Journal of Agricultural Science, Cambridge (2000), **134**, 305–317. *Printed in the United Kingdom*

Solubilization and degradation of ribulose-1,5-*bis*phosphate 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

B. R. MIN[1,2], W. C. MCNABB[1]*, T. N. BARRY[2] AND J. S. PETERS[1]

[1] *Nutrition and Behavior Group, AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand*

[2] *Institute of Veterinary, Animal & Biomedical Science, Massey University, Palmerston North, New Zealand*

(*Revised MS received 18 December 1999*)

SUMMARY

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-*bis*phosphate 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 solubilization 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 solubilization 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.

* To whom all correspondence should be addressed. Email: McNabbw@agresearch.cri.nz

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 (20–35% N intake) and the absorption of that ammonia from the rumen (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 affect 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 ribulose-1,5-*bis*phosphate carboxylase/oxygenase (*EC* 4.1.1.39; Rubisco; 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.

MATERIALS AND METHODS

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.

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 (two 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 12 sheep (two 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 six 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.

PEG infusion

Throughout the experiments, one group of six sheep (three white clover; three *L. corniculatus*; CT-inactivated) received a continuous intraruminal infusion of PEG (molecular weight 3500, Union Carbride, 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.

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 oesphagus, after which the total rumen contents were returned to each animal. In addition, fresh white clover and *L. corniculatus* was frozen at −20 °C, freeze dried and ground to pass through a sieve with aperture size of 1 mm and stored at −20 °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 $> 2{\cdot}0$, 1·0, 0·5, 0·25 and $< 0{\cdot}2$ mm. Material passing the wet sieve ($< 0{\cdot}2$ mm) was centrifuged (2000 ***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.

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 three and then offered white clover. The sheep in the first group were infused with PEG (three PEG sheep) while the sheep in the second group were left without PEG (three 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 ($n = 2$).

In vitro *experiment*

Preparation of strained rumen fluid

Four sheep (two 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 cheesecloth into a Dewar flask flushed with CO_2 gas. This rumen fluid was maintained at 39 °C under an atmosphere of CO_2 and used immediately for *in vitro* incubations.

In vitro *rumen incubation procedures*

The two *in vitro* experiments (Experiments A and B) were conducted to determine the effect of CT on the *in vitro* degradation of Rubisco by the microorganisms present when forages where 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 Experiments A and B. Firstly, fresh white clover and *L. corniculatus* was minced (using an electronic meat

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

Table 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 performed 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 (two 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; McDougall 1948).

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 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.

Samples (200 µl) were removed from each digestion flask by pipette under a CO_2 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 µl 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 CO_2 after each sampling.

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 method of Bradford (1976), whilst DM was determined by drying at 95 °C for 16 h. The NDF was determined using the method of Robertson & 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).

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

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 µl 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 h to detect protein bands. Rubisco consists of 8 large subunits (LSU; MW 54000) and 8 small subunits (SSU; MW 16000; 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™/CP Imaging Analysis Software, USA).

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 (Ørskov & McDonald 1979):

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

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 solubilization (PS) and predicted degradability (PD) was calculated using the equation of Ørskov & McDonald (1979), where r is the rumen particulate dry matter fractional outflow rate.

$$\text{PS (PD)} = a + (bc/(c + r)) \quad (2)$$

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 solubilization (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 (S.E.); 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.

RESULTS

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 2. Freshly minced forage tended to be better correlated with chewed boli for particle distribution ($R^2 = 0{\cdot}17$ and 0·78 for white clover ($P = 0{\cdot}47$) and *L. corniculatus* ($P = 0{\cdot}11$), respectively) than freeze-dried and ground ($R^2 = -0{\cdot}073$ and 0·43 for white clover ($P = 0{\cdot}9$) and *L. corniculatus* ($P = 0{\cdot}77$), 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.

Chemical composition of forages

The chemical composition of white clover and *L. corniculatus* during *in situ* and *in vitro* experiments is shown in Table 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.

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. For DM, in the absence of PEG, the potential solubilization (a + b; 84·3 *v.* 93·5 %; $P < 0{\cdot}001$) and predicted rumen DM solubilization (PS; 77·4 *v.* 85·5 %; $P < 0{\cdot}001$) were lower for *L. corniculatus* than for the white clover. The addition of PEG significantly increased the predicted DM solubilization ($P < 0{\cdot}001$) in the *L. corniculatus* and tended to increase solubilization rate ($P = 0{\cdot}06$).

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

Table 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

	Preparation method						
	White clover ($n = 2$)			*Lotus corniculatus* ($n = 2$)			
Sieve sizes* (mm)	Freeze dried and ground	Minced forage	Chewed boli†	Freeze dried and ground	Minced forage	Chewed boli†	S.E. (D.F. = 6)
> 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 (two on each diet; neither animal was receiving PEG) were used for collection of chewed boli for the particle distribution experiments.

Table 3. *Chemical composition of white clover and* Lotus corniculatus *during both the* in situ *and the* in vitro *experimental periods* (*mean values* ± *S.E.*; n = *4*).

Components (g/kg DM)	White clover	*Lotus corniculatus*
Total N*	44·8 ± 0·43	38·4 ± 0·43
NDF†	244·3 ± 7·77	259·2 ± 7·80
Condensed tannins (CT)		
Extractable CT	ND‡	14·8 ± 0·18
Protein bound CT	0·3 ± 0·03	5·9 ± 0·03
Fibre bound CT	ND‡	1·5 ± 0·02
Total CT	0·3 ± 0·15	22·1 ± 0·16

* Total nitrogen.
† Neutral detergent fibre.
‡ Not detectable.

The forage × PEG and diet × 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), suggesting that at a concentration of 22 g CT/kg DM, CT did not affect ruminal NDF digestion.

The loss of N, and the LSU and SSU of Rubisco from polyester bags suspended in the rumen is shown in Table 5 and Table 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 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 × 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.

For both subunits of Rubisco (Table 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 peptide's 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 × 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.

In vitro *Experiment A*

Degradation of the LSU and SSU of Rubisco from either minced or extracted white clover and *L.*

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

Table 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-ruminally infused polyethylene glycol* (*+/− PEG; MW 3500*)

	Instantly solubilized* a (%)			Solubilization* rate c (% h)			Potential solubilization* a+b (%)			Predicted solubilization* PS %		
Diets and forages	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.
					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 constitutes 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.

Table 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 and forages	Instantly solubilized a (%)			Solubilization rate c (%/h)			Potential solubilization a+b (%)			Predicted solubilization PS (%)		
	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.
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 constitutes 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.

corniculatus during *in vitro* rumen incubation is shown in Table 7. In the absence of PEG, the rate of degradation (17·0 *v.* 29·0 %/h) and potential degradability (49·8 *v.* 70·6 %; $P < 0{\cdot}001$) were lower for the LSU from *L. corniculatus* than for white clover. Addition of PEG increased the degradation rate ($P < 0{\cdot}01$) and potential degradability ($P < 0{\cdot}001$) of the LSU and increased the degradation rate of the SSU ($P < 0{\cdot}01$), 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 × PEG interactions, suggesting that increases due to PEG addition occurred for *L. corniculatus* and not for white clover. There were also significant forage × 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 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.

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 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 ($P < 0{\cdot}01$) and also reduced its potential degradability ($P < 0{\cdot}001$). Addition of PEG increased both degradation rate and potential degradability of both Rubisco sub-units, but there were significant CT × 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 ($P < 0{\cdot}05$), 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 × PEG interactions.

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

Table 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-ruminally infused polyethylene glycol* (*+/− PEG; MW 3500*)

Diets and forages	Instantly solubilized a (%)			Solubilization rate c (%/h)			Potential solubilization a+b (%)			Predicted solubilization PS (%)		
	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.	−PEG	+PEG	S.E.
					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 constitutes 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.

Table 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 c (%/h) −PEG	Degradation rate c (%/h) + PEG	S.E. (D.F. = 6)	Potential degradability (PD) a+b (%) −PEG	Potential degradability (PD) a+b (%) + PEG	S.E. (D.F. = 6)
			LSU ($n = 4$)			
WC						
Minced	41·4	47·4	4·03	68·4	67·4	0·80
Extracted	15·7	26·8	3·80	72·8	71·2	2·15
Mean	28·6	37·1	—	70·6	69·3	—
LC						
Minced	16·6	27·8	2·45	54·9	72·5	2·50
Extracted	17·3	27·4	0·22	44·7	67·7	3·44
Mean	17·0	27·6	—	49·8	70·1	—
			SSU ($n = 4$)			
WC						
Minced	11·3	13·7	1·23	59·5	60·3	1·89
Extracted	6·9	11·0	0·60	61·1	59·6	2·39
Mean	9·1	12·3	—	60·3	59·9	—
LC						
Minced	5·3	8·2	0·62	58·5	61·0	2·10
Extracted	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.

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 solubilization 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 (Tanner *et al.* 1994; McNabb *et al.* 1996). 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 (Asquith & Butler 1986; Horigome *et al.* 1988; Spencer *et al.* 1988; 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; Nugent & Mangan 1978; Mahadevan *et al.* 1980; McNabb *et al.* 1994). Similar mechanisms may be involved in the reactions between forage protein and CT, with the proteins that are more

Table 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 c (%/h) −PEG	Degradation rate c (%/h) + PEG	S.E. (D.F. = 6)	Potential degradability (PD) a+b (%) −PEG	Potential degradability (PD) a+b (%) + PEG	S.E. (D.F. = 6)
			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.

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 & 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

https://doi.org/10.1017/S0021859699007698 Published online by Cambridge University Press

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.

REFERENCES

Asquith, T. N. & Butler, L. G. (1986). Interactions of condensed tannins with selected proteins. *Phytochemistry* **25**, 1591–1593.

Barry, T. N. & Forss, D. A. (1983). The condensed tannin content of vegetative *Lotus pedunculatus*, its regulation by fertiliser application, and effect upon protein solubility. *Journal of the Science of Food and Agriculture* **34**, 1047–1056.

Beever, D. E. & Siddons, R. C. (1986). Digestion and metabolism in the grazing ruminant. In *Control of Digestion and Metabolism in Ruminants* (Eds L. P. Milligan, W. L. Grovum & A. Dobson), pp. 479–497. Englewood Cliffs, New Jersey, USA: Prentice-Hall.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of dye-binding. *Analytical Biochemistry* **72**, 248–254.

Cohen, D. & Wales, W. J. (1994). Solubility and degradability of white clover in the lactating cow. *Proceedings of the Australian Society of Animal Production* **20**, 136–139.

Domingue, B. M. F., Dellow, D. W., Wilson, P. R. & Barry, T. N. (1991). Comparative digestion in deer, goats, and sheep. *New Zealand Journal of Agricultural Research* **34**, 45–53.

Fay, J. P., Cheng, K. J., Hanna, M. R., Howarth, R. E. & Costerton, J. W. (1980). *In vitro* digestion of bloat-safe and bloat-causing legumes by rumen microorganisms: gas and foam production. *Journal of Dairy Science* **63**, 1273–1281.

Horigome, T., Kumar, R. & Okamoto, K. (1988). Effects of condensed tannins prepared from leaves of fodder plants on digestive enzymes *in vitro* and in the intestine of rats. *British Journal of Nutrition* **60**, 275–285.

Jackson, F. S., McNabb, W., Barry, T. N., Foo, Y. L. & Peters, J. S. (1996). The condensed tannin content of a range of subtropical and temperate forages and the reactivity of condensed tannin with ribulose-1,5-*bis*-phosphate carboxylase (Rubisco) protein. *Journal of the Science of Food and Agriculture* **72**, 483–492.

Jones, W. T. & Mangan, J. L. (1977). Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein and their reversal by polyethylene glycol and pH. *Journal of the Science of Food and Agriculture* **28**, 126–136.

Kawashima, N. & Wildman, S. G. (1970). Fraction 1 protein. *Annual Reviews in Plant Physiology* **21**, 325–358.

Li, Y. G., Tanner, G. & Lark, P. (1996). The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture* **70**, 89–101.

MacRae, J. C. & Ulyatt, M. J. (1974). Quantitative digestion of fresh herbage by sheep. II. The sites of digestion of some nitrogenous constituents. *Journal of Agricultural Science, Cambridge* **82**, 309–319.

Mahadevan, S., Erfle, J. D. & Sauer, F. D. (1980). Degradation of soluble and insoluble proteins by *Bacteroides amylophilus* protease and by rumen microorganisms. *Journal of Animal Science* **50**, 723–728.

Mangan, J. L. (1972). Quantitative studies on nitrogen metabolism in the bovine rumen. The rate of proteolysis of casein and ovalbumin and the release and metabolism of free amino acids. *British Journal of Nutrition* **27**, 261–283.

Mangan, J. L. (1982). The nitrogenous constituents of fresh forages. In *Forage Protein in Ruminant Animal Production*

(Eds D. J. Thomson, D. E. Beever & R. G. Gunn), pp. 25–40. British Society of Animal Production's Occasional Publication Series No 6: Thomas Ditton.

Mathers, J. C. & Miller, E. L. (1980). A simple procedure using ^{35}S incorporation for the measurement of microbial and underacted food protein in ruminant digesta. *British Journal of Nutrition* **43**, 503–518.

McNabb, W. C., Spencer, D., Higgins, T. J. V. & Barry, T. N. (1994). *In vitro* rates of rumen proteolysis of Rubisco-1,5-*bis*phosphate carboxylase (Rubisco) from lucerne leaves, and of ovalbumin, vicilin and sunflower albumin 8 storage proteins. *Journal of the Science of Food and Agriculture* **64**, 53–61.

McNabb, W. C., Waghorn, G. C., Peters, J. S. & Barry, T. N. (1996). The effect of condensed tannins in *Lotus pedunculatus* upon the solubilisation and degradation of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein in the rumen and the sites of Rubisco digestion. *British Journal of Nutrition* **76**, 535–549.

McNabb, W. C., Peters, J. S., Foo, L. Y., Waghorn, G. C. & Jackson, F. S. (1998). Effect of condensed tannin prepared from several forages on the *in vitro* precipitation of ribulose-1,5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4.21.4) and chymotrypsin (E.C. 2.4.21.1). *Journal of the Science of Food and Agriculture* **77**, 201–212.

Mehrez, A. Z. & Ørskov, E. R. (1977). A study of the artificial fibre bag technique for determining the digestibility of feeds in the rumen. *Journal of Agricultural Science*, *Cambridge* **88**, 645–650.

Nugent, J. H. A. & Mangan, J. L. (1978). Rumen proteolysis of Fraction 1 leaf protein, casein and bovine serum albumin. *Proceedings of Nutritional Society* **37**, 48A.

Nugent, J. H. A., Jones, W. T., Jordan, D. J. & Mangan, J. L. (1983). Rates of proteolysis in the rumen of the soluble proteins casein, fraction 1 leaf protein, bovine serum albumin and bovine submaxillary mucoprotein. *British Journal of Nutrition* **50**, 357–368.

Ørskov, E. R. & McDonald, I. (1979). The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *Journal of Agricultural Science*, *Cambridge* **92**, 499–503.

Reid, C. S. W., Lyttleton, J. W. & Mangan, J. I. (1962). Bloat in cattle. XXIV. A method of measuring the effectiveness of chewing in the release of plant cell contents from ingested feed. *New Zealand Journal of Agricultural Research* **5**, 237–248.

Robertson, J. B. & Van Soest, P. J. (1981). The detergent system of analysis and its application in human food. In *The Analysis of Dietary Fibre in Food* (Eds W. P. T. James & O. Theander), pp. 123–158. New York: Marcel Dekker.

Statistical Analysis System (1985). User's guide: Statistics, Version 5. North Carolina, USA: SAS Institute.

Spencer, D., Higgins, T. J. V., Freer, M., Dove, H. & Coombe, J. B. (1988). Monitoring the fate of dietary proteins in rumen fluid using gel electrophoresis. *British Journal of Nutrition* **60**, 241–247.

Stern, M. D. & Satter, L. D. (1984). Evaluation of nitrogen solubility and the dacron bag technique as methods for estimating protein degradation in the rumen. *Journal of Animal Science* **58**, 714–724.

Tanner, G. J., Moore, A. E. & Larkin, P. J. (1994). Proanthocyanidins inhibit hydrolysis of leaf proteins by rumen microflora *in vitro*. *British Journal of Nutrition* **71**, 947–958.

Terrill, T. H., Rowan, A. M., Douglas, G. B. & Barry, T. N. (1992). Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *Journal of the Science of Food and Agriculture* **58**, 321–329.

Ulyatt, M. J. & MacRae, J. L. (1974). Quantitative digestion of fresh herbage by sheep. I. The sites of digestion of organic matter, energy, readily fermentable carbohydrate, structural carbohydrate, and lipid. *Journal of Agricultural Science*, *Cambridge* **82**, 295–307.

Ulyatt, M. J. & Egan, A. R. (1979). Quantitative digestion of fresh herbage by sheep. V. The digestion of four herbages and prediction of sites of digestion. *Journal of Agricultural Science*, *Cambridge* **92**, 605–616.

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

Waghorn, G. C., Barry, T. N. (1987). Pasture as a nutrient source. In *Feeding Livestock on Pasture* (Ed. A. M. Nicol), Occasional Publication No. 10, pp. 21–37. Hamilton, New Zealand: New Zealand Society of Animal Production.

Waghorn, G. C. & Shelton, I. D. (1997). Effect of condensed tannins in *Lotus corniculatus* on the nutritive value of pasture for sheep. *Journal of Agricultural Science*, *Cambridge* **128**, 365–372.

Waghorn, G. C., Ulyatt, M. J., John, A. & Fisher, M. T. (1987). The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on *Lotus corniculatus* L. *British Journal of Nutrition* **57**, 115–126.

Waghorn, G. C., Shelton, I. D. & Thomas, V. J. (1989). Particle breakdown and rumen digestion of fresh ryegrass (*Lolium perenne* L.) and lucerne (*Medicago sativa* L.) fed to cows during a restricted feeding period. *British Journal of Nutrition* **61**, 409–423.

Yu, F., Barry, T. N., McNabb, W. C., Moughan, P. J. & Wilson, G. F. (1995). Effect of bound condensed tannin from cottonseed upon *in-situ* protein solubility and dry matter digestion in the rumen. *Journal of the Science of Food and Agriculture* **69**, 311–319.