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Effect of Condensed Tannins Prepared from Several Forages on the *In Vitro* Precipitation of Ribulose-1,5-*bis*phosphate Carboxylase (Rubisco) Protein and its Digestion by Trypsin (EC 2.4.21.4) and Chymotrypsin (EC 2.4.21.1)

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Abstract: A series of *in vitro* experiments was undertaken to determine the extent to which Sephadex LH-20 treated extracts from a range of temperate forages precipitated ribulose-1,5-*bis*phosphate carboxylase (Rubisco) and affected the enzymatic hydrolysis of Rubisco protein by trypsin and chymotrypsin at a range of pH values. Rubisco was chosen because it represents the principal dietary protein for ruminants fed fresh forages. Condensed tannins (CT) or proanthocyanidins (PA) are routinely purified by chromatography using Sephadex LH-20 as a matrix. However, these extracts contained non-CT phenolics together with PA so the term 'CT extract' was preferred to 'PA' to describe the extracts. The *in vitro* precipitation of Rubisco provided a means to compare the reactivity of the CT extracts. The amount of CT extract required to precipitate all the Rubisco in 10 μg of total soluble leaf protein from white clover (*Trifolium repens*) when this protein was incubated with CT extracts of *Lotus corniculatus*, *L pedunculatus* and sainfoin (*Onobrychis viciifolia*) was similar, with between 25 and 50 μg of extract required. The CT extract of sulla (*Hedysarum coronarium*) also precipitated all the Rubisco, however this only occurred with 50 μg of the extract. The CT extract of dock (*Rumex obtusifolius*) precipitated all the Rubisco when 5 μg of extract or greater was incubated with total soluble leaf protein. However, the differences between the reactivity of all these CT extracts at a range of pH values appeared to be small. Condensed tannin extracts of *L corniculatus* and *L pedunculatus* partially inhibited the hydrolysis of Rubisco by trypsin and chymotrypsin to a similar extent, but the extent of the inhibition was affected by pH. The inhibition was greater at pH 6·0 than 7·0, whilst at pH 8·0, CT extracts had little or no affect on trypsin and chymotrypsin. It was concluded that, although the precipitation of Rubisco provided an ideal method for comparing CT extracts, reactivity alone was unlikely to account for the differences in nutritive value that occur with forages containing CT. © 1998 SCI.

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INTRODUCTION

Condensed tannins (CT) or proanthocyanidins (PA) are a unique group of phenolic compounds of relatively high molecular weight which occur in a range of herbaceous legumes (Sarkar *et al* 1976; Terrill *et al* 1992) and tree leaves (Kumar and Vaithiyanathan 1990). When fresh forages containing CT were fed to sheep, a higher proportion of non-ammonia-nitrogen (NAN) reached the small intestine than with similar forages without CT or where the CT had been rendered unreactive by addition of polyethylene glycol (PEG) to the diet (Barry and Manley 1984; Waghorn *et al* 1987a,b). This is probably due to the insoluble complexes which form between CT and plant proteins at pH values common in the rumen (6–7; Jones and Mangan 1977) and which reduce the microbial degradation of plant protein (Waghorn *et al* 1987a,b, 1989; McNabb *et al* 1993; Tanner *et al* 1994). The CT–protein complex dissociates at pH values below 3·5 (Jones and Mangan 1977), presumably allowing protein to be digested and absorbed from the small intestine.

The CT in *Lotus corniculatus* (22 g CT kg^{-1} dry matter (DM); Waghorn *et al* 1987b) fed to sheep decreased nitrogen (N) digestibility by 8 percentage units but increased the net apparent absorption of essential amino acids (EAA) from the small intestine by 0·63. Voluntary feed intake was unaffected by the CT in that study. In a similar experiment, the CT in *L pedunculatus* (55 g CT kg^{-1} DM; Waghorn *et al* 1994) reduced N digestibility by 12 percentage units and voluntary intake by 0·12. The net apparent absorption of EAA from the small intestine was unaffected by the CT in that study. In sheep fed a mixed diet consisting of ryegrass (*Lolium perenne*) and *Lotus pedunculatus* with a final CT concentration in the mixed diet of 18 g CT kg^{-1} DM, N digestibility was reduced by 13 percentage units (Waghorn and Shelton 1995) and the effects of CT were similar to *L pedunculatus* (55 g CT kg^{-1} DM) fed to sheep as their sole diet (Waghorn *et al* 1994). The substantial reductions in N digestibility caused by the CT in *L pedunculatus* in these studies did not cause a reduction in the nutritive value of this forage for sheep. The CT in *L pedunculatus* (90 g CT kg^{-1} DM; Barry *et al* 1986) reduced N digestibility by 21 percentage units and voluntary feed intake by 0·12 and depressed animal production.

Other forages whose high concentrations of CT (>50 g CT kg^{-1} DM) have resulted in anti-nutritional effects when consumed include serecia lespedeza (*Lespedeza cuneata*; Windham *et al* 1990), mulga (*Acacia aneura*; Pritchard *et al* 1988) and eucalyptus (*Eucalyptus melliodora*; Foley and Hume 1987). However, not all the anti-nutritional affects of CT can be attributed to their high concentration in the diet. Sainfoin (*Onobrychis viciifolia*; 50–80 g CT kg^{-1} DM), when fed to ruminants has a higher nutritive value than similar forages without CT (Ulyatt *et al* 1976).

Variation in nutritional responses to CT from different forages is affected by concentration and may be affected by the chemical structure which will affect the reactivity or astringency of the CT. Reactivity can be defined as the ability of CT to precipitate protein per unit weight (Bate-Smith 1973) and is reported to increase with increasing degree of polymerisation (Horigome *et al* 1988) and delphinidin: cyanidin (PD : PC) ratio (Jones *et al* 1976).

The reactivity of CT has been determined by a number of methods including the colorimetric estimation of haemoglobin remaining in solution after reaction of CT with the proteins of haemolysed blood (Bate-Smith 1973) but proteins differ greatly in their affinity for a particular CT (Hagerman and Butler 1981; Asano *et al* 1982). Therefore, it was desirable to develop a method for measuring the reactivity of CT towards the principal leaf protein, ribulose-1,5-*bis*phosphate carboxylase (Rubisco; Fraction 1 leaf protein). This would enable the reactivity of the CT in several forages grown in New Zealand (NZ) to be compared and to ascertain if differences in the reactivity of CT may be responsible for the different effects of *L corniculatus* (Waghorn *et al* 1987) and *L pedunculatus* (Waghorn *et al* 1994) on the apparent absorption of amino acids from the small intestine. Rubisco was chosen because it represents 30–50% of the protein in plants and is the major dietary protein for ruminants fed fresh forages (Mangan and West 1977).

MATERIALS AND METHODS

Experimental design

Five *in vitro* experiments were undertaken to determine the extent to which Sephadex LH-20 treated extracts from dock (DK; *Rumex obtusifolius*), *Lotus corniculatus* (LC), *Lotus pedunculatus* (LP), sainfoin (SN; *Onobrychis viciifolia*), sulla (SL; *Hedysarum coronarium*) and white clover (WC; *Trifolium repens*) precipitated Rubisco protein and affected its enzymatic hydrolysis. Proanthocyanidins are routinely purified using affinity chromatography with Sephadex LH-20 as a matrix (Porter *et al* 1986; Terrill *et al* 1992). However, Jackson *et al* (1996) reported that the Sephadex LH-20 treated extract of LC, although containing predominantly PA, also contained other non-CT phenolic compounds. Therefore, in the present study, the term 'CT extract' was preferred to reflect this composition rather than the more specific term 'PA' to describe the preparations that were used to precipitate protein.

In three of the experiments, the effect of the CT extracts was determined with or without the addition of polyethylene glycol (PEG). Polyethylene glycol binds to

CT and prevents the CT from binding to protein (Jones and Mangan 1977); hence the effect of the CT extracts can be deduced by comparing reactions without added PEG (CT active) to reactions with added PEG (CT inactivated). Rubisco and BSA were quantified in all experiments using imaging densitometry after separation of proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The *in vitro* precipitation of Rubisco provided a means to compare the reactivity of the CT extracts.

Preparation of CT extracts from plants

The CT extracts were prepared using the method of Porter *et al* (1986) but with the following modifications. The fresh leaves (about 3 kg) of each forage were extracted with acetone/H_2O (70 : 30 v/v) containing ascorbic acid (1 g litre^{-1}) and the resulting concentrated extracts washed five times with methylene chloride (industrial grade; Tergo Industries, Auckland, NZ) to remove chlorophyll and lipids. The aqueous defatted crude extracts were then freeze-dried and approximately 25 g of the dried products were redissolved in 150 ml of 1 : 1 methanol/H_2O (v/v). This material was placed on a column containing 200 ml of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and washed with 2 litres of 1 : 1 methanol/H_2O (v/v) before eluting the CT fractions with 200 ml of acetone/H_2O (70 : 30 v/v). The CT extracts were freeze-dried and stored at −20°C.

Analysis of CT extracts

The CT extracts were analysed by ^{13}C-nuclear magnetic resonance (NMR) and by thin layer chromatography (TLC) of the anthocyanidins produced following the reaction of the extracts with 50% HCl in 2,2-dimethyl propanol. The extracts were dissolved in acetone-D_2O and ^{13}C-NMR spectra were obtained using a Bruker AC 300 NMR spectrometer according to the method described by Foo *et al* (1996). The spectra were examined for the presence of carbon signals characteristic of CT chemical structures (Porter *et al* 1982). Anthocyanidins were generated by treating CT extracts (3 mg) with 2 ml of 5% HCl (v/v) in 2,2-dimethyl propanol at 100°C for 1 h in sealed tubes. The anthocyanidins produced were resolved by TLC on cellulose plates developed with Forestal solvent comprising acetic acid/conc HCl/water (30 : 30 : 10 v/v; Harborne 1967).

Extraction of total soluble protein from leaves

Total soluble protein was extracted from fresh WC by grinding 30 g of leaves with 90 ml protein extraction buffer (0·1 M TES; *N*-tris[hydroxymethyl] methyl-2-aminoethanesulphonic acid), pH 7·0; 1·16% NaCl; 0·04% EDTA in a mortar and pestle held on ice. The extract was centrifuged at 12 000 × *g* for 15 min at room temperature to remove all particulate plant material. The supernatant was then filtered through a 0·2 μm syringe filter (Minisart, Sartorius, Germany) to clarify the total plant protein extract. Total soluble leaf protein in the extract was determined using the Bradford (1976) protein assay.

Precipitation of protein by Sephadex LH-20 extracts

Experiment 1: The precipitation of Rubisco and BSA by CT extracts

Aliquots were dispensed to give 0, 0·1, 0·5, 1·0 and 10, 25 and 50 μg respectively, of the CT extract of LC or LP and then 50 μl McIlvaine's buffer pH 7·0 (Elving *et al* 1956) was added. An aliquot of total soluble leaf protein (10 μg) or BSA (10 μg; Sigma, USA) was added and all reactions were diluted to a final volume of 100 μl with McIlvaine's buffer (pH 7·0) and the *in vitro* protein precipitation assay undertaken.

The in vitro *protein precipitation assay.* All reactions were incubated at 39°C for 90 min. After incubation the tubes were centrifuged at 12 000 × *g* for 10 min and the resulting pellet, which contained precipitated protein, was resuspended three times in McIlvaine's buffer (pH 7·0) to remove all the protein that was not precipitated. Finally, the pellet was resuspended in 20 μl SDS-PAGE loading buffer (62·5 mM Tris-HCl, pH 6·8; 2% SDS; 10% glycerol; 0·005% bromophenol blue; 5% 2-mercaptoethanol) and stored at −20°C for SDS-PAGE. All incubations were carried out in duplicate.

Experiment 2: The precipitation of Rubisco by CT extracts from several forages

Aliquots from CT extracts (0·1 or 2 mg ml^{-1}) of DK, LC, LP, SN, SL and WC where dispensed to give 0, 0·1, 0·5, 1·0, 10, 25 or 50 μg of each CT extract. An aliquot of total soluble leaf protein (10 μg) was added to all CT extracts and the reactions diluted to a final volume of 100 μl with McIlvaine's buffer, pH 7·0. In Experiment 2, half the reactions also contained PEG. Barry and Forss (1983) reported that 2 mg PEG g^{-1} CT was required to inactivate CT so it was assumed that 100 μg of PEG (molecular weight (MW) 3500; Union Carbide, USA) was required and this was added to the appropriate reactions and the *in vitro* protein precipitation assay undertaken as described in Experiment 1.

Experiment 3: The solubility of Rubisco at different pH values

An aliquot of total soluble leaf protein (10 μg) was added to all mixtures and diluted to a final volume of 100 μl with McIlvaine's buffer at either pH 2·0, 3·0, 5·0, 5·5, 6·0, 7·0 or 8·0. The *in vitro* protein precipitation assay was then undertaken as described Experiment 1.

Experiment 4: The precipitation of Rubisco at different pH values by CT extracts

An aliquot from a stock solution (2 mg ml^{-1}) was dispensed to give reactions containing 50 μg of CT extract from DK, LC, LP, SN, SL and WC and then 50 μl of McIlvaine's buffer at either pH 2·0, 3·0, 5·0, 5·5, 6·0, 7·0 or 8·0 was added. An aliquot of total soluble leaf protein (10 μg) was added to all the reactions and PEG (100 μg) was added to half the reactions. Finally all the reactions were diluted to a final volume of 100 μl with McIlvaine's buffer at the appropriate pH. The *in vitro* protein precipitation assay was then undertaken as described in Experiment 1.

Experiment 5: The affect of CT extracts on the enzymatic hydrolysis of Rubisco by trypsin and chymotrypsin

Wang *et al* (1996) reported that the pH in the proximal part of the small intestine, where trypsin and chymotrypsin are secreted into the digesta, was about 5·0 and increased to about 8·0 by the distal part of the small intestine so trypsin and chymotrypsin assays were undertaken at a range of pH values between 5·0 and 8·0. An aliquot of total soluble leaf protein (10 μg) and 50 μl of McIlvaine's buffer at either 5·0, 6·0, 7·0 or 8·0 was added to all reactions. An aliquot of CT extract (50 μg) from LC and LP was added to half of the reactions and all the reactions were diluted to a final volume of 100 μl with McIlvaine's buffer of the appropriate pH. After incubating all the reactions at 39°C for 90 min, an aliquot (0·5 μg) of either trypsin (EC 3.4.21.4; Type 111, Bovine Pancreas; Sigma, USA) or chymotrypsin (EC 3.4.21.1; Type II, Bovine Pancreas; Sigma, USA), or trypsin or chymotrypsin preincubated at 39°C for 90 min with CT extracts from LC and LP (in the ratio of 10 μg of enzyme to 50 μg of CT extract) were added to 75% of the reactions. The remaining reactions received McIlvaine's buffer of the appropriate pH only. All reactions were incubated for a further 30 min at 39°C before enzymatic hydrolysis was stopped by the addition of 10 μl the trypsin/chymotrypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF; 10 mg ml^{-1} in propan-2-ol; Sigma, USA). Total protein was then precipitated at −20°C for 1 h following the addition of 500 μl of acetone. Following centrifugation at 12 000 × *g* for 10 min, pellets were dissolved in 20 μl of SDS-PAGE loading buffer and stored at −20°C for SDS-PAGE. All incubations were repeated in duplicate.

Analysis of samples

Rubisco analysis by SDS-PAGE

Prior to fractionation by SDS-PAGE, samples were heated for 5 min at 95°C to denature protein and dissociate all protein–CT complexes and then cooled on ice. After centrifugation at 12 000 × *g* for 5 min, the soluble protein in the supernatant was fractionated by SDS-PAGE using the method described by McNabb *et al* (1996). After SDS-PAGE, the protein in the gels was fixed by washing the gels in methanol/acetic acid/water (40 : 10 : 30, v/v) for 30 min and total protein was visualised by staining with Coomassie Brilliant Blue R-250 (0·1% w/v in methanol/acetic acid/water (40 : 10 : 50, v/v)) for 30 min. Gels were de-stained in methanol/acetic acid/water (10 : 7·5 : 82·5, v/v) for 48 h to detect protein bands. Developed gels were quantified by imaging densitometry (Bio-Rad, Model GS-670 Imaging Densitometer with Molecular Analyst™/CP imaging analysis software, USA). Rubisco consists of eight large subunits (LSU; molecular weight (MW) 54 000) and eight small subunits (SSU; MW 16 000; Kawashima and Wildman 1970) and accounts for 30–50% of total plant protein (Mangan and West 1977). The LSU and SSU represented the predominant proteins present in the total soluble leaf protein extract and were readily detectable on stained gels.

Calculation of data

The LSU and SSU behaved in an identical manner and so were added together and the results are presented as Rubisco.

The proportion of protein which was precipitated by CT extracts was calculated according to eqn (**1**).

$$\text{Proportion precipitated} = \left(\frac{X_{CT} - X_0}{T}\right) \qquad (1)$$

where X_{CT} is the intensity of protein bands on SDS-PAGE gels precipitated by CT extracts added to the *in vitro* protein precipitation assay; X_0 is the intensity of protein bands on SDS-PAGE gels precipitated when no CT extracts were added to the *in vitro* protein precipitation assay; and T is the intensity of protein bands when 10 μg of BSA or total soluble plant protein was fractionated on SDS-PAGE gels.

The proportion of Rubisco in 10 μg of total soluble leaf protein which was hydrolysed by either trypsin or chymotrypsin was calculated in a similar way.

RESULTS

Analysis of CT extracts by ^{13}C-NMR and TLC

The CT extract of LC consisted predominantly of CT, although other non-CT phenolics, including flavonoid glycosides were also present. The CT had predominantly procyanidin-type subunits although some prodelphinidin-type subunits were also present. In contrast, the CT in the CT extract of LP contained predominantly prodelphinidin-type subunits although some procyanidin-type subunits were also detected. The CT extracts of SN and SL also consisted predominantly

of CT, although some flavonoid glycosides were also present. The CT in both species were predominantly of the prodelphinidin-type, where the B-rings of the monomeric flavans possessed a pyrogalloyl oxidation pattern. The CT extract of DK, although predominantly CT, also contained some flavonoid glycosides. The CT in the extract from DK was relatively homogeneous consisting of mostly 2,3-*cis*-flavan units with catechol B-ring oxidation pattern or procyanidin-type subunits with epicatechin extender units. The CT extract of WC was predominantly a mixture of flavonoid glycosides, although some CT was present. The CT of this forage contained predominantly prodelphinidin-type subunits. As estimated from their ^{13}C-NMR spectra, the CT represented about 0·9 of the CT extracts of DK, LC, LP, SN and SL. This was a rough estimate made by comparing the signal size of carbons with similar chemical environments. No ^{13}C-NMR signals consistent with hydrolysable tannins were detected in any of the CT extracts although there was evidence of some gallate moieties in the CT extract of DK suggesting some of the CT was esterified by gallic acid.

Precipitation of protein by CT extracts

Experiment 1: The precipitation of Rubisco and BSA by CT extracts

The precipitation of Rubisco in total soluble leaf protein and BSA when these proteins were incubated with the CT extracts of LC and LP at pH 7·0 were similar. However, between 1 and 50 μg of added extract, both extracts precipitated more Rubisco than BSA per unit weight (Fig 1).

Experiment 2: The precipitation of Rubisco by CT extracts of several forages

The amount of CT extract required to precipitate all the Rubisco when total soluble leaf protein was incubated with extracts of LC, LP and SN at pH 7·0 was similar, with between 25 and 50 μg of the extract required (Fig 2). The CT extract of SL also precipitated all the Rubisco, however this only occurred when 50 μg of the extract was incubated with total soluble leaf protein. The CT extract of DK precipitated all the Rubisco when 5 μg of extract or greater was incubated with total soluble leaf protein. However, the differences between the reactivity of all these CT extracts appeared to be small (Fig 2). In all cases except LP, precipitation of Rubisco was completely reversible by the addition of PEG. About 20% of the Rubisco was still precipitated when 50 μg of the CT extract of LP was incubated with total soluble leaf protein and 100 μg of PEG.

The CT extract of white clover was also able to precipitate some Rubisco with 50 μg of this extract precipitating about 25% of the Rubisco added to the *in vitro* assays. This precipitation was also completely reversible by PEG, suggesting that it was the CT present in the extract which was responsible for this activity.

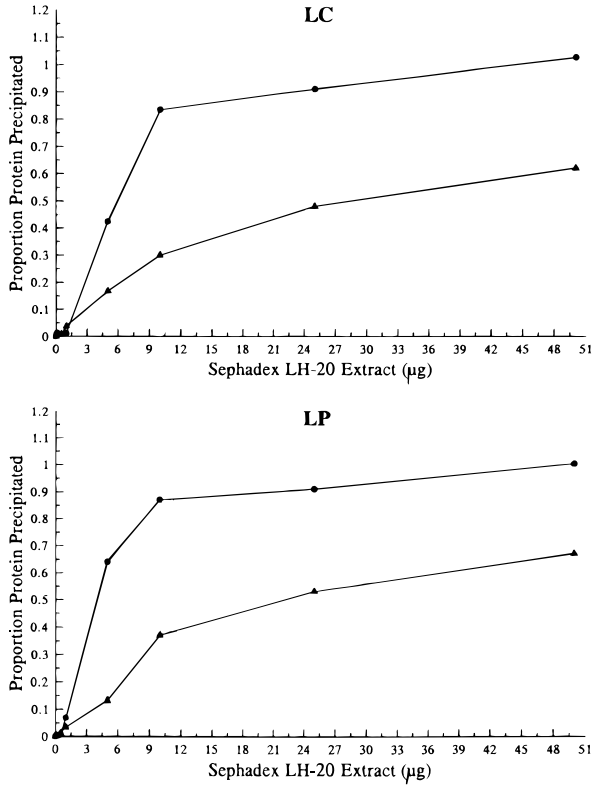

Fig 1. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco; ●) or bovine serum albumin (BSA; ▲) protein precipitated at pH 7·0 by the CT extracts of *Lotus corniculatus* (LC) and *Lotus pedunculatus* (LP) when total soluble leaf protein (extracted from white clover) or BSA was incubated with these extracts at 39°C for 90 min. All incubations were done in duplicate.

Experiment 3: The solubility of Rubisco at different pH values

When total soluble leaf protein was incubated in McIlvaine's Buffer, pH 2·0, 3·0, 5·0, 5·5, 6·0, 7·0 and 8·0 without any CT extracts, pH alone precipitate a proportion of the Rubisco and this varied with pH (Fig 3). At pH 2·0, about 0·2 of the Rubisco in total soluble leaf protein was precipitated, whilst at pH 3·0, this increased to about 0·25 and reached a maximum at pH 5·0, when 0·4 of the Rubisco in total soluble leaf protein was precipitated. At pH 5·5, 6·0, 7·0 and 8·0, the Rubisco precipitated was similar and represented about 0·05–0·1 of the Rubisco present in total soluble leaf protein.

Experiment 4: The precipitation of Rubisco at different pH values by CT extracts

When 50 μg of the CT extract of all forages except WC was incubated at pH 7·0, all the Rubisco present in 10 μg of total soluble leaf protein was precipitated (Fig 4). Therefore, repeating these incubations at different pH values enabled the affect of pH on the reactivity of CT extracts to be determined. The pH was varied

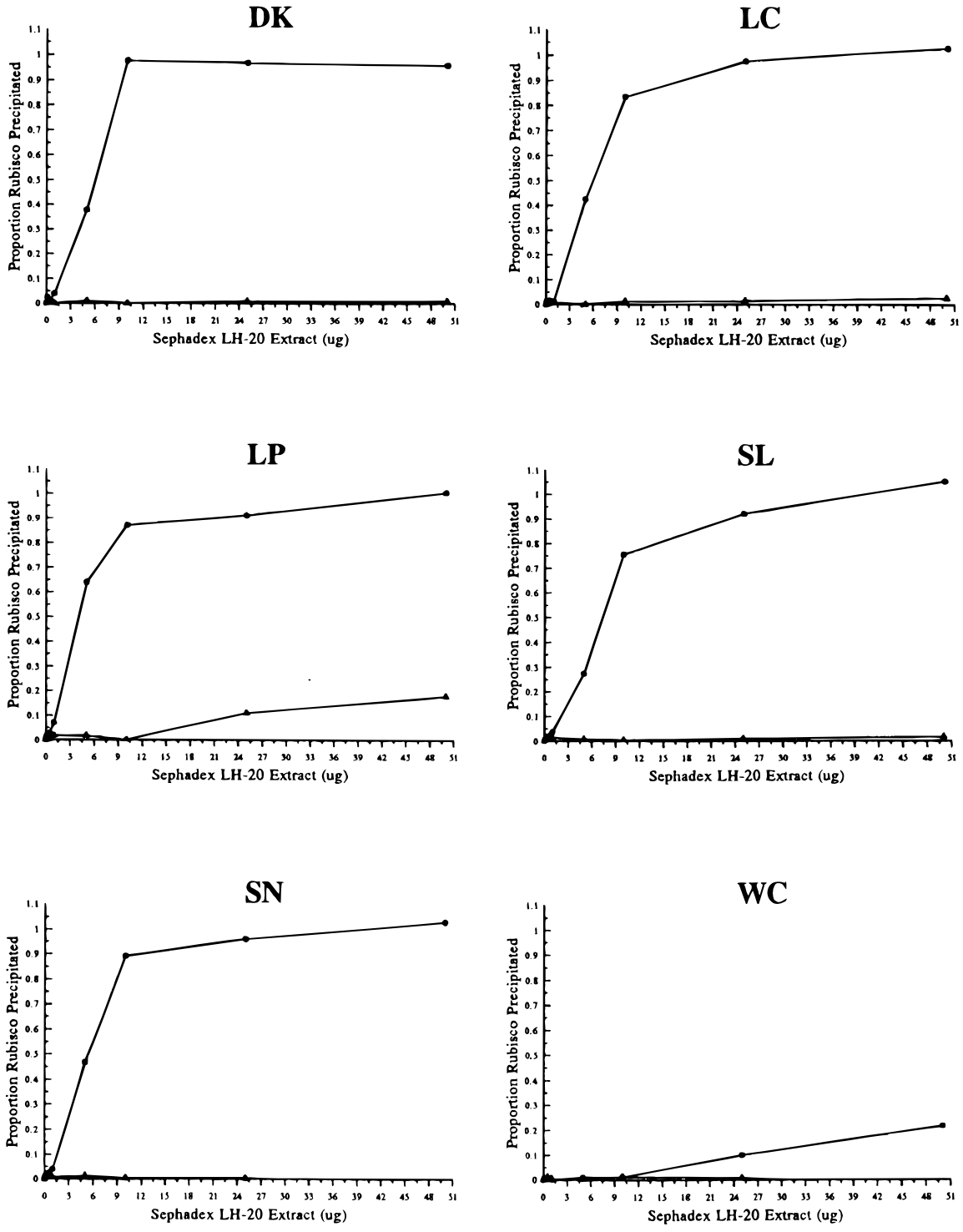

Fig 2. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein precipitated at pH 7·0 by the CT extracts of dock (DK), *Lotus corniculatus* (LC), *Lotus pedunculatus* (LP), sulla (SL), sainfoin (SN) and white clover (WC) when total soluble leaf protein (extracted from white clover) was incubated with these extracts. The incubations were done with (▲) and without (●) the addition of polyethylene glycol (PEG; molecular weight (MW) 3500). All incubations were done in duplicate.

between 2·0 and 8·0 because this is the pH range commonly encountered in the gastrointestinal tract of sheep (Wang *et al* 1996).

When total soluble leaf protein was incubated with the CT extracts of all forages at pH 2·0 and 3·0, the proportion of Rubisco precipitated by the extracts alone was small and never exceeded 0·1 except for the extract of SN, which precipated 0·28 of the Rubisco in total soluble leaf protein at pH 3·0 (Fig 4). At pH 5·0, all CT extracts except those of SN and WC, precipitated about 0·4 of the Rubisco in total soluble leaf protein. The CT extract of SN essentially precipitated all the Rubisco

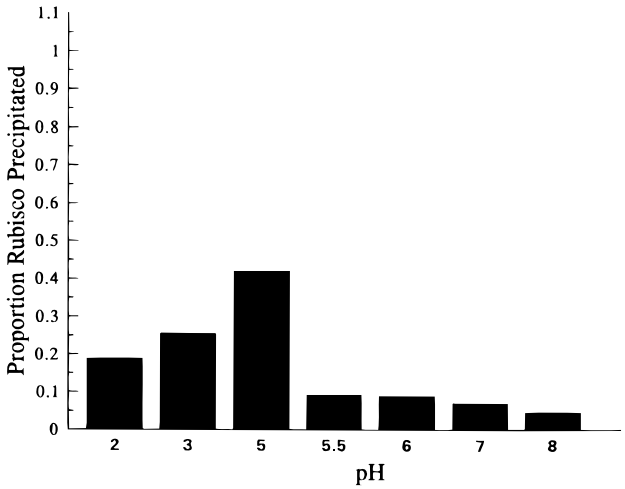

Fig 3. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein precipitated at pH 2·0, 3·0, 5·0, 5·5, 6·0, 7·0 and 8·0 when total soluble leaf protein (extracted from white clover) was incubated at 39°C for 90 min. All incubations were done in duplicate.

present at pH 5·0, 5·5, 6·0, 7·0 and 8·0. The Rubisco precipitated by the CT extract of WC never exceeded 0·1 until the reaction pH was 7·0 and 8·0, when it never exceeded 0·2. The Rubisco precipitated by CT extracts of DK, LC, LP and SL was similar and was between 0·8 and 0·95 at pH 5·5 and 6·0, whilst at pH 7·0 and 8·0, essentially all the Rubisco in total soluble leaf protein was precipitated (Fig 4).

In all cases except at pH 5·0 and for LP, the precipitation of Rubisco was completely reversible by PEG. At pH 5·0, between 0·1 and 0·2 of the Rubisco was precipitated by the CT extracts of DK, LC, LP and SL when PEG was present in the reaction mixture. The CT extract of LP precipitated Rubisco at all pH values except 2·0 and 3·0 when PEG was present, and this varied between 0·06 at pH 8·0 and 0·42 at pH 6·0.

Experiment 5: The effect of CT extracts on the digestion of Rubisco by trypsin and chymotrypsin

The percentage recovery of the Rubisco in 10 μg of total soluble leaf protein when that protein was precipitated with acetone at −20°C for 1 h was 98 (SE 0·03; $n = 14$). The effect of pH and CT extracts of LC and LP on the enzymatic hydrolysis of Rubisco by trypsin and chymotrypsin were similar (Figs 5 and 6). At all pH values there was no detectable hydrolysis of Rubisco when total soluble leaf protein was incubated without trypsin or chymotrypsin. The addition of trypsin or chymotrypsin resulted in the enzymatic hydrolysis of Rubisco and the extent to which Rubisco was hydrolysed was affected by pH. When total soluble leaf protein was incubated with trypsin or chymotrypsin at pH 5·0, the extent of Rubisco hydrolysis was similar and did not exceed 0·34 for trypsin and 0·57 for chymotrypsin. At pH 6·0, the proportion of Rubisco hydrolysed had increased but did not exceed 0·58 for trypsin and 0·88 for chymotrypsin. The proportion of Rubisco hydrolysed was similar at pH 7·0 and 8·0 and did not exceed 0·86 for trypsin, whilst chymotrypsin hydrolyzed all the Rubisco present in the total soluble leaf protein (Figs 5 and 6).

The enzymatic hydrolysis of Rubisco by trypsin and chymotrypsin was partially inhibited by the inclusion of CT extracts of LC and LP in the reaction mixtures and the extent of this inhibition was affected by pH. The activity of the two enzymes was similar, whether they were included in reactions with either CT extract or where preincubated with the CT extracts before being incubated with total soluble leaf protein (Figs 5 and 6). When total soluble leaf protein was incubated at pH 5·0 with either CT extract and either trypsin or chymotrypsin preparation, the reduction in the proportion of Rubisco hydrolysed was similar. At pH 6·0, although the proportion of Rubisco hydrolysed had increased, the CT extracts reduced this hydrolysis from a maximum of 0·58 to 0·19 for trypsin and from 0·88 and 0·38 for chymotrypsin. The proportion of Rubisco hydrolysed at pH 7·0 was also reduced by the CT extracts from a maximum of 0·83 to 0·66 for trypsin and from 1·0 to 0·67 for chymotrypsin. There was no effect of either CT extract on the enzymatic hydrolysis of Rubisco at pH 8·0.

DISCUSSION

Variation in nutritional responses to CT from different forages is certainly affected by concentration, although the impact of CT upon digestion may also depend on the source and chemical structure of the CT which could affect their reactivity. Reactivity has been defined as the ability of CT to precipitate protein per unit weight (Bate-Smith 1973). The principal objective of this study was to measure and compare the reactivity of CT from a range of forages commonly grown in New Zealand and to determine if differences in the reactivity of the CT from LC and LP contributes to the different effects that feeding these forages to sheep has on amino acid absorption from the small intestine.

The CT extracts from LC and LP had a much greater affinity for Rubisco than BSA in the present study. Several factors can influence the reactivity between CT and protein. Proteins differ greatly in their affinity for CT; those that bind strongly have properties which include high molecular weight (MW), open and flexible tertiary structures and high contents of proline and other hydrophobic amino acids (Hagerman and Butler 1981; Asano *et al* 1982; Asquith and Butler 1986). The difference in precipitation of BSA and Rubisco by CT extracts of LC and LP was of particular importance because the objective of this study was to evaluate the reactivity of CT extracted from forages which, in New

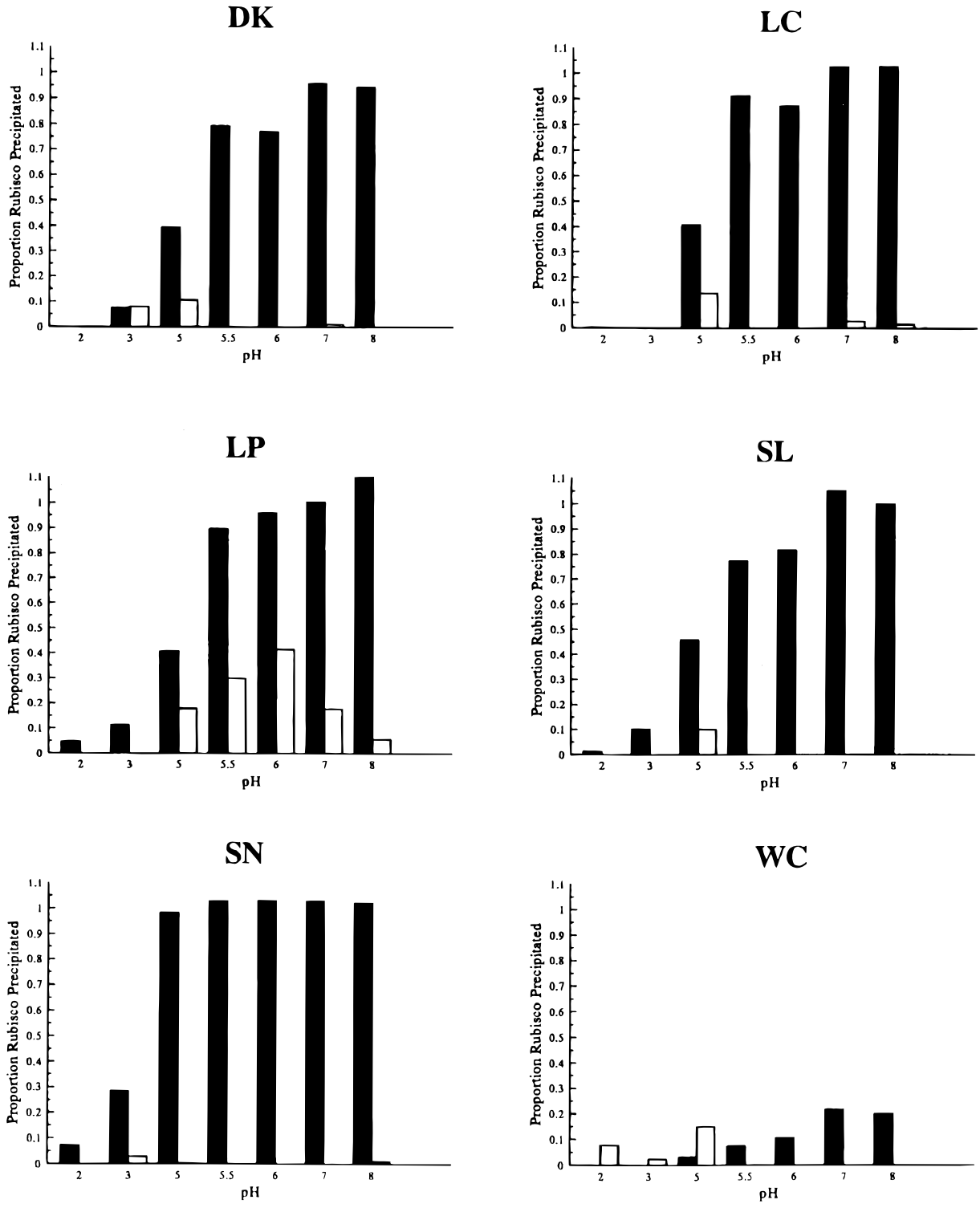

Fig 4. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein precipitated at pH 2·0, 3·0, 5·0, 5·5, 6·0, 7·0 and 8·0 by 50 μg of the CT extracts of dock (DK), *Lotus corniculatus* (LC), *Lotus pedunculatus* (LP), sulla (SL), sainfoin (SN) and white clover (WC) when total soluble leaf protein (extracted from white clover) was incubated with these extracts. The incubations were done with (□) and without (■) the addition of polyethylene glycol (PEG; molecular weight (MW) 3500). All incubations were done in duplicate.

Zealand, would predominantly be fed fresh to ruminants together with perennial ryegrass and white clover. Under these circumstances, Rubisco would represent the principal dietary protein (Mangan and West 1977) and so represents the most appropriate protein to use to measure and compare the reactivity of CT.

Reactivity has been reported to increase with increasing MW (Horigome et al 1988) and prodelphinidin

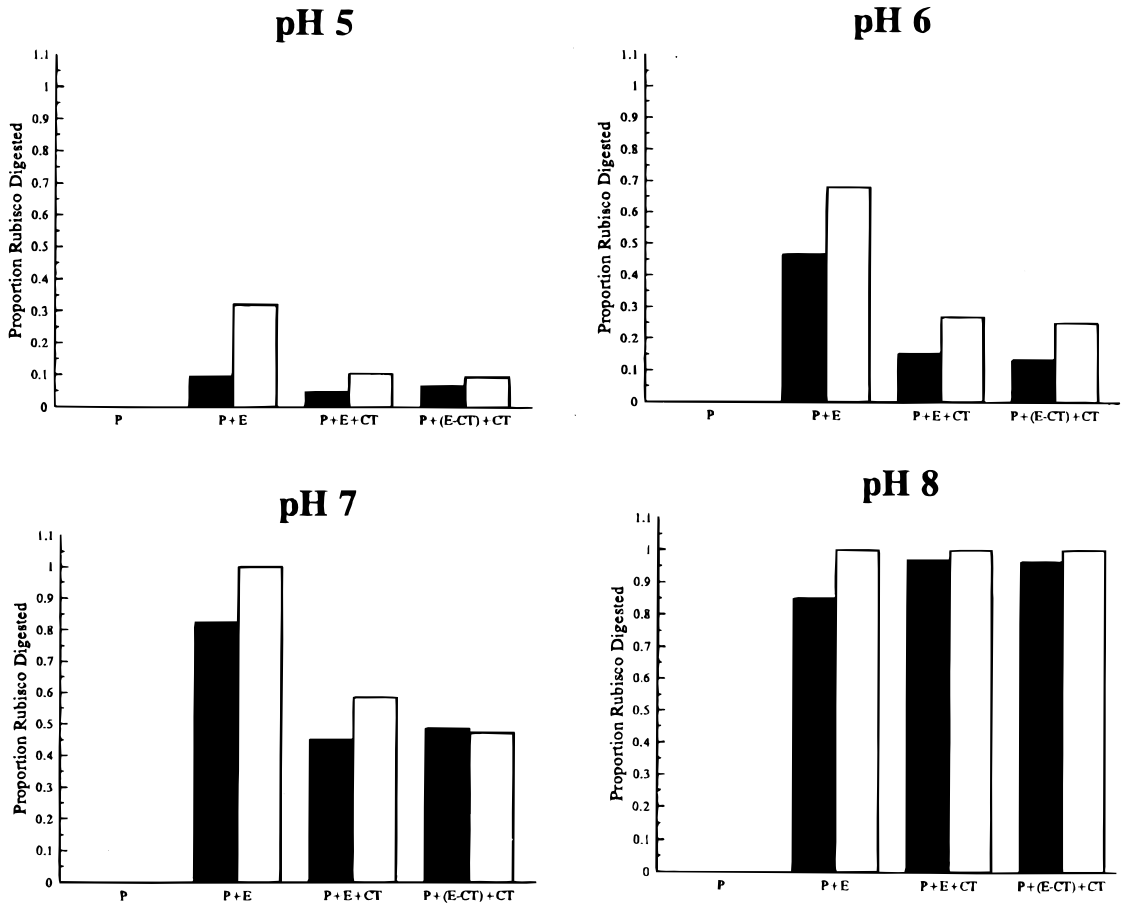

Fig 5. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein hydrolysed at pH 5·0, 6·0, 7·0 and 8·0 when total soluble leaf protein was either; (P), incubated at 37°C for 30 min; (P + E), incubated with trypsin (■) or chymotrypsin (○); (P + E + CT), incubated with the CT extract of *Lotus corniculatus* and trypsin (■) or chymotrypsin (□); (P + (E − CT) + CT), incubated with the CT extract of *Lotus corniculatus* and trypsin (■) or chymotrypsin (□) which had been preincubated with the CT extract of *Lotus corniculatus*. All incubations were done in duplicate.

content (Jones *et al* 1976). However, Jones *et al* (1976) reported that the CTs from sainfoin had a high MW (17 000–28 100) and a high prodelphinidin content but a low reactivity. Foo *et al* (1982), using ^{13}C-NMR, chiroptical and gel permeation chromatography, reported a considerably lower MW (2100–3300) for the CT in sainfoin. The MW of CT from LP (Jones *et al* 1976) was also higher than the estimates reported by Foo *et al* (1982, 1997) suggesting that the ultracentrifugation method used by Jones *et al* (1976) was not reliable for estimating the MW of CT. The CT in LP has an average MW of 2200 (Foo *et al* 1997) and contains predominantly prodelphinidin-type subunits, whilst the CT in LC has a slightly lower average MW (1900; Foo *et al* 1996) and contains predominantly procyanidin-type subunits. Foo *et al* (1996, 1997) reported that the dominant extender subunits in LC and LP were epicatechin (67%) and epigallocatechin (64%), respectively. Whilst the MW of the CT in the extracts from LC and LP were similar, the considerable difference in prodelphinidin content between these two and the other CT extracts caused little or no difference in their reactivity suggesting that MW rather than prodelphinidin content is more significant in determining reactivity.

Jones and Mangan (1977) reported that, when CT extracted from sainfoin was incubated with Rubisco purified from white clover, the insoluble CT–Rubisco complexes were stable between pH 3·0 and 7·0 but at pH < 3·0, about 95% of the Rubisco was released from the complex. At pH > 8·0, about 30% of the Rubisco was released from the complex. The results of the present study certainly support the observation that CT are unable to effectively precipitate Rubisco at pH < 3·0. However, all CT extracts used in the present study formed a complex with Rubisco which did not dissociate at 8·0. Although the interpretation of the work of Jones and Mangan (1977) suggests that CT can bind to protein in the rumen, reducing microbial proteolysis, and then release proteins in the acidic conditions of the abomasum, enabling enzymatic hydrolysis and absorption of amino acids from the small intestine, this relationship may not be strictly correct in the small intestine. Wang *et al* (1996) reported that the pH of digesta in the abomasum was about pH 3·0–4·0 and

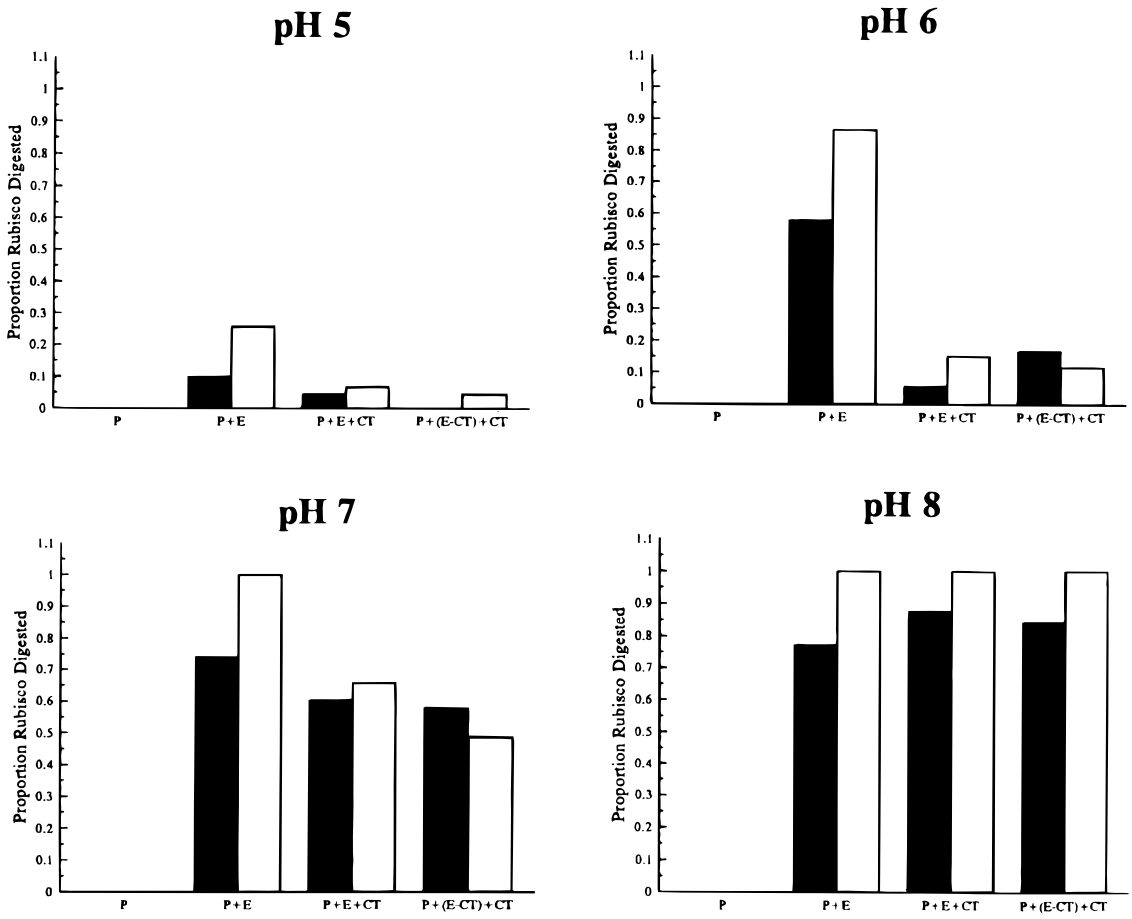

Fig 6. The proportion of ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein hydrolysed at pH 5·0, 6·0, 7·0 and 8·0 when total soluble leaf protein was either; (P), incubated at 37°C for 30 min; (P + E), incubated with trypsin (■) or chymotrypsin (□); (P + E + CT), incubated with the CT extract of *Lotus pedunculatus* and trypsin (■) or chymotrypsin (□); (P + (E − CT) + CT), incubated with the CT extract of *Lotus pedunculatus* and trypsin (■) or chymotrypsin (□) which had been preincubated with the CT extract of *Lotus pedunculatus*. All incubations were done in duplicate.

that the pH of digesta had increased to about 5·5 at the beginning of the small intestine. The pH of the digesta continued to increase and reached its maximum of about pH 8·0 by the fifteenth metre of the small intestine. The digesta in the abomasum will be in acidic conditions for only about 20–35 minutes and it is unlikely that proteolysis by pepsin will be completed in this short time before the pH of the digesta increases during passage into the small intestine. All CT extracts, except perhaps white clover, were able to form complexes with Rubisco at all pH values tested between 5·5 and 8·0 and, therefore, it is likely that significant reassociation of CT and protein will occur in the small intestine. The work of Terrill *et al* (1994), where it was reported that more than 75% of the CT recovered from duodenal digesta was associated with protein and fibre-bound fractions supports this hypothesis. However, the reactivity of CT extracts of LC and LP at all pH values between 5·0 and 8·0 were very similar. Therefore, it would seem unlikely that differences in the reactivities of CT in the small intestine are solely responsible for the different effects of LC (Waghorn *et al* 1987) and LP (Waghorn *et al* 1994) on the apparent absorption of EAA from the small intestine.

Wang *et al* (1996) reported that the CT in LC depressed the true digestibility of methionine and cysteine and altered the site of their digestion in the small intestine. The proportion digested in the proximal part of the small intestine was reduced and the proportion digested in the distal third of the small intestine was increased. The ability of CT to reduce trypsin and chymotrypsin activity was implicated as being partially responsible for this effect. Condensed tannins have been shown to inhibit endogenous enzyme activity (Griffith and Moseley 1980; Oh and Hoff 1986; Horigome *et al* 1988; Ahmed *et al* 1991; Longstaff and McNab 1991 a,b; Yuste *et al* 1992; Jansman *et al* 1993, 1994; Waghorn 1996). Although consistant inhibitory effects have been demonstrated for α-amylase and lipase, inhibition of pepsin, trypsin and chymotrypsin by CT has been more variable. Large reductions in enzyme activity have generally not resulted in major depressions in the digestibility of the diets concerned. In the present study, CT extracts of LC and LP partially inhibited the hydro-

lysis of Rubisco by trypsin and chymotrypsin to a similar extent, but the extent of the inhibition was affected by pH. The inhibition was greatest at pH 6·0. However, as the pH was increased, the inhibition declined such that, at pH 8·0, both CT extracts had little or no effect on trypsin and chymotrypsin irrespective of whether the enzymes had been prereacted with CT extracts or not. Ben-Ghedelai *et al* (1974) reported that most intestinal protease enzymes had a pH range of 7–8 for optimal activity and, in this pH range and in the present study, trypsin and chymotrypsin were able to overcome the inhibitory effects of CT that occurred at lower pHs.

Aerts *et al* (1998) measured the effect of CT extracts from LC and LP on the *in vitro* degradation of the LSU and SSU of Rubisco by rumen microorganisms when total soluble leaf protein was incubated with rumen fluid. The CT extract of LP reduced the degradation of the LSU and SSU of Rubisco to a much greater extent than the CT extract of LC. The ratio of CT extract to protein that was used in that study was about 1 : 1 and 2 : 1. In the present study, the precipitation of Rubisco by CT extracts of LC and LP, when the ratio of extract to protein was 1 : 1 and 1 : 2, was similar and about 0·9. Jones *et al* (1993) demonstrated that, after the addition of CT from sainfoin to a range of purified strains of proteolytic rumen bacteria grown *in vitro*, CT reduced growth rates and was strongly associated with the bacterial cell fraction rather than the culture supernatant suggesting that the CT became bound to the surface of cells. Therefore, in the rumen, CT probably associates with bacterial cell-surface features such as cell-bound extracellular enzymes, probably impeding their activity, and the extent of the inhibition may vary with the chemical structure of the CT. These results suggest that, at least with Rubisco, factors other than just the formation of insoluble CT-protein complexes probably influence the effect that CT has on the degradation of Rubisco in the rumen and that measurements of Rubisco degradation rather than precipitation may be a more relevant criteria for assessing the reactivity of CT extracts. These areas certainly warrant further research and are currently being pursued by this group.

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