairy cows fed fat and its relationship to milk protein depression. *Journal of Dairy Science* **76**:762-774

Cant, J.P.; McBride, B.W. 1995. Mathematical analysis of the relationship between blood flow and uptake of nutrients in the mammary glands of a lactating cow. *Journal of Dairy Research* **62**:405-422

Cary, C.A. 1920. Amino acids of the blood as the precursors of milk proteins. *Journal of Biological Chemistry* **43**:477-489

Casper, D.P.; Schingoethe, D.J. 1988. Protected methionine supplementation to a barley-based diet for cows during early lactation. *Journal of Dairy Science* **71**:164-172

Chalupa, W.; Sniffen, C.J. 1996. Protein and amino acid nutrition of lactating dairy cattle- today and tomorrow. *Animal Feed Science and Technology* **58**:65-75

Chamberlain, D.G.; Choung, J.J.; Robertson, S. 1992. Protein nutrition of dairy cows receiving grass silage diets: effects of feeding a protein supplement of unbalanced amino acid composition. *Journal of the Science of Food and Agriculture* **60**:425-430

Champredon, C.; Debras, E.; Mirand, P.P.; Arnal, M. 1990. Methionine flux and tissue protein synthesis in lactating and dry goats. *Journal of Nutrition* **120**:1006-1015

Choung, J.J.; Chamberlain, D.G. 1992. Protein nutrition of dairy cows receiving grass silage diets. Effects on silage intake and milk production of postruminal

supplements of casein or soya-protein isolate and the effects of intravenous infusions of a mixture of methionine, phenylalanine and tryptophan. *Journal of the Science of Food and Agriculture* **58**:307-314

Clark, D. A.; Penno, J. W.; Neil, P. G. 1997 Nutritional merits and problems of pasture. In Milk composition, Production and Biotechnology (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 397-418. Cambridge, UK.: CAB International.

Clark, J.H.; Davis, C.L.; Hatfield, E.E. 1974. Effects of formaldehyde treated soybean meal on nutrient use, milk yield and composition, and free amino acids in the lactating bovine. *Journal of Dairy Science* **57**:1031-1036

Clark, J.H.; Spires, H.R.; Davis, C.L. 1978. Uptake and metabolism of nitrogenous components by the lactating mammary gland. *Federation Proceedings* **37**:1233-1238

Clark, J.H.; Spires, H.R.; Derrig, R.G.; Bennink, M.R. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. *Journal of Nutrition* **107**:631-644

Cole, D J A; Van Lunen, T A. 1994 Ideal amino acids patterns. In Amino acids in farm animal nutrition (Ed. D'Mello, J. P. F.) 99-112. Wallingford, Oxon, U.K.: CAB International.

Colin-Schoellen, O.; Laurent, F.; Vignon, B.; Robert, J.C.; Sloan, B. 1995. Interactions of ruminally protected methionine and lysine with protein source or energy level in the diets of cows. *Journal of Dairy Science* **78**:2807-2818

Coulon, J.B.; Hurtaud, C.; Remond, B.; Verite, R. 1998 . Factors contributing to variation in the proportion of casein in cows' milk true protein: a review of recent INRA experiments. *Journal of Dairy Research* **65**:375-387

Coulon, J.B.; Remond, B. 1991. Variations in milk output and milk protein content in response to the level of energy supply to the dairy cow: a review. *Livestock Production Science* **29** :31-47

Creamer, L.K.; MacGibbon, A.K.H. 1996. Some recent advances in the basic chemistry of milk protein and lipids. *International Dairy Journal* **6**:539-568

Dalgleish, D.G. 1992. Bovine milk protein properties and the manufacturing quality of milk. *Livestock Production Science* **75**-93

Danfær, A.; Tetens, V.; Agergaard, N. 1995. Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. *Comparative Biochemistry and Physiology. B. Comparative Biochemistry* **111B**:201-210

Danilson, D.A.; Webb, K.E.Jr.; Herbein, J.H. 1987a. Transport and hindlimb exchange of peptide and serum protein amino acids in calves fed soy- or urea-based purified diets. *Journal of Animal Science* **64**:1852-1857

Danilson, D.A.; Webb, K.E.Jr.; Herbein, J.H. 1987b. Transport and hindlimb exchange of plasma and blood-cell amino acids in calves fed soy- or urea-based purified diets. *Journal of Animal Science* **64**:1842-1851

Davis, S.R.; Bickerstaffe, R.; Hart, D.S. 1978. Amino acid uptake by the mammary gland of the lactating ewe. *Australian Journal of Biological Sciences* **31**:123-132

Davis, S.R.; Collier, R.J. 1985. Mammary blood flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68**:1041-1058

DePeters, E.J.; Cant, J.P. 1992. Nutritional factors influencing the nitrogen composition of bovine milk: a review. *Journal of Dairy Science* **75**:2043-2070

Derrig, R.G.; Clark, J.H.; Davis, C.L. 1974. Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. *Journal of Nutrition* **104**:151-159

Dibley, G. 1997. Harnessing the nutritional power of milk. *Proceedings of the Nutrition Society of New Zealand* **22**:150-159

Donkin, S.S.; Varga, G.A.; Sweeney, T.F.; Muller, L.D. 1989. Rumen-protected methionine and lysine: Effects on animal performance, milk protein yield, and physiological measures. *Journal of Dairy Science* **72**:1484-1491

Drackley, J.K.; Schingoethe, D.J. 1986. Extruded blend of soybean meal and sunflower seeds for dairy cattle in early lactation. *Journal of Dairy Science* **69**:371-384

Eigel, W.N.; Butler, J.E.; Ernstrom, C.A.; Farrell, H.M.Jr.; Harwalkar, V.R.; Jenness, R.; Whitney, R.M. 1984. Nomenclature of proteins of cow's milk: Fifth revision. *Journal of Dairy Science* **67**:1599-1631

Erfle J.D.; Fisher, L.J. 1977. The effects of intravenous infusion of lysine, lysine plus methionine or carnitine on plasma amino acids and milk production of dairy cows. *Canadian Journal of Animal Science* **57**:101-109

Falconer, I.R. 1980. Aspects of biochemistry, physiology and endocrinology of lactation. *Australian Journal of Biological Sciences* **33**:71-84

Feagan, J.T. 1979. Factors affecting protein composition of milk and their significance to dairy processing. *Australian Journal of Dairy Technology* **34**:77-81

Fisher, L.J. 1972. Response of lactating cows to the intravenous infusion of amino acids. *Canadian Journal of Animal Science* **52**:377-384

FitzGerald, R. J. 1997 Exploitation of casein variants. In Milk composition, Production and Biotechnology (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 153-171. Cambridge, UK.: CAB International.



Foldager, J.; Huber, J.T.; Bergen, W.G. 1980. Factors affecting amino acids in blood of dairy cows. *Journal of Dairy Science* **63**:396-404

Fox, P. F.; Flynn, A. 1992 Biological properties of milk proteins. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 255-284. Essex, U.K.: Elsevier Science Publishers.

Fraser, D.L.; Ørskov, E.R.; Whitelaw, F.G.; Franklin, M.F. 1991. Limiting amino acids in dairy cows given casein as the sole source of protein. *Livestock Production Science* **28**:235-252

Fredeen, A.H. 1996. Considerations in the nutritional modification of milk composition. *Animal Feed Science and Technology* **59**:185-197

Gibson, J.P. 1989. Altering milk composition through genetic selection. *Journal of Dairy Science* **72**:2815-2825

Giese, J. 1994. Proteins as ingredients: types, functions, applications. *Food Technology* **48**:50-60

Gilles, J.; Lawrence, R.C. 1985. The yield of cheese. *New Zealand Journal of Dairy Science and Technology* **20**:205-214

Grandison A S; Anderson, M.; Ford, G.D. 1985. Interrelationships between the diet fed to cows, composition and properties of milk and composition and quality of Cheshire cheese from farmhouse manufacturers. *Journal of Dairy Research* **52**:587-593

Guinard J.; Rulquin, H. 1994. Effect of graded levels of duodenal infusions of casein on mammary uptake in lactating cows. 2. Individual amino acids. *Journal of Dairy Science* **77**:3304-3315

Gurr, M.I. 1992. Milk products: contribution to nutrition and health. *Journal of the Society of Dairy Technology* **45**:61-67

Hadsell, D.L.; Sommerfeldt, J.L. 1988. Chickpeas as a protein and energy supplement for high producing dairy cows. *Journal of Dairy Science* **71**:762-772

Hambling, S. G.; McAlpine, A. S.; Sawyer, L. 1992  $\beta$ -lactoglobulin. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 141-190. Essex, U.K.: Elsevier Science Publishers.

Hanigan, M.D.; Calvert, C.C.; DePeters, E.J.; Reis, B.L.; Baldwin, R.L. 1991. Whole blood and plasma amino acid uptakes by lactating bovine mammary glands. *Journal of Dairy Science* **74** :2484-2490

Hayssen, V. 1993. Empirical and theoretical constraints on the evolution of lactation. *Journal of Dairy Science* **76**:3213-3233

Heitmann, R.N.; Bergman, E.N. 1980. Transport of amino acids in whole blood and plasma of sheep. *American Journal of Physiology* **239**:E242-E247

Hill J.P. 1993. The relationship between  $\beta$ -lactoglobulin phenotypes and milk composition in New Zealand dairy cattle. *Journal of Dairy Science* **76**:281-286

Hill, J P; Trescher, W C; Boland, M J; Creamer, L K; Anema, S G; Manderson, G; Otter, D E; Paterson, G R; Lowe, R; Burr, R G; Motion, R L; Winkelman, A; Wickham, B. 1997 The polymorphism of the milk protein  $\beta$ -lactoglobulin. A review. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 173-202. Cambridge, UK.: CAB International.

Illg, D.J.; Sommerfeldt, J.L.; Schingoethe, D.J. 1987. Lactational and systemic responses to the supplementation of protected methionine in soybean meal diets. *Journal of Dairy Science* **70**:620-629

Jenness, R. 1974 The composition of milk . In *Lactation. A comprehensive treatise* (Ed. Larson, B. L. and Smith, V. R.) 3-107. New York, USA: Academic Press.

Jenness, R. 1986. Lactational performance of various mammalian species. *Journal of Dairy Science* **69**:869-885

Jones-Endsley, J.M.; Cecava, M.J.; Johnson, T.R. 1997. Effects of dietary supplementation on nutrient digestion and the milk yield of intensively grazed lactating dairy cows. *Journal of Dairy Science* **80**:3283-3292

Kaufmann, W. 1980. Protein degradation and synthesis within the reticulo-rumen in relation to milk protein synthesis. *Bulletin International Dairy Federation* **125**:152-158

Kaufmann, W.; Lüpping, W. 1982. Protected proteins and protected amino acids for ruminants. In Protein contribution of feedstuffs for ruminants: application to feed formulation (Ed. Miller, E. L., Pike, I. H., and van Es, A. J. H.) 36-75. London: Butterworth Scientific.

Kellaway, R.C.; Ranawana, S.S.E.; Buchanan, J.H.; Smart, L.D. 1974. The effect of nitrogen source in the diet on milk production and amino-acid uptake by the udder. *Journal of Dairy Research* **41**:305-315

Kitchen B.J. 1981. Review of the progress of Dairy Science: Bovine mastitis: milk compositional changes and related diagnostic tests. *Journal of Dairy Research* **48**:167-188

Kosikowski, F.V. 1981. Dairy foods of the world - Evolution, expansion, and innovation. *Journal of Dairy Science* **64**:996-1007

Kronfeld, D.S.; Raggi, F.; Ramberg, C.F.Jr. 1968. Mammary blood flow and ketone body metabolism in normal, fasted and ketotic cows. *American Journal of Physiology* **215**:218-227

Lanham, J.K.; Coppock, C.E.; Brooks, K.N.; Wilks, D.L.; Horner, J.L. 1992. Effects of whole cottonseed or niacin or both on casein synthesis by lactating Holstein cows. *Journal of Dairy Science* **75**:184-192

Lescoat, P.; Sauvant, D.; Danfær, A. 1996. Quantitative aspects of blood and amino acid flows in cattle. *Reproduction, Nutrition, Development* **36**:137-174

Linzell, J. L. 1974 Mammary blood flow and methods of identifying and measuring precursors of milk . In Lactation. A comprehensive treatise (Ed. Larson, B. L. and Smith, V. R.) 143-225. New York, USA: Academic Press.

Livestock Improvement Corporation. 1999. Dairy Statistics 1998-1999. *Dairy Statistics 1998-1999*

Lobley, G.E.; Connell, A.; Revell, D.K.; Bequette, B.J.; Brown, D.S.; Calder, A.G. 1996. Splanchnic-bed transfer of amino acids in sheep blood and plasma, as monitored through use of multiple U-13C-labelled amino acid mixture. *British Journal of Nutrition* **75**:217-235

Lobley, G.E.; Milne, V.; Lovie, J.M.; Reeds, P.J.; Pennie, K. 1980. Whole body and tissue protein synthesis in cattle. *British Journal of Nutrition* **43**:491-502

Maas, J.A.; France, J.; Dijkstra, J.; Bannink, A.; McBride, B.W. 1998. Application of a mechanistic model to study competitive inhibition of amino acid uptake by the lactating bovine mammary gland. *Journal of Dairy Science* **81**:1724-1734

Maas, J.A.; France, J.; McBride, B.W. 1997. Model of milk protein synthesis. A mechanistic model of milk protein synthesis in the lactating bovine mammary gland. *Journal of Theoretical Biology* **187**:363-378

Maas, J.A.; France, J.; McBride, B.W. 1998. Towards a mechanistic model of amino acid uptake and metabolism by the mammary gland of the lactating dairy cow: A literature review. *Journal of Animal and Feed Sciences* **7**:105-129

Mabjeesh, S.J.; Arieli, A.; Bruckental, I.; Zamwell, S.; Tagari, H. 1996. Effect of type of protein supplementation on duodenal amino acid flow and absorption in lactating dairy cows. *Journal of Dairy Science* **79**:1792-1801

Mackenzie, D. D. S. 1997. Milk composition as an indicator of mammary gland metabolism. *Proceedings of the Nutrition Society of New Zealand* **22**:126-136

MacRae, J.C.; Bruce, L.A.; Brown, D.S.; Calder, A.G. 1997. Amino acid use by the gastrointestinal tract of sheep given lucerne forage. *American Journal of Physiology* **273**:G1200-G1207

Madsen, J. 1987 Present situation of the modern protein systems: Nordic countries. In Feed evaluation and protein requirement systems for ruminants (Ed. Jarrige, R. and Alderman, G.) 29-39. Luxembourg: Commission of the European Communities.

Mann, E.J. 1996a. Dairy ingredients in foods - Part 1. *Dairy Industries International* **61**:14-15

Mann, E.J. 1996b. Dairy ingredients in foods - Part 2. *Dairy Industries International* **61**:10-11

Mayer, H.K.; Ortner, M.; Tschager, E.; Ginzinger, W. 1997. Composite milk protein phenotypes in relation to composition and cheesemaking properties of milk. *International Dairy Journal* **7**:305-310

McCormick, M.E.; Webb, K.E.J. 1982. Plasma free, erythrocyte free and plasma peptide amino acid exchange of calves in steady state and fasting metabolism. *Journal of Nutrition* **112**:276-282

McDowell, G.H.; Gooden, J.M.; Leenanuruksa, D.; Jois, M.; English, A.W. 1987. Effect of exogenous growth hormone on milk production and nutrient uptake by muscle and mammary tissues of dairy cows in mid-lactation. *Australian Journal of Biological Sciences* **40**:295-306

McLean, D.M.; Bruce Graham, E.R.; Ponzoni, R.W.; McKenzie, H.A. 1984. Effects of milk protein genetic variants on milk yield and composition. *Journal of Dairy Research* **51**:531-546

Meijer, G.A.L.; de Jonge, L.H.; Breuer, M.; van der Meulen, J.; van Vuuren, A.M. 1997. Peptide bound amino acids in arterial and mammary venous plasma of high yielding dairy cows. *Journal of Dairy Science* **80**, Suppl. 1:154

Meijer, G.A.L.; Van der Meulen, J.; Bakker, J.G.M.; Van der Koelen, C.J.; Van Vuuren, A.M. 1995. Free amino acids in plasma and muscle of high yielding dairy cows in early lactation. *Journal of Dairy Science* **78**:1131-1141

Meijer, G.A.L.; van der Meulen, J.; van Vuuren, A.M. 1993. Glutamine is a potentially limiting amino acid for milk production in dairy cows: a hypothesis. *Metabolism* **42**:358-364

Meisel, H. 1997. Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livestock Production Science* **50**:125-138

Mepham, T.B. 1982. Amino acid utilization by lactating mammary gland. *Journal of Dairy Science* **65**:287-298

Mepham, T. B. 1987. Physiology of lactation. Milton Keynes, UK: Open University Press.

Mepham, T. B.; Gaye, P.; Martin, P.; Mercier, J. C. 1992 Biosynthesis of milk protein. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 491-543. Essex, U.K.: Elsevier Science Publishers.

Mepham, T. B.; Gaye, P.; Mercier, J. C. 1982 Biosynthesis of milk proteins. In *Developments in Dairy Chemistry. 1.* (Ed. Fox P.F.) 115-156. London, U.K.: Applied Science Publishers.

Mercier, J. C.; Gaye, P. 1983 Milk protein synthesis. In *Biochemistry of lactation* (Ed. Mepham, T. B.) Amsterdam, The Netherlands: Elsevier.

Metcalf, J.A.; Wray-Cahen, D.; Chettle, E.E.; Sutton, J.D.; Beaver, D.E.; Crompton, L.A.; MacRae, J.C.; Bequette, B.J.; Backwell, F.R.C. 1996. The effect of dietary crude protein as protected soybean meal on mammary metabolism in the lactating dairy cow. *Journal of Dairy Science* **79**:603-611

Muir, D.D.; Banks, J.M.; Powell, A.K.; Sweetsur, A.W.M. 1983. Milk composition-manufacturing properties. *Proceedings of the Nutrition Society* 385-397

Munneke, R.L.; Schingoethe, D.J.; Casper, D.P. 1991. Lactational evaluation of ruminally protected methionine in diets containing extruded soybeans and urea. *Journal of Dairy Science* 74:227-233

Murphy, J.J.; O' Mara, F. 1993. Nutritional manipulation of milk protein concentration and its impact on the dairy industry. *Livestock Production Science* 35:117-134

Ng-Kwai-Hang, K. F.; Grosclaude, F. 1992 Genetic polymorphism of milk proteins. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 405-455. Essex, U.K.: Elsevier Science Publishers.

O'Connor, D. L.; Masor, M. L.; Paule, C.; Benson, J. 1997 Amino acid composition of cow's milk and human requirements. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 203-213. Cambridge, UK.: CAB International.

Oddy, V.H.; Lindsay, D.B.; Fleet, I.R. 1988. Protein synthesis and degradation in the mammary gland of lactating goats. *Journal of Dairy Research*. 55:143-154

Odle, J.; Zijlstra, R.T.; Donovan, S.M. 1996. Intestinal effects of milkborne growth factors in neonates of agricultural importance. *Journal of Animal Science* 74:2509-2522

Oldham, J. D. 1994 Amino acid nutrition of the dairy cow. In *Amino acids in farm animal nutrition* (Ed. D'Mello, J. P. F.) 351-375. Wallingford, Oxon, U.K.: CAB International.

Oldham, J. D. 1996 Protein requirement systems for ruminants. In *Progress in dairy science* (Ed. Phillips, C. J. C.) 3-27. Wallingford, Oxon, U.K.: CAB International.

Overton, T.R.; LaCount, D.W.; Cicela, T.M.; Clark, J.H. 1996. Evaluation of a ruminally protected methionine product for lactating dairy cows. *Journal of Dairy Science* **79**:631-638

Owens, F. N. 1987 The US-NRC approach to ruminant nitrogen usage. In Feed evaluation and protein requirement systems for ruminants (Ed. Jarrige, R. and Alderman, G.) 55-68. Luxembourg: Commission of the European Communities.

Papas, A.M.; Sniffen, C.J.; Muscato, T.V. 1984. Effectiveness of rumen-protected methionine for delivering methionine postruminally in dairy cows. *Journal of Dairy Science* **67**:545-552

Papas, A.M.; Vicini, J.L.; Clark, J.H.; Peirce-Sandner, S.B. 1984. Effect of rumen-protected methionine on plasma free amino acids and production by dairy cows. *Journal of Nutrition* **114**:2221-2227

Pearlman, W.H. 1991. Hormones and tissue growth factors in milk: evolutionary implications. *Endocrine Regulations* **25**:4-9

Peeters, G.; Houvenaghel, A.; Roets, E.; Massart-Leën, A.M.; Verbeke, R.; Dhondt, G.; Verschooten, F. 1979. Electromagnetic blood flow recording and balance of nutrients in the udder of lactating cows. *Journal of Animal Science* **48**:1143-1153

Petersen, M.K.; Clanton, D.C.; Britton, R. 1985. Influence of protein degradability in range supplements on abomasal nitrogen flow, nitrogen balance and nutrient digestibility. *Journal of Animal Science* **60**:1324-1329

Pisulewski, P.M.; Rulquin, H.; Peyraud, J.L.; Verite, R. 1996. Lactational and systemic responses of dairy cows to postruminal infusions of increasing amounts of methionine. *Journal of Dairy Science* **79**:1781-1791

Polan, C.E.; Cummins, K.A.; Sniffen, C.J.; Muscato, T.V.; Vicini, J.L.; Crooker, B.A.; Clark, J.H.; Johnson, D.G.; Otterby, D.E.; Guillaume, B.; Muller, L.D.; Varga, G.A.; Murray, R.A.; Peirce-Sandner, S.B. 1991. Responses of dairy cows to



supplemental rumen-protected forms of methionine and lysine. *Journal of Dairy Science* **74**:2997-3013

Pösö, A.R.; Lindberg, L.A. 1994. Plasma protein synthesis and serum amino acids in dry and lactating dairy cows. *Journal of Veterinary Medicine. Series A* **41**:72-75

Proenza, A.M.; Palou, A.; Roca, P. 1994. Amino acid distribution in human blood. A significant pool of amino acids is adsorbed onto blood cell membranes. *Biochemistry and Molecular Biology International* **34**:971-982

Prosser, C.G.; Davis, S.R.; Farr, V.C.; Lacasse, P. 1996. Regulation of blood flow in the mammary microvasculature. *Journal of Dairy Science* **79**:1184-1197

Rajczyk, Z. K.; Lean, I. J.; McKean, K.; Gooden, J. M. 1994. The effect of dietary protein on milk composition. *Proceedings of the Nutrition Society of Australia* **18**:178

Rasmussen, L.K.; Højrup, P.; Petersen, T.E. 1994. Disulphide arrangement in bovine caseins: localization of intrachain disulphide bridges in monomers of kappa- and alpha-s2-casein from bovine milk. *Journal of Dairy Research* **61**:485-493

Razooki Hasan, H.; White, D.A.; Mayer, R.J. 1982. Extensive destruction of newly synthesised casein in mammary explants in organ culture. *Biochemical Journal* **202**:133-138

Riis, P.M. 1988. Nitrogen balance, amino acid flux rates and rates of whole body protein synthesis in lactating and in pregnant goats at different energy intakes. *Journal of Animal Physiology and Animal Nutrition* **60**:86-5

Robitaille, G. 1995. Influence of  $\kappa$ -casein and  $\beta$ -lactoglobulin genetic variants on the heat stability of milk. *Journal of Dairy Research* **62** :593-600

Rogers, J.A.; Krishnamoorthy, U.; Sniffen, C.J. 1987. Plasma amino acids and milk protein production by cows fed rumen-protected methionine and lysine. *Journal of Dairy Science* **70**:789-798

Rulquin, H. 1981. Etude méthodologique sur la mesure des différences artérioveineuses mammaires chez la vache laitière. *Reproduction, Nutrition, Development* **21**:31-46

Rulquin, H. 1987. Détermination de certains acides aminés limitants chez la vache laitière par la méthode des administrations post-ruminales. *Reproduction, Nutrition, Development* **27 (1B)**:299-300

Rulquin, H.; Delaby, L. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. *Journal of Dairy Science* **80**:2513-2522

Rulquin, H.; Verite, R. 1996 Amino acid nutrition of dairy cows: productive effects and animal requirements. In *Recent Developments in Ruminant Nutrition 3* (Ed. Garnsworthy P.C. and Cole D.J.A.) 71-93. Nottingham, U.K.: Nottingham University Press.

Rulquin, H.; Verite, R.; Guinard, G.; Pisulewski, P. M. 1995 Dairy cows' requirements for amino acids. In *Animal Science Research and Development: Moving Towards a New Century* (Ed. Ivan, M.) 143-160. Ottawa, Canada: Centre for Food and Animal Research, Agriculture and Agri-Food Canada.

Schanbacher, F.L.; Talhouk, R.S.; Murray, F.A. 1997. Biology and origin of bioactive peptides in milk. *Livestock Production Science* **50**:105-123

Schingoethe, D.J. 1996a. Dietary influence on protein level in milk and milk yield in dairy cows. *Animal Feed Science and Technology* **60**:181-190

Schingoethe, D.J. 1996b. Balancing the amino acid needs of the dairy cow. *Animal Feed Science and Technology* **60**:153-160

Schwab, C.G.; Satter, L.D.; Clay, A.B. 1976. Response of lactating dairy cows to abomasal infusion of amino acids. *Journal of Dairy Science* **59**:1254-1270

Speckmann, E.W.; Brink, M.F.; McBean, L.D. 1981. Dairy foods in nutrition and health. *Journal of Dairy Science* **64**:1008-1016

Spires, H.R.; Clark, J.H.; Derrig, R.G.; Davis, C.L. 1975. Milk production and nitrogen utilisation in response to postruminal infusion of sodium caseinate in lactating cows. *Journal of Nutrition* **105**:1111-1121

Spörndly, E. 1989. Effects of diet on milk composition and yield of dairy cows with special emphasis on milk protein content. *Swedish Journal of Agricultural Research* **19**:99-106

Stem, M.D.; Santos, K.A.; Satter, L.D. 1985. Protein degradation in rumen and amino acid absorption in small intestine of lactating dairy cattle fed heat-treated whole soybeans. *Journal of Dairy Science* **68**:45-56

Storm, E.; Ørskov, E.R. 1984. The nutritive value of rumen micro-organisms in ruminants. 4. The limiting amino acids of microbial protein in growing sheep determined by a new approach. *British Journal of Nutrition* **52**:613-620

Subnel, A.P.J.; Meijer, R.G.M.; van Straalen, W.M.; Tamminga, S. 1994. Efficiency of milk protein production in the DVE protein evaluation system. *Livestock Production Science* **40**:215-224

Swaisgood, H. E. 1992 Chemistry of caseins. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 63-110. Essex, U.K.: Elsevier Science Publishers.

Swaisgood, H.E. 1993. Review and update of casein chemistry. *Journal of Dairy Science* **76**:3054-3061

Tagari, H.; Bergman, E.N. 1978. Intestinal disappearance and portal blood appearance of amino acid in sheep. *Journal of Nutrition* **108**:790-803

Tagari, H.; Webb Jr., K.; Theurer, B.; Huber, T.; Cuneo, P.; Deyoung, D.; Simas, J.; Delgado, A.; Sadik, M.; Alio, A.; Lozano, O. 1997. Portal-drained visceral (PDF) flux and mammary uptake of free (FAA) and peptide bound (PBAA) essential amino acids by lactating dairy cows fed diets containing dry rolled (DRS) or steam flaked (SFS) sorghum grain. *Journal of Dairy Science* **80**, **Suppl. 1**:213

Thomas, P.C. 1980. Influence of nutrition in the yield and content of protein in milk: Dietary protein and energy supply. *International Dairy Federation Bulletin* **125**:142-151

Thomas, P.C. 1983. Milk protein. *Proceedings of the Nutrition Society* **42**:407-418

Thomas P.C. and Chamberlain, D. G. 1984 Manipulation of milk composition to meet market needs. In *Recent Advances in Animal Nutrition* (Ed. Haresign, W. and Cole, D. J. A.) 219-243. Butterworths.

Tunncliffe, G. 1994. Amino acid transport by human erythrocyte membranes. *Comparative Biochemistry and Physiology. A. Comparative Physiology* **108A**:471-478

Ulyatt, M.J.; MacRae, J.C.; Clarke, R.T.J.; Pearce, P.D. 1975. Quantitative digestion of fresh herbage by sheep. IV. Protein synthesis in the stomach. *Journal of Agricultural Science, Cambridge* **84**:453-458

Valeur, J. 1997 Milk protein production and market prospects. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 93-103. Cambridge, UK.: CAB International.

Van Vuuren, A.M.; Krol-Kramer, F.; Van der Lee, R.A.; Corbijn, H. 1992. Protein digestion and intestinal amino acids in dairy cows fed fresh *Lolium perenne* with different nitrogen contents. *Journal of Dairy Science* **75**:2215-2225

Van Vuuren, A.M.; van der Koelen, C.J.; Vroons-de Bruin, J. 1993. Ryegrass versus corn starch or beet pulp fiber diet effects on digestion and intestinal amino acids in dairy cows. *Journal of Dairy Science* **76**:2692-2700

Verite, R. 1987 Present situation of protein evaluation for ruminants in France: the PDI system. In Feed evaluation and protein requirement systems for ruminants (Ed. Jarrige, R. and Alderman, G.) 11-20. Luxembourg: Commission of the European Communities.

Viatte, G. 1997 Major issues facing the world dairy economy. In Milk composition, Production and Biotechnology (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 1-11. Cambridge, UK.: CAB International.

Watson, D.L. 1980. Immunological functions of the mammary gland and its secretion-comparative review. *Australian Journal of Biological Science* **33**:403-422

Weaver, L.T. 1997. Significance of bioactive substances in milk to the human neonate. *Livestock Production Science* **50**:139-146

Webb, K.E.J. 1990. Intestinal absorption of protein hydrolysis products: A review. *Journal of Animal Science* **68**:3011-3022

Webb, K.E.Jr.; Matthews, J.C.; DiRienzo, D.B. 1992. Peptide absorption: a review of current concepts and future perspectives. *Journal of Animal Science* **70**:3248-3257

Webster, A. J. F. 1987 Metabolisable protein - the U.K. approach. In Feed evaluation and protein requirement systems for ruminants (Ed. Jarrige, R. and Alderman, G.) 47-52. Luxembourg: Commission of the European Communities.

Wilde, C.J.; Addey, C.V.P.; Knight, C.H. 1989. Regulation of intracellular casein degradation by secreted milk proteins. *Biochimica et Biophysica Acta* **992**:315-319

Wilson, G. F.; Moller, S.; Parker, W. J.; Hodgson, J. 1995. Seasonal differences in pasture composition and nutritional implications. *1995 Dairyfarming Annual* 47:46-56

Wong, D.W.S.; Camirand, W.M.; Pavlath, A.E. 1996. Structures and functionalities of milk proteins. *Critical Reviews in Food Science and Nutrition* 36:807-844

Wu, Z.; Polan, C.E.; Fisher, R.J. 1997. Adequacy of amino acids in diets fed to lactating dairy cows. *Journal of Dairy Science* 80:1713-1721

Xu, R.J. 1996. Development of the newborn GI tract and its relation to colostrum/milk intake: a review. *Reproduction, Fertility, Development* 8:35-48

Yang, C.M.J.; Schingoethe, D.J.; Casper, D.P. 1986. Protected methionine and heat-treated soybean meal for high producing dairy cows. *Journal of Dairy Science* 69:2348-2357

Zinn, S.A. 1997. Bioactive components in milk: introduction. *Livestock Production Science* 50:101-103

**CHAPTER 3.**

**THE EFFECTS OF METHIONINE SUPPLEMENTATION  
UPON MILK COMPOSITION AND PRODUCTION OF  
FORAGE-FED DAIRY COWS**

This chapter has been published in the *Canadian Journal of Animal Science* **79**: 235-241, (1999)

### 3.1. ABSTRACT

Two experiments (mid and late lactation) were conducted to test whether methionine (Met) limited milk production in Friesian cows fed sole diets of fresh perennial ryegrass-white clover pasture. In mid lactation (Experiment 1), twenty-six cows were assigned to one of three treatments: ruminally-protected oral DL-Met (15 g d<sup>-1</sup>), continuous intrajugular infusion of L-Met (15 g d<sup>-1</sup>) and control. Twelve animals from Experiment 1 were used during late lactation (Experiment 2) and distributed in two experimental groups: ruminally-protected oral DL-Met (15 g d<sup>-1</sup>) and control. Dry matter, metabolizable energy, crude protein and Met intakes, milk yield and composition and blood Met, cysteine and urea concentrations were measured. Oral and intravenous Met supplementation increased blood Met concentration by 50-90% compared to controls. Met supplementation did not alter the concentration of milk fat, protein or lactose in either experiment. Met supplementation had no significant effects on yields of fat, lactose, casein, whey proteins or non-protein nitrogen during mid lactation. In late lactation, Met supplementation did not affect milk protein composition or yield of milk components, with exception of a decrease ( $P < 0.05$ ) in the yield of  $\beta$ -casein. Intravenous Met supplementation increased ( $P < 0.05$ ) the efficiency of conversion of pasture N to milk protein in mid lactation.

**Key words:** dairy cows, methionine, ruminally protected methionine, milk protein, casein, fresh pasture diets

**Abbreviations:** AA, amino acid; AgR, AgResearch; CP, crude protein; DCRU, Dairy Cattle Research Unit; DM, dry matter; DMI, dry matter intake; DIM, days in milk; EAA, essential amino acid(s); Lys, lysine; ME, metabolizable energy; Met, methionine; NPN, non-protein nitrogen; RPM, ruminally protected DL-methionine



### 3.2. INTRODUCTION

Protein is one of the most valuable milk components, both in an economic and nutritional sense. The largest fraction of the milk proteins, casein, determines the yield and quality of numerous products, such as cheese and milk powder. Besides its economic and industrial value, the high biological value of casein makes it one of the most important protein sources for humans.

In the pasture-fed dairy cow, more than 50% of the protein ingested is degraded in the rumen into ammonia (Beever *et al.*, 1986) and this extensive protein degradation may limit the amount of protein passing to the small intestine (Black, 1990). It has been postulated that identifying and increasing post-ruminal supply of the most limiting amino acids would be an effective way to improve milk protein yield and quality (Erfle and Fisher, 1977).

In dairy cows, methionine (Met) has consistently been identified as one of the most limiting amino acids (Derrig *et al.*, 1974; Spires *et al.*, 1975; Clark *et al.*, 1977) as assessed from measurements of the amino acid uptake by the mammary gland. Several studies have been conducted to determine the effects of supplementation with Met upon milk protein yield and composition, with varied responses. In some cases an increase in milk protein and protein yield has been observed (Fisher, 1972; Koch *et al.*, 1996; Robert *et al.*, 1996), whilst in others there has been no response in milk protein yield (Papas *et al.*, 1984; Overton *et al.*, 1996). However, most of the studies have been undertaken with mixed rations, using different types of preserved forage and different ratios of forage to concentrate. The lack of experimental data on amino acid utilization by the dairy cow fed with fresh forage alone is noticeable.

In animals fed fresh ryegrass, the flow of Met has been reported to be less than 5 % of the essential amino acids flowing to the duodenum (Ulyatt *et al.*, 1975; Van Vuuren *et al.*, 1992; 1993). According to Schwab (1996), Met must contribute 5.3 to 5.6 % of the duodenal essential amino acids for optimal milk protein production. Therefore,

it would be expected that an extra supply of post-ruminal Met might have a positive effect on milk protein production of dairy cows fed fresh forages.

The responses to extra amino acid supply have been observed mostly in the casein fraction of milk protein (Schwab, 1996). In pasture-fed animals, the seasonal changes in pasture quality are associated with reductions in the proportion of casein in the milk of dairy cows in mid to late lactation (Auldist *et al.*, 1997). There is interest in developing strategies to increase the yield of casein in cows in advanced lactation.

The objective of this study was to determine whether Met supplementation affected the concentration and yield of individual milk proteins in dairy cows fed a diet of fresh perennial ryegrass-white clover pasture in mid and late lactation.

### **3.3. MATERIALS AND METHODS**

#### **3.3.1. Experimental Design**

Two experiments were conducted, each for 3 weeks, during mid lactation (Experiment 1) and late lactation (Experiment 2). The cows were fed diets of fresh perennial ryegrass (*Lolium perenne*; 80%) / white clover (*Trifolium repens*; 20%) pasture and the effects of Met supplementation on milk yield and composition, yield and concentration of milk proteins, feed intake and the efficiency of converting dietary protein to milk protein were studied.

#### **3.3.2. Animals and Diets**

Multiparous Friesian cows of similar body weight, parity and milk yield in the previous lactation were maintained and fed indoors. The cows were maintained in metabolism stalls and tethered using a head harness. The diet in both experiments consisted of fresh chopped pasture cut twice daily and was offered as two meals (0900 and 1500 h) at approximately 18 kg DM per cow per day. Water was available *ad libitum*. Animals were milked twice daily (0700 and 1500 h) and exercise was allowed

daily for two 2-h periods (after the morning and before the afternoon milkings) throughout both experiments.

### **3.3.2.1. Experiment 1**

Twenty six cows in mid lactation were maintained at two sites, 15 animals at the Massey University Dairy Cattle Research Unit (DCRU) and 11 at AgResearch Grasslands (AgR), both sites located in Palmerston North, New Zealand. The average body weight of the cows was 482 (SD 31.8) and 471 (SD 57.4) kg and the average days in milk (DIM) was 75 (SD 17.0) and 82 (SD 12.6) at DCRU and AgR, respectively. One jugular catheter was fitted to each animal on the first day of the experiment. Catheters were kept patent by flushing daily with sterile heparinised saline (200 IU mL<sup>-1</sup>).

### **3.3.2.2. Experiment 2**

Twelve cows were randomly selected from the initial group of twenty-six used in Experiment 1. Animals were maintained indoors in the cattle facilities at AgR. The average body weight of the animals at the beginning of the experiment was 460 (SD 29.0) kg and the average DIM was 200 (SD 10.9).

### **3.3.3. Treatments**

In both experiments, no treatments were applied during the first week. Data collected during this week were subsequently used as co-variates. In both experiments the treatments were applied from day 8-21.

#### **3.3.3.1. Experiment 1**

Three experimental groups were formed with cows of similar body weight and DIM. Treatments were (1) continuous intra-jugular infusion of 15 g d<sup>-1</sup> of L-Met (Rexim, 92400, Courbevoie, France) dissolved in sterile saline at a rate of 22.5 mL h<sup>-1</sup> (MET-I.V.); (2) oral administration of 15 g d<sup>-1</sup> of ruminally protected methionine in tallow (RPM; Lactet, 300 g D-L Met kg<sup>-1</sup>, Nippon Soda Co. Ltd., Tokyo 100, Japan)

and (3) control. For the MET-I.V. group, the daily dose of Met was infused over 22 hours, to allow for the exercise periods. The RPM was divided into two equal doses which were given at 0800 and 1600 h using a custom made drenching gun. Sterile saline was infused into the jugular vein of RPM and control cows at the same rate as the MET-I.V. group. All liquids were infused using a peristaltic pump (Desaga GmbH, D-6900 Heidelberg, Germany). Methionine-free capsules containing the equivalent amount of tallow in the ruminally-protected product were given to MET-I.V. and control animals at the same time that RPM cows were dosed.

Four animals showed signs of clinical mastitis during the third week of the experiment and all the values collected from these animals were not included in the analyses.

### **3.3.3.2. Experiment 2**

Two groups of six cows each were formed with cows of similar body weight and DIM. Treatments were (1) 15 g d<sup>-1</sup> of RPM and (2) control cows which received the Met-free capsules. The treatments were administered following the same protocol as described for Experiment 1.

### **3.3.4. Measurements**

Similar routines were used in both experiments. Feed offered was recorded twice daily at 0900 and 1500 h. Any feed refused, either as floor or bin refusals, was recorded daily to calculate the average feed intake. Feed was sampled and analysed twice daily for DM; the amount of fresh forage offered was calculated using the DM value from the previous day. Pooled samples were prepared for each week and analysed for the concentrations of crude protein (CP), metabolizable energy (ME) and Met.

Milk yields were recorded daily. Milk samples were taken once weekly on days 7, 14, and 21 of the experimental period. Morning and afternoon samples were collected, pooled according to yield and subsampled for protein, fat and lactose. Additionally, total nitrogen, non-casein nitrogen and non-protein nitrogen (NPN)

analyses were conducted on weekly samples during Experiment 1. In Experiment 2 nitrogen fractions were not determined, but the concentration of individual proteins was measured using electrophoretic techniques.

Blood samples were collected at 0700 h from the coccygeal vein or artery on days 8, 15, and 21 after the cows were milked in the morning. Samples were collected into evacuated tubes containing K-EDTA (Vacutainer, Becton Dickinson, NJ 07417, USA) then chilled. A subsample (1 mL) was hemolysed with deionised water (1:1), mixed with 0.1 mL of sodium-phosphate buffer (200 mM, pH 8.0) containing DL-dithiothreitol (80 mM) and deproteinized by ultrafiltration (Centrisart, molecular mass cut-off 10000; Sartorius AG, Goettingen D-37070, Germany). The supernatant was collected and stored at  $-85^{\circ}\text{C}$  until analysed for free Met. A second subsample (10 mL) was centrifuged at 3270 g for 15 min at  $4^{\circ}\text{C}$  and plasma harvested. Plasma (1 mL) was mixed with 0.5 mL of a solution containing sodium dodecyl sulphate ( $7.5\text{ g L}^{-1}$ ) and di-sodium EDTA (9 mM); and 0.1 mL of sodium phosphate buffer (200 mM, pH 8.0) containing DL-dithiothreitol (80 mM). After standing for 15 min at room temperature, the sample was centrifuged at 11250 g following deproteinization with 0.5 mL trichloroacetic acid ( $300\text{ g L}^{-1}$ ) and the supernatant stored at  $-85^{\circ}\text{C}$  until analysed for free cysteine. A plasma subsample was also stored for urea analysis.

### 3.3.5. Analytical Methods

Composition of the composited weekly feed samples was analysed by near-infrared reflectance spectroscopy (NIRSystems Inc., Silver Spring, MD 20904, USA) for CP and ME concentrations.

Milk protein, fat and lactose concentrations were analysed using an infrared milk analyser (Milk-O-Scan 104 A/B, Foss A/S, DK-3400 Hillerød, Denmark). Casein, whey protein and NPN concentrations in milk were determined by the Kjeldahl method as described by Hill (1993). Methionine concentration in blood was measured after reversed-phase HPLC separation of the phenylisothiocyanate derivative (Bidlemeier *et al.*, 1984) using a Waters Pico-Tag<sup>®</sup> column (3.9 x 300 mm; Waters Corporation,

Milford, MA 01757, USA) and a Shimadzu LC-10/A HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA) with a 90-minute running time between injections. Plasma cysteine was determined by spectrophotometry after reaction with acid ninhydrin using a continuous flow analyser (AutoAnalyzer II, Technicon, Dublin, Ireland) as described by Gaitonde (1967). Plasma urea was determined photometrically in a COBAS-Fara II Analyser, using a commercial kit with urease and glutamate dehydrogenase (Unimate 5 Urea, F.Hoffmann-La Roche AG, CH-4002 Basel, Switzerland).

In Experiment 2, individual milk proteins ( $\alpha$ -,  $\beta$ - and  $\kappa$ -caseins;  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin) were determined by electrophoresis using the methods described by Singh and Creamer (1991) and Anema and Creamer (1993). Milk proteins were separated using sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) in a vertical mini gel system (BioRad Corp., Richmond, CA 94547, USA) after the samples were reduced with 20  $\mu$ L of 2-mercaptoethanol. Protein concentration in the gels was quantified by laser densitometry (ImageQuant v. 3.3, Molecular Dynamics, Sunnyvale, CA 94086, USA) following Amido Black staining. Purified standards (Sigma Chemical Company, St Louis, MO 63178, USA) were used in each gel to prepare a standard curve, from which protein concentration of unknown samples was calculated.

### 3.3.6. Statistical Methods

Measurements obtained in week 3 were analysed as the dependent variable, with the value for the same variable in week 1 used as a co-variate. It was assumed that any response to Met supplementation should be observed after 2 weeks of treatment, as reported by Metcalf *et al.* (1996). All the data for milk yields and intakes were reduced to weekly averages for each cow before the analysis. Intake was adjusted using body weight at the beginning of the experiment as a co-variate. Results were analysed by ANOVA using the GLM procedure of SAS (SAS Institute, 1996). Each experiment was analysed separately, and the statistical model included the co-variate, treatment, and for Experiment 1, location and the interaction treatment by location as sources of variation. Differences between treatments were assessed by a multiple comparison test (PDIFF

option of PROC GLM, SAS Institute, 1996) of the calculated least square means. Significant effects were declared when  $P < 0.05$ , whereas trends are indicated when  $0.05 < P < 0.10$ .

### **3.3.7. Animal Care**

All procedures used were reviewed and approved by the Animal Ethics Committees of both Massey University and AgResearch, using guidelines that are comparable to those of the Canadian Council on Animal Care.

## **3.4. RESULTS**

In Experiment 1 no significant interactions between location and Met supplementation were found. Therefore, all the results are presented (Tables 1 and 3) as main treatment effects.

### **3.4.1. Feed Intake**

In Experiment 1, Met supplementation tended ( $P < 0.09$ ) to decrease DMI, which led to lower intakes of protein and energy. The ME intake for the MET-I.V. group was lower ( $P < 0.05$ ) than for the control group (Table 3.1). The DMI was lower during late lactation (Table 3.2), and was similar for control and Met supplemented cows.

**Table 3.1. Effect of methionine supplementation on feed intakes and blood methionine, cysteine and urea concentrations in mid lactation (Experiment 1).**

	Treatment			SEM
	Control (n=7)	RPM (n=9)	MET-I.V. (n=6)	
Initial body weight (kg)	477	485	472	
Intakes:				
Dry matter (kg d <sup>-1</sup> )	16.7	16.2	15.8	0.23
Crude protein (kg d <sup>-1</sup> )	1.81	1.74	1.72	0.039
Metabolisable energy (MJ d <sup>-1</sup> )	183 <sup>a</sup>	175 <sup>ab</sup>	172 <sup>b</sup>	2.9*
Methionine (g d <sup>-1</sup> )				
Pasture	31.1	31.3	30.4	0.65
Total <sup>1</sup>	31.1	46.3	45.4	
Blood methionine (μM)	24.5 <sup>a</sup>	36.5 <sup>b</sup>	42.2 <sup>b</sup>	3.32**
Plasma cysteine (μM)	123.1	118.5	125.9	3.20
Plasma urea (mM)	3.0	3.5	3.4	0.21

<sup>1</sup> Methionine intake from pasture and supplement.

<sup>a,b</sup> Different letters in the same row indicate a significant difference between means.

\*  $P < 0.05$

\*\*  $P < 0.01$

**Table 3.2. Effect of methionine supplementation on feed intakes and blood methionine, cysteine and urea concentrations in late lactation (Experiment 2).**

	Treatment		SEM
	Control (n=6)	RPM (n=6)	
Initial body weight (kg)	470	450	
Intakes:			
Dry matter (kg d <sup>-1</sup> )	13.3	14.1	0.35
Crude protein (kg d <sup>-1</sup> )	2.3	2.4	0.06
Metabolisable energy (MJ d <sup>-1</sup> )	147	156	3.8
Methionine (g d <sup>-1</sup> )			
Pasture	49.6	52.7	1.31
Total <sup>1</sup>	49.6	67.7	
Blood methionine (μM)	21.3 <sup>a</sup>	41.0 <sup>b</sup>	3.79
Plasma cysteine (μM)	81.0	88.3	2.35
Plasma urea (mM)	6.8	7.3	0.20

<sup>1</sup> Methionine intake from pasture and supplement

<sup>a,b</sup> Different letters in the same row indicate a significant difference between means ( $P < 0.01$ )



The energy content of the pasture was similar between Experiments 1 and 2 with 10.8 and 11.0 MJ ME per kg DM, respectively. However, CP concentration in the DM of the pasture was lower in Experiment 1 than in Experiment 2 (10.9 vs. 17.4 % CP). This resulted in higher CP intakes during late lactation, in spite of the lower DMI.

### **3.4.2. Blood Metabolites**

Methionine supplementation increased ( $P < 0.01$ ) the concentration of Met in whole blood in both experiments, with the response being similar for both RPM and MET-I.V. groups. The increase in concentration was of similar magnitude between experiments. Plasma cysteine concentration was not affected by Met supplementation during mid lactation, but tended ( $P = 0.06$ ) to be higher in Met supplemented animals in late lactation. Plasma urea concentration was not affected by the treatments during Experiment 1, but in late lactation, Met supplementation tended ( $P = 0.07$ ) to increase plasma urea concentration.

### **3.4.3. Milk Production and Composition**

Milk yield in mid lactation tended ( $P = 0.08$ ) to be higher for the MET-I.V. group (Table 3.3), but in late lactation the milk yield was similar for control and RPM cows (Table 3.4).

**Table 3.3. Effect of methionine supplementation on milk production, milk composition and efficiency of CP utilisation for milk protein synthesis in mid lactation (Experiment 1).**

	Treatment			SEM
	Control (n=7)	RPM (n=9)	MET-I.V. (n=6)	
Milk yield (kg d <sup>-1</sup> )	17.1	17.2	18.5	0.44
Milk composition (g kg <sup>-1</sup> ):				
Fat	41.8	39.2	39.1	1.01
Lactose	48.5	48.7	48.9	0.31
Total protein	30.2	30.7	30.0	0.42
Casein	22.6	24.0	23.0	0.55
Whey protein	4.8	5.1	4.7	0.23
Non-protein N	0.2	0.2	0.2	0.01
Yield of components (g d <sup>-1</sup> ):				
Fat	716	667	703	23.6
Lactose	833	835	903	23.8
Total protein	511	528	551	14.1
Casein	389	411	419	14.0
Whey protein	83	87	84	4.5
Non-protein N	3	3	3	0.3
Efficiency of CP utilisation: g milk protein/ g CP intake	0.283 <sup>a</sup>	0.302 <sup>ab</sup>	0.318 <sup>b</sup>	0.0070

<sup>a,b</sup> Different letters in the same row indicate a significant difference between means ( $P < 0.05$ )

Lactose concentration was not affected by Met supplementation in either experiment. In late lactation, Met supplemented cows tended ( $P = 0.09$ ) to have higher fat concentrations (+9.5%) in their milk (Table 3.4).

During mid lactation, Met supplementation did not alter the protein concentration of the milk, but tended ( $P = 0.07$ ) to increase it during late lactation. There were no treatment effects on the composition of the protein fractions (g kg<sup>-1</sup>), expressed as casein and whey in Experiment 1, or individual proteins ( $\alpha$ -,  $\beta$ -,  $\kappa$ -caseins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin) in Experiment 2. Milk protein concentration was higher (+13%) in late lactation compared with mid lactation.

**Table 3.4. Effect of methionine supplementation on milk production, milk composition and efficiency of CP utilisation for milk protein synthesis in late lactation (Experiment 2).**

	Treatment		SEM
	Control (n=6)	RPM (n=6)	
Milk yield (kg d <sup>-1</sup> )	10.3	9.9	0.32
Milk composition (g kg <sup>-1</sup> ):			
Fat <sup>1</sup>	49.7	54.4	1.76
Lactose <sup>1</sup>	47.1	46.9	0.19
Total protein <sup>1</sup>	33.4	35.2	0.62
α-Casein <sup>2</sup>	17.7	17.8	0.54
β-Casein <sup>2</sup>	10.6	10.2	0.46
κ-Casein <sup>2</sup>	2.9	3.0	0.13
α-Lactalbumin <sup>2</sup>	0.5	0.5	0.02
β-Lactoglobulin <sup>2</sup>	2.3	2.3	0.11
Yield of components (g d <sup>-1</sup> ):			
Fat <sup>1</sup>	508	531	31.8
Lactose <sup>1</sup>	487	464	15.3
Total protein <sup>1</sup>	341	344	9.5
α-Casein <sup>2</sup>	182	173	5.3
β-Casein <sup>2</sup>	110 <sup>a</sup>	99 <sup>b</sup>	3.1
κ-Casein <sup>2</sup>	30	29	1.2
α-Lactalbumin <sup>2</sup>	5	5	0.3
β-Lactoglobulin <sup>2</sup>	23	23	1.3
Efficiency of CP utilisation: g milk protein/ g CP intake	0.149	0.140	0.0032

<sup>1</sup> From Milk-O-Scan<sup>2</sup> From SDS-PAGE and laser densitometry<sup>a,b</sup> Different letters in the same row indicate a significant difference between means ( $P < 0.05$ )

Milk production was also expressed as the yield of individual components per day. No treatment effects were observed on the yields of milk fat or lactose in either experiment. In mid lactation, Met-supplemented animals yielded higher amounts of total protein and casein; however, these differences were not significant. Whey and NPN yields during mid lactation remained relatively constant across treatments. In Experiment 2, when individual proteins were quantified, Met supplementation was associated with a significant reduction in the yield of β-casein, without effects on any of the other milk proteins analysed.

The gross efficiency of dietary CP utilization was calculated as the yield of milk protein per unit of CP intake ( $\text{g g}^{-1}$ ). In general, the efficiency of protein utilization was higher in mid than in late lactation (0.28 vs. 0.15 g milk protein per g of CP intake). Intravenous infusion of Met increased ( $P < 0.05$ ) the gross efficiency of N utilization in mid lactation by 3%. During late lactation, utilization of CP in Met supplemented animals was not different from that of control animals

### 3.5. DISCUSSION

Although AA utilisation by dairy cows has received considerable attention from different research groups all over the world, there is relatively little published data on the effect of AA supplementation on milk production in pasture-fed animals. In New Zealand, experiments with Met and lysine (Lys) supplementation were conducted by Rusdi and Van Houtert (1997) with no significant effects on milk production associated with an extra supply of AA. However, the amount of AA offered in that experiment (5.3 and 3.3  $\text{g d}^{-1}$  of Met and Lys, respectively) may have been too small to be effective, with no evidence that the extra AA supplied was delivered to peripheral circulation.

Blood or plasma AA concentrations have been used to assess the effectiveness of ruminally-protected preparations in delivering amino acids to the systemic circulation (Papas *et al.*, 1984; Polan *et al.*, 1991). In the current study, the basal concentrations of Met in blood were similar to those reported elsewhere (Papas *et al.*, 1984; Illg *et al.*, 1987; Rogers *et al.*, 1987; Donkin *et al.*, 1989). Supplementation with RPM increased the basal concentration of blood Met by 50% (Experiment 1) and 90% (Experiment 2). Therefore, our results demonstrated that RPM effectively delivered Met post-rationally.

Additional Met supply to the peripheral blood during mid-lactation was associated with an increased efficiency of dietary CP utilization for milk protein synthesis (2% and 3.5% in the RPM and MET-I.V. groups, respectively), with the response being significant for the MET-I.V. group. This result is similar to those reported by Robinson *et al.* (1995) with ruminally protected Met and Lys. According to these authors, Met supplementation may improve the gross efficiency of dietary N

utilization in dairy cows given low-protein diets, such as those offered in Experiment 1 (approx. 11% CP). Such a response may arise from a better balance in the supply of AA to the small intestine. In studies with animals fed a variety of diets, Schwab (1996) concluded that an adequate AA supply to the duodenum must include 5.3 – 5.6 % of the total essential AA (EAA) as Met, and 14.7 – 16.7 % of EAA as Lys. The pasture composition data from the present study was combined with previous reports on protein degradability of pasture (Ulyatt *et al.*, 1975; Van Vuuren *et al.*, 1992; 1993) to obtain an estimate of the flux of Met and Lys to the duodenum (Schwab, 1996). In mid lactation, the average contributions of Met and Lys were estimated as 4.9 and 17.4 % of the total duodenal EAA, respectively. These values are comparable to those reported by Ulyatt *et al.*, (1975) in sheep fed fresh ryegrass and white clover. If the requirements mentioned above are applicable to pasture-fed cows, the results from the present study appear to indicate that the extra Met supply increased the efficiency of N utilisation by improving the balance of the AA mixture presented to the mammary gland. However the lack of significant increases in protein concentration or yield, which are the main responses observed in concentrate-fed animals (Rulquin and Verite, 1996; Schwab, 1996), suggests that Met was not the only limiting factor in the diet.

In late lactation, the estimated supply of duodenal Met and Lys exceeded the proposed requirement (5.9 and 17.5 % of the total duodenal EAA, respectively). These values suggest that neither Met nor Lys were limiting for milk protein synthesis in cows fed fresh-chopped pasture. Consequently, the estimates also provide a probable explanation for the failure of Met supplementation to elicit responses in lactational performance during late lactation. Although a 6% increase in total milk protein concentration was observed, it is likely the result of the lower milk volume recorded for the RPM group in late lactation (Mackenzie, 1997).

Except for the decreased yield of  $\beta$ -casein in methionine-supplemented cows, the results from SDS-PAGE analysis during late lactation did not reveal any effect on the concentration or yield of the milk proteins analysed ( $\alpha$ -,  $\beta$ - and  $\kappa$ -caseins,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin). This finding contrasts with the increase of  $\alpha$ - and  $\beta$ -caseins and the reduction of  $\kappa$ -casein when cows were supplemented with methionine

and lysine (Donkin *et al.*, 1989). Compared with the other major milk proteins,  $\beta$ -casein has a higher content of valine, leucine and phenylalanine (Mackenzie, 1997). These AA share neutral transport systems (namely L and ASC) with Met in the epithelial mammary cell (Christensen, 1990; Shennan *et al.*, 1997). It could be speculated that the increased concentration of Met competed with valine, leucine and phenylalanine for transport sites in the membrane of the mammary epithelial cells, thus reducing their availability for  $\beta$ -casein synthesis. However, the current experimental data does not enable confirmation of this hypothesis.

Direct comparisons between the mid- and late lactation data obtained in the current study are not straightforward, given the variation not only in stage of lactation but also the changing feed quality, particularly the concentration of CP. In experiments where mixed rations are used, it is possible to feed animals with diets of relatively constant composition. In contrast, pastoral systems, such as those used in New Zealand, are characterised by large fluctuations in feed quality between seasons and years (Wilson *et al.*, 1995). Nevertheless, this variation is a common feature of pastoral systems and as such is inherent to studies of this nature.

The results obtained during mid and late lactation in the present study contrast with other studies where Met supplementation increased milk protein synthesis throughout the full lactation in cows fed with concentrates and alfalfa (Robinson *et al.*, 1995). In pastoral dairy farming, seasonal changes in the CP and ME content of the pasture may modify the responses to supplementary AA in different stages of lactation. In the current study, the protein intake per unit of energy varied from 9.9 g CP per MJ ME (mid lactation) to 15.7 g CP per MJ ME (late lactation) with milk yields of 18 and 10 kg d<sup>-1</sup>, respectively. These fluctuations are likely to affect the amount and profile of the amino acid mixture reaching the duodenum, as estimated for Met and Lys. The lower efficiency of utilization observed between experiments (28% vs. 15% in mid and late lactation respectively) may indicate that CP was offered in excess of the requirements for milk protein synthesis in late lactation. This assumption is supported by the increased plasma urea concentration measured during late lactation compared with mid lactation (7.0 mM vs. 3.3 mM, respectively). High concentrations of urea in

blood and milk have been associated with crude protein supplied in excess of requirements (Wilson *et al.*, 1995). Similarly, the increase in blood cysteine concentration observed in Experiment 2, is likely to be the result of the catabolism of Met (Baker, 1994). Increases in the blood cysteine concentration have been reported previously in experiments using supplementary Met (Pisulewski *et al.*, 1996). These results support the suggestion that Met was not limiting protein synthesis during late lactation, as the extra supply of Met was apparently catabolized instead of being incorporated into extra production of milk proteins.

### 3.6. CONCLUSIONS

The results from the present studies suggest that post-ruminal AA supply may improve the efficiency of dietary CP utilization for milk protein synthesis in the mammary gland of pasture-fed dairy cows during mid lactation. In late lactation, Met from the digestion of fresh pasture seems sufficient to fulfil their requirements for milk production and additional post-ruminal Met is not required. Thus, the responses to supplemental Met in pasture-fed cows appear to be affected by quality of the forage consumed. Nevertheless, there are indications that Met is not the sole limiting amino acid for milk protein synthesis. Therefore, more research is required to define the limiting amino acid(s) necessary to improve the efficiency of protein utilization in the grazing dairy cow.

### 3.7. ACKNOWLEDGEMENTS

This work was financed by a grant from the Foundation for Science, Research and Technology. The authors thank the staff of the Nutrition Laboratory, Department of Animal Science and the Animal Research Unit, Massey University; the Analytical Laboratory, AgResearch Grasslands and the Food Science Section, New Zealand Dairy Research Institute for their contribution during the experimental and analytical phases of this work. The authors thank Dr. N. Roy for her comments on the preparation of this manuscript. The senior author thanks the National Autonomous University of Mexico for providing the scholarship for his studies.

### 3.8. REFERENCES

- Anema, S.G.; Creamer, L.K. 1993. Effect of the A and B variants of both  $\alpha$ s1- and  $\kappa$ -casein on bovine casein micelle solvation and  $\kappa$ -casein content. *Journal of Dairy Research* **60**:505-516
- Auldish, M.J.; Walsh, B.J.; Thompson, N.A. 1997. Seasonal and lactational influences on the composition of milk from pasture-fed dairy cows in New Zealand. *Journal of Dairy Science* **80**, Suppl. 1:203
- Baker, D. H. 1994 Utilization of precursors for L-amino acids. In Amino acids in farm animal nutrition (Ed. D'Mello, J. P. F.) 37-61. Wallingford, Oxon, U.K.: CAB International.
- Beever, D.E.; Dhanoa, M.S.; Losada, H.R.; Evans, R.T.; Cammell, S.B.; France, J. 1986. The effect of forage species and stage of harvest on the processes of digestion occurring in the rumen of cattle. *British Journal of Nutrition* **56**:439-454
- Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatisation. *Journal of Chromatography* **336**:93-104
- Black, J. L. 1990. Nutrition of the grazing ruminant. *Proceedings of the New Zealand Society of Animal Production* **50**:7-27
- Christensen, H.N. 1990. Role of amino acid transport and countertransport in nutrition and metabolism. *Physiological Reviews* **70**:43-77
- Clark, J.H.; Spires, H.R.; Derrig, R.G.; Bennink, M.R. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused posturally with sodium caseinate and glucose. *Journal of Nutrition* **107**:631-644
- Derrig, R.G.; Clark, J.H.; Davis, C.L. 1974. Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. *Journal of Nutrition* **104**:151-159



Donkin, S.S.; Varga, G.A.; Sweeney, T.F.; Muller, L.D. 1989. Rumen-protected methionine and lysine: Effects on animal performance, milk protein yield, and physiological measures. *Journal of Dairy Science* **72**:1484-1491

Erfle J.D.; Fisher, L.J. 1977. The effects of intravenous infusion of lysine, lysine plus methionine or carnitine on plasma amino acids and milk production of dairy cows. *Canadian Journal of Animal Science* **57**:101-109

Fisher, L.J. 1972. Response of lactating cows to the intravenous infusion of amino acids. *Canadian Journal of Animal Science* **52**:377-384

Gaitonde, M.K. 1967. A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. *Biochemical Journal* **104**:627-633

Hill J.P. 1993. The relationship between  $\beta$ -lactoglobulin phenotypes and milk composition in New Zealand dairy cattle. *Journal of Dairy Science* **76**:281-286

Illg, D.J.; Sommerfeldt, J.L.; Schingoethe, D.J. 1987. Lactational and systemic responses to the supplementation of protected methionine in soybean meal diets. *Journal of Dairy Science* **70**:620-629

Koch, K.L.; Whitehouse, L.; Garthwaite, B.D.; Wasserstrom, V.M.; Schwab, C.G. 1996. Production responses of lactating Holstein cows to rumen-stable forms of lysine and methionine. *Journal of Dairy Science* **79**, **Suppl. 1**:239

Mackenzie, D. D. S. 1997. Milk composition as an indicator of mammary gland metabolism. *Proceedings of the Nutrition Society of New Zealand* **22**:126-136

Metcalf, J.A.; Wray-Cahen, D.; Chettle, E.E.; Sutton, J.D.; Beever, D.E.; Crompton, L.A.; MacRae, J.C.; Bequette, B.J.; Backwell, F.R.C. 1996. The effect of dietary crude protein as protected soybean meal on mammary metabolism in the lactating dairy cow. *Journal of Dairy Science* **79**:603-611

Overton, T.R.; LaCount, D.W.; Cicela, T.M.; Clark, J.H. 1996. Evaluation of a ruminally protected methionine product for lactating dairy cows. *Journal of Dairy Science* **79**:631-638

Papas, A.M.; Sniffen, C.J.; Muscato, T.V. 1984. Effectiveness of rumen-protected methionine for delivering methionine postruminally in dairy cows. *Journal of Dairy Science* **67**:545-552

Pisulewski, P.M.; Rulquin, H.; Peyraud, J.L.; Verite, R. 1996. Lactational and systemic responses of dairy cows to postruminal infusions of increasing amounts of methionine. *Journal of Dairy Science* **79**:1781-1791

Polan, C.E.; Cummins, K.A.; Sniffen, C.J.; Muscato, T.V.; Vicini, J.L.; Crooker, B.A.; Clark, J.H.; Johnson, D.G.; Otterby, D.E.; Guillaume, B.; Muller, L.D.; Varga, G.A.; Murray, R.A.; Peirce-Sandner, S.B. 1991. Responses of dairy cows to supplemental rumen-protected forms of methionine and lysine. *Journal of Dairy Science* **74**:2997-3013

Robert, J.C.; Sloan, B.K.; Denis, C. 1996. The effects of graded amounts of rumen protected methionine on lactational responses in dairy cows. *Journal of Dairy Science* **79**, **Suppl.1**:194

Robinson, P.H.; Fredeen, A.H.; Chalupa, W.; Julien, W.E.; Sato, H.; Fujieda, T.; Suzuki, H. 1995. Ruminally protected lysine and methionine for lactating cows fed a diet designed to meet requirements for microbial and postruminal protein. *Journal of Dairy Science* **78**:582-594

Rogers, J.A.; Krishnamoorthy, U.; Sniffen, C.J. 1987. Plasma amino acids and milk protein production by cows fed rumen-protected methionine and lysine. *Journal of Dairy Science* **70**:789-798

Rulquin, H.; Verite, R. 1996 Amino acid nutrition of dairy cows: productive effects and animal requirements. In *Recent Developments in Ruminant Nutrition 3* (Ed. Garnsworthy P.C. and Cole D.J.A.) 71-93. Nottingham, U.K.: Nottingham University Press.

Rusdi; Van Houtert, M. J. F. 1997. Responses to protected amino acids or protected protein in dairy cows grazing ryegrass pastures in early or late lactation. *Proceedings of the New Zealand Society of Animal Production* **57**:120-125

SAS Institute Inc. 1996. *SAS User's Guide*. 6.11. Cary, NC

Schwab, C.G. 1996. Rumen-protected amino acids for dairy cattle: Progress towards determining lysine and methionine requirements. *Animal Feed Science and Technology* **59**:87-101

Shennan, D.B.; Millar, I.D.; Calvert, D.T. 1997. Mammary-tissue amino acid transport systems. *Proceedings of the Nutrition Society* **56**:177-191

Singh, H.; Creamer, L.K. 1991. Denaturation, aggregation and heat stability of milk protein during the manufacture of skim milk powder. *Journal of Dairy Research* **58**:269-283

Spires, H.R.; Clark, J.H.; Derrig, R.G.; Davis, C.L. 1975. Milk production and nitrogen utilisation in response to postruminal infusion of sodium caseinate in lactating cows. *Journal of Nutrition* **105**:1111-1121

Ulyatt, M.J.; MacRae, J.C.; Clarke, R.T.J.; Pearce, P.D. 1975. Quantitative digestion of fresh herbage by sheep. IV. Protein synthesis in the stomach. *Journal of Agricultural Science, Cambridge* **84**:453-458

Van Vuuren, A.M.; Krol-Kramer, F.; Van der Lee, R.A.; Corbijn, H. 1992. Protein digestion and intestinal amino acids in dairy cows fed fresh *Lolium perenne* with different nitrogen contents. *Journal of Dairy Science* **75**:2215-2225

Van Vuuren, A.M.; van der Koelen, C.J.; Vroons-de Bruin, J. 1993. Ryegrass versus corn starch or beet pulp fiber diet effects on digestion and intestinal amino acids in dairy cows. *Journal of Dairy Science* **76**:2692-2700

Wilson, G. F.; Moller, S.; Parker, W. J.; Hodgson, J. 1995. Seasonal differences in pasture composition and nutritional implications. *1995 Dairyfarming Annual* 47:46-56

**CHAPTER 4.**

**COMPARISON OF TWO VARIANTS OF THE FICK  
PRINCIPLE FOR ESTIMATION OF MAMMARY BLOOD  
FLOW IN DAIRY COWS FED TWO LEVELS OF DRY  
MATTER INTAKE**

This Chapter has been submitted for publication in the *Canadian Journal of Animal Science*

#### 4.1. ABSTRACT

The estimation of the mammary blood flow (MBF) is an important component of studies of the utilisation of metabolites by the mammary gland of lactating animals. Although there are several flowmeters available to measure MBF by direct contact with the vessels perfusing the mammary gland, their use is limited mainly due to the surgical preparation required for their implantation. For that reason, the application of the Fick principle was assessed as a means to estimate MBF as part of a study on amino acid utilisation by the mammary gland of pasture-fed dairy cows at either *ad libitum* or restricted dry matter intakes. Two methods based on the Fick principle were assessed, namely arterio-venous differences of amino acids (methionine: Met or phenylalanine+tyrosine: P+T) and tritiated water (TOH). The estimated MBF were not significantly different for the Met and P+T methods (average of 8.1 and 8.8 L min<sup>-1</sup>, respectively). The TOH method yielded a significantly lower ( $P < 0.05$ ) estimate of blood flow (average of 5.3 L min<sup>-1</sup>). Using the Met and P+T methods, the MBF was lower during the period of dietary restriction compared with the *ad libitum* treatment (averages 9.4 and 7.5 L min<sup>-1</sup>, respectively). In contrast, the TOH method resulted in a numerically higher MBF for the restricted group (5.7 vs. 4.9 L min<sup>-1</sup>). The short sampling period involved in the TOH method appears to be a disadvantage for extrapolating the estimated values to balance studies involving longer periods of time. The estimated values obtained using the Met or P+T appear to be representative of the MBF during the experimental period. Therefore, any of these methods may be used, alone or in combination, as an alternative to flowmeters in studies of mammary metabolism.

**Abbreviations:** AA, amino acid(s); A-V, arterio venous difference(s); MBF, mammary blood flow; Met, Methionine; PCV, packed cell volume; P+T, phenylalanine plus tyrosine; TOH, tritiated water..

## 4.2. INTRODUCTION

An initial understanding of the metabolism of a substrate by an organ can be obtained by measuring the balance between its uptake and the output of its various metabolites. In studies of mammary gland metabolism, the percentage uptake of precursors of milk constituents has been estimated by measuring the arterio-venous (A-V) differences across the udder. This technique is relatively straightforward and inexpensive to conduct in experiments with dairy cows (Bickerstaffe *et al.*, 1974; Linzell, 1971; Peeters *et al.*, 1979). However, the measured A-V difference has to be combined with an estimation of blood flow through the mammary gland to estimate the uptake (mass per unit of time) of metabolites in a given period (Linzell, 1971).

Mammary blood flow (MBF) has been estimated using a wide variety of methods (see Linzell, 1974; Lescoat *et al.*, 1996; Knight *et al.*, 1994). Ultrasonic and electromagnetic probes have been used to determine blood flow and its variation on a minute-to-minute basis (Peeters *et al.*, 1979; Gorewit *et al.*, 1989), but the cost and the surgical and technical preparation associated with their implantation and calibration have limited their use as a routine method for estimation of MBF, particularly in large animals. Several methods based on the injection of an indicator into the pudendal artery and measuring its dilution in a vein draining the gland have been used to estimate blood flow in different species of lactating animals (Reynolds *et al.*, 1968; Metcalf *et al.*, 1991). The Fick method states that the flow through an organ can be estimated if the inlet (artery) and outlet (vein) concentrations of an indicator can be measured together with its uptake (Lassen and Perl, 1979). Methods based on the Fick principle [i.e. tritiated water: McDowell *et al.* (1987), methionine (Met) A-V: Davis and Bickerstaffe (1978); phenylalanine plus tyrosine (Phe+Tyr) A-V: Davis *et al.*, (1988)] require simpler surgical preparation and are less expensive than alternative methods for estimation of blood flow through the mammary gland of lactating animals.

The Fick principle applied to amino acids for which the ratio of uptake by the gland to output in milk is close to unity (i.e. group I EAA: methionine, lysine,

phenylalanine+tyrosine) can theoretically provide an accurate estimate of MBF (Davis *et al.*, 1988). Furthermore, estimates of blood flow obtained from Phe+Tyr and electromagnetic probes were not significantly different (Davis *et al.*, 1988). However, the use of A-V differences of amino acids in plasma has been criticised, as the potential contribution of red blood cells is not considered (Hanigan *et al.*, 1991).

Besides amino acids, other indicators can be used to estimate MBF using the Fick principle. Several authors have used TOH for blood flow estimation in different organs, including the mammary gland of sheep (Pethick and Lindsay, 1982) and cows (McDowell *et al.*, 1987).

The purpose of this study was to assess the application of two different variants of the Fick principle (A-V of amino acids and tritiated water) for estimating MBF during a study on the amino acid metabolism of lactating dairy cows fed diets consisting solely of fresh forage. A comparison of sample preparation prior to determination of tritiated water in plasma was also conducted with the objective of simplifying the laboratory procedures associated with this method.

### **4.3. MATERIAL AND METHODS**

#### **4.3.1. Animals**

Mammary blood flow was estimated in four lactating mature Friesian cows, which were assigned to two levels of dry matter intake (DMI; *ad libitum* and 75% of *ad libitum*) and two feeding periods (16-d each) in a 2x2 crossover design. At the beginning of the experiment, the average days in milk of the cows was 44 (SD 14.5) with the average live weight being 498 (SD 64.2) kg. Animals were fed individually and maintained outdoors from day 1-5 of each period, and in individual metabolism stalls from day 6 to 16 at the Dairying Research Corporation in Hamilton, New Zealand. Cows were fitted on day 11 with custom-made polyvinyl chloride catheters (1.0 mm ID x 1.5 mm OD; Critchley Electrical Products, NSW, Australia) in one intercostal artery (Haibel *et al.*, 1989) and the jugular and mammary (caudal superficial epigastric) veins.



Animals were tranquillised with xylazine hydrochloride (0.8 ml Rompun 2%; Bayer New Zealand Ltd., Auckland, New Zealand). A local subcutaneous anaesthetic (lignocaine hydrochloride 2%, Ethical Agents Ltd, Auckland, New Zealand) was injected prior to catheterisation. Cows were allowed to recover from the surgery for one day before starting the MBF measurement protocols. The intercostal catheters were maintained during the two experimental periods, while both venous catheters were removed at the end of the first period and new catheters inserted on day 11 of the second period. Patency of all catheters was ensured by daily flushing with 3 ml of heparinised saline (200 IU heparin ml<sup>-1</sup>; New Zealand Pharmaceuticals, Linton, New Zealand).

#### 4.3.2. Diets

During the experimental period, fresh cut ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture was offered at 6 h intervals (0300, 0900, 1500 and 2100 h), at *ad libitum* and restricted intakes. Animals in the restricted group received 75% of the *ad libitum* intake, which was established during the first five days of each experimental period.

#### 4.3.3. Methods for estimating mammary blood flow

##### 4.3.3.1. Amino acid arterio-venous differences

On day 14 and 15, blood samples were collected from the intercostal artery and the mammary vein for each of two animals over a 12-h period. Blood samples were collected every two hours as 1-hour integrated samples (i.e. continuous sampling for one hour, no sampling for one hour) at a rate of 1 ml min<sup>-1</sup>, into plastic centrifuge tubes containing Na-EDTA and maintained in ice. Whole blood was haemolysed with deionised water (1:1 v/v) and mixed with 200 mM phosphate buffer containing 80 mM DL-dithiothreitol (0.1 ml of phosphate buffer per ml of whole blood) and stored at -85°C for amino acid analysis.

#### 4.3.3.2. *Tritiated water*

Mammary gland blood flow was measured on day 14 and 15 of each experimental period using a modification of the method described by Oddy *et al.*, (1981). A bolus infusion of 20 ml of sterile saline containing 17.4 MBq of tritiated water (TOH; ICN Biomedicals Australasia Pty. Ltd. Seven Hills, NSW, Australia) was followed by a 1-hour continuous infusion ( $1 \text{ ml min}^{-1}$ ) of saline containing 0.9 MBq of TOH. During the continuous infusion of TOH, blood was collected continuously, at 4-min intervals, from the intercostal artery and the mammary vein. Sample collection procedures were the same as described above. Blood was centrifuged at  $3\,270 \text{ g}$  for 15 min and plasma was harvested and stored for further analysis of TOH. Packed cell volume (PCV) was determined using microhaematocrit tubes on blood collected directly from the sampling lines.

#### 4.3.3.3. *Milk sampling and udder volume*

At the end of each blood sampling, cows were injected intravenously with 1 IU of oxytocin (Oxytocin EA; Ethical Agents Ltd.; Auckland, New Zealand) and machine milked. To ensure complete removal of milk, a second oxytocin injection (1 IU) was administered and residual milk was removed by hand-stripping. After milking, empty udder volumes were measured using the method described by Davis and Hughson (1988). Mammary gland weights were estimated from udder volumes, assuming a specific gravity of mammary tissue of 1.034 (Davis *et al.*, 1978).

#### 4.3.4. **Laboratory methods**

##### 4.3.4.1. *Amino acid(s) A-V*

Samples of whole haemolysed blood (2 ml) were mixed with 65  $\mu\text{l}$  of 3 mM methionine sulphone as an internal standard, and then deproteinised by ultrafiltration (Centrisart, molecular weight cut-off 10,000; Sartorius AG, Germany). The ultrafiltrate was collected and stored at  $-85^{\circ}\text{C}$  until it was analysed for free AA concentrations.

Methionine, phenylalanine and tyrosine concentrations in blood were measured after reverse phase HPLC separation of phenylisothiocyanate derivatives (Bidlingmeyer *et al.*, 1984) using a Waters PicoTag® column (3.9 x 300 mm; Waters Corporation, Milford, MA) and a Shimadzu LC-10/A HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD) with a 90-min running time between injections.

Phenylalanine and tyrosine concentration in milk were determined in freeze-dried skim milk samples, using post-column derivatisation with ninhydrin (PCX 3100 Post Column Reaction Module, Pickering Laboratories, Mountain View, CA 94043, USA) and a Shimadzu LC10Ai HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA) after HCl hydrolysis (AOAC, 1990). Similarly, methionine was determined in milk hydrolysed after oxidation with performic acid (AOAC, 1990).

#### **4.3.4.2. Tritiated water**

Tritium content of the plasma samples was analysed using three analytical approaches to account for possible interference of the sample matrix with the counting procedure. In all the cases, the samples were mixed with 2 ml of scintillation fluid (Starcint, Packard Instrument Company, 06450 Meriden, CT) and counted in glass vials in a scintillation counter (RackBeta 1219 Spectral, LKB Wallac, 20101 Turku 10, Finland). Counts were corrected for counting efficiency and expressed as disintegrations per minute (dpm). In the first method, plasma (0.25 ml) was mixed directly with scintillation fluid and counted. For the second method, 0.5 ml deproteinised plasma (0.5 ml of 0.6 M perchloric acid per 0.25 ml plasma) was used. While for the third method, TOH content was measured in water (0.25 ml) that was trapped under vacuum from frozen plasma samples. In all cases, specific activity was expressed on a per gram of plasma basis.

### 4.3.5. Calculations

#### 4.3.5.1. Amino acid(s) A-V difference

Mammary blood flow was calculated as described by Davis *et al.*, (1988) using the following equation:

$$MBF (L h^{-1}) = \frac{AA \text{ output in milk } (mg h^{-1})}{A - V \text{ of AA } (mg L^{-1} \text{ blood})}$$

Amino acid output was calculated assuming that 96% of milk protein is synthesised in the mammary gland (Swaisgood, 1992).

#### 4.3.5.2. Tritiated water

Estimates of MBF were obtained using a modification of the equation described by Pappenheimer and Setchell (1972):

$$MBF (ml \text{ min}^{-1} \cdot g) = \frac{SV_{eq} \times \frac{W_m}{W_p}}{\int_{=0}^{t=t_{eq}} (SA - SV) dt} \times \frac{100}{(100 - PCV)}$$

where  $SV_{eq}$  is the specific activity of venous plasma at equilibrium, corrected for the difference between water content of mammary tissue ( $W_m$ ) and plasma ( $W_p$ ). The denominator of the equation is the difference between areas under the curves of the specific activity of arterial ( $SA$ ) and venous ( $SV$ ) samples from the commencement of the infusion ( $t=0$ ) to the time in which arterial and venous specific activities reached equilibrium ( $t=t_{eq}$ ). Water in plasma was determined by drying (24 h at 70 °C) a bulked plasma sample for each animal. Water in mammary tissue was assumed to be 70% (Linzell, 1974). The resulting plasma flow was corrected to blood flow using the average PCV value measured for each animal during the sampling period.

#### 4.3.6. Statistical analysis

Mammary blood flow estimates for each animal and each method were obtained and then analysed using the GLM procedure of SAS (SAS Institute Inc., 1996) for a crossover design (Ratkowsky *et al.*, 1993). The model included the general mean, treatment (level of intake), period and sequence. Differences between treatments means were tested using a multiple comparison test (PDIFF option, SAS Institute, 1996). Differences between methods (amino acid A-V and tritiated water) were assessed by comparing the least squares means using *t*-test. Significant statistical differences between methods were declared at  $P < 0.05$ .

### 4.4. RESULTS

Due to problems with the arterial catheter in one of the animals, arterial samples were collected via a deep jugular catheter. However, during the analysis of the samples, it was apparent that there was incomplete mixing of the tritiated water in the deep jugular sample, and therefore, all the estimated values of blood flow for that animal were removed from the analysis. This situation also affected the arterio-venous differences of amino acids in that animal. Therefore, all the results presented refer to three cows.

The overall average values for PCV were  $27.7 \pm 0.67$  and  $27.9 \pm 0.65$  for arterial and venous samples (mean  $\pm$  SEM).

#### 4.4.1. TOH method: Effect of sample preparation

No differences ( $P > 0.10$ ) between the sample preparation methods were observed in terms of plateau values and areas under the curves of tritium activity (Figure 4.1). Therefore, the TOH activity values obtained from each type of sample (plasma, hydrolysed plasma and distilled plasma) were averaged within animal and the blood flow values were estimated as described in the Calculations section of this

chapter. In all cases, equilibrium between arterial and venous samples was first attained after approximately 10-16 minutes.

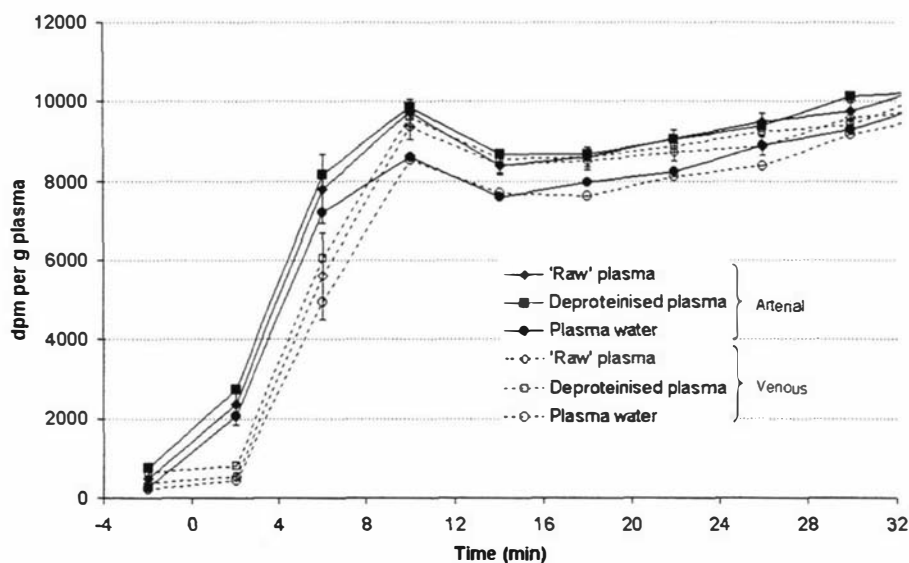


Figure 4.1. Curve of tritium activity in arterial and venous plasma samples processed using the different protocols described in the Material and Methods section (Means  $\pm$  SEM;  $n=3$ ).

#### 4.4.2. Comparison between the amino acid A-V and TOH methods

The estimates of mammary blood flow are presented in Table 4.1. There was no significant difference between the estimates obtained using either Met or Phe+Tyr arterio-venous differences. The estimated blood flow using TOH was significantly

Table 4.1. Estimated mammary blood flow (litres per minute) using amino acid arterio-venous differences and TOH.

Met A-V <sup>a</sup>		Method of BF estimation						
		Phe +Tyr A-V <sup>a</sup>			TOH <sup>b</sup>			
<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM
8.8 <sup>c</sup>	7.3 <sup>d</sup>	0.01	9.9	7.7	1.36	4.9	5.7	0.16

<sup>a, b</sup> Different superscripts indicate a difference between methods ( $t$ -test;  $P < 0.05$ ).

<sup>c, d</sup> Different superscript indicate a difference between ad libitum and restricted feed intake ( $P < 0.05$ ).

lower ( $P < 0.05$ ) than those obtained using the amino acid A-V differences. The ratios of blood flow to milk yield were, on average, 546, 602 and 395 for the Met, Phe+Tyr and TOH methods, respectively. The ratios obtained from amino acid A-V fell within the range of published values from different studies (450-900 litres of blood per litre of milk: Kronfeld *et al.*, 1968; Bickerstaffe *et al.*, 1974; Peeters *et al.*, 1979; McDowell *et al.*, 1987; Cant *et al.*, 1993). According to those values, the estimated flows using TOH in the present study seem to be an underestimation of the actual MBF in the experimental animals.

The MBF estimates (litres per minute) obtained from amino acid A-V differences were lower for the animals with the restricted feed intakes, with this result being significant ( $P < 0.05$ ) when Met was used as indicator. In contrast, the TOH method gave a numerically higher blood flow for the restricted animals; however, this difference was not statistically significant.

#### 4.5. DISCUSSION

Although not suitable for studies in which acute changes are likely to occur (Davis *et al.*, 1988), the Fick principle has been used to obtain an average estimate of the mammary blood flow over extended periods when relatively steady conditions applied. Such conditions were present in this study, and therefore, the measured MBF is considered to adequately define the average MBF over the experimental periods.

In the present study, the estimates obtained using amino acids as indicators of MBF were in close agreement, with similar trends for individual animals and dietary treatment groups. Estimates from Phe+Tyr were approximately 10% higher than those obtained using Met as the indicator amino acid. However, this difference did not reach statistical significance. The use of whole blood instead of plasma eliminates the possibility of this difference being the result of the contribution of red blood cells to amino acid exchange between blood and the mammary gland. The use of either Met or Phe+Tyr resulted in a lower estimate of MBF in the animals with the dietary restriction.

Reductions in MBF has been reported previously as a result of dietary restriction in several species (Davis and Collier, 1985; Sakanashi *et al.*, 1987; Lough *et al.*, 1990)

The Fick principle has been used with TOH as an indicator to estimate blood flow in several organs in different species (Pappenheimer and Setchell, 1972; Oddy *et al.*, 1981; Pethick and Lindsay, 1982; Brown *et al.*, 1982; Gooden *et al.*, 1986; McDowell *et al.*, 1987; Oddy *et al.*, 1988). To our knowledge, this technique has been used only once previously in lactating dairy cows (McDowell *et al.*, 1987). The estimated MBF from that study ranged between 6.9 and 10.4 L min<sup>-1</sup>. In the current study, the estimated MBF was approximately 60% of that value, although the experimental animals from both studies had similar liveweight and milk yield. In contrast, the estimates of blood flow per unit tissue mass estimated from that study (0.32 - 0.48 ml g<sup>-1</sup> min<sup>-1</sup>) are lower than those obtained in the present study using the TOH method (0.57 ml g<sup>-1</sup> min<sup>-1</sup>). The explanation for this apparent discrepancy resides in that MBF is measured, using TOH as indicator, as the flow in millilitres per gram of tissue mass. Therefore, the estimated total flow to the gland depends on its volume. McDowell *et al.*, (1987) assumed that the volume of the mammary gland was equal to the volume of the milk produced per day (*c.* 22 litres). In the present study, the volume of the emptied mammary gland ranged between 8.5 and 10.3 litres, and the milk yield ranged between 14.7 and 25.4 kg d<sup>-1</sup>. Thus, in the current study, the average milk yield was approximately 2.1 ml per gram of mammary tissue. This value is comparable to those reported by Davis and Bickerstaffe (1978) for domestic ruminants (1.75 – 1.90 ml per gram of tissue per day). Therefore, differences in the estimation of udder volume between the two studies may account for most of the differences in the estimated rates of MBF.

In both cases, the estimates of MBF from using the TOH method are lower than those obtained by other methods as reported in the literature and in the present experiment by using the A-V difference of amino acids. Several possible explanations can be given for the lower MBF values obtained using the TOH than the amino acid methods in the current study. The differences in the magnitude of the MBF estimates may be due, in part, to the losses of TOH through skin and lungs. The losses of



indicator through skin have been described as a potential cause for underestimation of blood flow using the Fick principle (Linzell, 1974). Another possibility is that the differences between the methods of MBF estimation may also be related to the time in which the measurements were made. The TOH method was carried out over a 1-h sampling period, whilst the amino acid methods involved sampling over 6-12 hours. Acute variations in MBF would have a greater effect in the shorter-term estimation, i.e. the TOH method, resulting in the contrasting estimates between methods reported here.

#### **4.6. CONCLUSIONS**

The use of arterio-venous difference of Met and Phe+Tyr provide an adequate estimate of MBF in those situations in which relatively stable conditions are maintained over a period of hours or days. The use of either Met or Phe+Tyr resulted in similar estimates of MBF and therefore, this MBF could be used to estimate the average uptake of amino acids across the mammary gland. The short sampling period involved in the TOH method may represent a disadvantage for extrapolating the estimated values to balance studies involving longer periods of time. The sample preparation for the TOH measurement in plasma did not affect the final estimate. Therefore, if the TOH method is used, the measurement of activity may be conducted directly on "raw" plasma, resulting in an easier and faster analysis.

#### 4.7. REFERENCES

AOAC. 1990. Official methods of analysis of the Association of Official Analytical Chemists (AOAC). *Official methods of analysis of the Association of Official Analytical Chemists (AOAC)*

Bickerstaffe, R.; Annison, E.F.; Linzell, J.L. 1974. The metabolism of glucose, acetate, lipids and amino acids in lactating dairy cows. *Journal of Agricultural Science, Cambridge*. **82** :71-85

Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatisation. *Journal of Chromatography* **336**:93-104

Brown, B.W.; Oddy, V.H.; Jones, A.W. 1982. Measurement of organ blood flow using tritiated water. II. Uterine blood flow on conscious pregnant ewes. *Australian Journal of Biological Sciences* **35**:25-31

Cant, J.P.; DePeters, E.J.; Baldwin, R.L. 1993. Mammary amino acid utilization in dairy cows fed fat and its relationship to milk protein depression. *Journal of Dairy Science* **76**:762-774

Davis, S.R.; Bickerstaffe, R. 1978. Mammary glucose uptake in the lactating ewe and the use of methionine arterio-venous difference for the calculation of mammary blood flow. *Australian Journal of Biological Sciences* **31**:133-139

Davis, S.R.; Bickerstaffe, R.; Hart, D.S. 1978. Amino acid uptake by the mammary gland of the lactating ewe. *Australian Journal of Biological Sciences* **31**:123-132

Davis, S.R.; Collier, R.J. 1985. Mammary blood flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68**:1041-1058

Davis, S.R.; Collier, R.J.; McNamara, J.P.; Head, H.H.; Sussman, W. 1988. Effects of thyroxine and growth hormone treatment of dairy cows on milk yield, cardiac output and mammary blood flow. *Journal of Animal Science* **66**:70-79

Davis, S.R.; Hughson, G.A. 1988. Measurement of functional udder capacity in lactating Jersey cows. *Australian Journal of Agricultural Research* **39**:1163-1168

Gooden, J.M.; Campbell, S.L.; Van der Walt, J.G. 1986. Measurement of blood flow and lipolysis in the hindquarter tissues of the fat-tailed sheep *in vivo*. *Quarterly Journal of Experimental Physiology* **71**:537-547

Gorewit, R.C.; Aromando, M.C.; Bristol, D.G. 1989. Measuring bovine mammary gland blood flow using a transit time ultrasonic flow probe. *Journal of Dairy Science* **72**:1918-1928

Haibel, G.H.; Guilbault L.A.; Villeneuve, P.; Thatcher, W.W. 1989. Aortic catheterization in cattle via the costoabdominal artery and validation for progesterone and estradiol-17 $\beta$  sample collection. *American Journal of Veterinary Research* **50**:1923-1925

Hanigan, M.D.; Calvert, C.C.; DePeters, E.J.; Reis, B.L.; Baldwin, R.L. 1991. Whole blood and plasma amino acid uptakes by lactating bovine mammary glands. *Journal of Dairy Science* **74** :2484-2490

Knight, C.H.; France, J.; Beever, D.E. 1994. Nutrient metabolism and utilization in the mammary gland. *Livestock Production Science* **39**:129-137

Kronfeld, D.S.; Raggi, F.; Ramberg, C.F.Jr. 1968. Mammary blood flow and ketone body metabolism in normal, fasted and ketotic cows. *American Journal of Physiology* **215**:218-227

Lassen, N. A. and Perl, W. 1979. Tracer kinetic methods in medical physiology. New York, NY, USA: Raven Press.

Lescoat, P.; Sauvant, D.; Danfær, A. 1996. Quantitative aspects of blood and amino acid flows in cattle. *Reproduction, Nutrition, Development* **36**:137-174

Linzell, J. L. 1971 Techniques for measuring nutrient uptake by the mammary glands. In Lactation (Ed. Falconer, I. R.) 261-279. London, U.K.: Butterworths.

Linzell, J. L. 1974 Mammary blood flow and methods of identifying and measuring precursors of milk . In Lactation. A comprehensive treatise (Ed. Larson, B. L. and Smith, V. R.) 143-225. New York, USA: Academic Press.

Lough, D.S.; Beede, D.L.; Wilcox, C.J. 1990. Effects of feed intake and thermal stress on mammary blood flow and other physiological measurements in lactating dairy cows. *Journal of Dairy Science* **73**:325-332

McDowell, G.H.; Gooden, J.M.; Leenanuruksa, D.; Jois, M.; English, A.W. 1987. Effect of exogenous growth hormone on milk production and nutrient uptake by muscle and mammary tissues of dairy cows in mid-lactation. *Australian Journal of Biological Sciences* **40**:295-306

Metcalf, J.A.; Sutton, J.D.; Cockburn, J.E.; Napper, D.J.; Beever, D.E. 1991. The influence of insulin and amino acid supply on amino acid uptake by the lactating bovine mammary gland. *Journal of Dairy Science* **74**:3412-3420

Oddy, V.H.; Brown, B.W.; Jones, A.W. 1981. Measurement of organ blood flow using tritiated water. I. Hind limb muscle blood flow in conscious ewes. *Australian Journal of Biological Sciences* **34**:419-425

Oddy, V.H.; Lindsay, D.B.; Fleet, I.R. 1988. Protein synthesis and degradation in the mammary gland of lactating goats. *Journal of Dairy Research*. **55**:143-154

Pappenheimer, J.R.; Setchell, B.P. 1972. The measurement of cerebral blood flow in the rabbit and sheep. *Journal of Physiology* **226**:48-50P

Peeters, G.; Houvenaghel, A.; Roets, E.; Massart-Leën, A.M.; Verbeke, R.; Dhondt, G.; Verschooten, F. 1979. Electromagnetic blood flow recording and balance of nutrients in the udder of lactating cows. *Journal of Animal Science* **48**:1143-11-53

Pethick, D.W.; Lindsay, D.B. 1982. Acetate metabolism in lactating sheep. *British Journal of Nutrition* **48**:319-328

Ratkowsky, D. A., Evans, M. A., and Alldredge, J. R. 1993. Cross-over Experiments: Design, Analysis, and Application. New York, NY.: Marcel Drekker, Inc.

Reynolds, M.; Linzell, J.L.; Rasmussen, F. 1968. Comparison of four methods for measuring blood flow in conscious goats. *American Journal of Physiology* 214:1415-1424

Sakanashi, T.M.; Brigham, H.E.; Rasmussen, K.M. 1987. Effect of dietary restriction during lactation on cardiac output, organ blood flow and organ weights of rats. *Journal of Nutrition* 117:1469-1474

Sandles, L.D.; Sun, Y.X.; D'Cruz, A.G.C.; McDowell, G.H.; Gooden, J.M. 1988. Responses of lactating ewes to exogenous growth hormone: short- and long-term effects on productivity and tissue utilization of key metabolites. *Australian Journal of Biological Sciences* 41:357-370

SAS Institute Inc. 1996. *SAS User's Guide*. 6.11. Cary, NC

Swaigood, H. E. 1992 Chemistry of caseins. In *Advanced Dairy Chemistry-1:Proteins* (Ed. Fox, P. F.) 63-110. Essex, U.K.: Elsevier Science Publishers.

**CHAPTER 5.**

**AMINO ACID UTILISATION BY THE MAMMARY  
GLAND OF DAIRY COWS FED FRESH PASTURE:  
PRECURSOR POOL AND EFFECT OF DRY MATTER  
INTAKE RESTRICTION**

This Chapter has been submitted for publication in the *Canadian Journal of Animal Science*

## 5.1. ABSTRACT

The utilisation of amino acids (AA) by the mammary gland of lactating dairy cows fed fresh forages has been studied to provide basic information on milk protein synthesis in pasture-fed cows. Arterio-venous differences for the AA in whole blood and plasma were measured across the mammary gland of four lactating cows maintained in metabolism stalls and fed fresh-cut pasture. The roles of whole blood and plasma as sources of free AA for milk protein synthesis were assessed in terms of the concentration, arterio-venous differences, extraction percentages and uptake of AA from these pools. Plasma was the major source of free AA for milk protein synthesis. The contribution of erythrocytes was small (5-15% of the total blood) and limited to isoleucine, leucine, phenylalanine and tyrosine. Extraction percentages and comparisons of the AA uptake and output in milk protein were used to indicate the AA that were potentially limiting milk protein synthesis. The data indicated that histidine, lysine, leucine, threonine and maybe methionine and phenylalanine were most likely to limit milk protein synthesis in lactating dairy cows fed fresh forages.

**Abbreviations:** AA, amino acid(s); A-V, arterio venous difference(s); BCAA, branched-chain amino acid(s); DIM, days in milk; DMI, dry matter intake; EAA, essential amino acid(s); FAA, free amino acid(s); NEAA, non-essential amino acid(s); PCV, packed cell volume.

## 5.2. INTRODUCTION

Global trends in the market for dairy products indicate an increase in the consumption of milk protein, both as food products and isolated compounds (Valeur, 1997; Viatte, 1997). This scenario has created an increasing interest in the mechanisms controlling milk protein synthesis. Particular attention has been given to the understanding of amino acid (AA) metabolism in dairy cows, as it appears to be fundamental to devise strategies to increase both the yield and the efficiency of production of milk protein. Several studies have been conducted to assess whether AA supply to the mammary gland limits milk protein synthesis (Rulquin, 1987; Metcalf *et al.*, 1994; Metcalf *et al.*, 1996; Schwab, 1996; Bauman and Mackle, 1997; Bequette and Backwell, 1997). However, most of the research on AA metabolism has been conducted with concentrate-fed animals. Before attempting to manipulate milk protein synthesis in New Zealand dairy cows, information on the utilisation of AA by the mammary gland of pasture-fed animals is required.

It is well recognised that dairy cows have a requirement for a specific amount and proportion of AA for optimised production of milk (Tamminga and Oldham, 1980; Rulquin *et al.*, 1995). However, there is no real consensus on the quantitative and qualitative definition of the AA requirements for dairy cows. In pasture-fed animals, supplementation with methionine resulted in numerically higher yields of casein during mid lactation, but not in late lactation (Pacheco-Rios *et al.*, 1999). In those studies, we demonstrated that single-AA supplementation was not an adequate way to increase protein synthesis in a consistent manner. Therefore, more research is required on the post-absorptive utilisation of AA in pasture-fed animals in order to elucidate their role as potentially limiting nutrients for milk protein synthesis.

Arterio-venous differences (A-V) of AA across the mammary gland are indicators of the amount of precursors available for the synthesis of milk protein. This measurement has been used to identify the AA which may potentially limit milk protein synthesis in lactating cows (Derrig *et al.*, 1974; Clark *et al.*, 1977) and ewes (Davis and



Bickerstaffe, 1978). Early research (Cary, 1920) demonstrated that free AA (FAA) in plasma were quantitatively the major precursors for milk protein synthesis. Some researchers have suggested, however, that there are significant contributions from other precursor pools (Bequette *et al.*, 1994; Lee *et al.*, 1999), including the erythrocyte (Hanigan *et al.*, 1991). There is still controversy about the relative contribution of plasma and erythrocytes to AA exchange between blood and the mammary gland (*cf.* Hanigan *et al.*, 1991; Cant *et al.*, 1993; Bequette *et al.*, 1997).

In this study, the AA uptake by the mammary gland of lactating cows offered two levels of dry matter intake (DMI) were compared with their output in milk protein as part of a research project on identifying the potential limiting AA in pasture-fed animals. Additionally, this study includes a comparison of the A-V measured in whole blood and plasma to elucidate the role of plasma and red blood cells as pools for AA exchange with the mammary gland.

### **5.3. MATERIALS AND METHODS**

#### **5.3.1. Animals and diets**

Four lactating Friesian cows in early lactation were assigned to a sequence of two levels of dry matter intake (DMI; *ad libitum* and restricted) in a 2x2 crossover design. The average days in milk of the cows was 44 (SD 14.5) with the average live weight being 498 (SD 64.2) kg at the beginning of the experiment. The two 16-day experimental periods comprised a 5-day period for diet adaptation and an 11-day period for measurements of milk production and composition, DMI, nitrogen balance and feed composition. Animals were fed individually and maintained outdoors from day 1 to 5, and in individual metabolism stalls from day 6 to 16 at the Dairying Research Corporation facilities in Hamilton, New Zealand.

The experimental diets consisted of fresh cut ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture which was offered at 6 h intervals (0300, 0900, 1500 and 2100 h). The two treatments consisted of pasture offered *ad libitum* and at 75% of

the *ad libitum* intake established during the 5-day period of diet adaptation. The chemical composition of the pasture offered is shown in Table 5.1

**Table 5.1. Chemical composition of the pasture offered during the experimental periods (% of DM, unless otherwise stated).**

	Period 1	Period 2
Organic matter	91.32	89.07
Crude protein	17.88	15.44
Soluble carbohydrates	19.90	19.90
Acid detergent fibre	29.77	27.63
Neutral detergent fibre	50.44	52.51
Metabolisable energy (MJ kg <sup>-1</sup> DM)	12.09	12.53

On day 11 of each period, the experimental animals were fitted with catheters in the costoabdominal (intercostal) artery, and jugular and mammary (caudal superficial epigastric) veins. The custom-made polyvinyl chloride catheters (1.0 mm ID x 1.5 mm OD; Critchley Electrical Products Pty. Ltd., NSW, Australia) were fitted following tranquillisation of the cows with xylazine hydrochloride (0.8 ml Rompun 2 %; Bayer New Zealand Ltd., Auckland, New Zealand) and local subcutaneous analgesia (lignocaine hydrochloride 2%, Ethical Agents, Auckland, New Zealand). Cows were allowed to recover from the surgery for one day before starting blood collections. The intercostal catheters were maintained during the two experimental periods, while both venous catheters were removed at the end of the first period and new catheters inserted on day 11 of the second period. Patency of all the catheters was ensured by daily flushing with 3 ml of heparinised saline (200 IU/ml). The arterial and mammary vein catheters were used to obtain simultaneous blood samples to assess the uptake of AA across the mammary gland. The jugular catheter was used to infuse a mixture of <sup>13</sup>C-AA to assess the fluxes of AA in the experimental animals (see Chapter 6).

Animals were milked twice daily during the whole experimental period, except during the last 24 hours of each period, when milking was conducted every two hours.

### 5.3.2. Measurements

On day 14 and 15, blood samples were collected from the intercostal artery and the mammary vein for each of two animals. Before starting the blood sampling, animals received a bolus dose of heparin intravenously (New Zealand Pharmaceutical Ltd., Linton, New Zealand; 150 IU kg<sup>-1</sup> liveweight) to minimise clotting in the collection catheter. Blood samples were collected every two hours as 1-hour integrated samples (i.e. continuous sampling for one hour, no sampling for one hour). Samples were collected at a rate of 1 ml/min using peristaltic pumps (Desaga GmbH, D-6900 Heidelberg, Germany) into plastic tubes kept on ice with Na-EDTA added as an anticoagulant. Blood was centrifuged at 3270 g at 4 °C for 15 minutes and plasma was harvested and mixed (10:1 v/v) with a solution of 200 mM phosphate buffer containing 80 mM DL-dithiothreitol. Whole blood was haemolysed with deionised water (1:1 v/v) and mixed with 200 mM phosphate buffer containing 80 mM DL-dithiothreitol (0.1 ml of phosphate buffer per ml of blood). Both whole blood and plasma samples were stored at -85 °C until processed for FAA analysis.

Samples of whole blood and plasma (2 ml) were mixed with 65 µl of 3 mM methionine sulphone as an internal standard, and then deproteinised by ultrafiltration (Centrisart, molecular weight cut-off 10,000; Sartorius AG, Goettingen D-37070, Germany). The ultrafiltrate was collected and stored at -85°C until it was analysed for free AA concentrations.

AA concentrations in the ultrafiltrates from whole blood and plasma were measured after reverse phase HPLC separation of phenylisothiocyanate derivatives (Bidlingmeyer *et al.*, 1984) using a Waters Pico-Tag® column (3.9 x 300 mm; Waters Corporation, Milford, MA 01757, USA) and a Shimadzu LC-10/A HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA) with a 90-minute running time between injections.

Packed cell volume (PCV) was determined using microhaematocrit tubes with blood samples collected directly from the sampling catheter.

At the end of each blood sampling, cows were injected with 1 IU of oxytocin (Oxytocin EA; Ethical Agents Ltd., Auckland, New Zealand) and machine milked. To ensure complete removal of milk, a second oxytocin injection (1 IU) was administered and residual milk was removed by hand-stripping. Milk samples were analysed for protein, fat and lactose using an infra-red analyser (Milk-O-Scan 133B; Foss Electric, Hillerød, Denmark). Total N (TN), non-protein N (NPN) and non-casein N (NCN) were measured using macro-Kjeldahl techniques (Barbano, 1994). These fractions were used to calculate true protein ((TN-NPN) x 6.38), casein protein ((TN-NCN) x 6.38) and whey protein (NCN-NPN) x 6.38).

AA output was determined by measuring the AA concentration in skim milk. A milk subsample was skimmed following centrifugation (200 g, 15 minutes) and stored at -85°C until analysed for AA composition. Freeze-dried skim milk samples were hydrolysed with HCl (AOAC, 1990) and acid stable AA were determined on the hydrolysate using post-column derivatisation with ninhydrin (PCX 3100 Post Column Reaction Module, Pickering Laboratories, Mountain View, CA 94043, USA) and a Shimadzu LC10Ai HPLC system (Shimadzu Scientific Instruments Ltd., Columbia, MD 21046, USA). Similarly, sulphur AA were determined in milk hydrolysed after oxidation with performic acid (AOAC, 1990).

### 5.3.3. Calculations

#### 5.3.3.1. AA concentrations in whole blood, plasma and erythrocyte

The AA concentrations measured in whole blood and plasma samples are expressed as nanomoles per gram of whole blood or plasma. AA concentrations in erythrocytes [ $AA_e$ ] were calculated as described by Lobley *et al.*, (1996):

$$[AA_e] = \frac{[AA_b] - ([AA_p] \times (1 - PCV))}{PCV} \quad (\text{Eq. 5.1})$$

where [ $AA_b$ ] and [ $AA_p$ ] are the concentration of the AA in whole blood and plasma, respectively.

The proportion of whole blood AA present in the erythrocytes was calculated using the erythrocyte concentrations corrected by PCV (Equation 5.2).

$$AA \text{ erythrocyte (\%)} = \frac{([AA_e] \times PCV)}{[AA_b]} \times 100 \quad (\text{Eq. 5.2})$$

### 5.3.3.2. *AA utilisation by the mammary gland*

AA uptake by the mammary gland is described in terms of their A-V (Equation 5.3 -blood- and 5.4 -plasma-), percent extraction (Equation 5.5) and net uptake by the mammary gland (Equation 5.6):

$$A - V = [AA_a] - [AA_v] \quad (\text{Eq. 5.3})$$

where  $[AA_a]$  and  $[AA_v]$  represent the concentration of AA in the arterial and venous samples, respectively. For plasma, the AA A-V were corrected for plasma volume to allow a direct comparison with the uptake calculated from whole blood (Equation 5.4).

$$A - V \text{ from plasma (nmol g}^{-1} \text{ whole blood)} = \text{Plasma } A - V \times (1 - PCV) \quad (\text{Eq. 5.4})$$

$$\text{Percent extraction} = \frac{A - V}{[AA_a]} \quad (\text{Eq. 5.5})$$

$$\text{Net uptake} = A - V \times \text{Mammary blood flow} \quad (\text{Eq. 5.6})$$

The mammary blood flow was obtained as the individual averages of the estimates reported in Chapter 4 using Met and Phe+Tyr as indicators for the Fick principle.

### 5.3.4. Statistical analysis

#### 5.3.4.1. *Differences between whole blood and plasma*

For each AA, the calculated A-V from whole blood and plasma were paired according to sampling time. The uptake of each AA across the mammary gland was tested to be significantly different from zero using Student's *t*-test. Similarly, the difference between the uptakes calculated from whole blood and plasma was tested by means of a paired *t*-test. Both tests were conducted using the PROC MEANS of SAS (SAS Institute Inc., 1996).

#### 5.3.4.2. *Dietary treatment effects*

Means of the concentration of individual AA for each cow were obtained and then analysed using the GLM procedure of SAS (SAS Institute Inc., 1996) for a crossover design (Ratkowsky *et al.*, 1993). Treatments were analysed as main effects and the sequence of treatment and the interaction sequence by treatment were also analysed. The effects of sequence were tested by using the mean square of the effect of cow within sequence as the residual error. Treatment, cow and interaction between treatment and sequence were tested using the residual error. Significant effects were declared when  $P < 0.05$  and trends at  $P < 0.10$ .

## 5.4. RESULTS

Due to problems with the implantation of one of the arterial catheters, deep jugular samples were collected from one animal and those samples treated as their arterial counterparts. However, from the analysis of AA concentrations and tritiated water (see Chapter 4) it was apparent that the deep jugular samples were not representative of the arterial supply. Therefore, all the results collected from one animal were not included in the ANOVA analysis, unless otherwise stated.

Dry matter intakes and milk yields are shown in Table 5.2. The restriction in DMI was achieved as planned, with the restricted group consuming on average 74% of the amount consumed by the *ad libitum* animals. The restriction in DMI tended to reduce both the concentration ( $P=0.07$ ) and yield ( $P=0.09$ ) of milk protein.

**Table 5.2. Dry matter intakes and milk yields during the experimental period ( $n=4$ ).**

	<i>Ad libitum</i>	Restricted	SEM
Dry matter intake (kg d <sup>-1</sup> )	16.7	12.3	0.26*
Milk yield (kg d <sup>-1</sup> )	21.6	19.8	0.52
Milk composition (%)			
Total protein	3.28	3.11	0.033‡
Casein	2.70	2.53	0.040‡
Whey	0.44	0.44	0.017
Fat	4.09	4.12	0.150
Lactose	5.01	5.08	0.038
Yield of components (kg d <sup>-1</sup> )			
Crude protein	0.70	0.61	0.021
Casein	0.58	0.49	0.018‡
Whey	0.10	0.09	0.006
Fat	0.87	0.81	0.054
Lactose	1.08	1.01	0.025

\*  $P < 0.05$

‡  $P < 0.10$

The mean  $\pm$  SE of the PCV for arterial and venous samples were  $27.7 \pm 0.67$  and  $27.9 \pm 0.65$ , respectively.

AA concentrations in whole blood, plasma and erythrocytes are shown in Table 5.3. In general, well-defined chromatograms were obtained from both types of sample. However, aspartic acid and tryptophan co-eluted with unidentified peaks in some cases. Therefore, caution should be exercised in interpreting data for those AA. In haemolysed whole blood samples, the quantification of arginine was hampered by hydrolysis caused by arginase present in the erythrocytes. Thus, whole blood concentration and A-V for this AA are not presented.

**Table 5.3. AA concentrations in arterial whole blood, plasma and red blood cells, together with the proportional contribution of erythrocytes to whole blood free AA in lactating dairy cows fed fresh pasture (Means  $\pm$  SE).**

AA	Concentration (nmol/g)			Contribution of erythrocytes to whole blood (%) <sup>2</sup>
	Whole blood	Plasma	Erythrocyte <sup>1</sup>	
<i>Essential</i>				
Arginine	nd	81 $\pm$ 3.1	nd	nd
Histidine	60 $\pm$ 1.9	36 $\pm$ 2.0	121 $\pm$ 1.8	58 $\pm$ 1.4
Isoleucine	99 $\pm$ 2.8	102 $\pm$ 3.6	92 $\pm$ 4.4	26 $\pm$ 1.0
Leucine	136 $\pm$ 4.9	121 $\pm$ 5.1	176 $\pm$ 6.0	37 $\pm$ 1.0
Lysine	97 $\pm$ 3.0	77 $\pm$ 2.9	149 $\pm$ 6.3	43 $\pm$ 1.3
Methionine	34 $\pm$ 1.4	28 $\pm$ 0.9	52 $\pm$ 4.6	40 $\pm$ 1.5
Phenylalanine	58 $\pm$ 1.7	50 $\pm$ 1.5	78 $\pm$ 3.1	38 $\pm$ 1.1
Threonine	90 $\pm$ 7.1	126 $\pm$ 6.6	1 $\pm$ 15.8	-11 $\pm$ 6.6
Tryptophan	13 $\pm$ 0.8	5 $\pm$ 0.2	33 $\pm$ 1.7	76 $\pm$ 1.1
Tyrosine	55 $\pm$ 1.5	44 $\pm$ 1.6	84 $\pm$ 2.4	43 $\pm$ 1.0
Valine	219 $\pm$ 8.5	231 $\pm$ 9.5	192 $\pm$ 11.5	24 $\pm$ 1.2
<i>Non-essential</i>				
Alanine	146 $\pm$ 5.9	154 $\pm$ 5.3	123 $\pm$ 9.5	23 $\pm$ 1.4
Asparagine	47 $\pm$ 1.4	48 $\pm$ 1.7	44 $\pm$ 2.7	27 $\pm$ 1.6
Aspartic acid	85 $\pm$ 10.3	5 $\pm$ 0.2	297 $\pm$ 37.0	93 $\pm$ 0.6
Glutamine	125 $\pm$ 5.5	227 $\pm$ 9.8	-140 $\pm$ 17.7	-32 $\pm$ 4.7
Glutamic acid	177 $\pm$ 8.1	60 $\pm$ 2.1	472 $\pm$ 22.0	75 $\pm$ 0.9
Glycine	388 $\pm$ 11.1	271 $\pm$ 8.3	698 $\pm$ 30.2	50 $\pm$ 1.0
Proline	99 $\pm$ 2.9	76 $\pm$ 3.0	158 $\pm$ 5.7	45 $\pm$ 0.9
Serine	91 $\pm$ 5.1	106 $\pm$ 5.1	56 $\pm$ 8.3	15 $\pm$ 1.8

<sup>1</sup> From Equation 4.1<sup>2</sup> From Equation 4.2

nd: Not determined

No consistent treatment effects were observed on arterial and venous AA concentrations. The *ad libitum* group had numerically higher arterial concentrations of isoleucine, methionine, tyrosine and valine; whilst the restricted group had numerically higher concentrations of arginine, histidine, leucine, lysine, phenylalanine, threonine and tryptophan. More consistent responses were observed in venous AA concentrations. The *ad libitum* cows had numerically higher venous concentrations of all the essential AA except arginine, histidine and threonine. However, none of these responses were significant.



Similar concentrations in plasma and whole blood (i.e. equilibrium between plasma and erythrocyte) were found for isoleucine, leucine, valine, and asparagine. The concentrations of histidine, lysine, phenylalanine, tryptophan, tyrosine, aspartic acid, glutamic acid and proline were higher in whole blood than in plasma (i.e. accumulation in the erythrocyte). For these AA, erythrocytes contained between 40-90% of the total amount of the AA in the whole blood. On the other hand, plasma concentrations were higher than whole blood for alanine, threonine, glutamine and serine.

**Table 5.4. Arterio-venous differences (nmol g<sup>-1</sup>) calculated from whole blood or plasma AA concentrations in lactating dairy cows fed fresh pasture (means ± SE).**

AA	Arterio-venous differences				
	Whole blood	<i>P</i> <sup>1</sup>	Plasma <sup>1</sup>	<i>P</i> <sup>1</sup>	<i>P</i> <sup>2</sup>
<i>Essential</i>					
Arginine	nd		19.4 ± 1.07	**	nd
Histidine	4.3 ± 0.45	**	4.9 ± 0.52	**	NS
Isoleucine	26.9 ± 1.40	**	23.3 ± 1.36	**	**
Leucine	36.8 ± 1.75	**	34.9 ± 1.87	**	*
Lysine	23.6 ± 1.27	**	22.9 ± 1.38	**	NS
Methionine	8.0 ± 0.43	**	7.8 ± 0.44	**	NS
Phenylalanine	12.2 ± 0.62	**	11.1 ± 0.60	**	NS
Threonine	8.9 ± 1.44	**	13.9 ± 1.50	**	*
Tryptophan	0.8 ± 0.44	NS	0.2 ± 0.11	NS	NS
Tyrosine	11.8 ± 0.65	**	10.3 ± 0.62	**	**
Valine	30.5 ± 1.28	**	29.9 ± 2.44	**	NS
<i>Non-essential</i>					
Alanine	12.8 ± 1.02	**	12.6 ± 1.85	**	NS
Asparagine	9.3 ± 0.39	**	9.7 ± 0.54	**	NS
Aspartic acid	-0.5 ± 1.39	NS	-0.1 ± 0.24	NS	NS
Glutamine	14.6 ± 2.23	**	16.9 ± 2.76	**	NS
Glutamic acid	30.3 ± 2.52	**	28.7 ± 1.28	**	NS
Glycine	2.5 ± 2.40	NS	2.6 ± 2.81	NS	NS
Proline	6.0 ± 0.81	**	6.9 ± 0.98	**	NS
Serine	7.1 ± 1.02	**	8.4 ± 1.59	**	NS

<sup>1</sup> From Equation 4.3

*P*<sup>1</sup> Probability that the uptake is not different from zero.

*P*<sup>2</sup> Probability that the difference between whole blood and plasma is not different from zero.

\*\* *P* < 0.01 Student's *t*.

\* *P* < 0.05 Student's *t*.

NS Non significant.

nd Not determined

Irrespective of the type of sample, the A-V indicated a significant ( $P < 0.01$ ) mammary uptake of all the AA reported, with exception of aspartic acid, glycine and tryptophan. For these AA, the A-V measured from both whole blood and plasma were not different from zero. For most AA, there was no difference in A-V calculated using whole blood or plasma (paired  $t$ -test; Table 5.4). Calculated plasma uptake was higher than that from whole blood for threonine (+56%;  $P < 0.05$ ). On the other hand, whole blood A-V were significantly higher than those from plasma for isoleucine (+15%;  $P < 0.01$ ); leucine (+5%;  $P < 0.05$ ) and tyrosine (+14%;  $P < 0.01$ ).

**Table 5.5. Least square means of the arterio-venous differences (nmol g<sup>-1</sup>) measured in blood or plasma of dairy cows offered *ad libitum* or restricted dry matter intakes.**

AA	Whole blood			Plasma		
	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM
<i>Essential</i>						
Arginine	nd	nd	nd	19.5	21.8	0.07*
Histidine	4.5	4.3	0.54	4.8	6.2	0.39
Isoleucine	31.1	28.1	0.08*	25.2	25.2	0.19
Leucine	40.1	39.8	0.34	36.5	38.7	0.98
Lysine	25.4	25.9	0.91	22.2	25.6	0.79
Methionine	8.0	8.3	1.13	8.4	8.3	0.27
Phenylalanine	12.8	12.8	0.35	11.2	12.5	0.52
Threonine	9.8	10.2	1.33	15.4	16.2	0.73
Tyrosine	12.3	13.2	0.67	10.2	11.8	0.22
Valine	31.7	33.3	1.01	33.1	33.4	0.47
<i>Non essential</i>						
Alanine	15.5	13.3	1.65	16.0	13.5	1.62
Asparagine	10.0	9.8	0.20	10.1	10.6	0.61
Aspartic acid	0.6	0.8	0.91	0.6	-0.3	0.25
Glutamine	14.2	16.1	1.97	14.3	22.6	5.62
Glutamic acid	24.5	36.7	4.57	25.0	33.0	0.27*
Glycine	-1.6	4.7	3.07	1.7	4.8	2.90
Proline	6.2	7.2	1.91	8.4	8.0	0.75
Serine	7.5	8.9	0.52	9.6	11.0	1.21

\*  $P < 0.05$  ANOVA for differences between dietary treatments.

nd: Not determined

Once the effect of pool (plasma vs. whole blood) was analysed, the effect of dietary treatment was assessed by ANOVA of the individual means over the sampling period. No significant effects ( $P > 0.10$ ) of dietary treatment were observed, with the exception of isoleucine in whole blood, and arginine and glutamic acid in plasma (Table 5.5). Similar trends between treatments were observed for whole blood and plasma.

Due to the poor resolution of the tryptophan chromatographic peak in some samples, the number of A-V observations for this amino acid did not allow the statistical analysis of treatment effects. Therefore, the data for tryptophan are not presented.

**Table 5.6. Least square means of the extraction percentage of AA from whole blood or plasma of cows offered *ad libitum* or restricted dry matter intakes.**

AA	Whole blood			Plasma			Prob <sup>2</sup> WB vs P
	<i>Ad libitum</i> <sup>1</sup>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM	
<i>Essential</i>							
Arginine	nd	nd		33.5	43.1	3.54	n/d
Histidine	7.9	7.0	1.53	19.5	25.7	3.60	**
Isoleucine	27.9	31.9	0.57	31.7	40.1	1.19	*
Leucine	26.9	29.8	0.25*	38.9	48.5	0.65†	**
Lysine	24.3	29.3	4.04	36.9	56.0	4.76	**
Methionine	22.7	32.2	0.66*	37.8	54.8	0.19*	*
Phenylalanine	20.8	22.4	0.97	29.4	36.3	1.15	**
Threonine	13.6	14.8	3.08	17.7	27.8	1.18	*
Tyrosine	19.6	27.7	0.28*	28.3	48.5	2.72	**
Valine	12.8	16.5	0.04*	17.4	23.3	0.64†	**
<i>Non essential</i>							
Alanine	10.3	11.1	0.07	13.4	16.0	0.28†	†
Asparagine	20.0	24.3	0.80	27.6	39.3	0.89†	**
Aspartic acid	0.5	0.6	0.79	7.6	-14.2	8.91	NS
Glutamine	11.1	17.4	2.44	8.5	20.1	1.57	NS
Glutamic acid	15.9	20.1	2.90	62.0	72.7	2.13	**
Glycine	-0.5	1.1	0.97	0.3	2.7	1.45	NS
Proline	6.3	8.8	1.20	15.9	19.9	0.55	**
Serine	10.0	11.9	1.07	14.5	19.4	0.15*	*

<sup>1</sup> Difference between treatments ANOVA

<sup>2</sup> Probability that the difference between whole blood and plasma percent extraction is not different from zero (paired *t*-test)

\*\*  $P < 0.01$

\*  $P < 0.05$

†  $P < 0.10$

nd: Not determined

The extraction percentages of AA from whole blood and plasma are presented in Table 5.6. For all the AA analysed, the extraction percentages from plasma were higher than those measured in whole blood. In terms of the dietary treatment effects, the values for the restricted animals were numerically higher than for *ad libitum* animals for both whole blood and plasma. However, for most of the AA, this effect was not statistically significant. The AA for which significant effects of dietary treatment were recorded included leucine, methionine and valine. These AA were extracted at a higher proportion ( $P < 0.10$ ) from both whole blood and plasma in animals under dietary restriction. Alanine, asparagine, serine and tyrosine also had higher extraction in restricted animals. However, this effect reached statistical significance for only one of the pools studied.

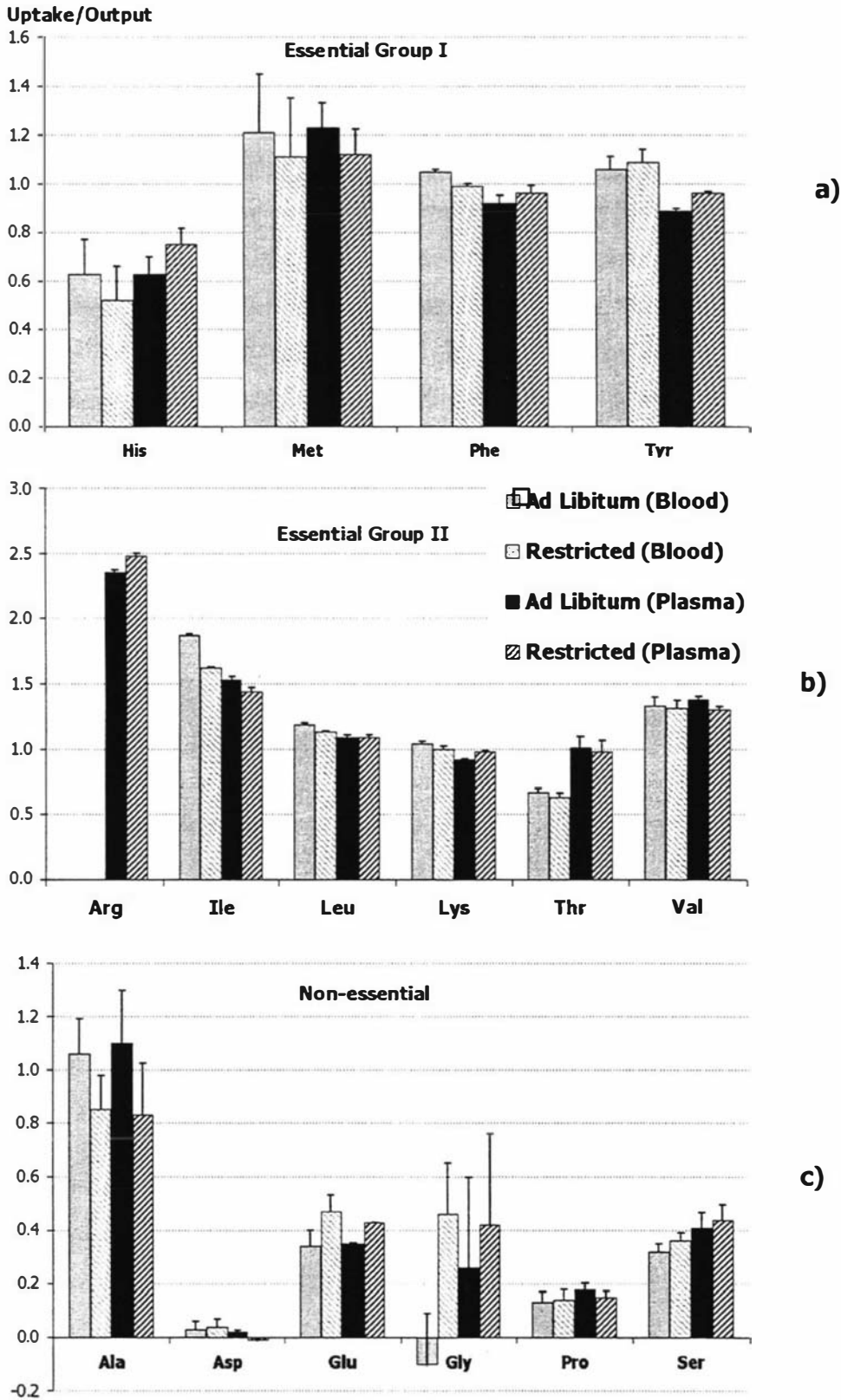
The estimated amounts of AA taken up by the mammary gland (net uptake), together with their corresponding outputs in milk protein of mammary origin are presented in Table 5.7. The amount of total AA taken up by the mammary gland (as calculated from plasma) accounted for 77% of the AA output in milk protein. For the individual EAA, the net uptake by the mammary gland represented between 60 to 250 % of their corresponding output in milk, with the extremes being histidine and arginine respectively.

**Table 5.7. Least square means of the net uptake of AA by the mammary gland from either whole blood or plasma, and their respective output in milk protein of dairy cows offered *ad libitum* (*Ad lib*) or restricted (*Rest*) dry matter intakes.**

	NET UPTAKE (mg h <sup>-1</sup> )						OUTPUT (mg h <sup>-1</sup> )		
	Whole blood			Plasma			<i>Ad lib</i>	<i>Rest</i>	SEM
	<i>Ad lib</i>	<i>Rest</i>	SEM	<i>Ad lib</i>	<i>Rest</i>	SEM			
<i>Essential</i>									
Arginine	nd	nd		1882	1706	102.3	805	692	27.4
Histidine	393	289	69.8	412	426	56.1	653	561	22.2
Isoleucine	2258	1669	128.5	1840	1473	88.8	1206	1036	41.1
Leucine	2912	2354	187.6	2652	2269	81.1	2434	2092	82.9
Lysine	2066	1694	51.5	1816	1668	52.8	1991	1712	67.8
Methionine	673	547	133.4	696	551	61.2	571	491	19.5
Phenylalanine	1180	951	37.4	1032	920	16.7	1128	969	38.4
Threonine	659	520	30.3	1021	856	105.8	1017	874	34.7
Tyrosine	1235	1086	139.8	1029	952	43.3	1164	1000	39.6
Valine	2067	1744	189.03	2154	1727	79.3	1559	1340	53.1
EAA (g d <sup>-1</sup> )	323	260		349	301		301	258	
<i>Non essential</i>									
Alanine	766	537	112.1	793	528	106.7	722	621	24.6
Asparagine	729	576	25.6	745	629	89.1	n/d	n/d	
Aspartic acid	53	48	45.6	40	-10	20.0	2030	1744	69.1
Glutamine	1159	959	77.8	1180	1442	532.1	n/d	n/d	
Glutamic acid	2026	2476	534.3	2066	2179	153.6	6039	5190	205.7
Glycine	-61	189	146.8	86	165	145.7	465	399	15.8
Proline	393	375	130.4	534	405	65.2	3031	2605	103.3
Serine	430	416	12.9	558	521	88.2	1332	1145	45.4
NEAA (g d <sup>-1</sup> )	132	134		144	141		261	224	
Total (g d <sup>-1</sup> )	455	394		485	442		562	482	

\* Difference between treatments  $P < 0.05$ † Difference between treatments  $P < 0.10$ 

nd: Not determined



**Figure 5.1.** Ratio of the net uptake to output of Group I EAA, Group II EAA and NEAA (see text for explanation) across the mammary gland of dairy cows fed *ad libitum* or restricted dry matter intakes. Uptake calculated from both whole blood and plasma.

The calculated net uptake of EAA from plasma was enough to account for their appearance in milk protein, with the exception of histidine, lysine, phenylalanine and tyrosine (Figure 5.1 a) and b). For these AA, the difference between net uptake and output was greater in the *ad libitum* animals. In the case of lysine, phenylalanine and tyrosine, the net uptake calculated from whole blood accounted for the amount secreted in milk protein, but there were small deficits when uptake was calculated from plasma. For histidine there was a deficit (ratio net uptake to output lower than unity) in the uptake irrespective of the pool considered (Table 5.7; Figure 5.1a). In contrast, the threonine uptake from whole blood was approximately 62% of its output. However, the plasma uptake accounted for the output of threonine in milk (Figure 5.1b). For the NEAA, the amounts taken from blood or plasma were lower than their respective secretion in milk protein in all cases (Figure 5.1c).

The relationship between uptake and output of AA across the mammary gland was also described by the regression analysis ( $y=bx$ ) of the output ( $y$ ) as a function of uptake ( $x$ ); the slope ( $b$ ) represents the proportion of the AA uptake appearing in milk protein. Under this premise, a slope higher or lower than unity would indicate a deficit or excess in AA uptake compared with output, respectively. Table 5.8 shows the regression parameters obtained for each AA. From the EAA studied, histidine was the only one exhibiting a slope significantly greater than unity for both whole blood ( $P < 0.10$ ) and plasma ( $P < 0.01$ ) in the current experiment.

**Table 5.8. Regression coefficients (slope  $\pm$  SE) and  $R^2$  values of the regression  $y=bx$ , where  $y$ =AA output and  $x$ =AA uptake from either whole blood or plasma by the mammary gland.**

AA	Blood			Plasma		
	Slope $\pm$ SE	$R^2$	$P \neq 1$	Slope $\pm$ SE	$R^2$	$P \neq 1$
<i>Essential</i>						
Arginine	nd			0.42 $\pm$ 0.013	0.99	< 0.01
Histidine	1.63 $\pm$ 0.261	0.89	< 0.10	1.44 $\pm$ 0.076	0.99	< 0.01
Isoleucine	0.57 $\pm$ 0.053	0.96	< 0.01	0.69 $\pm$ 0.031	0.99	< 0.01
Leucine	0.86 $\pm$ 0.061	0.98	< 0.10	0.94 $\pm$ 0.038	0.99	NS
Lysine	1.00 $\pm$ 0.047	0.99	NS	1.06 $\pm$ 0.037	0.99	NS
Methionine	0.85 $\pm$ 0.071	0.97	< 0.10	0.85 $\pm$ 0.039	0.99	< 0.05
Phenylalanine	0.98 $\pm$ 0.027	0.99	NS	1.09 $\pm$ 0.042	0.99	< 0.10
Threonine	1.42 $\pm$ 0.275	0.84	NS	1.02 $\pm$ 0.042	0.99	NS
Tyrosine	0.92 $\pm$ 0.081	0.96	NS	1.11 $\pm$ 0.052	0.99	< 0.10
Valine	0.77 $\pm$ 0.047	0.98	< 0.01	0.77 $\pm$ 0.037	0.99	< 0.01
<i>Non-essential</i>						
Alanine	1.01 $\pm$ 0.086	0.97	NS	0.96 $\pm$ 0.102	0.94	NS
Aspartic acid	4.35 $\pm$ 5.608	0.11	NS	6.88 $\pm$ 12.835	0.05	NS
Glutamic acid	2.26 $\pm$ 0.277	0.93	< 0.01	2.56 $\pm$ 0.236	0.96	< 0.01
Glycine	0.52 $\pm$ 0.643	0.11	NS	0.97 $\pm$ 0.630	0.32	NS
Proline	6.74 $\pm$ 0.893	0.92	< 0.01	6.03 $\pm$ 0.529	0.96	< 0.01
Serine	2.92 $\pm$ 0.237	0.97	< 0.01	2.20 $\pm$ 0.194	0.96	< 0.01

nd: Not determined

## 5.5. DISCUSSION

From the results obtained in this experiment, two major findings will represent the core of this discussion. Firstly, the results in the present study indicate that plasma is the major pool of FAA for the synthesis of milk protein in the mammary tissue. However, the erythrocytes appear to be involved in the transfer of some AA when the uptake from plasma seems insufficient to account for the AA secreted in milk protein. Secondly, there are indications that amino acid supply may be limiting milk protein synthesis in dairy cows fed fresh forages, as assessed by measurements of AA extraction percentages and the measurement of AA uptake and output as indicators of potentially limiting AA.

The concentrations of AA in either blood or plasma of lactating dairy cows have been well documented as part of numerous studies on AA metabolism. However, most of the data available have been obtained from animals fed different types and amounts



of concentrates. Furthermore, only a few reports have included comparisons between the AA concentrations measured in blood and plasma. This study represents a first step towards a better characterisation of the AA utilisation by lactating cows fed fresh pasture as the sole diet. The plasma AA concentrations measured in the present study are comparable to those of concentrate-fed cows (Pacheco-Rios, review of 37 experiments, Table 2.10) with the exception of methionine (higher in pasture-fed animals) and alanine, glutamate and glycine (higher in concentrate-fed animals). The differences in individual AA concentrations between blood and plasma are similar to those published overseas (Cant *et al.*, 1993).

Plasma is generally regarded as the blood compartment from which AA are extracted by the mammary gland for protein synthesis. However, erythrocytes have been reported as a major reservoir for some AA in blood. The results in the current study confirms previous reports that the erythrocyte accumulates, to a lesser or greater extent, AA present in the blood. The percent contribution of erythrocytes to whole blood AA found in this study (Table 5.3) is similar to that in previously published reports for ruminants (Danilson *et al.*, 1987) and monogastrics (Proenza *et al.*, 1994). For some AA, the accumulation can be related to particular requirements in the erythrocyte e.g. the accumulation of glutamic acid and glycine which are required for the intracellular synthesis of glutathione, an antioxidant tripeptide (Tunnicliff, 1994). However, the purpose of the accumulation of other AA in the erythrocyte remains obscure and it has given rise to the suggestion that erythrocytes contribute actively to the AA exchange between blood and tissues. Therefore, some authors have proposed that using plasma AA concentrations would underestimate their uptake by tissues, as the contribution from erythrocytes is not considered (Heitmann and Bergman, 1980; Danilson *et al.*, 1987; Hanigan *et al.*, 1991). Measurements of arterio-venous differences for whole blood and plasma have shown that erythrocytes are involved in AA transfer across the portal drained viscera of sheep (Heitmann and Bergman, 1980), the hindlimb of calves (Danilson *et al.*, 1987) and the mammary gland of dairy cows (Hanigan *et al.*, 1991).

In the current study, the percent extraction for all the essential AA and asparagine, glutamic acid, proline and serine was greater from plasma than whole blood. This finding is in agreement with previous reports in lactating dairy cows (Cant *et al.*, 1993). The higher percent extraction measured from plasma in this study is the reflection of a limited exchange of AA between the erythrocytes and the mammary tissue. The arterio-venous difference calculated from the erythrocyte AA concentrations in arterial and venous blood were not significantly different from zero ( $P > 0.10$ ) with exception of isoleucine ( $P < 0.01$ ), leucine ( $P = 0.05$ ), phenylalanine ( $P = 0.09$ ) and tyrosine ( $P < 0.01$ ). For these AA, the calculated arterio-venous differences from the erythrocyte were equal to 3.8, 1.8, 1.0 and 1.6 nmol per g of blood. Thus, the lower percent extraction from whole blood indicates that, given the same absolute A-V, a lower proportion of the AA concentration is involved in fluxes between blood and other tissues (i.e. compartmentalised AA in the erythrocyte that are not available for exchange). This finding is in contrast with the report of Hanigan *et al.*, (1991), who found that erythrocytes were involved in the transfer of most AA to the mammary gland, and stated that plasma A-V would underestimate the uptake compared with the A-V from whole blood. However, later research from the same laboratory (Cant *et al.*, 1993) found that plasma A-V, compared with whole blood, overestimated the uptake of some AA by the mammary gland. In those studies, "spot" samples were pooled and then assayed for AA concentrations, a methodological approach that may confound the results (Bequette *et al.*, 1999). In the current study, the data reflects the differences between whole blood and plasma within each hourly-integrated sample. Whole blood and plasma A-V were significantly different only for isoleucine, leucine, threonine and tyrosine. From these AA, the erythrocyte contributed 14% of the A-V of isoleucine and tyrosine across the mammary gland and 5% of the leucine A-V.

In the case of threonine, the plasma A-V was greater than that measured in whole blood. Higher plasma A-V has been described as a result of the transfer of AA from plasma to the red blood cell during the blood transit across tissue beds (Heitmann and Bergman, 1980; Hanigan *et al.*, 1992). The calculated arterio-venous difference of the erythrocyte concentrations indicated a significant (5.4 nmol per g of blood;  $P < 0.05$ ) movement of threonine into the erythrocyte. Alternatively, and by analogy with

the results obtained for arginine, the lower A-V measured in blood could be the result of threonine being degraded during the preparation of the haemolysed sample prior to the AA analysis.

The extraction percentage of AA calculated in this experiment for the restricted animals were similar to those published elsewhere for cows fed concentrate-based diets where either blood (Cant *et al.*, 1993) or plasma A-V were measured (Pacheco-Rios, review of 15 studies,  $n=36-44$ , unpublished data). On the other hand, the extraction percentage of AA in the *ad libitum* group were consistently lower than previous reports, with exception of leucine, glutamic acid and proline. Experiments with cows (Cant *et al.*, 1993) and goats (Nielsen and Jakobsen, 1993) suggested that the mammary gland may increase the extraction of nutrients as a compensatory response to reductions in MBF. In the current study, the restriction in DMI was associated with a 20% reduction in MBF (see Chapter 4). For most of the AA studied, the extraction percentage increased 25-30%, which could be explained as the compensatory response of the mammary gland to the reduction in MBF. However, for some amino acids (lysine, methionine, threonine and tyrosine) the extraction percentage was increased between 40-70% in the restricted cows.

It has been assumed that a relatively low concentration of a particular EAA together with a high extraction of that AA by the mammary gland identifies a potentially limiting AA (Clark *et al.*, 1977; Davis *et al.*, 1978). In the current study, the initial high extraction of leucine in the *ad libitum* group, together with the greater compensatory increases in extraction for lysine, methionine, threonine and tyrosine may suggest that those AA were the most likely to be limiting milk protein synthesis. Davis *et al.*, (1978) found a similar group of potentially limiting AA in their experiment with lactating sheep, while Clark *et al.*, (1977) reported that methionine and lysine were the most critical AA for lactating dairy cows.

Another approach to identifying the potentially limiting AA for milk protein synthesis consists of the comparison of the uptake of a particular AA by the mammary gland and its output in milk protein. The relationship between mammary uptake and

output in milk forms the base of the classification of AA in different groups (Mephram, 1982). Group I comprises the EAA with ratios of uptake to output close to unity: histidine, methionine, phenylalanine+tyrosine and tryptophan. This group is often referred to as a group of potentially limiting AA for milk protein synthesis (Mephram, 1982; Davis and Collier, 1985; Metcalf *et al.*, 1994). Group II includes the EAA with ratios of uptake to output greater than unity (arginine, isoleucine, leucine, lysine, threonine and valine), because of the excess in their uptake, this group is normally considered as secondary in terms of limitation for milk protein synthesis. The last group includes the NEAA, which are normally taken up in amounts that are only a fraction of their respective output in milk protein.

Bequette *et al.*, (1994) found that the uptake of free EAA was insufficient to account for the output of some AA in goat's milk protein. Similarly, in the current study the measured uptake from plasma of histidine, lysine, phenylalanine and tyrosine were lower than their corresponding output in milk protein. For these AA, the uptake by the mammary gland from plasma accounted, on average, for 0.69, 0.94, 0.92, and 0.90 of their output in milk protein, respectively. The differences between uptake and output of AA were greater in *ad libitum* animals. Several possible scenarios may, individually or in combination, explain the observed deficit between uptake and output of AA in the current experiment.

Firstly, AA uptake by the mammary gland is a function of the estimated MBF. In the current study, the MBF used for the calculation of uptake was the average of the MBF estimated from the arterio-venous difference and output of Met and Phe+Tyr (see Chapter 4). For these three AA, caution should be exercised interpreting the ratio of uptake to output, as it was determined *a priori* as close to unity. However, this does not limit the ability to identify, irrespective of the blood flow estimation, the likely order of the AA limitation for milk protein synthesis. In the current study, there was a 10% variation between the estimated MBF using methionine or phenylalanine+tyrosine as indicators. Although this difference did not attain statistical significance, the variation inherent in the estimation of MBF may explain the deficit observed in uptake, at least for lysine, phenylalanine and tyrosine.

A second possible explanation is the presence of alternative precursor pools other than plasma free AA, which may be involved in the transfer of AA to the mammary gland (Bequette *et al.*, 1996). In the current study, the uptake from whole blood accounted for the output of lysine, phenylalanine and tyrosine. Therefore, the deficit (6-10% of the output) in uptake measured from plasma for phenylalanine and tyrosine is in line with the calculated contribution of AA from the erythrocytes for these two AA.

A third explanation consists on the participation of blood-borne peptides, which have been described as sources of AA for milk protein synthesis of lactating dairy goats (Bequette *et al.*, 1999; Lee *et al.*, 1999). In their study, Bequette *et al.*, (1999) reported that for lysine, methionine, phenylalanine and tyrosine, vascular peptides contributed between 5-25% of their supply for casein synthesis in lactating dairy goats. This proportional contribution is compatible with the size of the deficit in AA uptake from plasma measured in the current study.

Histidine was the AA with the greatest proportional difference between uptake and output (a deficit equivalent to 30-40% of its output in milk, depending on the dietary treatment and the pool assayed) in the current study and also in studies with dairy goats (Bequette *et al.*, 1999). In contrast to lysine, phenylalanine and tyrosine, the analysis of histidine uptake from whole blood and plasma did not suggest any involvement of the erythrocyte as a supply of FAA for milk protein synthesis. This finding suggests that although the erythrocyte represents the major reservoir of histidine in whole blood (up to 60%; Table 5.3), this pool does not appear to be readily exchangeable with the mammary tissue.

In the current study, the difference between uptake of histidine by the mammary gland and its output in milk protein was equivalent to 4.2 g of histidine per day, which would have had to be supplied to the mammary gland from pools other than FAA. It is not clear whether the involvement of these alternative precursor pools is obligatory for milk protein synthesis, or it is an indicator of a limited supply of the FAA pool. Histidine has been nominated as the first limiting AA in diets consisting of grass silage

supplemented with cereal (Kim *et al.*, 1999; Korkohen *et al.*, 1999). Korkohen *et al.*, (1999) reported a linear increase in milk protein yield in response to supplementary post-ruminal infusions of histidine in the range of 2-6 g per day. It has been suggested that histidine limits milk protein synthesis in animals fed diets where the bulk of protein reaching the duodenum is of microbial origin (Kim *et al.*, 1999). In a companion experiment to the current study, (Kolver *et al.*, 1999) reported that histidine, arginine and methionine are the most limiting AA, based on their flows at the duodenum. Thus, possibly the low ratio of uptake to output for histidine in the current experiment is the result of a sub-optimal supply of histidine as FAA. More research is required to identify the source of histidine fulfilling the difference observed between its uptake by the gland and its output in milk protein.

Mammary uptake was greater than the corresponding output in milk protein for some AA, irrespective of the dietary treatment. Arginine, isoleucine, leucine, methionine and valine showed uptake in excess of their output in milk protein (2.4, 1.4, 1.1, 1.2 and 1.3 times, respectively). The excess uptake of arginine has been associated with its role as a precursor of proline in the mammary gland (Basch *et al.*, 1997). Proline uptake by the mammary gland accounted for less than 20% of its output in milk protein in the current study. In the case of the branched-chain AA (BCAA), the ratio of uptake to output observed in the current study is comparable to previous reports in lactating dairy goats (Bequette *et al.*, 1999) and dairy cows (Mabjeesh *et al.*, 1999). The excess uptake of BCAA is catabolised in the mammary gland to provide carbon skeletons and  $\alpha$ -amino nitrogen for the synthesis of NEAA, particularly glutamic acid and aspartic acid (Wohlt *et al.*, 1977). These AA, correspondingly, had ratios of uptake to output which were significantly lower than unity. In the current study, the mammary uptake of glutamic acid accounted for only 40% of its output in milk protein. In the case of aspartic acid, the uptake measured from either whole blood or plasma was not significantly different from zero. Assuming that the total excess of EAA is converted to NEAA, the surplus of EAA would account for only 20-25% (expressed on a molar basis) of the observed deficit of the NEAA. However, this proportional contribution is likely to be an overestimation, as the surplus in AA uptake by the mammary gland is also involved in glucogenic, lipogenic and miscellaneous functions (Bender, 1985).

Given this overall imbalance between uptake and output of AA, the role of putative sources of AA (peptides, tissue turnover; Bequette *et al.*, 1999; Lee *et al.*, 1999) appears to be likely.

From the different parameters studied, it can be concluded that some AA may be limiting milk protein synthesis in dairy cows. Histidine, lysine, leucine, methionine, phenylalanine and threonine are the AA which, based on their extraction percent and/or uptake by the mammary gland, are likely to be limiting. In general, the variation observed between the uptake measured from whole blood appears to be a result of the distribution of the AA concentrations between the erythrocyte and plasma. Erythrocytes appear to be involved in the transfer of small, but important amounts of lysine, phenylalanine and tyrosine to fulfil the requirements of the mammary tissue for milk protein synthesis, but conversely act as a non-exchangeable reservoir of histidine. Comparatively, the effect of dietary restriction was mediated mainly through changes in the MBF. Furthermore, dietary restriction did not affect the proportion of the AA uptake that was secreted as milk protein.

More detailed studies comparing the AA requirements for milk protein synthesis with their whole body fluxes could add to the understanding of the differences found using extraction percentages and the ratio of uptake to output in detecting potentially limiting AA for milk protein synthesis. The use of  $^{13}\text{C}$  labelled AA to measure the utilisation of AA in the whole body could provide more information to define limiting AA for milk protein synthesis in dairy cows fed fresh forages. In that way, once the understanding of the post-absorptive utilisation of AA by the lactating dairy cow is complete, it will be possible to devise feeding strategies for optimisation of milk protein synthesis.

## 5.6. ACKNOWLEDGEMENTS

This research was supported by the New Zealand Foundation for Research, Science and Technology. The authors thank the Dairying Research Corporation for providing the facilities and the skillful assistance of Vicki Carruthers, David Phipps,

Erna Jansen and Peter Neil (Dairying Research Corporation). The assistance of Tricia Harris, Jason Peters, and Sarah Cridland (Nutrition and Behaviour, AgResearch Grasslands). The senior author also thanks the National Autonomous University of Mexico for providing the scholarship for his studies.



## 5.7. REFERENCES

AOAC. 1990. Official methods of analysis of the Association of Official Analytical Chemists (AOAC). Arlington, VA, USA: AOAC.

Barbano, D.M. 1994. Reference and routine methods for measurement of N fractions. *Protein definition. International Dairy Federation Special Issue No. 9403* 40-55

Basch, J.J.; Wickham, E.D.; Farrell, H.M.Jr. 1997. Arginase in lactating bovine mammary glands: implications in proline synthesis. *Journal of Dairy Science* **80**:3241-3248

Bauman, D. E.; Mackle, T. R. 1997. Amino acid supply and the regulation of milk protein synthesis. *Proceedings Cornell Nutrition Conference for Feed Manufacturers* 196-207

Bender, D. A. 1985. Amino acid metabolism. Chichester, U.K.: John Wiley & Sons.

Bequette, B.J.; Backwell, F.R.C. 1997. Amino acid supply and metabolism by the ruminant mammary gland. *Proceedings of the Nutrition Society* **56**:593-605

Bequette, B.J.; Backwell, F.R.C.; Calder, A.G.; Metcalf, J.A.; Beever, D.E.; MacRae, J.C.; Lobley, G.E. 1997. Application of a U-<sup>13</sup>C-labeled amino acid tracer in lactating dairy goats for simultaneous measurements of the flux of amino acids in plasma and the partition of amino acids to the mammary gland. *Journal of Dairy Science* **80**:2842-2853

Bequette, B.J.; Backwell, F.R.C.; Dhanoa, M.S.; Walker, A.; Calder, A.G.; Wray-Cahen, D.; Metcalf, J.A.; Sutton, J.D.; Beever, D.E.; Lobley, G.E.; MacRae, J.C. 1994. Kinetics of blood free and milk casein-amino acid labelling in the dairy goat at two stages of lactation. *British Journal of Nutrition* **72**:211-220

Bequette, B.J.; Backwell, F.R.C.; Kyle, C.E.; Calder, A.G.; Buchan, V.; Crompton, L.A.; France, J.; MacRae, J.C. 1999. Vascular sources of phenylalanine, tyrosine, lysine and methionine for casein synthesis in lactating goats. *Journal of Dairy Science* **82**:362-377

Bequette, B.J.; Metcalf, J.A.; Wray-Cahen, D.; Backwell, F.R.C.; Sutton, J.D.; Lomax, M.A.; MacRae, J.C.; Lobley, G.E. 1996. Leucine and protein metabolism in the lactating dairy cow mammary gland: responses to supplemental dietary crude protein intake. *Journal of Dairy Research* **63**:209-222

Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. 1984. Rapid analysis of amino acids using pre-column derivatisation. *Journal of Chromatography* **336**:93-104

Cant, J.P.; DePeters, E.J.; Baldwin, R.L. 1993. Mammary amino acid utilization in dairy cows fed fat and its relationship to milk protein depression. *Journal of Dairy Science* **76**:762-774

Cary, C.A. 1920. Amino acids of the blood as the precursors of milk proteins. *Journal of Biological Chemistry* **43**:477-489

Clark, J.H.; Spires, H.R.; Derrig, R.G.; Bennink, M.R. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. *Journal of Nutrition* **107**:631-644

Danilson, D.A.; Webb, K.E.Jr.; Herbein, J.H. 1987. Transport and hindlimb exchange of peptide and serum protein amino acids in calves fed soy- or urea-based purified diets. *Journal of Animal Science* **64**:1852-1857

Davis, S.R.; Bickerstaffe, R. 1978. Mammary glucose uptake in the lactating ewe and the use of methionine arterio-venous difference for the calculation of mammary blood flow. *Australian Journal of Biological Sciences* **31**:133-139

Davis, S.R.; Bickerstaffe, R.; Hart, D.S. 1978. Amino acid uptake by the mammary gland of the lactating ewe. *Australian Journal of Biological Sciences* **31**:123-132

Davis, S.R.; Collier, R.J. 1985. Mammary blood flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68**:1041-1058

Derrig, R.G.; Clark, J.H.; Davis, C.L. 1974. Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. *Journal of Nutrition* **104**:151-159

Hanigan, M.D.; Calvert, C.C.; DePeters, E.J.; Reis, B.L.; Baldwin, R.L. 1991. Whole blood and plasma amino acid uptakes by lactating bovine mammary glands. *Journal of Dairy Science* **74** :2484-2490

Hanigan, M.D.; Calvert, C.C.; DePeters, E.J.; Reis, B.L.; Baldwin, R.L. 1992. Kinetics of amino acid extraction by lactating mammary glands in control and sometribove-treated Holstein cows. *Journal of Dairy Science* **75**:161-173

Heitmann, R.N.; Bergman, E.N. 1980. Transport of amino acids in whole blood and plasma of sheep. *American Journal of Physiology* **239**:E242-E247

Kim, C.H.; Choung, J.J.; Chamberlain, D.G. 1999. Determination of the first-limiting amino acid for milk production in dairy cows consuming a diet of grass silage and a cereal-based supplement containing feather meal. *Journal of the Science of Food and Agriculture* **79**:1703-1708

Kolver, E.S.; Carruthers, V.R.; Neil, P.G.; De Veth, M.J.; Jansen, E.B.L.; Phipps, D.E. 1999. Amino acid supply to the small intestine of dairy cows fed pasture. *Proceedings of the New Zealand Society of Animal Production* **59**:180-183

Korkohen, M.; Vanhatalo, A.; Varvikko, T.; Huhtanen, P. 1999. Lactation and metabolic responses to graded doses of postruminal histidine infusion in dairy cows fed grass silage-cereal diet. *Protein metabolism and nutrition. Proceedings VIIIth International Symposium* **79**

Lee, J.; Knutson, R.J.; Davis, S.R.; Louie, K.; Mackenzie, D.D.S.; Harris, P.M. 1999. Sulfur amino acid metabolism in the whole body and mammary gland of the lactating Saanen goat. *Australian Journal of Agricultural Research* **50**:413-423

Lobley, G.E.; Connell, A.; Revell, D.K.; Bequette, B.J.; Brown, D.S.; Calder, A.G. 1996. Splanchnic-bed transfer of amino acids in sheep blood and plasma, as monitored through use of multiple U-13C-labelled amino acid mixture. *British Journal of Nutrition* **75**:217-235

Mabjeesh, S.J.; Bruckental, I.; Arieli, A. 1999. Heat-treated whole cottonseed: effect of dietary protein concentration on the performance and amino acid utilization by the mammary gland of dairy cows. *Journal of Dairy Research* **66**:9-22

Mepham, T.B. 1982. Amino acid utilization by lactating mammary gland. *Journal of Dairy Science* **65**:287-298

Metcalf, J.A.; Beever, D.E.; Sutton, J.D.; Wray-Cahen, D.; Evans, R.T.; Humphries, D.J.; Backwell, F.R.C.; Bequette, B.J.; MacRae, J.C. 1994. The effect of supplementary protein on *in vivo* metabolism of the mammary gland in lactating dairy cows. *Journal of Dairy Science* **77**:1816-1827

Metcalf, J.A.; Crompton, L.A.; Wray-Cahen, D.; Lomax, M.A.; Sutton, J.D.; Beever, D.E.; MacRae, J.C.; Bequette, B.J.; Backwell, F.R.C.; Lobley, G.E. 1996. Responses in milk constituents to intravascular administration of two mixtures of amino acids to dairy cows. *Journal of Dairy Science* **79**:1425-1429

Nielsen, M.O.; Jakobsen, K. 1993. Changes in mammary glucose and protein uptake in relation to milk synthesis during lactation in high- and low-yielding goats. *Comparative Biochemistry and Physiology. A. Comparative Physiology* **106A**:359-365

Pacheco-Rios, D.; McNabb, W.C.; Hill, J.P.; Barry, T.N.; Mackenzie, D.D.S. 1999. The effects of methionine supplementation upon milk composition and production of forage-fed dairy cows. *Canadian Journal of Animal Science* **235**-241

Proenza, A.M.; Palou, A.; Roca, P. 1994. Amino acid distribution in human blood. A significant pool of amino acids is adsorbed onto blood cell membranes. *Biochemistry and Molecular Biology International* **34**:971-982

Ratkowsky, D. A., Evans, M. A., and Alldredge, J. R. 1993. Cross-over Experiments: Design, Analysis, and Application. New York, NY.: Marcel Drekker, Inc.

Rulquin, H. 1987. Determination de certain acides amines limitants chez la vache laitiere par la methode des administrations post-ruminales. *Reproduction, Nutrition, Development* **27 (1B)**:299-300

Rulquin, H.; Verite, R.; Guinard, G.; Pisulewski, P. M. 1995 Dairy cows' requirements for amino acids. In *Animal Science Research and Development: Moving Towards a New Century* (Ed. Ivan, M.) 143-160. Ottawa, Canada: Centre for Food and Animal Research, Agriculture and Agri-Food Canada.

SAS Institute Inc. 1996. *SAS User's Guide*. 6.11. Cary, NC

Schwab, C. G. 1996. Amino acid nutrition of the dairy cow: Current status. *Proceedings Cornell Nutrition Conference for Feed Manufacturers* **58**:184-198

Tamminga, S.; Oldham, J.D. 1980. Amino acid utilization by dairy cows. II. Concept of amino acid requirements. *Livestock Production Science* **7**:453-463

Tunnickliff, G. 1994. Amino acid transport by human erythrocyte membranes. *Comparative Biochemistry and Physiology. A. Comparative Physiology* **108A**:471-478

Valeur, J. 1997 Milk protein production and market prospects. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 93-103. Cambridge, UK.: CAB International.

Viatte, G. 1997 Major issues facing the world dairy economy. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 1-11. Cambridge, UK.: CAB International.

Wohlt, J.E.; Clark, J.H.; Derrig, R.G.; Davis, C.L. 1977. Valine, leucine and isoleucine metabolism by lactating bovine mammary tissue. *Journal of Dairy Science* **60**:1875-1882

**CHAPTER 6.**

**WHOLE BODY FLUXES AND PARTITIONING OF  
AMINO ACIDS TO THE MAMMARY GLAND OF COWS  
FED FRESH PASTURE AT TWO LEVELS OF INTAKE  
DURING EARLY LACTATION**

## 6.1. ABSTRACT

The utilisation of essential amino acids (EAA) by the mammary gland of lactating dairy cows fed fresh forages has been studied to provide basic information which can be used to design strategies to increase the production of milk protein in pasture-fed dairy cows. One of the aspects described in this Chapter is the relationship between the flux of EAA in the whole body and their uptake by the mammary gland as an indicator of the amino acid partitioning in lactating cows. Four cows in early lactation were maintained in metabolism stalls to measure the uptake of EAA by the mammary gland and their output in milk protein using an arterio-venous preparation. Concurrently, the whole body fluxes of EAA were estimated using an isotope-dilution method with a mixture of universally labelled  $^{13}\text{C}$ -AA, when the cows were fed *ad libitum* or restricted to 75% of *ad libitum*.

This method proved to be adequate to estimate simultaneously the whole body fluxes of 14 AA in lactating dairy cows. Mammary utilisation accounted for one third of the whole body flux of EAA, with individual AA ranging between 18-36%. Isoleucine, leucine, valine and lysine are the EAA with the greatest partitioning towards the mammary gland of lactating dairy cows (up to 36% of the ILR) and this could reflect a potentially limiting effect on milk protein synthesis. In the case of AA with low partitioning to the mammary gland (i.e. histidine) it is suggested that non-mammary tissues may have priority over the mammary gland and therefore the supply of this AA may also limit milk protein synthesis.

**Abbreviations:** AA, amino acid(s); A-V, arterio venous difference(s); BCAA, branched-chain amino acid(s); DIM, days in milk; DMI, dry matter intake; EAA, essential amino acid(s); FAA, free amino acid(s); IE, isotopic enrichment; ILR, irreversible loss rate; MPE, mole percent excess; NEAA, non-essential amino acid(s); PF; equivalent protein flux.



## 6.2. INTRODUCTION

The characterisation of amino acid (AA) utilisation by the mammary gland is an initial step towards the development of strategies for increasing the output or changing the relative proportions of proteins secreted in the milk of lactating dairy cows. In the previous chapter of this study, the AA utilisation by the mammary gland was described. That approach dealt with the characterisation of the AA utilisation by the mammary gland *per se*. However, it is well understood that the nutrients reaching the mammary gland are ultimately the result of complex interactions between organs and tissues in the whole body. With their concept of homeorhesis, Bauman and Currie (1980) described lactation as the result of a series of metabolic controls which orchestrate a prioritisation of tissues and organs in terms of the relevance of their metabolic function at a particular stage. A major effect of these controls is the diversion (partitioning) of nutrients from other tissues to the mammary gland to sustain lactation, sometimes at the expense of other bodily functions (Champredon *et al.*, 1990).

Although the concept of partitioning is generally accepted for various nutrients and physiological states, there is no quantitative assessment of its magnitude in terms of AA metabolism in lactating dairy cows, particularly when fed diets of fresh forage. Most of the current feeding systems and nutrient requirement guidelines (NRC, 1989; AFRC, 1993) propose that the rates of partitioning to the mammary gland and utilisation for milk protein synthesis is the same for all AA. Even in the most comprehensive studies in dairy cows the partitioning of only a limited number of AA, often in isolation, have been studied (Bruss and Black, 1982; Black *et al.*, 1990; Boirie *et al.*, 1995).

One way to measure the total body fluxes of AA in animals is by quantifying the dilution of a tracer substance (Reeds, 1992; Lobley, 1993). Both radioactive (Hammond *et al.*, 1987; Lobley *et al.*, 1987; Crompton and Lomax, 1993) and stable isotopes (Bequette *et al.*, 1994; Liu *et al.*, 1995; Lapierre *et al.*, 1996; Lapierre *et al.*, 1999) have been used to label AA for this purpose. A technique using the infusion of a mixture of <sup>13</sup>C-universally labelled AA of algal origin has allowed the simultaneous estimation of

the whole body fluxes of an array of amino acids (Lobley *et al.*, 1996; Bequette *et al.*, 1997). Using this technique, several studies have been conducted to study the proportional impact of specific organs and tissue beds on the whole body metabolism of AA in sheep (Lobley *et al.*, 1996) and dairy goats (Bequette *et al.*, 1997). However, no information is available on the simultaneous estimation of the partitioning of amino acids in lactating dairy cows.

This chapter describes the second part of our study, in which a measurement of the whole body AA fluxes was conducted and related to the mammary utilisation of AA described in the previous chapter, to estimate the partitioning of individual AA towards milk protein synthesis. In that way, it is expected to provide, firstly, a characterisation of the whole body AA fluxes in lactating cows fed fresh-forages and secondly, more information to identify those AA which are most likely to limit the synthesis of milk proteins.

### **6.3. MATERIALS AND METHODS**

#### **6.3.1. Animals and diets**

Four lactating Friesian cows in early lactation were assigned to a sequence of two levels of dry matter intake (DMI) in a 2x2 crossover design (*ad libitum* and 75% of *ad libitum* intakes). The average days in milk of the cows was 44 (SD 14.5) with the average live weight being 498 (SD 64.2) kg at the beginning of the experiment. The experiment consisted of two 16-day experimental periods comprised a 5-day period for diet adaptation and an 11-day period for measurement of milk production and composition, DMI, nitrogen balance and feed composition. Animals were fed individually and maintained outdoors from day 1 to 5, and in individual metabolism stalls from day 6 to 16 at the Dairying Research Corporation facilities in Hamilton, New Zealand.

**Table 6.1. Chemical composition of the pasture offered during the experimental periods (% of DM unless otherwise stated).**

	Period 1	Period 2
Organic matter	91.32	89.07
Crude protein	17.88	15.44
Soluble carbohydrates	19.90	19.90
Acid detergent fibre	29.77	27.63
Neutral detergent fibre	50.44	52.51
Metabolisable energy (MJ kg <sup>-1</sup> DM)	12.09	12.53

The experimental diets consisted of fresh cut ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture which was offered at 6 h intervals (0300, 0900, 1500 and 2100 h). The composition of feed samples, which was determined by near-infrared reflectance spectroscopy (NIRSystems Inc., Silver Spring, 20904, MD, USA), is shown in Table 6.1.

On day 11 of each period, the experimental animals were fitted with custom-made polyvinyl chloride catheters (1.0 mm ID x 1.5 mm OD; Critchley Electrical Products, NSW, Australia) in the costoabdominal (intercostal) artery, and jugular and mammary (caudal superficial epigastric) veins. The catheters were fitted in the afore mentioned vessels following tranquillisation with xylazine hydrochloride (0.8 ml Rompun 2%; Bayer New Zealand Ltd., Auckland, New Zealand) and local subcutaneous anaesthesia (lignocaine hydrochloride 2%, Ethical Agents, Auckland, New Zealand). Cows were allowed to recover from the surgery for one day before starting blood sampling and tracer infusions. The intercostal catheters were maintained during the two experimental periods, while both venous catheters were removed at the end of the first period and new catheters inserted on day 11 of the second period. The catheters in the artery and mammary vein allowed simultaneous blood sampling to assess the uptake of AA across the mammary gland, whilst the jugular catheter was used for infusing the isotopic tracer. A bolus injection of heparin (150 IU kg<sup>-1</sup> liveweight; New Zealand Pharmaceuticals Ltd., Linton, New Zealand) was administered before starting blood collection to minimise clot formation in the sampling catheter. Patency of all the catheters was ensured by daily flushing with 3 ml of heparinised saline (200 UI/ml).

Animals were milked twice daily during the whole experiment, except during the last 24 hours of each period, when milking was conducted every two hours. Animals were machine milked after an intravenous injection of 1 IU of oxytocin (Oxytocin EA, Ethical Agents Ltd., Auckland, New Zealand). To ensure complete removal of milk a second oxytocin injection (1 IU) was administered and residual milk was removed by hand stripping.

### 6.3.2. Measurements

Whole body irreversible loss rates (ILR; flux) were measured at the end of each experimental period. Amino acid uptake by the mammary gland was measured (see Chapter 5) and compared to the whole body flux to obtain an estimation of the partitioning of amino acids towards milk protein synthesis. Dry matter intake (DMI) was recorded daily. Milk yield during the infusion period was recorded and samples were collected every two hours and analysed for amino acid composition.

#### 6.3.2.1. Determination of whole body irreversible loss rate of AA

Whole body irreversible loss rates of AA was measured using  $^{13}\text{C}$ -universally labelled AA of algal origin as markers, as described by Lobley *et al.*, (1996).

##### 6.3.2.1.1. Preparation of the mixture of $^{13}\text{C}$ -universally labelled amino acids

The  $^{13}\text{C}$ -AA infusates were prepared using a modification of the procedure described by Lobley *et al.*, (1996). Briefly, the mixture of U- $^{13}\text{C}$ -labelled amino acids was prepared by hydrolysis of de-starched and de-lipidated labelled *Spirulina* (Martek Biosciences Corporation, Columbia MD 21045, USA; enrichment > 98%). Four 18.75-gram batches of *Spirulina* were hydrolysed separately in 3.75 L of boiling 6 M HCl containing 25 mg of phenol crystals and 500 mg of DL-dithiothreitol (DTT) per litre. The hydrolysis was conducted for 22 h under continuous nitrogen flow. The resulting hydrolysate was taken to near-dryness (approx. 15 mL final volume) in a rotary evaporator and diluted in 0.1 M sodium phosphate buffer (approximately 60 ml of buffer per gram of *Spirulina*) and pH adjusted to 7.4. The final infusate was prepared by

diluting this solution (1:2 v/v) with sterile saline and sodium heparin (400 IU g<sup>-1</sup> of the final infusate) and filtering through a 0.2 µm filter prior to storage at -20°C. Infusates were thawed and re-filtered (0.2µm filter) before infusion into the experimental animals.

#### 6.3.2.1.2. Infusions

On day 14 and 15,, the mixture of U-<sup>13</sup>C-labelled amino acids was infused into the jugular vein of two cows for 12 h (day 14) and 2 cows for 6 h (day 15) to determine the whole body ILR of AA in the experimental animals. The isotope mixture was infused at a rate of 1 ml/min. The composition of the infusates is presented in Table 6.2.

**Table 6.2. Concentration (mean ± SD) of amino acids (mmol per ml) of infusates.**

Amino acid	Concentration
<i>Essential</i>	
Histidine	1.23 ± 0.049
Isoleucine	5.64 ± 0.150
Leucine	8.80 ± 0.277
Lysine	3.72 ± 0.128
Methionine	2.23 ± 0.057
Phenylalanine	3.55 ± 0.125
Threonine	5.11 ± 0.160
Tyrosine	1.84 ± 0.069
Valine	6.11 ± 0.197
<i>Non-essential</i>	
Alanine	10.91 ± 0.403
Aspartic acid	8.99 ± 0.279
Glutamic acid	9.80 ± 0.342
Glycine	8.38 ± 0.279
Proline	3.88 ± 0.115
Serine	5.53 ± 0.173

Prior to the infusion, arterial samples were collected to correct for background abundance of <sup>13</sup>C in the blood of the experimental animals. During the isotope infusion, blood was sampled from the intercostal artery and the mammary catheters every two hours as 1-hour integrated samples (i.e. continuous sampling for one hour, no sampling for one hour). Samples were collected at a rate of 1 ml/min using peristaltic pumps (Desaga GmbH, D-6900 Heidelberg, Germany) into plastic tubes kept on ice with Na-EDTA added as an anticoagulant. The blood was subdivided in two and one sample

was centrifuged at 3270 g at 4 °C for 15 minutes. Plasma was harvested and mixed (10:1 v/v) with a solution of 200 mM phosphate buffer containing 80 mM DL-dithiothreitol (DTT). Whole blood was haemolysed with deionised water (1:1 v/v) and mixed with 200 mM phosphate buffer containing 80 mM DL-dithiothreitol (0.1 ml of phosphate buffer per ml of blood). Both whole blood and plasma samples were stored at -85 °C until processed for amino acid concentration and isotopic enrichment analyses.

### 6.3.3. Laboratory methods

#### 6.3.3.1. *Sample preparation for stable isotope determination*

Concentration of  $^{13}\text{C}$  in the AA of whole blood and plasma was determined in deproteinised samples. Samples of whole blood (3 ml) and plasma (1.5 ml) were deproteinised by ultrafiltration (Centrisart, molecular weight cut-off 10,000; Sartorius AG, Goettingen D-37070, Germany). Amino acids in the ultrafiltrate were concentrated and cleaned of interfering ions using ion exchange columns (SCX strong cation exchanger, Alltech Associates, Inc. Deerfield, IL 60015, USA). Columns were conditioned with a sequence of washes (three  $\times$  1-ml of deionised water, two  $\times$  1-ml of 0.005 M HCl, one  $\times$  1-ml of methanol and four  $\times$  1-ml of deionised water). After conditioning, 1 ml of ultrafiltrate was added to the column and washed with 2 ml of 0.01 M HCl. The column was washed with 5 ml of deionised water to remove the acid and the amino acids were eluted and collected in 2 ml of 4 M  $\text{NH}_4\text{OH}$ .

#### 6.3.3.2. *Determination of $^{13}\text{C}$ concentration in samples*

Isotopic enrichment of the samples was measured by gas chromatography-mass spectrometry (Gas chromatograph Model 17A and Mass Selective Detector Model QP5050A, Shimadzu Scientific Instruments Ltd., Columbia, MD, 21046, USA) equipped with a capillary column (DB-5MS; 30 m, 0.25 mm ID, 0.25  $\mu\text{m}$  film thickness; J&W Scientific, Folsom, CA, 95630-4714, USA) and using helium as the carrier gas. The resulting extract from the ion-exchange column was freeze dried and treated with *N-tert.*-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (Fluka; Sigma-Aldrich Pty. Ltd. Castle Hill, NSW, Australia) to yield the *N,O-tert.*-butyldimethylsilyl

(TBDMS) AA derivatives. The mass selective detector was operated in electron impact (EI) mode and selected ion monitoring was performed for the TBDMS (M-15)<sup>+</sup> and (M-159)<sup>+</sup> m/z <sup>12</sup>C- and <sup>13</sup>C-AA derivative ion fragments as outlined by (Chaves das Neves and Vasconcelos, 1987). Analysis of the TBDMS-amino acid derivatives was performed using the mass selective detector in electron impact (EI) mode. The retention times and ions monitored for each amino acid are presented in Table 6.3.

**Table 6.3. Chromatograph retention times of essential amino acids studied and ions monitored using the mass selective detector.**

Amino acid	Retention time (minutes)	m/z ions monitored
<i>Essential</i>		
Histidine	17.50	446/440
Isoleucine	8.82	308/302
Leucine	8.60	308/302
Lysine	15.50	437/431
Methionine	10.58	325/320
Phenylalanine	11.72	345/336
Threonine	10.98	408/404
Tyrosine	18.08	475/466
Valine	8.26	293/288
<i>Non-essential</i>		
Alanine	7.22	263/260
Glutamic acid	13.97	437/432
Glycine	7.51	248/246
Proline	9.11	291/286
Serine	10.72	393/390

#### 6.3.4. Calculations

The isotopic enrichment (IE) of the amino acids was calculated using Equation 6.1 (Lobley *et al.*, 1996):

$$IE = \frac{(R_t - R_0)}{1 + (R_t - R_0)} \quad (\text{Eq. 6.1})$$

where *IE* is expressed as mole percent excess (mpe) and *R* is the ratio of the mass of the monitored fragment containing the <sup>13</sup>C over the mass of the unlabelled molecule. The subscript *t* refers to the enrichment measured at time *t* over the infusion

period, while the subscript 0 indicates the enrichment measured in background samples (arterial) obtained before the beginning of the isotope infusion.

The values obtained from the different sampling times for each cow were then used to calculate the isotopic enrichment of amino acids in whole blood and plasma at steady state by fitting the exponential model defined by Equation 6.2 to the mean of  $IE$  obtained from duplicate samples for each amino acid:

$$IE = A \times (1 - e^{-kt}) \quad (\text{Eq. 6.2})$$

where  $A$  defines the plateau value of  $IE$  assuming the enrichment increases at a rate  $k$  over time  $t$  (Bequette *et al.*, 1998).

The whole-body ILR ( $\text{mmol h}^{-1}$ ) for each amino acid was determined using the model described in Equation 6.3:

$$ILR = \left( \frac{IE_{\text{inf}}}{IE_{b \text{ or } p}} - 1 \right) \times IR \quad (\text{Eq. 6.3})$$

in which  $IR$  is the infusion rate ( $\text{mmol h}^{-1}$ ) for each amino acid and  $IE_{\text{inf}}$  is the isotopic enrichment of the infusate (98%) and  $IE_{b \text{ or } p}$  is the isotopic enrichment of the corresponding amino acid in blood or plasma at plateau (Lobley *et al.*, 1996).

The ILR of essential AA were transformed to equivalent daily protein fluxes (PF;  $\text{g d}^{-1}$ ) by:

$$PF = \frac{ILR \times 2.4 \times MW_{aa}}{[AA]_{\text{whole body}}} \quad (\text{Eq. 6.4})$$

where  $MW_{aa}$  is the molecular mass of the AA, and  $[AA]_{\text{whole body}}$  is the percentage of the AA in whole body protein (calculated from Gibb *et al.*, 1992; MacRae *et al.*, 1993).



Partitioning of each amino acid to the mammary gland was expressed as the percent contribution of net uptake of amino acid to the whole body ILR, as described by Bequette *et al.*, (1997):

$$\frac{\text{Net uptake (NU)}}{\text{Whole body ILR}} \times 100 \quad (\text{Eq. 6.5})$$

where net uptake is:

$$NU = [AA_a] - [AA_v] \times MBF \quad (\text{Eq.6.6})$$

given that  $[AA_a]$  and  $[AA_v]$  represent the concentrations of unlabelled AA in the arterial and venous samples, respectively, from either whole blood or plasma.

### 6.3.5. Statistical methods

#### 6.3.5.1. Estimation of plateau of enrichment in samples

Plateau values for the ILR calculation were obtained from the exponential model described in Eq. 6.2. For calculation purposes, it was assumed that pseudo-plateau were attained when the slope of the regression of enrichment from time 6-12 was not significantly different from zero.

#### 6.3.5.2. Treatment and pool effects

Means of the concentration of individual EAA for each cow were obtained and then analysed using the GLM procedure of SAS (SAS Institute Inc., 1996) for a crossover design (Ratkowsky *et al.*, 1993). Treatments were analysed as main effects and the sequence of treatment and the interaction (*sequence x treatment*) were also analysed. The effects of sequence were tested by using the mean square of the effect of cow within sequence as the residual error. Treatment, cow and interaction between treatment and sequence were tested using the residual error. Because of the reduced number of animals used in this analysis, significant effects were declared when  $P < 0.05$  and trends are discussed when  $P < 0.10$ .

Comparison between whole blood and plasma (where applicable) were conducted by means of paired *t*-test using the MEANS procedure of SAS (SAS Institute Inc., 1996). Significant effects were declared when  $P < 0.05$ .

### 6.3.5.3. Ranking of partitioning

The analysis of the partitioning of EAA to the mammary gland was performed on the ranked values using non-parametric ANOVA (SAS Institute Inc., 1996). For each animal and period, the amino acids were ranked according to their partitioning percentage (i.e. the amino acid with the highest partition to the mammary gland had a score of 1, the second highest 2, and so on).

## 6.4. RESULTS

Compared with the *ad libitum* period, animals consumed 26% less DM during the period of DMI restriction (12.3 vs. 16.7 kg DM d<sup>-1</sup>;  $P < 0.01$ ). The restriction in dry matter intake was associated with a 13% reduction in the yield of protein (0.61 vs. 0.70 kg d<sup>-1</sup>;  $P = 0.09$ ). The reduction in protein yield was, in turn, the combined effect of both a reduction in milk yield (19.8 vs. 21.6 kg d<sup>-1</sup>;  $P = 0.14$ ) and a decrease in the concentration of milk protein (31.1 vs. 32.8 g kg<sup>-1</sup>;  $P = 0.09$ ).

**Table 6.4. Least square means of the different components of the nitrogen balance measurements during the experimental periods (days 6 to 11;  $n=4$ ).**

	<i>Ad libitum</i>	Restricted	SEM	P
N intake (g d <sup>-1</sup> )	0.445	0.328	0.0083	*
Milk N (g d <sup>-1</sup> )	0.111	0.097	0.0039	NS
Faecal N (g d <sup>-1</sup> )	0.143	0.082	0.0094	*
Urine N (g d <sup>-1</sup> )	0.173	0.141	0.0055	‡
Total N excretion (g d <sup>-1</sup> )	0.426	0.320	0.0091	*
N balance (g d <sup>-1</sup> )	0.019	0.009	0.0072	NS
N intake to milk (%)	24.9	29.3	0.51	*
N intake to faeces (%)	31.7	24.8	1.90	NS
N intake to urine (%)	38.8	43.3	2.73	NS
N retained (%)	4.5	2.6	1.75	NS

\*  $P < 0.05$ ; ANOVA; *ad libitum* vs. restricted

‡  $P < 0.10$ ; ANOVA; *ad libitum* vs. restricted

The dietary restriction caused a lower N balance (Table 6.4). There was a significant ( $P < 0.05$ ) reduction in N intake as a consequence of the dietary restriction. This resulted in decreased ( $P < 0.05$ ) N excretion in faeces and urine.

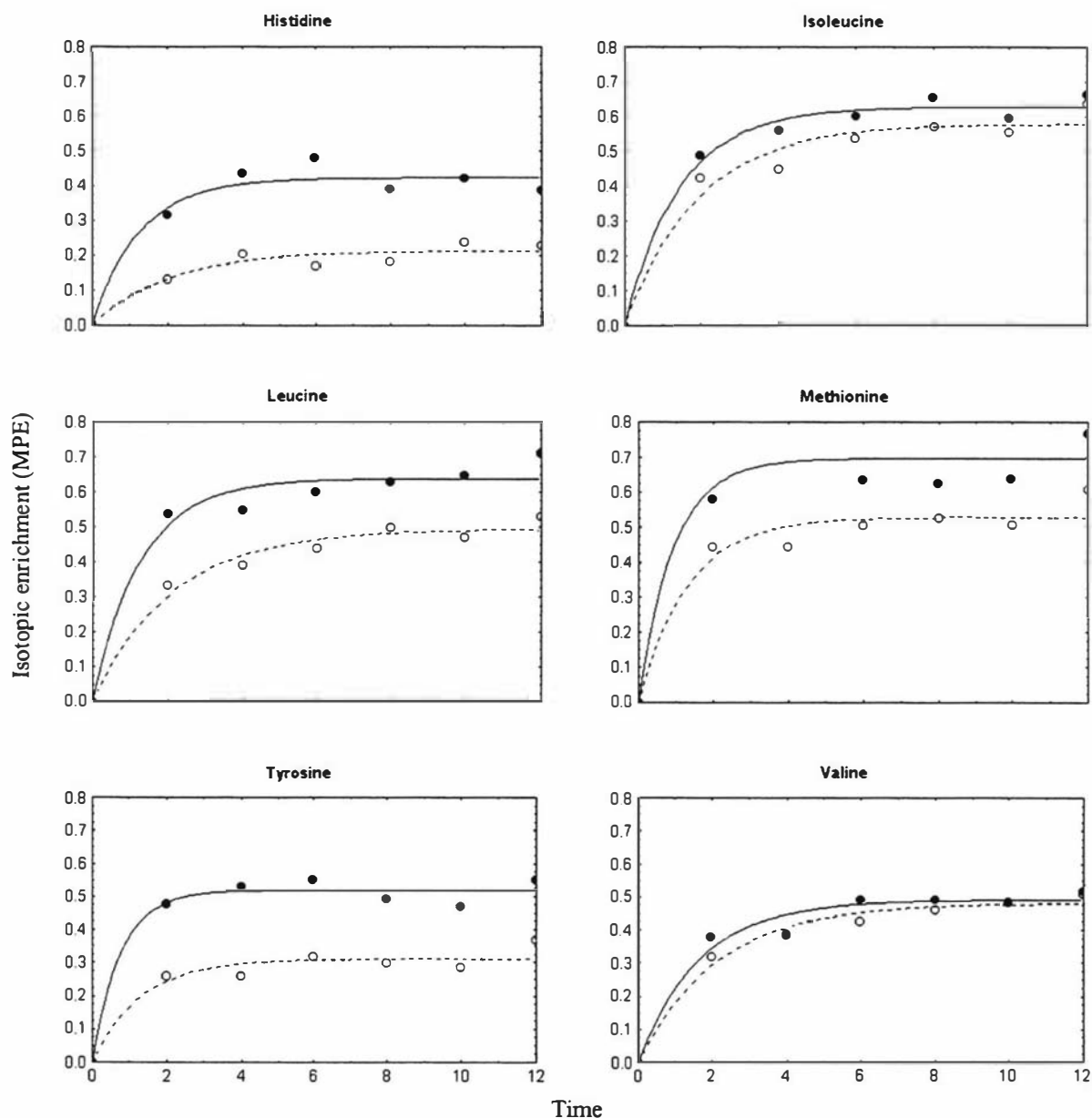


Figure 6.1. Examples of the isotopic enrichment curves for His, Ile, Leu, Met, Tyr and Val, as measured in whole blood (○) or plasma (●) in one cow during the course of infusion in Period 1, together with the fitted lines from  $MPE = A(1 - e^{-kt})$  to determine the plateau values in whole blood (broken line) and plasma (solid line).

No adverse reactions were observed as result of the infusion of the *Spirulina* hydrolysate. Most of the amino acids studied exhibited a rapid increase in  $^{13}\text{C}$  enrichment over the first 4-h of the infusion period, with a discernible plateau attained after this time (Figure 6.1).

The data collected from the animals with the 12-h infusion showed that the coefficient of variation of the *IE* was less than 10% over the period 6-12 h for most of the essential amino acids studied, with exception of methionine. The regression analysis of *IE* versus time (6-12 hours) revealed that, in most cases, the slope was not significantly different from zero ( $P > 0.05$ ; i.e. plateau were attained).

**Table 6.5. Plateau isotopic enrichment ( $^{13}\text{C}$  MPE) of amino acids in whole blood or plasma as determined from the model  $IE=A(1-e^{-kt})$ .**

	Whole blood			Plasma			WB vs. P
	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM	
<i>Essential</i>							
Histidine	0.22	0.26	0.009	0.46	0.52	0.043	§
Isoleucine	0.63	0.87	0.052	0.71	0.95	0.052	§
Leucine	0.57	0.71	0.012‡	0.74	0.94	0.003*	§
Lysine	0.37	0.45	0.012‡	0.54	0.72	0.015‡	§
Methionine	0.59	0.97	0.086	0.78	0.97	0.024‡	
Phenylalanine	0.62	0.69	0.021	0.73	0.87	0.052	§
Threonine	0.46	0.52	0.058	0.57	0.81	0.052	
Tyrosine	0.35	0.44	0.012‡	0.52	0.73	0.040	§
Valine	0.50	0.68	0.028‡	0.55	0.73	0.012‡	§
<i>Non-essential</i>							
Alanine	0.35	0.57	0.006*	0.46	0.66	0.021‡	§
Glutamic acid	0.28	0.28	0.009	0.85	0.71	0.147	§
Glycine	0.24	0.37	0.012‡	0.45	0.60	0.028	§
Proline	0.49	0.65	0.003*	0.86	1.10	0.024‡	§
Serine	0.39	0.45	0.098	0.37	0.50	0.037	

§  $P < 0.01$ ; *t*-test; whole blood vs. plasma

\*  $P < 0.05$ ; ANOVA; *ad libitum* vs. restricted

‡  $P < 0.10$ ; ANOVA; *ad libitum* vs. restricted

The  $^{13}\text{C}$ -enrichment measured in whole blood ranged from 0.22 to 0.97 with the values for histidine and methionine being the extremes. In plasma, the *IE* ranged from 0.37 (Pro) to 1.10 (Ser). Plasma had higher *IE* for all the amino acids studied, although the effect was not significant ( $P > 0.05$ ) for methionine, threonine and serine. Irrespective of the pool studied, the restricted animals had numerically higher *IE* than

the cows with *ad libitum* intakes, however the treatment effect attained statistical significance for only some amino acids (Table 6.5).

The ILR of the AA studied is presented in Table 6.6. The ILR calculated using whole blood isotopic enrichments were higher than those calculated from plasma. Irrespective of the type of sample analysed, the animals undergoing the dietary restriction had ILR that were lower than those measured in animals fed *ad libitum*.

**Table 6.6. Whole body irreversible loss rates (ILR; mmol h<sup>-1</sup>) of amino acids, calculated from arterial whole blood or plasma <sup>13</sup>C isotopic enrichments, of lactating dairy cows fed *ad libitum* or restricted dry matter intakes (n=3).**

	Whole blood			Plasma			WB vs. P
	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM	
<i>Essential</i>							
Histidine	33.5	29.1	1.07	14.9	14.4	0.43	§§
Isoleucine	52.3	38.6	0.84‡	46.8	35.3	0.20*	§
Leucine	88.0	72.8	1.44‡	68.7	55.1	2.75	§§
Lysine	59.6	47.9	1.43	40.1	31.8	0.07*	§§
Methionine	22.8	13.5	1.66	16.8	14.3	0.31‡	
Phenylalanine	35.6	30.7	2.92	28.7	24.5	0.98	§§
Threonine	71.2	59.3	8.28	50.6	36.1	0.63*	§
Tyrosine	29.6	26.3	0.87	19.5	15.9	1.56	§§
Valine	71.8	52.7	3.86	63.4	49.8	1.94‡	§
<i>TEAA</i>	464.4	370.9		349.5	277.2		
<i>Non-essential</i>							
Alanine	169.6	119.3	6.52	132.8	101.0	1.69*	§§
Glutamic acid	192.0	200.6	11.44	69.8	75.5	24.77	§§
Glycine	202.4	137.4	7.47‡	121.5	85.2	17.54	
Proline	45.4	36.3	1.58	25.2	20.0	0.64‡	§§
Serine	101.1	73.0	33.00	78.6	66.4	9.60	
<i>TNEAA</i>	710.5	566.6		427.9	348.1		
<i>TAA</i>	1174.9	937.5		777.4	625.3		

§§  $P < 0.01$ ; *t*-test; whole blood vs. plasma

§  $P < 0.05$ ; *t*-test; whole blood vs. plasma

\*  $P < 0.05$ ; ANOVA; *ad libitum* vs. restricted

‡  $P < 0.10$ ; ANOVA; *ad libitum* vs. restricted

Depending on the AA and pool analysed (whole blood or plasma) the ILR of AA in restricted animals was 65-95% of that observed in *ad libitum* animals (average 89% and 80% using whole blood and plasma *IE*, respectively). However, this effect was statistically significant for isoleucine and leucine only ( $P < 0.10$ ; whole blood) and for

isoleucine, threonine, and alanine ( $P < 0.10$ ; plasma). Glutamic acid was the only AA for which the ILR was numerically increased in restricted animals. In those cases, the ILR of glutamic acid in restricted animals was 104% and 108% (whole blood and plasma, respectively) of that measured in the *ad libitum* group.

The equivalent protein fluxes (uncorrected for oxidation) calculated from the ILR of EAA are presented in Table 6.7.

**Table 6.7. Whole body equivalent protein fluxes (PF; g d<sup>-1</sup>); as calculated from the ILR of essential amino acids of lactating dairy cows fed *ad libitum* or restricted dry matter intakes ( $n=3$ ).**

	Whole blood			Plasma		
	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM
Histidine	4920	4274	202.9	1795	1809	95.9
Isoleucine	4097	2923	109.5†	3579	2602	10.5*
Leucine	3243	2658	96.8	2343	1827	157.8
Lysine	2623	2062	108.3	1512	1148	30.3‡
Methionine	4238	2274	400.9	2901	2464	39.4‡
Phenylalanine	3181	2744	348.7	2423	2058	134.4
Threonine	3983	3301	544.5	2672	1830	58.4‡
Tyrosine	3499	3134	98.0	1977	1560	201.7
Valine	4336	3062	320.2	3712	2846	177.9

\*\*  $P < 0.01$

\*  $P < 0.05$

‡  $P < 0.10$

Due to catheter problems, it was not possible to sample arterial blood from one cow. Therefore, the results obtained from that animal are not included in the statistical analysis and are presented separately in the Table A.5.1 of the Appendix.

The partitioning of amino acids to the mammary gland ranged from 6-33% and 18-36% as calculated from whole blood or plasma ILR, respectively (Table 6.8). For the non-essentials amino acids (NEAA), the partitioning to the mammary gland was between 0-20%, and for most of those AA it was below 10%. Partitioning for most EAA was remarkably similar for *ad libitum* and restricted animals, and in many cases was similar when the calculation was done using analysis of whole blood or plasma. Isoleucine, lysine, leucine and valine, in that order, were the amino acids with the highest partitioning to the mammary gland. This finding was consistent between animals and treatments, as reflected by their ranks.

**Table 6.8. Partitioning (net uptake/ILR\*100) of amino acids from whole blood and plasma to the mammary gland of lactating dairy cows fed fresh pasture at *ad libitum* and restricted intakes (*n*=3).**

	Whole blood			Plasma			Rank <sup>1</sup>
	<i>Ad libitum</i>	Restricted	SEM	<i>Ad libitum</i>	Restricted	SEM	
<i>Essential</i>							
Histidine	8	6	2.3	18	19	3.4	9 <sup>d</sup>
Isoleucine	33	33	1.7	30	32	1.4	1 <sup>a</sup>
Leucine	25	25	2.0	30	31	2.1	3 <sup>ab</sup>
Lysine	24	24	1.5	31	36	1.1	2 <sup>ab</sup>
Methionine	20	29	6.3	28	26	2.6	6 <sup>bc</sup>
Phenylalanine	20	19	2.6	22	23	0.7	7 <sup>cd</sup>
Threonine	9	7	1.8	17	20	2.5	8 <sup>d</sup>
Tyrosine	23	23	1.6	29	34	1.5	5 <sup>ab</sup>
Valine	25	28	3.9	29	29	2.4	4 <sup>ab</sup>
<i>Non-essential</i>							
Alanine	5	5	0.7	7	6	1.2	
Glutamic acid	7	9	2.7	22	20	8.4	
Glycine	0	2	1.0	1	3	2.0	
Proline	7	9	3.8	18	18	3.5	
Serine	4	6	1.6	7	7	0.7	

<sup>1</sup> For the EAA different letters indicate a significant difference  $P < 0.05$ . Non-parametric ANOVA of ranked values.

## 6.5. DISCUSSION

The use of *Spirulina* <sup>13</sup>C-AA has been a valuable tool to estimate simultaneously the fluxes of AA in different tissues and species (Lobley *et al.*, 1996; Bequette *et al.*, 1997). To our knowledge, the current study represents the first report of such measurements in lactating dairy cows fed fresh forages.

The uptake of AA by the mammary gland for milk protein synthesis represented up to 36% of the whole body flux of AA in lactating cows fed fresh forages. The implications of such high demand in terms of the potentially limiting AA for milk protein synthesis are discussed.

### 6.5.1. Methodological considerations

The validity of the ILR estimation relies on the assumption that the isotopic enrichment of plasma is representative of steady state conditions during the

measurement period (Lobley, 1993). The data collected from the animals with the 12-h infusion had a coefficient of variation for the *IE* of less than 10% over the period 6-12 h for most of the AA studied, with the exception of methionine (CV = 17%). This finding, together with the sharp increase in  $^{13}\text{C}$ -enrichment measured during the first four hours of the infusion (Figure 6.1) indicated that enrichment plateau were attained also in the cows receiving the 6-hour infusion.

The reported presence of D-isomers of AA in the *Spirulina* hydrolysates has to be borne in mind when interpreting the ILR values obtained in the current study. Using a similar technique to produce the algal infusates, Bequette *et al.* (1997) reported that up to 20% of the lysine in the hydrolysate is present as D-isomer, while for the rest of the AA, the contribution of D-isomers is less than 4%. Similar results were obtained by Lobley (pers. comm.). In the current study it was not possible to analyse the infusates for the presence of D-isomers due to analytical limitations. Given the results observed by other researchers using similar techniques, the value of ILR for lysine may be an underestimation of the true value, as suggested by the low equivalent protein flux obtained from lysine ILR. For the rest of the AA, the bias arising from the presence of D-isomers would be less than 5%.

The variation in *IE* observed between individual AA and between whole blood and plasma is comparable to that in previous studies using the same technique with sheep and goats (Table 6.9).



**Table 6.9. Comparison of the ratio of isotopic enrichments measured in whole blood and plasma in this study and previously published studies with sheep (Lobley *et al.*, 1996) and goats (Bequette *et al.*, 1997).**

	<i>IE</i> Whole blood : Plasma		
	Dairy cow (this study)	Sheep (Lobley <i>et al.</i> , 1996)	Goats (Bequette <i>et al.</i> , 1997)
<i>Essential</i>			
Histidine	0.49	0.44	0.46
Isoleucine	0.90	0.93	0.96
Leucine	0.76	0.85	0.86
Lysine	0.66	0.75	0.61
Methionine	0.88	0.77	0.80
Phenylalanine	0.82	0.86	0.91
Threonine	0.72	0.76	0.68
Tyrosine	0.64	0.75	0.77
Valine	0.92	0.95	0.94
<i>Non-essential</i>			
Alanine	0.81	0.77	0.81
Glutamic acid	0.36	0.50	-
Glycine	0.58	0.66	-
Proline	0.58	0.61	0.69
Serine	0.98	0.73	0.83

Assuming that the composition of the *Spirulina* hydrolysates used in the current study was similar to those used in sheep (Lobley *et al.*, 1996) and goats (Bequette *et al.*, 1997), the variation in *IE* between the studies is most likely due to inter-species variability in AA concentrations. In this study, the measured *IE* is compatible with the relative AA concentrations measured in whole blood and plasma, (i.e. the higher the concentration of unlabelled amino acid, the lower the measured enrichment). As the isotopes are infused into the plasma, it is expected that a higher *IE* would be measured in this pool. The lower isotopic enrichment in whole blood than plasma samples has been attributed to either an incomplete equilibrium across the erythrocyte membrane, or the dilution of intracellular AA with unlabelled molecules originating from peptide hydrolysis inside the erythrocyte (Backwell *et al.*, 1994).

Another methodological consideration is related with the site of infusion of the tracer. It has been reported that up to 65% of the AA-N appearing in the portal circulation is removed by the liver (Lescoat *et al.*, 1996). When the tracer is infused

directly into peripheral circulation (as in this study: jugular vein) it is not possible to account for the modulator effect of the liver as a result of the first-pass removal of AA from the portal circulation (Bruss and Black, 1982). The unaccounted variations due to the hepatic uptake or release of AA could be responsible for some of the variation observed in the equivalent protein flux calculated from ILR of individual AA.

### 6.5.2. Irreversible loss rates

The ILR of AA measured in this study are comparable when expressed on a metabolic weight basis to those obtained in lactating dairy goats (Bequette *et al.*, 1997) using the same type of algal tracer. Studies in dairy cows had been limited to the single AA-tracer approach (histidine: Bruss and Black (1982); leucine: Bequette *et al.* (1996b)). Thus, the current study represents, to our knowledge, the first report of simultaneous estimation of an array of AA in lactating dairy cows. Despite the differences in methodology and amount of tracer used, the results obtained here are similar to those reported in the forementioned studies (Table 6.10).

**Table 6.10. Comparison of the ILR ( $\mu\text{mol h}^{-1}$  per kg  $\text{LW}^{0.75}$ ) of selected EAA measured in lactating dairy cows.**

Method	Irreversible loss rate		
	This study	(Bruss and Black, 1982)	(Bequette <i>et al.</i> , 1996b)
	Continuous infusion	Flooding dose	Continuous infusion
Histidine	133	89	
Leucine (whole blood)	770		890
Leucine (plasma)	590		740

For all AA, dietary restriction resulted in a numerical decrease in ILR irrespective of the type of sample (whole blood or plasma) analysed. Similar effects of feed intake on leucine ILR have been reported in several species (beef cattle: Hammond *et al.*, 1987; Lobley *et al.*, 1987; Lapierre *et al.*, 1996; Lapierre *et al.*, 1999); sheep: Liu *et al.*, 1995; and dairy goats: Riis, 1988). The only exception to this finding was glutamic acid, for which the ILR in restricted animals was numerically increased.

Although the transfer of  $^{13}\text{C}$  from glutamine occurring during the sample preparation prior to the GC-MS analysis may confound the *IE* for glutamic acid (Tavendale, pers. comm.), this effect would appear equally in both treatments. Thus, this finding could be explained in terms of the important role of glutamic acid and glutamine in transport of N between organs (Lacey and Wilmore, 1990; Black *et al.*, 1990). In contrast to the rest of the AA, the liver releases glutamic acid and glutamine to the peripheral circulation (Lobley *et al.*, 1996; Lopez *et al.*, 1998). During diet deprivation, the hepatic release of these two AA has been reported to increase in rats (Lopez *et al.*, 1998) as means of channelling N originating from tissue protein mobilisation (particularly from the muscle). Therefore, the numerical increase in the whole body flux of glutamic acid measured in the current experiment could be attributed to the mobilisation of body reserves as a result of the dietary restriction.

### 6.5.3. Partitioning

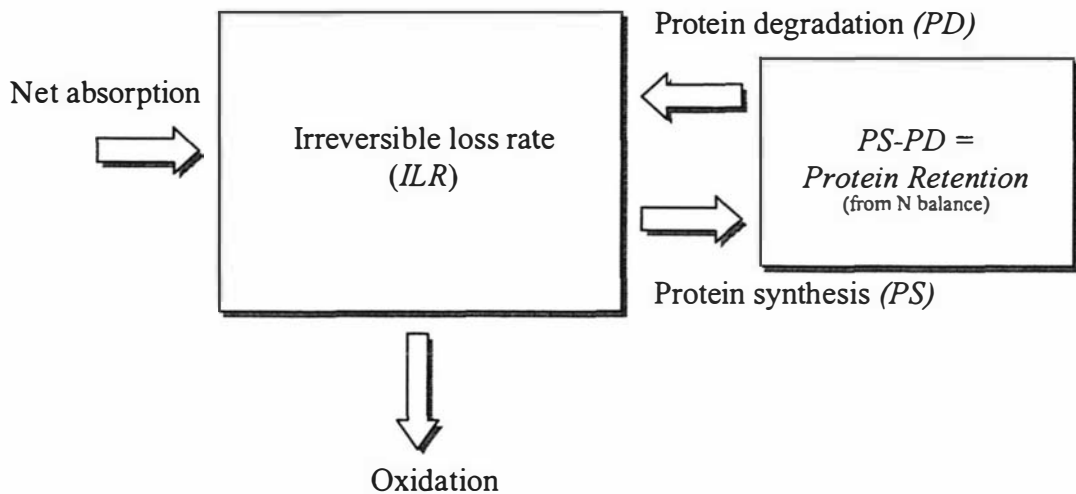


Figure 6.2. Schematic representation of the two-compartment model used to describe the whole body flux (ILR) of amino acids (modified from Lobley, 1993).

Based on the two-compartment model described by Lobley (1993; Figure 6.2), the ILR represents the sum of the net fluxes of AA utilised for protein synthesis and oxidation. Under steady state conditions, the ILR can be equated to the sum of AA absorbed from the lumen of the gastrointestinal tract (GIT) and those originating from protein breakdown in the tissues (protein turnover). This model was used to estimate the contribution of each of those four processes (absorption from the GIT, oxidation, whole body protein synthesis and degradation) in the protein metabolism of the experimental animals. For discussion purposes, the calculated contribution to, and partitioning of, the AA flux is presented in Table 6.11.

**Table 6.11. Estimation of the contribution of EAA absorbed or protein breakdown to the AA flux and the partitioning of this flux between protein synthesis and oxidation in lactating dairy cows fed fresh pasture<sup>a</sup>.**

	Proportion of the ILR contributed by :			
	Net absorption <sup>b</sup>		Protein breakdown <sup>c</sup>	
	<i>Ad libitum</i>	Restricted	<i>Ad libitum</i>	Restricted
Histidine	0.17	0.16	0.83	0.84
Isoleucine	0.26	0.24	0.74	0.76
Leucine	0.31	0.29	0.69	0.71
Lysine	0.33	0.31	0.67	0.69
Methionine	0.22	0.29	0.78	0.71
Phenylalanine	0.27	0.25	0.72	0.75
Threonine	0.34	0.24	0.66	0.70
Tyrosine	0.27	0.24	0.74	0.76
Valine	0.29	0.28	0.71	0.72

	Proportion of ILR partitioned to:			
	Protein synthesis <sup>d</sup>		Oxidation <sup>e</sup>	
	<i>Ad libitum</i>	Restricted	<i>Ad libitum</i>	Restricted
Histidine	0.98	0.98	0.02	0.02
Isoleucine	0.94	0.97	0.06	0.03
Leucine	0.93	0.93	0.07	0.07
Lysine	0.94	0.94	0.06	0.06
Methionine	0.97	0.97	0.03	0.03
Phenylalanine	0.94	0.95	0.06	0.05
Threonine	0.82	0.83	0.08	0.17
Tyrosine	0.98	0.98	0.02	0.02
Valine	0.92	0.94	0.08	0.06

<sup>a</sup> Calculated from the ILR measured using whole blood as precursor pool

<sup>b</sup> Calculated from the duodenal AA flows reported by Kolver *et al.* (1999) and the intestinal absorption coefficient reported by Armstrong (1973) and Stern *et al.* (1985).

<sup>c</sup> Calculated as ILR minus net absorption. Whole body AA composition from Gibb *et al.* (1992); MacRae *et al.* (1993).

<sup>d</sup> Calculated from nitrogen retention (N balance) plus milk protein output plus protein breakdown

<sup>e</sup> Calculated as ILR minus protein synthesis.

Concurrent with this study, Kolver *et al.*, (1999) measured the flows of AA to the small intestine of dairy cows using the same forages, treatments and experimental design as those described here. Based on their measurement of duodenal flow of AA and using an average of the intestinal absorption rates of AA in ruminants (Armstrong, 1973; Stern *et al.*, 1985), it is possible to obtain an estimate of the net absorption of AA and by difference, the contribution of tissue turnover to ILR. Absorption of AA from the GIT accounted, on average, for 30 % (range 17-34 %; Table 6.11) of the ILR measured from whole blood AA isotopic enrichment. The results obtained are comparable to those reported in the few studies in which GIT flows and ILR of AA were measured simultaneously. For instance, Egan *et al.*, (1983) reported that absorption from GIT represented 30% of the threonine ILR measured in mature sheep. Liu *et al.*, (1995) reported that, for leucine, the contribution of GIT absorption ranged from 2 to 34 % of the ILR measured in lambs. Comparing the amounts absorbed from the GIT and partitioned to the mammary gland (Table 6.8), it appears that mammary utilisation by itself may account for a large proportion of the absorbed histidine (78 %), isoleucine (76 %), lysine (75 %), methionine (81 %) and tyrosine (86 %). Although the process of tissue turnover represents a major contributor of the total body flux of these AA (up to 85 %), it could be hypothesised that their dietary supply could be limiting milk protein synthesis.

The other two terms in the model, namely protein synthesis and oxidation, can be calculated using the nitrogen retention estimated during the nitrogen balance measurements [(Lapierre *et al.*, 1996); -corrected to estimate total protein accretion (MacRae *et al.*, 1993)]. Black *et al.*, (1990) and Bequette *et al.*, (1998) have suggested that the AA that are most likely to be limiting for milk protein synthesis will be conserved relative to others and thus, least oxidised. Based on that premise, histidine, methionine and tyrosine are the AA most likely to be limiting protein synthesis in this study. In spite of the limitations of the oxidation estimates described in this study, the results were comparable to studies in which individual radioactive tracers were used to estimate oxidation of AA to CO<sub>2</sub> (Black *et al.*, 1990).

Kolver *et al.*, (1999) reported a 23% (range 18-30 %, depending on the AA) reduction in AA flow to the small intestine of restricted animals in the study parallel to this one. In the current study, the reduction in ILR associated with the dietary restriction was, on average, 21% (range 11-41%) and 18% (range 3-30 %) as calculated from whole blood and plasma, respectively.

Partitioning of AA to the mammary gland ranged from 19-34% of the whole body ILR depending on the AA. This finding is similar to the partitioning values reported for dairy goats (Bequette *et al.*, 1997) and congruent with the major role of the mammary gland in the AA economy of the lactating ruminant (Champredon *et al.*, 1990; Baracos *et al.*, 1991).

Total EAA uptake by the mammary gland was, on average, 28% of the EAA whole body flux. Deviations on the partitioning from these percentages can depict the AA requirements of the mammary gland relative to the whole body flux of that AA. For instance, the branched-chain AA (BCAA) and lysine utilised by the mammary gland represented up to 36% of the whole body ILR (Table 6.8). These four AA account for approximately 60% of the total EAA in milk protein. Besides their role in protein synthesis, the BCAA are also required in the mammary gland to yield carbon skeletons and  $\alpha$ -amino groups for the synthesis of non-essential amino acids, mainly glutamic and aspartic acids (Wohlt *et al.*, 1977; Lobley, 1992). These two major metabolic pathways (milk protein synthesis plus mammary catabolism) may explain the high partitioning to the mammary gland observed for these AA. Experiments with supplementary BCAA have failed to show a positive effect of extra supply of BCAA on milk protein synthesis (Mackle *et al.*, 1999). However, the objective of their experiment was to assess the effect of BCAA supplementation with concentrate diets formulated to exceed the cows' requirements of metabolisable protein and energy. Similarly, studies on the leucine metabolism of lactating ruminants (Bequette *et al.*, 1996a; Bequette *et al.*, 1996b) have been conducted using concentrate-rich diets. More research is required to quantify the major processes of the BCAA metabolism in pasture-fed animals, in order to ascertain their role as potentially limiting amino acids.

In contrast to the BCAA, threonine and histidine are present in smaller proportions in milk protein, which may explain their lower partitioning to the mammary gland. However, in the case of histidine, for which measured uptake by the mammary gland did not account for its output in milk protein (see Chapter 5), the low partitioning to the mammary gland may indicate that functions in the body have priority over the lactating mammary gland. Histidine is not a major precursor for the synthesis of NEAA (Bruss and Black, 1982) or a major energy contributor (Black *et al.*, 1990). In fact, according to the latter authors, and also as calculated in this study, histidine is the amino acid with the lowest oxidation in the lactating dairy cow. These findings would suggest that histidine is spared for the synthesis of protein and specific metabolites (e.g. histamine involved in mammary blood flow regulation: Bequette *et al.*, 1998). More research is required to determine whether the low partitioning of histidine to the mammary gland is the result of competition by other metabolic pathways or rather the reflection of limited ability by the appropriate AA transport system in the mammary epithelial cell (Baumrucker, 1985)

In the current study it has been shown that it is possible to obtain adequate estimates of the whole body AA fluxes in large ruminants using relatively low amounts of tracers. More research is still required on the metabolic fate of certain AA (i.e. histidine, isoleucine, phenylalanine-tyrosine) to increase the understanding of the metabolic processes underlying milk protein synthesis.

## 6.6. ACKNOWLEDGEMENTS

This research was supported by the New Zealand Foundation for Research, Science and Technology. The authors thank the Dairying Research Corporation for providing the facilities and the skillful assistance of Vicki Carruthers, David Phipps, Erna Jansen and Peter Neil (Dairying Research Corporation). The assistance of Tricia Harris, Jason Peters and Penny Back (Nutrition and Behaviour, AgResearch Grasslands) is gratefully appreciated. Also thanks to Michael Tavendale (Nutrition and Behaviour, AgResearch for his technical expertise and useful discussions on GC-MS. The comments and questions of Dr. H el ene Lapierre (Agri-Food and Agriculture Canada)

are very much appreciated. The senior author also thanks the National Autonomous University of Mexico for providing the scholarship for his studies.



## 6.7. REFERENCES

AFRC. 1993. Energy and protein requirements of ruminants. An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients. Wallingford, U.K.: CAB International.

Armstrong, D. G. 1973 The uptake of amino acids by the small intestine of the ruminant animal. In Production disease in farm animals (Ed. Payne, J. M., Hibbitt, K. G., and Sansom, B. F.) 43-53. London, U.K.: Baillière Tindall.

Backwell, F.R.C.; Bequette, B.J.; Wilson, D.; Calder, A.G.; Metcalf, J.A.; Wray-Cahen, D.; MacRae, J.C.; Beever, D.E.; Lobley, G.E. 1994. Utilization of dipeptides by the caprine mammary gland for milk protein synthesis. *American Journal of Physiology*. **267**:R1-R6

Baracos, V.E.; Brun-Bellut, J.; Marie, M. 1991. Tissue protein synthesis in lactating and dry goats. *British Journal of Nutrition* **66**:451-465

Bauman, D.E.; Currie, W.B. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. *Journal of Dairy Science* **63**:1514-1529

Baumrucker, C.R. 1985. Amino acid transport systems in bovine mammary tissue. *Journal of Dairy Science* **68**:2436-2451

Bequette, B.J.; Backwell, F.R.C.; Calder, A.G.; Metcalf, J.A.; Beever, D.E.; MacRae, J.C.; Lobley, G.E. 1997. Application of a U-<sup>13</sup>C-labeled amino acid tracer in lactating dairy goats for simultaneous measurements of the flux of amino acids in plasma and the partition of amino acids to the mammary gland. *Journal of Dairy Science* **80**:2842-2853

Bequette, B.J.; Backwell, F.R.C.; Crompton, L.A. 1998. Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. *Journal of Dairy Science* **81**:2540-2559

Bequette, B.J.; Backwell, F.R.C.; Dhanoa, M.S.; Walker, A.; Calder, A.G.; Wray-Cahen, D.; Metcalf, J.A.; Sutton, J.D.; Beever, D.E.; Lobley, G.E.; MacRae, J.C. 1994. Kinetics of blood free and milk casein-amino acid labelling in the dairy goat at two stages of lactation. *British Journal of Nutrition* **72**:211-220

Bequette, B.J.; Backwell, F.R.C.; MacRae, J.C.; Lobley, G.E.; Crompton, L.A.; Metcalf, J.A.; Sutton, J.D. 1996. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. *Journal of Dairy Science* **79**:2217-2224

Bequette, B.J.; Metcalf, J.A.; Wray-Cahen, D.; Backwell, F.R.C.; Sutton, J.D.; Lomax, M.A.; MacRae, J.C.; Lobley, G.E. 1996. Leucine and protein metabolism in the lactating dairy cow mammary gland: responses to supplemental dietary crude protein intake. *Journal of Dairy Research* **63**:209-222

Black, A.L.; Anand, R.S.; Bruss, M.L.; Brown, C.A.; Nakagiri, J.A. 1990. Partitioning of amino acids in lactating cows: Oxidation to carbon dioxide. *Journal of Nutrition* **120**:700-710

Boirie, Y.; Fauquant, J.; Rulquin, H.; Maubois, J.L.; Beaufre, B. 1995. Production of large amounts of [<sup>13</sup>C]leucine-enriched milk proteins by lactating cows. *Journal of Nutrition* **125**:92-98

Bruss, M.L.; Black, A.L. 1982. Estimation of plasma histidine flux in cows. *Journal of Nutrition* **112**:574-583

Champredon, C.; Debras, E.; Mirand, P.P.; Arnal, M. 1990. Methionine flux and tissue protein synthesis in lactating and dry goats. *Journal of Nutrition* **120**:1006-1015

Chaves das Neves, H.J.; Vasconcelos, A.M.P. 1987. Capillary gas chromatography of amino acids, including asparagine and glutamine: sensitive gas chromatographic-mass spectrometric and selected ion monitoring gas chromatographic-mass spectrometric detection of the N,O(S)-*tert*-butyldimethylsilyl derivatives. *Journal of Chromatography* **392**:249-258

Crompton, L.A.; Lomax, M.A. 1993. Hindlimb protein turnover and muscle protein synthesis in lambs: a comparison of techniques. *British Journal of Nutrition* **69**:345-358

Egan, A.R.; MacRae, J.C.; Lamb, C.S. 1983. Threonine metabolism in sheep. 1. Threonine catabolism and gluconeogenesis in mature Blackface wethers given poor quality hill herbage. *British Journal of Nutrition* **49**:373-383

Gibb, M.J.; Ivings, W.E.; Dhanoa, M.S.; Sutton, J.D. 1992. Changes in body components of autumn-calving Holstein-Friesian cows over the first 29 weeks of lactation. *Animal Production* **55**:339-360

Hammond, A.C.; Huntington, G.B.; Reynolds, P.J.; Tyrrell, H.F.; Eisemann, J.H. 1987. Absorption, plasma flux and oxidation of L-leucine in heifers at two levels of intake. *Journal of Animal Science* **64**:420-425

Kolver, E.S.; Carruthers, V.R.; Neil, P.G.; De Veth, M.J.; Jansen, E.B.L.; Phipps, D.E. 1999. Amino acid supply to the small intestine of dairy cows fed pasture. *Proceedings of the New Zealand Society of Animal Production* **59**:180-183

Lacey, J.M.; Wilmore, D.W. 1990. Is glutamine a conditionally essential amino acid? *Nutrition Reviews* **48**:297-309

Lapierre, H.; Bernier, J.F.; Dubreuil, P.; Reynolds, C.K.; Farmer, C.; Ouellet, D.R.; Lobley, G.E. 1999. The effect of intake on protein metabolism across splanchnic tissues in growing beef steers. *British Journal of Nutrition* **81**:457-466

Lapierre, H.; Pelletier, G.; Ouellet, D.R.; Lobley, G.E.; Bernier, J.F. 1996. Measuring leucine flux and protein metabolism with carbon-13 isotope in growing cattle. *Canadian Journal of Animal Science* **76**:259-262

Lescoat, P.; Sauvant, D.; Danfær, A. 1996. Quantitative aspects of blood and amino acid flows in cattle. *Reproduction, Nutrition, Development* **36**:137-174

Liu, S.M.; Lobley, G.E.; MacLeod, N.A.; Kyle, D.J.; Chen, X.B.; Ørskov, E.R. 1995. Effects of long-term protein excess or deficiency on whole-body protein turnover in sheep nourished by intragastric infusion of nutrients. *British Journal of Nutrition* **73**:829-839

Lobley, G.E. 1992. Control of the metabolic fate of amino acids in ruminants: a review. *Journal of Animal Science* **70**:3264-3275

Lobley, G. E. 1993 Protein metabolism and turnover. In Quantitative aspects of ruminant digestion and metabolism (Ed. Forbes, J. M. and France, J.) 313-339. Wallingford, UK: CAB International.

Lobley, G.E.; Connell, A.; Buchan, V. 1987. Effect of food intake on protein and energy metabolism in finishing steers. *British Journal of Nutrition* **57**:457-465

Lobley, G.E.; Connell, A.; Revell, D.K.; Bequette, B.J.; Brown, D.S.; Calder, A.G. 1996. Splanchnic-bed transfer of amino acids in sheep blood and plasma, as monitored through use of multiple U-13C-labelled amino acid mixture. *British Journal of Nutrition* **75**:217-235

Lopez, H.W.; Moundras, C.; Morand, C.; Demigné, C.; Rémésy, C. 1998. Opposite fluxes of glutamine and alanine in the splanchnic area are an efficient mechanism for nitrogen sparing in rats. *Journal of Nutrition* **128**:1487-1494

Mackle, T.R.; Dwyer, D.A.; Bauman, D.E. 1999. Effects of branched-chain amino acids and sodium caseinate on milk protein concentration and yield from dairy cows. *Journal of Dairy Science* **82**:161-171

MacRae, J.C.; Walker, A.; Brown, D.; Lobley, G.E. 1993. Accretion of total protein and individual amino acids by organs and tissues of growing lambs and the ability of nitrogen balance techniques to quantitate protein retention. *Animal Production* **57**:237-245

<01 Author, Analytic>. 89 <04 Title, Analytic>. Washington, DC. USA: National Academy Press.

Ratkowsky, D. A., Evans, M. A., and Alldredge, J. R. 1993. Cross-over Experiments: Design, Analysis, and Application. New York, NY.: Marcel Drekker, Inc.

Reeds, P. J. 1992 Isotopic estimation of protein synthesis and proteolysis *in vivo*. In Modern methods in protein nutrition and metabolism (Ed. Nissen, S.) 249-273. San Diego, CA.: Academic Press.

Riis, P.M. 1988. Nitrogen balance, amino acid flux rates and rates of whole body protein synthesis in lactating and in pregnant goats at different energy intakes. *Journal of Animal Physiology and Animal Nutrition* **60**:86-5

SAS Institute Inc. 1996. *SAS User's Guide*. 6.11. Cary, NC

Stem, M.D.; Santos, K.A.; Satter, L.D. 1985. Protein degradation in rumen and amino acid absorption in small intestine of lactating dairy cattle fed heat-treated whole soybeans. *Journal of Dairy Science* **68**:45-56

Wohlt, J.E.; Clark, J.H.; Derrig, R.G.; Davis, C.L. 1977. Valine, leucine and isoleucine metabolism by lactating bovine mammary tissue. *Journal of Dairy Science* **60**:1875-1882

## **CHAPTER 7.**

# **GENERAL DISCUSSION AND CONCLUSIONS**

## 7.1. INTRODUCTION

In a global and local scale, there has been a growing interest in increasing milk protein output from dairyfarming systems. In order to attain this goal, scientific efforts have been conducted to unveil the metabolic mechanisms controlling milk protein synthesis in lactating ruminants.

The general objective of this thesis was to characterise the utilisation of amino acids by the mammary gland of lactating dairy cows fed fresh forages. Through the completion of this objective, it is expected ultimately to identify which amino acids may limit the capacity of the mammary gland to synthesise milk protein. The results of the different metabolic parameters measured have been described in detail in each of the preceding chapters. This General Discussion will focus on 1) some methodological considerations pertaining to the interpretations of the results, 2) the identification of potentially limiting amino acids based on different aspects of the mammary metabolism of amino acids and 3) the implications of the findings of this thesis on future research.

## 7.2. METHODOLOGICAL CONSIDERATIONS

Metabolism studies such as those described in this thesis (Chapters 4, 5 and 6) may impose limitations in terms of the number of animals that can be used. This shortcoming is related to the cost of the isotopic tracers, the requirement of adequate facilities to maintain the animals in controlled environments (metabolism stalls) and the labour-demanding sampling protocols. Therefore, two important aspects of the experimental design were used to help to counterbalance the statistical limitations associated with small numbers of animals. Firstly, a crossover design was used to reduce the unaccounted variation between treatments, as each animal acts as its own control (Ratkowsky *et al.*, 1993). Secondly, the blood sampling protocol had the purpose to adequately represent the utilisation of a particular compound by the mammary gland (i.e. integrated samples; Bequette *et al.*, 1999). One of the main

advantages of the use of integrated samples is the reduction in the variation between repeated sampling from the same animal (Pacheco-Rios, unpublished observations).

Mammary blood flow, together with the concentrations in arterial and venous blood, are the factors determining the measurement of AA uptake by an organ (Davis and Collier, 1985). From the technical point of view, the adequate measurement of blood flow is perhaps the more challenging issue in studies on organ metabolism. Therefore, a whole chapter in this thesis was dedicated to the assessment of MBF comparing different methods for its estimation. One of the methods used (arterio-venous differences across the mammary gland and the output of AA in milk) has been criticised (Bequette *et al.*, 1998) as there is growing evidence of the involvement of pools other than free AA as part of the supply available to the mammary gland for milk protein synthesis. In fact, this study offers support for such hypothesis (*vide infra*). It has to be recognised that any bias in the estimation of MBF (Chapter 4) would affect the absolute values for uptake described in Chapters 5 and 6. Based on the estimated contribution of blood non-free AA (peptides?) to the mammary intracellular pool of methionine and phenylalanine, the estimated MBF reported in Chapter 4 could be overestimated by 5-18%. Nevertheless, the relative order of AA limitation for milk protein synthesis in the mammary gland and the subsequent discussion of results (Chapters 5 and 6) would hold irrespective of the bias in the estimation of MBF.

### 7.3. PRECURSOR POOLS FOR MILK PROTEIN SYNTHESIS

Results from the current study supported the concept that plasma is the main pool of free AA (FAA) for milk protein synthesis in the mammary gland. The comparison of whole blood vs. plasma samples showed that the contribution of erythrocytes is relatively small (5-14%) and confined only to a small group of AA. However, the involvement of pools different from FAA was also evident. Measurement of the arterio-venous difference of histidine across the mammary gland showed that its estimated uptake accounted for only 60-75% of the amount secreted in milk (Chapter 5). It was demonstrated that availability is not the limiting factor in this case. For instance, concentration of histidine in the erythrocyte was four times that of plasma (121 vs. 36



nmol g<sup>-1</sup>) and accounted for 60% of the total amount in whole blood. Interestingly, the mammary gland appeared to depend on sources other than the seemingly available histidine in erythrocytes. Therefore, several areas of further research can be proposed to identify a) the factors limiting the uptake of histidine by the mammary gland and b) the source of histidine used by the mammary gland to meet the deficit uptake measured in this study and elsewhere (Chapter 5; Bequette *et al.*, 1999). The understanding of the regulatory mechanisms controlling these factors may be useful to determine the best strategy to deliver AA to the mammary gland (i.e. supplementing peptides instead of free AA).

**Table 7.1. Percent contribution of plasma peptides to the total plasma concentrations (free amino acid plus peptide-bound amino acid) of selected amino acids in lactating ruminants.**

Amino acid	Dairy goat (Backwell <i>et al.</i> , 1996)	Dairy cow (Meijer <i>et al.</i> , 1997)
Aspartic/Asparagine		22
Glycine	nd	32
Alanine	25	12
Proline	12	15
Leucine	15	12
Phenylalanine	10	11
Lysine	nd	22
Histidine	30	35

nd: not determined

Peptides in blood have been postulated as one of the pools of AA for protein synthesis in the mammary gland. Technical difficulties associated with the accurate isolation and measurement of peptides in blood samples have limited obtaining reliable data on their mammary utilisation (Backwell *et al.*, 1996; Backwell *et al.*, 1997). Using an improved methodology, Backwell *et al.* (1996) reported the proportion of the total AA in arterial blood present as peptides for an array of AA in dairy goats. Similar values were reported by Meijer *et al.* (1997) in dairy cows (Table 7.1). However, the unequivocal demonstration of peptide utilisation by the mammary gland is still to be obtained.

Additionally, the use of arterial AA profiles to determine the potentially limiting amino acids for dairy cows (Derrig *et al.*, 1974; Spires *et al.*, 1975; Clark *et al.*, 1977)

can be questioned in the light of the results obtained in the current study. Arterial concentrations, as discussed above, may not necessarily represent the available supply of AA to the mammary gland. More research is required, combining the isotope approach and the isolation of intracellular tRNA (Cant *et al.*, 1999) to quantify the fluxes of AA to the actual site of protein synthesis. In that way, it could be possible to positively identify the limitations in the transfer of AA from the arterial blood to the secretory mammary cell.

#### 7.4. LIMITING AMINO ACIDS FOR MILK PROTEIN SYNTHESIS

Most of the research conducted on determining the requirements for optimal milk protein production has been orientated to assess the effects of the postruminal supply of AA upon the amount of protein synthesised in the mammary gland. By that means, estimates of the AA requirements for dairy cows have been proposed in terms of their duodenal appearance across a range of dietary conditions and limiting AA nominated (Schwab, 1996; Rulquin and Verite, 1996; Iburg and Lebzien, 2000). However, there is evidence from the current study that duodenal AA may not represent adequately the requirements of lactating dairy cows for milk protein synthesis. Based on estimation of duodenal flow of AA in pasture-fed cows, methionine was identified as the first limiting amino acid for milk protein synthesis (Chapter 3). These calculations were later supported by *in vivo* results reported by (Kolver *et al.*, 1999). However, no consistent responses were observed as a result of extra post-ruminal supply of methionine. In fact, an unexpected reduction in the yield of  $\beta$ -casein in methionine-supplemented cows in late lactation was observed and it was speculated that the extra supply of methionine could have had deleterious effects on others AA (namely phenylalanine, leucine and valine). Recent research has demonstrated that the extra supply of post-ruminal methionine reduced the arterial concentrations of BCAA (isoleucine, leucine and valine) in cows fed diets based on grass silage and barley/oats (Varvikko *et al.*, 1999).

Therefore, it seems necessary that research on AA metabolism becomes more comprehensive, exploring simultaneously the interactions among the whole array of amino acids. From that perspective, the characterisation of some aspects of mammary metabolism described in this thesis (Figure 7.1) represent a benchmark which future research can be compared against.

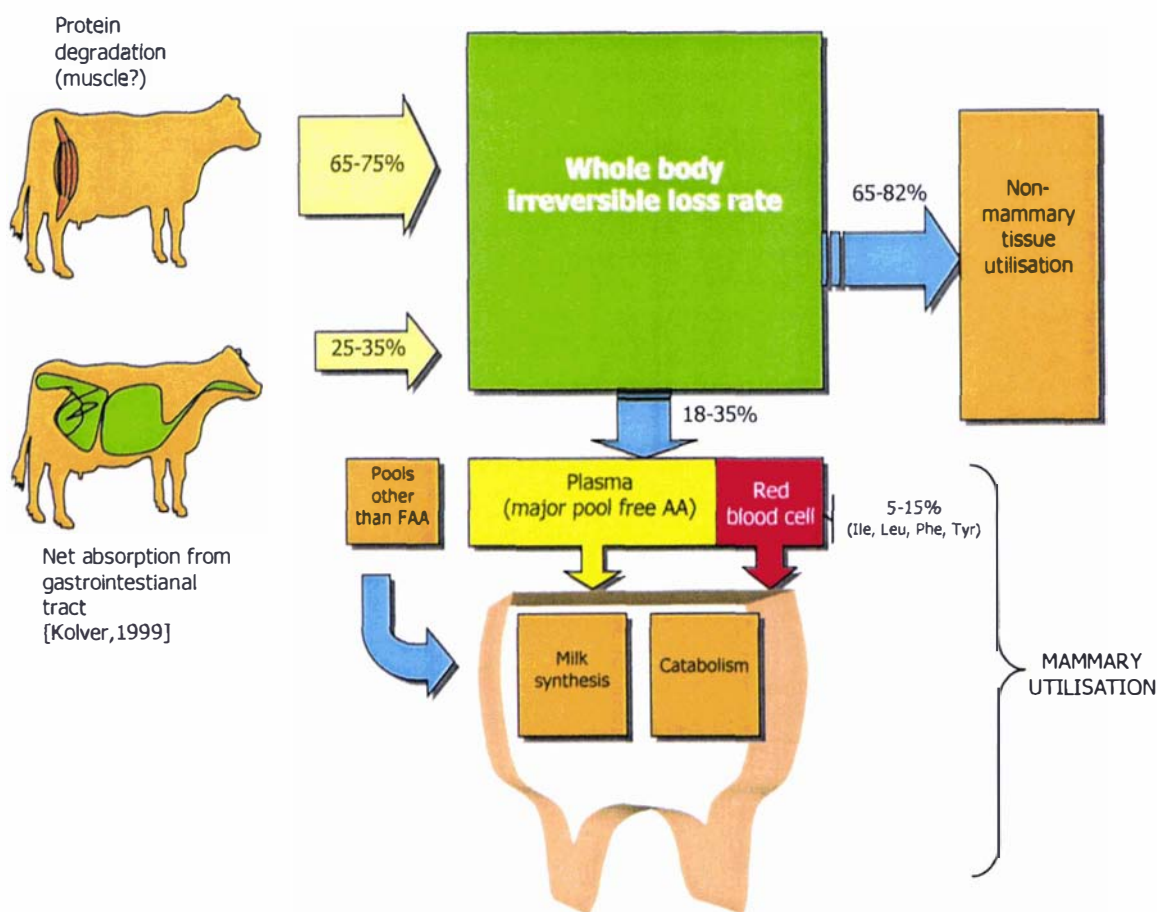


Figure 7.1. Diagram summarising the characterisation of the metabolism of amino acids by the lactating dairy cow fed fresh-forages.

**Table 7.2. Summary of potentially limiting amino acids for dairy cows fed fresh forage or concentrate-based mixed rations, as assessed from the study of different metabolic parameters.**

Method for assessment of limiting AA (AA in order of limitation from left to right)	Diet <sup>1</sup>	Source:
Extraction percentage by the mammary gland:		
<i>Leu, Lys, Met, Thr</i>	Pasture RG-WC	This study (Chapter 4)
<i>Met, Lys, Leu</i>	Pasture	(Davis <i>et al.</i> , 1978) <sup>2</sup>
<i>Met, Lys, Phe, Leu</i>	LH-MG-SBM	(Spires <i>et al.</i> , 1975)
<i>Met, Lys, Phe, Leu, Ile</i>	TMR: MS-MG-SBM	(Yang <i>et al.</i> , 1986)
Comparison of arterial and milk protein AA profiles:		
<i>Met, Lys, Phe</i>	LH-MG-SBM	(Clark <i>et al.</i> , 1977)
<i>Met, Lys, Phe, Leu</i>	LH-MG-SBM	(Spires <i>et al.</i> , 1975)
<i>Met, Lys, Phe, Thr, Leu</i>	TMR: MS-MG-SBM	(Yang <i>et al.</i> , 1986)
<i>Met, Lys, Phe, Leu</i>	TMR: MS-MG-SBM	(Illg <i>et al.</i> , 1987)
<i>Met, Lys, Phe, Leu, Thr</i>	TMR: MS-MG-SBM	(Drackley and Schingoethe, 1986)
<i>Met, Lys, Phe, Thr, Leu</i>	TMR: MS-MG-SSF	(Drackley and Schingoethe, 1986)
<i>Met, Phe, Lys, Leu</i>	TMR:MS-MG-SBM/CP	(Hadsell and Sommerfeldt, 1988)
AA supplementation and/or removal:		
<i>Lys, Met, His</i>	ST-CN-SyntAA	(Fraser <i>et al.</i> , 1991)
<i>Lys, Met</i>	TMR: GS-B	(Metcalf <i>et al.</i> , 1996)
<i>Met, Lys</i>	Maize based	(Rulquin, 1987)
Comparison of mammary uptake vs. output in milk protein		
<i>His, Lys, Thr, Phe, Leu</i>	Pasture: RG-WC	This study (Chapter 4)
<i>His, Lys, Met, Phe, Thr</i>	TMR:MS-MG-SBM	(Yang <i>et al.</i> , 1986)
<i>Thr, Met, Phe, Lys, Trp</i>	TMR:MS-MG-SBM	(Illg <i>et al.</i> , 1987)
<i>Met, Thr, Phe, Tyr, Lys, Leu</i>	TMR:MS-MG-SBM	(Drackley and Schingoethe, 1986)
<i>Met, Phe, Tyr, Leu, His, Thr</i>	TMR:MS-MG-SSF	(Drackley and Schingoethe, 1986)
<i>Met, Lys, Phe, Leu</i>	TMR: LH-MG-SBM	(Austin <i>et al.</i> , 1991)
<i>Met, Lys, Phe, Thr</i>	TMR: MS-MG-SBE	(Munneke <i>et al.</i> , 1991)
<i>His, Leu</i>	GS-B/O	(Varvikko <i>et al.</i> , 1999)
Partitioning to the mammary gland		
<i>Ile, Lys, Leu, Val</i>	Pasture RG-WC	This study (Chapter 5)
Partitioning between protein synthesis and oxidation		
<i>His, Met, Tyr</i>	Pasture RG-WC	This study (Chapter 5)

<sup>1</sup> B: barley; CN: casein; CP: chickpeas; GS: grass silage; LH: lucerne hay; MG: maize grain; MS: maize silage; O: oats RG: ryegrass; SBE: extruded soybean; SBM: soybean meal; SSF: sunflower seeds; ST: straw; SyntAA: synthetic amino acids; TMR: Total mixed ration; WC: white clover.

<sup>2</sup> lactating sheep was used in this study

The main objective of this study was to provide information on limiting AA for milk protein synthesis based on the measurement of different parameters of mammary metabolism (i.e. extraction percentage across the mammary gland, ratio uptake to output, partitioning of AA towards the mammary gland). A summary of these findings is presented in Table 7.2, together with the limiting amino acids as reported for concentrate-fed cows.

From the methods of assessment used in the current study, it can be concluded that histidine, lysine, phenylalanine, threonine and leucine appear to be the EAA most likely to be limiting milk protein secretion in the dairy cow fed sole diets of fresh forage. Comparatively, in concentrate-fed animals, methionine, lysine and leucine have been identified as limiting EAA, with histidine sometimes ranked as one of the least likely to be limiting, based on its low extraction percentage by the mammary gland (Derrig *et al.*, 1974; Spires *et al.*, 1975; Clark *et al.*, 1977) or the lack of response to extra post ruminal supply (Rulquin, 1987).

As demonstrated in this study, the identification and order of limitation of amino acids varies depending on the assumptions made to define the conditions of a limiting amino acid. Given the variation between methods of assessment, it seems unlikely that a proper identification of limiting AA can be derived from a single approach. Rather, the different metabolic parameters described in Chapters 4, 5 and 6 of this thesis could illustrate "metabolic bottlenecks" which may cause an AA to become limiting for milk protein synthesis.

A limiting AA has been defined as the one present in the least amount relative to the requirement for a particular productive process involving protein synthesis (Cole and Van Lunen, 1994). According to this definition, the individual or collective denomination of limiting AA would depend on the particular type of protein being synthesised (in the case of this study, milk protein).

From the methods used in this study to assess the limiting AA for lactating dairy cows, only the comparison between mammary uptake and output in milk protein relates

directly AA supply with the milk protein output. Using this approach for limiting AA identification, histidine was found to be the first limiting AA, as its mammary uptake only met 60% of its output in milk protein. Paradoxically, the partitioning of the whole body flux of histidine to the mammary gland ranked as one of the lowest. Thus, histidine was present in the body but for unknown reasons, it was not available to the mammary gland. If the extraction percentage from arterial blood is an indicator of the transfer efficiency of AA to the mammary gland (Linzell and Mepham, 1974; Mepham, 1982), it could be speculated that, in the case of histidine, the transport into the secretory cell is the limiting step as denoted by its low extraction rate (Chapter 5). Mutual inhibition between glutamine, asparagine and histidine has been described for amino acid transporters in the rat jejunum *in vitro* (Taylor *et al.*, 1989). Such a competitive inhibition can not be ruled out as a factor limiting the availability of substrates for milk protein synthesis in the mammary gland.

Also, it is important to emphasise that milk protein synthesis is only one of several metabolic functions in which AA are involved. There is growing evidence that the impact of AA use for functions other than direct incorporation in milk protein (i.e. constitutive tissue protein, energy supply, synthesis of NEAA, tissue signalling; Bender, 1985; Lobley, 1992) needs to be assessed in terms of their potentially limiting role in milk protein synthesis.

For instance, due to their excess uptake the branched-chain AA (BCAA) were not identified as limiting amino acids by means of the comparison of their mammary uptake and output in milk protein (Chapter 5). The efficiency of transfer from the arterial supply does not seem to be a limiting factor, as the percent extraction for this group reached 40-50% of their arterial concentration. The measurement of the whole body fluxes of BCAA indicated that these AA, together with lysine, were partitioned towards the mammary at significantly higher proportions than the rest of EAA (Chapter 6; Bequette *et al.*, 1997). Is this higher partitioning to the mammary gland a reflection of the mammary requirement of BCAA? Bequette *et al.*, (1996b) found that the extra supply of leucine associated with a higher dietary protein content increased the fractional oxidation (5 vs. 14 %) of this AA in the mammary gland of dairy cows.

Research by the same group demonstrated that leucine oxidation is not an obligatory process for milk protein synthesis, but a means of disposal of the extra mammary uptake relative to the requirement for synthesis of mammary proteins (Bequette *et al.*, 1996a). The idea of having up to 36 % of the whole body flux of these AA diverted to the mammary gland where a sizeable portion (i.e. up to 24 % of the mammary leucine; Roets *et al.*, 1983) is catabolised to CO<sub>2</sub> may appear inefficient. More research is required to identify unknown effects of BCAA metabolism on the AA economy of the mammary gland, which could translate into a higher efficiency of AA utilisation. Given the growing concerns associated with nitrogen pollution in dairyfarming, the research on AA metabolism may provide leads to increase the efficiency of dietary nitrogen utilisation in lactating dairy cows, with subsequent positive environmental effects.

This study also provided support for the concept that the mammary gland is able to exert self-control to respond to changing conditions of AA delivery. For instance, the mammary gland increased the extraction percentage of amino acids as a response to reductions in blood flow (Chapter 5). Similarly, Varvikko *et al.*, (1999) reported a reduction in extraction percentage of methionine as the concentrations in blood increases as a result of abomasal supplementation with methionine. Although this intrinsic control of nutrient supply to the mammary gland has received more experimental support ("pull theory": see Lescoat *et al.*, 1996; Bequette and Backwell, 1997), there is no conclusive identification of the factors involved in its regulation. Additional research has to be conducted to identify the mechanisms (more likely at intracellular level; Cant *et al.*, 1999) underlying the control of AA utilisation in the mammary secretory cell.

### 7.5. CONFIRMING LIMITING EAA AND PRACTICAL IMPLICATIONS

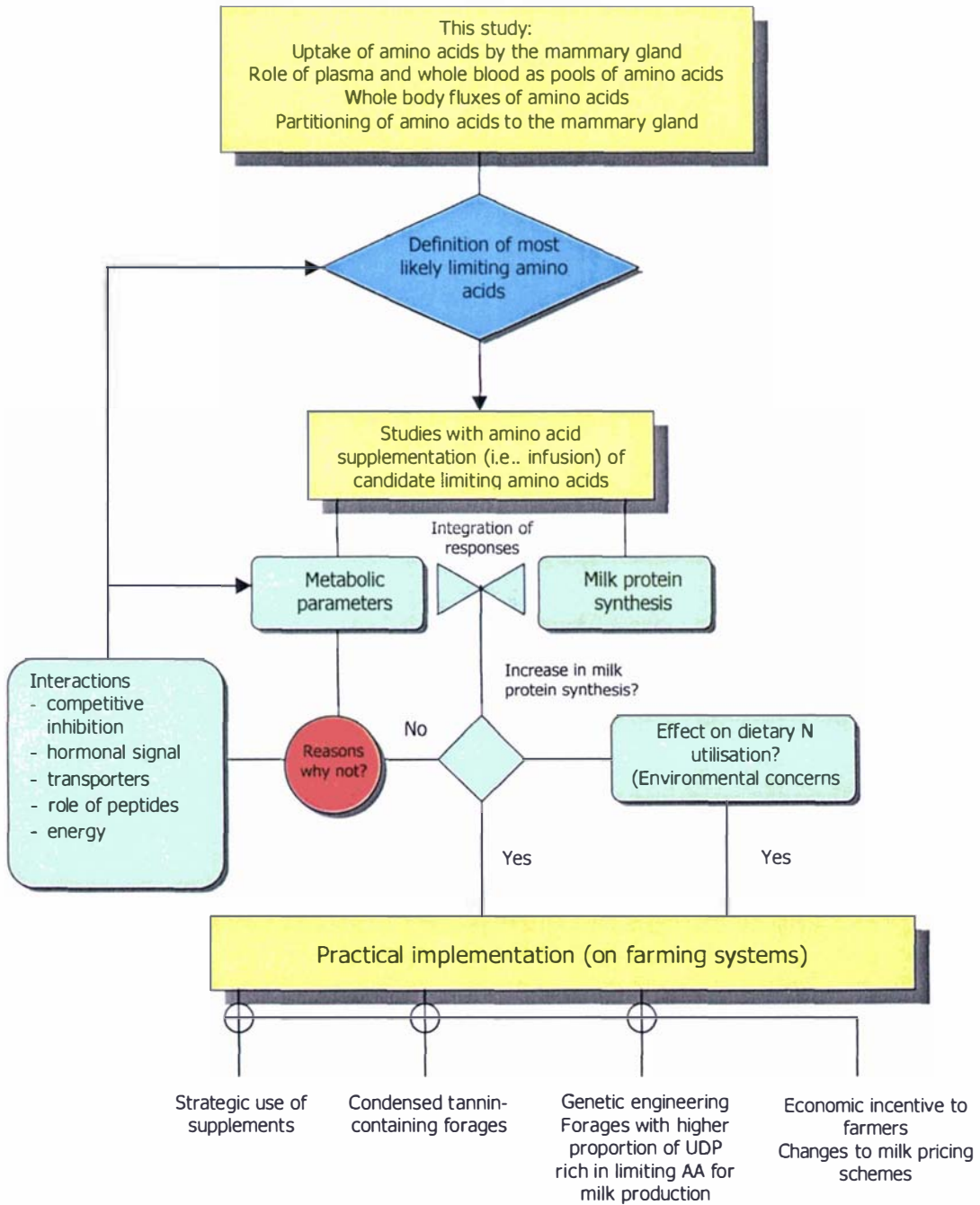


Figure 7.2. Flowchart representing the results described in this thesis in relationship with future research needs and their practical implementation in pastoral dairyfarming in New Zealand.



The research described in this thesis represents the first step on characterising some of the metabolic pathways determining the amount and profile of the AA supply to the mammary gland of the lactating cow fed fresh forages. As part of this understanding, several AA have been postulated as potentially limiting for milk protein synthesis. Further research has to be carried out to test whether or not the extra supply of these candidate AA may translate to a higher yield of protein (particularly casein), which is one of the main goals of the New Zealand dairy industry. However, it is proposed that forthcoming research on AA metabolism includes the assessment of both productive and metabolic responses, as outlined in Figure 7.2.

In that way, if the productive responses are positive, it would be possible to plan strategies to modify milk protein output with the support of a mechanistic model. Colaterally, and even if the responses in milk protein output are null or negligible from an economic point of view, there is still the opportunity to explore mechanisms leading to possible increases in the efficiency of production from pasture-fed ruminants.

Finally, if any beneficial effects of the research on AA metabolism are derived, they have to be implemented in on-farm practice. In concentrate-fed animals, the modification of the AA supply to the mammary gland can be relatively straightforward (ruminally protected AA, use of protein supplements of low ruminal degradability). In pastoral dairyfarming, the modification of AA supply offer different challenges. On the one hand, 50-60% of the protein in forages is the photosynthetic enzyme ribulose 1-5 bis-phosphate carboxylase-oxygenase (Rubisco: Aufrère *et al.*, 1994). This alleviates the concern about the variation in dietary protein supply which has been described frequently as one of the reasons for the lack of consistent responses to AA supplementation in concentrate-fed cows (Mepham, 1982). On the other hand, economic and management issues may limit the ability to change the AA supply in grazing animals, as discussed below. However, there are various strategies, as seen on Figure 7.2, that can be used if needed.

In the case of sheep, it has been demonstrated that sulphur amino acids (SAA; methionine, cysteine) are the main limiting AA for wool production in pasture-fed

animals (Wang *et al.*, 1994), This case can be used to discuss some of the approaches that can be used to increase the postruminal supply of limiting AA in grazing animals. Similar strategies could be used once the limiting AA for milk protein synthesis are properly identified.

One of the attempts to increase the supply of SAA to grazing ruminants involves the use of genetic engineering of species of agronomic interest (Hancock *et al.*, 1994). SAA-rich proteins have been identified and their expression in transgenic plants has been obtained (Tabe *et al.*, 1993). In a similar way, research efforts could be conducted towards the identification of proteins of low ruminal degradability and high intestinal digestibility containing high proportions of the limiting AA for milk protein synthesis. Current technology has been used to successfully express foreign proteins in transgenic plants. Tabe *et al.* (1993) reported expressions of 0.3% of sunflower albumin 8 (a SAA-rich protein) in transgenic tobacco plants. However, this value is still remote from the 3.3% of soluble leaf protein required to increase by 2 g d<sup>-1</sup> the supply of SAA in sheep.

The use of condensed tannin (CT)-containing forages has provided a tool for increasing the net flow of AA to the small intestine by means of a reduction in the ruminal degradation of dietary proteins (Barry, 1989). Sheep fed *Lotus corniculatus* had higher wool production, liveweight gains and milk production (Wang *et al.*, 1994; Wang *et al.*, 1996). Grazing trials with dairy cows have also demonstrated the beneficial effects of *L. corniculatus* on milk protein production (Woodward *et al.*, 1999). Studies on AA metabolism in *L. corniculatus*-fed dairy cows could help to provide a better understanding of the mechanisms eliciting the positive responses observed in these experiments with lactating sheep and cows.

Considering the increasing public awareness about the use of genetically-modified organisms, and the agronomic issues still to be solved in the case of CT-containing forages (i.e. low grazing resilience), the strategic use of supplementary AA (either as protein or synthetic AA) is perhaps the alternative more immediately acceptable. In New Zealand, the high cost of supplements has been an obstacle to their routine use in pastoral dairyfarming. Nevertheless, if the demand for protein in the

---

overseas market maintains its growing trend (Valeur, 1997; Viatte, 1997), the use of supplements may be justified. The preferential pricing of commodity products in the international dairy market (Livestock Improvement Corporation, 1999) may offer the incentive to explore new pricing schemes (i.e. based on casein content of milk) which could translate to higher income for farmers producing more milk protein. In fact, the use of such pricing strategies could be used to encourage the implementation of any of the alternatives (alone or in combination) discussed herein if the research on AA metabolism shows that it is possible to increase milk protein output from pastoral dairyfarming in a consistent and predictable fashion.

## 7.6. CONCLUSIONS

- Although research on amino acid metabolism and milk protein synthesis is almost 80 years old (Cary, 1920), there are still many gaps in the knowledge on the factors controlling amino acid utilisation and protein synthesis. These gaps need to be researched before establishing whether or not it is possible to modify favourably milk protein synthesis.
- Methionine supplementation did not appear as an effective way to increase milk protein output in a consistent manner in pasture-fed cows. In fact, it seems unlikely that supplementation with a single amino acid can be used successfully to increase milk protein output in dairy cows.
- Plasma was identified as the major pool of free amino acids used in the mammary gland. Small, but significant, contributions from erythrocytes were measured for isoleucine, leucine, phenylalanine and tyrosine. Although the pool of free amino acids in blood appears to be the major source of amino acids for the mammary gland, an unidentified pool also appeared to be involved as a source of precursors for milk protein synthesis.
- The study on arterio-venous differences proved that the mammary gland is able to compensate for reductions in the supply of nutrients (i.e. reduction in blood flow was compensated by increasing the extraction of AA).
- Based on the analysis of uptake of amino acids by the mammary gland and their corresponding output in milk protein, histidine appears to be the first limiting amino acid for milk protein synthesis in pasture-fed dairy cows. Together with the data on percentage extraction and partitioning of whole body flux, it can be speculated that the efficiency of transfer from the arterial blood to the mammary gland is the limiting factor for histidine uptake.

- The high percentage extraction of leucine, methionine and lysine from arterial plasma suggests that the mammary gland has a considerable demand for these amino acids. Because of their high extraction rate, it can be concluded that the efficiency of transfer is not a rate limiting step. However, conditions reducing the arterial supply of these amino acids, or increasing their demand for non-synthetic processes in the mammary gland may create limiting conditions for milk protein synthesis.
- The study of the whole body fluxes of amino acids indicated that mammary utilisation accounted for up to 30-35% of the whole body utilisation of branched-chain amino acids and lysine. Methionine, phenylalanine and tyrosine utilisation by the mammary gland represented between 25-30% of the whole body flux. In the case of histidine and threonine, mammary utilisation was less than 20% of the whole body flux.
- The use of a single metabolic parameter to determine limiting amino acids for milk protein synthesis is not considered an adequate approach. The study of several parameters (i.e. extraction rate, ratio uptake to output) has to be integrative to identify different metabolic steps in which amino acids are potentially limiting.
- Considering the different approaches used in this study identify limiting amino acids for dairy cows, histidine, lysine, phenylalanine, threonine and leucine appear to be the amino acids most likely to become limiting for milk protein synthesis in pasture-fed dairy cows.

## 7.7. REFERENCES

Aufrère, J.; Boulberhane, D.; Graviou, D.; Andrieu, J.P.; Demarquilly, C. 1994. Characterisation of *in situ* degradation of lucerne proteins according to forage type (green forage, hay and silage) using gel electrophoresis. *Animal Feed Science and Technology* **50**:75-85

Austin, C.L.; Schingoethe, D.J.; Casper, D.P.; Cleale, R.M. 1991. Influence of bovine somatotropin and nutrition on production and composition of milk from dairy cows. *Journal of Dairy Science* **74**:3920-3932

Backwell, F.R.C.; Bequette, B.J.; Wilson, D.; Metcalf, J.A.; Franklin, M.F.; Beever, D.E.; Lobley, G.E.; MacRae, J.C. 1996. Evidence for the utilization of peptides for milk protein synthesis in the lactating dairy goat *in vivo*. *American Journal of Physiology* **271**:R955-R960

Backwell, F.R.C.; Hipolito-Reis, M.; Wilson, D.; Bruce, L.A.; Buchan, V.; MacRae, J.C. 1997. Quantification of circulating peptides and assessment of peptide uptake across the gastrointestinal tract of sheep. *Journal of Animal Science* **75**:3315-3322

Barry, T. N. 1989 Condensed tannins: Their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem. In *The roles of protozoa and fungi in ruminant digestion* (Ed. Nolan, J. V., Leng, R. A., and Demeyer, D. I.) 153-169. Armidale, NSW, Australia: Penambul Books.

Bender, D. A. 1985. Amino acid metabolism. Chichester, U.K.: John Wiley & Sons.

Bequette, B.J.; Backwell, F.R.C. 1997. Amino acid supply and metabolism by the ruminant mammary gland. *Proceedings of the Nutrition Society* **56**:593-605

Bequette, B.J.; Backwell, F.R.C.; Calder, A.G.; Metcalf, J.A.; Beever, D.E.; MacRae, J.C.; Lobley, G.E. 1997. Application of a U-<sup>13</sup>C-labeled amino acid tracer in

lactating dairy goats for simultaneous measurements of the flux of amino acids in plasma and the partition of amino acids to the mammary gland. *Journal of Dairy Science* **80**:2842-2853

Bequette, B.J.; Backwell, F.R.C.; Crompton, L.A. 1998. Current concepts of amino acid and protein metabolism in the mammary gland of the lactating ruminant. *Journal of Dairy Science* **81**:2540-2559

Bequette, B.J.; Backwell, F.R.C.; Kyle, C.E.; Calder, A.G.; Buchan, V.; Crompton, L.A.; France, J.; MacRae, J.C. 1999. Vascular sources of phenylalanine, tyrosine, lysine and methionine for casein synthesis in lactating goats. *Journal of Dairy Science* **82**:362-377

Bequette, B.J.; Backwell, F.R.C.; MacRae, J.C.; Lobley, G.E.; Crompton, L.A.; Metcalf, J.A.; Sutton, J.D. 1996a. Effect of intravenous amino acid infusion on leucine oxidation across the mammary gland of the lactating goat. *Journal of Dairy Science* **79**:2217-2224

Bequette, B.J.; Metcalf, J.A.; Wray-Cahen, D.; Backwell, F.R.C.; Sutton, J.D.; Lomax, M.A.; MacRae, J.C.; Lobley, G.E. 1996b. Leucine and protein metabolism in the lactating dairy cow mammary gland: responses to supplemental dietary crude protein intake. *Journal of Dairy Research* **63**:209-222

Cant, J. P.; Qiao, F.; Toerien, C. A. 1999. Regulation of mammary metabolism. *Protein metabolism and nutrition. Proceedings VIIIth International Symposium*. EAAP Publication No. 96 Wageningen Pers: 203-219

Cary, C.A. 1920. Amino acids of the blood as the precursors of milk proteins. *Journal of Biological Chemistry* **43**:477-489

Clark, J.H.; Spires, H.R.; Derrig, R.G.; Bennink, M.R. 1977. Milk production, nitrogen utilization and glucose synthesis in lactating cows infused postruminally with sodium caseinate and glucose. *Journal of Nutrition* **107**:631-644

Cole, D J A; Van Lunen, T A. 1994 Ideal amino acids patterns. In Amino acids in farm animal nutrition (Ed. D'Mello, J. P. F.) 99-112. Wallingford, Oxon, U.K.: CAB International.

Davis, S.R.; Bickerstaffe, R.; Hart, D.S. 1978. Amino acid uptake by the mammary gland of the lactating ewe. *Australian Journal of Biological Sciences* **31**:123-132

Davis, S.R.; Collier, R.J. 1985. Mammary blood flow and regulation of substrate supply for milk synthesis. *Journal of Dairy Science* **68**:1041-1058

Derrig, R.G.; Clark, J.H.; Davis, C.L. 1974. Effect of abomasal infusion of sodium caseinate on milk yield, nitrogen utilization and amino acid nutrition of the dairy cow. *Journal of Nutrition* **104**:151-159

Drackley, J.K.; Schingoethe, D.J. 1986. Extruded blend of soybean meal and sunflower seeds for dairy cattle in early lactation. *Journal of Dairy Science* **69**:371-384

Fraser, D.L.; Ørskov, E.R.; Whitelaw, F.G.; Franklin, M.F. 1991. Limiting amino acids in dairy cows given casein as the sole source of protein. *Livestock Production Science* **28**:235-252

Hadsell, D.L.; Sommerfeldt, J.L. 1988. Chickpeas as a protein and energy supplement for high producing dairy cows. *Journal of Dairy Science* **71**:762-772

Hancock, K.R.; Ealing, P.M.; White, D.W.R. 1994. Identification of sulphur-rich proteins which resist rumen degradation and are hydrolysed rapidly by intestinal proteases. *British Journal of Nutrition* **72**:855-863

Iburg, M.; Lebzien, P. 2000. Requirements of lactating dairy cows for leucine and methionine at the duodenum. *Livestock Production Science* **62**:155-168

Illg, D.J.; Sommerfeldt, J.L.; Schingoethe, D.J. 1987. Lactational and systemic responses to the supplementation of protected methionine in soybean meal diets. *Journal of Dairy Science* **70**:620-629



Kolver, E.S.; Carruthers, V.R.; Neil, P.G.; De Veth, M.J.; Jansen, E.B.L.; Phipps, D.E. 1999. Amino acid supply to the small intestine of dairy cows fed pasture. *Proceedings of the New Zealand Society of Animal Production* **59**:180-183

Lescoat, P.; Sauvant, D.; Danfær, A. 1996. Quantitative aspects of blood and amino acid flows in cattle. *Reproduction, Nutrition, Development* **36**:137-174

Linzell, J.L.; Mepham, T.B. 1974. Effects of intramammary arterial infusion of essential amino acids in the lactating goat. *Journal of Dairy Research* **41**:101-109

Livestock Improvement Corporation. 1999. Dairy Statistics 1998-1999. *Dairy Statistics 1998-1999*

Lobley, G.E. 1992. Control of the metabolic fate of amino acids in ruminants: a review. *Journal of Animal Science* **70**:3264-3275

Meijer, G.A.L.; de Jonge, L.H.; Breuer, M.; van der Meulen, J.; van Vuuren, A.M. 1997. Peptide bound amino acids in arterial and mammary venous plasma of high yielding dairy cows. *Journal of Dairy Science* **80**, Suppl. 1:154

Mepham, T.B. 1982. Amino acid utilization by lactating mammary gland. *Journal of Dairy Science* **65**:287-298

Metcalf, J.A.; Crompton, L.A.; Wray-Cahen, D.; Lomax, M.A.; Sutton, J.D.; Beever, D.E.; MacRae, J.C.; Bequette, B.J.; Backwell, F.R.C.; Lobley, G.E. 1996. Responses in milk constituents to intravascular administration of two mixtures of amino acids to dairy cows. *Journal of Dairy Science* **79**:1425-1429

Munneke, R.L.; Schingoethe, D.J.; Casper, D.P. 1991. Lactational evaluation of ruminally protected methionine in diets containing extruded soybeans and urea. *Journal of Dairy Science* **74**:227-233

Ratkowsky, D. A. *et al.*. 1993. Cross-over Experiments: Design, Analysis, and Application. New York, NY.: Marcel Drekker, Inc.

Roets, E.; Massart-Leen, A.M.; Peeters, G.; Verbeke, R. 1983. Metabolism of leucine by the isolated perfused goat udder. *Journal of Dairy Research* **50**:413-424

Rulquin, H. 1987. Determination de certain acides amines limitants chez la vache laitiere par la methode des administrations post-ruminales. *Reproduction, Nutrition, Development* **27 (1B)**:299-300

Rulquin, H.; Verite, R. 1996 Amino acid nutrition of dairy cows: productive effects and animal requirements. In *Recent Developments in Ruminant Nutrition 3* (Ed. Gamsworthy P.C. and Cole D.J.A.) 71-93. Nottingham, U.K.: Nottingham University Press.

Schwab, C.G. 1996. Rumen-protected amino acids for dairy cattle: Progress towards determining lysine and methionine requirements. *Animal Feed Science and Technology* **59**:87-101

Spires, H.R.; Clark, J.H.; Derrig, R.G.; Davis, C.L. 1975. Milk production and nitrogen utilisation in response to postruminal infusion of sodium caseinate in lactating cows. *Journal of Nutrition* **105**:1111-1121

Tabe, L.M.; Higgins, C.M.; McNabb, W.C.; Higgins, T.J.V. 1993. Genetic engineering of grain and pasture legumes for improved nutritive value. *Genetica* **90**:181-200

Taylor, P.M.; Egan, C.J.; Rennie, M.J. 1989. Transport of glutamine across blood-facing membranes of perfused rat jejunum. *American Journal of Physiology* **256**:E550-E558

Valeur, J. 1997 Milk protein production and market prospects. In *Milk composition, Production and Biotechnology* (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 93-103. Cambridge, UK.: CAB International.

Varvikko, T.; Vanhatalo, A.; Jalava, T.; Huhtanen, P. 1999. Lactation and metabolic responses to graded abomasal doses of methionine and lysine in cows fed grass silage diets. *Journal of Dairy Science* **82**:2659-2673

Viatte, G. 1997 Major issues facing the world dairy economy. In Milk composition, Production and Biotechnology (Ed. Welch, R. A. S., Burns, D. J. W., Davis, S. R., Popay, A. I., and Prosser, C. G.) 1-11. Cambridge, UK.: CAB International.

Wang, Y.; Douglas, G.B.; Waghorn, G.C.; Barry, T.N.; Foote, A.G. 1996. Effect of condensed tannins in *Lotus corniculatus* upon lactation performance in ewes. *Journal of Agricultural Science, Cambridge* **126**:353-362

Wang, Y.; Waghorn, G. C.; Douglas, G. B.; Barry, T. N.; Wilson, G. F. 1994. The effects of condensed tannins in *Lotus corniculatus* upon nutrient metabolism and upon body and wool growth in grazing sheep. *Proceedings of the New Zealand Society of Animal Production* **54**:219-222

Woodward, S.L.; Auldist, M.J.; Laboyrie, P.J.; Jansen, E.B.L. 1999. Effect of *Lotus corniculatus* and condensed tannins on milk yield and milk composition of dairy cows. *Proceedings of the New Zealand Society of Animal Production* **59**:152-155

Yang, C.M.J.; Schingoethe, D.J.; Casper, D.P. 1986. Protected methionine and heat-treated soybean meal for high producing dairy cows. *Journal of Dairy Science* **69**:2348-2357

# APPENDIX

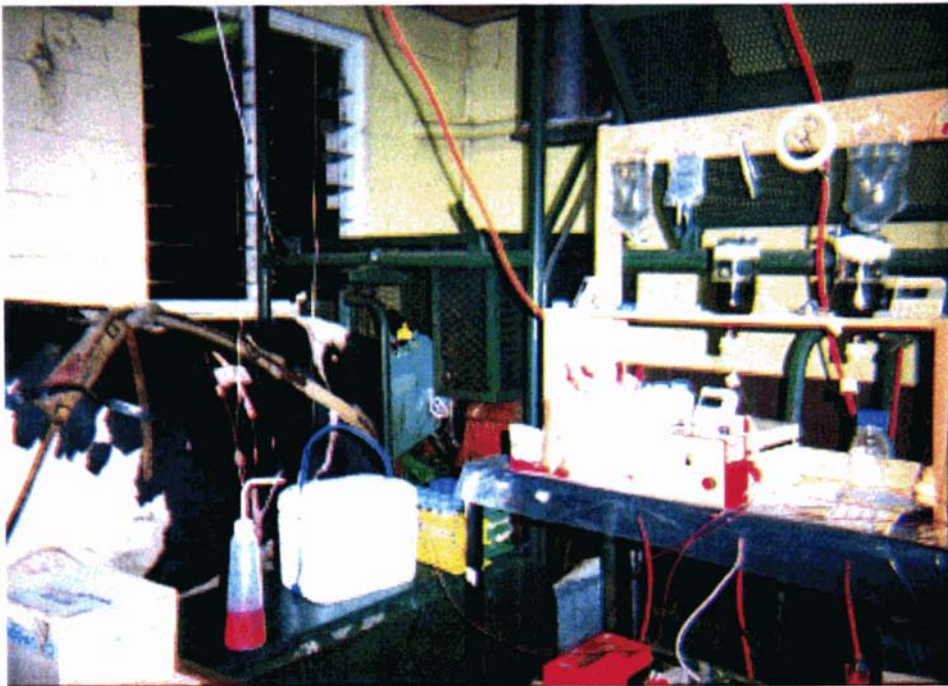
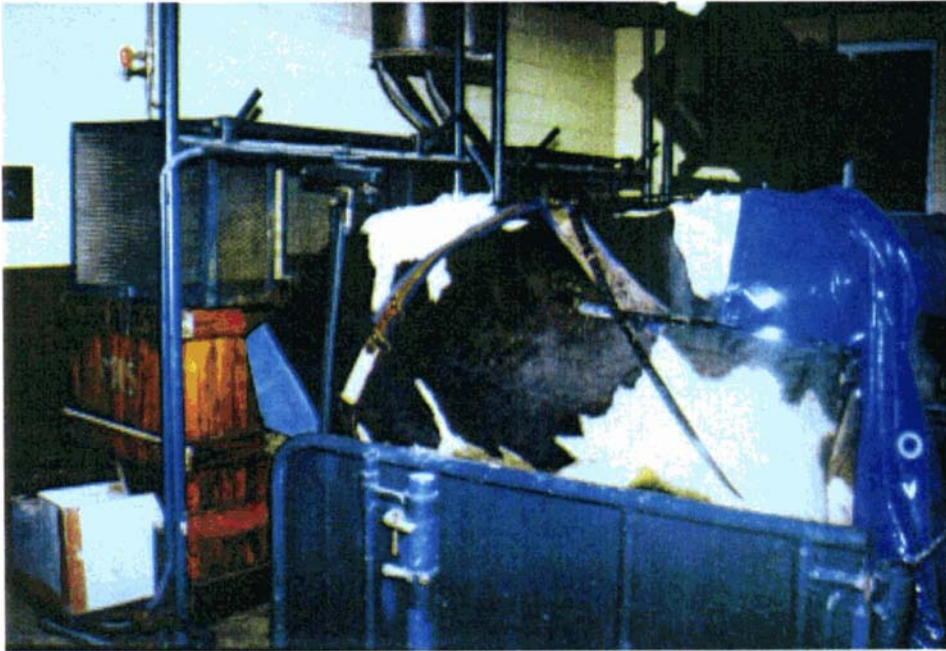
**Appendix A.1.**

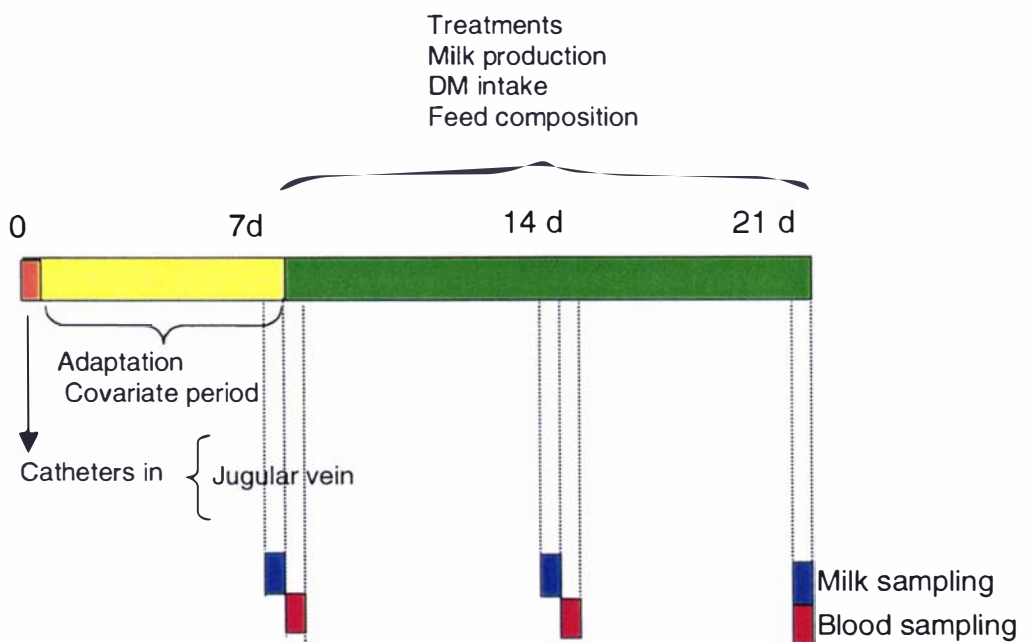
**Image of the experimental conditions described in Chapter 3**



**Appendix A.2.**

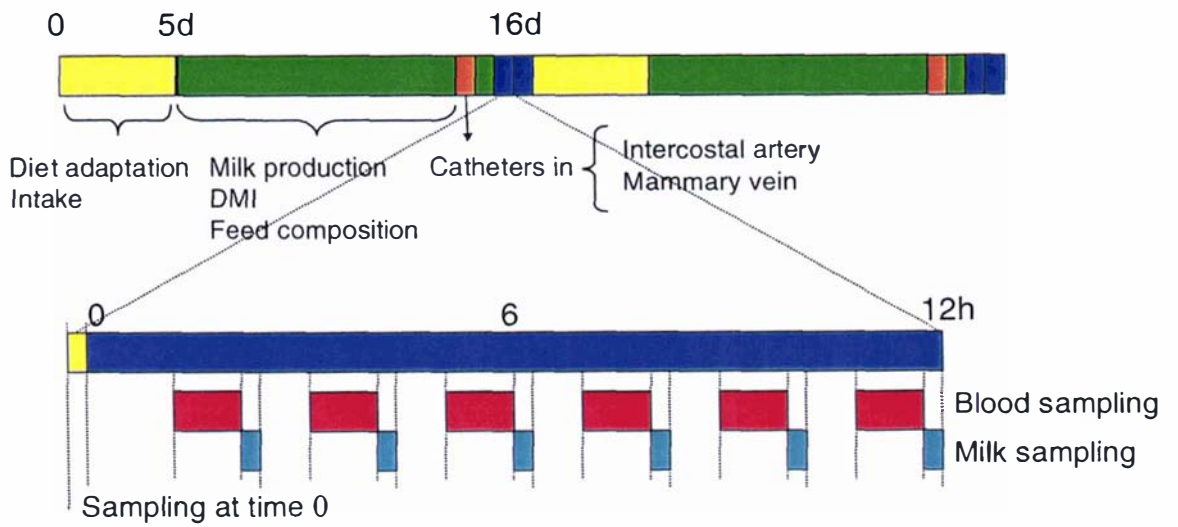
**Image of the experimental conditions described in Chapters 4, 5 and 6**



**Appendix A.3.****Diagram of the experimental protocol used in the methionine supplementation trials (Chapter 3)**

**Appendix A.4.**

**Diagram of the experimental protocol described in Chapters 4, 5, and 6**





## Appendix A.5.

## Irreversible loss rates of amino acids measured in deep jugular sample (Cow 2235; Chapter 6)

**Table A.5.1. Whole body irreversible loss rates (ILR; mmol h<sup>-1</sup>) of amino acids in deep jugular whole blood and plasma. Results from animal no. 2235.**

	Whole blood		Plasma	
	<i>Ad libitum</i>	Restricted	<i>Ad libitum</i>	Restricted
<i>Essential</i>				
Histidine	25.3	24.8	8.1	10.5
Isoleucine	27.0	39.0	22.4	37.2
Leucine	49.6	70.4	28.2	52.4
Lysine	32.5	43.5	15.2	33.2
Methionine	11.6	16.9	4.8	11.3
Phenylalanine	17.5	27.1	8.9	29.8
Threonine	49.9	69.6	36.7	46.7
Tyrosine	12.0	30.3	4.8	17.2
Valine	47.6	51.3	42.9	49.6
<i>TEAA</i>	273.0	372.9	172.0	287.9
<i>Non-essential</i>				
Alanine	79.8	103.0	58.5	94.0
Glutamic acid	127.1	211.6	31.5	86.3
Glycine	173.9	181.3	60.2	131.0
Proline	27.2	31.5	13.9	18.1
Serine	32.8	85.3	29.6	84.3
<i>TNEAA</i>	440.8	612.7	193.7	273.0
<i>TAA</i>	713.8	985.6	365.7	560.9