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**THE EFFECT OF CONDENSED TANNINS UPON PROTEIN
DEGRADATION IN THE RUMEN AND
ON ANIMAL PRODUCTION IN SHEEP FED FRESH *LOTUS*
*CORNICULATUS***

A thesis in partial fulfillment of the requirements for the degree
of DOCTOR OF PHILOSOPHY in the Institute of Food, Nutrition
and Human Health, College of Sciences at Massey University

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1999

DECLARATION

The studies presented in this thesis were completed by the author while a post-graduate student in the Institute of Food, Nutrition and Human Health, College of Science, Massey University, Palmerston North, New Zealand. This is all my own work and the views presented are mine alone. Any assistance received is acknowledged in the thesis.

I certify that the substance of this thesis has not been already 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 used, have been acknowledge in this thesis.



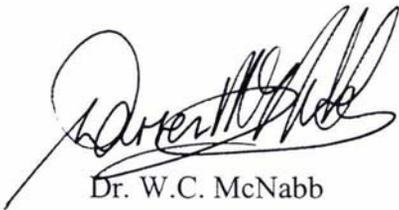
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ABSTRACT

A series of *in vitro*, *in sacco* and *in vivo* indoor and grazing experiments were conducted at Massey University and AgResearch Grasslands, Palmerston North, New Zealand to study the effect of condensed tannins (CT) in *Lotus corniculatus* (Birdfoot trefoil; CV. Grasslands Goldie) upon protein digestion in the rumen and on animal production. Aspects studied included effects of CT upon proteolytic bacterial activity, protein solubilization and degradation in the rumen and wool production and reproduction in grazing sheep. The studies also investigated the potential of *L. corniculatus* compared with perennial ryegrass/white clover pasture (hence referred as to pasture).

The nutritional effects of CT in *L. corniculatus* were assessed by administering polyethylene glycol (PEG; MW 3500) into the rumen of one group of sheep (PEG sheep; CT-inactivated), whilst a separate group of sheep received water (control sheep; CT-acting). PEG selectively binds with CT, preventing the CT from binding plant proteins in the rumen, so that effects of CT can be determined by comparing CT-acting sheep with PEG sheep. The productivity of mixed age ewes in grazing trials was measured in two experiments in the summer of 1995/1996 (Chapter 2) and the summer/autumn of 1997 (Chapter 3), to evaluate the effects of CT in *L. corniculatus* upon efficiency of animal production. A rotational grazing system with restricted feed allowance was used in both experiments.

1. During 1995/1996 (Chapter 2), a grazing trial was conducted to evaluate the effect of CT in *L. corniculatus* on wool growth and on wool processing characteristics in sheep fed close to maintenance for 125 days during summer and autumn (20 December 1995 until 25 April 1996). Half the ewes received twice daily supplements of PEG. The *Lotus corniculatus* contained 32 g total nitrogen (N) and 28 g total CT/kg dry matter (DM) and had an *in vitro* organic matter digestibility of 0.70. Action of CT reduced rumen ammonia concentration ($P < 0.05$) and reduced blood plasma urea concentration ($P < 0.01$) but increased blood plasma cysteine concentration ($P < 0.05$) compared to their counterparts receiving PEG supplementation. The concentration of blood plasma methionine was unaffected by CT. The CT had no effect on voluntary feed intake (VFI) and average liveweight gain ($P > 0.05$) but increased both clean fleece weight ($P < 0.05$) and staple length ($P < 0.001$). The CT also reduced dag percentage ($P < 0.05$) and tended to reduce wool yellowness ($P = 0.07$) relative to sheep receiving PEG. There were no significant effects of CT on fiber diameter (μm),

staple breaking force (Newtons), bulk density (cm^3/g) or wool resilience (cm^3/g). It was concluded that the action of CT in sheep fed *L. corniculatus* increased the efficiency of wool production, with more wool being produced at the same feed intake.

2. Another grazing trial (Chapter 3) was conducted to study the effects of CT in *L. corniculatus* upon VFI, concentration of plasma metabolites, reproductive efficiency and wool production in ewes during two synchronised oestrous cycles in autumn 1997. The ewes were restricted to maintenance feeding for the first 12 days of each oestrous cycle and then increased to *ad-libitum* for the last five days before ovulation. The experiment was of 2 x 2 factorial design, using two types of forage (*L. corniculatus* vs. pasture), with half the ewes grazing each forage being given twice daily oral PEG supplementation. A rotational grazing system with 200 mixed aged dry ewes (52 ± 0.88 kg/ewe) was used. The *Lotus corniculatus* contained 17 g total CT/kg DM in the diet selected, with only trace amounts of total CT present in pasture. Ewes grazing *L. corniculatus* had higher plasma concentrations of branched chain amino acids (BCAA; 57 %) and essential amino acids (EAA; 52 %) than sheep grazing pasture. Again CT in *L. corniculatus* had no effect on mean VFI. The PEG supplementation had no effect upon ovulation rate (OR; 1.33 vs. 1.35) and lambing percentage (1.36 vs. 1.36 %) of the ewes grazing pasture. The CT increased both OR (1.78 vs. 1.56) and lambing percentage (1.70 vs. 1.42 %) in the ewes grazing *L. corniculatus* relative to sheep supplemented with PEG. Increases in OR and lambing % of ewes grazing *L. corniculatus* were due to increases in fecundity (more multiple ovulations and less single ovulations), with no effect on ewes cycling/ewes mated. Compared to ewes grazing pasture, ewes grazing *L. corniculatus* had increased clean fleece weight (19 %). It was concluded that action of CT in the lotus diet was partly responsible for the increased efficiency of reproduction, with more lambs being produced at the same VFI.

3. *In situ* and *in vitro* rumen incubations (Chapter 4) were used to determine the effect of CT on both the solubilization and degradation of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39; fraction 1 leaf protein) from white clover (*Trifolium repens*; 0.3 g CT/kg DM) and *Lotus corniculatus* (22.1 g CT/kg DM). The sheep used for the experiments were fed either white clover or *L. corniculatus*. The loss of DM and neutral detergent fibre (NDF), total N and Rubisco from polyester bags suspended in the rumen of sheep was used as a measurement of solubilisation. The effect of CT extracted from *L. corniculatus* on the degradation of Rubisco from white clover was measured by *in vitro*

incubations with rumen fluid obtained from the same fistulated sheep fed either white clover or *L. corniculatus*.

In the absence of PEG, the solubilisation of Rubisco from *L. corniculatus* was less rapid than the solubilisation of this protein from white clover when each forage was incubated in the rumen of sheep fed the same diet. Addition of PEG tended to increase the solubilisation of Rubisco from *L. corniculatus*, suggesting that CT slowed the rates of solubilization of Rubisco from this forage. The action of CT did not inhibit the *in situ* loss of NDF from either white clover or *L. corniculatus*. In the absence of PEG, the *in vitro* degradation of Rubisco from *L. corniculatus* was slower when compared to the degradation of this protein from white clover; PEG addition increased the degradation of Rubisco from *L. corniculatus*, but not from white clover, showing that CT was the causal agent. The addition of CT extracted from *L. corniculatus* markedly depressed the degradation of Rubisco from white clover, with the effect being completely reversible by PEG. The large subunit (LSU) of Rubisco was consistently degraded at a faster rate than the small subunit (SSU) and added CT had a greater effect in slowing the degradation of the LSU compared to the SSU. It was concluded that the action of CT from *L. corniculatus* reduces the digestion of protein in the rumen of sheep through a minor effect on solubilization and a major effect on degradation. The main effects of CT on protein solubilization and degradation seemed to be produced locally by CT present in plant tissue.

4. Eleven strains of proteolytic rumen bacteria (Chapter 5) were used to determine the effect of CT extracted from *Lotus corniculatus* on the *in vitro* proteolysis of Rubisco protein, bacterial specific growth rate and maximum optical density (OD_{max}). Effects of CT on the rate of Rubisco proteolysis (%/h) were determined through making measurements in the presence and absence of PEG. *Streptococcus bovis* strain NCFB 2476 and B315, *Butyrivibrio fibrisolvens* strain WV1 and C211a, *Prevotella ruminicola* strain 23 and C21a, *Clostridium proteoclasticum* B316^T, *Ruminococcus albus* 8, *Fibrobacter succinogenes* S-85, *Eubacterium* sp. strain C12b and C124b were tested against 1.5 mg CT/ml for Rubisco proteolysis and were examined with 0, 50, 100, 200, 400, and 600 µg CT/ml for bacterial growth measurements.

In general, the presence of CT markedly depressed the degradation of both the LSU and SSU of Rubisco, with the effect being completely reversible by PEG. However, the rates of proteolysis per hour for both sub-units of Rubisco varied considerably between individual bacterial species and subunits of Rubisco. In the absence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola* like-strain C21a appeared to be most active in both LSU and

SSU degradation, while *P. ruminicola* 23, *Eubacterium* sp. strain C12b and C124b, *C. proteoclasticum* B316^T, *B. fibrisolvans* strain WV1 and C211a had moderate to lower rates of LSU and SSU degradation. In the presence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola*-like strain C21a appeared to be most active in both LSU and SSU breakdown.

Most bacterial strains showed significantly ($P < 0.05-0.01$) decreased specific growth rate and OD_{max} with increasing CT concentrations. However, some of the strains, *C. proteoclasticum* B316^T and *R. albus* 8 showed transient increases in specific growth rate at low concentrations of CT (between 50 to 100 µg CT/ml), but not at high concentrations of CT. In terms of specific growth rate, addition of CT at low concentrations (50-200 µg CT/ml), *S. bovis* NCFB 2476, *Eubacterium* sp. C124b and *F. succinogenes* S-85 were most affected compared to the minus CT controls, while *P. ruminicola* sp. C21a and *C. proteoclasticum* B316^T were not greatly inhibited at the highest concentrations of CT. The degree of inhibition of both bacterial growth and Rubisco degradation in the presence of CT varied considerably between individual bacterial species and will be discussed in Chapter 5. It was concluded that action of CT from *L. corniculatus* reduces both the rate of Rubisco proteolysis and the growth rate of proteolytic rumen bacteria, but the magnitude of the CT effect differed between strains used.

5. Twelve six month old Romney sheep were fistulated in the rumen and abomasum and fed *Lotus corniculatus* (32 g CT/kg DM), to examine the effects of CT on proteolytic rumen bacterial populations and on quantitative N digestion in the rumen. Half the animals were given continuous intraruminal infusions of PEG. In the first part of the experiment, the populations of four proteolytic rumen bacteria were enumerated directly from rumen samples using a competitive polymerase chain reaction (cPCR) technique. During pre-feeding on a perennial ryegrass/white clover pasture diet, populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvans* C211a were 1.6×10^8 , 2.7×10^8 , 7.1×10^6 and 1.2×10^6 per ml respectively. When the diet was changed from pasture to *L. corniculatus* (average of 8 h to 120 h), the average populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvans* C211a from the same animals were decreased significantly ($P < 0.001$) to 5.1×10^7 , 1.5×10^8 , 2.6×10^6 and 1.0×10^6 per ml, respectively. When the PEG was infused into the rumen of sheep fed *L. corniculatus*, the populations of proteolytic bacteria were significantly increased ($P < 0.01-0.001$) compared to the CT-acting group. Rumen proteinase activity, concentrations of rumen ammonia and

soluble N were decreased significantly ($P < 0.05-0.001$) in the CT-acting compared to the PEG treatment group.

In the quantitative N studies, the principal effects of CT were to reduce rumen N digestibility ($P < 0.05$) and ammonia pool size, and to increase the flow of non-ammonia nitrogen (NAN) to the abomasum. Dry matter intake and DM digestibility were unaffected. The N intake, rumen NAN and microbial NAN pool sizes were similar in both CT-acting and PEG sheep. Non-microbial NAN fluxes to the abomasum were significantly higher ($P < 0.01$) in the CT-acting sheep than in the PEG sheep, but microbial NAN flux to the abomasum was unaffected by treatment. It was concluded that *L. corniculatus* CT reduced forage protein degradation in the rumen, and increased the flow of undegraded feed NAN to the abomasum. Proteolytic bacterial populations seemed to be reduced by CT, but these changes did not effect the total rumen microbial NAN pool or abomasal microbial NAN flux. Therefore, more protein was potentially available for absorption from the small intestine.

6. This study is the first to report that action of CT increased reproductive efficiency in grazing ewes. It is also the first study to show that action of CT decreased proteolytic bacterial populations measured directly from rumen samples using cPCR techniques. Feeding forages containing CT such as *L. corniculatus* has been shown to reduce proteolysis in the rumen, with the mechanisms being to slightly reduce protein solubilization, to markedly reduce protein degradation and to reduce the populations of proteolytic bacteria. CT increased NAN flux into the abomasum (in indoor studies) and increased animal production in grazing ewes without affecting VFI, thus improving the efficiency of animal production. It is concluded that forage CT can be used to increase the efficiency and sustainability of livestock production from grazed forages.

ACKNOWLEDGEMENTS

I would like to express special thanks to my supervisors Professor T.N Barry, Dr. W.C. McNabb and Dr. G.T. Attwood for their constant inspiration, valuable guidance, patience and encouragement throughout this study.

I also wish to thank Dr. A.L. Molan and Mr. J. S. Peters, AgResearch and Mr. G. S. Purchas and Nutrition and Wool Laboratory staff, Massey University. Their skilled assistance was greatly appreciated. Mr. G.T. Harvey; Professor M.F. McDonald, Dr. P. D. Kemp and Dr. G. C. Waghorn are thanked for their help and guidance. Dr. P.C.H. Morel and Professor D. J. Garrick are thanked for advice with statistical analysis.

Wools of New Zealand and New Zealand Department of Education are acknowledged for their financial support.

I sincerely acknowledge the technical assistance in various ways from:

Ms F. S. Jackson; Miss M. L. Zou; Miss K. Reilly; Miss S. April, Associate Prof. W. Sun; Prof. J.M. Fernandez; Mr. C. Parsons; Ms P. Back; Mr. Luque; Mr. W.C.L. Howell.

Finally, I would like to express my special thanks to my wife, Hae Young Min who has been both a mother and father of our daughter Yun Kyeng Min for many years, for love, support and tolerance during my study.

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LIST OF ABBREVIATIONS

AA	Amino acids
BCAA	Branched amino acids
BCF	Bound condensed tannins
BSA	Bovine serum albumin
BW	Body weight
Cr-EDTA	Chromium ethylenediaminetetra acetic acid
CT	Condensed tannins
DM	Dry matter
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
EAA	Essential amino acids
ECT	Extractable condensed tannins
FCT	Fibre bound condensed tannins
FSH	Follicle stimulating hormone
FV	Feeding value
g	Gram
GLM	General linear model
ha	Hectare
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HT	Hydrolysable tannins

IRL	Irreversible loss rate
K _f	Efficiency of utilization of ME for fattening
K _g	Efficiency of utilization of ME for growth
K _l	Efficiency of utilization of ME for lactation
K _m	Efficiency of utilisation of ME for maintenance
LH	Luteinizing hormone
LWG	Liveweight gain
ME	Metabolisable energy
MW	Molecular weight
N.Z.	New Zealand
N	Nitrogen
NAN	Non ammonia nitrogen
NV	Nutritive value
OF	Oesophageal fistulae
OM	Organic matter
OMD	Organic matter digestibility
OR	Ovulation rate
cPCR	Competitive polymerase chain reaction
PRG	Perennial ryegrass/white clover pasture
PEG	Polyethylene glycol
RPM	Revolutions per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39)
SE	Standard error
SEM	Standard error of mean
SAS	Statistical analysis system
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
UK	United Kingdom
VFA	Volatile fatty acid
VFI	Voluntary feed intake
V/V	Volume by volume
W/V	Weight by volume

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CHAPTER 1

A REVIEW OF LITERATURE

1.1 INTRODUCTION

Livestock numbers in New Zealand (NZ) are large relative to the human population (3.7 million) and pastoral animal products represent about 40 % of NZ's total export earnings (Table 1.1), which demonstrates the importance of pastoral agriculture to the NZ economy.

Table 1.1. Livestock numbers (millions) and value of pastoral industry exports as a percentage of total New Zealand export (\$) receipts.

Animal species	Livestock numbers	Commodities	% of total NZ (\$) exports
Sheep	47.4	Wool	5.0
Beef	5.0	Meat & edible offal ¹	13.0
Dairy	4.2	Dairy products	16.8
Deer and goats	1.4	Animal originated products ²	4.6
Total	58.0		39.4

Human population	3.7		

¹ Includes beef, sheep, venison and venison offal, goat and others.

² Includes hides, thins, leather and others.

³ Statistics New Zealand (year ended June), 1997

This review, based on published literature, summarises data on the digestion and absorption of protein in ruminant animals fed forage diets and on the feeding value (FV) of grazed forages. Secondly, structure and reactivity of forage condensed tannins (CT) with various substances will also be reviewed. Finally detailed consideration is given to the effects of CT on forage FV, involving effects on voluntary fed intake (VFI), nutrient digestion, rumen microbial activity and effects on animal production.

1.2 DIGESTION AND ABSORPTION OF FORAGE PROTEIN IN RUMINANT ANIMALS

The sections of the digestive tract where different feed components are digested and the products of digestion and absorbed are summarised in Figure 1.1. The major sites of organic matter (OM) digestion in ruminants are:

- the rumen (60-70 % OM digestion; microbial fermentation)
- the small intestine (20-30 % OM digestion; animal enzymes)
- the large intestine (10 % OM digestion; microbial fermentation)

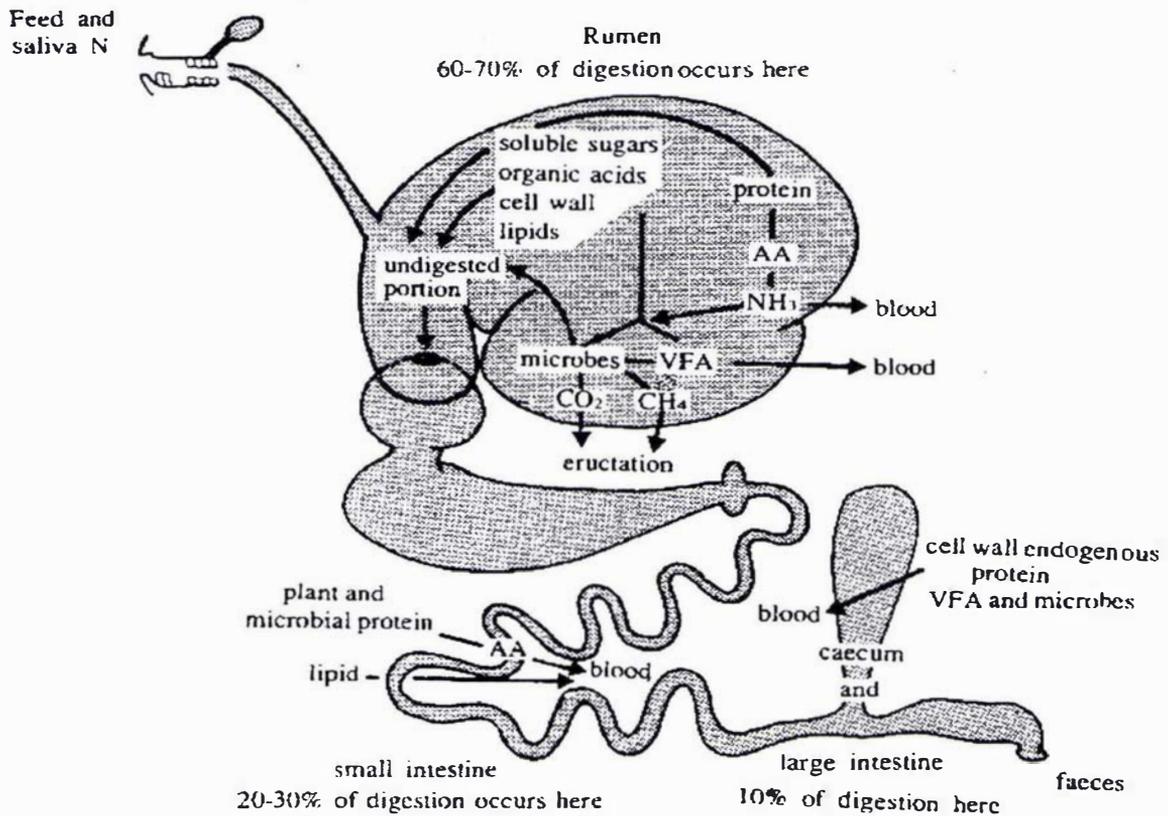


Figure 1.1. The ruminant digestive system and the products of digestion when offered fresh forage (From Waghorn and Barry 1987).

1.2.1 Protein breakdown in the rumen

Ruminants depend for their amino acid supply on the dietary protein which escapes ruminal degradation and on the microbial protein which is formed as the result of rumen fermentation. In the rumen there is rapid breakdown of the protein contained in forage and a large proportion is absorbed as ammonia (MacRae & Ulyatt 1974; Siddons *et al.* 1985). Excess ammonia is absorbed through the rumen wall, transported via the blood to the liver where it is converted to urea. Urea is then excreted in the urine (MacRae & Ulyatt 1974). This results in an inefficient use of nitrogen by ruminants fed high quality forages and ruminant nutrition research has focused on improving the efficiency of nitrogen digestion in the rumen. Rubisco protein, one of the major plant leaf proteins, is probably the most important and wide occurring protein in all fresh forages of high nutritive value (Nugent & Mangan 1981), indicating considerable nutritional advantage to reducing proteolysis in the rumen by compounds such as condensed tannins (McNabb *et al.* 1996).

The rate of protein breakdown in the rumen is correlated with protein solubility, the degree of secondary and tertiary protein structure, the ^{number} ~~amount~~ of disulphide bonds and the presence of inhibiting compounds such as condensed tannins (Chalmers & Syngé 1954; Kaufmann & Luppig 1982; McNabb *et al.* 1994, 1996). The level of disulphide bond and cross linkage of protein in the diet were relatively resistant to rumen microbial degradation (Nugent & Mangan 1978; Mahadevan *et al.* 1980) in proteins such as ovalbumin and bovine serum albumin (BSA). Sunflower albumin 8 (half-life of 69.3 h; 8000 mol wt polypeptide) and ovalbumin (half-life of 8.7 h), for example, were relatively more resistant to rumen degradation than the Rubisco protein (half-life of 1.5 h; Mangan 1972; McNabb *et al.* 1994). However, when these proteins were treated with ^{ribulose 1,5 bisphosphate carboxylase/oxygenase, EC 4.1.1.39} β -mercaptoethanol, dithiothreitol, or performic acid, resulting in the breaking of disulphide bonds, they ^{were} ~~are~~ susceptible to rumen proteolysis (Mahadevan *et al.* 1980; Nugent *et al.* 1983). For example, performic acid-treated

albumin was hydrolyzed as rapidly as casein, in comparison with native albumin which was hydrolyzed at 7 % of the rate of casein (Wallace 1988). Conversely, disulphide bonding is not the only factor determining resistance to rumen protein degradation, as the small sub-unit (SSU) of ribulose-1,5-bisphosphate/carboxylase (Rubisco; fraction 1-leaf protein), which contains no disulphide bonds (Kawashima & Wildman 1970) is relatively resistant to rumen degradation. Previous studies suggested that the small sub-unit (SSU) of Rubisco protein from lucerne leaf (*Medicago sativa*) degraded much slower than the large sub-unit (LSU) of Rubisco in the rumen (Kawashima & Wildman 1970; Nugent & Mangan 1981; McNabb *et al.* 1994). Therefore, the rate of degradation of different soluble proteins by rumen microorganisms varies considerably, indicating that not only protein solubility, but also content of sulphur amino acid (disulphide linkages) and protein structure, influence the rate of rumen proteolysis.

1.2.1.1 Microbial breakdown of protein

Both the bacterial and protozoal fractions have proteolytic activity, but the specific activity of the bacterial fraction was measured at 6-10 times greater than that of the protozoal fraction (Brock *et al.* 1982). Overall, three major changes occur to dietary plant protein in the rumen due to the action of microbes (see Fig. 1.2; Nolan 1975; Nolan *et al.* 1976; Waghorn & Barry 1987; Wolin *et al.* 1997):

- The first step in protein breakdown in the rumen is hydrolysis of proteins by microbial proteinases to peptides and amino acids. These products are either utilized directly by the microbes or degraded further by peptidases and deaminating enzymes to short chain fatty acids and ammonia.
- Microbial protein is the most abundant form of protein nitrogen leaving the rumen.

- Ammonia is the major product of protein catabolism and main substrate for microbial protein synthesis. However, ammonia overflow in the rumen leads to inefficient nitrogen retention.

1.2.1.2. Rumen microbial ecosystem

The rumen microbial ecosystem is characterized by an extreme variety and density of microbial cells. The rumen contains large numbers of bacteria (up to 10^{11} per ml, comprising approximately 200 species), ciliate protozoa ($10^4 - 10^6$ per ml, spread over 25 genera), anaerobic rumen fungi (10^3-10^5 fungal zoospores per ml, divided into 4 genera) and bacteriophage particles (10^7-10^9 particles per ml) (Orpin & Joblin 1988; Stewart & Bryant 1988; Williams & Coleman 1988; Hespell *et al.* 1996). The general pathways and microbial interactions involved in degradation and metabolism of plant protein in the rumen are shown in Figure 1.2. Figure 1.2 shows that the major significant protein metabolism in the rumen is breakdown of proteins to peptides and amino acids by microbial proteases. These nitrogen fractions are degraded further to mainly ammonia and short-chain fatty acids by deaminating enzymes and peptidases. This ammonia is the main ^{nitrogenous} substrate for microbial protein synthesis (see also 1.2.2). In addition, moderate and minor important protein metabolism is interactions involved in nitrogen metabolism and the use of branched chain acids. *P. ruminicola*, for example, degraded the casein and produced ammonia and *iso*-butyrate or 2- methyl butyrate, which are required by *R. albus* for growth (Wolin *et al.* 1997).

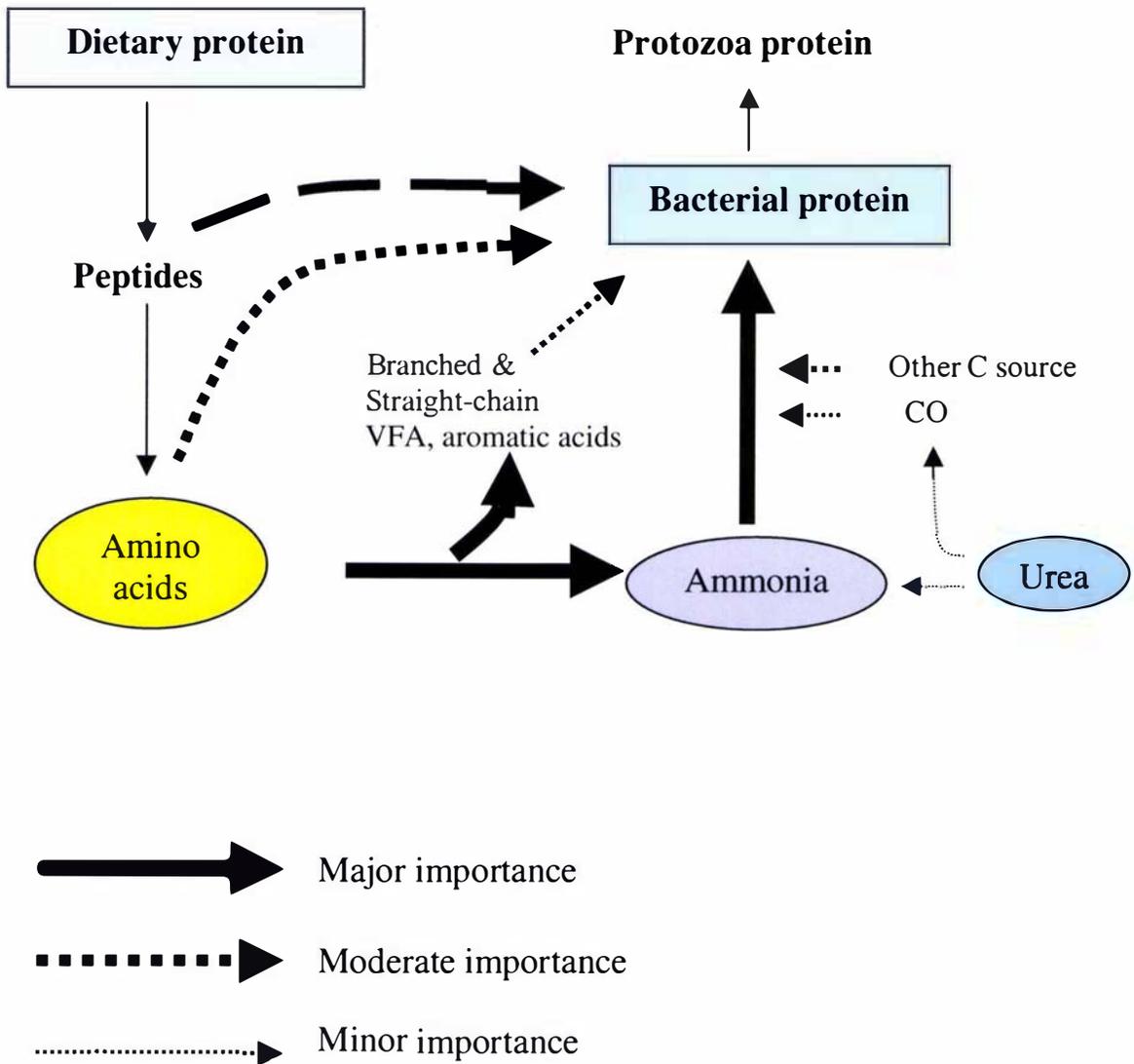


Figure 1.2. Protein metabolism in the rumen (adapted from Wolin *et al.* 1997).

1.2.1.2.2. Protein degradation

Bacteria.

Bacteria are the most active proteolytic organisms in the rumen (Brock *et al.* 1982), and many species of rumen bacteria are known to be proteolytic (12-38%; Blackburn *et al.* 1962; Fulghum & Moore 1963; Abou *et al.* 1965; Russell *et al.* 1981). Most bacterial protease activity is cell-associated, as little proteolytic activity can be detected in cell-free rumen fluid (Blackburn & Hobson 1960; Brock *et al.* 1982).

There are many commonly isolated proteolytic bacteria and their occurrence and predominance is largely influenced by the diet of the animal. Proteolytic activity in the rumen of cows fed fresh lucerne is about 2-3 times higher than that found when fed dry rations (hay + concentrates) are fed (Nugent *et al.* 1983), and the higher content of Rubisco (Fraction 1 leaf protein) in the forage enhanced the number of proteolytic bacteria (*S. bovis*; 84 vs 44 %) compared to dry rations (Hazlewood *et al.* 1983). More studies showed that proteases activity could be altered significantly by changing the dietary protein source in the medium or by altering specific growth rate of proteolytic bacterial species such as *B. fibrisolvans* and *P. ruminicola* (Hazlewood *et al.* 1983; Cotta & Hespell 1986).

The most commonly isolated proteolytic bacteria under high fibre or high concentrate dietary conditions are *Ruminobacter (Bacteroides) amylophilus*, *Prevotella ruminicola (Bacteroides ruminicola)*, *Streptococcus bovis* and *Butyrivibrio* spp. (Blackburn & Hobson 1962; Hazlewood & Nugent 1978; Hazlewood *et al.* 1983). In addition, the proteolytic bacteria in fresh forage-fed New Zealand ruminants are dominated by species of the genera *Streptococcus*, *Eubacterium*, *Butyrivibrio*, *Prevotella* and *Clostridium* sp. (Attwood & Reilly 1995). It seems probable that the most significant factor affecting the proteolysis of rumen contents is diet.

The overall role of bacteria in the conversion of protein to ammonia in the rumen is shown in Figure 1.3. A constriction at the step of oligopeptide breakdown is apparent, where very few species have been identified (Hazlewood & Nugent 1978; Wallace & Brammall 1985; Van Gylswyk 1990).

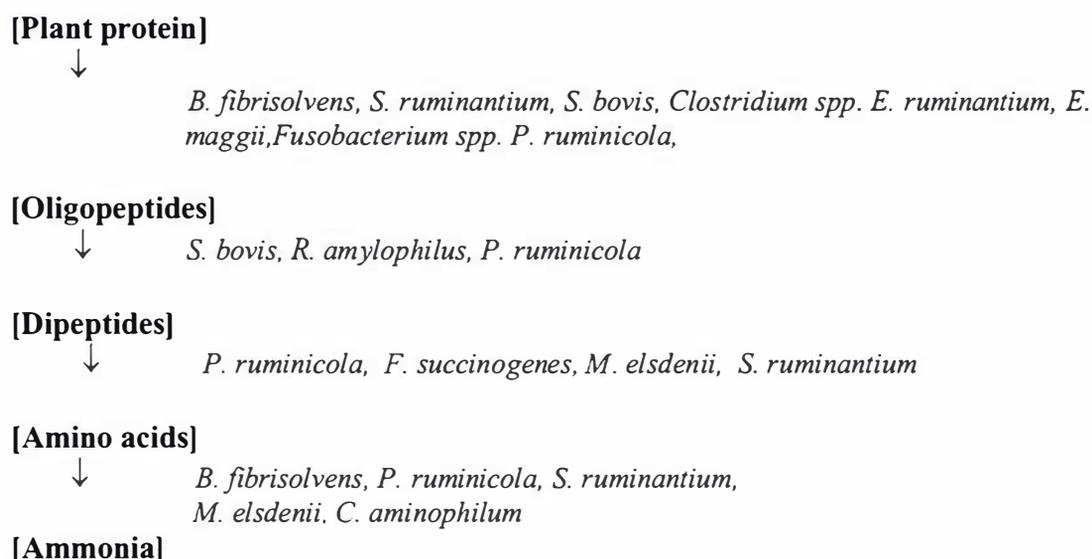


Figure 1.3. A summary of the major microorganisms carrying out the various steps of the catabolic sequence from protein to ammonia in the rumen (from Wallace *et al.* 1997).

Ciliate protozoa. Protozoa are divided morphologically into two major subclasses: flagellates and ciliates (Hungate 1966; Russell & Hespell 1981). Flagellates mainly occur in young calves, and the major protozoal population in adults is ciliate, which is further subdivided into two major groups: holotrichs and oligotrichs (Hungate 1966; Russell & Hespell 1981).

The rumen ciliates have been known to be proteolytic. Holotrichs (isotrichid protozoa) utilise both soluble and insoluble protein (including bacterial cells) and degrade protein

internally (Heald & Oxford 1953; Coleman 1980). Entodiniomorphs (oligotrichs) appear to utilise insoluble proteins associated with particulate matter, engulfing bacteria and chloroplasts (Coleman 1983; Lockwood *et al.* 1988). Although various proteolytic and peptidolytic activities have been demonstrated (Forsberg *et al.* 1984; Wallace *et al.* 1990; Nagasawa *et al.* 1992), such studies do not generate information on the proteolytic capabilities of the individual members of the consortia.

Maximum proteolytic activity of intact mixed protozoa occurred at pH 5.8-5.9 (Forsberg *et al.* 1984). However, the role of ciliate protozoa in the digestion of soluble proteins is not entirely clear. Nugent and Mangan (1981) reported that protozoa were responsible for only 10-20 % of the total proteolytic activity in the rumen, measured with substrate of ¹⁴C-labelled Rubisco protein from lucerne. Brock *et al.* (1982) also estimated that rumen ciliates were one-tenth as active (specific activity basis) in azocasein breakdown as rumen bacteria. In contrast, Ushida & Jouany (1985) observed in an *in vitro* system that protozoa increased ammonia production when fish meal, soybean meal, lupin grains or peanut meal were added, while no effect of protozoa was detected when casein was used. This can be explained by the fact that insoluble proteins are the first target for degradation by protozoa. It has been observed that entodiniomorphid protozoa did not metabolise soluble protein and were unable to grow unless insoluble proteins were supplied (Musynski *et al.* 1985; Michalowski (1989). Therefore, the main role of the protozoa is likely to be in the hydrolysis of particulate proteins of an appropriate particle size, due to the engulfment of feed particles by protozoa and ~~these~~ ^{their} subsequent intracellular degradation (Naga & El-Shazly 1968; Mangan & West 1977).

Fungi. The rumen fungi have been reported to have proteolytic activity with isolates of *Neocallimastix frontalis*, *Piromyces spp.* and *Orpinomyces joyonii* showing low protease activity (Wallace & Joblin 1985, Asao *et al.* 1993, Yanke *et al.* 1993). Fungal proteolytic

activity was highest in animals fed corn compared to animals receiving wheat or barley (Yanke *et al.* 1993). However, Michel *et al.* (1993) found little proteolytic activity in their isolates, and experiments with gnotobiotic lambs suggested that fungal protease made a minor contribution to ruminal proteolysis (Bonnemoy *et al.* 1993).

1.2.1.2.3 Peptide breakdown

The microbial breakdown of protein leads to the formation of oligopeptides, peptides, amino acids and ammonia. When the rate of peptide breakdown exceeds the rate which it can be assimilated, peptide catabolism leads to excessive ammonia production and poor nitrogen utilization. Using ^{14}C -labelled amino acids and peptides, it has been shown that ~~the~~ mixed or individual strains ^{of} rumen bacteria use peptides for growth more efficiently than free AA (Pittman *et al.* 1967; Wright 1967; Cooper & Ling 1985). However, Armstead & Ling (1993) found that AA were preferred compared with peptides in rumen fluid from some sheep. Furthermore, Ling and Armstead (1995) found that free AA were preferentially incorporated by *S. bovis*, *Selenomonas ruminantium*, *Fibrobacter succinogenes* and *Anaerovibrio lipolytica*, while Pittman *et al.* (1967) reported that *P. ruminicola* preferred peptides for growth. The majority of peptidase activity in the rumen is of the amino-peptidase type (Wallace *et al.* 1990) specifically of the dipeptidyl peptidase class (Wallace *et al.* 1993; Depardon *et al.* 1995). *P. ruminicola* is the only common rumen bacterium with significant dipeptidyl peptidase activity (Wallace & McKain 1991; McKain *et al.* 1992; Wallace 1997). Dipeptidase activity is also present and found in many species of bacteria. The rumen ciliate protozoa also have high dipeptidase activity (Newbold *et al.* 1989; Wallace *et al.* 1996), and while rumen fungi are known to possess aminopeptidase activity, their contribution of peptide degradation in the rumen is unknown.

1.2.1.2.4 Amino acid breakdown

The first step in the catabolism of most AA involves removal of the α -amino group, converting the AA to the corresponding keto acid. The conversion of AA to keto acid is usually catalyzed by aminotransferase enzymes (Brody 1994). The final product of AA degradation is acetyl Co-A, which is then processed *via* the tricaboxylic acid (TCA) to yield energy (McDonald *et al.* 1988). One of the consequences of AA catabolism is the production of ammonia which can also be used for microbial protein synthesis (Lewis & Elsdon 1955; Al-Rabbat *et al.* 1971; Chalupa 1974).

The products of the break-down of trypticase, an enzymatic digest of casein (15 g/l), by mixed rumen microorganisms *in vitro* were (mmol/l): acetate (35.7), propionate (1.0), butyrate (14.1), valerate (5.4), *iso*-butyrate (4.3), *iso*-valerate and 2-methylbutyrate (10.2; Hino & Russell 1985). The low propionate and high acetate and butyrate values suggest that the metabolic fate of most AA is acetyl-coA, and that little is converted to pyruvate, the precursor of propionate. Furthermore, about 40 % of total ATP expenditure was used for active AA transport, especially for arginine and lysine (Van Kessel & Russell 1992). This suggests that AA break-down in the rumen may be nutritionally expensive not only because AA are lost as ammonia, but also because of the high energetic costs of their breakdown.

Different AA are broken down at different rates and to different products. The most rapidly degraded in a mixture of AAs were the non-essential AA (NEAA), glutamic acid, aspartic acid, ornithine and alanine (Chalupa 1976). Essential AA (EAA) were broken down at different rates. Arginine and threonine, for example, were rapidly degraded (0.5 to 0.9 mmol/l/h). Lysine, *iso*-leucine, leucine and phenylalanine were apparently degraded at 0.2 to 0.3 mmol/l/h, while methionine and valine were more resistance (0.09 and 0.14 mmol/l/h; Chalupa 1976).

Different bacterial species degrade different AA during growth. Scheifinger *et al.* (1976) reported that the genera *Megasphaera*, *Eubacterium* and *Streptococcus* degraded all 14 AA tested. However, *Butyrivibrio* degraded only serine, asparagine and glutamine. *Selenomonas ruminantium* and *P. ruminicola* (*B. ruminicola*) have been shown to breakdown cysteine and casein, respectively (Bryant 1956; Bryant *et al.* 1986). Furthermore, *Peptostreptococcus elsdenii* degraded L-serine, L-threonine, and L-cysteine, with the production of ammonia and VFAs (Lewis & Elsdén 1955). *Megasphaera elsdenii* and *P. ruminicola* can degrade branched chain amino acids (BCAA) to branched chain fatty acids (BCFA; Hespell & Smith 1983) which are required by the cellulolytic rumen bacteria (*F. succinogenes*, *R. albus* & *R. flavefaciens*) for the synthesis of BCAA (Allison & Bryant 1963; Dehority *et al.* 1967). Thus it appears that ruminal degradation of dietary AA may occur as a result of extensive bacterial interactions.

In the rumen, free AA are rapidly broken down to ammonia. Russell *et al.* (1988) reported the isolation of three amino acid-fermenting rumen bacteria which had significantly higher rates of ammonia production than previously isolated rumen bacteria (Chen & Russell 1988; Paster *et al.* 1993). These organisms, *Peptostreptococcus anaerobius*, *Clostridium sticklandii* and *Clostridium aminophilum* (Russell *et al.* 1988; Chen & Russell 1989; Paster *et al.* 1993) were also sensitive to monensin and this may explain why ammonia concentrations are lower when monensin is fed to ruminants. Recently, Attwood *et al.* (1998) tested pasture-grazed dairy cows, deer, and sheep using a medium in which tryptone and casamino acids were the sole nitrogen and energy sources and have observed the presence of hyper ammonia producing bacteria.

Protozoa also have significant deaminase activities. Experiments with rumen microbial cell extracts (disrupted cell) indicated that the protozoal extracts had a several-fold greater specific deaminase activity than the bacterial extracts (Hino & Russell 1985), but their capacity to

transport (into cytosol) and deaminate amino acid sources is limited (Forsberg *et al.* 1984). However, the bacteria have a high capacity to take up amino acids sources and deaminase them. Bacterial numbers often increase after defaunation (Demeyer & Van Nevel 1979), and bacteria were more active at deaminating amino acid sources than protozoa (Hino & Russell 1987). This apparent contradiction may be related to the level of protein.

1.2.2 Microbial protein synthesis

Microbial flow from the rumen can meet 50 percent or more of the AA requirements of ruminants in various states of production (Ørskov 1982). Microbial protein synthesis is known to be influenced by following factors (NRC 1985; Nolan 1993):

- source of energy and fermentation rate
- source of rumen degradable protein
- source of diets (quantity and quality)
- rumen outflow rate

A source of sulphur, BCFA, minerals and vitamins are also required for microbial growth (Scott & Dehority 1965; Hungate 1966; Nolan 1993).

Ammonia is the main source of nitrogen for microbial protein synthesis in the rumen. ¹⁵N-labelling studies show that, depending on the animal diet, between 40% and 100 % of rumen microbial nitrogen is derived from ammonia (Pilgrim *et al.* 1970; Nolan *et al.* 1976; Wallace *et al.* 1996). Incorporation of ammonia was greater in the bacterial fraction (50-70 %) than in protozoa (31-64 %; Pilgrim *et al.* 1970).

It is well established that rumen bacteria can synthesis protein from ammonia (Satter & Slyter 1974; Schaefer *et al.* 1980). Ciliate protozoa are also able to synthesis AA (Wallace *et*

al. 1998). Previous research has shown that carbon from ^{14}C -labelled monosaccharides was incorporated into the protein of holotrichs, and was specifically incorporated into alanine, histidine, threonine, glutamate and aspartate (Harmeyer 1965; Williams 1979). Recently, Wallace *et al.* (1998) reviewed that ammonia may be required for protozoal protein synthesis, and this assimilation mechanism, has not been described. Lowe *et al.* (1985) reported that the anaerobic fungus *Neocallimastix frontalis* grew in a defined medium without preformed AA, suggesting that it uses ammonia for protein synthesis *in vivo*, but the enzymatic mechanism on these organism is lacking.

The low K_s (ammonia saturation constants) of rumen bacteria ($< 50 \mu\text{M}$) for ammonia means that virtually any excess of ammonia present in rumen contents provides sufficient ammonia for microbial growth (Satter & Slyter 1974; Schaefer *et al.* 1980). Once rumen ammonia concentration attains 50 mg N (3 mM) per litre or greater, then there is no restriction on microbial protein synthesis (Satter & Slyter 1974; Schaefer *et al.* 1980). Typical rumen ammonia concentration values for ruminants fed fresh forage diets in NZ are 200-500 mg N per litre (Waghorn *et al.* 1994; Wang *et al.* 1996a). Therefore, under NZ grazing conditions, rumen ammonia supply is unlikely to restrict microbial protein synthesis.

Another important characteristic controlling rumen microbial protein output to the intestine is the dilution rate of fluid in the rumen. Data summarised by Van Soest *et al.* (1982) also indicated that microbial protein synthesis is significantly and positively correlated with rumen dilution rate ($R^2 = 0.76$). This means that as dilution rate increases, there is a corresponding increase in the efficiency of microbial protein synthesis and microbial outflow from the rumen. However, microbial protein synthesis is not consistently increased when fractional passage rate increases (Hoover *et al.* 1984), presumably due to microbial associations with both particles and fluid in the rumen. Oldham (1984) proposed that microbial protein synthesis (MPS) can be calculated as shown in equation 1.

$$\text{MPS} = P_s K_s + P_l K_l \quad (1)$$

Where, P_s and P_l are the proportions of microbial population associated with the solid and liquid fractions, respectively, and K_s and K_l are the fractional outflow rates for solid and liquid, respectively.

Most reviews of microbial growth efficiency have considered Y_{ATP} (microbial cells/mole ATP), protein or N synthesized/unit of fermentable organic matter, or mole of hexose fermented (Hespell & Bryant 1979; Stern & Hoover 1979). ATP is used for microbial cell growth with an efficiency in the range 10-28 g DM/cells synthesised/mol ATP (Y_{ATP}) (Harrison & McAllan, 1980). These Y_{ATP} yields correspond to microbial protein yields of 20 to 63 g N/kg digestible organic matter apparently digested in the rumen (DOMR; ARC 1984).

Diets can be classified according to the efficiency of rumen microbial protein synthesis (ARC 1984). The efficiency of microbial protein synthesis was low for concentrate and for silage diets, probably due to the low rumen dilution rate of high concentrate diets (about 6 %/h for concentrate diets and 12 %/h for forage) and to extensive degradation of N during the silage fermentation (ARC 1984). Low efficiency of microbial protein synthesis is also found with forages containing less than 100 g crude protein/kg DM, possibly because there is insufficient AA and ammonia to match the energy available for microbial growth. For optimum efficiency of microbial protein production the diet should contain at least 170 g rumen degradable protein/kg rumen degradable OM (McMeniman & Armstrong 1977).

Various methods have been used to estimate the quantity of microbial protein in digesta leaving the ruminant stomach. The majority of these techniques are based on determination of compounds naturally present as microbial cell constituents, e.g. ribonucleic acid (RNA), 2, 6-diaminopimelic acid (DAPA), 2-aminoethylphosphonic acid (AEPA), D-alanine or urinary

excretion of purine derivatives (PD) or use of isotopes to specifically mark rumen microorganisms (^{35}S , ^{15}N , ^{32}P) (Nolan & Leng 1972; Stern & Hoover 1979; Chen *et al.* 1990; McNabb 1993; Broudiscou & Jouany 1995; Lee *et al.* 1995). The most popular microbial markers currently used are purine bases and ^{35}S and ^{15}N incorporation. The main characteristics of the above markers are briefly summarized in Table 1.2. There are no studies at present on the effect of CT from *L. corniculatus* upon the rumen proteolytic bacterial populations and microbial protein synthesis in sheep, and this area requires further study.

Table 1.2. Characteristics of the methods generally used for measuring protein synthesis in the rumen.

Method	Main characteristics
DAPA	Costly analysis, marks mainly the bacteria, tendency to overestimate the microbial N flow.
Total purines	Simple, cost effective, marks all the microbes, differences in purine/N ratios among microbial pools.
^{15}N incorporation	Stable isotope, marks all the microbes, microbial isolates more representative, costly analysis.
^{35}S incorporation	Bacterial and protozoal sulphur AA labelled; requires separation of microbial fraction and assay of radio-activity in (a) methionine, (b) cysteine or (c) organic sulphur of microbial fraction and duodenal digesta.

Cited by Broudiscou and Jouany (1995).

1.2.3 Digestion and absorption in the abomasum and small intestine

The quantity of protein (non-ammonia nitrogen; NAN) leaving the rumen is the sum of the undegraded feed protein, microbial protein, and endogenous NAN flow. Microbial protein comprises between 0.3 to 1.0 of the total NAN flow from the rumen and undegraded feed protein comprises 0 to 0.7 (Smith 1975).

Digestion of protein in the abomasum and small intestine appears to be similar for ruminants as in non-ruminants, except for the slow neutralization of digesta in the small intestine and the abundance of pancreatic ribonuclease (Armstrong & Hutton 1975). Considerable proteolysis in the duodenum is due to gastric protease and pepsin enzymes. Optimal activity for trypsin, chymotrypsin and carboxypeptidase does not occur until the middle jejunum, and peak activity of exopeptidases and dipeptidases is found in the middle ileum (Ben Ghedalia *et al.* 1974). The proteolysis of microbial nucleic acids is done by RNAses, DNAses, phosphodiesterases and phosphomonesterases (Bergen 1978; Roth & Kirchgessner 1980).

The mucosa of the small intestine contains uptake systems for free AA, peptides, nucleotides and nucleosides (Armstrong & Hutton 1975; Bergen 1978). When endogenous loss from the small intestine is expressed as a proportion of the N supply to the duodenum, true absorption is the sum of apparent absorption and endogenous loss. With 23 diets, true absorption of NAN from the small intestine of sheep was 0.76-0.80; true absorption of EAA was 0.80-0.86 (Hogan & Weston 1970; Nolan 1975; Tas *et al.* 1981), suggesting that true absorption of NAN and AA from the small intestine are 0.78 and 0.83 of amounts entering the duodenum. In addition, absorption of leaf protein labeled with ^{15}N was 0.85 (Salter & Smith 1977), while 0.73 to 0.82 of ^{14}C -labeled chloroplast protein was absorbed (Smith *et al.* 1974). True absorption of rumen bacterial and protozoal protein was 0.66 and 0.88, respectively (Chalupa 1972; Zin & Owens 1982).

The most active site for AA absorption in sheep is the mid to lower ileum (Phillips *et al.* 1976), but the highest rate of AA disappearance *in situ* from the digesta in the small intestine occurs in the mid jejunum (Ben-Ghedalia *et al.* 1974). In the small intestine, the sulphur AA (cysteine and methionine) are absorbed less efficiently than the other AA (Armstrong & Hutton 1975). Apparent absorption of EAA is about 0.05 greater than NEAA (Armstrong *et al.* 1977; Tamminga 1980). Tamminga (1980) suggest that absorption of lysine and arginine is greater while absorption of threonine, valine and phenylalanine is less than the absorption of total AA. However, there is a wide range across the values presented by authors (MacRae & Ulyatt 1974; MacRae & Reeds 1980)

The importance of effective nucleic acid degradation in the small intestine to the phosphorus pathway is well recognised (Armstrong & Hutton 1975). Armstrong and Hutton (1975) reported that proportionate digestion within the small intestine of nucleic acid N was in the range 0.75-0.95.

1.3 FORAGE FEEDING VALUE (FV)

1.3.1 Ruminant animal production from grazing forage in New Zealand

Ruminants in NZ are farmed year round in pasture-based grazing systems, with little or no supplementary feeding and no indoor housing in a low cost animal production system. This system is seasonal as determined by the pasture growth pattern, where the efficiency of production is a function of the utilization of pasture and the feed conversion efficiency into animal products. Most NZ pastures contain high concentrations of metabolizable energy (ME; 11.5 MJ/kg DM) and total N (30 g/kg DM; Waghorn & Barry 1987). Rumens digestion of carbohydrate (readily fermentable and structural carbohydrate) is efficient on such diets, but with N digestion duodenal NAN flow is only 65 % of the N eaten (MacRae & Ulyatt 1974; Ulyatt & MacRae 1974) This is mainly due to the excessive degradation (about 70 %) of

forage protein to ammonia by rumen micro-organisms, with only 30 % escaping to the small intestine for absorption. Of the N degraded in the rumen, a large proportion is absorbed as ammonia and excreted as urea in the urine. There also appears to be a shortage of AA supply from such forages for high producing animals, such as high wool and milk producing animals or fast growing animals, since supplementing protected protein or abomasal infusion of AA has been shown to improve productivity of these animals (Barry 1980, 1981; Rogers *et al.* 1980). Therefore, with such high quality fresh forage, a major issue is how to utilize protein efficiently to maximise animal production. This reviews focuses on forage CT for fulfilling this objective.

1.3.1 Definition of FV

Feeding value (FV) is defined as the animal production response to grazing a specified forage under unrestricted conditions and is determined by voluntary feed intake (VFI) and nutritive value (NV) per unit of intake (Ulyatt 1973). This is shown in equation 2.

$$\begin{aligned} \text{FV} &= \text{Animal production} && (2) \\ &= \text{VFI} \times \text{NV} \end{aligned}$$

The FV of forage species can be expressed in units such as liveweight gain (LWG) per day for growing animals or milk production for dairy cows. Nutritive Value is defined as the concentration of nutrients in a forage or the animal production response (meat, milk and wool production) per unit DM eaten. Thus, FV is a function of both NV and VFI (Ulyatt 1973).

1.3.2. Factors affecting FV of fresh forages

Grasses and legumes are the main temperate forage sources and FV of legumes is generally superior to that of grasses (Table 1.3). The FV of annual and short-rotation grasses are also

higher than those of perennial ryegrass. White clover is superior to any other legume. The FV of annual (i.e. Italian ryegrass) and short-rotation grasses are also higher than those of perennial ryegrass.

Table 1.3. The comparative feeding value (FV) in terms of sheep LWG of some forage species grown in New Zealand. All values relative to white clover (Grasslands Huia) and all plants grown under high soil fertility conditions.

Forage species	Relative FV	Growth rate (g/d)
Legumes		
White clover, 'Grasslands Huia'	100	250
Lotus pedunculatus		
'Grasslands Maku'	84	210
Sainfoin, 'Melrose'	84	210
Lucerne, 'Wairau'	82	205
Red clover, 'Grasslands Hamua'	71	178
Red clover, 'Red West'	69	173
Red clover, 'Grasslands Pawera'	65	163
Grasses		
Italian ryegrass, 'Grasslands Paroa'	83	208
Short-rotation ryegrass		
'Grasslands Manawa'	77	193
Perennial ryegrass, 'Grasslands Ruanui'	52	130

(From Ulyatt 1981).

About 50-70 % of the variation in FV between different temperate forages can be explained by differences in VFI (Ulyatt 1973, 1984), therefore any factors affecting VFI will significantly effect FV. In grazing ecosystems, such as in NZ, VFI is influenced by both nutritional and non-nutritional factors.

The relative importance of these factors varies with pasture structure, pasture species and physiological state of the animal (Poppi *et al.* 1987). It has been shown that when pasture is offered to an animal in increasing quantities, VFI increases curvilinearly to reach a plateau (Poppi *et al.* 1987). In the ascending part of the curve, the ability of the grazing animal to harvest pasture is affected by non-nutritional factors such as grazing behaviour of the animal (bite size & rate of biting), pasture structure and environment (temperature, day length etc). At the plateau section of the curve, nutritional factors are important such as digestibility, crude protein content, concentration of metabolic end products and retention time of digesta. The major nutritional factor influencing VFI is the digestibility of the pasture eaten such that as digestibility increases so does intake (Hodgson 1977). Digestibility varies from 0.4 (dead material) to 0.85 (grass or legume leaf), with intake of legumes being up to 40 % greater than for grasses (Ulyatt 1971; Cruickshank *et al.* 1985). The relationship between intake and physical conditions (rumen fill and digesta passage rates) is not always consistent for controlling intake, and in certain situations (during lactation or cold stress) animals can greatly increase intake by increasing digesta passage rates (Ketelaars & Tolkamp 1992). Therefore, the important concept is that no one factor (nutritional and non-nutritional) adequately explains intake regulation and that a combination of factors, both nutritional and non-nutritional, are integrated to control intake.

NV is influenced by both the digestive process (including apparent digestibility, rumen retention time and sites of digestion) and by the efficiency of utilization of absorbed nutrients.

The efficiency of utilization of absorbed nutrients can best be described as the efficiency of utilization of metabolizable energy (ME), which is defined as equation 3.

$$K = \frac{\Delta \text{Energy retained}}{\Delta \text{Me intake}} \quad (3)$$

The efficiency of utilization of ME is denoted by the coefficient K , with a suffix m, f or g depending on whether K refers to the use of the feed for maintenance (K_m) and fattening/growth (K_f or K_g ; Waghorn & Barry 1987). The utilization of the end products of digestion depends both on the type of animal used and on the forage quality. The K_m is relatively similar between fresh legumes (white clover; 0.67) and fresh grasses (Ruanui perennial ryegrass; 0.62), but K_g is higher for legumes (white clover; 0.51) than for grass (Ruanui ryegrass; 0.33; Rattray & Joyce 1974). Season also has a great effect on ME utilization from forage diets, with K_g being higher for forage grown in spring than in autumn, while K_m is similar between seasons (Waghorn & Barry 1987). Forage-grown during autumn was associated with a lower soluble carbohydrate content and a lower proportion of propionic acid in rumen VFA than for spring-grown forage. Sheep fed autumn forages had lower rumen microbial protein synthesis and lower K_g values than sheep fed spring forages (MacRae *et al.* 1985). Whilst K values can be used to describe the overall utilization of ME derived from a feed, diet also affects the efficiency with which individual nutrients are used. The effect of forage CT content on some of these processes is covered in Section 1.5.4.

Condensed tannins have their effect on FV by influencing both VFI and NV. The effect of CT on NV is mainly mediated through its effects on protein digestion (see section 1.5).

1.4 STRUCTURE AND REACTIVITY OF FORAGE CONDENSED TANNINS

1.4.1 INTRODUCTION

Tannins are phenolic plant secondary compounds and are widely distributed through the plant kingdom. Tannins exist primarily in condensed and hydrolyzable forms (1.4.2; Fig. 1.4).

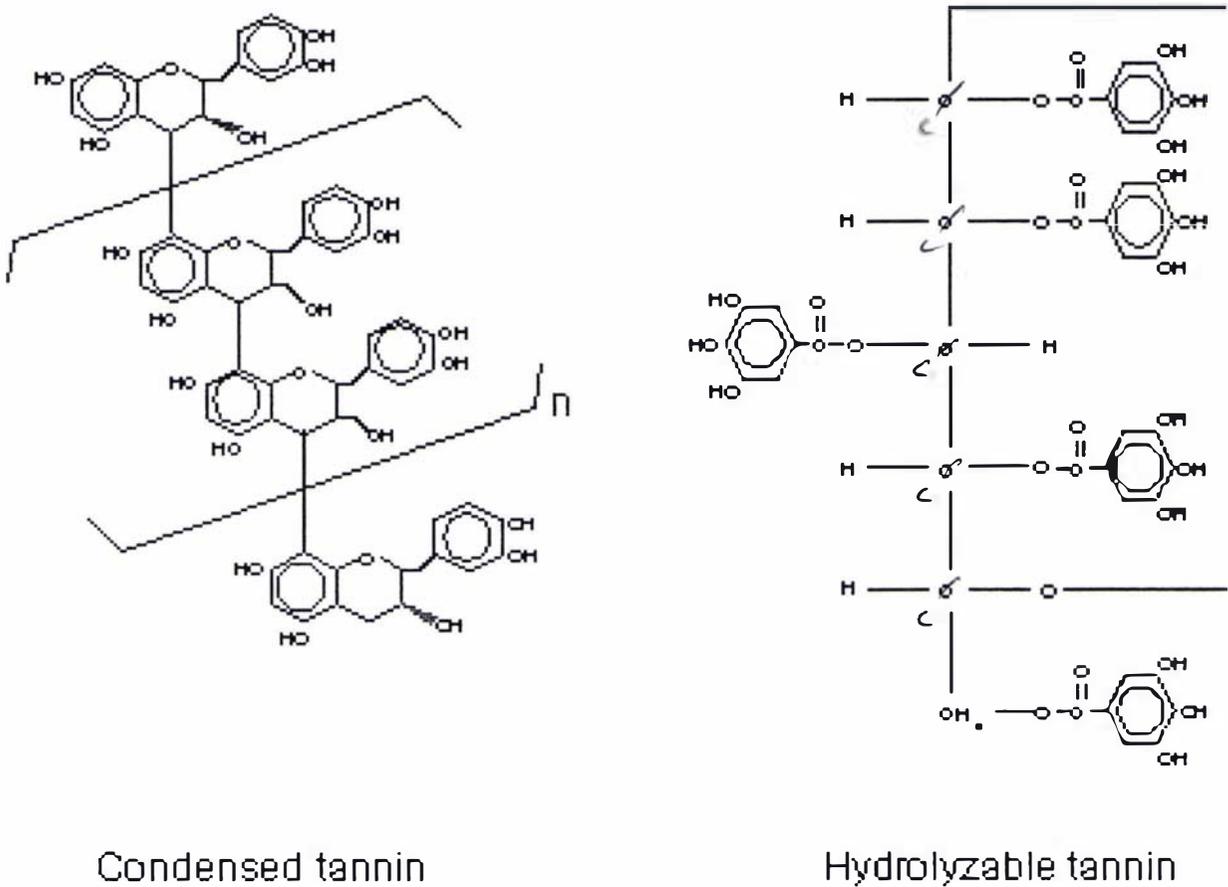


Figure 1.4. Condensed tannins and hydrolysable tannins in plants (From Swain 1979; Zucker 1983).

Swain (1979) and Zucker (1983) proposed the theory that plants evolved tannin production as a chemical defense mechanism against attack by pathogenic bacteria and fungi, and against being eaten by insects and grazing herbivores. This review will concentrate upon the effect of tannins on forage NV.

1.4.2 Structure of plant CT

Tannins are usually subdivided into two groups (Fig. 1.4):

- hydrolyzable tannins (HT)
- condensed tannins (CT) or proanthocyanidins

Hydrolyzable tannins (HT) are molecules with a carbohydrate (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups such as gallic acid (gallotannins) or ellagic acid (ellagitannins) (Haslem 1989). The HT occur mainly in fruit pods and plant galls and unlike CT their degradation products are absorbed from the small intestine of animals (McLeod 1974).

The CT or proanthocyanidine (PA) are the most common type of tannin found in forage legumes, trees and shrubs. This thesis deals only with CT, as HT rarely occur in temperate forages. Structurally, CT are complexes of oligomers and polymerers of flavanoid units (i.e. flavan-3-ols, flavan-3,4-diols & biflavans) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis (Fig. 1.4; Morris & Robbins 1997; Hagerman & Butler 1991). The CT accumulate in the vacuoles of cells in various tissues of many forage species and the range of molecular weight and structure found for forage CT is summarized in Table 1.4.

Table 1.4. Types of condensed tannins (CT) found in some forage legumes

Species	MW range	CT structure		References
		PC:PD	Tissue	
Lotus sp.				
<i>L. corniculatus</i>	1800-2100	67:30	leaf	Foo <i>et al.</i> (1996)
<i>L. corniculatus</i>	-	80:20	leaf	Morris & Robbins (1992)
<i>L. pedunculatus</i>	2200	19:64	leaf	Foo <i>et al.</i> (1997)
Sainfoin (<i>Onobrychis</i> sp.)				
<i>O. vicifolia</i>	2040-3060	81:19	leaf	Koupai-Abyazani <i>et al.</i> (1993)
<i>O. arenaria</i>	1560-3300	75:25	leaf	Koupai-Abyazani <i>et al.</i> (1993)
<i>O. antasiatica</i>	1650-2490	76:24	leaf	Koupai-Abyazani <i>et al.</i> (1993)
<i>O. vaginalis</i>	1980-2700	80:20	leaf	Koupai-Abyazani <i>et al.</i> (1993)
<i>Desmodium</i> sp.	-	PC & PD	leaf	McLeod (1974)
<i>Leapedeza cuneata</i>	14000-20000	Main PD	leaf	McLeod (1974)

PC, procyanidin; PD, prodelphinidin.

1.4.3 The occurrence of CT in plants

Condensed tannins are found in

- plant leaf and stem tissues
- bud, flower and seed tissues
- root tissues

The amount and type of CT synthesized by forages vary considerably depending on forage species, cultivars, soil type, stage of development, and environment conditions (Table 1.5 and 1.6; Broadhurst & Jones 1978; Terrill *et al.* 1992; Barry & Forss 1983; Iason *et al.* 1995; Jackson *et al.* 1996b). Therefore, the study of the nutritional effects of CT on animals requires identification of the type of CT present in a particular forage. In most cases CT are present in the leaves and stems of plants whilst in some forages, such as white clover and red clover, CT

occur only in the flower petals (Barry 1989; Iason *et al.* 1995). Table 6 lists the concentrations of CT in some plants (leaf and stem parts). Of the samples containing CT, total CT content varied widely between and within a species (Table 1.5). The extractable fraction generally contained the highest CT concentration.

Table 1.5. Concentrations (g/kg DM) of extractable condensed tannins (ECT), protein-bound condensed tannins (PCT), fibre-bound condensed tannins (FCT) and total condensed tannins (TCT) of the leaf and stems in a range of temperate and tropical plants.

Forages	ECT	PCT	FCT	TCT	Reference
Temperate forages					
Birdsfoot trefoil	7.0	13.0	1.0	21.0	1
Birdsfoot trefoil ¹					
(Grasslands Goldi)	35.8	8.6	1.8	46.2	2
Birdsfoot trefoil	36.1	10.9	1.2	49.2	3
Narrow leaf birdsfoot trefoil	2.0	3.0	1.0	6.0	1
Big trefoil	61.0	14.0	1.0	76.0	1
Sulla	33.0	9.0	3.0	45.0	1
Sulla	27.5	8.1	0.65	36.2	4
Sainfoin	29.0	nd	nd	nd ²	5
Crownvetch	16.0	13.0	2.0	31.0	1
Hairy canary clover	121.0	65.0	1.0	187.0	1
Prostrate clover	100.0	23.0	3.0	126.0	1
Canary clover	83.0	54.0	6.0	143.0	1
Red clover	0.4	0.6	0.7	1.7	2
Sheep's burnet	1.0	1.4	1.0	3.4	1
Yorkshire fog (wild ecotype)	1.1	0.3	0.4	1.8	1
Lucerne	0.0	0.5	0.0	0.5	2
Perennial ryegrass	0.8	0.5	0.5	1.8	2
Chicory	1.0	0.4	0.3	1.7	2
Chicory (<i>Cichorium intybus</i>)	1.7	1.2	0.2	3.1	3
Dock	11-23				6
Tropical tree and legume forages (leaf only)					
Acacia mangium	71.8	25.1	3.5	100.4	7
Arachis pintoi	31.7	1.2	0.7	33.6	7
Senna velutina	54.0	nd	nd ³	54.0	7
Calliandra sp	158.1	28.4	7.8	194.3	7
Desmodium ovalifolium	196.9	30.5	10.1	237.5	7
Leucaena diversifolia	75.8	13.2	3.5	95.5	7

¹Diet selected samples. nd², no determined. nd³, non-detectable. All samples were freeze dried and ground. CT concentration determined by the Butanol-HCl method of Terrill *et al.* 1992. (1) Terrill *et al.* (1992), (2) Jackson *et al.* (1996b), (3) Min *et al.* (1997), (4) Douglas *et al.* (1993), (5) Barry & Manley (1986), (6) Waghorn & Jones (1989), (7) Jackson *et al.* (1996a).

The effect of soil fertility on CT concentration is summarized in Table 1.6. In cultivars of *L. pedunculatus* and *L. corniculatus* that produced substantial amounts of CT, growing the plants under conditions of low soil fertility substantially increased CT concentration.

Table 1.6. The condensed tannin concentration (g/kg DM) of vegetative Lotus species as affected by soil fertility. (All determined by the vanillin-HCl procedure of Broadhurst & Jones 1978).

Soil	<i>Lotus pedunculatus</i>	<u><i>Lotus corniculatus</i></u>		Author
Fertility	cv Maku	cv Empire	cv Maitland	
High soil fertility¹				
	20.0	3.0	15.0	John & Lancashire (1981)
	32.0	NA	NA	Barry & Forss (1983)
	45.6	NA	NA	Barry & Duncan (1984)
Low soil fertility²				
	94.5	2.8	28.1	Lowther <i>et al.</i> , (1987)
	78.0	NA	NA	Barry & Forss (1983)
	105.9	NA	NA	Barry & Duncan (1984)

¹ High soil fertility pH>5.3; Olsen P > 18 g/ml; SO₄-S >12 µg/g.

² Low soil fertility pH <5.2; Olsen P > 8g/ml; SO₄-S > 5 µg/g.

NA, Not applicable.

1.4.4 Properties of CT

Condensed tannins can complex with numerous types of molecules including:

- proteins
- polysaccharides
- nucleic acids & minerals
- bacterial cell membranes and enzymes involved in digestion
- other compounds such as polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP)

1.4.4.1 Interaction of CT with protein

CT:protein interactions are most frequently based on:

- Hydrophobic and hydrogen bonding
- Ionic and covalent bonding which occurs less frequently

CT:protein complexes are specific and depend on the structure of both the CT and the protein (molecular weight, tertiary structure, isoelectric point, and compatibility of binding sites; Van Sumere *et al.* 1975; Asquith & Butler 1986; Asquith *et al.* 1987). Reactivity can be defined as the ability of CT to precipitate protein per unit weight (Bate-Smith 1973) and is reported to increase with increasing degree of polymerisation (Horigome *et al.* 1988) and with increasing delphinidin: cyanidin ratio (Jones *et al.* 1976). It has been suggested that the interactions between CT and protein are most frequently based on hydrophobic and hydrogen bonding (Spencer *et al.* 1988; Haslam 1989). Covalent and ionic bonding occur less frequently (Kawamoto *et al.* 1997).

CT-protein interactions occur highly selectively in the region of aromatic groups of hydrocarbon side chains (formation of a hydrophobic pocket; Spencer *et al.* 1988). The second stage is firmly reinforced by appropriate hydrogen bonds between tannin residues and polar groups (e.g. guanidino, amide and peptide, amino hydroxyl and carboxyl groups) of amino acids. The process of complex formation is normally reversible, and both proteins and CT can be recovered unchanged from the complex. However, if the protein and CT are brought into contact under some conditions (e.g. alkaline, O₂) then the CT may become oxidized or denatured over time and then form covalent bondings with nucleophilic amino acid side chains such as lysine or cysteine of protein, making the association irreversible (Fig. 1.5; Haslam 1989; Kawamoto *et al.* 1997).

1.4.4.1.1 Factors influencing CT-forage protein interactions

1.4.4.1.1.1 pH

It is generally accepted that the interaction between protein and CT are pH dependent: each protein has a distinctive pH optimum (4.1 for BSA and 6.1 for Rubisco) which usually lies at or close to the isoelectric point of the particular proteins (Martin & Martin 1983). In accordance with those results, globular proteins with acidic isoelectric points like BSA have greater affinities for tannin at pH 4.0-5.0 than pH 7.0, and basic proteins like lysozyme have higher affinities at a higher pH (Hagerman & Butler 1981). CT extracted from sainfoin and lotus forages complexed strongly with plant Rubisco protein at pH 5.0-7.0 but weakly at pH < 3.0 and pH > 8.0 (Jones & Mangan 1977; McNabb *et al.* 1998).

1.4.4.1.1.2 Chemical and physical properties of protein

Proline-rich proteins, high molecular weight (MW) polymers and other hydrophobic amino acids have a high affinity for CT, while tightly coiled globular proteins have much lower affinities for CT than conformationally loose proteins (Hagerman & Butler 1981; Asquith & Butler 1986). Recently, It has been confirmed that CT extracted from lotus forages had a much greater affinity for plant soluble leaf protein (Rubisco) than bovine serum albumin (BSA; McNabb *et al.* 1998). However, Mole *et al.* (1990) observed that proline richness *per se* is not the only reason for high protein affinity for CT and suggested that post-translational modifications such as glycosylation may enhance the affinity towards CT by converting the protein to a more open conformation. It is therefore reasonable to believe that interactions of CT with proteins might depend on the shape of the protein.

1.4.4.1.1.3 Sites of action of CT

From the research reviewed to date (Fig. 1.5), it is evident that CT can bind to plant protein in the rumen (pH 6.0-7.0), reducing microbial proteolysis, and then release proteins in the acidic conditions of the abomasum (pH 2.5-3.5), enabling enzymatic hydrolysis and absorption of AA from the small intestine. This forms the basis of how low concentrations of CT can be used to increase amino acid absorption in forage-fed ruminants. The pH of the small intestine increases from 5.5 at the beginning of the small intestine and reaches its maximum of pH 8.0 between 10 and 21 metres from the duodenum (Terrill *et al.* 1994; Wang *et al.* 1996c). McNabb *et al.* (1998) reported that re-association of CT and protein will occur in the distal small intestine.

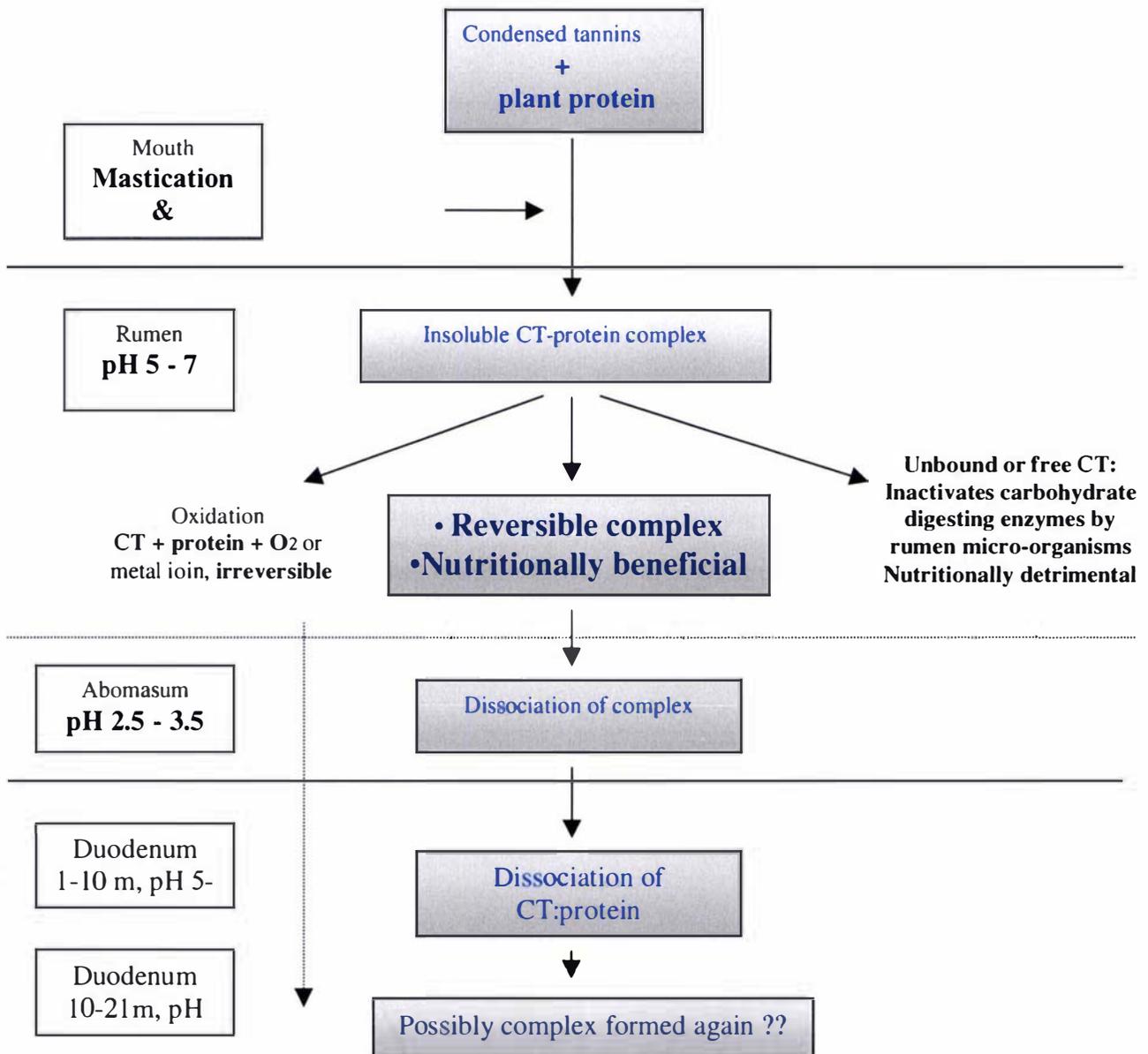


Figure 1.5 Proposed mechanism of CT reaction with plant proteins and free CT formation during cell disruption (adapted from Jones & Mangan 1977; Barry & Manley 1986; Haslam 1989, 1993; McNabb *et al.* 1998).

1.4.4.1.2 Interaction with other macromolecules

CT and carbohydrates: Condensed tannins are known to interact with carbohydrates, particularly starch. However, their affinity seems to be much less than for proteins. These interactions are probably based on hydrophobic and hydrogen linkages (Haslam 1989). Precipitation of five different legume starches by tannin has been reported and different phenolic compounds precipitated different starches and starch fractions (Deshpande & Salunkhe 1982). More fundamental research should be carried out to get information about the nature of the interactions of CT with starch and other polysaccharides.

CT and minerals: CT can also react with metal ions, such as Fe^{++} , Mg^{++} , and other metal ions to form complexes which are insoluble or partly soluble (Garcia-Lopez *et al.* 1990; Scalbert 1991; Haslam 1993). The mechanism may involve chelation but is not fully understood.

CT and binding agents: Strong complexes are formed between CT and agents like polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Both the PEG and PVP have been widely used to study the interaction between CT and protein/carbohydrate (see section 1.5; Jones and Mangan 1977; Barry & Manly 1986). Barry and Foss (1983) showed that 1.7 g of PEG (MW 3,350) was required to complex one gram of CT in *L. pedunculatus*, such that the CT did not precipitate forage protein in this species.

1.4.5 Effects of CT on enzyme activity, rumen microorganisms and intestinal proteases

The effect of CT in inhibiting the growth of rumen microorganisms and modifying microbial proteolysis has been described for several bacteria species including *S. bovis*, *B. fibrisolvens*, *Fibrobacter succinogenes*, *P. ruminicola*, and *Ruminobacter amylophilus* (Bae *et al.* 1993; McAllister *et al.* 1993; Jones *et al.* 1994). CT from sainfoin (*Onobrychis viciifolia* Scop.) inhibited the growth in *B. fibrisolvens* A38 at 200 µg CT/ml and *S. bovis* 45S1 at 100 µg CT/ml, but had little effect on *P. ruminicola* B14 or *R. amylophilus* WP 225 up to 600 µg CT/ml (Jones *et al.*, 1994). They also found that CT from sainfoin caused the normally single rods of *B. fibrisolvens* A 38 to become filamentous and inhibited the separation of cells after division, implying that the cell wall of proteolytic bacteria was the target of sainfoin CT and that the reduced bacterial growth may reflect reduced proteolysis in the rumen in the presence of CT.

CT have been shown to inhibit rumen bacterial enzyme activities. The CT in sainfoin (25 µg CT/ml) reduced the protease enzyme activity in cultures of *B. fibrisolvens* and *S. bovis* by 48 and 92 %, respectively (Jones *et al.*, (1994). In contrast, the total protease enzyme activity in cultures of *P. ruminicola* was 36 % higher in the presence of 100 µg CT/ml than in the control, and there was no reduction in the cell-associated protease activity of *P. ruminicola* up to 300 µg CT/mg. Bae *et al.* (1993) also found that cell-associated endoglucanase activity in *F. succinogenes* S85 increased in concentrations of CT from *L. corniculatus* between 100 and 300 µg/ml, but suppressed by 400 µg of CT/ml.

It can be shown that CT could possibly reduce breakdown of soluble protein by forming CT-protein or CT-bacterial cell complexes, and also inactivate cell-bound extracellular enzymes, thus inhibiting proteolysis (Bae *et al.* 1993; Jones *et al.* 1994). However, these authors did not conclude the reason rumen bacterial activity in both *P. ruminicola* and *F.*

succinogenes is enhanced at the low level of CT concentrations and this requires further study. It may be due to enzyme induction. These results also shown that rumen bacteria vary widely in their growth and enzyme activity response to the presence of CT. Marked resistance of *P. ruminicola* or *R. amylophilus* WP 225 up to 600 µg CT/ml (Jones *et al.* 1994) in sainfoin CT is of particular interest because this strain represents one of the most significant bacterial species responsible for the hydrolysis of soluble protein in the rumen. The effect of CT upon these rumen bacterial populations in the rumen, and rate of proteolysis of each bacterial strains have not been investigated yet and research needs to conducted in this area.

In *in vitro* studies, McNabb *et al.* (1998) reported that added CT had the greatest inhibiting effect on intestinal protease enzymes (trypsin and chemotrypsin) digesting Rubisco at pH 6.0. However, at pH 8.0, CT had little or no effect on these enzymes, suggesting that trypsin and chemotrypsin were able to overcome the inhibitory effects of CT at this pH. In addition, digestive enzymes such as alkaline phosphatase and 5'-nucleotide phosphodiesterase, solubilized from bovine intestinal mucosa and purified to homogeneity, were found to be strongly inhibited *in vitro* by CT purified from sorghum seeds and from quebracho (Blytt *et al.* 1988). In addition, digestive enzymes such as trypsin and α -amylase have been reported to ^{be} inhibited *in vitro* by CT extracted from field beans (Griffith & Moseley 1980). However, when tested under *in vivo* conditions, both enzymes were much less inhibited than under *in vitro* conditions, suggesting that small intestine digestive enzymes may be relatively insensitive to inhibition by CT. Compared to knowledge of the effect of CT on rumen proteolytic enzymes, the effect of CT on intestinal protease enzymes is less well known and requires further study.

1.4.6. Potential value of CT in ruminant nutrition

The presence of CT in diets for monogastric animals is generally viewed adversely (Jansman 1993), because of CT reducing protein digestion in the small intestine. However, in ruminants, low dietary concentrations of CT can induce beneficial effects under certain well defined conditions and these will be described in the following section.

1.5 CONDENSED TANNINS AND FORAGE FEEDING VALUE

The effect of CT upon forage FV can be defined as the animal production response (see section 1.3) and is determined by the combined effects of CT upon VFI and NV (digestion process and the efficiency of utilization of digested nutrients).

Effects of CT have been widely investigated by comparing unsupplemented sheep (CT-acting) with a group of sheep supplemented with PEG (MW 3,350), as PEG specifically binds and inactivates CT without affecting microbial or digestive enzymes (Jones & Mangan 1977; Barry & Manley 1986). Therefore, comparing animals not receiving PEG with animals given PEG can be used to quantify the effects of CT in ruminants.

1.5.1 Effects of CT on forage VFI

High CT concentrations in *L. pedunculatus* (55-100 g CT/kg DM) have been shown to depressed VFI in sheep (-12 ~ -27 %; Barry & Duncan 1984; Waghorn *et al.* 1994). However, Terrill *et al.* (1992) and Wang *et al.* (1996a) reported that medium concentrations of CT in sulla (45 g CT/kg DM) and in *L. corniculatus* (34 & 44 g CT/kg DM) had no effect upon VFI of grazing sheep. It would appear that CT concentrations below about 40g CT/kg DM in forage legumes have no effect upon VFI, whereas higher CT concentrations depress VFI, in line with plant CT production being a defence against overgrazing by herbivores.

1.5.2. Effects of CT in digestion in the rumen and small intestine

1.5.2.1 Carbohydrate digestion

Low concentrations of CT (22g CT/kg DM) in *Lotus corniculatus* have been reported to have no effects on the digestion of both water soluble and structural carbohydrates in sheep (Ulyatt & Egan 1979; Waghorn *et al.* 1987b). Observations by Barry and Manley (1984), however, showed an 18 % reduction in cellulose and a 23 % reduction in hemicellulose digestibility when sheep were fed *L. pedunculatus* containing 106 g CT/kg DM compared with lotus containing 46 g CT/kg DM. Furthermore, Barry *et al.* (1986) found that increasing the CT concentration from 14g/kg DM to 95g/kg DM in *L. pedunculatus* lowered the proportion of hemicellulose, cellulose and lignin digestion taking place in the rumen, with the reduction being greatest for lignin. Recently, however, Waghorn *et al.* (1994) reported that the fibre digestibility was not affected by 55 g CT/kg DM in *L. pedunculatus*. The main effects of CT on carbohydrate digestion may be due to CT inactivating carbohydrate-digesting enzymes secreted by rumen micro-organisms. It is probable that this occurs when *L. pedunculatus* containing high concentrations of CT is fed to sheep, but not when *L. corniculatus* containing medium CT concentrations is fed.

1.5.2.2 Nitrogen digestion

Non ammonia nitrogen (NAN) flowing post the abomasum/duodenum sheep fed fresh forages is positively related to total N intake and to digestible organic matter intake (Waghorn & Barry 1987) and also to legume CT concentration (Barry *et al.* 1986; Fig. 1.6).

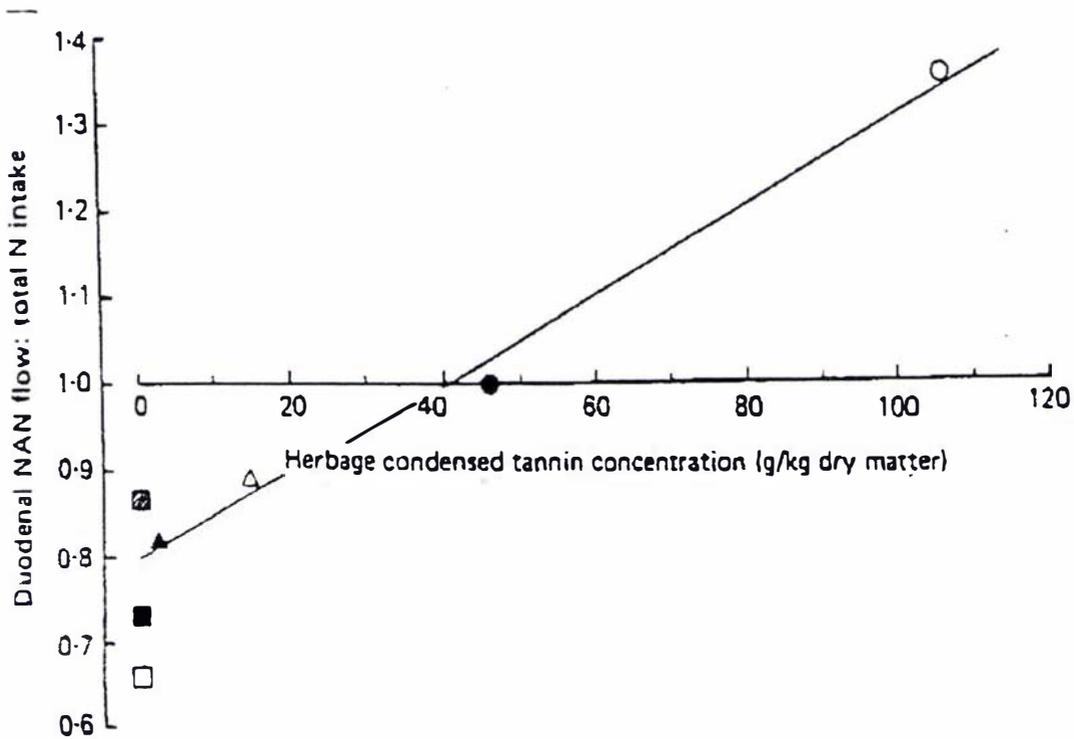


Figure 1.6. Duodenal non-ammonia nitrogen (NAN) flow per unit total N intake as a function of herbage condensed tannin concentration in sheep fed on lotus species. (o) high- and (•) low-tannin *Lotus pedunculatus*. (Δ) high- and (▲) low-tannin *Lotus corniculatus*. Results are compared with the non-tannin containing herbages (■) short rotation ryegrass, (□) perennial ryegrass, and (▨) white clover. All results are for an N intake of 28 g/d and refer to fresh forages. From Barry *et al.* (1986).

However, this relationship differs between herbage, with ruminant animals consuming conventional forages (i.e. perennial ryegrass, short-rotation ryegrass and white clover which contain only trace amount of CT), duodenal NAN flow is only about 0.75 of N intake (MacRae & Ulyatt 1974), indicating extensive absorption of ammonia from the rumen. However, for ruminants consuming CT-containing fresh forages, duodenal NAN flow increased linearly with increasing CT concentration and equalled N intake at a CT concentration of approximately 40 g/kg DM (Fig. 1.5). For fresh sainfoin (about 39 g CT/kg DM) or a mixture of sainfoin plus red clover (60:40 %; DM basis), microbial protein synthesis (g/g degraded AA) was higher (10-25 %) than when red clover was fed as the sole diet (Beever & Siddons 1986), with the authors attributing these effects to the CT in sainfoin.

The effect of CT upon apparent absorption of EAA from the small intestine differed between *L. corniculatus* and *L. pedunculatus*, and is shown in Table 1.7. When expressed as a proportion of N intake, action of CT in *L. corniculatus* increased absorption of EAA (65 %) and BCAA (51 %) from the small intestine, mainly due to the effect of CT in increasing abomasal AA flow (Barry & Manley 1984; Barry *et al.* 1986; Waghorn *et al.* 1987b). This is due to the action of CT reducing N degradation in the rumen, which is clearly shown by the ammonia concentrations in the rumen fluid sheep fed on CT-containing diets being lower than that of PEG-supplemented sheep (Table 1.7). For the non-EAA, however, apparent absorption from the small intestine was decreased by 10 %.

Experiments carried out with *L. pedunculatus* also showed a similar increase in amino acids flux leaving the rumen, but only an 11 % increase in apparent absorption of EAA from the small intestine due to action of CT (Table 1.7).

Table 1.7. Effect of CT upon rumen ammonia concentration and upon the intake and absorption of amino acid (AA; g/d) in sheep fed fresh *Lotus corniculatus* and *Lotus pedunculatus*.

	CT-acting		PEG-supplemented		Author	
Rumen ammonia (mg N/l)						
<i>L. corniculatus</i>	367		504		1,3	
<i>L. pedunculatus</i>	175		458			
Absorption of amino acids						
PEG supplementation	EAA		BCAA		NEAA	
	-	+	-	+	-	+
<i>L. corniculatus</i> (22 g CT/kg DM)						
AA intake (g/d)	112	112	41	41	98	98
Flux to SI	96	64	35	23	69	60
Apparent absorption from SI:	59	36	23	15	37	41
Proportion intake	0.53	0.32	0.56	0.37	0.38	0.42
Response to CT (%)	+ 65		+ 51		-9	
<i>L. pedunculatus</i> (55 g CT/kg DM)						
AA intake (g/d)	103	117	42	47	88	99
Flux to SI	121	106	45	40	84	78
Apparent absorption from SI:	81	83	30	32	51	57
Proportion intake	0.79	0.71	0.71	0.68	0.58	0.58
Response to CT (%)	+ 11		+ 4		0	

Abbreviations: AA; amino acids; EAA, essential AA; BCAA, branched chain AA, NEAA. Non-EAA; PEG, polyethylene glycol; SI, small intestine. Sources: (1) Waghorn *et al.* (1987b), (2) Wang *et al.* (1996c), (3) Waghorn *et al.* (1994), (4) McNabb *et al.* (1993).

These effects with *L. pedunculatus* could be due to effects of the CT in not releasing some AA in the small intestine, increasing endogenous protein secretion or inactivating digestive enzymes (Barry & McNabb 1998). The reason why CT preferentially increased abomasal flow and absorption of EAA from the small intestine relative to NEAA is not clear and requires further study.

1.5.3 Digestion of CT

Terrill *et al.* (1994) reported that there was no absorption of ¹⁴C-labelled CT in the small intestine and large intestine of sheep fed the temperate legume *L. pedunculatus*. In contrast, Perez-Maldonado & Norton (1996) found that 45 % of the CT eaten was lost from the rumen and 40 % of that flowing at the abomasum was lost (probably absorbed) from the lower tract in sheep and goats fed the tropical legume *Desmodium intortum*. These differences are probably due to differences in structure and reactivity between the two types of CT.

1.5.4 Utilization of absorbed nutrients

McNabb *et al.* (1993) found CT in *L. pedunculatus* (55g CT/kg DM) reduced the proteolysis of forage sulphur AA in the rumen. They found that CT in *L. pedunculatus* (55 g extractable CT/kg DM) had no effect upon irreversible loss (IRL) of methionine from blood plasma but markedly increased the IRL of cystine (56 %) and reduced the IRL of inorganic sulphate from blood plasma.

Inter-conversion of three metabolites are shown in Fig. 1.7. The ³⁵S studies with *L. corniculatus* (Wang *et al.* 1994, 1996c) and with *L. pedunculatus* (McNabb *et al.* 1993) showed that CT significantly increased trans-sulphuration of ^{methionine} methionine to cystine (28-56 %) and increased plasma cystine flux to body synthetic reactions (46-47 %) and reduced the quantity of inorganic sulphate excreted in the urine or recycled to the gut.

These data indicate the importance of cysteine as a limiting AA in these NZ sheep selected for high rates of wool growth, and show that forage CT can be used to increase the AA availability for animal production.

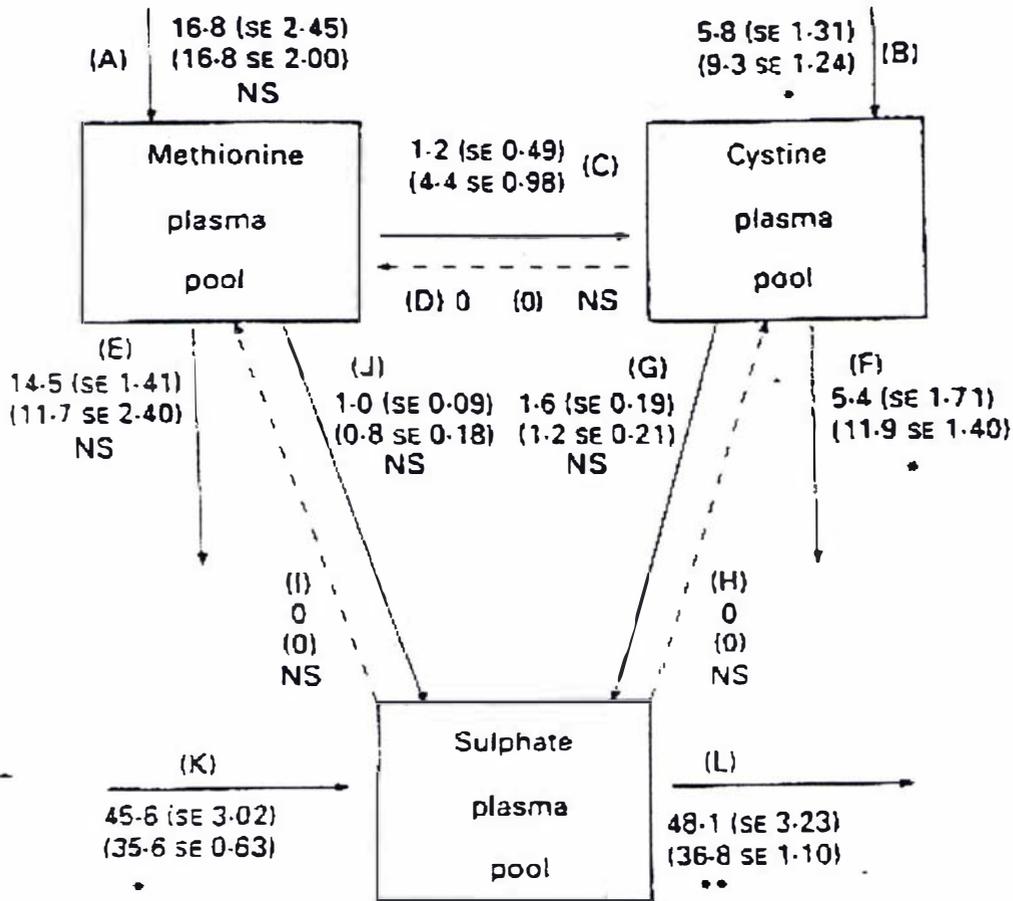


Figure 1.7. A general three-pool, compartmentalized model for amino acids transactions in the posthepatic plasma of sheep fed on fresh *Lotus corniculatus*, with and without an intra ruminal infusion of polyethylene glycol (PEG). Mean values with their standard errors are shown for six PEG-infused sheep, with the corresponding values for six CT-acting sheep given in parentheses. ↓↓, rates of flow ($\mu\text{mol}/\text{min}$). From Wang *et al.* (1994).

1.5.5 The effect of CT on animal production (Liveweight gain, wool and milk production)

In growing lambs grazing *L. corniculatus* for four months, action of CT increased wool growth and improved the efficiency of wool production (12 %) without affecting rate of LWG or VFI (Table 1.8) indicating that supply of EAA (probably sulphur containing AA) was limiting wool growth but not body growth in these lambs. The VFI and body growth data (Table 1.8) suggested that feed conversion efficiency of lambs grazing lotus was higher than for lambs grazing lucerne. However, the cause of the better feed conversion in lambs grazing lotus is not clear.

Table 1.8. Voluntary feed intake, liveweight gain (LWG), carcass gain and wool growth in lambs (n = 20) grazing the forage legumes *Lotus corniculatus* (34 g CT/kg DM) and lucerne (0.3 g CT/kg DM).

	<i>L. corniculatus</i>		Lucerne		S.E.
	CT-acting	PEG-group	CT-acting	PEG-group	
Rumen ammonia (mg N/l)	255	370	555	535	
VFI (hg OM/head/d)	1.19	1.20	1.32	1.34	0.056
LWG (g/d)	203	188	185	178	4.8
Carcass gain (g/d)	79	75	68	63	2.9
Wool growth (g/d)	12.1	10.9	10.8	10.2	0.39
Efficiency of wool production (g wool growth/kg OMI/d)	10.2	9.1	8.2	7.6	

From: Wang *et al.* (1996a)

Figure 1.8 shows that action of CT in lactating Romney ewes (aged 3 to 5 years) grazing *L. corniculatus* (44.5 g/kg CT) had no effect upon milk secretion at peak lactation (weeks 3 and 4), but increased the efficiency of milk production and increased the secretion rates (g/h) of whole milk (21 %) and of lactose (12 %) and protein (14 %) in mid and late lactation (weeks 6-11). The diet selected was of high NV, containing 35.5 g total N/kg DM, with an *in vitro* digestibility of 73 %. Milk protein concentration (g/kg) was unaffected by CT, but action of CT substantially reduced milk fat content ($P < 0.1$). These results have implications for the dairy industry, as a nutritional treatment which increases protein secretion but reduces milk fat concentration would be value for human nutrition.

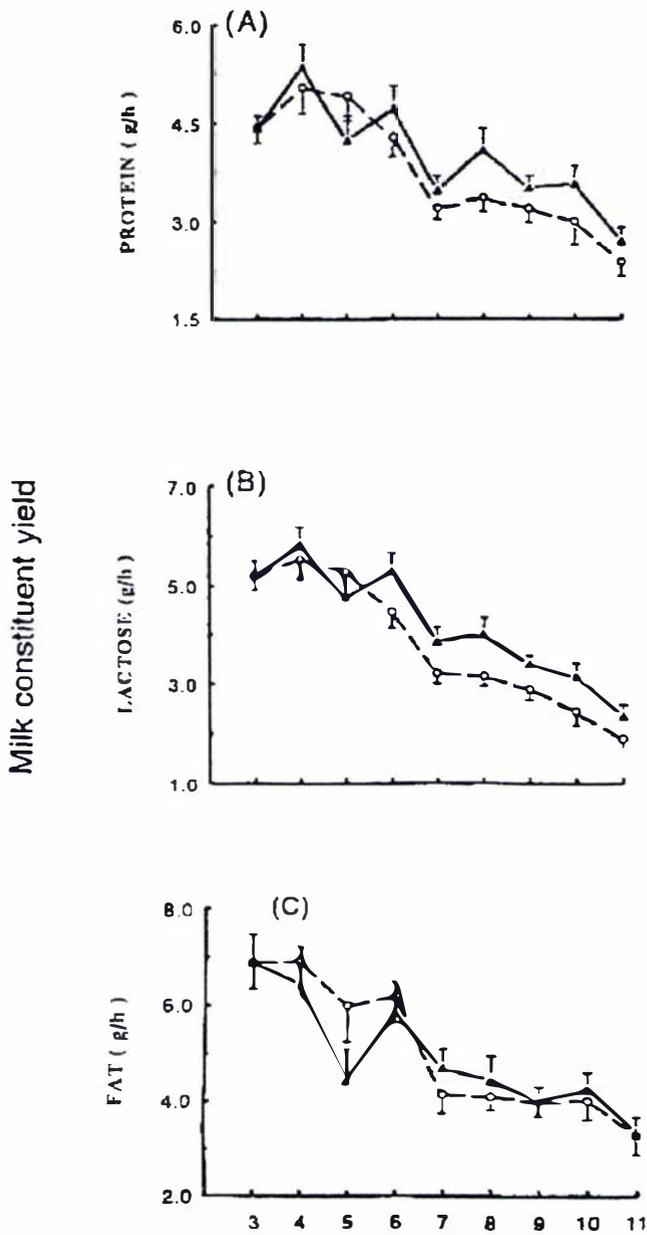


Figure 1.8. Yields (g/h) of (a) protein, (b) lactose and (c) fat in the milk of twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; CV. Grasslands Goldie). CT acting ewes (—▲—); ewes given twice-daily oral administration of polyethylene glycol (PEG; MW 3500) (-O-). From Wang *et al.* (1996b).

1.6 CONCLUSIONS AND NEED FOR FUTURE RESEARCH

1.6.1 Temperate fresh forages are the main feed source for grazing ruminant animals, especially in New Zealand. However, when ruminants are fed on high quality fresh forages containing high concentration of N, about 70% of the forage N is degraded to ammonia in the rumen with only 30 % escaping to the small intestine for absorption. Therefore, inefficient use of nitrogen by ruminants needs research to focus on improving nitrogen retention in the rumen by using natural compounds with known ability to reduce proteolysis such as CT.

1.6.2. The amount and type of CT synthesized by forages vary considerably depending on forage species, soil type, stage of development, and environment conditions. Therefore, the study of the effects of CT on ruminants nutrition requires identification of both type and amount of CT present in a particular forage.

1.6.3. Condensed tannins can bind with many types of molecules such as proteins, carbohydrates, rumen micro-organisms and other compounds. The CT bind with proteins mainly by hydrophobic and hydrogen bonding and this binding is influenced by many factors such as pH, structure and molecular weights of both the CT and the proteins. These interactions occur in the rumen at the isoelectric point (pH 6.0-7.0) and then release proteins in the acidic conditions of the abomasum (pH 2.5-3.5), indicating that CT can be used to reduce microbial proteolysis and increasing absorption of AA from the small intestine.

1.6.4. The CT reduced the growth of rumen micro-organisms and modifying microbial proteolysis. CT behave differently with different bacterial strains. For examples, CT inhibit

rumen protease enzyme activities in cultures of *B. fibrisolvans* and *S. bovis* by 50 to 90%. While the protease enzyme activity in *P. ruminicola* and endoglucanase enzyme activity in cultures of *F. succinogenes* were higher in the presence of CT (100-300 µgCT/ml) relative to control incubations (no CT). However, the reason behind enhancement of rumen bacterial activity in both strains at lower concentrations of CT was unknown and this requires further study. Furthermore, the effect of CT upon these proteolytic bacterial populations in the rumen and the rate of proteolysis of plant protein by these strains have not been investigated yet and research needs to be conducted in this area.

1.6.5. There is strong evidence that improved rate of nutrition, especially protein content in the diet, can lead to increased multiple ovulation rate (OR). This multiple OR is significantly correlated with the plasma concentration of BCAA and EAA. Low levels of CT in forage plants have shown to offer advantages for sheep production by protecting plant protein from rumen degradation, increasing the flow of EAA to the small intestine, and increasing the absorption of EAA from the small intestine. BY increasing EAA absorption, CT may be beneficial for increasing OR in grazing ruminants. However, little information on this aspect are available and research in these areas needs to be conducted.

1.6.6. The CT reduced rumen ammonia concentration and linearly increased abomasal NAN flow and the maximum nutritional benefit can be obtained at a concentrations of up to 40 g/kg DM . Studies also are required to investigate the effects of CT on the outflow of nitrogen fractions from the rumen in sheep.

1.6.7. It has been suggested that CT at the level of 20-40 g total CT/kg DM can be beneficial for the grazing sheep without depressing VFI. This hypothesis needs to be tested by using *in-*

vitro and *in-vivo* metabolism and grazing experiments to evaluate the effect of CT upon animal production.

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CHAPTER 2

The effect of condensed tannins on the production of wool and on its processing characteristics in sheep grazing *Lotus corniculatus*

(Short title: Condensed tannin and wool production)

This chapter has been published in *Australian Journal of Agricultural Research* (1998) 49, 597-605

2.1 ABSTRACT

The effect of condensed tannins (CT) in *Lotus corniculatus* upon voluntary feed intake (VFI), concentration of rumen and plasma metabolites, production of wool and wool processing characteristics was measured in grazing ewes restricted to maintenance feeding for 125 days during summer. A rotational grazing system with restricted feed allowance with 40 mixed aged dry ewes (54 ± 1.0 kg) was used. Half of the ewes were selected randomly for twice daily oral polyethylene glycol (PEG; MW 3,500) supplementation (condensed tannins; CT-inactivated), with the remainder being CT-acting animals. Measurements of the diet selected, VFI, wool production and wool processing characteristics were made. The concentration of ammonia in rumen fluid and the concentrations of methionine, cysteine and urea in blood plasma were also measured.

The diet selected contained 32 g total nitrogen (N) and 28 g total CT/kg dry matter (DM) and had an *in vitro* organic matter digestibility of 0.70. Action of CT reduced rumen ammonia concentration (256 v. 302 mg N/L), reduced blood plasma urea concentration (7.3 v. 8.9 μ M), and increased blood plasma cysteine concentration (30 v. 27 μ M) compared to sheep receiving PEG supplementation. CT had no effect on VFI but increased clean fleece weight (2.53 v. 2.28 kg/ewe), increased staple length (7.7 v. 6.9 cm), but did not affect live weight gain (54 v. 67 g/day). The CT reduced dag weight and tended to reduce wool yellowness relative to sheep receiving PEG. There were no significant effects of CT on fibre diameter, staple strength, bulk density and wool resilience. It was concluded that action of CT in sheep fed *L. corniculatus* reduced the degradation of forage protein in the rumen and the principal effect of this was to increase the efficiency of wool production, with more wool being produced at the same feed intake and the wool being of reduced yellowness.

Additional keywords: Romney sheep, VFI, clean wool, cysteine, methionine.

2.2 INTRODUCTION

Clean wool is predominantly protein, with a very high cystine content (13 %), and L-cyst(e)ine is often the first limiting amino acid (AA) for both skin growth and wool production (Black & Reis 1979; Reis 1979; Lee *et al.* 1993; Wilson *et al.* 1996). Therefore dietary conditions which enhance the supply of sulphur AA (SAA) to the body should achieve gains in wool growth (Reis 1986; Reis *et al.* 1989). Wool growth is seasonal in New Zealand (NZ) crossbred sheep, with the highest rates of wool growth occurring during the summer months (November to February; Bigham *et al.* 1978; Sumner *et al.* 1994). However, protein digestion is inefficient in ruminants grazing high quality fresh NZ forages, with duodenal flow of non-ammonia nitrogen (NAN) being only about 65% of the total nitrogen (N) eaten (MacRae & Ulyatt 1974). Hence, techniques that reduce the very high degradation rates (70-80%) of forage proteins in the rumen could increase wool growth.

The legume *Lotus corniculatus* (birdsfoot trefoil) contains condensed tannins (CT; 34 g total CT/kg dry matter), which can be used to reduce rumen protein digestion (Terrill *et al.* 1992b; McNabb *et al.* 1996; Waghorn & Shelton 1996). CT are secondary plant compounds which can bind with protein by hydrogen bonding at near neutral pH (pH 4.0-7.0) to form CT-protein complexes, but dissociate and release protein at pH less than 3.5 (Jones & Mangan 1977). Much work has been conducted in NZ upon the effects of CT on nutrient supply of sheep fed fresh forage diets, using controlled indoor experiments with the animals individually fed. CT in both *L. corniculatus* and *L. pedunculatus* plants can be used to reduce the degradation of plant protein by rumen micro-organisms, with the overall effect of CT upon digestion being related to its concentration in the forage, structure and degree of polymerisation. High levels of CT in LP (75-90 g CT/kg DM) decrease voluntary feed intake

(VFI) and reduce rumen fibre digestion (Barry & Duncan 1984; Barry & Manley 1984). However, low concentrations of CT in *L. corniculatus* (20-40 g/kg DM) appear to be beneficial through protecting dietary protein against degradation in the rumen (Barry 1989; Tanner *et al.* 1994), increasing essential amino acid (EAA) absorption from the small intestine (Waghorn *et al.* 1987; Wang *et al.* 1996b), and increasing cysteine flux to body synthetic reactions (Wang *et al.* 1994), with little effect on VFI and rumen carbohydrate digestion.

Abomasal and parenteral administration of methionine and cysteine have been shown to be effective in stimulating wool growth (4-38 %), fibre diameter (0.5-2 μm) and live-weight gain (30 – 115 %) in sheep fed on maintenance diets (Mata *et al.* 1995). It seems probable that the increased supply of EAA and, especially of SAA, from the action of CT can be used to increase productivity in sheep grazing *L. corniculatus*. Objectives of the present experiment were to determine the effect of CT in *L. corniculatus* upon the production of wool, wool processing characteristics, and VFI and the concentration of rumen metabolites in grazing ewes feed at close to maintenance energy intake for four months during summer, in the period between weaning and mating.

2.3. MATERIALS AND METHODS

2.3.1 Experimental design

A grazing trial using mixed age ewes was conducted at Massey University, Palmerston North, New Zealand, from 20 December 1995 until 25 April 1996 (125 days). Forty ewes were grazed on *L. corniculatus*, with half being orally supplemented with polyethylene glycol (PEG; MW 3,500) twice daily (PEG-supplemented group) and the remaining animals forming the CT-acting group. The PEG binds with CT, preventing the CT from binding with protein (Jones & Mangan 1977; Barry & Manley 1986). Therefore, comparing ewes not receiving

PEG (CT-acting) with ewes given PEG (CT-inactivated) can be used to quantify the effects of CT in sheep grazing *L. corniculatus*. Wool production was determined by shearing the ewes at the end of the experiment; initial wool weight was predicted by shearing non-experimental animals at the start of the experiment and using this information to calculate the initial wool weight of the experimental ewes. Earlier studies showed that effects of PEG supplementation were specific for forages containing CT, with there being no responses to PEG in sheep grazing forages where the CT concentration was close to zero (Wang *et al.* 1996a).

2.3.2. Forages

Pure vegetative *L. corniculatus* (Birdsfoot trefoil; cv. Grasslands Goldie; Plate 2.1) was grazed in breaks (i.e. small areas) by the ewes, with each break lasting 3 or 4 days. Herbage mass and botanical composition were determined weekly, immediately before and after grazing, by cutting 6 x 0.125 m² quadrats per sward to ground level and drying for 16h in a forced air oven at 80°C. Herbage samples for determination of pre-grazing herbage quality (feed on offer) were collected weekly (0.4-0.5 ha/paddock) by cutting to ground level from 6 sites and pooling. Three wire mesh cages measuring c. 1.4 x 0.9 m were placed in each break before ewes commenced grazing. At the end of grazing that break, the cages were removed and the forage cut and samples taken corresponding to what animals were observed to be eating. These are referred to as diet selected (hand plucked). Samples of diet selected were also taken using sheep fistulated in the oesophagus (OF sheep), referred to as diet selected (OF extrusa). These were taken at the beginning and end of grazing each break. Samples were analysed for chemical composition from both hand plucked and OF extrusa samples.



Plate 2.1 *Lotus corniculatus*

(Birdsfoot trefoil; cv. Grasslands Goldie)

2.3.3. Animals

Forty mixed age dry ewes with an initial weight of 54 kg (s.e. 1.0) were used as the experimental animals. An extra twelve ewes with a mean live-weight of 53 kg (S.E. 1.3) were weighed and shorn at the beginning of the experiment, to predict initial clean fleece weight (CFW). The value of CFW was recorded and used to estimate the initial CFW of the 40 experimental ewes. The 40 selected experimental ewes were weighed at fortnightly intervals and shorn when the experiment concluded. All ewes were treated with IVOMEK (Merck, Sharp & Dohme, NZ Ltd; 12 mL) and deltamethrin (10 mL; Wipe-out; Coopers Animal Health NZ) to control internal and external parasites at the beginning and mid-point of the experiment. Half of the ewes were randomly selected for oral PEG supplementation (CT-inactivated) and the remainder being CT-acting animals. The PEG was administered orally twice daily, at 0800 and at 1800 hours. The PEG was administered as a 50 % w/v solution, with 60 g PEG given daily to each ewe. The dose of PEG was calculated on the basis of

estimated daily voluntary feed intake (VFI) and the CT content in *L. corniculatus*, with the objective of administering 1.7 g PEG/g CT consumed (Barry & Forss 1983).

VFI was measured using slow releasing chromium capsules (Nufarm, Auckland, NZ), as described by Parker *et al.* (1989). Chromium ^{chromium oxide (Cr₂O₃)} capsules were orally administered to each ewe in week 5 of the experiment. Faeces sampling from the rectum commenced 8 days after chromium capsules had been given and was continued every 2 days during 2 weeks. Faeces samples were dried at 80 °C for 3 days and then bulked for each ewe over the 14 day sampling period for chromium analysis. A group of 6 rumen fistulated sheep (3 control and 3 PEG; orally supplemented), were grazed with the ewes over this period (14 days), with chromium capsules suspended in the rumen, and used to measure chromium release rate from the capsules.

2.3.4. Grazing management

The ewes were rotationally grazed on the LC over summer, with forage supply restricted so that the ewes were close to the maintenance level of energy intake (allowance 1.3 kg DM/ewe/day), using small breaks which had electric fences at the front and rear. CT-acting and PEG ewes grazed together on each sward. Total grazing days were calculated using Equation 4.

$$\text{Total grazing days} = \frac{\text{Herbage mass (kg DM/ha)} \times \text{Paddock area (ha)}}{\text{Number of animals} \times \text{feed allowance/hd/day}} \quad (4)$$

This management was intended to provide vegetative high quality forage at all times. The relatively short period of grazing each break (3-4 days) was in order to provide reasonably constant levels of available feed at all times. Ewes had free access to water throughout the experiment.

2.3.5. Sample preparation

Rumen fluid (15 mL/time) was sampled between 1300 and 1500 hours from 12 ewes/group on days 1, 30 and 100 after the PEG supplementation started using a stomach tube, with the same ewes sampled on each occasion. The rumen fluid was used to determine rumen ammonia (NH₃; 10 mL) and volatile fatty acid (VFA; 5 mL) concentration. These samples were deproteinized immediately, using 1 ml 50% HCl for NH₃ samples and 1 ml of a mixture of metaphosphoric acid (187.5 g/L) and formic acid (250 ml 100 %/L) for VFA samples, with 1 ml of *iso*-caproic acid (0.52 % v/v) added as internal standard for VFA samples. The samples were then centrifuged at 17,000 g for 15 min and the deprotenised supernatant stored at -20°C for analysis.

Blood samples (2 x 7 mL) were taken from the same 12 ewes on days 2, 31 and 101 to measure the concentrations of methionine, cysteine and urea. The blood samples were collected into vacutainers with Na-EDTA as an anticoagulant and placed on ice. One vacutainer was held on ice and centrifuged (3,200 g for 20 min at 4 °C) to obtain blood plasma for cysteine and urea analysis. One portion (for cysteine; 1 mL of blood plasma) was treated with 0.5 mL 0.75 % sodium dodecyl sulfate (SDS), 9 mM Na-EDTA, 0.1 mL 200 mM phosphate buffer pH 8.0 containing 80 mM dithiothreitol (DTT), vortexed and held at room temperature for 15 minutes so as to release the protein-exchangeable cysteine. Then 500 µL of chilled 30 % (w/v) trichloroacetic acid (TCA) was added to precipitate the protein, vortexed and centrifuged at 11,000 g for 20 minutes at 4 °C. The supernatant was then filtered (0.2 µm; Sartorius AG, Goettingen, Germany) and stored at - 80 °C for analysis. A second portion (for urea; 2 mL) of plasma was stored at - 20 °C for analysis. Whole blood (1 mL) from the second vacutainer tube for methionine analysis was held on ice, with 1 mL 200 µM norleucine in milliQ water added as internal standard, and 0.1 mL 200 mM phosphate buffer

pH 8.0 containing 80 mM DTT then added. The combined solution was then centrifuged (Sartorius; MW cut-off 10,000; Gottingen, Germany) at 1,000 x g for 15 min followed by 6,000 x g for 85 min. The clear deproteinised supernatant was passed through a 0.2 µm syringe filter and stored at – 85 °C prior to analysis.

2.3.6. Laboratory analyses.

2.3.6.1. Forage and faeces

All samples of feed offered and diet selected were stored at – 20 °C, freeze-dried, and ground to pass through a 1 mm diameter sieve, prior to laboratory analyses. Acetone/water-extractable, protein-bound and fibre-bound CT fractions were determined using the butanol-HCl procedure of Terrill *et al.* (1992a), total N by the Kjeldahl method, OM by ashing samples for 16 h at 550 °C and *in vitro* organic matter digestibility (OMD) by the enzymatic method of Roughan and Holland (1977). Because extractable and protein-bound CT would be dissolved in the initial *in vitro* extraction steps, but are known to be indigestible *in vivo* (Terrill *et al.* 1994), extractable and protein-bound CT (% OM) values were deducted from all *in vitro* OM digestibility (OMD) determinations. Chromium in faeces was determined by the method of Costigan and Ellis (1987).

2.3.6.2. Rumen fluid and plasma samples

Rumen VFA was determined by capillary gas chromatography (Carlo Erba GC-5380, Italy) using a 15 m x 0.53 mm i.d. stabilway-DA (1.0 µm coat thickness column, Restek Corporation, Bellefonte, PA, USA). The procedure used a split injection mode (approximately 2:1 split ratio) and a 5 °C/min temperature programme from 105 to 132 °C, with H₂ as carrier gas (pressure 0.5 kg/cm²), and a flame ionisation detector. Internal standardisation was used and the data were calculated using a Maxima 820 chromatography workstation (Waters

Associates, Milford, MA, USA). Methionine concentration was determined using high performance liquid chromatography (HPLC; Waters Associates, USA) equipped with an ion-exchange column (Na⁺-form AA analysis column; Waters associates, USA). Methionine was detected using post column derivatisation with ninhydrin and detection using absorbance at 570 nm. The concentration of cysteine was determined by continuous flow auto analyser (Technicon, Dublin, Ireland), using a modified method described by Gaitonde (1967) using acid ninhydrin reagent, followed by incubation at 95 °C. Optical density was read at 570 nm. Plasma urea and rumen ammonia concentrations were determined using an enzymatic ultraviolet colorimetric assay that utilises glutamate dehydrogenase and NADH. Both the urea and ammonia concentrations were measured by auto analyser (COBAS, FARA, Basel, Switzerland), using commercially available diagnostic kits obtained from Roche (Basel, Switzerland) for urea and Sigma (St. Louis, USA) for ammonia.

2.3.6.3. *Wool samples*

Fleeces were weighed at shearing to determine greasy fleece weight. Samples of 200-300 g were taken from each of the left and right mid-side areas just prior to shearing. Fleece yield (%) and clean fleece weight (CFW) were determined using a standard greasy wool washing procedure (36 L water/bowl; 60, 55, 50 °C and cold water and each bowl containing 32, 16, 16 mL of detergent (TERIC GN9) and clean water respectively), drying in an oven (10 min at 80 °C) to remove excess moisture and then in a humidity room (24 h at 21°C, 65 % humidity). Wool staple length (cm) was determined by measuring the length of 30 randomly chosen staples. Mean fibre diameter (MFD; μm) was measured using an Optical Fibre Diameter Analyser (OFDA 100; Melden Laboratory, Western Australia) according to the procedure described by the International Wool Testing Organisation (IWTO, 1995). A 4 g sample was taken from the scoured, carded and conditioned wool and measured for colour according to

the method described in the Standards Association of NZ (SANZ, 1984) using a colorimeter (Hunter Lab, Model D25 Optical sensor, Virginia, USA). Loose wool bulk and resilience (cm^3/g) were measured according to the method developed by the Wool Research Organisation of NZ (WRONZ, 1977). Staple strength was determined on both left and right mid-side staples of greasy wool sample at the NZ Wool Testing Authority (NZWTA Ltd; Newtons/ktex), using an Automatic Length and Strength measurement system.

2.3.7. Calculation of data and statistical analyses

The VFI was calculated as follows in Equation 5.

$$\mathbf{VFI} = \frac{F}{1 - D} \quad (5)$$

Where D is the *in vitro* OMD of the diet selected from the both hand plucked and OF samples, and F is faeces OM output, which was calculated as capsule chromium release rate divided by chromium concentration in faeces.

A regression equation was established of CFW (kg) upon live-weight, using data from the initial non-experimental shearing group. The initial CFW of the 40 experimental animals were then estimated using Equation 6. The CFW from the initial shearing group was related to LW (kg) by the following regression equation:

$$\text{CFW (kg)} = 0.344 + 0.0108\text{LW} \quad (r = 0.540) \quad (6)$$

(S.E. 0.0015)

Analysis of variance to test for differences between treatment means was done using GLM (general linear models) procedures (SAS 1985).

2.4. RESULTS

2.4.1. Forage mass

The LC was in the early flowering state throughout the experiment. Leaf mass was greatly reduced ($P < 0.001$) by grazing (Table 2.1), showing that the diet selected comprised mainly of green leaf with some stem.

Table 2.1. Pre-grazing and post-grazing forage mass (t dry matter (DM)/ha) of *Lotus corniculatus* (cv. Grasslands Goldie). Mean values with their S.E. (standard error for 16 samples)

	Pre-grazing	S.E..	Post-grazing	S.E..
	(n = 16)		(n = 16)	
Leaf	1.50	0.290	0.11	0.051
Stem	1.00	0.289	0.83	0.051
Dead + weed	0.24	0.028	0.15	0.050
Total	2.74		1.09	

2.4.2. Chemical composition

Total CT concentrations in feed on offer and diet selected (hand-plucked and early OF extrusa) were similar at c. 27-30 g/kg DM (Table 2.2). Most CT in both feed on offer and hand-plucked samples was readily extractable, with smaller amounts being protein-bound or fibre-bound, whereas in OF extrusa (early and late; samples taken at the start and the end of

grazing each break) a much lower component was readily extractable, with the largest component being protein-bound. Small amounts of CT were detected in the late OF extrusa. *In vitro* OMD and total N concentrations for both feed on offer and diet selected (hand-plucked and early OF extrusa samples) were similar, but late diet selected OF extrusa samples had lower OMD ($P < 0.001$) and total N ($P < 0.05$) concentrations compared to early OF samples.

2.4.3. Rumen metabolites

Supplementation with PEG increased rumen ammonia concentration at all sampling times ~~for~~ of ewes grazing *L. corniculatus* ($P < 0.05$; Table 2.3). After one day of PEG supplementation, molar % of acetate was higher ($P < 0.001$) and that of propionate ($P < 0.01$), *iso*-valerate and *n*-valerate ($P < 0.001$) were lower for CT-acting than for PEG-supplemented sheep (Table 2.3). This trend progressively diminished with time, with the effects being significant ($P < 0.05$) by day 35, but non-significant by day 100.

Table 2.2. Organic matter (g/kg DM), total N (g/kg DM) and condensed tannin (CT; g/kg DM) contents and *in vitro* OM digestibility (% OM) of feed offered and diet selected by sheep grazing *Lotus corniculatus* (cv. Grasslands Goldie). Oesophageal fistulae (OF): Early, samples taken at the start of grazing each break; Late, samples taken at the end of grazing each break; S.E.M., standard error of the mean.

	Feed on offer	Diet selected		S.E.M..	
		hand-plucked	OF extrusa		
			Early	Late	
	(n = 12)	(n = 12)	(n = 6)	(n = 6)	
Organic matter (OM)	909	905	871	862	4.90
<i>In vitro</i> OMD					
Unadjusted	67.7	69.6	71.6	61.3	1.56
Adjusted ^A	65.0	66.6	69.0	61.2	2.19
Total N	31.4	32.4	34.2	27.8	1.71
Condensed tannin^B	(n = 8)	(n = 8)	(n = 8)	(n = 8)	-
Extractable CT	14.1	17.0	2.8	ND	0.41
Protein bound CT	12.9	12.3	22.8	0.6	0.22
Fibre bound CT	0.7	0.5	1.2	0.2	0.02
Total CT	27.7	29.8	26.8	0.8	0.41
Bound CT (% total)	49.1	42.9	89.7	100	-

^A After deducting extractable and protein-bound CT, as described in laboratory analysis.

^B Butanol/HCl method. N.D., Not detectable.

Table 2.3. Concentration of ammonia (mg N/l) and molar percentages of volatile fatty acids (VFA) in the rumen fluid of sheep grazing *Lotus corniculatus* (cv. Grasslands Goldie), with and without twice-daily oral administration of polyethylene glycol (PEG; MW 3500). S.E.M., standard error of the mean.

	Day 1			Day 35			Day 100		
	-	+	S.E.M.	-	+	S.E.M.	-	+	S.E.M.
Ammonia	(n = 16)			(n = 16)			(n = 16)		
(mg N/l)	234.2	342.9	10.34	221.2	278.0	8.51	256.0	302.8	7.15
VFA (%)									
Acetate	68.5	65.1	1.57	72.1	70.3	1.38	64.3	63.4	1.97
Propionate	19.5	21.3	1.26	17.7	19.5	1.54	22.2	22.0	1.42
<i>Iso</i> -butyrate	1.2	1.4	0.65	1.0	1.1	0.60	1.2	1.2	0.59
<i>n</i> -butyrate	9.8	10.8	1.51	8.7	8.8	1.26	10.3	10.9	1.43
<i>iso</i> -valerate	0.6	0.8	0.40	0.4	0.5	0.32	1.6	1.6	0.73
<i>n</i> -valerate	0.6	0.8	0.35	0.5	0.6	0.27	1.2	1.3	0.63

2.4.4. Plasma metabolites

Plasma concentrations of urea were lower for CT-acting than for PEG-supplemented sheep (Table 2.4) on days 31 ($P < 0.05$) and 101 ($P < 0.01$). Plasma methionine concentration was not significantly different between treatments, whilst plasma cysteine concentration was higher for CT-acting than for PEG sheep (Table 2.4; $P < 0.05$).

Table 2.4. Plasma concentrations of urea, methionine and cysteine (μM) in ewes grazing *Lotus corniculatus* (cv. Grasslands Goldie), with and without twice-daily oral administration of polyethylene glycol (PEG; MW 3,500). S.E.M., standard error of the mean.

	CT-acting sheep (n = 16)	PEG-supplemented sheep (n = 16)	S.E.M.
PEG	-	+	
Urea (mM)			
Day 2	8.4	7.8	0.38
Day 31	6.7	7.7	0.30
Day 101	7.3	8.9	0.39
Methionine			
(μM)	18.7	18.2	1.07
Cysteine			
(μM)	30.0	27.1	0.97

2.4.5. Voluntary feed intake and live-weight gain

The VFI of CT-acting and PEG-supplemented ewes, calculated using OMD from both hand plucked and the mean of early and late OF extrusa samples, were not significantly different ($P > 0.05$; Table 2.5). Ewes in both groups had a similar mean live-weight (LW) and similar low levels of LW gain (Table 2.5).

2.4.6. *Wool growth and wool processing characteristics*

The CFW ($P < 0.05$), rate of clean wool growth (g/day; $P < 0.05$), efficiency of wool production (g clean wool/kg OMI; $P < 0.05$) and staple length ($P < 0.001$) were all significantly higher for CT-acting than for PEG-supplemented sheep, with greatly reduced dag weight ($P < 0.05$) and reduced wool yellowness ($P = 0.07$) in CT-acting sheep (Table 2.5). There were no significant differences in MFD, staple strength, loose wool bulk and wool resilience between treatments.

Table 2.5. Organic matter intake, wool production and wool processing characteristics in sheep grazing *Lotus corniculatus*, with or without polyethylene glycol (PEG; MW 3,500) supplementation. S.E.M., standard error of the mean.

PEG	CT-acting sheep -	PEG-supplemented sheep +	S.E.M.
	(n= 16)	(n=16)	
OMI (kg/ewe.d)^A			
Adjusted			
Hand plucked	1.31	1.23	0.037
Mean OF extrusa	1.23	1.20	0.051
Live-weight (LW; kg/ewe)			
Start LW	54.2	54.3	1.00
Final LW	61.0	62.6	1.49
LW gain (g/day)	54.4	66.5	9.32
Wool weight (kg)			
Greasy fleece	3.03	2.79	0.094
Clean fleece	2.53	2.28	0.083
(g/day)	13.2	11.1	0.66
Clean belly	0.24	0.28	0.023
Dag wt (g)	6.7	38.6	10.92
Efficiency of wool production			
(g clean wool/kg OMI/day)	10.5	8.8	1.45
Wool characteristics	(n = 32)	(n = 32)	
Colour:			
Y value ^B	58.7	59.1	0.31
Y-Z value ^C	0.22	0.91	0.263
Staple length (cm)	7.7	6.9	0.17
Mean fibre diameter (µm)	42.8	42.4	0.69
Staple strength (Newtons/ktex)	62.7	68.9	5.88
Loose wool bulk (cm ³ /g)	18.9	18.9	0.41
Wool resilience (cm ³ /g)	7.0	7.3	0.27

^A Calculation of OMI based on OMD of diet selected from hand plucked or the mean of Oesophageal fistulae (OF) samples taken at the beginning and end of grazing each break.

^B Y value: wool brightness.

^C Y-Z value: wool yellowness

2.5. DISCUSSION

The most significant finding in this experiment was that the CT in *L. corniculatus* increased wool production (10 %) without changing VFI, resulting in a significant increase in the efficiency of wool production (16.2 %). Calculation of VFI in grazing animals is very sensitive to the OMD of diet selected; in this study the effects upon VFI were checked by sampling diet selected using two methods (hand plucked and OF extrusa). The present study showed that the action of CT also improved wool processing characteristics, producing wool of increased length and reduced yellowness. ^(y-z) The latter characteristic is particularly important for improving dye uptake (Thompson 1989; Reid 1993; Atkins 1997).

The reduced concentration of ammonia and molar percentages of *iso*-valerate and *n*-valerate (days 35) in the rumen fluid, together with the lower plasma urea concentration in CT-acting sheep compared with PEG-supplemented sheep indicated that CT in LC reduced protein degradation in the rumen. This is a similar result to other grazing studies with LC and probably results in more EAA being absorbed from the small intestine (Waghorn *et al.* 1987; Wang *et al.* 1994). The lower proportions of *iso*-valerate and *n*-valerate in CT-acting compared to PEG-supplemented sheep on days 1 and 35 can be explained by CT reducing deamination of specific EAA, with *iso*-valerate originating from the deamination of leucine and *n*-valerate from deamination of arginine, ornithine, proline, σ -aminovaleric acid and lysine (Van Soest 1982; El-Shazly 1952). In the present study, major VFA resulting from carbohydrate digestion showed minor differences between CT-acting and PEG-supplemented ewes on day 1, but this had disappeared by day 100. This suggests that in the present study *L. corniculatus* containing 28 g CT/kg DM had little effect upon major VFA formation during rumen carbohydrate fermentation.

The ^{35}S studies with *L. corniculatus* (Wang *et al.* 1994) and with *L. pedunculatus* (McNabb *et al.* 1993) showed that CT reduced the loss of sulphur AA (SAA; 30 %) in the rumen, and increased blood plasma concentration and irreversible loss (IRL) of cystine. Due to the high cystine content in wool, SAA play a major role in stimulating wool growth, which is influenced by diet and the wool-producing capacity of the sheep (Reis 1979; Reis *et al.* 1989; Mata *et al.* 1995). The increase in wool growth due to action of CT in the present study is probably due its effect on increasing EAA absorption from the small intestine (Waghorn *et al.* 1987; Wang *et al.* 1996b), and increasing cysteine flux to body synthetic reactions (Wang *et al.* 1994).

Wool colour, especially yellowness, is influenced by photo-oxidation of aromatic AA (Goddinger *et al.* 1994), bacterial stain (Thompson 1989), high temperature, humidity, fleece architecture (Henderson 1968) and propensity of wool to yellow (Wilkinson 1981; Reid 1993). Action of CT tended to reduce wool yellowness (0.22 v. 0.91) in the present study relative to sheep receiving PEG. Probably CT altered the chemical composition of wool, specially changing the relative proportion of individual proteins in scoured wool. An increase in the supply of SAA (methinine and cysteine) stimulates the synthesis of the high-sulphur protein (especially cyst(e)ine) in the wool and skin (Reis 1979; Lee *et al.* 1993; Mata *et al.* 1995). Sulphur-enriched wools (4.3 % S) are generally more difficult to solubilise and more poorly penetrated by alkaline reagents (0.8 M potassium thioglycollate; pH 10.3) than wools with a lower content of sulphur. This is related to the higher cross-linking density of disulphide bonds in wool (Broad *et al.* 1970). Similar mechanisms may inhibit microbial activity in high SAA-containing wool and so reduce yellowness, which is normally caused by colonies of bacteria when conditions are warm, wet and damp (Thompson 1989).

The aromatic amino acids phenylalanine and *p*-tyrosine are degraded in wool irradiated with UV-light and these AA produce photo-oxidation products such as *o*-, *m*- and *p*-tyrosine

(derived from phenylalanine), and the *p*-tyrosine photoproducts dihydroxyphenylalanine (DOPA) and dityrosine. These AA might be involved in the formation of photoyellowing or yellowing precursors in UV-irradiated wool (Inglis & Lennox 1963; Asquith & Rivett 1969; Goddinger *et al.* 1994). Continuous intravenous infusions into a jugular vein of either L-cysteine or DL-methionine with methioxinine (*O*-methyl-DL-homoserine) caused a substantial reduction (40-45 %) in the content of high tyrosine proteins in wool, whereas the high sulphur proteins were usually slightly increased (Reis & Gillespie 1985). Studies of the relative proportion of high and low sulphur wool proteins and of high tyrosine proteins are required, to see how these are affected by the action of dietary CT, and to examine their relationship with wool yellowness.

Sheep grazing the CT-containing forages *L. corniculatus* (Leathwick & Atkinson 1995, 1996) and sulla (*Hedysarum coronarium*; Niezen *et al.* 1995) showed significantly reduced dag formation and flystrike compared with sheep grazing lucerne or ryegrass/white clover pasture, which contain only traces of CT. The present study confirmed that action of CT in *L. corniculatus* dramatically reduced dag formation, but the mechanism of how CT accomplishes this is unknown.

This study has shown that the action of CT in *L. corniculatus* in increasing the supply of EAA (including SAA) has important effects on the productivity of grazing ewes fed at close to maintenance during summer. These include increases in wool production and improved wool quality without affecting VFI and rumen fermentation of carbohydrate to major VFA, thereby increasing the efficiency of wool production. The mechanism of how CT reduced wool yellowness is of major interest to the NZ wool industry and warrants further research.

This study was supported by a grant from Wools of New Zealand. The authors wish to thank Mr G. S. Purchas and Mr W. C. L Howell, who assisted with data collection. The skilled assistance from the Wool and Nutrition Laboratory staff, Massey University is greatly

acknowledged. Dr. P. C. H. Morel is thanked for advice with statistical analysis and Mr T. G. Harvey is thanked for advice on grazing management. Scholarship support to B. R. Min from the New Zealand Department of Education is acknowledged.

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CHAPTER 3

The effect of condensed tannins in *Lotus corniculatus* upon reproductive efficiency and wool production in sheep during late summer and autumn

(Short title: *Condensed tannins and reproduction*)



This chapter has been published in *Journal of Agricultural Science, Cambridge* (1999)

132, 323-334.

3.1 ABSTRACT

A grazing experiment, conducted for 55 days (from 4 March to 29 April) in the late summer/autumn of 1997, at Massey University, Palmerston North, New Zealand, compared the reproductive efficiency and wool growth of ewes grazing *Lotus corniculatus* (birdsfoot trefoil) or perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) dominant pasture (pasture). Half the ewes grazing each forage were given daily oral polyethylene glycol (PEG: molecular weight 3500) supplementation to inactivate the condensed tannins (CT) in lotus. A rotational grazing system with 200 mixed age ewes (54.2 ± 0.88 kg/ewe; 50 ewes/treatment) was used.

The effect of forage species and PEG supplementation upon voluntary feed intake (VFI), concentration of plasma metabolites, reproductive efficiency, wool production and wool characteristics was measured during two synchronized oestrous cycles. The ewes were restricted to maintenance feeding for the first 12 days of each oestrous cycle and then increased to *ad libitum* for the 6 days prior to and including ovulation. Lotus contained 17 g total CT/kg dry matter (DM) in the diet selected. There were only trace amounts of total CT in pasture. *In vitro* organic matter digestibility (OMD) was higher for lotus (0.82 v. 0.74) than for pasture, whilst lotus contained less nitrogen (N; 37.8 v. 44.5 g/kg OM).

Mean ovulation rates (OR) for CT-acting and PEG sheep grazing pasture and lotus were respectively 1.33 v. 1.35 and 1.78 v. 1.56, with corresponding lambing percentages being 1.36 v. 1.36 and 1.70 v. 1.42. Fecundity (number of corpora lutea/ewe ovulating) was greater for ewes grazing lotus than pasture ($P < 0.01$), and tended to be greater for CT-acting than for PEG sheep grazing lotus ($P = 0.06$). In unsupplemented sheep, ewes grazing lotus had increased plasma concentrations of branched chain amino acids (BCAA; 57 %) and essential amino acids (EAA; 52 %) compared to ewes grazing pasture.

In ewes grazing pasture, PEG administration had no effect on plasma concentrations of urea and free amino acids, VFI, reproductive efficiency and wool production. However, in sheep grazing lotus, plasma concentrations of urea were significantly lower and concentrations of most amino acids were significantly higher for CT-acting than for PEG supplemented ewes (CT not acting); there was no difference in VFI between these two groups. Compared to ewes grazing pasture, ewes grazing lotus had similar VFI but produced more wool with longer staples and thicker fibre diameter, with there being no effect of PEG supplementation.

It was concluded that feeding lotus increased the efficiency of both reproduction and wool production without an increase in VFI, and that a possible cause was the action of CT in increasing plasma EAA and especially BCAA concentration.

3.2. INTRODUCTION

Most New Zealand (NZ) vegetative pastures contain high concentrations of nitrogen (N; 25-35 g/kg dry matter (DM)) and metabolizable energy (ME; 11-12 MJ/kg DM), and carbohydrate digestion is efficient on such diets (Ulyatt & MacRae 1974; Waghorn & Barry 1987). However, 25-30 % of the N eaten is lost across the rumen because the rapid degradation of soluble protein to ammonia-N exceeds the capacity for microbial protein synthesis (MacRae & Ulyatt 1974; Ulyatt *et al.* 1975; Beever 1993). Therefore the performance of ruminants grazing on fresh pasture could be limited by protein supply, because protein absorption from the small intestine is low in relation to ME intake (Barry *et al.* 1982; Waghorn & Barry 1987).

Condensed tannins (CT) are polyphenolic compounds known to precipitate dietary proteins, with the extent of this reaction being dependent on the concentration, molecular weight and structure of the CT and on protein structure (Jones & Mangan 1977; Martin & Martin 1983; Asquith & Butler 1986; Spencer *et al.* 1988). The CT:protein complex is stable and insoluble at pH 3.5-7.0 and medium concentrations of CT in *Lotus corniculatus* (30-35 g/kg DM) have reduced protein solubility and degradation in the rumen (Min *et al.* 1998a), increased the absorption of essential amino acids (EAA) from the small intestine by 62 % (Waghorn *et al.* 1987a) and increased the flow of cysteine to body synthetic reactions (Wang *et al.* 1994). In long term grazing experiments with sheep, the increase in EAA absorption caused by the action of CT in *L. corniculatus* increased wool growth by 12 % during summer (Wang *et al.* 1996a; Min *et al.* 1998b) and increased milk protein secretion by 14 % in mid and late lactation during spring (Wang *et al.* 1996b).

Increased protein absorption has been implicated in increasing the ovulation rate (OR) of ewes (Smith 1991), and this was illustrated by an increase in ewes showing multiple

ovulations when given abomasal infusions of lactalbumin and soya protein isolate (73 v. 55 %; Cruickshank *et al.* 1988). Subsequent work correlated this response to an increase in plasma concentration of branched chain amino acids (BCAA; valine + leucine + *iso*-leucine; Waghorn *et al.* 1990). It therefore seems possible that the increased supply of protein and, especially of BCAA, caused by the action of CT could be used to increase reproductive efficiency in grazing ewes.

The objectives of the present investigation were to measure effects of CT in *L. corniculatus* upon reproductive efficiency and wool production in grazing ewes during autumn. Perennial ryegrass/white clover dominant pasture, containing only trace amounts of CT, was grazed by similar animals as a control diet.

3.3 MATERIALS AND METHODS

3.3.1 *Experimental design*

A grazing trial involving 200 mixed age Romney ewes, including 100 rising 2-year-olds mated for the first time, was conducted at Massey University, Palmerston North, NZ, from 4 February 1997 (late summer) to 29 April (autumn) 1997 (85 days). The experiment was a 2 x 2 factorial design, using two types of forage (*L. corniculatus* v. perennial ryegrass/white clover pasture), with half the ewes grazing each forage receiving a twice daily oral supplement of polyethylene glycol (PEG; MW 3500; PEG-supplemented group). The PEG binds with CT, preventing the CT from binding with protein (Jones & Mangan 1977; Barry & Manley 1986). Effects of CT can be quantified by comparing unsupplemented ewes (CT-acting) with ewes given PEG (CT-inactivated). The experiment was conducted over three oestrous cycles, with oestrus being synchronized in each cycle for all ewes. Reproductive efficiency was measured as OR in three synchronized oestrous cycles using laparoscopy and as lambs born/ewe; data are expressed in terms of fertility (ewes cycling/ewes mated) and

fecundity (number ovulations/ewe ovulating). Wool production was determined by shearing the ewes at the end of the experiment (from 4 February to 29 April 1997); both fleece weight and wool processing characteristics were measured.

3.3.2. Forages

Pure vegetative *L. corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) and pasture were grazed in breaks by the ewes, with each break lasting 3 or 4 days. Measurement of herbage mass before and after grazing and collection of samples of feed on offer (cut to soil level) and diet selected (using sheep fistulated in the oesophagus; OF) were performed as described by Min *et al.* (1998b). Pre-and post-grazing herbage mass were determined weekly, immediately before and after grazing, by cutting eight random quadrats (6 x 0.125 m²) per paddock to ground level and drying at 90 °C for 17 hours. A further eight samples per paddock were cut to ground level, pooled, and stored at -20 °C for nutritive value analysis of feed on offer. The diet selected was determined using six OF Romney sheep, which allowed sampling for organic matter digestibility (OMD), total nitrogen (N) and CT. Samples were stored -20 °C, and then freeze dried and ground for chemical analysis.



Plate 3.1 Ewes grazing on perennial ryegrass/white clover pasture during autumn 1997



Plate 3.2 Ewes grazing on *L. corniculatus* during autumn 1997

3.3.3. *Grazing management*

Total grazing days (TGD; Min *et al.*, 1998b) were calculated using Equation 7.

$$\text{TGD} = \frac{\text{HM} \times \text{PA}}{\text{n} \times \text{FA}} \quad (7)$$

Where HM is herbage mass (kg DM/ha), PA is paddock area (ha), n is number of animals, and FA is feed DM allowance/head per day (kg). This management was intended to provide vegetative high quality forage at all times. The relatively short period of grazing each break (3-4 days) was in order to provide reasonably constant levels of available feed at all times.

All 200 ewes were rotationally grazed on the pasture during the first 30 days, which included the first ovulation cycle, with feed allowance restricted to 1.5 kg DM/ewe per day, so that the ewes were fed at close to the maintenance level of energy intake. They were then randomly allocated to treatment groups, which were balanced for age of ewe, and grazed on either pasture (100 ewes) or lotus (100 ewes) during the second and third ovulation cycles (34 days), with and without PEG supplementation (n=50 ewes/group). During ovulation cycles 2 and 3 the feed allowance was kept at maintenance for the first 12 days of each cycle, and then increased to *ad libitum* allowance (c. 2.2 kg DM/ewe) for the 6 days prior to and including ovulation. Day of ovulation was defined as day zero. After ovulation, the feed allowance was reduced to maintenance again. The rationale for this was that a minimum of 6 days of increased protein supply immediately prior to ovulation was required to increase ovulation rate in sheep (Smith *et al.* 1983; Stewart & Oldham 1986). *Ad libitum* feeding over this period was designed to give the action of CT in *L. corniculatus* maximum opportunity for increasing EAA supply over this critical period, whilst restricted feeding ensured that all ewes on both forages were kept close to the maintenance level of energy intake at all other times. At the end of oestrous cycle 3, the ewes continued grazing either lotus or pasture for a further 21 days,

with and without PEG supplementation, taking the total period of lotus feeding to 55 days. The groups were then joined and grazed on pasture until lambing.

3.3.4. *Animals*

Mean initial liveweight (LW) was 52.1 kg (S.D. 0.38) for rising 2-year-old ewes and 56.3 kg (S.D. 0.49) for ewes aged 3 years or older. At the start of the experiment, all ewes were weighed, tagged and drenched with anthelmintic (Ivomec; Merck, Sharp & Dohme, NZ Ltd) to control internal parasites, and treated for external parasites (Wipeout; Coopers Animal Health NZ). Anthelmintic (Ivomec) was then given at monthly intervals to all ewes. The animals were weighed at fortnightly intervals, and feed supply adjusted if needed to keep the ewes at maintenance. All ewes were shorn before the experiment commenced and again at the conclusion of the grazing experiment (29 April). The dose of PEG was calculated on the basis of estimated daily VFI and the CT content in lotus, with the objective of administering 1.8 g PEG/g CT, this being the minimum amount to bind all the CT and prevent binding with soluble protein (Barry & Forss 1983). The PEG (71 g/d during maintenance and 110 g/d during *ad libitum* feeding) was administered as a 50 % w/v solution, given daily as two equal doses at 08.00 and 16.00 h. The VFI was measured using slow release chromium capsules (Cr₂O₃ matrix, Nufarm, Auckland, NZ), according to the method described by Parker *et al.* (1989) and Min *et al.* (1998b). Sixteen ewes per treatment group were used to estimate VFI. Four rumen-fistulated Romney sheep were grazed on each forage for 27 days to measure the Cr release rate of capsules suspended in the rumen. Measurements started on day 5 after chromium capsules insertion, and proceeded at 3-day intervals until day 27.

3.3.5. *Synchronization of oestrus and determination of ovulation rate*

Ovulation was synchronized using controlled release intravaginal devices (CIDR; type G; Carter Holt Harvey; containing 0.3 g progesterone). They were inserted for 12 days of the first ovulatory cycle and were inserted for 8 days of the second and the third cycles. Ewes were mated with vasectomized teaser rams fitted with tugging crayons (four rams per group; during 8 days) at the first and the second ovulatory cycles and then entire rams fitted with tugging crayons (five rams per group; during 25 days) were mated with ewes at the third cycle. Ovulation rate was determined by counting corpora lutea (CL) using laparoscopy (Kelly & Allison 1976) *c.* 7 days after oestrus started. A total of three laparoscopy measurements were made (4 March, cycle 1; 20 March, cycle 2 and 7 April, cycle 3), following the initial synchronization. Subsequent lambing records were collected, including birth rank and birth weight.



Plate 3.3 Mating during the third ovulatory cycles.



Plate 3.4 Lambs & ewes

3.3.6. Plasma samples

Blood samples (2 x 7 ml) were taken from the jugular vein of 80 sheep grazing each forage (40 PEG and 40 CT-acting) on day -8 (maintenance feed) and on day -1 (*ad libitum*) before ovulation during each oestrous cycle to measure plasma urea concentration. Samples for plasma amino acid analysis were taken from the same sheep on day -1 at the third cycle only. The blood samples were collected into vacutainers with Na-EDTA (Becton Dickinson, USA) as an anticoagulant and placed on ice. Sample preparation for cysteine and urea were performed using the methods described by Min *et al.* (1998b). One vacutainer was held on ice and centrifuged (3200 g for 20 min at 4 °C) to obtain blood plasma for cysteine and urea analysis. The samples for cysteine analysis was treated with 0.5 ml 0.75% sodium dodecyl sulphate (SDS), 9 mM Na-EDTA, 0.1 ml 200 mM phosphate buffer pH 8.0 containing 90 mM dithiothreitol (DTT) in order to release the protein-exchangeable cysteine. The concentration of cysteine was determined by Continuous Flow Auto Analyzer (CFAA; Technicon, Dublin, Ireland) using the method described by Gaitonde (1967) modified to include acid ninhydrin reagent, following incubation at 95 °C.

Whole blood from the second vacutainer tube was used for the analysis of all other amino acids. Whole blood (1ml) was mixed with 1 ml 200 µM norleucine in milliQ water (added as internal standard) and 0.1 ml 200 mM phosphate pH 8.0 containing 80 mM DTT in a Centrisart tube (Sartorius; MW cut-off 10000; Gottingen, Germany), vortexed and then centrifuged at 1000 g for 15 minutes followed by 6000 g for 85 min. The clear deproteinized supernatant was filtered through a 0.2 µm syringe filter into an micro-centrifuge tube and stored at -85 °C for analysis. All amino acid concentrations except cysteine were determined using a HPLC (Waters Associates, USA) equipped with an ion-exchange column (Na⁺-form AA analysis column; Waters Associates, USA). Amino acids were detected using post-

column derivatization with ninhydrin. Absorbance was measured at 570 nm. Plasma urea concentration was determined with an enzymatic ultraviolet colourimetric assay that utilizes glutamate dehydrogenase and NADH, using an Auto-Analyzer (COBAS FARA, Basel, Switzerland).

3.3.7 Laboratory analyses

3.3.7.1 Forage and faeces

Samples of feed offered and diet selected were stored at -20 °C, freeze-dried, and ground to pass through a 1 mm diameter sieve for laboratory analysis. Acetone/water-extractable, protein-bound and fibre-bound CT fractions in forages and OF extrusa were determined using a butanol-HCl colorimetric procedure (Terrill *et al.* 1992). All CT concentrations were determined using CT extracted from *L. pedunculatus* as a reference standard (Jackson *et al.* 1996). Total N was determined by the Kjeldahl method, OM by ashing samples for 16 h at 550 °C and *in vitro* OMD by the enzymatic method of Roughan & Holland (1977). Chromium in faeces was determined by the method of Costigan & Ellis (1987). As extractable and protein-bound CT would be dissolved in the initial *in vitro* extraction steps, but are known to be indigestible *in vivo* (Terrill *et al.* 1994), extractable and protein-bound CT (% OM) values were deducted from all *in vitro* OM digestibilities.

3.3.7.2. Wool samples

Fleeces were weighed at shearing to determine greasy fleece weight, with samples of 200-300 g being taken from both the left and right mid-side areas for laboratory analyses. A standard greasy wool washing procedure and measurements of staple length (cm), mean fibre diameter (MFD; μm) and wool colour were made using the methods described by Min *et al.* (1998b).

3.3.7.3. *Calculation of data and statistical analyses*

Ovulation data was analyzed in terms of fertility (ewes ovulating/ewes mated) and fecundity (CL/ewe ovulating). The fecundity and lambing percentage of ewes giving birth are presented in the form of a percentage of ewes in the group having multiple (one, two or more ovulations). For statistical analysis the data were transformed using a Logistic Regression Model (SAS 1995) and treatment effects established using the Chi-squared procedure, as described by Smith (1985). The other treatment means for plasma urea and plasma AA concentrations were analyzed by General Linear Models procedures using the Statistical Analysis System package (SAS 1985), with the factors fitted being forage type, PEG supplementation and the interaction. Data are presented as mean values, together with the standard error of the mean (SE); the number of observations contributing to each mean is denoted in all tables by the letter n.

3.4. RESULTS

3.4.1. *Forages*

Both pasture and lotus were in a vegetative state throughout the experiment. Pre- and post-grazing herbage mass were generally similar for both forages and were higher during *ad libitum* than maintenance feeding (Table 3.1).

Table 3.1. Pre-grazing and post-grazing forage mass (dry matter; DM (t/ha)) of *Lotus corniculatus* (cv. Grasslands Goldie) and perennial ryegrass/white clover pasture.

	Forage mass (t DM/ha)				S.E.
	Pasture		Lotus		
	Pre-grazing	Post-grazing	Pre-grazing	Post-grazing	
Maintenance intake period*					
n	8	8	5	5	
DM (t/ha)	2.49	1.12	2.06	0.61	0.144
<i>Ad libitum</i> period†					
n	3	3	3	3	
DM (t/ha)	2.80	1.44	3.23	1.40	0.208

* Fed for the initial 12 days in each cycle (8 Feb - 20 Feb, 27 Feb - 8 Mar and 14-25 Mar) before *ad libitum* feeding started.

† Fed for the final 5 days in each cycle (21-26 Feb, 9-14 Mar and 25-30 Mar), with day of ovulation = day 0.

3.4.2. Chemical composition

Total CT concentration in the lotus was 23 g/kg DM for feed offered and 17 and 9 g/kg DM for OF extrusa representing the diet selected at the beginning and end of grazing each break (Table 3.2). Only trace amounts of total CT were detected in pasture. Most CT in the lotus on offer was readily extractable (65 %), with much smaller amounts being protein-bound (32 %) or fibre-bound (3 %; Table 3.2). In the diet selected (OF extrusa), a much lower component was readily extractable (23-35 %), with the largest component being protein-bound (55-62 %).

For both forages, *in vitro* OMD and total N concentration were higher for the diet selected than for feed on offer. *In-vitro* OMD was slightly higher for lotus than for pasture in both feed

on offer and OF extrusa. Lotus contained lower contents of N in both feed on offer and early OF extrusa than pasture, but was similar for late OF extrusa.

Table 3.2. Organic matter (OM), total N (N) and condensed tannin (CT) contents and in vitro OM digestibility of feed offered and diet selected by sheep grazing *Lotus corniculatus* (cv. Grassland Goldie) and perennial ryegrass/white clover pasture.

	Feed on		Diet selected (OF extrusa)*				S.E. (D.F. 28)
	offer		Early†		Late†		
	Pasture	Lotus	Pasture	Lotus	Pasture	Lotus	
n	7	7	6	6	4	4	
OM (g/kg DM)	893	900	851	844	857	848	6.88
<i>In vitro</i> OMD (% OM)							
Unadjusted	64.9	78.7	81.8	84.4	76.3	86.8	3.60
Adjusted†	64.8	76.4	81.6	82.9	76.0	86.0	3.59
Total N (g/kg OM)	41.5	29.0	49.6	41.1	42.3	43.3	0.43
Condensed tannin (g/kg DM)							
Extractable CT	0.3	14.9	1.1	5.7	1.6	2.1	0.49
Protein bound CT	0.5	7.4	1.2	9.0	1.5	5.6	0.43
Fibre bound CT	0.3	0.8	1.8	1.8	1.0	1.3	0.26
Total CT	1.1	23.1	4.1	16.5	4.1	9.0	0.62
% bound CT	73.0	35.5	73.1	65.4	61.0	77.0	-

* OF: Oesophageal fistulae.

† Early: samples taken at the start of grazing each break;

Late: samples taken at the end of grazing each break.

† After deducting extractable and protein-bound CT, as described in laboratory analysis.

3.4.3. Plasma metabolites

There were no differences in plasma urea concentration between the four groups of animals during ovulatory cycle 1, when all animals grazed pasture without PEG administration. Plasma urea concentrations in sheep grazing lotus during ovulatory cycles 2 and 3 were significantly lower for CT-acting than for PEG-supplemented animals (Table 3.3) during both maintenance and *ad libitum* ($P < 0.01$) feeding, but PEG did not affect plasma urea concentrations of sheep fed pasture.

For sheep grazing pasture, PEG supplementation generally had no effect on plasma amino acid concentration (Table 3.4). Comparing the unsupplemented groups, plasma concentration of ~~most~~^{total} amino acids were significantly higher ($P < 0.01$) in sheep grazing lotus than those grazing pasture, with the increase being 52 % for total EAA, 57 % for BCAA and 25 % for methionine. Most of the lotus effect can be explained by the action of CT, which significantly increased plasma concentration of all EAA except arginine ($P < 0.01$).

Table 3.3. Plasma concentration of urea (mM) in ewes grazing perennial ryegrass/white clover pasture and *Lotus corniculatus* (cv. Grasslands Goldie), with and without twice-daily oral administration of polyethylene glycol (PEG; MW 3500)

Oestrous cycles	Pasture		Lotus		S. E. (D.F. 76)
	PEG-sheep	CT-acting	PEG-sheep	CT-acting	
n	20	20	20	20	
1st cycle (Both groups grazed on pasture)					
Maintenance intake period	6.9	6.5	7.1	6.9	0.22
<i>Ad libitum</i> intake	8.4	8.3	8.5	8.4	0.25
2nd cycle (Ewes grazed on either pasture or lotus)					
Maintenance intake period	4.6	4.5	5.4	4.5	0.19
<i>Ad libitum</i> intake	5.1	4.8	9.2	8.3	0.20
3rd cycle (Ewes grazed on either pasture or lotus)					
Maintenance intake period	6.1	6.2	8.6	7.7	0.22
<i>Ad libitum</i> intake	4.5	4.1	7.5	6.6	0.23

Table 3.4. Plasma concentration of amino acids (μM) in ewes grazing perennial ryegrass/white clover pasture and *Lotus corniculatus* (cv. Grasslands Goldie) ad libitum, with and without twice-daily oral administration of polyethylene glycol (PEG; MW 3500).

Amino acids	Pasture		Lotus		S. E. (D.F. 69)
	PEG- sheep	CT-acting sheep	PEG- sheep	CT-acting sheep	
n	18	18	19	18	
EAA*					
Valine	138	126	161	199	5.2
Leucine	77	69	93	108	3.8
Iso-leucine	52	45	58	72	3.3
Tyrosine	46	41	41	49	2.8
Phenylalanine	30	30	30	38	2.7
Histidine	54	56	59	67	3.8
Tryptophan	106	105	177	247	6.4
Lysine	103	89	100	129	5.2
Arginine	34	51	71	68	5.7
Threonine	114	99	77	106	5.5
Methionine	12	12	12	15	1.8
Cysteine (n = 10)	28	33	33	35	1.5
NEAA†					
Asparagine	17	27	21	25	3.2
Serine	85	85	62	86	4.7
Glutamate	188	186	192	254	6.4
Proline	61	68	69	96	5.3
Glycine	366	338	330	480	10.8
Alanine	139	116	120	149	5.5
BCAA‡	267	241	312	379	12.8
EAA*	786	742	894	1128	35.9
NEAA†	856	819	793	1091	35.8

* Essential amino acids (including BCAA and cysteine).

† Non-essential amino acids.

‡ Branched-chain amino acids (valine, leucine and iso-leucine).

3.4.4. Reproductive rate

Effects of the nutritional treatments upon mean ovulation rate, lambing percentage and lamb birth weight are shown in Table 3.5, whilst treatment effects upon ewe fecundity are given in Table 6. Mean birth weight of single, twin and triplet lambs was respectively 5.2, 4.4 and 3.9 kg per lamb, and these were not affected by the nutritional treatments (Table 3.5).

Table 3.5. The effect of grazing ewes on *Lotus corniculatus* or perennial ryegrass/white clover pasture, and of supplementation with polyethylene glycol (PEG; MW 3500), on ovulation rate (CL/ewe mated), lambing (lambs born/ewe mated) and lamb birth weight (kg/lamb)

	Pasture		Lotus		S. E. (D.F. 196)
	PEG- sheep	CT-acting	PEG- sheep	CT-acting	
Ovulation rate (OR)					
n	50	50	50	50	
1 st cycle	1.29	1.42	1.34	1.45	0.090
2 ^d cycle	1.23	1.35	1.32	1.43	0.081
3 ^d cycle	1.35	1.33	1.56	1.78	0.080
Lambing (lambs born/ewe mated)					
	1.36	1.36	1.42	1.70	0.097
Birth weight					
Single					
n*	29	32	27	21	
Mean value	5.21	5.28	5.12	5.00	0.153
Twin					
n*	28	24	40	42	
Mean value	4.44	4.16	4.60	4.35	0.184
Triplet					
n*	3	3	-	15	
Mean value	4.93	3.03	-	3.80	1.031

* Number of lambs.

Ewe fertility increased from 92 % in cycle 1 to 98 % in cycle 2 and then 100 % in cycle 3, and was not affected by forage type, PEG supplementation or age of ewe (2 years *v.* 3 years and older). Fecundity at ovulation was consistently less for rising 2-year-old than for older ewes ($P < 0.01$), but there were no interactions between age of ewe and the nutritional treatments. PEG supplementation had no effect upon the fecundity of ewes grazing pasture (Table 3.6). Fecundity of ewes grazing lotus was greater than that of ewes grazing pasture during cycle 3 ($P < 0.01$), with this trend becoming apparent during cycle 2 ($P < 0.11$). During cycle 3, fecundity of CT-acting ewes grazing lotus was greater than that of PEG-supplemented ewes (CT not acting; $P = 0.06$).

Fertility at lambing was 90.1 % for rising 2-year-old ewes and 95.1 % for older ewes ($P > 0.05$), and was not affected by the nutritional treatments. Fecundity at lambing was less for rising 2-year-old than for older ewes ($P < 0.001$), with no interactions between age and nutritional treatments, and was greater for ewes that grazed lotus than pasture during cycles 2 and 3 of ovulation ($P < 0.05$), with a component of this due to action of CT in the ewes that grazed lotus ($P = 0.12$; Table 3.6). The increased fecundity of ewes grazing lotus is indicated by fewer ewes having only one CL and giving birth to one lamb, and to more ewes having multiple ovulations and giving birth to two or more lambs. These effects were apparent in both nutritional treatment groups and both ages of ewe grazing lotus, but the effect was of greater magnitude in the CT-acting than the PEG supplemented ewes and the CT effect tended to be greater in older ewes.

Table 3.6. The effect of grazing ewes on *Lotus corniculatus* or perennial ryegrass/white clover pasture, and of supplementation with polyethylene glycol (PEG; MW 3500), on fecundity at ovulation (number of CL/ewe cycling) and at lambing (lambs born/ewe lambing).

	Pasture			Lotus			S. E. (D.F. 97)
	Fecundity			Fecundity			
	1	2	3-4	1	2	3-4	

Ovulation rate (% OR)							
n	50/group			50/group			
Cycle 1							
PEG-sheep	59.6	40.4	0.0	51.1	48.9	0.0	0.40
CT-acting	52.2	45.7	2.2	51.1	46.8	2.1	0.40
Cycle 2							
PEG-sheep	74.0	26.0	0.0	62.5	35.4	2.1	0.45
CT-acting	69.4	26.5	4.1	59.2	38.9	2.0	0.45
Cycle 3							
PEG-sheep	66.7	31.4	2.0	40.8	55.1	4.1	0.60
CT-acting	69.4	28.6	2.0	30.6	61.2	8.2	0.60
Lambing (%)							
n	25/group			25/group			
2 years old							
PEG	81.8	18.2	0.0	68.2	31.8	0.0	0.77
CT-acting	82.6	17.4	0.0	63.6	31.8	4.6	0.77
3 years & older							
PEG	50.0	45.5	4.5	48.0	52.0	0.0	0.61
CT-acting	59.1	36.4	4.6	29.2	58.3	12.5	0.61

3.4.5. *Voluntary feed intake, liveweight gain, wool production and wool processing characteristics*

VFI was similar for ewes grazing pasture and lotus, and was not affected by PEG supplementation (Table 3.7). Liveweight gain was low, but was higher in sheep grazing lotus than pasture ($P < 0.001$). Clean fleece weight ($P < 0.001$), efficiency of wool production (g clean wool/kg OMI per eaten; $P < 0.001$), staple length (cm; $P < 0.01$) and mean fibre diameter ($P < 0.01$) were all significantly higher in ewes grazing lotus than those on pasture, with no effects due to PEG supplementation. In ewes grazing lotus, PEG supplementation significantly increased wool yellowness ($P < 0.05$) and reduced brightness ($P < 0.01$), but had no effect in ewes grazing pasture.

Table 3.7. Organic matter intake (OMI), liveweight gain (LWG), wool production and wool processing characteristics of sheep grazing *Lotus corniculatus* and perennial ryegrass/white clover pasture, with or without polyethylene glycol (PEG; MW 3500) supplementation (85 days).

	Pasture		Lotus		S. E. (D.F. 195)
	PEG- sheep	CT-acting	PEG- sheep	CT-acting	
OMI (kg/ewe/d)					
n	16	13	16	16	
Mean value	1.98	1.83	1.85	1.70	0.087 (D.F. 57)
LWG					
n	50	50	49	50	
Mean value (g/day)	4.5	18.6	33.8	40.3	6.88
Wool weight					
n	50	49	49	49	
Greasy fleece (kg)	1.32	1.38	1.64	1.56	0.029
Clean fleece (kg)	1.14	1.09	1.31	1.35	0.027
(g/day)	13.5	12.9	15.4	15.9	0.32
Clean belly (g)	86.5	84.6	86.1	95.9	4.49
Efficiency of wool production (g clean wool/kg OMI/day)	7.35	7.01	8.38	8.67	0.175
Wool characteristics					
Staple length (cm)	4.12	4.00	4.27	4.37	0.082
(mm/day)	4.85	4.70	5.02	5.14	0.092
Mean fibre diameter (μm)	38.7	39.7	40.1	40.8	0.50
Colour					
Brightness*	65.0	64.9	63.8	64.9	0.25
Yellowness†	0.18	0.17	0.49	0.15	0.078

* Measured as Y value.

† Calculated as Y-Z values.

3.5. DISCUSSION

The most significant findings in this study were that ewes grazing lotus rather than pasture for two oestrus cycles during autumn increased both lambing percentage (25 %) and wool production (14 %) with no changes in VFI, thereby increasing the efficiency of both lamb and wool production. The increase in reproductive rate was due to increases in fecundity at both ovulation and birth, with no effect on fertility. Responses to PEG supplementation showed that a major reason for the effect of lotus on reproductive rate was its CT content. The CT in the diet probably increased EAA absorption (Waghorn *et al.* 1987a, b, 1990). The reduced plasma urea concentration and increased plasma concentration of most free amino acids in CT-acting compared to PEG supplemented sheep grazing lotus in the present experiment is consistent with these findings. This is the first report of forage CT increasing reproductive rate in ewes. The close similarity between cycle 3 OR and lambing data suggests that there was minimal embryonic loss in this experiment.

There is strong evidence showing that an improved rate of nutrition, especially protein content in the diet, can result in an increased OR (Smith 1991). The importance of protein nutrition was confirmed by Cruickshank *et al.* (1988), who found that abomasal infusions of lactalbumin and soya protein isolate increased the number of ewes having multiple ovulations from 55 to 73 %. Subsequent work reported that the strongest correlation was found between OR and the plasma concentration of BCAA ($r = 0.95$) and EAA ($r = 0.61$) (Waghorn 1986; Waghorn *et al.* 1990). This has been confirmed by the findings that intravenous infusion of a BCAA mixture (33.1 g total BCAA/ewe per day) over a 5 day period in the late stages of the oestrous cycle (before luteolysis), produced an increase in OR (2.4 v. 1.5; Downing & Scaramuzzi 1991; Downing *et al.* 1995). One of the explanations for the increased fecundity of ewes grazing lotus in the present experiment may be their higher circulating plasma concentration of BCAA relative to pasture-fed ewes, especially in the CT-acting group. The

critical period for protein supplementation to increase OR is the last 6 days before ovulation (Stewart & Oldham 1986) which is why the feeding level was increased to *ad libitum* over this critical period in this study. An additional possibility is that rumen ammonia production on the pasture and lotus + PEG diets may have elevated plasma ammonia concentration to the point where it reduced the survival of ova (Kaur & Arora 1995) and that action of CT in lotus reduced this. Plasma ammonia concentration needs to be measured in future experiment of this type.

Nutrition (protein and energy) influences ovarian function (including OR) through modulating the secretion of gonadotrophins (i.e. follicular stimulating hormone (FSH) (Davis *et al.* 1981; McNatty *et al.* 1985; Thompson & Smith 1988), but studies in sheep (Cruickshank *et al.* 1990; Downing & Scaramuzzi 1991; Downing *et al.* 1995) have not shown consistent effects of protein nutrition on peripheral concentrations of gonadotropins in ovariectomized ewes. It has been suggested that changes in metabolic hormones such as growth hormone (GH), insulin and insulin-like growth factor (IGF), which consistently accompany the nutrition-induced alteration in body energy, protein balance and muscle protein synthesis (Garlick *et al.* 1983; Pell & Bates 1990; Downing *et al.* 1995), can affect ovarian function, either directly or by modulating gonadotropin actions at the ovarian level (Smith 1991; Gong & Webb 1996). Furthermore, both insulin and EAA, especially intravenous infusion of BCAA, have been shown to increase the sensitivity of muscle protein synthesis *in vivo* to insulin (Garlick & Grant 1988; Biolo & Wolfe 1993). However, it has been suggested that the sensitivity of the muscle to insulin might be facilitated by EAA (including BCAA) and that the increase in protein synthesis after feeding might be associated with the simultaneous presence of both these factors. In addition, branched-chain amino transferase iso-enzyme (cytosolic) is found only in the ovaries, placenta and brain (Hutson *et al.* 1988, 1992), whereas mitochondrial iso-enzyme (branched-chain amino transferase) is

expressed most in tissue. Therefore BCAA may have a direct stimulating effect on the ovaries, increasing OR, by an as yet unknown mechanism. Further studies are needed on the uptake of BCAA by the ovary and on the action of BCAA within the ovary, including any increase in the numbers of CL released.

Although wool growth was greater in sheep grazing lotus than pasture, there was no wool growth response on lotus due to the action of CT in this study. Wool growth is well known to respond slowly to changes in nutrition and it may well be that a longer period of PEG supplementation is needed. Min *et al.* (1998b) reported that 18 weeks of PEG supplementation ^{produced on} ~~was required, before~~ the effect of CT on wool characters ~~was apparent.~~

The availability of sulphur-containing AA (SAA) and post-ruminal supplementation with SAA has markedly increased wool growth (Black & Reis 1979; Reis 1979). More recently, studies with *L. pedunculatus* (McNabb *et al.* 1993) and with *L. corniculatus* (Wang *et al.* 1994) showed that the action of CT increased the irreversible loss rate (ILR) of cystine from blood plasma, mainly due to reducing the loss of SAA (30 %) in the rumen. The increase in clean fleece weight (19 %) from feeding lotus in the present experiment could be due to CT increasing the absorption of SAA and also that of all other EAA. This is similar to the result obtained by Min *et al.* (1998b), who found CT in lotus increased the efficiency of wool production, suggesting that CT from lotus may have altered the chemical composition of wool, especially changing the proportion of individual proteins in wool. Lotus feeding in the present experiment also increased length of wool growth and fibre diameter.

In ewes grazing lotus, PEG supplementation significantly increased wool yellowness and reduced brightness, whilst this effect was not apparent in ewes grazing pasture. Therefore, it appears that an interaction between PEG and lotus caused increased wool yellowness and reduced brightness. This effect was eliminated by the action of CT. However, the exact nature of the interaction between PEG and lotus which lead to changes in wool colour is unclear and

further research is necessary to resolve this question. Min *et al.* (1998b) reviewed factors influencing yellowness in NZ crossbred wool and concluded that a major likely cause was degradation products of aromatic amino acids in the fleece and their subsequent adsorption onto wool fibres. Whilst, in part, this may have contributed to changes in wool colour in ewes fed lotus and supplemented with PEG, other factors must also be involved because similar changes in wool colour in ewes fed pasture with and without PEG supplementation did not occur, yet degradation of aromatic amino acids would also have occurred in those animals.

This study has shown that feeding the CT-containing legume *Lotus corniculatus* can be used to increase both the efficiency of reproduction and wool production in grazing ewes, with more lambs and wool being produced without any change in feed intake. Further research is required to define the length of feeding time on lotus to give maximum response in reproductive rate. The action of CT in lotus also improved wool quality and reduced yellowness.

This study was supported by a grant from Wools of New Zealand. The authors wish to thank G. S. Purchas, W. C. L. Howell and C. Parsons, who assisted with data collection. The skilled assistance from the Wool and Nutrition Laboratory staff, Massey University and J. S. Peters, AgResearch, is greatly acknowledged. P. C. H. Morel and D. J. Garrick are thanked for advice with statistical analysis and T. G. Harvey is thanked for advice on grazing management. J. Smith and T. Knight, AgResearch, are thanked for their advice and assistance with the experimental design. Scholarship support to B. R. Min from the New Zealand Department of Education is acknowledged.

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CHAPTER 4

Solubilization and degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco) protein from white clover (*Trifolium repens*) and *Lotus corniculatus* by rumen microorganisms and the effect of condensed tannins on these processes

(Short title: *Condensed tannins and protein degradation*)

This chapter has been submitted to the *Journal of Agriculture Science, Cambridge*

(in press)

4.1. ABSTRACT

In situ and *in vitro* rumen incubations were used to determine the effect of condensed tannins (CT) on the solubilization and degradation of the plant protein from white clover (*Trifolium repens*) and *Lotus corniculatus*. These forages contained, respectively 0.3 and 22.1 g CT/kg dry matter (DM). The sheep used for the experiments were also fed either white clover or *L. corniculatus*. Effects of CT were determined by making measurements in the presence and absence of polyethylene glycol (PEG; molecular weight 3500), which binds and inactivates CT. The loss of DM, neutral detergent fibre (NDF), total nitrogen (N) and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39; fraction I leaf protein) from polyester bags suspended in the rumen of sheep was measured. The loss of these constituents from polyester bags suspended in the rumen was used as a measurement of their solubilization. Degradation was defined as the disappearance of Rubisco from white clover and *L. corniculatus* added to *in vitro* incubations with rumen fluid obtained from the same fistulated sheep fed either white clover or *L. corniculatus*.

In the absence of PEG, the *in situ* loss of Rubisco from *L. corniculatus* was less rapid than the loss of this protein from white clover when each forage was incubated in the rumen of sheep fed the same diet. Addition of PEG tended to increase the loss of Rubisco from *L. corniculatus*, suggesting that CT slowed the rates of solubilization of Rubisco from this forage. Effects of rumen fluid were small, but there was some evidence that the rumen fluid in sheep fed *L. corniculatus* reduced the solubilisation of Rubisco from white clover. The action of CT did not inhibit the *in situ* loss of NDF from either white clover or *L. corniculatus*.

In the absence of PEG, the *in vitro* degradation of Rubisco from *L. corniculatus* was slower when compared to the degradation of this protein from white clover; PEG

addition increased the degradation of Rubisco from *L. corniculatus*, but not from white clover, showing that CT was the causal agent. The addition of CT extracted from *L. corniculatus* markedly depressed the degradation of Rubisco from white clover, with the effect being completely reversible by PEG. The large subunit (LSU) of Rubisco was consistently degraded at a faster rate than the small subunit (SSU) and added CT had a greater effect in slowing the degradation of the LSU compared to the SSU. There was little difference in the degradation of Rubisco when rumen fluid from sheep fed either white clover or *L. corniculatus* was used for *in vitro* incubations.

It was concluded that the action of CT from *L. corniculatus* reduces the digestion of protein in the rumen of sheep. This effect is predominantly due to the action of CT reducing the degradation of plant protein, although CT also reduced the solubilisation of plant protein. The main effects of CT on protein solubilization and degradation seemed to be produced locally by CT present in plant tissue; transfer of these effects through rumen fluid was small in magnitude.

4.2. INTRODUCTION

Ruminal degradation of plant protein and carbohydrates must be synchronized for optimal microbial protein synthesis in order to make efficient use of dietary nitrogen (N) in productive ruminants (Waghorn & Barry 1987). When ruminants are fed on high quality fresh forages containing high concentrations of N (25-35 g N/kg dry matter (DM)) and metabolizable energy (10.0-11.5 MJ/kg DM), carbohydrate digestion in the rumen is efficient; however about 70 % of the forage N is degraded to ammonia in the rumen with only 30 % escaping to the small intestine for absorption (MacRae & Ulyatt 1974; Ulyatt & MacRae 1974; Waghorn & Barry 1987). This large loss of N across the rumen increases with increasing N intake (Ulyatt & Egan 1979), and is associated with the excessive degradation of soluble protein to ammonia and the absorption of that ammonia from the rumen (NH₃; 20-35 % N intake; Ulyatt *et al.* 1975; MacRae & Ulyatt 1974; Beever & Siddons 1986).

Rapid and indirect methods for assessing the degradability of protein in the rumen tend to depend on either their solubility in buffered rumen fluid or the disappearance of protein from synthetic-fibre bags suspended in the rumen (Mehrez & Ørskov 1977; Ørskov & McDonald 1979). Although estimates of degradability using the synthetic-fibre bag technique are better correlated with *in vivo* measures of degradability than *in vitro* estimates of protein solubility (Mathers & Miller 1980), there is now increasing evidence that loss from synthetic-fibre bags and degradation are not always similar (Stern & Satter 1984; McNabb *et al.* 1996).

The digestion of fresh forage protein in the rumen is the result of the combined processes of solubilization and degradation. For the purposes of this study, protein

solubilization (total-N loss) was defined as the release of protein from plant cells following chewing and ruminating of fresh forages, and this is an important prerequisite of protein degradation (Mangan 1972, 1982; Nugent *et al.* 1983). In the present study solubilization was estimated by measuring the loss of plant constituents from white clover and *L. corniculatus* contained in polyester bags suspended in the rumen of sheep. Degradation was defined as the rate of disappearance of individual proteins from these forages during *in vitro* incubation with rumen fluid. The degradation of individual proteins by rumen micro-organisms has been successfully studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and imaging densitometry (McNabb *et al.* 1996). Condensed tannins (CT) could conceivably effect either or both these processes.

The objective of this study was to determine the effects of diet and CT on the *in situ* solubilization and *in vitro* rumen degradation of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39; fraction I leaf protein) from white clover (little or no CT) or *L. corniculatus* (CT-containing legume). The specific effects of CT were assessed using polyethylene glycol (PEG; presence or absence) which binds and inactivates CT.

4.3. MATERIALS AND METHODS

4.3.1. *Experimental design*

Two experiments were conducted to determine what effect CT had on the solubilization and degradation of plant protein in the rumen using *in situ* and *in vitro* methods. Two forages were used in this study; the first, *L. corniculatus* (birdfoot trefoil; cv. Grasslands Goldi) contains CT, whilst the second, white clover (*Trifolium repens*) contains little or no CT. The effect of CT was also assessed by making

measurements in the presence (+ PEG sheep) and absence (- PEG sheep) of polyethylene glycol (PEG). The PEG binds strongly to CT and can be used to displace protein from CT-protein complexes (Jones & Mangan 1977). Therefore, effects of CT can be quantified by comparing controls (CT-acting) with PEG treatments (CT-inactivated). An initial study was also conducted to compare minced fresh plant material with freeze dried and ground plant material, to determine which method was most suitable for preparing plant material for *in situ* and *in vitro* rumen incubations.

4.3.2. *Animals*

Twelve rumen fistulated (90 mm ID flexible rumen cannula; Beruc Equipment Ltd, Republic of South Africa) Romney sheep (mean live-weight 74.3 (S.D. 8.0) kg; aged between 20 and 30 months) were used for *in situ* experiments. These sheep were housed indoors in metabolism crates and offered fresh forage hourly from overhead belt-feeders at 900 g DM/day. Four of these sheep (2 on each diet) were also used to determine particle size distribution in chewed boli before the *in situ* experiments commenced. After the *in situ* experiments were completed, four of the twelve sheep (2 on each diet) were used for collection of rumen fluid for the *in vitro* rumen incubations.

Pure swards of vegetative white clover and *L. corniculatus* were harvested daily at about 08.00 h using a sickle-bar mower and each forage fed to 6 sheep. Restricted intakes (900 g DM/day) enabled polyester bags (37 μm pore size; Estal Mono; Swiss Screens (Aust) Pty Ltd) to be suspended in, and removed from the rumen with relative ease. Water was freely available. All sheep were orally treated with an anthelmintic to control internal parasites (12 ml Ivomec; Merck Sharp and Dohme (NZ) Ltd) and were treated to control external parasites (10 ml Wipeout; Coopers Animal Health (NZ) Ltd) prior to the experiment commencing.

4.3.3. PEG infusion

Throughout the experiments, one group of six sheep (3 white clover; 3 *L. corniculatus*; CT-inactivated) received a continuous intraruminal infusion of PEG (molecular weight 3500, Union Carbide, Danbury, CT, USA; 100 g/day in 300 ml water;), whilst the remaining group of six sheep (control sheep; CT-activated) received an intraruminal infusion of water.

4.3.4. Particle size distribution

Four of the rumen fistulated sheep were used for the collection of chewed boli samples before commencing the *in situ* and *in vitro* experiments. The sheep were fasted for 12 h and then offered either fresh white clover or *L. corniculatus* (900 g DM/animal). After about 1 h, total rumen contents were removed from each animal to enable collection of chewed boli. The samples were collected from the rumen via the oesophagus, after which the total rumen contents were returned to each animal. In addition, fresh white clover and *L. corniculatus* was frozen at $-20\text{ }^{\circ}\text{C}$, freeze dried and ground to pass through a sieve with aperture size of 1 mm and stored at $-20\text{ }^{\circ}\text{C}$ for 3 days prior to wet sieving. On the same day as chewed boli were collected, fresh white clover and *L. corniculatus* was minced in an electronic meat mincer (Kenwood Electronics, UK) containing a sieve with holes of 1.2 cm diameter. The particle size distribution of forage samples (freeze dried and ground, minced forage and chewed boli) was determined by wet sieving, following the procedure described by Waghorn *et al.* (1989). A re-circulating flow of water was directed through a series of rotating sieves for 5 min, separating material according to sieve aperture size. Sieve sizes (length of the sides of square holes) and particle fractions used in the present study were > 2.0 ,

1.0, 0.5, 0.25 and < 0.2 mm. Material passing the wet sieve (< 0.2 mm) was centrifuged (2,000 x g for 20 min) and DM of the pellet determined. The dry weight of material was determined by difference from the initial sample dry weight and the sum of recovered particulate DM fractions.

4.3.5. *In situ Experiment*

An *in situ* experiment was conducted with the 12 sheep in a cross-over design to determine the loss of DM, neutral detergent fibre (NDF), N and Rubisco from polyester bags containing either freshly minced white clover or freshly minced *L. corniculatus* (about 5 g DM per bag). Rubisco was studied because it is the principal leaf protein, representing 30-40 % of the total protein present in plants (Mangan 1982). Fresh white clover and *L. corniculatus* were harvested from the same area on the same day and were minced.

Six sheep were divided into two groups of 3 and then offered white clover. The sheep in the first group were infused with PEG (3 PEG sheep) while the sheep in the second group were left without PEG (3 control). The remaining six sheep were treated similarly except for the diet which was *L. corniculatus*. One week was allowed for readjustment after changing over the forage diets, but keeping the same sheep as PEG or control animals, and the experiment repeated. Therefore, the polyester bags were suspended (twice) in the rumen of all sheep. Bags were removed from the rumen after 2, 4, 6, 8, 12, 24 and 48 h of the start of incubation and were thoroughly washed by hand in cold tap water for approximately 3 min until no further colour could be washed out of the bags. In addition, two further bags of minced white clover and *L. corniculatus* which were not incubated in the rumen, were washed to give residues at 0 h. After completion of the two periods, samples were bulked for each animal for

chemical analysis. About 0.5-1.0 g of the plant residue collected from nylon bags was bulked for each treatment and was immediately frozen in liquid-nitrogen and used to extract total plant protein for Rubisco analysis by SDS-PAGE and imaging densitometry. The remaining residue from the *in situ* experiment was weighed, freeze-dried, re-weighed (DM residue; n=6), ground to pass through a 1-mm diameter sieve and bulked to obtain 2 samples per treatment and used to determine N and NDF (n=2).

4.3.6. In vitro *Experiment*

4.3.6.1. *Preparation of strained rumen fluid*

Four sheep (2 on each diet; neither animal was receiving PEG) were used for collection of rumen fluid. Rumen fluid was collected at 08:00 h, and quickly strained through cheese cloth into a Dewar flask flushed with CO₂ gas. This rumen fluid was maintained at 39 °C under an atmosphere of CO₂ and used immediately for *in vitro* incubations.

4.3.6.2. *In vitro rumen incubation procedures*

The two *in vitro* experiments (Experiments A & B) were conducted to determine the effect of CT on the *in vitro* degradation of Rubisco by the microorganisms present when forages were incubated with rumen fluid. *In vitro* incubations were performed using the method described by McNabb *et al.* (1994). Sixteen *in vitro* rumen incubations for each of Experiments A and B were undertaken. Each incubation was undertaken in duplicate and are shown in Table 1.

Two forage preparation methods were used for Experiment A and B. Firstly, fresh white clover and *L. corniculatus* was minced (using an electronic meat mincer). Secondly, total soluble protein was extracted from fresh white clover and *L. corniculatus* using the method described by McNabb *et al.* (1994).

In Experiment A, either minced or extracted total soluble protein from white clover and *L. corniculatus* was incubated in flasks with rumen fluid from sheep fed either white clover or *L. corniculatus*. All incubations were undertaken in the presence and absence of PEG (110 mg). To this either 6.5 g minced white clover (45 mg total N and 0.3 mg total CT/g DM) or *L. corniculatus* (37.4 mg total N and 22.4 mg total CT/g DM) or total soluble protein extracted from white clover (51 mg total soluble N and no CT/g DM) or *L. corniculatus* (23 mg total soluble N and 15 mg free CT/g DM) were added. Rumen fluid (7.5 ml) was placed in flasks and the flasks were adjusted to a constant weight with artificial saliva (see Table 4.1).

For Experiment B, CT was extracted from *L. corniculatus* using the method described by Jackson *et al.* (1996). Either minced white clover (6.5 g; 0 extractable CT and 17 mg total N) or total soluble protein extracted from white clover (20 ml; 51 mg total soluble N) was incubated. Incubations were undertaken with the addition of CT (55 mg) or without added CT. Incubations were also undertaken with or without the addition of PEG (110 mg). Total volume of each flask was adjusted to a final volume of 37.5 g with artificial saliva. Each flask was fitted with a Bunsen valve and shaken (90/min) at 39 °C for 24 h.

Table 4.1. Quantities of plant material, purified condensed tannin (CT), polyethylene glycol (PEG; MW 3500), rumen fluid and artificial saliva added to each flask for the *in vitro* protein degradation experiments. Each incubation was undertaken with rumen fluid from sheep fed white clover (WC) or *Lotus corniculatus* (LC). Each incubation was undertaken in duplicate.

Minced material (g)	Total soluble plant protein extract (ml)	CT (LH-20 extracted) (g)	PEG (g)	Rumen* fluid (ml)	Artificial saliva† (g)
Experiment A					
6.5 WC	-	-	-	7.5	23.5
6.5 WC	-	-	0.11	7.5	23.4
-	20 WC	-	-	7.5	10.0
-	20 WC	-	0.11	7.5	9.9
6.5 LC	-	-	-	7.5	23.5
6.5 LC	-	-	0.11	7.5	23.4
-	20 LC	-	-	7.5	10.0
-	20 LC	-	0.11	7.5	9.9
Experiment B					
6.5 WC	-	-	-	7.5	23.5
6.5 WC	-	-	0.11	7.5	23.4
6.5 WC	-	0.055	-	7.5	23.5
6.5 WC	-	0.055	0.11	7.5	23.4
-	20 WC	-	-	7.5	10.0
-	20 WC	-	0.11	7.5	9.9
-	20 WC	0.055	-	7.5	9.9
-	20 WC	0.055	0.11	7.5	9.8

* Four sheep (2 on each diet; neither animal was receiving PEG) were used for collection of rumen fluid for the *in vitro* experiments.

† Total volume of each flask was adjusted to 37.5 ml with artificial saliva (pH 6.8).

Samples (200 μ l) were removed from each digestion flask by pipette under a CO₂ gas stream, prior to and after 2, 4, 6, 8, 12 and 24 h of incubation. These samples were added to micro-centrifuge tubes containing 50 μ l of protein digestion buffer (64 mM Tris-HCl, pH 6.8 and 20 g SDS/l, 50 ml β -mercaptoethanol/l, 0.05 g bromophenol/l; McNabb *et al.* 1996). All samples were immediately frozen, stored at -20 °C and used for SDS-PAGE. The flasks were re-flushed with CO₂ after each sampling.

4.3.6.3. *Laboratory measurements*

Samples of feed and feed residues in polyester-bags were freeze dried and ground before analysis of N, NDF and CT. Total nitrogen was determined by the Kjeldahl digestion procedure using a Kjeltec Auto Analyser (Tecator, Hoganas, Sweden). Total soluble plant protein (N) was determined by the methods of Bradford (1976), whilst DM was determined by drying at 95 °C for 16 h. The NDF was determined using the method of Robertson and Van Soest (1981). The concentration of CT in these samples was determined using the three stage butanol-HCl method described by Terrill *et al.* (1992).

4.3.6.4. *Rubisco analysis by SDS-PAGE.*

Rubisco analysis for both *in situ* and *in vitro* experiments were performed using the method described by McNabb *et al.* (1996). All samples were heated at 95 °C for 5 min to denature protein and dissociate CT-protein complexes and the soluble protein in 30 μ l was fractionated by SDS-PAGE. After electrophoresis (about 3-4 hours at 65 V; 11.8 V/cm), the gels were washed (twice for 15 min) in 40 % methanol; 10 % acetic acid (v/v) and proteins were visualized by staining in Coomassie Brilliant Blue R-250 (5 g/l ethanol/acetic acid (40: 25, v/v)) for 30 min and destained in 10 % methanol; 7.5

% acetic acid (v/v) for 24 hours to detect protein bands. Rubisco consists of 8 large subunits (LSU; MW 54,000) and 8 small subunits (SSU; MW 16,000; Kawashima & Wildman 1970). Rubisco represents 30-50 % of the total protein present in plants (Mangan 1982), therefore the LSU and SSU were easily detected on stained gels and were quantified by imaging densitometry (Model GS-670, BioRad, Hercules, USA). The data were processed using image analysis software (Bio-Rad Molecular Analyst^{TM/CP} Imaging Analysis Software, USA).

4.3.6.5. Calculation of data and statistical analysis

The *in situ* DM, NDF digestion and N solubilization rate in the rumen was calculated using the following exponential equation 8 (Ørskov & McDonald 1979):

$$Y = a + b(1 - e^{-ct}) \quad (8)$$

Where Y was defined as DM, NDF or N disappearance (% added) in time t ; a , b and c being constants of the exponential equation, respectively, the instantly soluble fraction at time 0 (a), the proportion degraded (b) during time (t) and the rate of degradation of the 'b' fraction (c). Potential solubilization and potential digestibility were calculated as $a + b$. Predicted rumen solubilisation (PS) and predicted degradability (PD) was calculated using the equation 9 of Ørskov and McDonald (1979), where r is the rumen particulate dry matter fractional outflow rate.

$$PS(PD) = a + (bc/(c + r)) \quad (9)$$

Domingue *et al.* (1991) determined that in sheep fed forage (chaffed lucerne), r was 0.033/h for DM. The same procedures were used to quantify N, NDF and solubilization and degradation of Rubisco.

The constants a , b , c for each animal were calculated with the method described by Yu *et al.* (1995) using Non-Linear Regression (NLIN) procedures from the Statistical Analysis System package (SAS 1985). The significance of differences between data for c , a and $a+b$ and predicted rumen solubilisation (PS) were assessed by using General Linear Models (GLM) procedures from SAS (1985), with the factors examined being forage (white clover *v.* *L. corniculatus*; in the bag residues), sheep diet (white clover *v.* *L. corniculatus*), PEG treatment and any interactions. Data are presented as mean values, together with the standard error of the mean (SE); the number of observations contributing to each mean is denoted in all tables by the letter n .

The *in vitro* degradation of Rubisco during incubation with rumen fluid was calculated using the equations previously described, with the factors examined for *in vitro* Experiment A being forage type, processing method, source of rumen fluid, PEG treatment and any interactions. Factors examined for *in vitro* Experiment B were CT addition, PEG addition and source of rumen fluid.

4.4 RESULTS

4.4.1 Particle size distribution

The particle size distribution of freshly minced forage and freeze-dried and ground white clover and *L. corniculatus*, and that of chewed-boli are shown in Table 4.2. Freshly minced forage tended to be better correlated with chewed boli for particle distribution ($R^2 = 0.17$ and 0.78 for white clover ($P = 0.47$) and *L. corniculatus* ($P = 0.11$)),

respectively) than freeze-dried and ground ($R^2 = -0.073$ and 0.43 for white clover ($P = 0.9$) and *L. corniculatus* ($P = 0.77$), respectively). Therefore, minced forage approximated better to the particle size distribution of chewed-boli than did freeze-dried and ground, hence minced forage was chosen as the forage preparation method for all experiments.

Table 4.2. The particle distribution (g/kg dry matter (DM)) of freshly minced forage, freeze-dried and ground white clover and *Lotus corniculatus* used for wet sieving and chewed boli from sheep fed on fresh white clover and *Lotus corniculatus*.

Sieve sizes* (mm)	Preparation method						S.E. (D.F.=6)
	White clover (n = 2)			<i>Lotus corniculatus</i> (n = 2)			
	Freeze dried & ground	Minced forage	Chewed boli [†]	Freeze dried & ground	Minced forage	Chewed boli [†]	
> 2	1	144	401	1	209	370	3.5
1	5	136	99	7	106	58	3.9
0.5	448	228	98	332	139	96	3.3
0.25	158	107	105	155	185	139	2.7
< 0.2	414	386	351	479	359	336	1.1

* Aperture size of sieves.

† Four sheep (2 on each diet; neither animal was receiving PEG) were used for collection of chewed boli for the particle distribution experiments.

4.4.2. Chemical composition of forages

The chemical composition of white clover and *L. corniculatus* during *in situ* and *in vitro* experiments is shown in Table 4.3. Total N content was generally lower for *L. corniculatus* than white clover, but NDF content was higher in *L. corniculatus* than white clover. Total CT in the *L. corniculatus* was 22 g CT/kg DM, and 67 % of this was readily extractable. Only trace amounts of CT were detected in white clover.

Table 4.3. Chemical composition of white clover and *Lotus corniculatus* during both the *in situ* and the *in vitro* experimental periods (mean values \pm S.E.; n = 4).

Components (g/kg DM)	White clover	<i>Lotus corniculatus</i>
Total N*	44.8 \pm 0.43	38.4 \pm 0.43
NDF [†]	244.3 \pm 7.77	259.2 \pm 7.80
Condensed tannins (CT)		
Extractable CT	ND [‡]	14.8 \pm 0.18
Protein bound CT	0.3 \pm 0.03	5.9 \pm 0.03
Fibre bound CT	ND [‡]	1.5 \pm 0.02
Total CT	0.3 \pm 0.15	22.1 \pm 0.16

* Total nitrogen.

[†] Neutral detergent fibre.

[‡] Not detectable.

4.4.3. In situ *Experiment*

In situ DM and NDF digestion of minced white clover and *L. corniculatus* in the rumen of sheep, with or without an intraruminal infusion of PEG, is shown in Table 4.4. For DM, in the absence of PEG, the potential solubilization (a+b; 84.3 v. 93.5 %; $P < 0.001$) and predicted rumen DM solubilization (PS; 77.4 v. 85.5 %; $P < 0.001$) were lower for *L. corniculatus* than for the white clover. The addition of PEG significantly increased the predicted DM solubilization ($P < 0.001$) in the *L. corniculatus* and tended to increase solubilization rate ($P = 0.06$).

The forage x PEG and diet x PEG interactions were significant ($P < 0.01$) for predicted rumen DM solubilization, suggesting that PEG addition mainly increased solubilization when *L. corniculatus* forage was suspended in the rumen of sheep fed the *L. corniculatus* diet but not under other conditions. Comparing diets suggests that in sheep fed *L. corniculatus*, the rate of solubilization (c) and predicted rumen DM solubilization were significantly lower ($P < 0.01$), but potential solubilization was unaffected.

There were no effects of forage species, PEG or the diet fed to the sheep on any of the *in situ* measurements of NDF solubilization (Table 4.4), suggesting that at a concentration of 22 g CT/kg DM, CT did not affect ruminal NDF digestion.

Table 4.4. *In-situ* Experiment. Effect of condensed tannins (CT) upon the *in situ* rates of disappearance of dry matter (DM) and neutral detergent fibre (NDF) from white clover (WC) and *Lotus corniculatus* (LC) suspended in polyester bags in the rumen of sheep, either with or without intra-rationally infused polyethylene glycol (+/- PEG; MW 3500)

Diets & forages	Instantly solubilized*		S.E.	Solubilisation*		S.E.	Potential solubilisation*		S.E.	Predicted solubilisation*		S.E.
	a (%)			c (%/h)			a + b (%)			PS (%)		
	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG		
DRY MATTER (n = 6; D.F. = 4)												
WC DIETS [†]												
WC in bags [‡]												
	42.3	44.6	1.41	22.9	26.1	2.91	94.2	91.0	2.45	86.2	86.4	0.51
LC in bags [‡]												
	40.4	40.4	0.34	18.3	19.3	1.11	92.8	93.9	0.80	84.7	86.1	0.59
Mean	41.4	42.5	-	20.6	22.7	-	93.5	92.5	-	85.5	86.3	-
LC DIETS [†]												
WC in bags [‡]												
	43.2	43.5	0.14	25.6	27.3	0.61	85.5	86.0	0.18	80.6	81.5	0.26
LC in bags [‡]												
	39.1	42.0	0.55	13.9	22.4	2.40	83.2	87.7	0.91	74.2	82.3	1.23
Mean	41.2	42.8	-	19.9	24.9	-	84.3	86.9	-	77.4	81.9	-
NEUTRAL DETERGENT FIBRE (n = 2; D.F. = 2)												
WC DIETS [†]												
WC in bags [‡]												
	43.1	39.3	8.43	27.4	35.4	0.12	68.1	68.3	4.00	65.2	65.6	3.55
LC in bags [‡]												
	47.7	38.0	8.25	12.2	24.5	0.65	71.2	73.1	4.65	65.7	68.8	2.65
LC DIETS [†]												
WC in bags [‡]												
	43.5	44.8	7.05	22.2	22.3	0.01	72.9	72.1	6.00	69.2	68.7	4.30
LC in bags [‡]												
	38.9	37.5	5.30	28.0	30.2	7.25	65.9	61.3	2.53	59.1	58.6	5.70

* Solubilization was estimated by measuring the loss of plant constituents from polyester bags, suspended in the rumen of sheep.

[†] Fresh forage diet fed to rumen fistulated sheep used in the *in situ* experiment.

[‡] Fresh minced forage in the polyester bag suspended in the rumen.

The loss of N, and the LSU and SSU of Rubisco from polyester bags suspended in the rumen is shown in Table 4.5 and Table 4.6. Nitrogen solubilization in general (mean values), the proportion of N instantly solubilized (a; 23.5 v. 27.4 %; $P < 0.01$) and N solubilization rate (c; 8.0 v. 15.0 %/h; $P < 0.01$) were lower for *L. corniculatus* than for white clover (Table 4.5). The PEG infusion significantly increased the rate of N solubilization (c; $P < 0.01$) and predicted N solubilization (PS; $P < 0.05$), with there being significant forage x PEG interactions, suggesting that PEG increased N solubilization more when *L. corniculatus* forage was suspended in the rumen of sheep fed the *L. corniculatus* diet compared to sheep fed the white clover diet.

Table 4.5. *In-situ* Experiment. Effect of condensed tannins (CT) upon rates of solubilization* of nitrogen (N) during *in situ* incubation of white clover (WC) and *Lotus corniculatus* (LC) suspended in polyester bags in the rumen of sheep, either with or without intra-ruminally infused polyethylene glycol (+/- PEG; MW 3500). (n = 2; D.F. = 2).

Diets & forages	Instantly Solubilized		S.E.	Solubilization rate		S.E.	Potential solubilization		S.E.	Predicted solubilization		S.E.
	a (%)			c (%/h)			a + b (%)			PS (%)		
	-PEG	+PEG		-PEG	+PEG		-PEG	+PEG		-PEG	+PEG	
WC DIETS†												
WC in bags‡												
	30.7	27.9	4.25	7.8	15.8	3.60	66.6	70.3	5.40	46.3	51.5	4.47
LC in bags‡												
	28.9	22.1	3.34	9.4	26.9	4.52	68.6	68.1	2.94	48.2	58.3	6.11
Mean	27.4	-	-	15.0	-	-	68.4	-	-	51.1	-	-
LC DIETS†												
WC in bags‡												
	23.5	26.6	1.77	11.7	14.3	2.84	69.7	77.7	8.74	51.9	57.8	2.60
LC in bags‡												
	21.7	22.1	1.07	2.0	4.0	0.32	61.0	88.0	10.25	32.6	47.7	2.35
Mean	23.5	-	-	8.0	-	-	74.1	-	-	47.5	-	-

* Solubilization was estimated by measuring the loss of plant constituents from polyester bags, suspended in the rumen of sheep.

† Fresh forage diet fed to rumen fistulated sheep used in the *in situ* experiment.

‡ Fresh minced forage in the polyester bag suspended in the rumen.

For both subunits of Rubisco (Table 4.6), in the absence of PEG, all measures were lower for *L. corniculatus* than for white clover forage. For the LSU, PEG infusion increased the proportion of that peptides solubilization rate (c; $P < 0.001$), potential solubilization (a+b; $P < 0.001$) and predicted solubilization (PS; $P < 0.001$). There was a significant diet x PEG interaction ($P < 0.05$), suggesting that there were no responses to PEG when polyester bags containing white clover were suspended in the rumen of sheep fed white clover. However, there were responses to PEG when polyester bags containing white clover were suspended in the rumen of sheep fed *L. corniculatus*: additionally, responses to PEG tended to be greater when polyester bags containing *L. corniculatus* were suspended in the rumen of sheep fed *L. corniculatus*. For the SSU, responses to PEG tended to be in a similar direction as was observed for the LSU, but these responses were much smaller in magnitude, and few attained statistical significance.

Table 4.6. *In-situ* Experiment. Effect of condensed tannins (CT) upon rates of solubilization* of the large subunit (LSU) and small subunit (SSU) of Rubisco during *in situ* incubation of white clover (WC) and *Lotus corniculatus* (LC) suspended in polyester bags in the rumen of sheep, either with or without intra-rationally infused polyethylene glycol (+/- PEG; MW 3500).

Diets & forages	Instantly solubilized		S.E.	Solubilization rate			S.E.	Potential solubilization		S.E.	Predicted solubilization		S.E.
	a (%)	+PEG		c (%/h)	-PEG	+PEG		a + b (%)	-PEG		+PEG	PS (%)	
LSU (n = 3; D.F. = 4)													
WC DIETS [†]													
	WC in bags [‡]												
	32.6	30.8	0.60	15.5	30.8	1.43	99.0	101.3	1.42	87.8	90.5	1.50	
	LC in bags [‡]												
	24.5	26.8	0.61	8.4	26.8	3.35	88.6	99.7	1.01	70.5	88.4	1.32	
	Mean	28.7	-	20.4	-	-	97.2	-	-	84.3	-	-	
LC DIETS [†]													
	WC in bags [‡]												
	32.6	29.1	4.10	8.4	12.4	1.60	101.0	103.3	2.52	80.7	87.4	3.65	
	LC in bags [‡]												
	8.4	17.7	1.20	6.2	7.1	0.15	77.4	104.4	2.76	57.0	75.3	1.65	
	Mean	24.5	-	8.5	-	-	96.5	-	-	75.1	-	-	
SSU (n = 3; D.F. = 4)													
WC DIETS [†]													
	WC in bags [‡]												
	28.1	22.6	0.70	15.7	16.7	2.35	94.8	96.1	1.35	83.3	83.9	1.83	
	LC in bags [‡]												
	11.1	22.6	2.50	15.7	17.1	3.15	99.3	99.9	1.00	83.7	87.2	3.05	
	Mean	21.1	-	16.3	-	-	97.5	-	-	84.5	-	-	
LC DIETS [†]													
	WC in bags [‡]												
	9.8	12.4	2.50	11.3	14.2	1.72	100.1	100.3	1.10	80.1	83.6	2.80	
	LC in bags [‡]												
	10.8	9.33	0.35	5.8	9.7	0.65	102.6	100.3	1.38	69.6	77.1	0.89	
	Mean	10.58	-	10.25	-	-	100.8	-	-	77.5	-	-	

* Solubilization was estimated by measuring the loss of plant constituents from polyester bags, suspended in the rumen of sheep.

[†] Fresh forage diet fed to rumen fistulated sheep used in the *in situ* experiment.

[‡] Fresh minced forage in the polyester bag suspended in the rumen.

4.4.4. In vitro *Experiment A*

Degradation of the LSU and SSU of Rubisco from either minced or extracted white clover and *L. corniculatus* during *in vitro* rumen incubation is shown in Table 4.7. In the absence of PEG, the rate of degradation (17.0 v. 29.0 %/h) and potential degradability (49.8 v. 70.6 %; $P < 0.001$) were lower for the LSU from *L. corniculatus* than for white clover. Addition of PEG increased the degradation rate ($P < 0.01$) and potential degradability ($P < 0.001$) of the LSU and increased the degradation rate of the SSU ($P < 0.01$), with the LSU (mean values of white clover and *L. corniculatus*) being degraded at a faster rate than the SSU (32.3 v. 14.8 %/h). For the LSU, however, there were significant forage x PEG interactions, suggesting that increases due to PEG addition occurred for *L. corniculatus* and not for white clover. There were also significant forage x processing interactions, suggesting that the LSU and SSU from minced white clover had a much faster rate of degradation than the same proteins in the total soluble protein extracts. The reverse occurred for *L. corniculatus* forage preparations. For the SSU (Table 4.7) responses to PEG were in the same direction as for the LSU, but were much smaller in magnitude, and few attained statistical significance, suggesting that the SSU of Rubisco was more resistant to rumen degradation than the LSU. Source of rumen fluid had little effect upon the degradation of Rubisco.

Table 4.7. *In-vitro* Experiment A. Effect of condensed tannins (CT) and adding polyethylene glycol (PEG; MW 3500) upon degradation* of the large subunit (LSU) and small subunit (SSU) of Rubisco from *Lotus corniculatus* (LC) and white clover (WC) during *in vitro* incubation with rumen fluid.

Forages	Degradation rate			Potential degradability (PD)			
	c (%/h)		S.E. (D.F. = 6)	a + b (%)		S.E. (D.F. = 6)	
	- PEG	+ PEG		- PEG	+ PEG		
LSU (n = 4)							
Minced	WC	41.4	47.4	4.03	68.4	67.4	0.80
Extracted	WC	15.7	26.8	3.80	72.8	71.2	2.15
	Mean	28.6	37.1	-	70.6	69.3	-
Minced	LC	16.6	27.8	2.45	54.9	72.5	2.50
Extracted	LC	17.3	27.4	0.22	44.7	67.7	3.44
	Mean	17.0	27.6	-	49.8	70.1	-
SSU (n = 4)							
Minced	WC	11.3	13.7	1.23	59.5	60.3	1.89
Extracted	WC	6.9	11.0	0.60	61.1	59.6	2.39
	Mean	9.1	12.3	-	60.3	59.9	-
Minced	LC	5.3	8.2	0.62	58.5	61.0	2.10
Extracted	LC	7.9	26.4	5.80	53.1	60.5	2.40
	Mean	8.5	17.3	-	55.8	60.8	-

* Degradation was defined as the rate of disappearance of the individual proteins from *in vitro* rumen incubations.

4.4.5. *In vitro Experiment B*

The effect of adding purified CT and PEG upon the degradation of the LSU and SSU of Rubisco from white clover during *in vitro* incubation with rumen fluid obtained from sheep fed either white clover or *L. corniculatus* is shown in Table 4.8. The SSU was degraded at a lower rate than the LSU (10.4 v. 31.3 %/h). In the absence of PEG, addition of purified CT to white clover significantly reduced the rate of LSU degradation ($P < 0.01$) and also reduced its potential degradability ($P < 0.001$). Addition of PEG increased both degradation rate and potential degradability of both Rubisco sub-units, but there were significant CT x PEG interactions. This suggests that PEG addition increased the rate of Rubisco degradation and its potential degradability in the presence, but not in the absence of added CT extracts. Similar effects were observed with the SSU, but the response to CT and PEG were of smaller magnitude than found for the LSU. Changing the source of rumen fluid from white clover fed sheep to *L. corniculatus* fed sheep significantly reduced the rate of LSU degradation ($P < 0.05$), but increased the rate of SSU degradation. However, source of rumen fluid had no effect upon potential degradability, suggesting that the effects of CT in rumen fluid were small. There were no rumen fluid x PEG interactions.

Table 4.8. *In-vitro* Experiment B. Effect of (1) adding condensed tannins (CT) extracted from *Lotus corniculatus* and (2) adding rumen fluid from sheep fed either white clover (WC) or *Lotus corniculatus* (LC) upon the degradation* of the large subunit (LSU) and small subunit (SSU) of Rubisco (extracted from WC). All incubations were undertaken with or without polyethylene glycol (PEG; MW 3500).

Treatments	Degradation rate		S.E. (D.F. = 6)	Potential degradability (PD)		S.E. (D.F. = 6)
	c (%/h)			a + b (%)		
	- PEG	+ PEG		- PEG	+ PEG	
LSU (n = 4)						
¹ Effect of CT						
No CT	30.4	38.6	4.09	70.3	69.0	1.41
With CT	16.6	39.4	2.40	53.6	65.6	1.14
Mean		31.3	3.24		64.6	1.28
² Rumen fluid						
WC	26.2	43.0	3.28	61.9	66.7	2.44
LC	20.7	35.0	3.38	62.0	67.8	1.25
Mean		31.2	3.33		64.6	1.85
SSU (n = 4)						
¹ Effect of CT						
No CT	9.1	12.1	0.85	60.0	60.1	1.40
With CT	7.4	13.1	0.89	48.9	57.8	1.85
Mean		10.4	0.87		56.7	1.63
² Rumen fluid						
WC	7.0	10.4	0.85	56.8	58.6	2.26
LC	9.7	14.8	0.62	52.3	59.3	1.68
Mean		10.5	0.74		56.8	1.97

* Degradation was defined as the rate of disappearance of the individual proteins from *in vitro* rumen incubations.

4.5. DISCUSSION

The principal objective of the present study was to determine how the CT in *L. corniculatus* affected both the solubilization and degradation of plant protein by rumen microorganisms. The digestion of forage protein in the rumen can be attributed to the combined processes of solubilization and degradation. Solubilization can be defined as the release of protein from plant cells into the rumen environment during chewing and it is an important prerequisite for degradation (Mangan 1982; Nugent *et al.* 1983). Degradation is the catabolism of protein by microbial proteolysis resulting in the formation of peptides, amino acids and ammonia. Recent studies have shown that CT from *L. pedunculatus* depressed the digestion of Rubisco by rumen microorganisms principally through reducing degradability, with little effect on the initial solubilisation of Rubisco (McNabb *et al.* 1996). The main findings of the current study support that work but suggest that the action of CT from *L. corniculatus* also had a small but consistent effect on reducing both the initial and subsequent rate of solubilization of Rubisco.

The CT from *L. pedunculatus* reduced the degradation of Rubisco from lucerne leaves by 3 to 4-fold after 2 and 4 h of incubation (McNabb *et al.* 1996; Tanner *et al.* 1994). The rate of binding between CT and protein is dependent on the type of protein as well as the type of CT, and is mainly by hydrogen bonding and hydrophobic interactions (Spencer *et al.* 1988; Horigome *et al.* 1988; Asquith & Butler 1986; McNabb *et al.*, 1998). Previous studies have shown that proteolysis of the LSU of Rubisco from lucerne occurs relatively quickly, but that the SSU of Rubisco was more resistant to rumen degradation (26.0 v. 4.0 %/h; McNabb *et al.* 1994). Results from the current study show that the LSU of Rubisco was consistently solubilized and degraded faster than the SSU, and adding purified CT from *L. corniculatus* had a larger effect on

reducing the degradability of the LSU. Recent studies have shown that vicilin from pea seeds (containing no sulphur amino acid; SAA) was rapidly hydrolysed by rumen micro-organisms, whereas sunflower albumin 8 (SFA 8; 24 % SAA) from sunflower seeds and ovalbumin (6 % SAA) from chicken egg white were relatively resistant to rumen degradation (McNabb *et al.* 1994; Mangan 1972). This suggests that the rate of degradation of proteins by rumen microorganisms is influenced not only by the solubilization of these proteins but also by their protein structure (level of crosslinking, disulphide linkages etc; McNabb *et al.* 1994; Mahadvan *et al.* 1980; Nugent & Mangan 1978). Similar mechanisms may be involved in the reactions between forage protein and CT, with the proteins that are more resistant to rumen degradation (such as SSU) offering less opportunity for degradation to be slowed through the action of CT.

In the absence of CT, 27 % of the total N in white clover forage was instantly solubilized (Table 5), with the rate of rumen solubilization of the insoluble component being 15 %/h. These values show that the total N in white clover forage was rapidly solubilized compared to the CT-containing forage (23 % total N instantly solubilized and 8 %/h for the rate of rumen solubilization, respectively). In ruminants fed fresh forages most proteins are rapidly solubilized and release between 56 and 65 % of the N concentration in the rumen during mastication; consequently large losses of N (25-35 %) as ammonia absorbed from the rumen occur (Reid *et al.* 1962; Mangan 1972; MacRae & Ulyatt 1974; Ulyatt & Egan 1979). The minimum concentration of CT (g/g protein) needed to inhibit proteolysis in laboratory studies is 1:10 (w/w; Tanner *et al.* 1994) or 1:12 (w/w; Jones & Mangan 1977), with 5 mg CT/g DM or greater being required to prevent bloat in cattle (Li *et al.* 1996). The present study indicated that the trace amounts of CT in white clover (0.3 mg CT/g DM) were not sufficient to reduce solubilization and degradation of plant protein.

In laboratory studies, homogenates of CT-containing plants can be used to partially precipitate soluble protein in low CT-containing plants (Barry & Forss 1983; Waghorn & Shelton 1997). However, when similar mixtures were fed to sheep, CT had little effect on rumen fermentation. The reason for these differences could be that only partial disruption of leaf tissue occurs when a mixture of plants is chewed by animals, compared to complete disruption when plants are homogenized in the laboratory. Fay *et al.* (1980) have shown that higher rates of gas production were detected when plant leaves were homogenized rather than chewed by cattle.

Cohen and Wales (1994) reported that cutting forages into 1 cm lengths, freeze drying and oven drying as preparation methods had a marked effect on protein solubilization and degradation in cows. Fresh cut 1 cm lengths had a lower initial solubilization, higher potential degradability and were generally degraded at a faster rate than any of the dried preparations. Mastication increased degradability compared to cutting into 1 cm lengths, as it increased the initial solubilization of plant protein. Recently, McNabb *et al.* (1996) compared fresh minced and freeze-dried and ground *L. pedunculatus* as forage preparation methods for *in situ* polyester bag incubations in the rumen and reported similar results to this study. Mincing fresh *L. pedunculatus* resulted in a particle distribution which more closely resembled the particle distribution of *L. pedunculatus* in boli which had been chewed by sheep than did freeze-drying and grinding the forage. The freeze-dried and ground preparation method reduced the loss of N and Rubisco from synthetic fibre bags at all sampling times relative to the fresh minced preparation (McNabb *et al.* 1996). These results suggest that mincing fresh plant material should be the preparation method of choice when evaluating fresh forages using *in situ* studies of this type.

The design of the present experiments makes it possible to deduce if the source of rumen fluid had any influence on protein solubilization and degradation and in particular if the inhibiting effects of CT could be transferred through rumen fluid. One of the best measures of the latter is responses to PEG infusion when white clover was incubated *in situ* in the rumen of sheep fed *L. corniculatus*. Whilst PEG infusion did increase the solubilization of both total N (Table 5) and the LSU of Rubisco (Table 6) under these conditions, the magnitude was small suggesting that the ability to transfer protein-inhibiting properties of CT through rumen fluid is minimal. This is supported by the *in vitro* studies, where using rumen fluid from sheep fed either white clover or *L. corniculatus* produced similar results for protein degradation (Table 8).

This study has highlighted that CT from *L. corniculatus* can be used to inhibit the digestion of forage proteins by rumen micro-organisms in sheep fed fresh white clover and *L. corniculatus*, and that it was effective at reducing protein degradation and to a lesser extent solubilization. The effect of CT on protein solubilization and degradability were established using mixed microorganisms in whole rumen fluid from sheep fed on a *L. corniculatus* diet, but the effect of individual rumen bacterial strains upon proteolysis and their response to CT have not been defined and should be studied in future work.

This study was supported by the New Zealand (NZ) Foundation of Research, Science and Technology. The authors wish to thank I. D. Shelton (AgResearch Grasslands, Palmerston North, NZ), F. S. Jackson and P. D. Pearce (Institute of Food, Nutrition & Human Health, Massey University, NZ) for their skilled technical assistance. D. J. Garrick and P. C. H. Morel are thanked for advice with statistical

analysis. Scholarship support to B. R. Min from the NZ Department of Education is acknowledged.

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CHAPTER 5

The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and growth of rumen bacteria

(Short title: *Condensed tannins and bacterial growth*)

5.1 ABSTRACT

Eleven strains of ruminal bacteria were used to determine the effect of various concentrations of condensed tannins (CT) extracted from *Lotus corniculatus* on the proteolysis of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco, EC 4.1.1.39) protein, bacterial specific growth rate (SGR) and maximum optical density (OD_{max}) *in vitro*. The effect of CT on the rate of Rubisco proteolysis (%/h) was determined by making measurements in the presence and absence of polyethylene glycol (PEG; MW 3500), which binds and inactivates CT. *Streptococcus bovis* strain NCFB 2476 and B315, *Butyrivibrio fibrisolvens* strain WV1 and C211a, *Prevotella ruminicola* strain 23, *Prevotella*-like strain C21a, *Ruminococcus albus* strain 8, *Fibrobacter succinogenes* S-85, *Eubacterium* sp. strain C12b and C124b, and *Clostridium proteoclasticum* B316^T were tested against 1.5 mg CT/ml for Rubisco proteolysis. The growth of these strains were also tested against 0, 50, 100, 200, 400, and 600 µg CT/ml in a medium containing Rubisco as the sole nitrogen (N) source. In general, the presence of CT markedly depressed the degradation of both the large sub-unit (LSU) and small sub-unit (SSU) of Rubisco, with the effect being completely reversible by PEG. However, the rate of proteolysis varied considerably between individual bacterial species and between the LSU and SSU of Rubisco.

In the absence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola* like-strain C21a appeared to be most active in both LSU and SSU degradation. *P. ruminicola* strain 23, *Eubacterium* sp. strain C12b and C124b, *C. proteoclasticum* B316^T, *B. fibrisolvens* strain WV1 and C211a had moderate to lower rates of LSU and SSU degradation. Proteolysis of the LSU occurred more rapid than the SSU for *S. bovis* strain NCFB 2476 and B315, *Prevotella*-like strain C21a, *B. fibrisolvens* strain WV1 and C211a. However, *P. ruminicola* strain 23, *Eubacterium* sp. strain C12b and C124b and *C. proteoclasticum* B316^T degraded the SSU faster than the LSU. The non-proteolytic bacterial strains *R. albus* 8 and *F. succinogenes* S-85 appeared to have the slowest rates of degradation of both the LSU and SSU of Rubisco.

In the presence of CT, *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola*-like strain C21a appeared to be most effective at degrading LSU and SSU. CT had a greater effect on the proteolysis of the SSU compared to the LSU in *S. bovis* strain

NCFB 2476 and B315, *P. ruminicola* strain 23, *Eubacterium* sp. strain C12b and C124b.

Most of the bacterial strains showed significantly ($P < 0.05-0.01$) decreased specific growth rate and OD_{max} with increasing CT concentrations. However, some of the strains, *C. proteoclasticum* B316^T and *R. albus* 8 showed transient increases in specific growth rate at low concentrations of CT (between 50 to 100 $\mu\text{g CT/ml}$), but not at high concentrations of CT. In terms of specific growth rate, addition of CT at 50-200 $\mu\text{g CT/ml}$ affected *S. bovis* NCFB 2476, *Eubacterium* sp. C124b and *F. succinogenes* S-85 the most compared to the minus CT controls. Higher concentrations of CT had a smaller effect on the specific growth rate of *P. ruminicola* sp. C21a and *C. proteoclasticum* B316^T when these strains were compared to incubations without CT. In the presence of 100 to 200 $\mu\text{g CT/ml}$, *P. ruminicola* strain 23, *Eubacterium* sp. C124b, *B. fibrisolvans* strain WV1, *C. proteoclasticum* B316^T, *R. albus* 8 and *F. succinogenes* S-85 reached a significantly ($P < 0.01-0.001$) lower OD_{max} compared to minus CT controls. However, the OD_{max} of *Prevotella*-like strain C21a and *Eubacterium* sp. strain C12b appeared to be least affected by increasing CT concentration.

It was concluded that the action of CT from *L. corniculatus* reduces both the rate of Rubisco proteolysis and the growth rate of proteolytic rumen bacteria, but the magnitude of the CT effect differed between the bacterial strains used.

Key words: Condensed tannins, proteolytic rumen bacteria, Ribulose-1,5-bisphosphate carboxylase (Rubisco).

5.2 INTRODUCTION

The breakdown of soluble plant protein in ruminants fed fresh forage is often too rapid and inefficient for microbial protein synthesis to be optimal. In production animals this can cause a serious loss of ingested nitrogen (25-30 %; N) (Beever & Siddons, 1986; MacRae & Ulyatt, 1974; Mangan 1972). This is due to the rate of material protein degradation being much faster than the rate at which ammonia can be reincorporated into microbial protein (Ulyatt *et al.* 1975). The presence of condensed tannins (CT) in legumes such as *Lotus corniculatus* can reduce protein degradation in the rumen (McNabb *et al.* 1998; Min *et al.* 1999). This is probably due to stable CT:protein complexes forming at pH 4.0 - 7.0 in the rumen, but subsequently releasing protein in the acidic conditions of the abomasum. This enables protein to bypass degradation in the rumen and undergo enzymatic hydrolysis and be absorbed from the small intestine (Jones & Mangan 1977; Martin & Martin 1983; McNabb *et al.* 1998). Furthermore, CTs form complexes with both the surface of bacterial cells and with bacterial enzymes. Consequently, this may alter bacterial growth and reduce proteolytic enzyme activities (Jones *et al.* 1993).

The relative proteolytic activities of rumen micro-organisms and their responses to CT are influenced by diet (Nugent *et al.* 1983; Tsai & Jones 1975; Osawa 1992). The effect of CT on the growth of rumen bacterial strains and on microbial proteolysis has been described for only a few bacterial species (Bae *et al.* 1993; McAllister *et al.* 1993; Jones *et al.* 1994). Little information exists on the effects of CT upon rumen microbial growth or its effect on ribulose-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39; Fraction 1 leaf protein; the principal plant protein) degradation.

The objective of the present study was to determine the effect of *L. corniculatus* CT on the proteolytic activities and growth of eleven rumen bacterial strains using Rubisco from white clover as the substrate in anaerobic *in vitro* incubations.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains and media.

The bacterial strains used in this study are listed in Table 5.1.

Table 5.1. Bacterial strains

Species	Strain	Source
<i>Streptococcus bovis</i>	NCFB ¹ 2476	Rowett Research Institute, Aberdeen, UK
<i>Streptococcus bovis</i>	B315	Attwood & Reilly 1995
<i>Eubacterium</i> sp.	C12b	Attwood & Reilly 1995
<i>Eubacterium</i> sp.	C124b	Attwood & Reilly 1995
<i>Butyrivibrio fibrisolvens</i>	WV1	AgResearch, Grasslands, New Zealand
<i>Butyrivibrio fibrisolvens</i>	C211a	Attwood & Reilly 1995
<i>Prevotella (Bacteroides)</i>		
<i>ruminicola</i>	23	NCFB 2202 (=ATCC ² 19189)
<i>Prevotella</i> sp.	C21a	Attwood & Reilly 1995
<i>Clostridium proteoclasticum</i>	B316 ^T	Attwood <i>et al.</i> 1996
<i>Ruminococcus albus</i>	8	R. Mackie, University of Illinois
<i>Fibrobacter succinogenes</i>	S-85	R. Mackie, University of Illinois

¹ NCFB, National Collection of Food Bacteria, Reading Laboratory.

² ATCC, American Type Culture Collection.

All strains were grown anaerobically in a Plant Protein medium (PPM) which was the same as the low-nitrogen medium of Hazlewood and Nugent (1978), except that it contained soluble plant protein extracted from white clover (1.7 mg total N/ml) as the sole nitrogen (N) source. The preparation, distribution and inoculation of PPM was carried out under a stream of CO₂ or in an anaerobic hood with a 95 % CO₂/ 5% H₂ atmosphere (Coy Laboratory Products Inc. Grass Lake, Mich.). Total soluble plant protein was extracted from fresh white clover forage by blending 100 g of plant material in 300 ml artificial saliva (AS; pH 6.8; McDougal 1948), and squeezing the extract through 4 layers of cheese cloth. The filtrate was collected and centrifuged at 17,000 x g for 20 min at 5 °C and the supernatant fraction was filter-sterilized by passing it through a 0.22 µm filter. Dithiothreitol, glucose, cellobiose, NaHCO₃, and vitamins (Bryant & Robinson 1962) were prepared anaerobically and filter-sterilized before addition to the basal medium.

CT and polyethylene glycol (PEG; MW 3,500) were prepared as concentrated stock solutions using anaerobic distilled water and were filter sterilized under anaerobic conditions. CT was extracted from *L. corniculatus* leaves by the method of Terrill *et al.* (1992) with the following modifications: *L. corniculatus* leaves were homogenized in 70 % acetone containing 0.1 % ascorbic acid, and plant pigments and lipids were removed by extraction with methylene chloride three times. The remaining CT fraction was freeze-dried, redissolved in 50 % methanol (vol/vol) and purified using the method of Jackson *et al.* (1996). The purified CT fractions were freeze-dried and stored in the dark at 4 °C.

5.3.2 *In vitro* incubations

The effect of CT upon bacterial proteolysis was followed by adding CT to *in vitro* incubations of rumen bacteria growing on PPM and measuring the disappearance of Rubisco over time. All *in vitro* incubations were conducted at a CT:N ratio of 1:1.1 (1.5 mg CT and 1.7 mg total N/ml) to mimic the CT:protein ratio found in *L. corniculatus* under field growth conditions (28 g total CT and 32 g total N/kg DM; Min *et al.* 1997; 1998). Firstly, CT was added to PPM prior to the addition of PEG (3 mg/mL) and an insoluble CT-protein complex was formed (Jones & Mangan, 1977). The complex was incubated for 10 min at room temperature and then PEG was added (CT+PEG). Aliquots of cultures were removed from *in vitro* reactions prior to and after 2, 4, 6, 8, 12 and 24 h (or 36 h) incubation, mixed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (McNabb *et al.* 1996) and stored at -20°C for SDS-PAGE analysis.

The effect of CT on the specific growth rate and maximum optical density (OD_{max} at 600 nm) of rumen bacterial strains was determined by adding a range of CT concentrations (0, 50, 100, 200, 400, and 600 µg CT/ml) to *in vitro* cultures of each bacterial strain and measuring their growth spectrophotometrically at 600 nm. However, CT concentrations in excess of 600 µg CT/ml caused high background precipitation of Rubisco in PPM and could not be used to determine their effect on bacterial growth. However, CT concentrations of 600 µg CT/ml or below gave acceptable background turbidity and were used in this study. Uninoculated tubes which received equivalent concentrations of CT served as blanks for background turbidity caused by CT:protein interactions. CT or CT plus PEG were added to bacterial *in vitro* incubations after the cells had entered early-logarithmic growth (2 hours for proteolytic strains, 4 hours for cellulolytic strains). Specific growth rate

was calculated (Equation 10) as the maximum specific growth rate achieved by each culture, while OD_{max} was the maximum OD achieved by each culture. The specific growth rate (SGR or μ) was calculated using following the equation 10 (Staneir *et al.* 1977):

$$\text{SGR (h}^{-1}\text{)} = (\log Z - \log Z_0)/(t-t^0) \quad (10)$$

Where Z and Z_0 correspond to the amount of any bacterial component of the culture at times t and t^0 respectively.

5.3.3 SDS-PAGE analysis of Rubisco degradation

Samples were thawed and heated at 95 °C for 5 min to denature protein and dissociate CT-protein complexes and then cooled on ice. The samples were centrifuged at 12,000 x g for 5 min at room temperature and aliquots of the supernatant were analyzed in 20 % SDS-PAGE mini gels with 4 % stacking gels at a constant current of 80 V for approximately 3 h (McNabb *et al.* 1996). After electrophoresis, the gels were fixed by washing twice for 15 min in 40 % methanol; 10 % acetic acid and proteins were visualized by staining with 0.5 % Coomassie Brilliant Blue R-250 in 62.5 % ethanol (37.5 % acetic acid) for 30 min and de-staining in 10 % methanol; 7.5 % acetic acid solution for over night.

Both the LSU (MW 54,000) and SSU (MW 16,000; Kawashima & Wildman 1970) were detected on stained gels and quantified using a Imaging Densitometer (Model GS-670) and the image analysed using Molecular Analyst Image Analysis software (Bio-Rad, Hercules, CA, USA) as described previously (McNabb *et al.* 1996).

5.3.4 Statistical analysis

The significance of differences between means and standard error were calculated using GLM (General Linear Model) procedures (SAS 1985).

5.4 RESULTS

5.4.1 Effects of CT on bacterial proteolysis of Rubisco

The effect of adding 1.5 mg CT/ml of *L. corniculatus* on the proteolysis of the LSU and SSU of Rubisco during *in vitro* rumen incubation of eleven rumen bacterial strains is shown in Figs 5.1 to 5.5. The results are also summarised as rate of Rubisco proteolysis and presented in Table 5.2.

In proteolytic bacterial cultures without added CT or with CT plus PEG, both the LSU and SSU of Rubisco degraded rapidly so that 50 to 90 % disappeared within 4 hours of incubation (Figs 5.1 to 5.5; Table 5.2). However, the degree of Rubisco degradation and the rates of proteolysis of Rubisco varied considerably between individual bacterial species and between the large and small sub-units proteins (Figs. 5.1 to 5.5; Table 5.2). In the absence of CT, *S. bovis* NCFB strain 2476 and B315 and *Prevotella*-like strain C21a appeared to be highly active at degrading Rubisco, particularly the LSU, while strains of *P. ruminicola* strain 23, *C. proteoclasticum* B316^T, *Eubacterium* sp. C12b and C124b showed the opposite trend (Table 5.2). *B. fibrisolvens* strain WV1 and C211a had moderate to low rates of Rubisco degradation. The non-proteolytic bacteria *F. succinogenes* S-85 and *R. albus* 8 appeared to have the slowest rates of degradation of both the LSU and SSU of Rubisco.

Bacterial cultures grown in the presence of CT showed a marked decrease in degradation of both the LSU and SSU of Rubisco (Figs 5.1 to 5.5; Table 5.2). This

effect was most obvious within the first 12 hours of incubation. *S. bovis* strain NCFB 2476 and B315 and *P. ruminicola* C21a appeared to be most active in both LSU and SSU Rubisco degradation in the presence of CT. In contrast, *P. ruminicola* strain 23, *C. proteoclasticum* B316^T, *B. fibrisolvens* strain WV1 and C211a had slow rates of Rubisco degradation. *Eubacterium* sp. strains C12b and C124b had moderate rates of Rubisco degradation in the presence of CT.

In the presence of CT, *Prevotella*-like strains C21a, *C. proteoclasticum* B316^T, *B. fibrisolvens* strain WV1 and C211a degraded the LSU of Rubisco faster than SSU. However, *S. bovis* strain NCFB 2476 and B315, *P. ruminicola* strain 23, *Eubacterium* sp. strains C12b and C124b degraded the SSU of Rubisco faster than the LSU (Table 5.2).

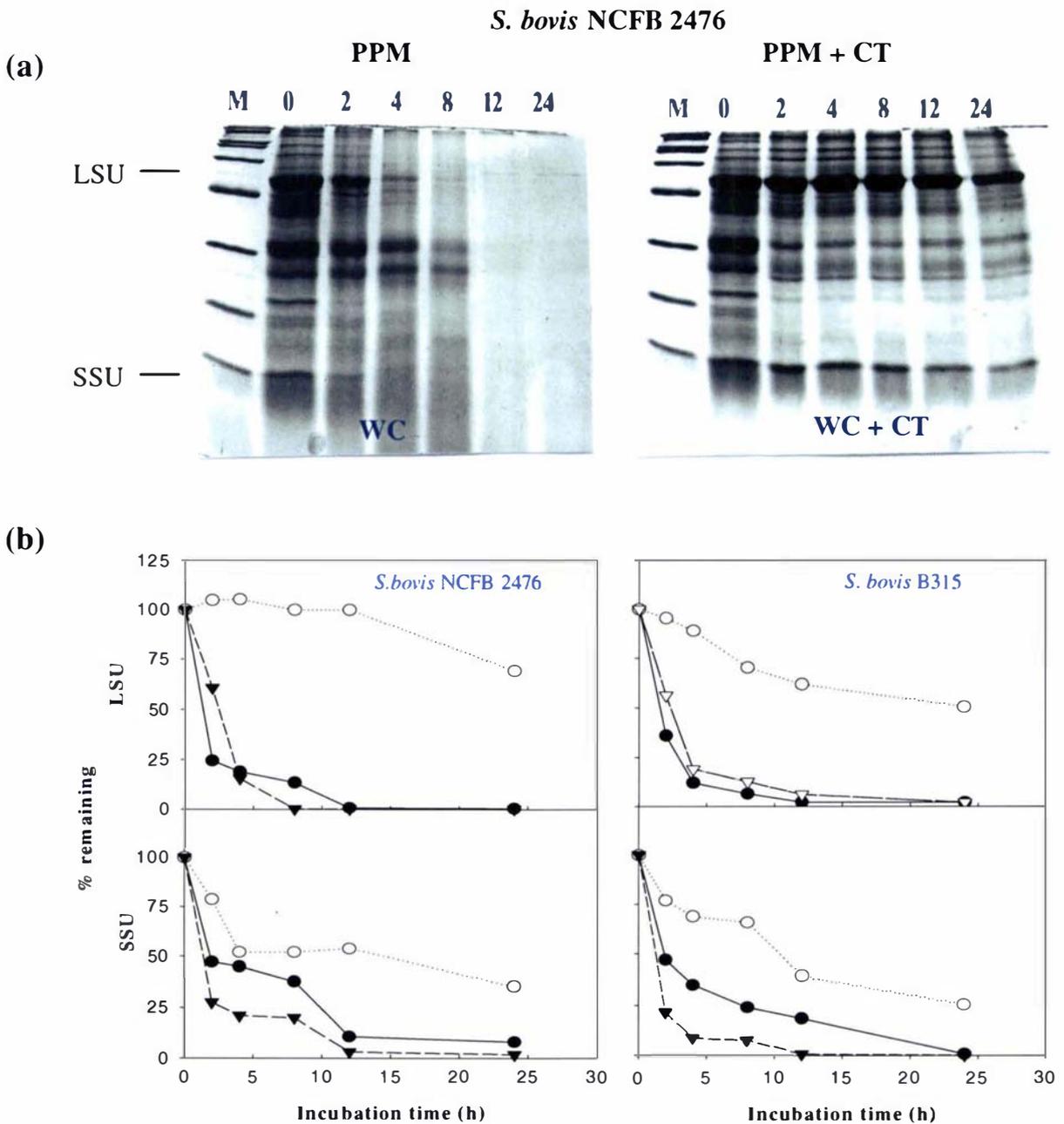


Figure 5.1 Degradation of both the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco). (a) SDS-PAGE gels showing LSU and SSU degradation in an incubation of *S. bovis* NCFB 2476, with and without added CT. (b) Percentage LSU and SSU remaining in incubations of *S. bovis* strain NCFB 2476 and B315 in the presence or absence of CT and CT+PEG. Control (plant protein medium; PPM ●--●), PPM + CT (○--○), PPM + CT + PEG (▲--▲).

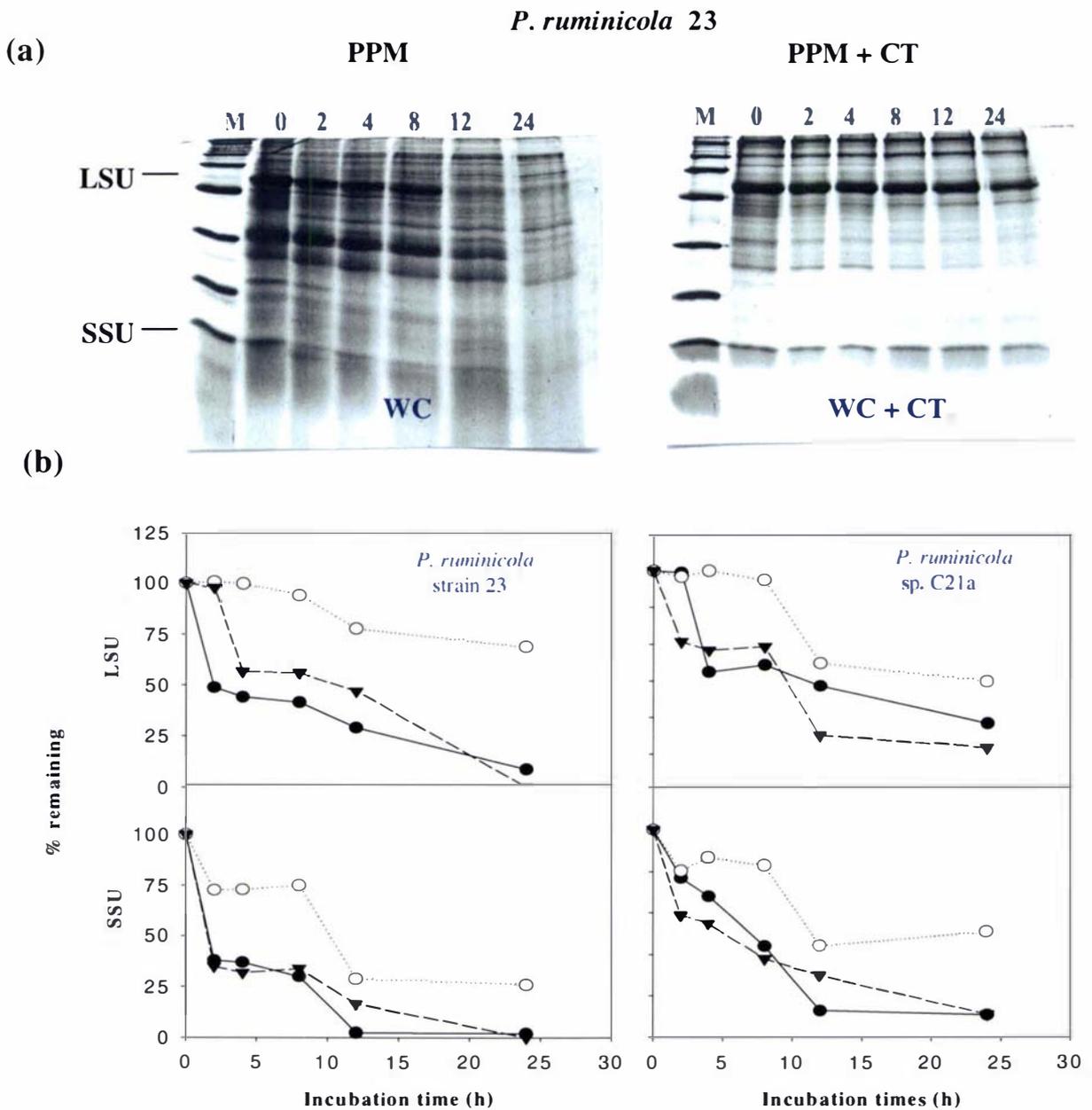


Figure 5.2 Degradation of both the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxigenase (EC 4.1.1.39; Rubisco). (a) SDS-PAGE gels showing LSU and SSU in an incubation of *P. ruminicola* strain 23, with and without added CT. (b) Percentage LSU and SSU remaining in incubations of *P. ruminicola* strain 23 and C21a in the presence or absence of CT and CT+PEG. Control (plant protein medium; PPM; ●--●), PPM + CT (○--○), PPM + CT + PEG (▲--▲).

Eubacterium sp. C12b

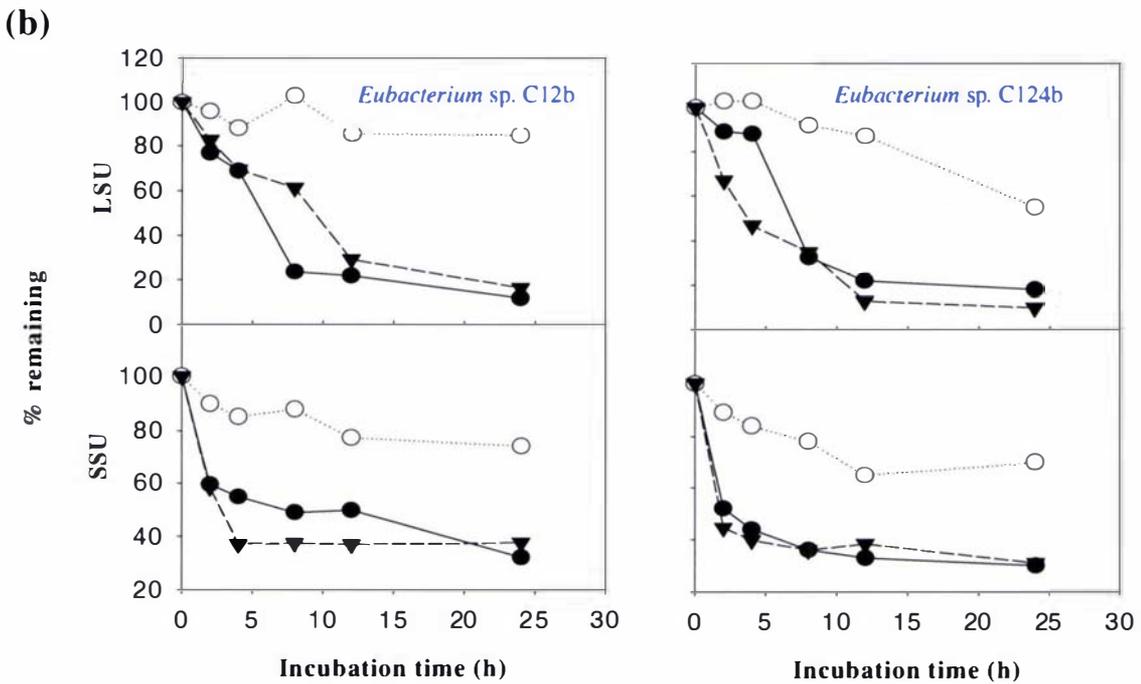
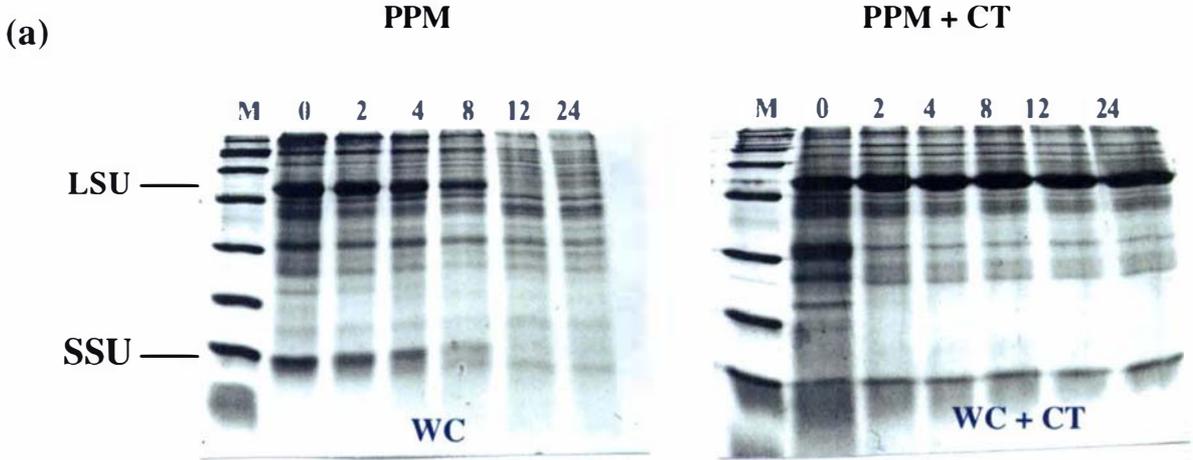


Figure 5.3 Degradation of both the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco). (a) SDS-PAGE gels showing LSU and SSU in incubation of *Eubacterium* sp. C12b, with and without added CT. (b) Percentage LSU and SSU remaining in incubations of *Eubacterium* sp. strain C12b and C124b in the presence or absence of CT and CT+PEG. Control (plant protein medium; PPM; ●--●), PPM + CT (○--○), PPM + CT + PEG (▲--▲).

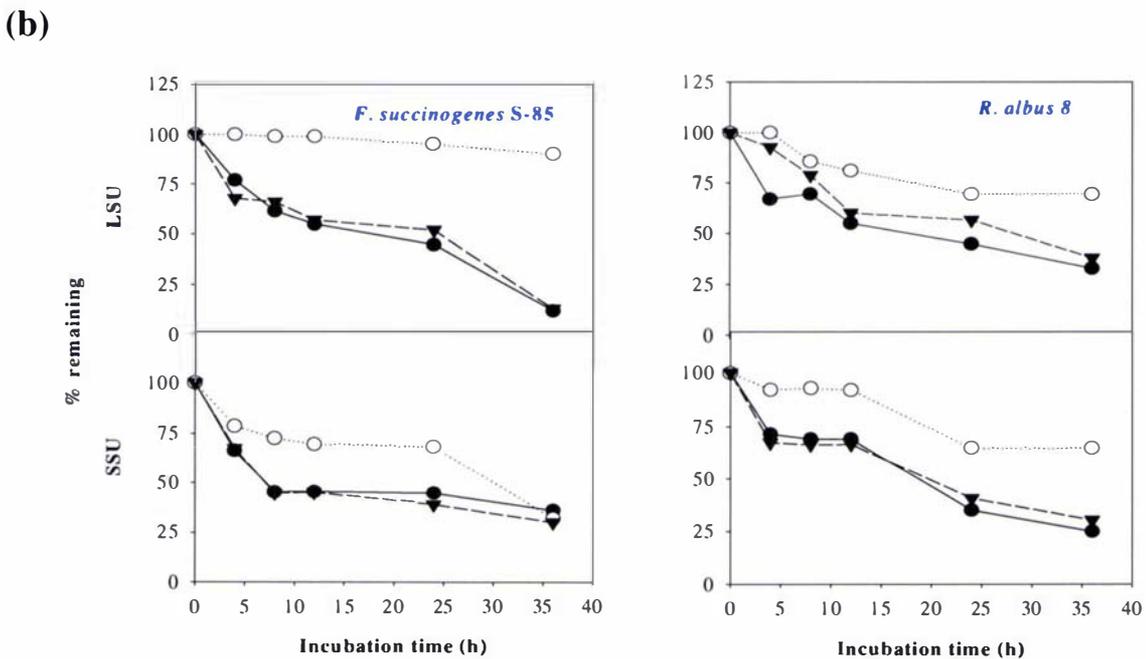
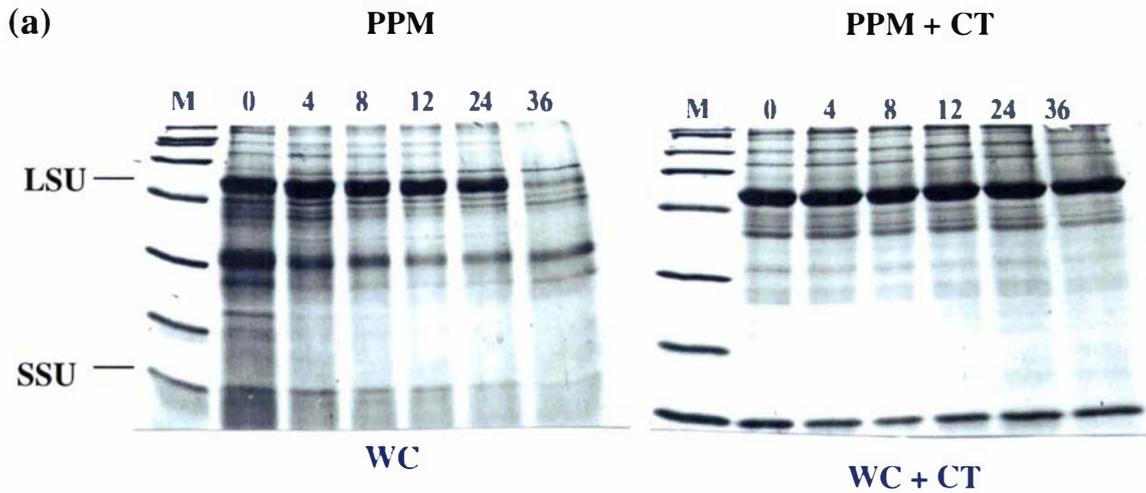
F. succinogenes S-85

Figure 5.4 Degradation of both the large (LSU) and small (SSU) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco). (a) SDS-PAGE gels showing LSU and SSU in an incubation of *F. succinogenes*, with and without added CT. (b) Percentage LSU and SSU remaining in incubation of *F. succinogenes* S-85 and *R. albus* 8 in the presence or absence of CT and CT+PEG. Control (plant protein medium; PPM; ●--●), PPM + CT (○--○), PPM + CT + PEG (▲--▲).

Table 5.2 The effect of CT extracted from *L. corniculatus* upon the rates of Rubisco proteolysis during *in vitro* rumen incubations of rumen bacterial strains.

	<u>Rubisco proteolysis (%/h)¹</u>				<u>Inhibition by CT (%)²</u>	
	<u>Without CT</u>		<u>With CT</u>			
	LSU	SSU	LSU	SSU	LSU	SSU
<i>F. succinogenes</i> S-85	6.3	7.8	0.3	1.8	95.2	76.9
<i>S. bovis</i> B315	37.8	26.5	2.6	11.5	93.1	56.6
<i>S. bovis</i> NCFB 2476	32.1	26.4	2.3	10.6	92.8	59.8
<i>P. ruminicola</i> strain 23	12.8	15.5	1.4	3.8	89.1	75.5
<i>C. proteoclasticum</i> B316 ^T	8.3	14.1	2.0	1.3	75.9	90.8
<i>Eubacterium</i> sp. C12b	11.3	20.2	3.8	5.1	66.4	74.7
<i>B. fibrisolvens</i> WV1	8.0	7.5	2.9	2.3	63.8	69.3
<i>Prevotella</i> -like strain C21a	27.0	11.5	11.3	9.8	58.1	14.8
<i>Eubacterium</i> sp. C124b	5.5	24.0	2.6	5.5	52.7	77.1
<i>B. fibrisolvens</i> C211a	5.8	4.0	2.8	2.4	51.7	40.0
<i>R. albus</i> 8	2.2	0.8	1.3	1.9	40.9	-1.3
Mean	14.3	14.4	3.0	5.1	70.9	57.7

¹ The rates of Rubisco proteolysis were summarized from Figs 5.1 to 5.5 using the following equation: Rubisco proteolysis (%/h) = $\{(\log Z - \log Z_0)/\log Z\}/(t-t^0) \times 100$. Where Z and Z₀ correspond to the amount of LSU and SSU remaining at times t (maximum) and t⁰ respectively.

² Calculated as:

$$\frac{\text{Mean proteolysis (\%/h) without CT} - \text{mean proteolysis (\%/h) in presence of CT}}{\text{Mean proteolysis (\%/h) without CT}}$$

5.4.2 Effects of CT on bacterial growth

The growth of eleven proteolytic rumen bacteria in the absence or presence of different concentrations of CT extracted from *L. corniculatus* are shown in Figs 5.6 to 5.8. The results are also summarised as inhibitory effect of CT upon rumen bacterial

growth and presented in Table 5.3. Varying concentrations of CT were added to bacterial cultures growing in a medium in which a plant protein extract was the sole N source. Bacterial growth was measured and the resulting growth curves were summarised by calculating specific growth rate and maximum optical density (OD_{max}) and plotting these values against the CT concentrations tested (Figs. 5.6, 5.7 & 5.8).

In the absence of CT, all the bacterial strains showed typical growth reaching their respective OD_{max} within 24 hours. Addition of 200, 400 and 600 µg CT/ml significantly ($P < 0.05-0.01$) reduced the specific growth rate of most of the bacterial strains tested compared to the minus CT controls (Figs. 5.6, 5.7 & 5.8). However, *C. proteoclasticum* B316^T (Fig. 8 a; $P < 0.05$) and *R. albus* 8 (Fig. 5.8 b; $P = 0.09$) showed transient increases in their specific growth rate at 100 µg CT/ml, after which their growth rate declined with increasing CT concentration. In terms of specific growth rate, *S. bovis* strain NCFB 2476 (Fig. 5.6a), *F. succinogenes* S-85 (Fig. 5.8b) and *Eubacterium* sp. C124b (Fig. 5.7a) were most affected ($P < 0.05-0.01$) at the concentrations of 50, 100 and 200 µg CT/ml, respectively. *Prevotella*-like strain C21a (Fig. 5.6b) and *C. proteoclasticum* B316^T (Fig. 5.8a) appeared to be the least affected by increasing CT concentrations.

The degree of inhibitory effect of CT on bacterial growth varied considerably between individual bacterial species (Table 5.3). *S. bovis* strain NCFB 2476 and B315, *F. succinogenes* S-85, *R. albus* 8, *Eubacterium* sp. C12b and *Prevotella*-like strain C21a appeared to be most inhibited (up to 50 %) in their growth rate by CT. In contrast, *C. proteoclasticum* B316^T, *P. ruminicola* strain 23, *Eubacterium* sp. C124b, *B. fibrisolvans* strain C211a WV1 were moderately inhibited (less than 50 %) by CT (Table 5.3).

In general, the OD_{max} of the bacterial strains tested decreased as CT concentration increased (Figs. 5.6, 5.7 and 5.8). In the presence of 100 to 200 µg CT/ml, *P. ruminicola* strain 23, *Eubacterium* sp. C124b, *B. fibrisolvens* WV1, *C. proteoclasticum* B316^T, *R. albus* 8 and *F. succinogenes* S-85 had significantly ($P < 0.01-0.001$) lower maximum growth compared to minus CT controls. However, *Prevotella*-like strain C21a (Fig. 5.6b) and *Eubacterium* sp. C12b (Fig. 5.7a) did not have significantly different maximum OD values at CT concentrations between 50-200 µg CT/ml compared to minus CT cultures. However, above 200 µg CT/ml the differences became significant.

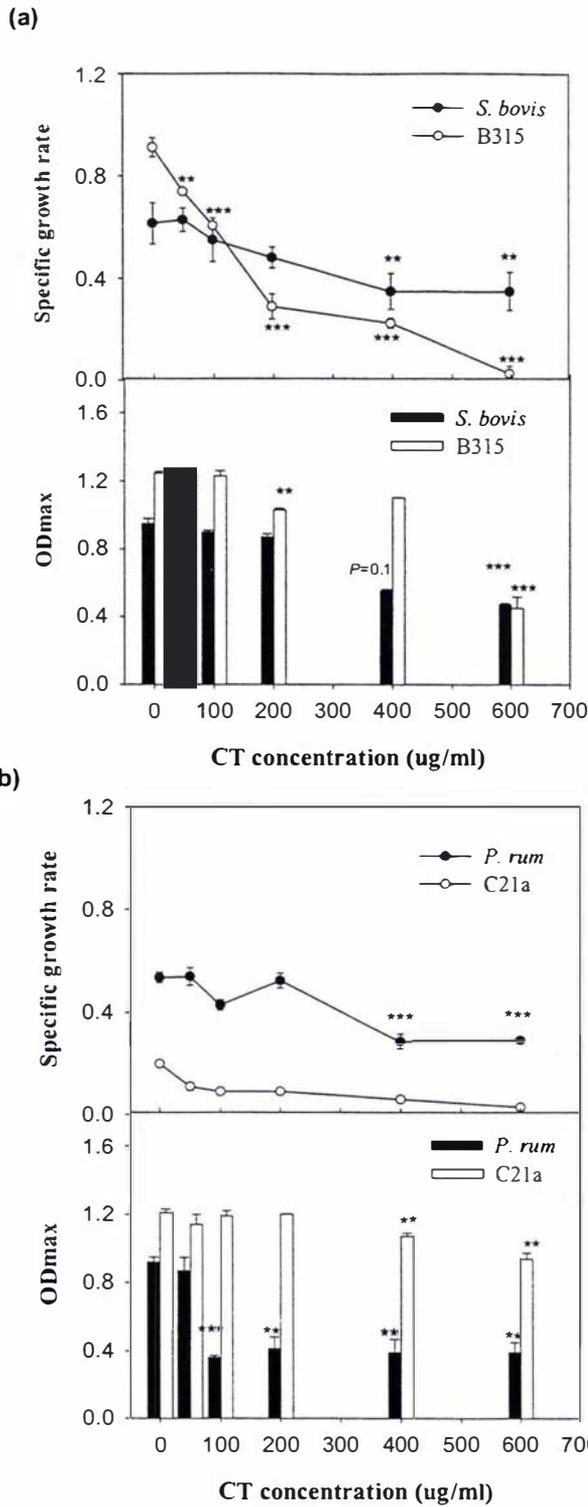
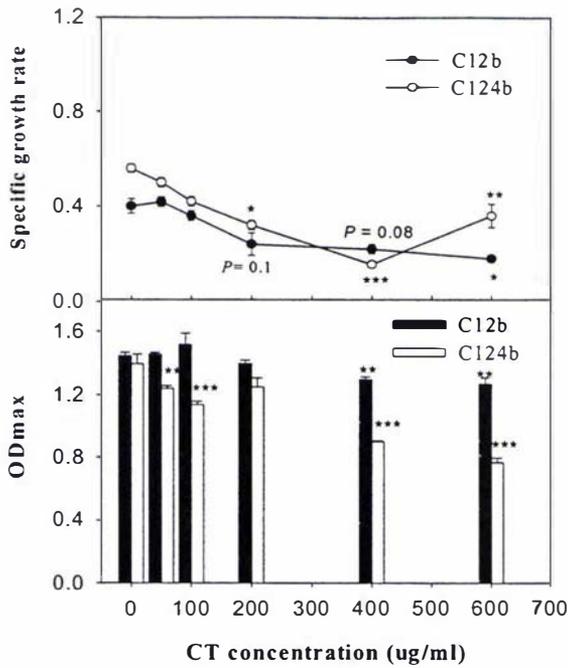


Figure 5.6 Effects of condensed tannins (CT) extracted from *L. corniculatus* on the specific growth rate and maximum optical density (ODmax) of (a) *S. bovis* strain NCFB 2476 and B315, (b) *P. ruminicola* strain 23 and *Prevotella*-like strain C21a; Max. specific growth rate (-●- -○-); OD_{max} (■, □). Within strain comparison (control vs each CT concentration); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(a)



(b)

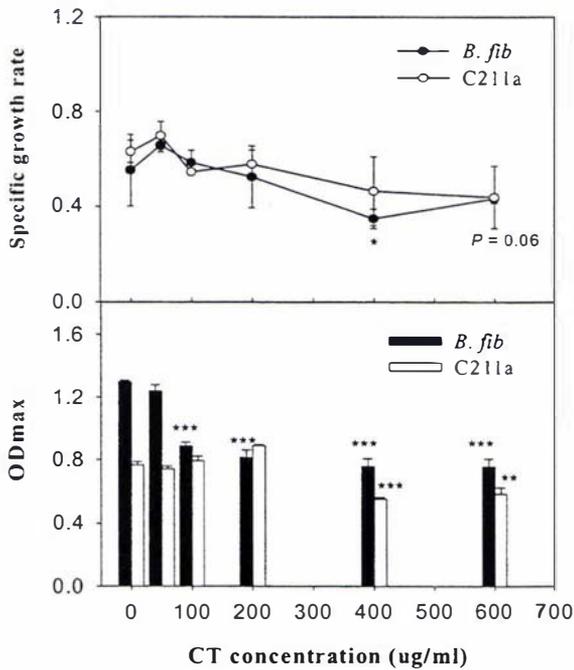
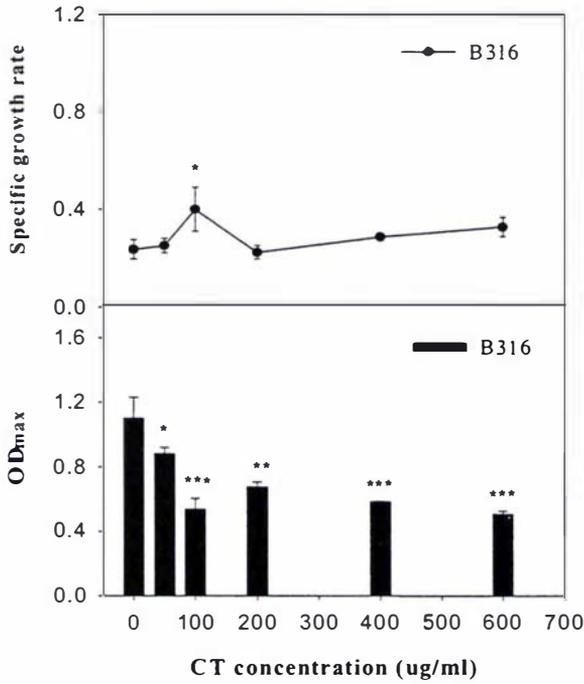


Figure 5.7. Effects of condensed tannins (CT) extracted from *L. corniculatus* on the specific growth rate and maximum optical density (OD_{max}) of (a) *Eubacterium* sp. strain C12b and C124b, (b) *B. fibrisolvens* strain WV1 and C211a; Max. specific growth rate (●-●, ○-○); OD_{max} (■, □). Within strain comparison (control vs each CT concentration); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(a)



(b)

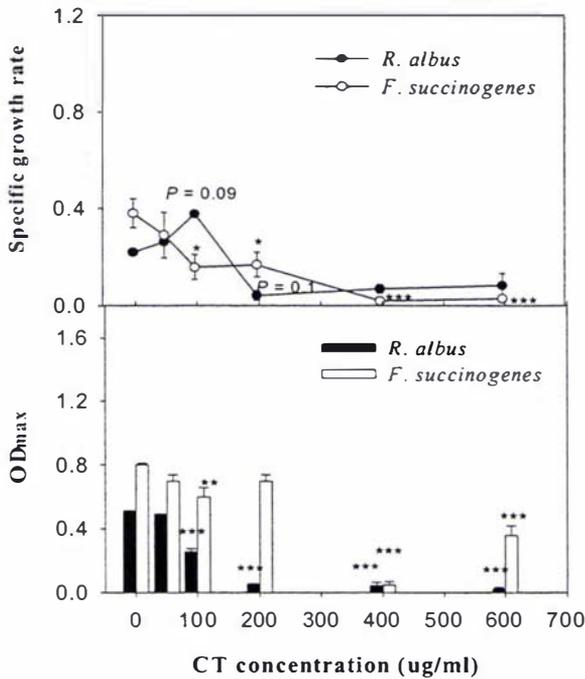


Figure 5.8 Effects of condensed tannins (CT) extracted from *L. corniculatus* on the specific growth rate and maximum optical density (OD_{max}) of (a) *C. proteoclasticum* B316^T, (b) *R. albus* 8 and *F. succinogenes* S-85; Max. specific growth rate (-●- -○-); OD_{max} (■, □). Within strain comparison (control vs each CT concentration); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5.3 Inhibitory effect of CT extracted from *L. corniculatus* upon rumen bacterial growth *in vitro*.

	<u>Inhibition by CT (%)¹</u>	
	Specific growth rate	ODmax
<i>S. bovis</i> NCFB 2476	95.5	50.5
<i>S. bovis</i> B315	73.6	35.8
<i>F. succinogenes</i> S-85	64.3	32.9
<i>R. albus</i> 8	60.2	96.3
<i>Eubacterium</i> sp. C12b	56.0	11.8
<i>Prevotella</i> -like strain C21a	55.9	16.8
<i>P. ruminicola</i> strain 23	45.4	57.1
<i>Eubacterium</i> sp. C124b	35.9	47.3
<i>B. fibrisolvens</i> C211a	30.5	56.9
<i>B. fibrisolvens</i> WV1	21.4	43.8
<i>C. proteoclasticum</i> B316 ^T	-39.7	52.2
Mean	45.4	43.8

¹ The inhibition rate of bacterial growth was summarised from Figs. 5.6 to 5.8 and was calculated using the following equation: The inhibition rate of bacterial growth (%) =

$$\frac{\text{Mean growth rate without CT} - \text{mean growth rate with CT (600 } \mu\text{g CT/ml)}}{\text{mean growth rate without CT}} \times 100$$

5.5 DISCUSSION

The principal objectives of the present study were to determine how CT from the forage legume *L. corniculatus* affected the proteolytic activity and growth rate of rumen proteolytic bacteria. The main findings show that *L. corniculatus* CT reduced both the proteolysis of Rubisco protein and the rate and extent of growth of various proteolytic rumen bacteria. The rates of LSU and SSU Rubisco proteolysis varied considerably between individual bacterial strains.

The CT:N ratio of 1:1.1 (1.5 mg CT: 1.7 mg total N/ml) used in this study was that found in *L. corniculatus* grown under field conditions (28 g total CT and 32 g total N/kg DM; Min *et al.*, 1997; 1998) during a typical New Zealand summer. The results of the *in vitro* experiments on selected proteolytic strains suggest that the levels of CT:N ratios (1:1.1) found under field conditions significantly reduce bacterial proteolysis and probably inhibit bacterial growth. Tanner *et al.* (1994) have shown that *L. corniculatus* CT: protein ratios of 1:1 or 1:2 significantly reduced the rate of Rubisco proteolysis relative to CT-free control. However, no significant effect was detected when the CT:protein ratio was over 1:5 (Jones & Mangan 1977; Perez-Maldonado *et al.* 1995).

In the present study, *in vitro* proteolysis of the LSU and to a lesser degree the SSU of Rubisco was generally reduced by CT. These effects were reversed by the addition of PEG or in the absence of CT. These observations suggest that *L. corniculatus* CT would reduce protein degradation in the rumen. Previous work has shown that in the presence of CT, the LSU of Rubisco was susceptible to proteolysis by mixed rumen micro-organisms, but that the SSU had lower rates of proteolysis (McNabb *et al.* 1994; Tanner *et al.* 1994; Min *et al.* 1999). This was thought to be due to either the

rate of solubilisation of SSU, to its disulphide crosslinking (high content of methionine & cysteine) or tertiary structure (Nugent & Mangan 1978; McNabb *et al.* 1994). Previous research has shown that amino acid composition was similar in the LSU Rubisco from various plant species, but that it varied in the SSU (Sugiyama *et al.* 1971; Mangan 1982). Mangan (1982) and McNabb *et al.* (1994) reported that the sulphur amino acid (methionine & cystein) composition of the LSU of Rubisco was lower than the SSU of Rubisco, indicating that the breakdown of plant proteins by proteolytic rumen bacteria depends on the protein solubility, sulphur amino acid content and protein structure, possibly explaining the slower SSU breakdown of Rubisco.

In the present study, bacterial strains showed differential rates of proteolysis of the LSU and SSU Rubisco in the absence or presence of CT. A partial explanation for the different rates of proteolysis in Rubisco sub-units may be different affinities and structures of the sub-units of Rubisco which influence how they bind with CT and the way individual strains interact with the Rubisco subunits and CT. The relative affinities of proteins and polypeptides for CT are influenced by the size of the CT polymer and the proline content in the substrate (Muenzer *et al.* 1979; Siebert *et al.* 1996). However, Mole *et al.* (1990) observed that proline richness *per se* is not the only reason for high protein affinity for CT and suggested that post-translational modification such as glycosylation may enhance the affinity towards CT by converting the protein to a more open conformation. Also loosely structured globular proteins, such as BSA and histone F1, have very high affinities for CT compared with the compact structured globular proteins such as ovalbumin, lysozyme, ribonuclease and myoglobin (Hagerman & Butler 1981; Privalov 1979). Chapman *et al.* (1988) reported that the SSU contains a high percentage of α -helix (35-40 %) with very little

β -structure, but that the LSU contains long COOH-terminal extensions and β or α/β -structure of the open-face sandwich type (Salnikow & Vater 1984; Chapman *et al.* 1988). The *in vitro* study reported here has found that CT had a more inhibitory effect on the proteolysis of the LSU compared to the SSU of Rubisco against *S. bovis* strains NCFB 2476 and B315, *P. ruminicola* strains 23 and C21a and *B. fibrisolvans* C211a, while strains of *C. proteoclasticum* B316^T, *Eubacterium* sp. C12b and C124b showed the opposite trend. It appears that the loosely structured LSU probably has more affinity for CT compared with the compactly structured SSU of Rubisco and this explains the greater inhibitory effect of CT on the proteolysis of the LSU. The opposite trend detected in strains of *C. proteoclasticum* B316^T, *Eubacterium* sp. C12b and C124b is more difficult to explain. However, the greater inhibitory effect of CT upon proteolysis of the SSU by these strains may be explained by their apparent preference for SSU proteolysis in the absence of CT (Table 5.2). It may be that the proteolytic enzymes of these strains have a greater affinity for the SSU compared to the LSU and therefore in the presence of CT, SSU degradation is most greatly affected. These observations suggest that the preferential interaction of bacterial proteolytic enzymes with either the LSU or SSU will influence the effect of CT upon Rubisco degradation.

Previous studies have shown that Sainfoin (*Onobrychis viciifolia* Scop.) and *L. corniculatus* CT inhibit the growth and proteolysis of several bacterial species including *S. bovis*, *B. fibrisolvans*, *F. succinogenes*, *P. ruminicola* and *Ruminobacter amylophilus* (Bae *et al.* 1993; McAllister *et al.* 1993; Jones *et al.* 1994). The results of the present study using CT extracted from *L. corniculatus* are in agreement with those studies. The specific growth rate of *S. bovis* strain NCFB 2476, *Eubacterium* sp. C124b and *F. succinogenes* S-85 were strongly inhibited by CT addition. There is

evidence that CT extracted from sainfoin caused the normally single rods of *B. fibrisolvens* A 38 to become filamentous and inhibited the separation of cells after division (Jones *et al.* 1994). They also found that CT binds to cell coat polymers of *B. fibrisolvens* A 38 (200 µg CT/ml) and *S. bovis* 45S1 (100 µg CT/ml) and also inactivated cell-bound proteinase (Jones *et al.* 1993, 1994). The first step in plant protein breakdown in the rumen was hydrolysis of proteins by proteolytic rumen bacteria to peptides and amino acids (Hazlewood *et al.* 1981). These products are either utilised directly by the microbes or degraded further to short chain fatty acids and ammonia (Nolan *et al.* 1976). The *L. corniculatus* CT could possibly reduce breakdown of plant protein by forming CT-protein or CT-bacterial cell complexes, and also inactivate cell-associated proteinase. Consequently, this may inhibit proteolysis, bacterial incorporation of amino acids and modify bacterial growth.

While the growth of some strains was specifically inhibited by CT, the specific growth rate of *P. ruminicola* strain 23, *Eubacterium* sp. C124b, *B. fibrisolvens* strain WV1 and C211a and *C. proteoclasticum* B316^T were not greatly affected. Jones *et al.* (1994) have shown that rumen bacterial strains *P. ruminicola* B14 and *Ruminobacter amylophilus* WP225 had considerable resistance to even high CT concentrations (up to 600 µg CT/ml). This implies that these bacterial species perhaps play important roles in protein breakdown in ruminants consuming CT-containing diets.

Previous studies have shown that a low level of CT (100- 200 µg CT/ml) from *L. corniculatus* increased bacterial growth and enzyme activity of *F. succinogenes* S-85, but these activities were suppressed by 400 µg CT/ml (Bae *et al.* 1993). Jones *et al.* (1994) also showed that the total protease enzyme activity (using azocasein as a substrate) in cultures of *P. ruminicola* was 36 % higher in the presence of 100 µg CT/ml than the control, and that there was little effect on the cell-associated

proteinase activity of *P. ruminicola* up to 300 µg CT/ml. In addition, CT had different effects on digestive enzymes. Oh and Hoff (1986) reported that CT added to bovine serum albumin (BSA) inhibited pepsin and chymotrypsin activity. However, low concentrations of CT (0.1 %; w/w) increased trypsin activity by about 80 %, apparently because of an alteration in the conformation of the BSA due to binding with CT. In the present experiment we observed that the rumen bacterial strains *C. proteoclasticum* B316^T and *S. bovis* NCFB 2476 showed an increase in their specific growth rates at low levels of CT (50-200 µg CT/ml). The reason for the enhancement of bacterial growth is not clear, but it may result from CT-induced structural changes in the substrate protein. Low concentrations of CT may partially denature the plant protein, which is perhaps a more easily degraded substrate for the proteinases of the rumen proteolytic bacteria (Dellert & Stahman 1955; Mole & Waterman, 1985). No further stimulation of growth rate was observed when higher concentrations of CT (greater than 200 µg CT/ml) were added to bacterial cultures, which supports the hypothesis that CT might be promoting hydrolysis of protein at low CT concentrations.

This study has highlighted that CT from *L. corniculatus* reduced the bacterial proteolysis of Rubisco protein and inhibited the growth of various proteolytic rumen bacteria. The effect of CT upon proteolysis of individual bacterial strains and their growth response to CT were established, but the effect of CT upon proteolytic rumen bacterial populations *in vivo* has not been defined and should be studied in future work. There are likely to be many other factors involved in CT-bacterial interactions *in vivo* and these need to be addressed.

This study was supported by Public Good Science Funding from the Foundation of Research Science and Technology (FORST) in New Zealand. The authors wish to

thank K. Reilly and J. S. Peters (AgResearch Grasslands, Palmerston North, NZ) for their skilled technical assistance. Scholarship support to B.R. Min from the New Zealand Department of Education is acknowledged.

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CHAPTER 6

Effect of condensed tannins upon four proteolytic bacterial populations in the rumen and upon nitrogen fluxes to the abomasum of sheep fed

Lotus corniculatus.

This chapter will be submitted to *Journal of Agricultural Science, Cambridge*.

6.1 ABSTRACT

Twelve six month old Romney sheep were fed *Lotus corniculatus* (32 g CT/kg DM) to examine the effects of condensed tannins (CT) on proteolytic rumen bacterial populations. Nitrogen (N) fluxes to the abomasum was also examined after the sheep became adapted to the *L. corniculatus*.

In the first part of the experiment, the populations of four proteolytic rumen bacteria were enumerated directly from rumen samples using a competitive polymerase chain reaction (cPCR) technique. When feeding perennial ryegrass/white clover (referred to as Past-I) diet, populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315, *B. fibrisolvens* C211a were 1.6×10^8 , 2.7×10^8 , 7.1×10^6 and 1.2×10^6 per ml respectively. When the diet was changed from pasture (Past-I) to *L. corniculatus*, the populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315, *B. fibrisolvens* C211a from the same animals decreased significantly ($P < 0.001$) to 5.1×10^7 , 1.5×10^8 , 2.6×10^6 and 1.0×10^6 per ml, respectively. When PEG was infused into the rumen of sheep fed *L. corniculatus*, the populations of proteolytic bacteria increased significantly ($P < 0.01-0.001$) compared to the CT-acting group.

The action of CT in *L. corniculatus* reduced rumen proteinase activity, and the concentrations of rumen ammonia and soluble N were significantly decreased ($P < 0.05-0.001$) compared to the PEG treatment group. The principal effects of CT were to reduce rumen N digestibility ($P < 0.05$) and ammonia pool size, and to increase the flow of undegraded feed nitrogen (N) to the abomasum. Dry matter intake (DMI) and DM digestibility were unaffected. The N intake, rumen non-ammonia nitrogen (NAN) pool size, rumen microbial NAN pool size and abomasal microbial NAN fluxes were

similar in both CT-acting and PEG sheep, but non-microbial NAN flux to the abomasum was higher ($P < 0.01$) in the CT-acting sheep than in the PEG sheep.

L. corniculatus CT protected protein against degradation in the rumen, and increased the flow of undegraded feed N to the abomasum. Proteolytic bacterial populations were reduced by CT, but these changes did not effect the total rumen microbial NAN pool size or abomasal microbial NAN flux. Consequently, more protein was available for hydrolysis in the small intestine in sheep.

6.2 INTRODUCTION

New Zealand ruminants graze high quality fresh forages which are high in protein. On these diets there is substantial breakdown of protein to ammonia in the rumen with only a small proportion of undigested dietary N (about 30 %) flowing to the small intestine for absorption. The rate of ammonia production exceeds the capacity of rumen micro-organisms to synthesize microbial protein from ammonia (Ulyatt *et al.* 1975). This process represents the sum of a number of microbial processes, and as protein synthesis by rumen microbes plays an important role in ruminant nutrition (Broudiscou & Jouany 1995), controlling this process is essential for optimizing production in grazing ruminants.

The proportion of individual proteolytic rumen bacteria appear to change with diet (Hazlewood *et al.* 1983). The proteolytic bacteria in hay-concentrate fed cattle were dominated by species of *Butyrivibrio fibrisolvens* (58 %) and *Streptococcus bovis* (44 %), but changed to predominantly *S. bovis* when the diet was changed to fresh lucerne forage (Hazlewood *et al.* 1983). Furthermore, the proteolytic bacteria in fresh forage-fed New Zealand cattle were also variable and were dominated by species of the genera *Streptococcus* (61 %), *Eubacterium* (23 %), *Butyrivibrio* (23 %) and *Clostridium* (7 %; Attwood & Reilly 1995). In addition, proteolytic rumen bacteria vary widely in their growth and proteinase activity in response to the presence of condensed tannins (CT; Jones *et al.* 1994; Nelson *et al.* 1998; Odenyo & Osuji 1998). However, the effects of CT from *Lotus corniculatus* on individual proteolytic activities, bacterial populations and total microbial N flow from the rumen *in vivo* has not been determined.

CT are polyphenolic compounds and are beneficial to ruminants in some instances. When sheep were fed on *L. corniculatus* containing moderate levels of CT (20-40g

CT/kg DM), the rumen degradation of plant soluble proteins was reduced (pH 5.5-7.0; McNabb *et al.* 1996). CT have also increased the absorption of essential amino acids from the small intestine (Waghorn *et al.* 1987; Wang *et al.* 1994). It seems probable that reducing the rate of protein degradation in the rumen and increasing escape of dietary plant proteins from the rumen, using CT will improve N retention in the grazing animal.

The objectives of the present investigation were to determine the effects of CT from *L. corniculatus* upon the population of four proteolytic bacterial species, examine the effects of CT on proteinase activities in the rumen and to evaluate the impact of CT upon the outflow of nitrogen fractions from the rumen using a ^{15}N tracer technique.

6.3 MATERIALS AND METHODS

6.3.1 Experimental design

Sheep were fed the CT-containing legume *L. corniculatus*, with and without an intraruminal infusion of polyethylene glycol (PEG; MW 3500). In the first part of the experiment, the populations of four proteolytic rumen bacteria were determined and the proteinase activities of total rumen contents and particulate matter, protozoal, bacterial and supernatant fractions was measured. Total soluble N and $\text{NH}_3\text{-N}$ concentration in the rumen were also measured. In the second part of the experiment, the proportion of microbial-non ammonia nitrogen (NAN) in whole rumen digesta-NAN was measured by ^{15}N ammonium chloride infusion into the rumen of sheep. The inert liquid phase marker, chromium ethylene diaminetetraacetic acid (Cr-EDTA) and the solid phase marker, natural plant alkanes, were used to estimate the flux of digesta at the abomasum. In both experiments, one group of six sheep received a continuous intraruminal infusion of PEG (PEG sheep; MW 3500; Union Carbide, Danbury, CT,

USA), while the remaining six sheep received an intraruminal infusion of water (CT-acting sheep). PEG preferentially binds CT, preventing it from binding with protein (Jones & Mangan, 1977; Barry & Manley, 1984). Therefore, comparing unsupplemented sheep with sheep given PEG can be used to quantify the effects of CT.

6.3.2 Animals and diets

Twelve six month old Romney sheep with mean live-weight of 32.5 kg (SD 2.25), were prepared with rumen fistulae (63 mm ID) and abomasal canulae (10 mm ID; 'T' piece) approximately 40 days prior to the start of the experiment. All sheep were treated for external parasites (10 ml Wipeout; Coopers Animal Health Ltd, Wellington, NZ), and drenched with anthelmintic to control internal parasites (12 ml Ivomec; Merc Sharp & Dohme (NZ) Ltd) prior to the start of the experiment. The sheep were housed indoors in metabolism crates after surgery and fed freshly harvested perennial ryegrass/white clover pasture (referred to as Past-I) hourly from overhead belt feeders. The sheep were fed the pasture diet for the first 12 days, then *L. corniculatus* for 18 days and finally all sheep were grazed outdoors on perennial ryegrass/white clover pasture for 12 days (referred as Past-II).

While indoors, the animals were offered 1.0 kg DM/sheep/day. One group of six sheep received intraruminal infusion of PEG (100 g/day; dissolved in 300 ml water; infused at 12.5 ml/sheep/h) and the other six sheep received intraruminal infusions of water. This quantity of PEG is sufficient to bind about 70 g CT per day (Barry & Forss 1983). Water was available at all the times. Pasture and *L. corniculatus* were cut daily at 8:30 h. Two thirds of the daily feed was placed on overhead belt feeders at

9:00 h, and the remaining one third held at 4 °C until 17:00 h when it was placed on the belt feeders.

6.3.3 Measurement sequence

The sequence of events during the trial is shown in Table 6.1.

Table 6.1 Time sequence of events during experimental period.

Day	Event
1	Commence PEG infusion until day 31. Feed freshly cut perennial ryegrass/white clover pasture (referred as Past-I) until day 11.
4	Weigh sheep.
5	Record intakes of sheep.
12	Rumen bailing & sampling (Past-I) for ammonia, bacterial population, soluble N & proteinase activity.
12	Change feed from Past-I to <i>L. corniculatus</i> and take samples at 8, 12, 24, 48, 96 & 120 h after starting <i>L. corniculatus</i> feeding for ammonia, bacterial population, soluble N and proteinase activity.
20	Second rumen bailing, fit collection harnesses and attach faeces collection bags.
21	Commence digestion trial. Cr-EDTA infused into the rumen and feeds and feed residual samples from individual sheep collected for CT, alkane, N analysis & DM determination.
24-26	Commence 6-8 hourly abomasal sampling for digesta flow.
27	Finish digestion trial. Commence Group A (3 PEG sheep & 3 water infused sheep) intraruminal ¹⁵ N infusion.
28	Commence Group B (another 3 PEG sheep & 3 water infused sheep) intraruminal ¹⁵ N infusion.
29	Group A sampling (rumen and abomasum for ¹⁵ N at 36, 39 & 42 h after commencing infusion).
30	Group B sampling (rumen and abomasum for ¹⁵ N at 36, 39 & 42 h).
31	All sheep grazed on perennial ryegrass/white clover (referred as Past-II) paddock, without PEG supplementation.
44	Rumen sampling for bacterial populations (Past-II) .

6.3.4 Rumen emptying (bailing)

Rumen emptying was done through a large rumen fistulae (ID 63 mm). All animals were bailed prior to *L. corniculatus* feeding on day 12 (Past-I) at the beginning and at end of feeding *L. corniculatus* on day 20 and day 31 respectively. The rumen contents were emptied into a bucket, kept warm by placing in a water bath (70 °C). The digesta was weighed (to estimate total rumen volume), sampled and returned to the animal. Sub-samples of rumen digesta were taken for determination of DM, DNA extraction for microbial population determination, proteinase activity, total N, total soluble N and ammonia concentrations

6.3.5 *Proteolytic bacterial population and proteinase activities rumen samples*

Rumen samples were collected from all 12 sheep while still on the perennial ryegrass/white clover pasture diet (Past-I). Rumen fluid samples were taken at 8, 12, 24, 48, 96 and 120 h after transfer to the *L. corniculatus* diet (Table 1). The rumen samples were also collected after the animals were grazed outside on a perennial ryegrass/white clover pasture for 12 days (Past-II). Ten ml of the whole rumen sample was diluted with 10 ml mineral salts buffer (Attwood & Reilly 1996), homogenised using an Ultra-Turrax (IKA-Werk, Netzstecker Ziehen) and stored at -80 °C for DNA extraction.

Rumen contents for proteinase assays were taken to the laboratory in a prewarmed (37 °C) container and bulked within each treatment (CT-acting vs. PEG-sheep). The rumen samples were homogenised (Fraction 1), wrapped in two layers of cheese cloth and placed on top of a test tube insert holder inside a centrifuge bucket (MSE Mistral, 6 L, Measuring & Scientific Equipment Ltd, London). The samples were centrifuged at 1,000 x g for 5 min at 20 °C to standardize the amount of liquid which was filtered

from the homogenised rumen contents. The filtrate (Fraction 2) and the residual particulate matter (Fraction 3) were adjusted to their original weights with CO₂ saturated-artificial saliva (McDougall 1948). The filtrate (Fraction 2) was centrifuged at 375 x g for 5 min at 20 °C to sediment the protozoa and small feed particles. The sediment was resuspended in 100 ml of artificial saliva and was called the protozoal fraction (Fraction 4), while the supernatant was centrifuged at 16300 x g for 15 min at room temperature. The resulting pellet was resuspended in 100 ml of artificial saliva and called the bacterial fraction (Fraction 5), while the remaining fluid was called the supernatant fraction (Fraction 6).

Rumen samples for total N determination were freeze dried and stored for chemical analysis. For soluble N, rumen contents were centrifuged at 10,000 x g for 30 min, the clear supernatant was taken and stored at -20 °C for analysis (Mangan & West 1977). Strained rumen fluid was analyzed for ammonia as described by Min *et al.* (1997).

6.3.6 Proteinase enzyme assays

Proteinase activity was assayed using azocasein (Sigma Chemical Co, St. Louis, Mo) as substrate, at a final concentration of 1 % (wt/vol; 1 mg/100 µl) in 0.1 M mineral salts buffer (pH 6.8; Brock *et al.* 1982; Attwood & Reilly 1995). The assays were performed in triplicate at 39 °C in CO₂-saturated buffer after a 2 h incubation. Reactions were stopped by the addition of an equal volume of 10 % (wt/vol) trichloroacetic acid (TCA). After incubation on ice for 30 min, the tubes were centrifuged at 27,000 x g for 10 min at 4 °C. One ml of the supernatant fluid was removed from each tube and mixed with 1 ml of 1M NaOH, and the absorbance of

the mixture was measured at 450 nm. Samples in which substrate was added at the end of the incubation period after the addition of TCA acted as controls.

6.3.7 Bacterial strains and growth.

The rumen proteolytic bacterial strains *Streptococcus bovis* B315, *Clostridium proteoclasticum* B316, *Butyrivibrio fibrisolvens* C211a and *Eubacterium* sp. C12b were obtained from the Rumen Microbiology Unit, Nutrition and Behaviour Group, AgResearch, Grasslands Research Centre, Palmerston North, New Zealand. All bacterial strains were grown in CC medium (Leedle & Hespell 1980) as modified by Attwood and Reilly (1995). Bacterial cells were grown in 100 ml overnight cultures and samples were counted by phase contrast microscopy using a counting chamber (Weber Scientific International Ltd., Middlesex, England). Cells were pelleted by centrifugation and resuspended to a final concentration of 1×10^{10} cells per ml and their DNA extracted using a bead-beating technique described previously (Reilly & Attwood 1998). Ten fold serial dilutions of this DNA were amplified to determine the detection limit.

6.3.8 DNA extraction.

The extraction of DNA from rumen samples was performed using a modified cetyltrimethylammonium bromide (CTAB) method (Johnson 1991). One ml of the diluted and homogenized rumen samples were added to 1.2 g of sterile zirconia beads (Biospec Products Ltd., OK., USA) and centrifuged at 12,000 x g for 10 min at 20 °C. The supernatant was removed and the sample plus beads were resuspended in 500 µl of 2x CTAB lysing buffer (100 mM Tris-hydrochloride (pH 8.0); 1.4 M NaCl; 20 mM EDTA; 2 % (wt/vol) CTAB; 2% (vol/vol) 2-mercaptoethanol). The samples

were shaken for 5 min, then physically disrupted using a Mini-beadbeater (Biospec Products) at maximum speed for two intervals of 2 min each, with a 1 min incubation on ice between each treatment. The bead-beaten mixture was centrifuged at 12,000 x g for 10 min at 20 °C and the upper aqueous phase was recovered. The interface layer was re-extracted with 1 x CTAB buffer and the resulting upper aqueous phase pooled with the first aqueous phase. Proteinase K was added to a final concentration of 50 µg/ml and the mixture was incubated for 1 hour at 60 °C. A final chloroform-isoamyl alcohol (24:1; wt/vol) extraction was performed before the nucleic acids were precipitated with an equal volume of CTAB-precipitation buffer (50 mM Tris-hydrochloride; 10 mM EDTA and 1 % (wt/vol) CTAB). The precipitate was recovered by centrifugation at 8,000 x g for 10 min at room temperature. The pellet was dispersed with a pipette tip and washed 3 times with 500 µl of cold 0.4 M NaCl. The DNA pellet was dissolved in 100 µl of 2.5 M ammonium acetate at 50 °C. RNase A was added to a final concentration of 1 mg/ml and the tubes incubated at 37 °C for 30 min. RNase A was removed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction followed by ethanol precipitation and centrifugation at 12,000 x g for 20 min at 4 °C. The air-dried DNA pellet was resuspended in 100 µl of sterile water and was stored at -20 °C until required for cPCR.

6.3.9 Polymerase chain reactions

Both specific PCR primers and internal control DNA for *C. proteoclasticum* B316, *S. bovis* B 315, *B. fibrisolvens* C211a and *Eubacterium* sp. C12b have been described previously (Attwood & Klieve 1999).

PCRs contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer and 0.5 U of *Taq* DNA polymerase (Gibco BRL, Auckland, New Zealand). PCR reactions were in a final volume of 20 μl and sealed in a capillary tip and thermocycled using a Corbett FTS-1 Capillary Thermal Sequencer (Corbett Research, Australia). PCR amplification conditions for B316 830, B315 454, C211a 458 and C12b 744 with fD1* primers were denaturation at 95 °C for 3 min, followed by 6 cycles of 95 °C for 30s, 62 °C for 15s, 72 °C for 30s and 25 cycles of 95 °C for 15s, 62 °C for 5s and 72 °C for 30s and a final cycle of 72 °C for 3 min (Reilly & Attwood 1998). Amplification with the universal primers, fD1* and rD1* differed only in annealing temperature, which was 55 °C. PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide and visualized by UV trans-illumination.

6.3.10 Quantitation of PCR products

PCR products were quantitated by photographing agarose gels using Polaroid film (St. Albans, Hertfordshire, England) which produces a negative image of the photograph. The negative was scanned using a GS-670 Imaging Densitometer (BioRad, Hercules, CA, USA). The relative amplification efficiencies of target and internal control DNAs were determined from the co-amplification of target DNA from 1×10^3 bacterial cells and dilution of internal control DNA (Reilly & Attwood 1998). The optimal concentrations of internal control DNA were used to construct standard curves (Figs. 1, 2, 3 and 4) by amplification with target DNA extracted from a known number of cells. PCR products were quantitated by comparing the amount of amplified target DNA with the amount of amplified internal control DNA using the following equation (11):

$$\log (Nn_1/Nn_2) = \log (No_1/No_2) + n \log (eff_1/eff_2) \quad (11)$$

Where eff_1 and eff_2 are the amplification efficiencies of target and internal control respectively, n is the number of cycles, and Nn_1/Nn_2 and No_1/No_2 are the ration of amplified products and initial templates (starting products) respectively (Zachar *et al.* 1993).

6.3.11 Marker infusion procedure

The inert liquid phase marker, Cr-EDTA, and the solid phase marker, plant alkanes were used for abomasal flow rate determination. The Cr-EDTA was prepared following the method of McNabb *et al.* (1993). The Cr-EDTA was made up to 10 litres with a final Cr concentration of 2 mg/g of solution. The Cr-EDTA and PEG were infused together at a rate of 17.46 mg/h using a peristaltic pump (PLG-multipurpose pump, Desaga Heidelberg, German Federal Republic), fitted with silicone rubber tubing. The Cr-EDTA marker was infused at a constant rate into the rumen via the rumen cannula for 6 days.

Flux of microbial NAN at the abomasum was determined by infusing ^{15}N ammonium chloride into the rumen of each sheep for 42 h (Waghorn *et al.* 1994a). The ammonium chloride, containing 93 atoms $^{15}\text{N}/100$ atoms N, was dissolved in water (290 mg $\text{NH}_4\text{-N/l}$) and infused into the rumen at 12.1 ml/h so that each sheep received approximately 65 mg $^{15}\text{N}/24\text{h}$.

6.3.12 Nitrogen digestion and sampling

Sheep were fitted with harnesses on day 20 so that faecal collection bags could be attached to measure digestibility. During the digestibility period (Table 1), faeces were

weighed daily and sampled for DM determination (100 g) and for N analysis (10 % wet weight). Urine was channeled down a plastic chute into buckets containing 100 ml of 50 % (vol:vol) HCl to reduce ammonia loss (pH < 3). The urine was weighed and samples (10 % by volume) frozen for N determination so that N retention could be calculated. Both faecal and urine samples for analysis were frozen and accumulated for each animal during each collection period. Feeds and feed refusals were collected from individual sheep and weighed; samples were frozen (-20 °C) for CT, N and alkane analyses and DM was determined.

N digestion was determined by measuring digesta flow at the abomasum between days 21 and 26 of the experimental period. The first 3 days were allowed for marker equilibrium with the digesta, then samples of abomasal digesta were collected during the subsequent 3 days (Binnerts *et al.* 1968).

Abomasal digesta samples (50 ml) were collected at the following times spread over a 3 day period: 08.00, 14.00, 20.00, 02.00, 10.00, 16.00, 22.00, 04.00, 12.00, 18.00, 24.00 and 06.00 h, beginning on day 24. The samples were bulked for determination of Cr and alkane concentration, to measure flow of total DM, N and nitrogen kinetics.

6.3.13 Flux of microbial NAN

Two hundred grams of rumen and abomasal contents for chemical analyses were collected and passed through two layers of cheese cloth (Waghorn *et al.* 1994a). The contents of rumen and abomasum were collected for ¹⁵N analyses which were taken as follows: 100 g rumen and abomasal contents for NAN enrichment and 300 g rumen and abomasal contents for microbial N enrichment. All samples were stored at -20 °C, except those used for microbial determinations. Rumen and abomasal microbes were

isolated by centrifuging at 300 x g for 5 min to remove feed particulate material and lysed protozoa. The supernatant was decanted, centrifuged (30,000 x g, 30 min), the pellet was resuspended in 100 ml ice-cold physiological saline and again centrifuged twice (30,000 x g, 30 min), after which the pellet was frozen prior to analysis.

6.3.14 Chemical analyses

Dry matter was determined after oven drying at 85 °C for 24 hours (feeds, feed refusals) or 48 hours (faeces, digesta samples). Feed, feed refusals, faeces and digesta samples were freeze-dried and ground to pass through a 1 mm sieve prior to analysis.

Abomasal samples from each sheep were bulked on an equal weight basis for the determination of digesta flow rate using Cr-EDTA and alkanes. For Cr analysis, freeze-dried and ground whole abomasal digesta samples (0.5 g) were digested in concentrated nitric acid at 40 °C for 96 h and dried at 100 °C for 24 h. The samples were re-dissolved in 10 ml of 2M HCl and stored at -20 °C prior to Cr analysis (Grace 1983). Strained digesta samples were centrifuged at 3,000 x g for 30 minutes and 8 ml of the supernatant added to 1.8 ml of concentration HCl and stored at -20 °C for Cr analysis. Chromium concentrations in digesta samples and their supernatants were determined by Inductively Coupled Argon Plasma Spectrometry (ICAPS; Lee 1983). Alkane concentrations in freeze-dried digesta samples and their supernatant were determined using Gas Chromatography (GC; Hewlett Packard 5, 95, USA) fitted with a HP1 Hewlett Packard column as described by Dove (1993).

Samples of rumen and abomasal digesta and bacteria for ¹⁵N-enrichment were determined at the Waikato Stable Isotope Unit (WSIU), New Zealand, using a Dumas elemental analyzer (Europa Scientific ANCA-SL, Crewe, U.K.) interfaced to a isotope mass spectrometry (Europa Scientific Ltd, Crewe, U.K.). The N in feed, feed refusals,

faeces and digesta samples were determined following the Kjeldahl digestion (Williams & Twine 1967), while ammonia concentration in rumen fluid was determined using the method of Chaney and Marbach (1962). CT was determined as extractable, protein-bound and fibre-bound fractions using the butanol-HCl procedure (Terrill *et al.* 1992).

6.3.15 Calculation of data

¹⁵N enrichment values in rumen bacteria and rumen digesta samples, after 42h of infusion, were used in all calculations. Calculations of N partitioning between microbial and feed pools, and fluxes to the abomasum are based on ¹⁵N-enrichment of appropriate pools (Nolan & Leng 1972; Nolan 1975; Waghorn *et al.* 1994).

6.3.16 Statistical analysis

All comparisons between treatments were made using the General Linear Model (GLM) of the SAS program (SAS 1985). Initial perennial ryegrass/white clover pasture (Past-I) data and nitrogen intake for N balance were used as covariates.

Fitted regression lines were used to compare rate of change with time (8-120 h) and difference between treatments (PEG-infused *vs.* CT-acting sheep) for individual proteolytic bacterial populations, concentrations of rumen ammonia and soluble N, rumen pH and proteinase activity; there were analysed using a Least-Squares Estimation (Proc REG) of the SAS program (SAS 1985).

6.4 RESULTS

6.4.1 Standard curves and sensitivity testing

The results of the standard curve constructions for *C. proteoclasticum* B316, *S. bovis* B 315, *B. fibrisolvens* C211a and *Eubacterium* sp. C12b are illustrated in Figs 6.1, 6.2, 6.3, and 6.4. The intensities of DNA from *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315, *B. fibrisolvens* C211a cells gave linear responses ($R^2 = 0.96$ to 0.99) from 6.7×10^3 to 1.0×10^7 and could be used for quantitation of samples which fell within this range (Figs. 6.1, 6.2, 6.3 & 6.4).

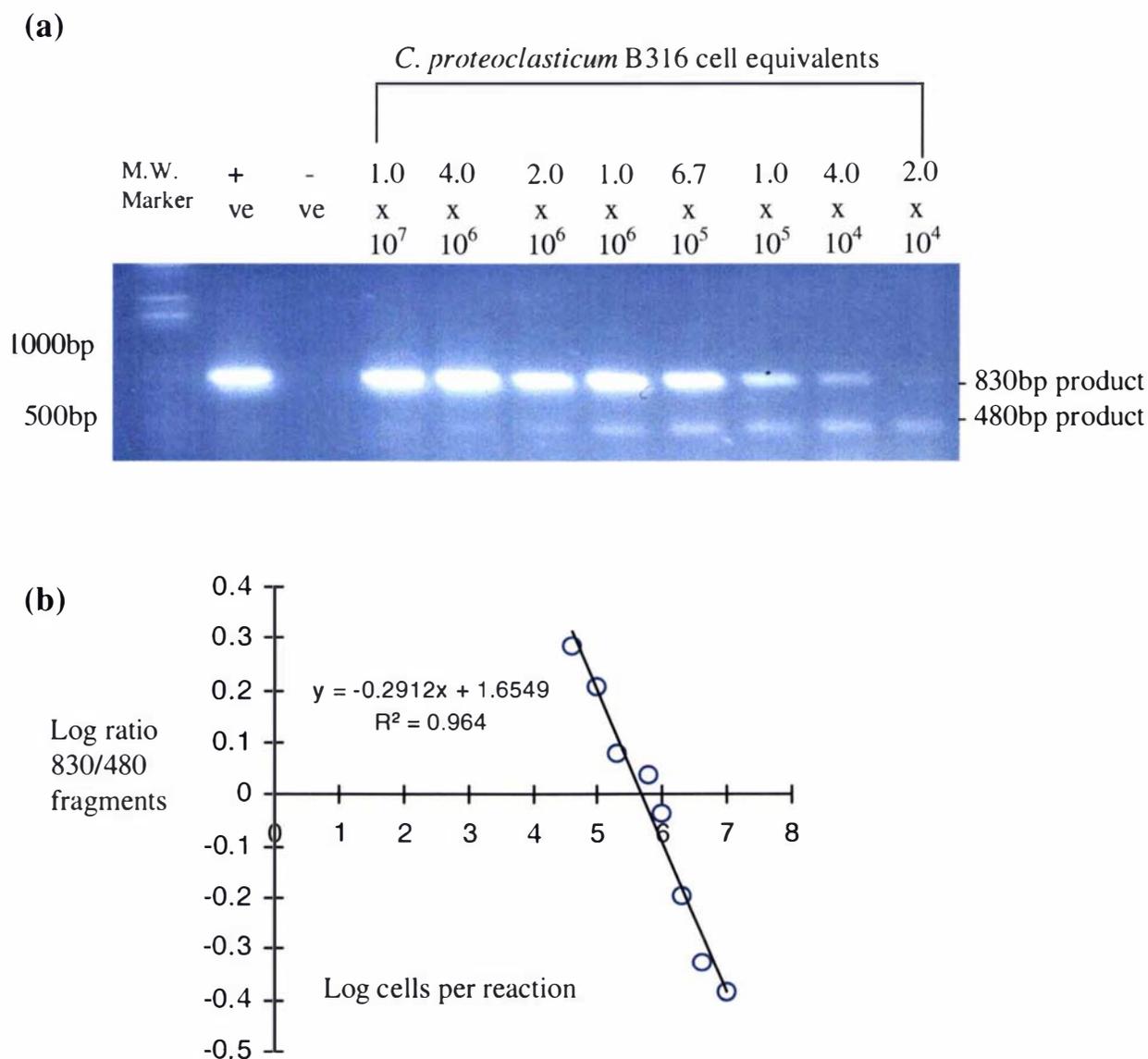


Figure 6.1 Standard curve construction; a) DNA extracted from 1×10^{10} *C. proteoclasticum* B316 cells/ml was serially diluted and co-amplified with a 1×10^9 dilution of the internal control. b) Ratios of the intensities of internal control to target DNA were quantified by scanning densitometry and plotted on a log scale.

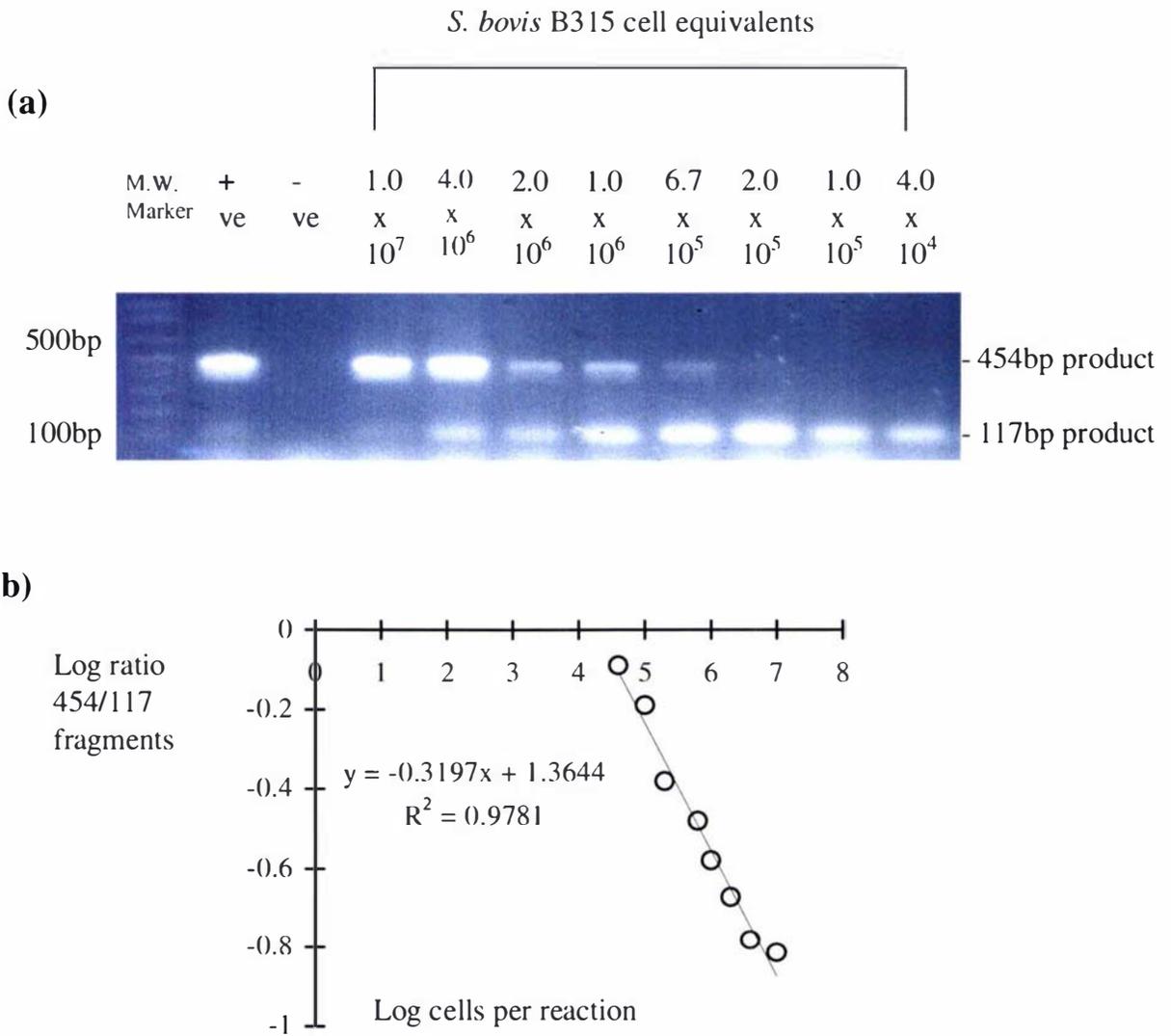


Figure 6.2 Standard curve construction; a) DNA extracted from 1×10^{10} *S. bovis* B315 cells/ml was serially diluted and co-amplified with a 1×10^9 dilution of the internal control. b) Ratios of the intensities of internal control to target DNA were quantified by scanning densitometry and plotted on a log scale.

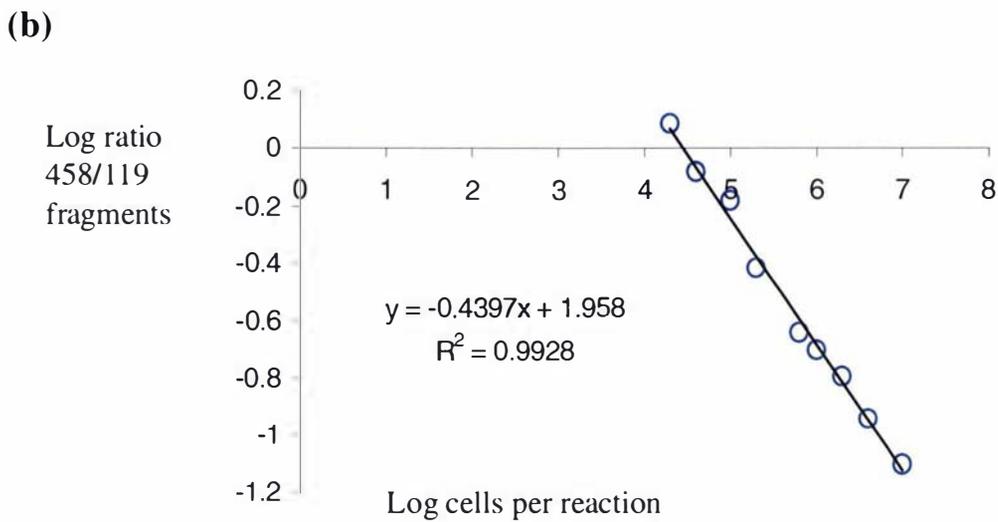
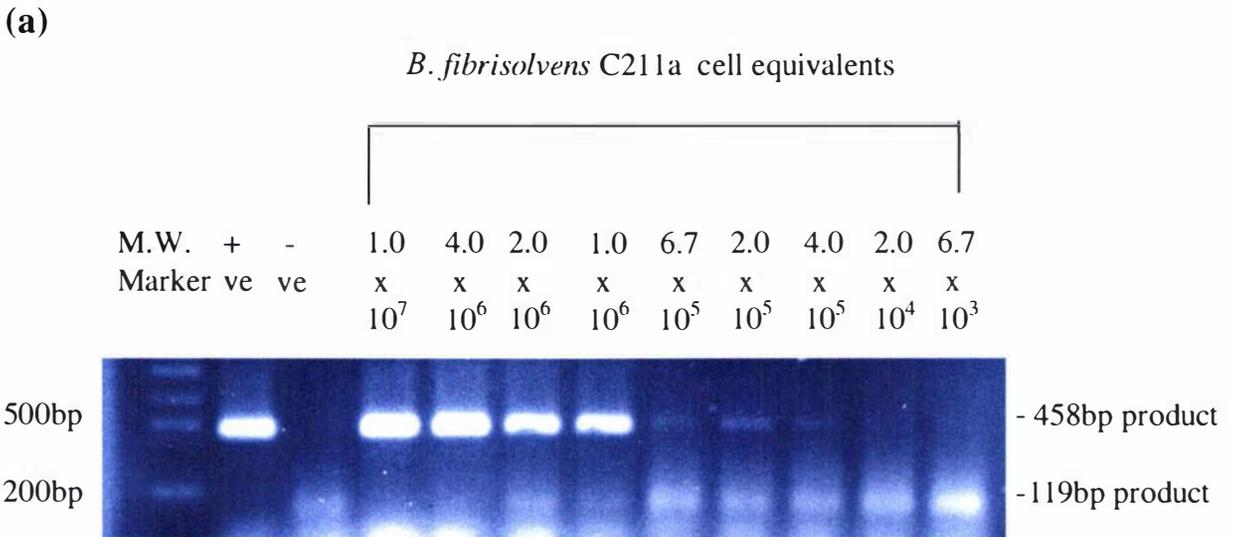


Figure 6.3 Standard curve construction; a) DNA extracted from 1×10^{10} *B. fibrisolvens* C211a cells/ml was serially diluted and co-amplified with a 1×10^7 dilution of the internal control. b) Ratios of the intensities of internal control to target DNA were quantified by scanning densitometry and plotted on a log scale.

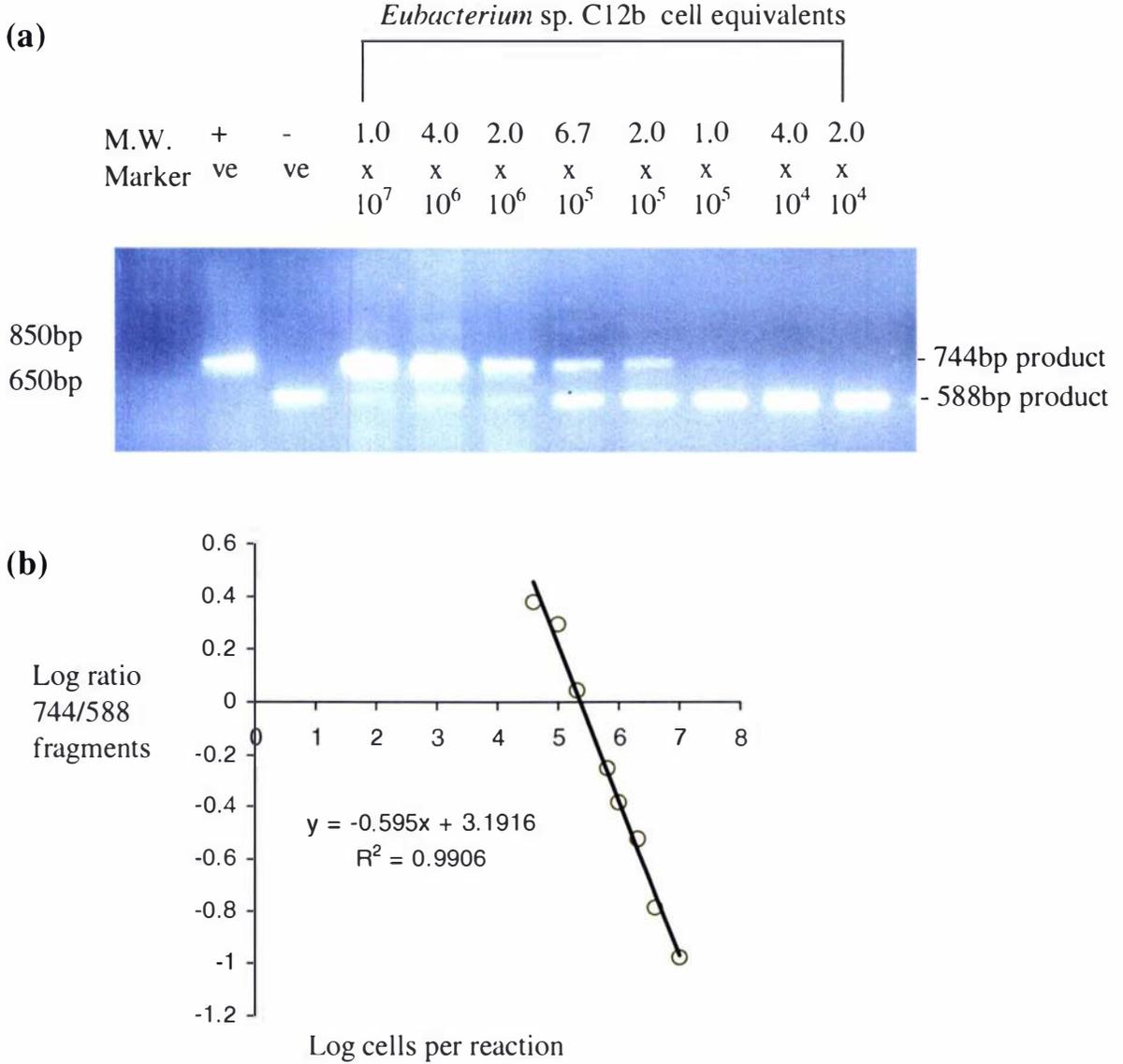


Figure 6.4 Standard curve construction; a) DNA extracted from 1×10^{10} *Eubacterium* sp. C12b cells/ml was serially diluted and co-amplified with a 1×10^6 dilution of the internal control. b) Ratios of the intensities of internal control to target DNA were quantified by scanning densitometry and plotted on a log scale.

6.4.2 Detection of proteolytic rumen bacteria *in vivo*.

The cPCR assay was used to enumerate four proteolytic bacterial populations directly from rumen samples (Fig. 6.5). During perennial ryegrass/white clover (Past-I) feeding, the populations of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvans* C211a were 1.6×10^8 , 2.7×10^8 , 7.1×10^6 and 1.2×10^6 respectively. When the diet changed from pasture (Past-I) to *L. corniculatus*, the average populations (8 to 120 h) of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvans* C211a decreased significantly ($P < 0.001$) to 5.1×10^7 , 1.5×10^8 , 2.6×10^6 and 1.0×10^6 , respectively.

Animals fed on the *L. corniculatus* diet, in the absence of PEG, had populations (8-120 h) of *C. proteoclasticum* B316, *Eubacterium* sp. C12b, *S. bovis* B315 and *B. fibrisolvans* C211a of 3.4×10^7 , 1.2×10^8 , 2.6×10^5 and 1.1×10^6 , respectively. Animals fed *L. corniculatus* plus PEG, had significantly higher ($P < 0.01-0.001$) populations of the same strains; 6.9×10^7 , 1.9×10^8 , 1.9×10^6 and 4.0×10^6 , respectively. Overall comparisons using separate fitted lines for PEG-infused and CT-acting sheep showed that the intercept values were significantly different ($P < 0.012-0.001$) between treatments.

After *L. corniculatus* feeding the sheep were transferred outside and allowed to graze a perennial ryegrass/white clover pasture (Past-II) for 15 days. The sheep were re-sampled in order to evaluate the effect of reversing the diet on the populations of proteolytic bacteria. Significant differences were still detected in populations of *C. proteoclasticum* B316 ($P < 0.05$) and *B. fibrisolvans* C211a ($P < 0.0001$) between treatment groups, but smaller differences were present in populations of *S. bovis* B315 ($P = 0.09$) and *Eubacterium* sp. C12b ($P > 0.05$).

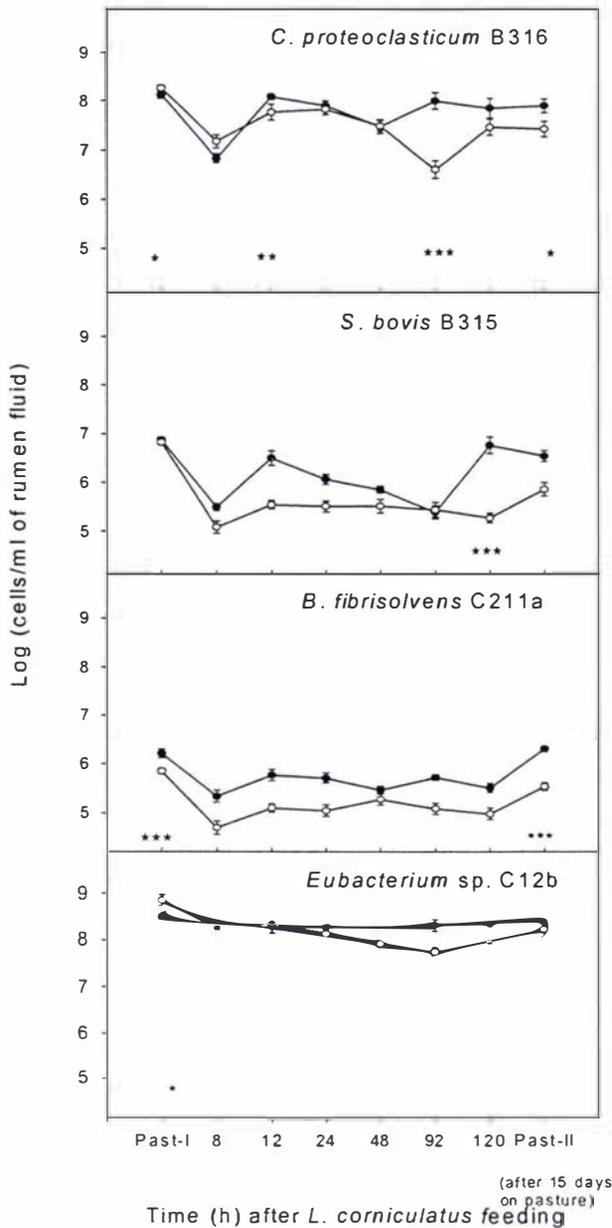


Figure 6.5 cPCR quantitation of proteolytic bacterial populations *in vivo*. The sheep were fed perennial ryegrass/white clover pasture during the first 12 days (referred as Past-I) and then transferred to *L. corniculatus* as the sole diet; -O- CT-acting; -●- PEG supplemented sheep (CT-inactivated). Rumen samples were taken at -1 h (Past-I) and 8, 12, 24, 48, 96 and 120 h after *L. corniculatus* feeding commenced. After *L. corniculatus* feeding, all sheep were transferred to grazing perennial ryegrass/white clover pasture for 15 days (Past-II) and the last rumen samples taken (referred as Past-II). Results are the mean of triplicate determinations and error bars represent standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.4.3 Rumen ammonia, soluble N and rumen pH

The concentrations of rumen ammonia, soluble N and pH were measured in sheep during feeding of the two different diets (Past-I vs *L. corniculatus*) under the two treatments (PEG-infused sheep vs CT-acting sheep; Fig. 6.6). When diets changed from Past-I to *L. corniculatus* (average of 8-120h), average rumen ammonia concentrations decreased significantly (171 vs 133 mg/l; $P < 0.001$). When PEG was infused intraruminally to animals fed the *L. corniculatus* diet, the ammonia concentrations generally increased compared to the CT-acting group ($P < 0.001$). When analyzed with fitted lines to each treatment, there was a significant ($P < 0.001$) difference between the PEG and CT-acting group in the intercept values.

Rumen total soluble N concentrations (0.66 vs 0.39 mg/ml) and pH (7.13 vs 6.84) dropped significantly ($P < 0.001$) when diets changed from Past-I to *L. corniculatus* (average of 8-120 h). No difference in pH was detected in the presence or absence of PEG, while the average soluble N concentration was significantly lower (0.45 vs 0.34 mg/ml; $P < 0.001$) in sheep fed *L. corniculatus* compared with those fed *L. corniculatus* plus PEG. When treatments were analyzed using fitted lines, there was a significant difference between the PEG-infused and CT-acting sheep for soluble N ($P < 0.001$) in the intercept values, but not for pH.

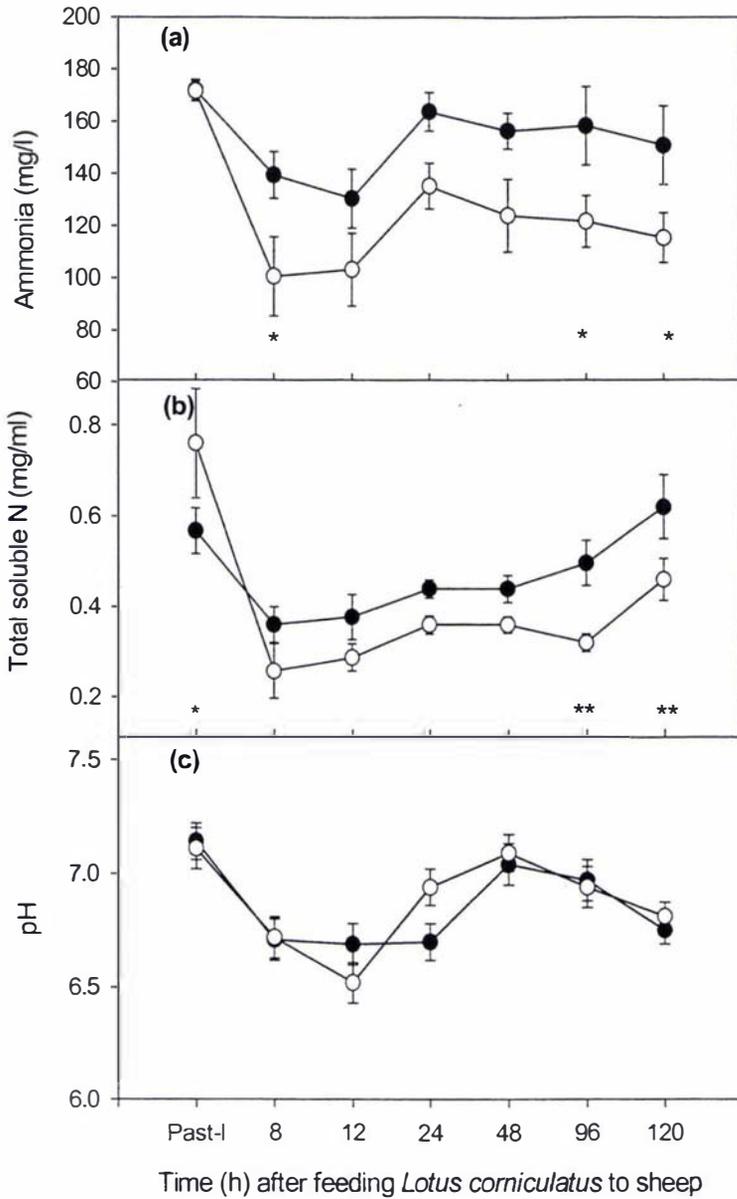


Figure 6.6 The effect of CT on the ruminal concentrations of (a) ammonia, (b) soluble N and (c) pH in sheep fed fresh *L. corniculatus*; -O- CT-acting; -●-PEG-supplemented sheep (CT-inactivated). The sheep were fed perennial ryegrass/white clover (Past-I) pasture during the first 12 days and then transferred to *L. corniculatus* as a sole diet. Results are the means of triplicate determinations and error bars represent standard error of the mean. * $P < 0.05$, ** $P < 0.01$.

6.4.4 Proteinase activity

Proteolytic enzyme activities of the mixed rumen microorganisms in sheep were assayed using azocasein as the substrate (Fig. 6.7). When diets changed from Past-I to *L. corniculatus* (average of 8-120 h), average proteinase activity of six fractions decreased significantly (366 vs 164 μg azocasein hydrolysed/h), but increased significantly when the diet changed from *L. corniculatus* to pasture (Past-II; 164 vs 239.2 μg azocasein hydrolysed/h). Among the six rumen fluid fractions assayed from sheep fed *L. corniculatus* (average of 8-120 h), fraction 1 (229 μg azocasein hydrolysed/h) had significantly higher activity ($P < 0.01-0.001$) compared to the other fractions (from 85 to 187 μg azocasein hydrolysed/h). Proteinase activity in the filtrate (fraction 2), particulate matter (fraction 3), protozoal (fraction 4) and bacterial fractions (fraction 5) were relatively similar, but the activity detected in the supernatant fraction (fraction 6) was consistently lower than other fractions. When PEG was infused intraruminally into sheep fed *L. corniculatus* (8-120 h), the proteinase activity overall increased ($P < 0.05-0.001$) compared to the CT-acting group. For rumen proteinase activity, there were no significant responses to PEG in samples taken at 4 to 12 h from animals fed *L. corniculatus*, but there were significant ($P < 0.05-0.001$) responses to PEG after 12 and 24h compared to the CT-acting group except for fraction 2.

Statistical analyses using fitted lines to compare between the two treatments suggested that proteinase activity in whole rumen fluid (fraction 1), filtrate, particulate matter, bacterial, protozoal and supernatant fractions were significantly higher ($P < 0.01-0.001$) in PEG sheep than the CT-acting group in the intercept values.

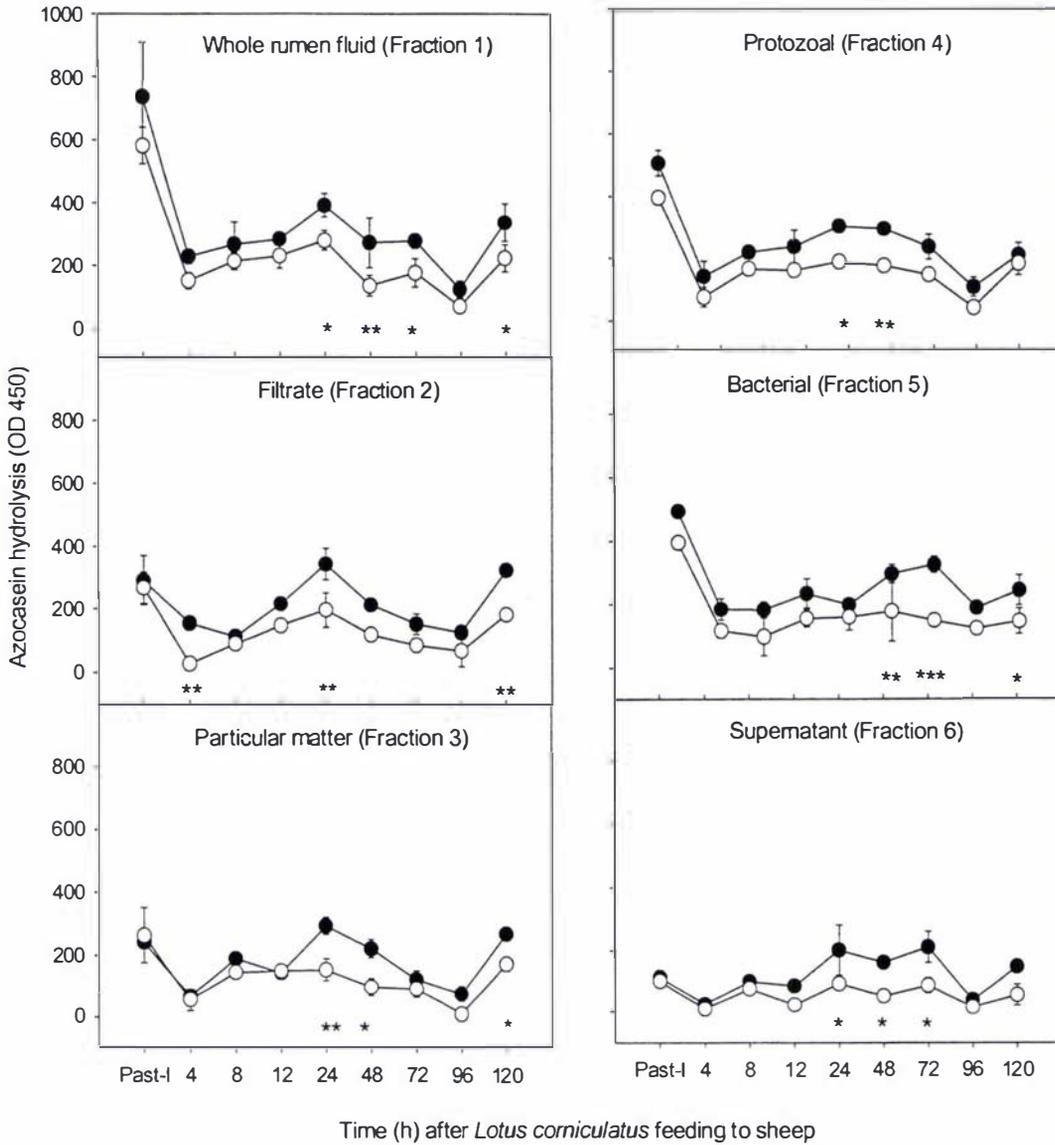


Figure 6.7 Hydrolysis of azocasein by whole and fractionated samples of rumen contents prepared from sheep fed *L. corniculatus*; -O-CT-acting sheep; -●- PEG-supplemented sheep (CT-inactivated). The sheep were fed perennial ryegrass/white clover (Past-I) pasture during the first 12 days (0 h) and were then transferred to *L. corniculatus* as a sole diet. Results are the mean of triplicate determinations and error bars represent standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.4.5 Nitrogen digestion

Nitrogen (N) and dry matter (DM) intake, and site of digestion in sheep fed *L. corniculatus* with and without an intraruminal infusion of PEG are presented in Table 6.2. Nitrogen and DM intake, N balance and DM digestibility were similar in CT-acting sheep and PEG-infused sheep. However, N digestibility was lower ($P < 0.05$) in CT-acting sheep than PEG-infused sheep.

Table 6.2 Nitrogen (N) and dry matter intake (DMI), and ~~site of~~ ^{of DM and N} digestion in sheep fed *L. corniculatus* with and without an intraruminal infusion of polyethylene glycol (PEG).

	- PEG (N = 6)	+ PEG (n = 6)	S.E.M. (D.F. = 10)	P value
Intake (g/day)				
N intake	30.1	28.2	2.48	NS
DMI	811	761	68.5	NS
N balance (g/day)*	0.77	0.79	0.01	NS
Digestibility (%) [†]				
Dry matter [‡]	0.67	0.62	0.060	NS
N	0.72	0.80	0.024	$P < 0.05$

* Data was adjusted using initial N intake as covariate.

† Data was adjusted using initial DM intake or N intake as covariate.

‡ PEG was deducted from the data.

6.4.6 ^{15}N -kinetics

Nitrogen fluxes to the abomasum of sheep used in the present study are presented in Table 6.3. In the rumen, non-ammonia N (NAN) and microbial NAN pools were similar in the both treatments group, but the CT-acting group had a lower ammonia pool ($P < 0.01$) than in PEG-infused sheep. In the abomasum, microbial NAN fluxes were similar for both treatments, but the CT-acting group had a lower ammonia flux and a greater non-microbial NAN flux ($P < 0.01$) than PEG-sheep (Table 6.3). The rumen microbial N turnover rate was similar for both treatments.

Table 6.3 Nitrogen dynamics (based on ^{15}N enrichment) in sheep fed *Lotus corniculatus* with and without an intraruminal infusion of polyethylene glycol (PEG)

	CT-acting sheep (n = 5)	PEG-sheep (n = 6)	S.E.M (D.F.=9)	P value
Rumen pools (g)				
NH ₃ -N	0.4	0.6	0.04	$P < 0.01$
Non-microbial NAN	10.6	10.3	1.18	NS
Microbial NAN	5.3	3.9	1.32	NS
Total NAN	15.9	14.2	2.35	NS
Abomasal fluxes (g/day)*				
NH ₃ -N	0.5	0.8	0.06	$P < 0.01$
Non-microbial NAN	21.0	15.9	1.00	$P < 0.01$
Microbial NAN	10.4	10.2	0.77	NS
Total NAN	31.4	26.1	1.73	$P < 0.05$
Rumen microbial N turnover (per day)				
	2.5	2.9	0.54	NS

All data was adjusted using initial nitrogen intake as covariate.

* PEG was deducted from the data.

6.5 DISCUSSION

There is strong evidence that when forages containing CT are fed to sheep a significant reduction in proteolysis of plant protein and in the concentration of ammonia in rumen fluid occurs relative to similar forages without CT (Waghorn *et al.* 1987a, McNabb *et al.* 1993). The observed change in N metabolism in the rumen (such as reduced N digestibility and decreased ammonia, soluble N, proteinase activity and proteolytic bacterial populations), together with the higher flux of total NAN to the abomasum of CT-acting sheep compared to the PEG infused sheep reported in this study indicated that CT in *L. corniculatus* protects soluble plant protein in the rumen and enhances NAN flow to the abomasum.

Polyphenolic compounds in plants serve as a natural defense mechanism against microbial infections (Bate-Smith & Swain 1962). The antimicrobial activity of tannins has been well documented. Fungi, various yeasts, bacteria and viruses have been inhibited by tannins (Henis *et al.* 1964; Scalbert 1991; Chung *et al.* 1993). Furthermore, CT in the diet reduced the growth of several rumen bacterial species, including *S. bovis*, *B. fibrisolvans*, *Fibrobacter succinogenes*, *P. ruminicola* and *Ruminobactor amylophilus* (Bae *et al.* 1993; McAllister *et al.* 1993; Jones *et al.* 1994). CT can, perhaps, theoretically serve as natural regulators of microbial populations in different habitats (Chung *et al.* 1998).

Using a cPCR technique, results presented here have shown that changing the diet from Past-I to the *L. corniculatus* reduces the populations of several proteolytic bacteria occur. Furthermore, due to the action of CT in the *L. corniculatus* diet, the populations of proteolytic bacterial strains *S. bovis* B315 (51 %), *B. fibrisolvans*

C211a (72 %), *C. proteoclasticum* B316 (51 %) and *Eubacterium* sp. C12b (34 %) were also reduced compared to PEG-infused sheep. This *in vivo* study is the first to show that CT in *L. corniculatus* has the ability to modify individual proteolytic bacterial populations in the rumen of sheep. Several possible explanations of those effects can be proposed which are mainly due to CT-protein or CT-bacterial cell complexing (Jones *et al.* 1994; Barry & McNabb 1999). During the process of proteolysis in the rumen, soluble plant proteins adsorb to the surface of proteolytic bacteria (Wallace 1985) and are hydrolyzed by cell-associated proteases (Wallace & Brammall 1985). When CT-containing forage is masticated, insoluble CT-substrate complexes are formed due to hydrogen bonds and hydrophobic interactions (Haslam 1989, 1996; Jones & Mangan 1977; Jones *et al.* 1993). CT can also become bound to rumen bacterial cell coat polymers and induce changes in morphology of several bacterial species (Jones *et al.* 1994). CT can also reduce the absorption of soluble plant protein to the bacteria cell surface and decrease proteinase activity (Jones *et al.* 1993, 1994). These mechanisms are presumably involved in the reduction of proteolysis and reduced proteolytic rumen bacterial populations caused by CT in the rumen of sheep. However, the exact interactions between CT:bacterial cells and rumen digesta components (such as protein) in the rumen are not clear and these require further study.

Rumen samples collected from sheep fed the Past-I or *L. corniculatus* diet showed that the populations of *C. proteoclasticum* B316 were 1.6×10^8 and 5.1×10^7 , respectively. These findings support the original studies in which *C. proteoclasticum* was isolated from a 10^8 dilution of rumen contents from cattle, fed a fresh ryegrass/white clover diet (Attwood *et al.* 1996). Subsequent work with dairy cows fed four different N diets reported that *C. proteoclasticum* was present at 2.01×10^6 to

3.12×10^7 per ml of rumen contents (Reilly & Attwood, 1998). Attwood and Reilly (1995) and Attwood *et al.* (1996) also found that *C. proteoclasticum* had a high chymotrypsin-like proteinase activity and strong casein hydrolysing activity. Furthermore, *in vitro* (Chapter 5) and *in vivo* studies reported here have found that *C. proteoclasticum* B316 appeared to be least affected by *L. corniculatus* CT compared with other proteolytic bacterial strains. Taken together with previous studies, it appears that *C. proteoclasticum* is a common proteolytic organism in New Zealand ruminants grazing fresh forages.

Previous studies have shown that *S. bovis* isolates represented 61 % of the 43 proteolytic isolates characterized from cattle grazing ryegrass/white clover pasture (Attwood & Reilly 1996). While *S. bovis* does not have high proteinase activity, it does have a high leucine aminopeptidase activity in the rumen (Wallace & Brammall 1985). This low proteolytic, but high leucine aminopeptidase activity may explain the organism's ability to take up amino acids but not peptides (Ling & Armstead 1995). *S. bovis* was also the most rapidly acting rumen amylolytic bacteria in the cereal diets tested by McAllister *et al.* (1990). In Chapter 5, *S. bovis* cultures grown with Rubisco protein extracted from white clover appeared to be highly active at degrading large subunit Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39). However, in the presence of *L. corniculatus* CT, the growth and degradation of Rubisco by this bacteria were significantly reduced. These findings support the present study in which *S. bovis* B315 was the proteolytic organism largely effected by CT (92 %) in the rumen when compared to the PEG-infused sheep. Relatively low populations of *S. bovis* B315 were detected in the present study compared with *C. proteoclasticum* B316 and *Eubacterium* sp. C12b when sheep were fed *L. corniculatus*.

It has been found that the flow of NAN entering the duodenum was highly correlated to insoluble N or to total N intake and to digestible organic matter intake (ARC 1984). The daily flow of NAN to the intestine of sheep relative to N intake is higher when fed *L. corniculatus* than forages without CT (Waghorn *et al.* 1987); this advantage increased as dietary CT concentration increased. Our results are similar to the results of other studies on lotus cultivars and probably indicate that more N fractions are available for absorption from the small intestine for use in anabolic reactions in the animal (Waghorn *et al.* 1987; Waghorn *et al.* 1994; Wang *et al.* 1994).

The effects of CT on total microbial protein flow to the intestine are variable. Previous research has found that there is a higher duodenal microbial protein flow in sheep fed a CT-containing forage diet (sainfoin) than the CT-free diet (red clover; Beaver & Siddons, 1985). A recent *in vitro* study has found that sainfoin (110 g CT/kg DM) had a much greater ^{15}N microbial mass enrichment than lucerne alone (McMahon *et al.* 1999). In addition, Waghorn *et al.* (1994b) reported a small reduction in microbial N flow and an increase in plant protein flow to the small intestine due to CT in sheep fed fresh *L. pedunculatus* (55 g CT/kg DM). Furthermore, CT in *L. pedunculatus* did not affect rumen microbial NAN pool size (Waghorn *et al.* 1994b). This is similar to the results of the present study on *L. corniculatus* (32 g CT/kg DM) and indicates that CT reduced the growth of individual bacteria rather than total microbial protein synthesis in the rumen.

Previous studies have shown that addition of purified CT from *L. corniculatus* or sainfoin to bacterial cultures resulted in a substantial reduction in proteinase and endoglucanase (cellulase) activity (Bae *et al.* 1993; Jones *et al.* 1994; McMahon *et al.* 1999). In addition, CT in sainfoin did not affect xylanase activity but proteinase and endoglucanase activities decreased significantly with increasing amount of sainfoin in

the diet compared with lucerne (McMahon *et al.* 1999). Furthermore, the extracellular cellulases of *F. succinogenes* S-85 were more susceptible to inhibition by CT from *L. corniculatus* than the cell-associated cellulases and were completely inactivated at concentrations greater than 400 µg CT/ml (Bae *et al.* 1993). In the present study, the proteinase activities detected in rumen fluid during feeding of *L. corniculatus* ~~was~~ ^{was} generally lower in CT-acting compared to PEG-infused sheep. The alteration in proteinase activity may contribute to the beneficial effect of CT on forage protein digestion in ruminants.

This study has shown that feeding the CT-containing legume *L. corniculatus* can protect soluble plant protein in the rumen and increases non-microbial NAN and total NAN flows to the abomasum. Some rumen proteolytic bacterial populations and protease enzyme activities are affected by CT, but these did not lead to reductions in rumen microbial NAN pool size or abomasal microbial NAN flux. This indicates that more rumen by-pass protein is available for absorption from the small intestine. However, the actual mode of action of CT upon CT:substrates (protein or carbohydrate etc.) or CT:rumen bacterial cell interactions are not clear. Future studies should therefore focus on the mode of action of CT in the rumen.

This study was supported by Public Good Science Funding from the Foundation of Research Science and Technology (FORST) in New Zealand. The authors wish to thank Dr. A. Molan and J. S. Peters (AgResearch Grasslands, Palmerston North, NZ) for their skilled technical assistance. Dr. P.C.H. Morel is thanked for advice with statistical analysis. Scholarship support to B.R. Min from the New Zealand Department of Education is acknowledged.

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CHAPTER 7

GENERAL DISCUSSION

7.1. INTRODUCTION

Nutritional benefits of forage CT in ruminant animals probably depends upon its concentration, molecular weight and structure (McNabb *et al.* 1998; Barry & McNabb 1999). High CT concentrations in *L. pedunculatus* (95 to 106 g CT/kg DM) depressed rumen digestion of readily fermentable carbohydrate (soluble sugar + pectin) and depressed both VFI and animal production (Reed *et al.* 1982; Barry & Duncan 1984; Barry & Manley 1984). However, moderate CT concentrations in *L. corniculatus* (20-40 g/Kg DM) have been suggested to have beneficial effects (Waghorn *et al.* 1987; Barry & McNabb 1999; Min *et al.* 1999 a & b).

The effects of CT upon protein digestion, rumen proteolytic bacteria in the rumen and upon animal performance have not been examined simultaneously in animals fed a single CT-containing forage. In this thesis the effect of CT in the legume *L. corniculatus* (25-30 g extractable CT/kg DM) upon protein degradation in the rumen and on sheep production have been studied.

7.2. THE EFFECT OF CT ON RUMEN MICROORGANISMS

7.2.1. Relationships between CT, rumen bacteria and plant protein

It has been found that CT in *L. corniculatus* inhibited the growth of several proteolytic bacterial species (Chapter 5) and reduced proteolytic activity and bacterial populations in the rumen (Chapter 6) compared with *L. corniculatus* plus PEG, and this inhibitory effect was dependent on the amount of CT added (Fig. 7.1). The general relationship

between *in vitro* growth of proteolytic rumen bacteria and CT concentration is summarised in Fig. 7.1. This figure shows that the reduction in proteolytic bacterial growth is related to CT concentrations until a concentration of 400 μg CT/ml is reached, after which growth remains stable at a low level. Similarly, other studies have reported that CT in the diet (about 400 μg CT/ml) inhibited growth of several bacterial species and inhibited endoglucanase (cellulase) activity (Bae *et al.* 1994; Jones *et al.* 1994). Recently, Aerts *et al.* (1999) found that CT extracted from *L. corniculatus* and *L. pedunculatus* (237-477 μg CT/ml; equivalent with 890-1790 μg CT/ μg soluble leaf protein; Rubisco) markedly protected Rubisco from degradation by mixed rumen microorganisms. Taken together these and the results of present study showed that the concentration of CT needed to produce maximum inhibition of proteolysis in *in vitro* studies is probably 400 μg CT/ml or greater; however CT concentrations lower than this will reduce the growth of proteolytic rumen bacteria but to a lower extent.

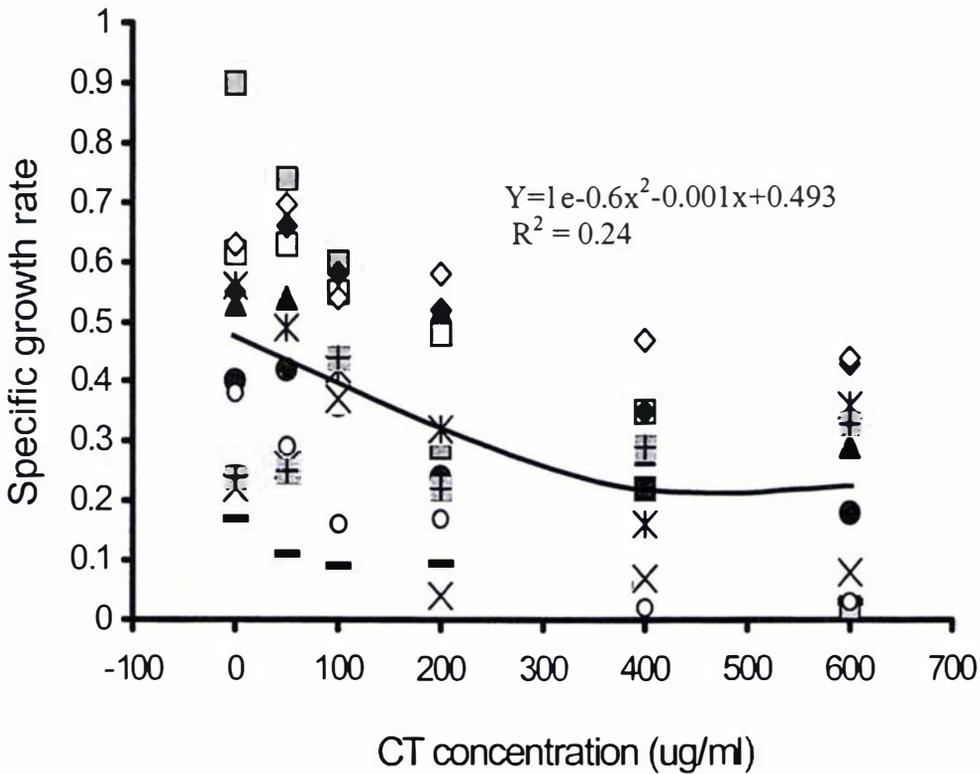


Figure 7.1. The effect of increasing concentration of condensed tannins (CT) in *L. corniculatus* upon specific growth (SGR) *in vitro* of strains of proteolytic rumen bacteria. □, *S. bovis*; ■, *S. bovis* B315; ▲, *P. ruminicola*; --, *P. ruminicola* C21a; ●, *Eubacterium* sp C12b; *, *Eubacterium* C124b; X, *R. albus*; O, *F. succinogenes*; ◆, *B. fibrisolvens*; ◇, *B. fibrisolvens* C21 1a; +, *C. proteoclasticum* B316. (Data are taken from Chapter 5).

The effect of CT in forages such as *L. corniculatus* upon the interactions between rumen bacteria and plant protein are proposed in Fig. 7.2.

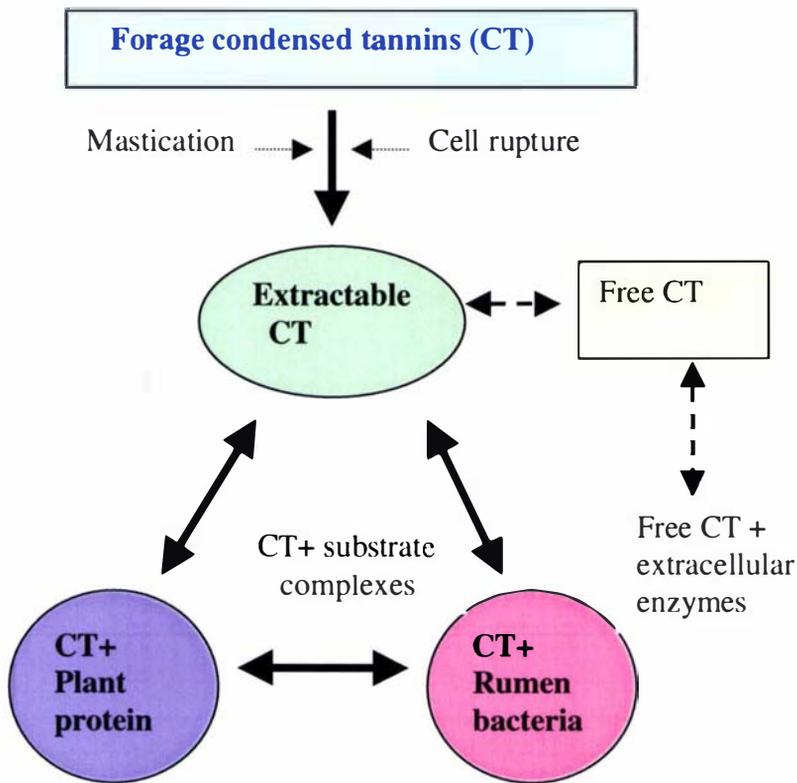


Figure 7.2. Proposed effect of condensed tannins (CT) on rumen bacteria and plant protein in the rumen and their interactions. Sources: Barry & Manley (1986); Mangan (1988); Haslam (1993); Jones *et al.* (1994).

Barry and Forss (1983) defined CT associated with plant protein after mastication as bound CT, and the CT remaining in the supernatant after high speed centrifugation as free CT (Fig. 7.2). It has been suggested that free CT can react with other sources of protein after chewing by animals, such as enzymes secreted by rumen bacteria and so inhibit rumen carbohydrate fermentation (Barry & Manley 1986). Previous research has suggested that the interaction between CT and proteins involves both hydrogen bonds and hydrophobic interactions (Haslam 1989, 1996). The principal driving forces towards these interactions are hydrophobic effects, and these are enhanced by hydrogen bonding of the CT to points on the protein, in particular, the carbonyl group of tertiary peptides (Haslam 1989).

The proteolysis of soluble proteins in the rumen is effected, primarily by cell-associated proteinases on the rumen bacteria (Nugent & Mangan 1981; Wallace & Brammall 1985). When CT-containing forage is masticated, insoluble CT-substrate complexes are formed (Jones & Mangan 1977) and then CT in the rumen become bound to cell coat polymers of bacterial cells (Jones *et al.* 1994). Furthermore, CT in the diet (*L. corniculatus* & sainfoin) also induce changes in morphology of several species of rumen bacteria (Chiquette *et al.* 1988; Jones *et al.* 1994). It has been shown in Chapters 5 and 6 that the proteolytic rumen bacteria are also inhibited by CT, indicating that this is probably due to the CT:substrate (protein or bacterial cell etc.) interactions (Scalbert 1991; Bae *et al.* 1993; Jones *et al.* 1994). The reactions between CT and bacterial cells are less well understood than reactions between CT and forage proteins. The CT probably associates with the bacterial cell surface such as cell-bound extracellular enzymes, inhibiting their activity, and the extent of the inhibition may differ with different types of CT. However, our understanding of physical and chemical associations between CT, rumen microbes and plant protein is compromised by our inability

to quantify the CT content of digesta (Terrill *et al.* 1994) and further studies are required on CT:bacteria interactions.

7.2.2 Action of CT on the solubility & degradability of protein in temperate forages

At equivalent concentrations, CT extracted from *L. pedunculatus* was more effective at reducing the degradation of Rubisco protein by rumen microorganisms than CT extracted from *L. corniculatus* (Fig. 7.3). This effect of CT on protein degradation may be due to differences in the chemical structure influencing the reactivity of the CT. The average MW of CT in *L. corniculatus* is 1,900, whilst in *L. pedunculatus* it is 2,200. In addition, the CT from *L. pedunculatus* contains a predominance of prodelphephidin type subunits. Conversely, the CT from *L. corniculatus* comprises predominantly procyanidin subunits with epicatechin (67 %) dominating this CT (Foo *et al.* 1996, 1997). The large number of free hydroxyl groups enables hydrogen bonding with proteins and other molecules but the extent of the association appears to be affected by the size of the polymer, the predominance of prodelphephidin relative to procyanidin units, the types of terminal groups and the structure of potential binding sites (Hagerman & Butler 1981; Asquith *et al.* 1987; Mangan 1988; Reed 1995; Foo *et al.* 1996). Therefore, chemical structure of CT as well as CT concentration need to be considered in studies involving protein degradation.

Recently, McNabb *et al.* (1998) reported that the amount of CT extract required to precipitate all the Rubisco in 10 µg of total soluble leaf protein from white clover was similar for CT extracted from *L. corniculatus*, *L. pedunculatus* and sainfoin, with between 25 and 50 µg of the CT required. This finding suggested that measurements of degradation of Rubisco in the rumen rather than precipitation may be a more relevant method for assessing the action of CT in temperate forages.

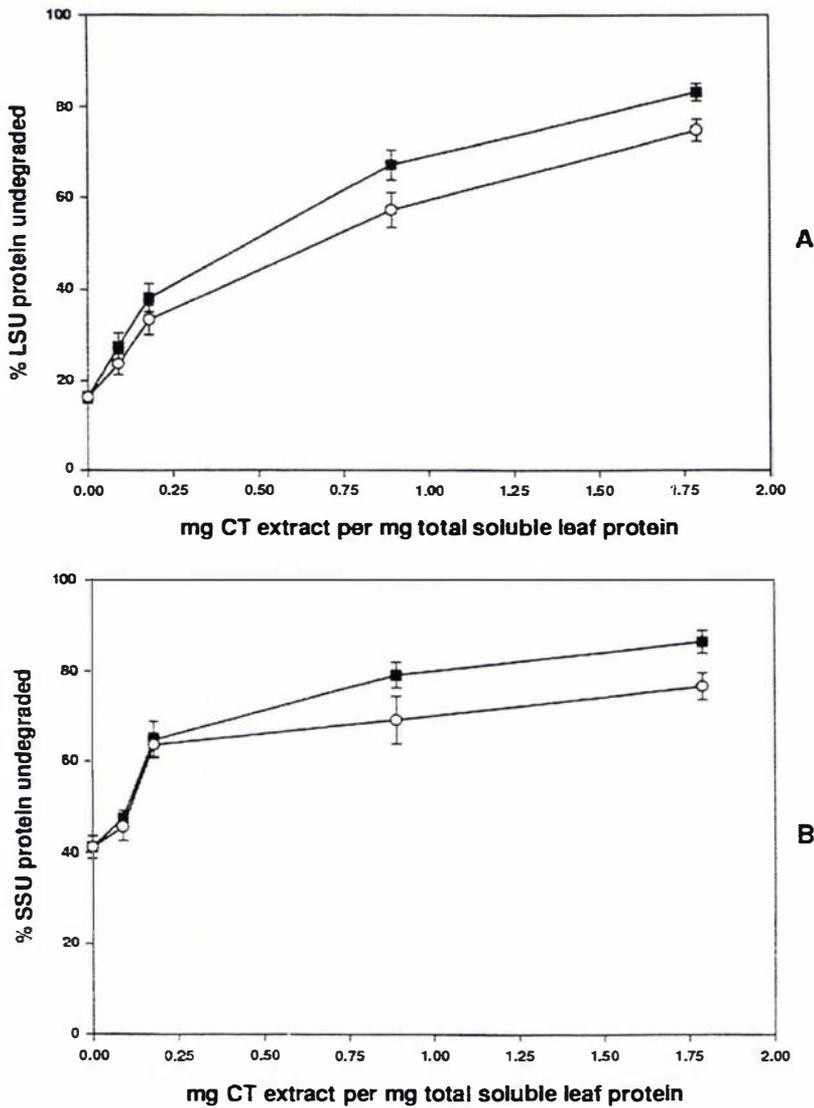


Figure 7.3. The percentage of (A) the LSU and (B) the SSU of Rubisco protein which remained undegraded when total soluble leaf protein from white clover (*Trifolium repens*) was incubated with rumen fluid from sheep. The *in vitro* rumen incubations were undertaken without and with the addition of CT extracts from either (O) *L. corniculatus* or (■) *L. pedunculatus*. The percentage of undegraded protein at the end of the incubation period was calculated as the average for the 6.5 and 8 h time points. Means of quadruplicate incubations are shown. From Aerts *et al.* (1999).

Several factors can affect the reactivity between CT and protein. It has been found that the rate of affinity between CT and protein is dependent on the type of protein as well as the concentration and type of CT (Hagerman & Butler 1981, Asquith & Butler 1986; McNabb *et al.* 1998). A recent study has found that CT extracted from *L. corniculatus* and *L. pedunculatus* had a much greater affinity for Rubisco extracted from white clover than for bovine serum albumin (BSA) protein at pH 7.0 (McNabb *et al.* 1998). Other studies reported that proteolysis of the LSU of Rubisco from lucerne occurs relatively fast, but that the SSU of Rubisco was more resistant to rumen degradation (McNabb *et al.* 1994). Results presented in Chapter 4 show that the LSU of Rubisco was consistently solubilised and degraded faster than the SSU, and CT extracted from *L. corniculatus* had more effect on reducing the degradability of the LSU. It has been suggested that CT bind strongly with proteins having high molecular weight, open and flexible tertiary structure and high contents of proline and other hydrophobic amino acids (Hagerman & Butler 1981; Asquith & Butler 1986). Similar mechanisms may be involved in the reactions between CT and plant protein, with the proteins that are more resistant to rumen degradation (such as SSU) offering less opportunity for degradation to be slowed by CT. Rubisco was used in the present study because it represents the principal forage protein (Mangan & West 1977) and hence represents the most appropriate protein to measure and compare the reactivity of CT.

7.3. THE EFFECT OF CT ON THE NUTRITIVE VALUE OF TEMPERATE FORAGES

It has been shown in Chapters 4, 5 & 6 that action of CT in *L. corniculatus* markedly reduced protein solubilization measured *in sacco*, reduced rumen protein degradation measured *in vitro*, and reduced rumen proteolytic activity and proteolytic bacterial

populations measured *in vivo*. Moreover in sheep, it reduced rumen ammonia concentration, but increased plasma essential amino acid (EAA) concentrations (including branched chain amino acids) and non-ammonia-nitrogen (NAN) outflow from the rumen. This implied that the action of CT in *L. corniculatus* reduced rumen degradation of forage protein by a variety of mechanisms.

To further understand the effect of CT concentration, duodenal (abomasal) NAN fluxes per unit of N eaten have been plotted against CT concentration for sheep fed lotus species (Fig. 7.4; Chapter 6); these duodenal NAN flows can be used as an index of protein leaving the rumen. This shows total NAN flux progressively increased ($R^2 = 0.65$; $P = 0.08$) with increasing CT concentration, whereas rumen microbial outflow was little affected ($R^2 = 0.08$). This indicates that increasing CT concentration progressively increased the amount of undegraded feed protein flowing out of the rumen. *In situ* and *in vitro* experiments (Chapter 4) have shown that this effect is due to the action of CT in *L. corniculatus* slowing the rates of degradation and to a lesser extent, solubilization, of plant proteins (Rubisco) by rumen microorganisms. McNeill *et al.* (1998) found that action of CT in the tropical legume *Leucaena leucocephala* increased the flow of undegraded dietary protein out of the rumen but did not affect the efficiency of rumen microbial synthesis. Possible mechanisms include the reduced growth of proteolytic rumen bacteria and the CT:protein complex reducing the susceptibility of forage protein to microbial degradation in the rumen.

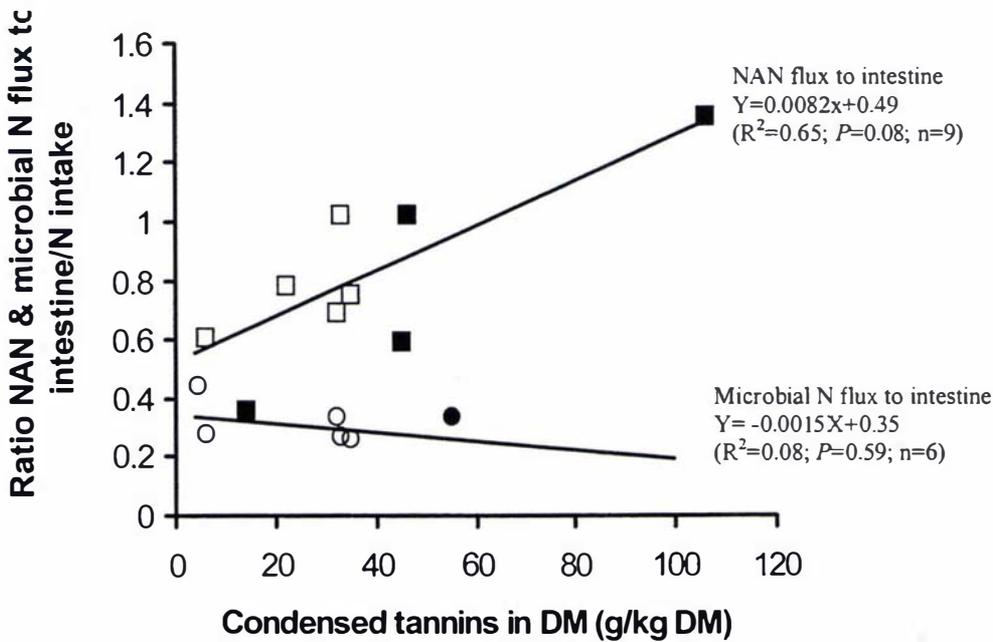


Figure 7.4. The relationship between condensed tannins concentration in lotus species dry matter (X), and the ratio of non-ammonia-nitrogen (NAN) flowing at the abomasum or duodenum (\square , *L. corniculatus*; \blacksquare , *L. pedunculatus*) and microbial N (O, *L. corniculatus*; \bullet , *L. pedunculatus*) per unit of N eaten by sheep. Sources: Barry & Manley 1984; Barry *et al.* 1986; Waghorn *et al.* 1987a & b, 1994; McNabb *et al.* 1993; Wang *et al.* 1996b, Chapter 6.

It has been found that CT in *L. corniculatus* fed to sheep increased absorption of EAA from the small intestine by 62 %, relative to no effect for *L. pedunculatus* (Table 7.1). Of equal importance is the effect of CT in releasing protein and making it available for digestion in the small intestine. Whilst the action of CT in both *L. corniculatus* and *L. pedunculatus*

increased abomasal/duodenal NAN flow, relative to PEG supplemented sheep, only CT in *L. corniculatus* significantly increased the absorption of EAA from the small intestine. The principle reason for the different effects of the two plant species is that action of CT did not affect the apparent digestibility (% abomasal flow) of EAA in the small intestine in sheep fed *L. corniculatus*, whereas it markedly reduced apparent digestibility in the small intestine in sheep fed *L. pedunculatus*. It is not yet fully understood how CT affects amino acid absorption from the small intestine and this area needs further study. One reason may be that CT from *L. pedunculatus* does not release EAA in the small intestine for digestion to the same extent that the CT in *L. corniculatus* does, probably reflecting the difference in CT structure.

Table 7.1. Effect of condensed tannins (CT) upon the intake and absorption of essential amino acids (EAA) from the small intestine of sheep fed on fresh *L. corniculatus* and *L. pedunculatus*, containing respectively 22 and 55 g CT/kg DM.

	<i>L. corniculatus</i> ¹		<i>L. pedunculatus</i> ¹	
	CT-acting	PEG supplemented	CT-acting	PEG supplemented
Rumen ammonia (mgN/l)	367	504	175	460
CT intake (g/d)	98.9	98.9	103.2	116.8
EAA				
Abomasal flow:				
g/d	84.7	55.5	121.1	105.6
Proportion intake	0.86	0.56	1.17	0.90
Apparent absorption				
from small intestine (g/d)	58.8	36.2	81.4	83.5
Proportion abomasal flow	0.67	0.67	0.66	0.79
Proportion intake	0.59	0.37	0.79	0.72

From Waghorn *et al.* (1987b, 1994).

¹ Excluding arginine

7.4. THE EFFECT OF CT IN *LOTUS CORNICULATUS* UPON ANIMAL PRODUCTION

7.4.1. Wool production

Clean wool is mainly protein, with a high cystine content, and the availability of sulphur-containing amino acids (SAA) has significantly affected wool production (Black & Reis 1979; Reis 1979). Previous studies have shown that action of CT in *L. pedunculatus* and *L. corniculatus* increased the irreversible loss rate of cystine from blood plasma, mainly due to reducing the loss of SAA in the rumen (McNabb *et al.* 1993; Wang *et al.* 1994). However, recent study with rumen-protected methionine (Mepron®) oral supplementation showed that simple deficiencies in sulphur-containing amino acids were not limiting for wool production in grazing sheep (Purchas *et al.* 1998).

Wool growth responses to action of CT depends upon both the concentration and type of CT (Fig 7.5; Chapter 2 & 3), with increases over 10 % occurring in the range 22-38 g CT/kg DM for *L. corniculatus*. When CT concentration increased above 50 g/kg DM, the responses became negative, especially for *L. pedunculatus* and sulla, as shown in Fig. 7.5. However, when CT concentration decreased below 22 g CT/kg DM, the wool growth response was variable. Therefore, beneficial effects of CT in *L. corniculatus* for wool production occur in the range 22-38 g CT/kg DM. Although the concentration of CT in the forage is important, however, more recent research suggests that the chemical structure and source of CT may be equally important when evaluating specific forages for use in grazing systems (Aerts *et al.* 1999; Barry & McNabb 1999).

It has been found that clean fleece weight, efficiency of wool production and staple length were higher in ewes grazing *L. corniculatus* than those grazing pasture, with no PEG supplementation effects in Chapter 3, but substantial effect of PEG in Chapter 2.

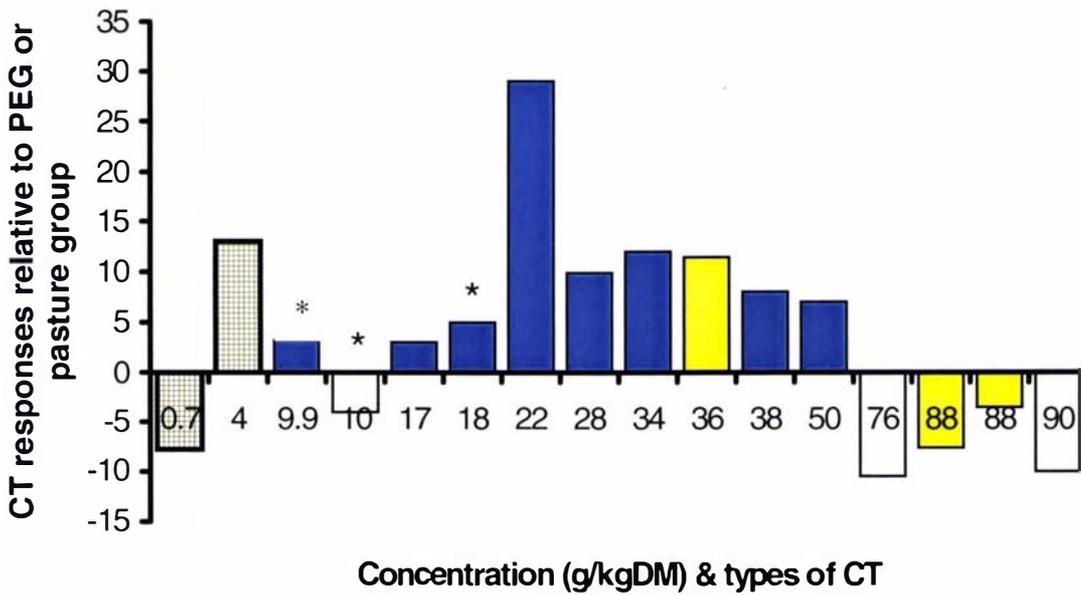


Figure 7.5. The effect of forage CT concentration on wool production of grazing sheep, calculated as the wool production response of control sheep (CT-acting) relative to PEG-supplementation or non-CT containing pasture sheep (CT-inactivated); , temperate grass or temperate grass/white clover; * mixture of lotus species + lucerne or grass; , *L. corniculatus*; , *L. pedunculatus*; , Sulla; Sources: Chapters 2 & 3; Barry 1985; Terrill *et al.* 1992b; Lee *et al.* 1995; Waghorn & Shelton 1995, 1997; Douglas *et al.* 1995, 1999; Montossi *et al.* 1996; Wang *et al.* 1996a.

7.4.2 Reproduction

One of the main findings of this study was that action of CT in *L. corniculatus*, compared with PEG supplemented animals as controls, increased ovulation rate (OR) and lambing percentage, but had only minor effects on liveweight gain (Chapter 3). Nutrition is one of the most significant factors that influences the OR in sheep (Downing & Scaramuzzi 1991), but

mechanism(s) for the effect of nutrition on OR are not clear. The effect of nutrition on OR is probably connected with the metabolic hormonal control of the ovary (Downing & Scaramuzzi 1991). Changes in plasma growth hormone, insulin and insulin-like growth factor (IGF) concentrations consistently accompany nutrient-induced alterations in body energy and protein balance and muscle protein synthesis (Garlic *et al.* 1983; Downing *et al.* 1995), and can affect ovarian function, either directly or by modulating gonadotrophin actions at the ovarian level (Smith 1991; Gong & Webb 1996). Other studies reported that plasma BCAA and EAA concentration were positively correlated to increases in OR (Waghorn 1996; Waghorn *et al.* 1990). Intravenous infusion of BCAA also increased OR (Downing & Scaramuzzi 1991; Downing *et al.* 1995). In addition, BCAA have been shown to increase the sensitivity of muscle protein to insulin *in vitro* (Garlic & Grant 1988; Biolo & Wolfe 1993). Also BCAA transferase iso-enzyme has been found in the ovaries only (Hutson *et al.* 1988, 1992) indicating that BCAA (including EAA) may have a direct stimulating effect on the ovaries, increasing OR by an as yet unknown mechanism(s).

The action of CT in *L. corniculatus* (22 g CT/kg DM) fed to sheep increased abomasal flux of EAA by 50 %, especially of BCAA, increased EAA absorption from the small intestine (Waghorn *et al.* 1987b) and increased cysteine used in body synthetic reactions (Wang *et al.* 1996b), with no effect on VFI. Similarly, the grazing studies reported in this thesis have shown that action of CT in *L. corniculatus* increases animal production without increasing VFI. Thus, action of CT in *L. corniculatus* increases the efficiency of nutrient utilisation and increases the efficiency of animal production. Similar effects of CT in *L. corniculatus* (27g CT/kg DM) have been observed in dairy cows where the efficiency of milk production was increased by 16 % (Fig. 7.4; Woodward *et al.* 1999).

Relative to ewes grazed on perennial ryegrass/white clover pasture, grazing on *L. corniculatus* increased OR by an average of 22 % (Table 7.2). Table 7.2 shows that the

magnitude of the response was related to the duration of *L. corniculatus* feeding, with maximum response occurring after feeding for 2 to 3 cycles (5-7 weeks). Compared with PEG supplemented sheep, the OR response in Chapter 3 and in the experiment of Min *et al.* (1999c) could be partially explained by the action of CT in *L. corniculatus*, but not in the experiment of Luque (1999). A possible reason for this difference is that the lighter ewes used in the present experiment and by Min *et al.* (1999c) were more responsive to the additional protein absorption caused by the action of CT than were the heavier ewes used by Luque (1999).

Table 7.2. The effect of grazing ewes on *L. corniculatus* or perennial ryegrass/white clover (pasture), and of supplementation with polyethylene glycol (PEG), on maximum ovulation rate (OR).

Authors		n ¹	LW ² (kg)	Maximum OR			Response to lotus feeding (%) ³
				Pasture +/- PEG	<i>L. corniculatus</i> + PEG	-PEG	
Chapter 3	(2 nd cycle)	200	54	1.33	1.56	1.76	32.3
Luque 1999	(2 nd cycle)	240	60	1.45	1.66	1.64	13.1
Min <i>et al.</i> 1999c	(3 rd cycle)	225	51	1.48	1.66	1.80	21.6
Mean		222	55	1.42	1.62	1.73	22.3

¹ n = number of experimental animal. ² LW = mean live-weight.

³ Calculated as $\frac{\text{OR lotus (-PEG)} - \text{OR pasture (-PEG)}}{\text{OR pasture (-PEG)}} \times 100$

7.5. TOWARDS THE PRACTICAL APPLICATION OF CT IN SUSTAINABLE ANIMAL PRODUCTION FROM TEMPERATE FORAGES

Lotus corniculatus grows well on acidic (pH 5.0-5.5), dry and medium fertility soils in NZ and may well find application in NZ in low cost development programmes (Scott & Charlton 1983). From an agronomic point of view, *L. corniculatus* grows best in dry East Coast areas of NZ, where ewe live-weights are also likely to be lowest. Therefore, it could have an application for increasing lambing percentage in light ewes grazed in these environments, especially if only 5–7 weeks of grazing during mating is required. However, difficulties with establishment, low competitive ability in high fertility soil conditions, poor winter growth and other agronomic problems (Waghorn *et al.* 1999) currently limits its widespread use in temperate grazing systems. On a wider basis the CT-containing forages are seen as having potential for increasing sustainable ruminant production from grazed forages due to beneficial effects of CT on protein digestion, animal production, animal health and perhaps methane production, without affecting VFI, thereby increasing the efficiency of animal production.

The effect of CT-containing forages such as *L. corniculatus* on protein metabolism, animal production, animal health (eg. parasite infection & fly strike) and methane formation are proposed in Fig. 7.6.

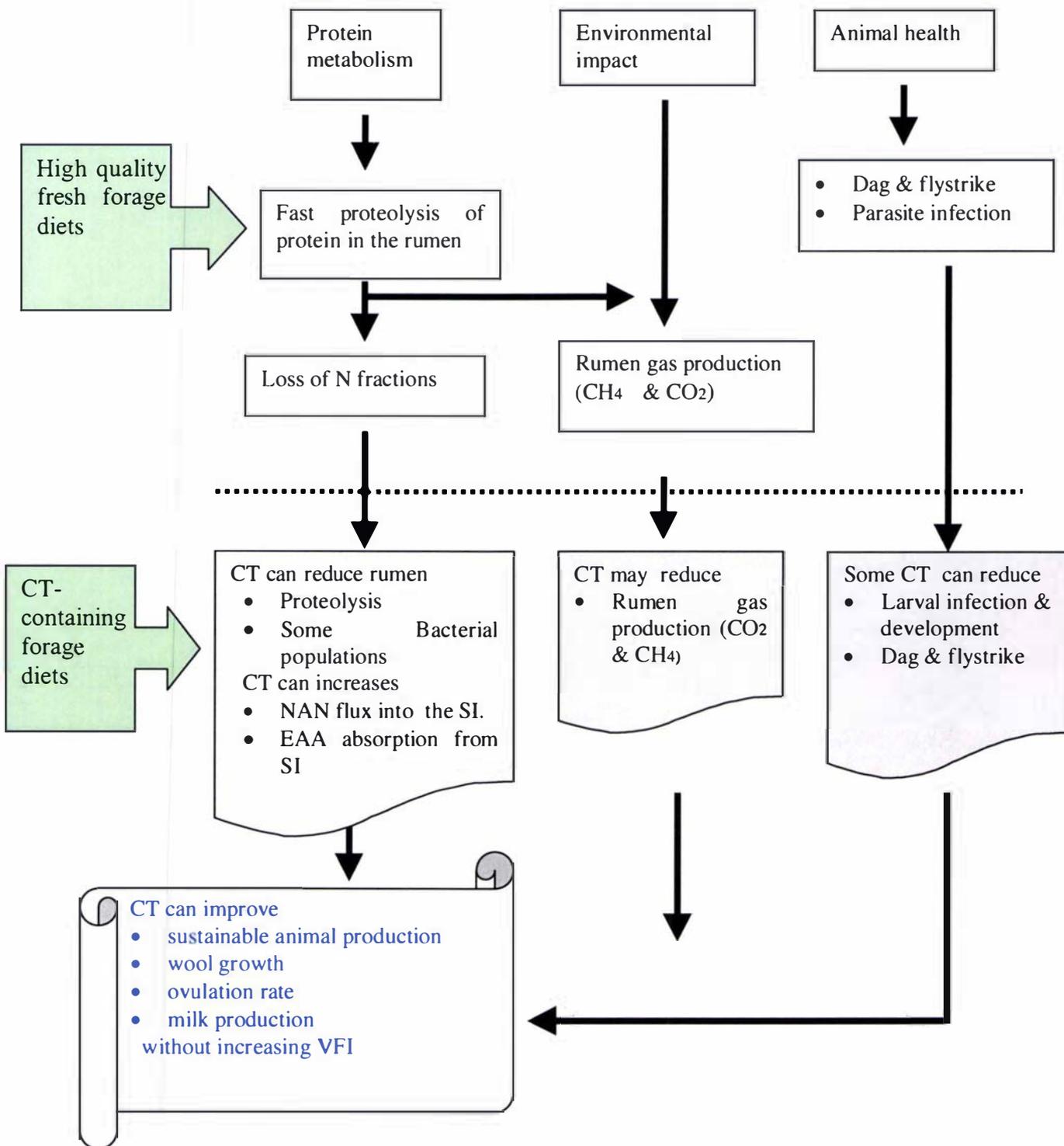


Figure 7. 6 Proposed effect of condensed tannin-containing forages on rumen proteolysis, methane formation in the rumen, animal health and productivity, and their integration into new systems of sustainable productivity. Abbreviations: SI, small intestine, CT, condensed tannins. Sources: This thesis Chapters 2, 5, 6 and Fig. 7.1; Bathe (1951); Jones & Lyttleton (1971); Attwood & Reilly (1995); Niezen *et al.* (1995); Waghorn (1996); Wang *et al.* (1996a & b); Stewart *et al.* (1997); Nollet *et al.* (1998); Molan *et al.* (1999 a & b); Lopez *et al.* (1999).

Ruminants grazing forage diets are subject to a number of diseases, some of which have a nutritional and metabolic component. There are also environmental impacts. Two major components are rumen gas formation (causing bloat & methane environmental problems) and parasite infection in grazing ruminants (Fig. 7.6). Methane and carbon-dioxide gas contribute to both the greenhouse heating effect (19%) and to energy losses (Leng 1992). Methane gas is produced by rumen methanogenic micro-organisms as a metabolic end product ($\text{CO}_2 + \text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$; Lajoie *et al.* 1998; Nollet *et al.* 1998). It has been suggested that a minimum reduction of both CO_2 (50-80 %) and CH_4 (10-20 %) gas emissions is required to stabilise atmospheric concentrations at current levels (Gibbs *et al.* 1989; Leng 1992). Therefore, the development of strategies to reduce methane production in ruminants, without causing a negative impact on ruminant production, continues to be a major challenge for ruminant nutritionists and microbiologists.

It has been found that CT-containing legumes fed to ruminants markedly reduced rumen gas formation (Chiquette *et al.* 1988) and prevented bloat due to action of CT (Jones & Lyttleton 1971; Jones *et al.* 1973; Waghorn & Jones 1989). Furthermore, recent measurements of methane production in sheep fed either fresh ryegrass, lucerne or *L. pedunculatus* (Waghorn 1996) has shown that methane production from the respective feeds was 29.2, 25.3 and 17.0 g/kg digestible DM intake (42 % reduction), or 6.2, 5.9 and 3.9 % of gross energy intake (37 % reduction). However, the mechanism for the low methane production in sheep grazing *L. pedunculatus* is unknown at this time, and the potential value of CT containing forages for reducing rumen methane production needs to be tested experimentally.

Parasitism of the abomasum and small intestine causes extensive protein losses in sheep (Kimambo *et al.* 1988; MacRae 1993) and a significant economic burden to the animal

industries in New Zealand (McAunty *et al.* 1982). However, of greater importance is the development of parasite resistance to anthelmintic drenches (Waller 1994) that has been reported in sheep, goats and cattle in NZ. Alternative non-chemical parasite strategies have recently been suggested based on using temperate forages which contain CT (Niezen *et al.*, 1995; Molan *et al.* 1999a). Recently, Molan *et al.* (1999 b) have shown that CT extracted from four forages had the ability to inhibit the development of *Trichostrongylus colubriformis* eggs to infective larvae. They suggested that the CT containing forages may have the ability to break the life cycle of sheep nematodes and reduce the contamination of pasture with infective larvae. This may reduce dependence on anthelmintic drugs as the main method of controlling internal parasites in grazing ruminants.

It can be concluded that medium concentrations of CT in forages can be used to improve the efficiency of N digestion in ruminants grazing on fresh forage diets. As a result, CT improved wool growth, OR and lambing percentage in sheep grazing *L. corniculatus* (Chapters 2 & 3). This is related to the reduction in proteolytic bacterial activity and protein digestion in the rumen and to enhanced NAN flow to the abomasum (Chapters 4, 5 & 6). The beneficial effects of medium concentrations of forage CT (*L. corniculatus*, sulla & sainfoin) in high fertility grazing ecosystems may be better expressed through genetic engineering programmes combined with traditional plant selection techniques to increase CT concentration in common legumes such as white clover, red clover and lucerne that have widespread application in animal production from forage diets. This offers exciting future possibilities.

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