The effect of condensed tannins in *Lotus pedunculatus* on the solubilization and degradation of ribulose-1,5-*bis*phosphate carboxylase (*EC* 4.1.1.39; Rubisco) protein in the rumen and the sites of Rubisco digestion

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Three experiments were undertaken to determine the effect of condensed tannin (CT) in *Lotus pedunculatus* (45–55 g extractable CT/kg DM) on the digestion of the principal leaf protein, ribulose-1,5-*bis*phosphate carboxylase (*EC* 4.1.1.39; Rubisco; fraction 1 leaf protein). In two of the experiments *Lotus pedunculatus* was fed to sheep, with one group receiving a continuous intraruminal infusion (*per fistulum*) of PEG (molecular weight 3500) to bind and inactivate the CT (PEG group). The other group, which did not receive PEG, was termed the control sheep (CT acting). Expt 3 involved *in vitro* incubations of *Lotus pedunculatus* in buffered rumen fluid, with and without PEG added. In all experiments the results have been interpreted in terms of the effects of CT on Rubisco solubilization and degradation. Disappearance of N and Rubisco from *Lotus pedunculatus* suspended in polyester bags in the rumen was used as a measure of solubilization. Degradation was defined as the disappearance of Rubisco from *in vitro* incubations of *Lotus pedunculatus* in rumen fluid. In Expt 1, CT reduced the digestion of Rubisco in the rumen from 0·96 to 0·72 of intake ($P < 0·01$). Rubisco digestion in the small intestine was 0·27 of intake in control sheep and 0·04 of intake in PEG sheep. In Expt 2, PEG had no effect on the loss of Rubisco from *Lotus pedunculatus* contained in polyester bags which were incubated in the rumen, hence CT did not affect the solubilization of Rubisco. Observations in Expt 1 were confirmed by *in vitro* incubations in Expt 3, where PEG addition substantially increased the rate of degradation of plant protein to NH_3. Addition of PEG decreased the period of time taken to degrade 50% of the Rubisco from about 13·8 h to about 3·0 h. It was concluded that the action of CT reduced the digestion of Rubisco in the rumen of sheep fed on fresh *Lotus pedunculatus*, and that this was primarily due to the ability of CT to slow its degradation by rumen micro-organisms, without affecting its solubilization. Both fresh-minced, and freeze-dried and ground lotus were used for *in sacco* and *in vitro* incubations; however, fresh-minced lotus was more suitable for the evaluation of protein solubilization and degradation in fresh forages.

Condensed tannin: Rumen degradation: Ribulose-1,5-*bis*phosphate carboxylase

When ruminants fed on fresh forage were given abomasal infusions of protein or fed on protein protected from rumen degradation, production responses included increased wool growth (Reis, 1979), milk production in dairy cattle (Rogers *et al.* 1980; Hamilton *et al.* 1992) and sheep (Penning *et al.* 1988), and live-weight gain (Barry, 1981; Stock *et al.* 1981; Hogan, 1982; Poppi *et al.* 1988; Fraser *et al.* 1990). These results suggest that productivity is reduced because insufficient essential amino acids (EAA) are absorbed from the small intestine relative to energy. The low absorption of amino acids (AA) is due in part to the

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substantial degradation of plant proteins to NH_3 in the rumen (MacRae & Ulyatt, 1974; Beever & Siddons, 1986) and the use of AA in the conversion of NH_3 to urea (Lobley *et al.* 1995).

A number of studies have shown that a low concentration of proanthocyanidin or condensed tannin (CT) in the diet can increase the flow of non-NH_3-N (NAN) to the intestine, relative to N intake (for review, see Mangan, 1988). CT can also reduce N degradation in the rumen (Waghorn *et al.* 1994*a*), increase EAA absorption from the small intestine (Waghorn *et al.* 1987; McNabb *et al.* 1993) and increase N retention (John & Lancashire, 1981) in sheep fed on forage diets. The improved productivity of sheep fed on forages containing low concentrations of CT, including lower carcass fat (Purchas & Keogh, 1984) and increased live-weight gain and wool growth (Wang *et al.* 1994), may be attributed to increased abomasal flow of NAN and increased absorption of EAA from the small intestine. Therefore, CT may provide a practical means to increase the absorption of AA from the small intestine in ruminants grazing fresh forages.

Rapid and indirect methods for assessing the degradability of protein in the rumen tend to depend on either the solubility of protein in rumen fluid or the disappearance of protein from synthetic-fibre bags suspended in the rumen. Although Mangan (1972) demonstrated that a soluble protein, ovalbumin, was degraded very slowly in the rumen, the disappearance of protein from feed incubated in the rumen in a synthetic-fibre bag has gained wide acceptance as a measure of its degradability (Mehrez & Ørskov, 1977; Ganev *et al.* 1979). Nevertheless, Spencer *et al.* (1988*b*) reported that individual pea (*Pisum sativum*)-seed proteins differed widely in their relative resistances to rumen degradation despite almost complete loss of total pea-seed N from polyester bags suspended in the rumen. Although estimates of degradability from the synthetic-fibre-bag technique are better correlated with *in vivo* measures of degradability than laboratory measurements of protein solubility (Mathers & Miller, 1980), there is now increasing evidence that loss from synthetic-fibre bags and degradation are not always interdependent. Additionally, very little research has been published using the synthetic-fibre-bag technique to evaluate the degradability of the protein from fresh forages.

The digestion of fresh-forage protein in the rumen is the result of the combined processes of solubilization and degradation. Solubilization of plant protein in the rumen, which occurs following the chewing of fresh forage, is here defined as the release of protein from cells into the fluid environment of the rumen and is an important determinant of its susceptibility to degradation (Mangan, 1972, 1982; Nugent *et al.* 1983; Wallace, 1983, 1985). In the present study we have used the disappearance of plant protein from *Lotus pedunculatus* suspended in polyester bags in the rumen as a measure of solubilization. Degradation is defined as the catabolism of plant protein by microbial proteases which yields peptides, AA and NH_3. It has been successfully studied *in vitro* and *in vivo* by direct measurement of a decreasing protein content using SDS–PAGE (Nugent & Mangan, 1981; Spencer *et al.* 1988*b*; McNabb *et al.* 1994). CT could potentially affect either or both these processes. The principal objective of the present study was to determine whether the CT in *Lotus pedunculatus* affected the digestion of ribulose-1,5-*bis*phosphate carboxylase (*EC* 4.1.1.39; Rubisco; fraction 1 leaf protein) in the rumen and whether changes in Rubisco digestion were due to changes in protein solubilization or degradation or a combination of both processes. Rubisco was studied because it is the principal leaf protein, representing 30–50% of the total protein present in plants (Mangan, 1982).

MATERIALS AND METHODS

Experimental design

Three experiments were undertaken to determine how the CT in *Lotus pedunculatus* affected the digestion of Rubisco in the rumen. In Expt 1 the effect of CT on the digestion and disappearance of Rubisco from the rumen and small intestine was measured in sheep fed on *Lotus pedunculatus*. Expts 2 and 3 were undertaken to determine whether CT affected Rubisco solubilization or degradation or both. In the present study the loss of N, Rubisco and AA from *Lotus pedunculatus* contained in polyester bags suspended in the rumen of sheep fed on *Lotus pedunculatus* was used to determine whether CT affected the solubilization of these constituents (Expt 2). In the third experiment the effect of CT on the degradation of Rubisco was determined using *in vitro* incubations of *Lotus pedunculatus* in rumen fluid. In each of these experiments the effect of CT was determined with or without the presence of PEG (molecular weight (MW) 3500), which binds and inactivates CT (Jones & Mangan, 1977). Rubisco was quantified in all experiments by SDS–PAGE and imaging densitometry.

Animals and forages

Sheep used in Expts 1 and 2, and those which provided rumen fluid for Expt 3 were fed on a diet of only the CT-containing legume, *Lotus pedunculatus* (cv. 'Grasslands Maku'). They were aged between 18 and 30 months, and were held indoors in metabolism crates for the duration of each trial. The animals were treated to remove internal parasites (12 ml Ivomec; Merck Sharp and Dohme (NZ) Ltd, Auckland, New Zealand) and external parasites (10 ml Wipeout; Coopers Animal Health (NZ) Ltd, Wellington, New Zealand). The eight sheep used in Expt 1 weighed 48·3 (SD 8·66) kg and were fitted with rumen (55 mm i.d.) and abomasal (10 mm i.d.) cannulas. A separate group of sheep (live weight 55·0 (SD 2·33) kg) was used in Expts 2 and 3, and each was fitted with a large (90 mm i.d.) rumen cannula to enable suspension of polyester bags in the rumen. In Expts 1 and 2, one group of sheep received an intraruminal infusion of PEG (PEG sheep; CT inactivated; 100 g/d in 300 g water), while the remaining group (control sheep; CT effective) received an intraruminal infusion of water. The effect of CT and of the PEG infusion was monitored by measuring the NH_3 concentration in rumen fluid.

The *Lotus pedunculatus* was harvested daily at about 08.00 hours from a vegetative stand (300–400 mm high), using a sickle-bar mower. Immediately after harvest it was further cut into 50 mm lengths with a chaff cutter and one-third of the daily allowance was placed on belt feeders by 10.00 hours and fed hourly for 6 h. The remaining two-thirds was stored at 4° until 16.00 hours, when it was placed on belt feeders and fed hourly for 18 h so that all the sheep were fed at hourly intervals each day in all experiments.

The results in Expt 1 were obtained from the same experiment reported by McNabb *et al.* (1993) and Waghorn *et al.* (1994*b*). The sheep used in Expts 2 and 3 have been described by McNabb *et al.* (1993). Hence, only brief details of the experimental conditions are given here.

Expt 1

In Expt 1 the effect of CT on the digestion of Rubisco in the rumen and small intestine was determined in four control and four PEG sheep. Daily DM allowance was about 1400 g for all sheep, and *Lotus pedunculatus* had been fed for about 25 d before measurements of Rubisco disappearance. Digesta flow-rates were determined at the abomasum over a 3 d period, and at the ileum at slaughter using ^{51}Cr EDTA (Binnerts *et al.* 1968) and ^{103}Ru phenanthroline (Tan *et al.* 1971) according to the method of Faichney (1975). PEG was

given to four sheep for 26 d until slaughter, whilst all the sheep received a continuous intraruminal infusion of the two indigestible markers for the last 7 d before slaughter. Digesta flow-rates (Waghorn *et al.* 1994*b*) in conjunction with Rubisco concentration in feed and digesta samples enabled the net disappearance from the rumen and small intestine to be quantified.

Expt 2

In Expt 2 the effect of CT on the digestion of DM and on the solubilization of N, Rubisco and protein-bound AA was measured in twelve sheep given about 900 g DM/d (maintenance requirements) for the 30 d trial. Restricted intakes enabled polyester bags (37 μm pore size; Estal Mono; Swiss Screens (Aust) Pty Ltd, Sydney, Australia) to be suspended in and removed from the rumen with relative ease. Six of the sheep received an intraruminal infusion of PEG for 14 d before and during the incubation period.

The solubilization of N, Rubisco and protein-bound AA was determined with both freshly-minced (FM) and freeze-dried and ground (FD) *Lotus pedunculatus* grown outdoors. Freeze-drying and grinding is a common method used for preparing feeds for *in situ* incubation in synthetic-fibre bags in the rumen and so FD lotus was included in the present study. However, in New Zealand, forages are predominantly fed fresh to grazing ruminants. Therefore, FM lotus was included in the present study in an effort to prepare a feed for *in situ* incubation in the rumen which more closely resembled fresh forage which had been chewed and swallowed by sheep. The lotus used for the FD preparation was freeze-dried for 45 h and ground through a 1 mm screen. Fresh lotus was harvested about 48 h later from the same plot and minced to achieve a particle size distribution similar to that in swallowed boluses collected from a sheep which had been bailed (rumen contents removed) and fed on fresh *Lotus pedunculatus*. Several mincers were evaluated and a model of kitchen mincer (Moplen, Italy) was found to give an appropriate particle size distribution as determined by wet sieving (Waghorn *et al.* 1989). The FM and FD lotus were weighed (about 30 g wet weight and 5 g DM respectively) into polyester bags and suspended in the rumen of sheep by 10.00 hours for *in sacco* incubation.

Determination of N and protein-bound AA losses from FM and FD material required correction for microbial N colonization of the plant residues. This was done by labelling microbial protein with an intraruminal infusion of $(NH_4)_2{}^{35}SO_4$ according to the method described by Mathers & Aitchison (1981). The $(NH_4)_2{}^{35}SO_4$ (925 MBq, 925–1480 GBq/mg) was infused at 7·4 MBq/d in 230 ml water containing 0·5 g inert Na_2SO_4 (BDH, Poole, Dorset) for 7 d. The infusion began 3 d before commencing the polyester-bag incubations in the rumen. It was assumed that plateau specific activity (SA) of reducible-S (i.e. sulphide) was attained after 18 h infusion (Kennedy *et al.* 1975).

Twelve sheep were used to determine losses from both FM and FD lotus by placing five bags of prepared lotus in the rumen of each sheep on each of 4 d. On the first day, three control and three PEG sheep were used to incubate FM lotus, whilst an additional three control and three PEG sheep were used to incubate FD lotus. Bags were held in the rumen by large brass weights. The treatments were reversed on the following day and the sequence repeated on the third and fourth days.

Bags were removed from the rumen after 2, 4, 6·5, 11 and 24 h of incubation and were thoroughly washed in tap water for approximately 2 min until no further colour could be washed out of the bags. In addition, two further bags of FM and FD lotus which were not incubated in the rumen, were washed to give residues at 0 h. Plant residues from the bags were freeze-dried, weighed and stored at $-20°$ for analysis.

Expt 3

In Expt 3 the effect of CT on the degradation of Rubisco was measured using *in vitro* rumen incubations with and without added PEG and using the same FM and FD lotus preparations as in Expt 2. Two sheep with large rumen cannulas were fed on *Lotus pedunculatus* for 30 d before the experiment, and supplied rumen fluid for the *in vitro* incubations, which were carried out using the method described by McNabb *et al.* (1994). Either FM (7·0 g) or FD (0·9 g) *Lotus pedunculatus* was added to conical flasks (250 ml) containing 60 ml artificial saliva, pH 6·8 (McDougall, 1948) and 15 ml rumen fluid collected after a 15 h fast. Twelve flasks per incubation were prepared for each lotus preparation, with 70 mg PEG added to six flasks per treatment. One control and one PEG flask was removed at 0 h and at 1, 2, 4, 8 and 24 h after the start of incubation. Incubations were undertaken on two occasions. A 10 ml portion was rapidly frozen in ethanol–solidified CO_2, freeze-dried, ground (1 mm sieve size) and stored at −20° for the analysis of Rubisco. An additional 10 ml portion was taken after 0 and 24 h incubation, acidified with 0·3 ml H_2SO_4 (300 ml/l) and stored at 4° for NH_3 analysis.

Chemical analysis

The concentration of CT in freeze-dried feeds was determined colorimetrically following reaction of the end hydroxyl groups of the terminal monomers of CT with vanillin to form a red colour which is detectable at 500 nm, according to the method of Broadhurst & Jones (1978). Samples of feed, feed refusals and abomasal and ileal digesta were freeze-dried and ground before analysis of N by automated analysis of NH_3 following Kjeldahl digestion (Williams & Twine, 1967). NH_3 was determined by automated analysis (Technicon Industrial Systems, 1973), whilst the AA concentration in polyester-bag residues and microbial extracts was determined by ion-exchange chromatography as described by Waghorn *et al.* (1994*a*).

Expt 2. Microbial-N colonization of polyester-bag residues. Rumen microbes were isolated from rumen digesta taken from each sheep at 10.00 and 16.00 hours on the final day of the $(NH_4)_2{}^{35}SO_4$ infusion to determine microbial N and ^{35}S content. Rumen whole digesta (about 200 g) was stored at −20°, whilst strained rumen fluid was centrifuged at 1000 ***g*** for 1 min to sediment plant debris. The supernatant fraction was decanted and centrifuged at 20000 ***g*** for 20 min to obtain a microbial pellet, which was washed three times with ice-cold saline (9 g NaCl/l) and freeze-dried for analysis. Freeze-dried polyester-bag residues (300 mg) and rumen microbes (150 mg) were oxidized with performic acid to determine the radioactivity of ^{35}S-labelled amino acids (SAA; Mathers & Miller, 1980). The N in bag residues and rumen microbes was determined by a micro-Kjeldahl procedure (Tecator Kjeltec Auto 1030 Analyser; Tecator AB, Hoganas, Sweden).

Rubisco analysis by SDS–PAGE. Protein digestion buffer (400 μl; 62·5 mM-Tris–HCl, pH 6·8, 100 g glycerol/l, 20 g SDS/l, 0·05 g bromophenol blue/l, 50 ml 2-mercaptoethanol/l) was added to 20 mg freeze-dried and ground (1 mm sieve size) feed, abomasal and ileal digesta (Expt 1), polyester bag residues (Expt 2) and *in vitro* rumen incubation (Expt 3) samples. The samples were denatured by heating at 95° for 5 min and the soluble protein in 20 μl was fractionated by SDS–PAGE using a modification of the method described by Laemmli & Favre (1973). The gels (0·75 × 75 × 100 mm) consisted of a stacking gel (12·5 mM Tris–HCl, pH 6·8; and (g/l): acrylamide 38·9 , bisacrylamide 1·1, SDS 1, ammonium persulphate 1, tetramethyl-ethylenediamine 1) approximately 22 mm high, layered over a separating gel (37·5 mM-Tris–HCl, pH 8·8; and (g/l): acrylamide 155·7, bisacrylamide 4·3, SDS 1, ammonium persulphate 0·5, tetramethyl-ethylenediamine 0·5). Electrophoresis was carried out for about 3 h at 80 V, after which the gels were washed in

methanol–acetic acid–water (40:10:50, by vol.) for 30 min and total soluble protein was visualized by staining with Fast Green FCF (1 g Fast Green FCF/l methanol–acetic acid–water (40:10:50, by vol.)) for 30 min. The gels were destained in 10% methanol; 7·5% acetic acid for 24 h and Rubisco was quantified by imaging densitometry (Model GS-670 Imaging Densitometer with Molecular Analyst$^{TM/CP}$ imaging analysis software; BioRad, Hercules, CA, USA). Rubisco consists of eight large subunits (LSU; MW 54000) and eight small subunits (SSU; MW 16000; Kawashima & Wildman, 1970). Rubisco accounts for 30–50% of total plant protein (Mangan, 1982); therefore, LSU and SSU are readily detectable on stained gels as they represent the predominant proteins present. Rubisco was purified from lucerne (*Medicago sativa*) using the method of Jones & Lyttleton (1972) and was used to generate a standard curve for converting the density of the LSU and SSU which were determined by densitometry, to the concentration of LSU and SSU in samples. The LSU and SSU were added together and the results are presented as Rubisco. Purified Rubisco (10 μg) also was run on all gels as an internal standard for standardizing between gels.

Calculation of data and statistical analysis

Rubisco flux through the abomasum and ileum (Expt 1) was calculated as:

Rubisco flux (g/d) = Rubisco concentration in digesta (g/g N) × true N flux (g/d).

The plant N in bag residues (Expt 2) was calculated as total N less microbial N colonization. The microbial N colonization of bag residues was calculated according to the equation described by Mathers & Aitchison (1981).

Values are presented as means and standard deviation, standard error or standard error of the difference as appropriate. Comparison between control and PEG treatments was by ANOVA.

RESULTS

Expt 1

The *Lotus pedunculatus* fed during Expt 1 contained 55 g extractable CT and 38·6 g N/kg DM respectively. Rubisco represented 0·39 (SE 0·005) of the crude protein (N × 6·25) intake in control and PEG sheep. Before PEG infusion, rumen NH_3 concentrations were similar in the control and PEG sheep, but PEG infusion resulted in a significantly higher rumen NH_3 concentration (Waghorn *et al.* 1994*b*). During the 5 d digesta flow period, the DM and N intakes of control sheep (1165 (SE 20·8) g DM/d; 42·4 (SE 1·79) g N/d) were lower ($P < 0·01$) than those of sheep receiving PEG (1288 (SE 36·9) g DM/d; 47·6 (SE 0·90) g N/d).

Rubisco intake, flow and digestion are shown in Table 1. Although the lower DM intakes of control sheep resulted in a lower Rubisco intake than that of sheep receiving PEG, substantially more Rubisco passed through the abomasum of control sheep (29·8 g/d) than PEG sheep (5·3 g/d; $P < 0·001$). Rumen digestion of Rubisco was extensive in both treatments, but was less so in control (0·72) than PEG sheep (0·96; $P < 0·01$). Virtually all the Rubisco protein had disappeared from digesta collected at slaughter from the ileum of sheep in both treatments, so that CT enabled more Rubisco to be hydrolysed in the small intestine than when its effects were removed by PEG infusion. Digestibility of Rubisco in the small intestine exceeded 90% for both groups of sheep.

Expt 2

The *Lotus pedunculatus* fed during Expts 2 and 3 contained 134 g DM/kg and had a DM composition (g/kg) of: N 33·3, hemicellulose 94·4, cellulose 182·1, lignin 76·8, ash 82·2, extractable CT 45·0. The *Lotus pedunculatus* comprised 0·55 leaf and 0·45 stem, on a DM basis. Feed DM intakes were restricted to facilitate suspension of polyester bags in the

Table 1. *Expt 1*† *The intake and digestion of ribulose-1,5-*bis*phosphate carboxylase* (EC *4.1.1.39; Rubisco*) *by sheep fed on* Lotus pedunculatus, *with and without an intraruminal infusion of PEG*

(Mean values for four animals per treatment)

	Control sheep	PEG-infused sheep	SED	Statistical significance of difference
Rubisco				
Intake (g/d)	106·5	118·9	2·43	**
Abomasal flux:				
g/d	29·8	5·3	5·15	***
g/g eaten	0·28	0·05	0·051	**
Ileal flux:				
g/d	0·9	0·4	0·45	NS‡
g/g eaten	0·01	0·01	0·041	NS‡
Digestion in small intestine:				
g/d	28·9	4·9	4·90	***
g/g eaten	0·27	0·04	0·054	**
g/g entering SI	0·97	0·92	0·020	NS‡
Digestibility (proportion of intake)				
Rumen	0·72	0·96	0·046	**
Total	0·99	1·00	0·004	NS‡

SI, small intestine.
** $P < 0·01$, *** $P < 0·001$.
† For details of procedures, see pp. 537–540.
‡ $P > 0·05$.

Table 2. *Expt 2*. The particle size distribution* (*g/kg DM*) *of freshly-minced, and freeze-dried and ground* Lotus pedunculatus *used for* in situ *polyester-fibre-bag incubations, and of swallowed boli from sheep fed on fresh* Lotus pedunculatus

	Preparation method		
Particle size†	Freshly-minced	Freeze-dried and ground	Chewed boli
> 1 mm	267	22	351
0·25–1 mm	223	452	162
< 0·25 mm	212	175	155
Soluble	298	351	332

* For details of procedures, see pp. 537–538.
† Aperture size of sieves.

rumen, with control sheep eating 818 (SE 50·9) g DM/d and PEG sheep eating 893 (SE 48·6) g DM/d ($P > 0·05$). The concentration of NH_3 in rumen fluid (mg NH_3-N/l) before PEG infusion was 186 (SE 4·6) in control sheep and 152 (SE 4·2) in PEG sheep ($P < 0·05$). However, after 3 d of PEG infusion, rumen NH_3 concentration (mg NH_3-N/l) increased to 398 (SE 7·3; $P < 0·001$), whereas it was not significantly different in control sheep (204 (SE 6·8); $P > 0·05$).

The particle size distribution of FM and FD *Lotus pedunculatus* used for polyester bag incubations and that of chewed *Lotus pedunculatus* is given in Table 2. The mincing of fresh

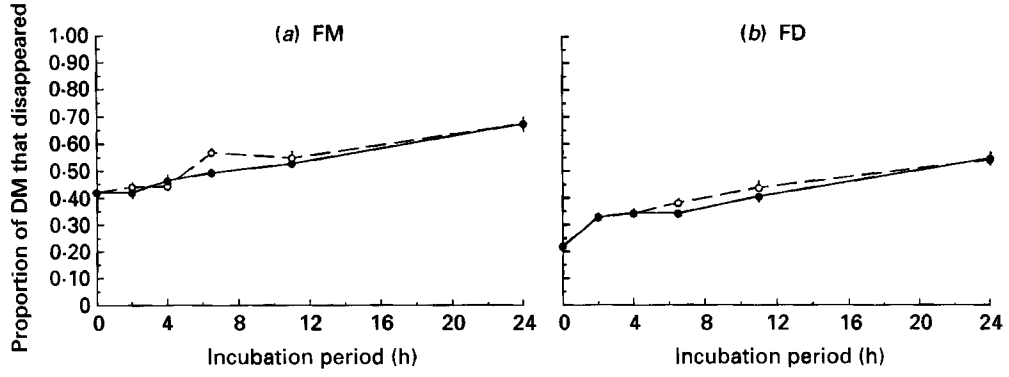

Fig. 1. Expt 2. The loss of DM from freshly-minced (FM) or freeze-dried and ground (FD) *Lotus pedunculatus* contained in polyester bags incubated in the rumen of control sheep and sheep given an intraruminal infusion of PEG. (●—●), Control sheep; (○---○), PEG sheep. Values are means with their standard errors represented by vertical bars for six sheep per treatment. For details of experimental procedures, see pp. 537–538.

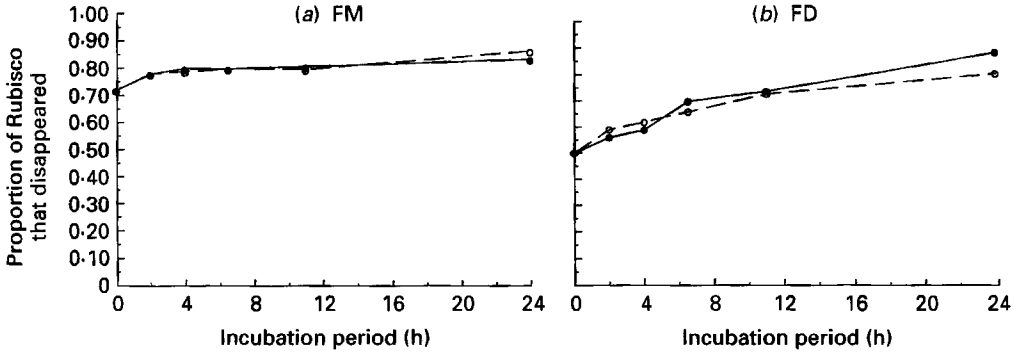

Fig. 2. Expt 2. The loss of ribulose-1,5-*bis*phosphate carboxylase (*EC* 4.1.1.39; Rubisco) from freshly-minced (FM) or freeze-dried and ground (FD) *Lotus pedunculatus* contained in polyester bags incubated in the rumen of control sheep and sheep given an intraruminal infusion of PEG. (●—●), Control sheep; (○---○), PEG sheep. Polyester bag residues for the analysis of Rubisco were bulked within treatments at each sampling time. For details of experimental procedures, see pp. 537–540.

Lotus pedunculatus resulted in a smaller mean particle size than chewing; however, mincing was more representative of chewed and swallowed *Lotus pedunculatus* than the FD preparation.

For both preparations (FM and FD), DM (Fig. 1) and Rubisco (Fig. 2) losses from polyester bags incubated in the rumen were similar for control and PEG sheep; however, the DM lost from FM lotus was always greater ($P < 0{\cdot}01$) than that lost from FD lotus. The initial solubilization of Rubisco was greater from FM lotus (0·72) than that from FD lotus (0·50; Fig. 2), but after 24 h incubation the losses were similar.

The solubilization of N from *Lotus pedunculatus* (corrected for microbial N colonization) is given in Fig. 3. For the FM preparation there was no overall difference ($P > 0{\cdot}05$) between control and PEG treatments, apart from a significantly ($P < 0{\cdot}001$) lower value for the control preparation after 11 h incubation in the rumen. However, for the FD lotus there was a significant ($P < 0{\cdot}05$) difference between control and PEG treatments at all sampling times. In the absence and presence of PEG, N solubilization was greater for FM than for FD lotus at all sampling times ($P < 0{\cdot}001$).

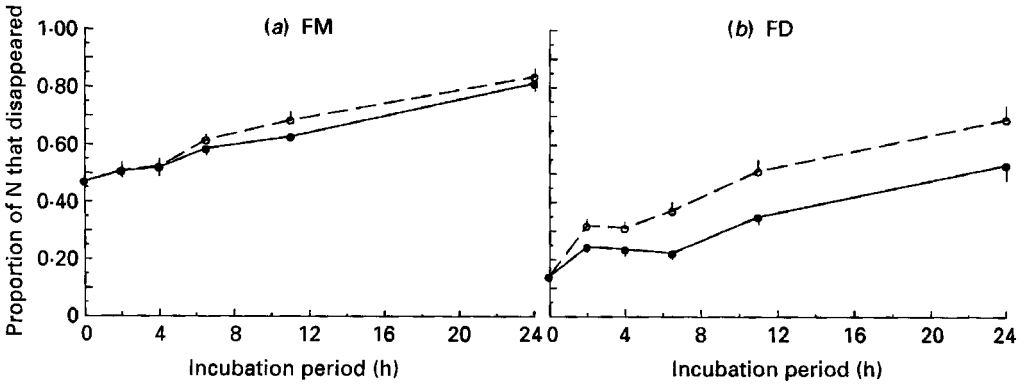

Fig. 3. Expt 2. The loss of N corrected for microbial N colonization, from freshly-minced (FM) or freeze-dried and ground (FD) *Lotus pedunculatus* contained in polyester bags incubated in the rumen of control sheep and sheep given an intraruminal infusion of PEG. (●—●), Control sheep; (○---○), PEG sheep. Values are means with their standard errors represented by vertical bars for six sheep per treatment. For details of experimental procedures, see pp. 537–540.

Table 3. *Expt 2.* The amino acids† (AA, mg) in freshly-minced (FM) and freeze-dried and ground (FD)* Lotus pedunculatus *in plant residues after 0 and 24 h incubation in the rumen of sheep fed on* Lotus pedunculatus *with and without an intraruminal infusion of PEG*

(The concentration of AA was determined on polyester-bag residues which were pooled within treatment groups before and after 24 h incubation)

	Control sheep	PEG-infused sheep
FM:		
Feed	815·0	815·0
Residues after 0 h‡	483·2	483·2
Residues after 24 h§	95·8	35·5
Proportion of AA lost		
After 0 h	0·407	0·407
After 24 h	0·882	0·956
FD:		
Feed	932·0	932·0
Residues after 0 h‡	805·9	805·9
Residues after 24 h§	381·5	238·4
Proportion of AA lost		
After 0 h	0·135	0·135
After 24 h	0·591	0·744

* For details of procedures, see pp. 537–540.

† Cysteine, methionine, proline and tryptophan are not included.

‡ The AA content in polyester bags before incubation, after washing in running water to remove soluble plant material.

§ The AA content in polyester bag residues remaining after 24 h incubation in the rumen have been corrected for microbial AA contamination.

The loss of protein-bound plant AA after 24 h incubation in the rumen was calculated by subtracting microbial AA (314 mg/g microbial DM) from the total AA in bag residues. The true loss of protein-bound AA from lotus suspended in the rumen in polyester bags was much greater from FM than FD lotus initially (0 h), and after 24 h incubation the losses of protein-bound AA remained higher for FM than FD lotus (Table 3). Control sheep had

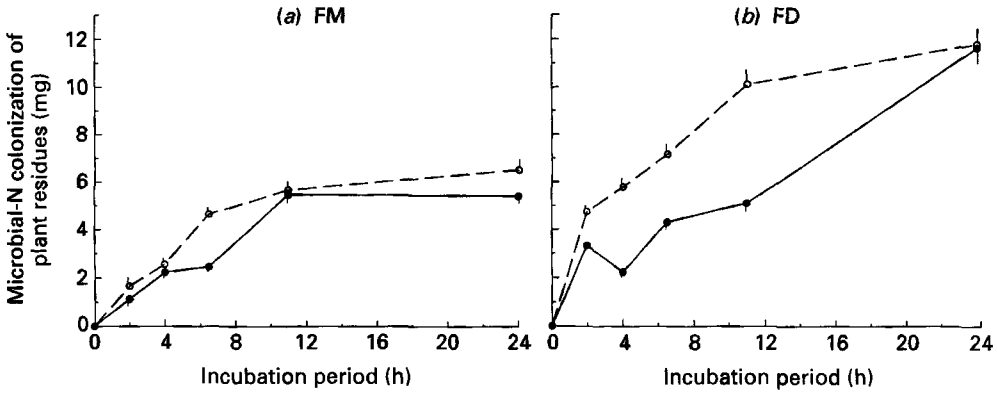

Fig. 4. Expt 2. Microbial N colonization of plant residues in polyester bags after *in sacco* incubation of freshly-minced (FM) and freeze-dried and ground (FD) *Lotus pedunculatus* in the rumen of control sheep and sheep given an intraruminal infusion of PEG. (●—●), Control sheep; (○---○), PEG sheep. Values are means with the standard errors represented by vertical bars for six sheep per treatment. For details of experimental procedures, see pp. 537–549.

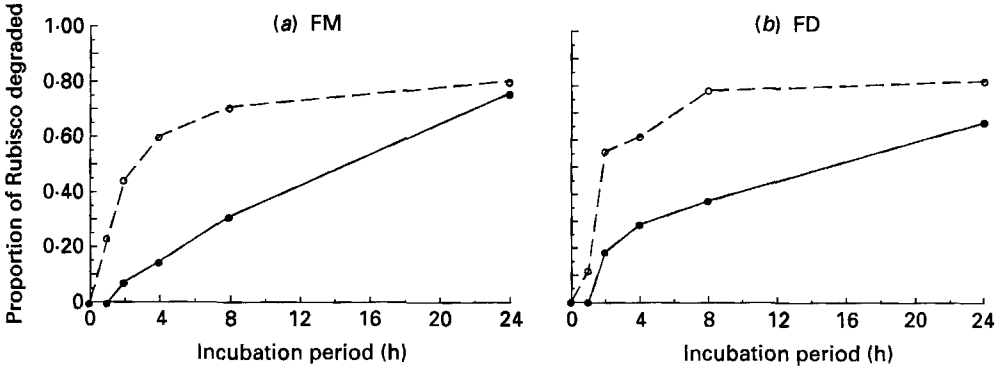

Fig. 5. Expt 3. The *in vitro* degradation of ribulose-1,5-*bis*phosphate carboxylase (*EC* 4.1.1.39; Rubisco) from freshly-minced (FM) or freeze-dried and ground (FD) lotus in an incubation with rumen fluid from sheep fed on *Lotus pedunculatus*. (●—●), Control incubation; (○---○), PEG added to incubation. Values are means for two incubations per treatment. For details of experimental procedures, see pp. 537–540.

a lower true loss of protein-bound AA after 24 h incubation than PEG sheep, with the effect being much smaller for FM than FD lotus.

Total microbial N colonization of FM and FD *Lotus pedunculatus* residues remaining in polyester bags at 0, 2, 4, 6·5, 11 and 24 h of incubation in the rumen are shown in Fig. 4. The microbial mass associated with FM lotus residues increased until plateau was reached after 11 h of incubation. Microbial N in FM lotus residues was always somewhat lower in control than PEG sheep with the difference being significant after 6·5 ($P < 0·001$) and 24 ($P < 0·05$) h incubation. A similar pattern was seen with FD lotus, but the differences were much more striking. Less microbial N was associated with residues in control than PEG sheep at 2 ($P < 0·01$), 4 ($P < 0·001$), 6·5 ($P < 0·001$) and 11 ($P < 0·001$) h of incubation.

Expt 3

The *in vitro* degradation of Rubisco in rumen fluid from sheep fed on *Lotus pedunculatus* is given in Fig. 5. In the absence of PEG the net release of NH_3 (mg NH_3-N/g DM) after 24 h was similar for FM (2·76) and FD (2·18) lotus, but the addition of PEG to the

incubations increased the net release of NH_3 to 14·71 and 15·03 mg NH_3-N/g DM for FM and FD lotus respectively. Rubisco was readily detectable in both FM and FD preparations before incubation; however, the action of CT delayed the onset of Rubisco degradation by about 1 h for both feed preparations compared with when PEG was added. It was calculated that 50% of Rubisco was degraded after 14·4 and 13·2 h incubation of FM and FD lotus respectively. The addition of PEG to *in vitro* rumen incubations reduced the period of time required for 50% of the Rubisco to be degraded to 3·1 and 2·9 h for FM and FD lotus respectively.

DISCUSSION

The principal objective of the present study was to determine how the CT in *Lotus pedunculatus* affected the digestion of Rubisco in the rumen. Chloroplasts contain 69% of their dry weight as protein (Kirk & Tilney-Bassett, 1967) and account for 75% of the total leaf protein (Mangan, 1982). Chloroplast protein consists of two major fractions: the soluble stromal protein, Rubisco, and the structural lamellar proteins (Mangan & West, 1977). Chloroplasts are the major source of dietary protein for herbage-fed ruminants, and Rubisco is the most abundant protein in chloroplasts, representing 0·39 of the crude protein eaten in the present study. The present study has shown that the action of CT reduced the disappearance of Rubisco in the rumen of sheep fed on fresh *Lotus pedunculatus*, enabling more Rubisco to be digested in the small intestine. This was due to CT reducing the rate of Rubisco degradation in the rumen, whilst solubilization was unaffected.

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 fluid phase of the rumen following chewing and it is an important determinant of its susceptibility to degradation (Mangan, 1972, 1982; Nugent *et al.* 1983; Wallace, 1983, 1985). Degradation is the catabolism of protein by microbial proteases resulting in the formation of peptides, AA and NH_3. The increase in rumen NH_3 concentration with PEG infusion (Expts 1 and 2) showed that PEG was able to reduce the effects of CT, and increase the degradation of forage protein to NH_3. However, the objective of the present study was to ascertain whether CT affected protein solubilization, protein degradation or both. The loss of N from synthetic-fibre bags has gained wide acceptance as an index of protein degradability in the rumen (Mehrez & Ørskov, 1977). However, Spencer *et al.* (1988*b*) reported that individual pea-seed proteins (albumins) were relatively resistant to rumen degradation, despite the almost complete loss of pea-seed N from synthetic-fibre bags suspended in the rumen. In the present study we have defined the loss of N and Rubisco from *Lotus pedunculatus* suspended in polyester bags in the rumen as solubilization. Protein solubilization and degradation in the rumen may be independent processes and solubilization, defined as the loss of N from synthetic-fibre bags suspended in the rumen, may not always be a good index of dietary protein degradation. In the present study the rate of N, protein-bound AA and Rubisco loss from *Lotus pedunculatus* in polyester bags suspended in the rumen was used to indicate how CT affected protein solubilization. Protein degradation, either *in vitro* or *in vivo* can be quantified by identification of individual proteins using SDS–PAGE (Nugent & Mangan, 1981; Spencer *et al.* 1988*b*; McNabb *et al.* 1994) and measuring their rate of disappearance.

Previous studies have shown that CT in *Lotus pedunculatus* reduced rumen NH_3 concentration, increased the flow of NAN and AA to the small intestine and often resulted in an increased absorption of EAA from the small intestine (Barry & Manley, 1984; Barry *et al.* 1986; Waghorn *et al.* 1987; McNabb *et al.* 1993). Work reported here has shown that these effects were due, in part, to a reduction in the disappearance of Rubisco in the rumen and an increase in the digestion of Rubisco in the small intestine when CT was present. This

was due to the ability of CT to reduce microbial degradation of Rubisco, whilst the solubilization of Rubisco was unaffected by CT. However, solubilization of Rubisco was affected by the method of feed preparation. Rumen degradability of Rubisco was calculated to be 0·72 and 0·96 of intake for control and PEG sheep respectively, showing that the action of CT had slowed rather than prevented Rubisco degradation. This confirms the general observation of a slower rumen turnover rate in sheep fed on *Lotus pedunculatus* alone compared with *Lotus pedunculatus* with PEG (Waghorn *et al.* 1994*b*).

The action of CT reduced the solubilization of N and protein-bound AA from FD *Lotus pedunculatus* incubated in the rumen in polyester bags, but CT did not affect losses from FM lotus to the same extent. The very different responses are of particular importance when evaluating fresh forages using both *in sacco* and *in vitro* incubation techniques. FM *Lotus pedunculatus* was included in the present study in an attempt to mimic the degree of particle breakdown which occurs during chewing. It is clear that FM material closely resembled the particle size distribution of boluses swallowed by sheep eating fresh *Lotus pedunculatus* (Table 2), red (*Trifolium pratense*) and white (*Trifolium repens*) clover (Waghorn & Shelton, 1988) and of rumen digesta (Ulyatt *et al.* 1986), especially with regard to the release of soluble constituents. When the composition of bag residues taken before rumen incubation (0 h) were compared, it was evident that freeze-drying and grinding reduced the solubilization of N and Rubisco relative to FM plant material. Therefore, the chemical and physical composition of the plant residues in polyester bags which were available for microbial colonization and fermentation in the rumen were quite different for the two feed preparations. It is unlikely that PEG *per se* affected microbial colonization or fermentation, because previous studies when lucerne (*Medicago sativum*) and PEG were fed to sheep demonstrated that PEG had no effect on the digestion of that non-CT-containing forage (Wang *et al.* 1994). In the present study we did not have a non-CT-containing forage with and without PEG as a control, although the effects of PEG certainly warrant future research as they are pivotal to our interpretation of CT. However, it would seem illogical to evaluate forages which are fed fresh to ruminants by first freeze-drying and grinding them, because freeze-drying appeared to exaggerate the effect of CT on N solubilization.

In contrast to the effects on N and protein-bound AA, CT did not appear to have any effect on the solubilization of Rubisco from either FM or FD lotus incubated in the rumen. Although in the present study we were able to use SDS–PAGE and imaging densitometry successfully to identify and quantify the effect of CT on the solubilization of the LSU and SSU of Rubisco, other less-abundant plant proteins remaining in polyester bag residues were not resolved by SDS–PAGE. Plant proteins exhibit a wide range of solubilities (Mangan, 1982), therefore the solubilization of these other proteins may have been affected by CT. This may explain why the loss of N and total AA from polyester bags was affected by CT, even though Rubisco loss was unaffected and this should be considered for future research.

Tanner *et al.* (1994) have shown that proanthocyanidin (CT), purified from *Lotus pedunculatus*, reduced the degradation of Rubisco from lucerne leaves by 3-fold when added to *in vitro* incubations containing rumen fluid. In the present study, CT resulted in a 4-fold reduction in the degradation of Rubisco at 2 and 4 h of incubation. The extent to which CT reduced Rubisco degradation was similar for both FM and FD feed preparations; therefore, once solubilization of protein occurred, it was degraded at a similar rate irrespective of whether it was derived from FM or FD lotus.

In the present study the action of 55 g extractable CT/kg DM in *Lotus pedunculatus* increased the abomasal flux of both Rubisco and total AA. However, whilst this level of CT increased the digestion of Rubisco in the small intestine, total AA absorbed was unchanged. This was due to the action of CT in reducing the apparent digestibility of most

AA in the small intestine (Waghorn *et al.* 1994*a*). CT are able to bind to protein by H bonding at multiple sites, and by hydrophobic and covalent interactions (Spencer *et al.* 1988*a*). The H bonds are continuously broken and reformed randomly (McLeod, 1974), whilst the degree of bonding between CT and protein is also affected by the chemical structure of the protein (Asquith & Butler, 1986; Horigome *et al.* 1988). During the process of protein digestion in the small intestine, CT may form stronger bonds with small peptides and even individual AA than with intact proteins such as Rubisco, so as to reduce the apparent digestibility of many individual AA in the small intestine when sheep are fed on *Lotus pedunculatus*, despite an apparent increase in the amount of Rubisco digested. However, in sheep fed on *Lotus corniculatus* (with a lower CT content; 22 g CT/kg DM), CT increased the apparent absorption of EAA from the small intestine (Waghorn *et al.* 1987), suggesting that CT from that forage reacts differently in the small intestine, so it may provide one practical means of increasing the digestion of protein in the small intestine. Further research is necessary to define better the relationship between CT concentration and reactivity on protein digestion and AA absorption from the small intestine.

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