THE EFFECTS OF CONDENSED TANNINS UPON NUTRIENT DIGESTION AND METABOLISM AND UPON ANIMAL PRODUCTION IN SHEEP FED *LOTUS CORNICULATUS*

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ABSTRACT

(Yuxi Wang, Department of Animal Science, Massey University, Palmerston North, NEW ZEALAND. *The effects of condensed tannins upon nutrient digestion and metabolism and upon animal production in sheep fed* Lotus corniculatus)

A series of indoor metabolism and grazing experiments were conducted at AgResearch Grasslands and Massey University, Palmerston North, New Zealand, to study the effects of condensed tannins (CT) in *Lotus corniculatus* (birdsfoot trefoil; *cv*. Grasslands Goldie) upon nutrient digestion and metabolism and upon animal production using sheep as the experimental animal. Half of the animals in each experiment were supplemented with polyethylene glycol (PEG; MW 3500). PEG specifically binds and inactivates CT without affecting other nutrients and is indigestible; hence effects of CT were specifically defined by comparing control sheep (CT acting) with PEG supplemented sheep (CT inactivated). A rotational grazing system with some restriction in feed allowance was used in most of the grazing experiments. For the indoor experiments, sheep were held in metabolism crates and fed fresh *L. corniculatus* from overhead belt feeders at hourly intervals.

1. The effects of CT in *L. corniculatus* (35 g total CT/kg DM) upon nutrient digestion and upon metabolism of methionine, cystine and inorganic sulphate in plasma were determined. PEG was continuously infused into the rumen of half the sheep. Principal measurements in the two groups were plasma irreversible loss rate (IRL) and interconversions of methionine, cystine and inorganic sulphate using ^{35}S labelling. Action of CT considerably reduced the concentration of ammonia and molar proportions of *iso*-butyric acid, *iso*-valeric acid and *n*-valeric acid in rumen fluid, but had no effects upon total volatile fatty acid (VFA) concentration and molar proportions of major VFAs. CT greatly increased the IRL of plasma cystine (13.1 *vs* 7.0 μmol/min) and reduced IRL of plasma inorganic sulphate (36.8 *vs* 48.1 μmol/min) but had no effect upon methionine IRL. Action of CT increased transulphuration of methionine to cystine (4.37 *vs* 1.24 μmol/min) and increased cystine flux to body synthetic reactions (11.89 *vs* 5.41 μmol/min). CT had no effect upon the proportion of methionine total flux transferred to sulphate (0.05 *vs* 0.06; i.e. oxidation) but markedly reduced the proportion of cystine flux transferred to sulphate (0.09 *vs* 0.27). CT had no effect upon the apparent digestion of cellulose and minerals, slightly depressed DM, OM and hemicellulose apparent digestion and markedly reduced the apparent digestion of nitrogen (N). Action of CT also reduced protozoa numbers in rumen fluid.

2. The productivity of lactating ewes (Exp 1) and weaned lambs (Exp 2) grazing swards of *L. corniculatus*, lucerne (*Medicago sativa; cv.* Grasslands Oranga) and a mixture of lucerne and lotus were compared in two grazing experiments in the 1991/1992 summer. Total CT content was 32-57 g/kg DM for lotus, 8-10 g/kg DM for the mixture and was negligible for lucerne (< 2 g/kg DM). In Experiment 1, ewe wool production and lamb live weight gain (LWG) did not differ between forages, but ewe LWG was greater on lotus than on lucerne (251 vs 65 g/d), with the mixture being intermediate (115 g/d). In Experiment 2, voluntary feed intake (VFI; 1.76 vs 1.65 kg OM/d), LWG (228 vs 183 g/d), wool production (2.78 vs 2.25 kg) and carcass weight (20.4 vs 17.8 kg) were greater for lambs grazing lotus than lucerne; lambs grazing the mixture had similar VFI (1.63 kg OM/d) to those grazing lucerne, but wool production (2.49 kg) was intermediate between lucerne and lotus lambs. Lotus did not affect carcass fatness (GR 13.1 vs 12.8 mm). It was concluded that *L. corniculatus* supported high levels of sheep productivity, with the results suggesting that part of the response may be due to increased protein supply from action of CT in the digestive system.

3. A grazing experiment was conducted for 22 weeks in the 1992/93 summer to study the effects of CT upon lamb LWG, wool growth and rumen metabolism, and to compare the productivity of lambs grazing *L. corniculatus* and lucerne. PEG was given orally twice daily to half of the lambs grazing each sward. Lotus contained 34 g total CT/kg DM in the diet selected, whilst there was essentially no CT in lucerne. Compared to lambs grazing lucerne, lambs grazing lotus had slightly lower VFI, and higher LWG, carcass weight gain, carcass dressing-out percent and wool growth. PEG supplementation had no effect on these measurements or upon the composition of rumen fluid in lambs grazing lucerne. However, in lambs grazing lotus, PEG supplementation reduced wool growth (10.9 *v.* 12.1 g/day), slightly reduced LWG (188 *v.* 203 g/day), increased rumen ammonia concentration, and increased the molar proportions of *iso*-butyric, *iso*-valeric and *n*-valeric acids and protozoa numbers in rumen fluid. PEG supplementation did not affect carcass gain, carcass fatness or the molar proportion of rumen acetic, propionic or *n*-butyric acids in lambs grazing lotus.

4. The effects of CT in *L. corniculatus* upon the lactation performance of ewes rearing twin lambs was measured in an 8 week grazing experiment in the spring/summer of 1993. Half of the ewes were supplemented orally twice daily with PEG. Lotus contained

45 g total CT/kg DM in the diet selected, with an *in vitro* digestibility of 73%. The results showed that action of CT slowed down the decline in milk production and secretion rates of protein and lactose after the attainment of peak lactation, resulting in more milk (21%), more milk protein (14%) and more milk lactose (12%) secretion in mid and late lactation compared to CT inactivated ewes. CT reduced milk fat percentage but not fat secretion rate. CT had no effect upon VFI, LWG and wool growth of lactating ewes rearing twin lambs. Plasma urea and glucose concentrations were reduced due to action of CT. CT had no effect upon concentrations of non-esterified fatty acids (NEFA), growth hormone and insulin in the plasma, had no effect upon molar proportions of acetic, propionic and *n*-butyric acids in rumen fluid, but markedly reduced concentrations of ammonia and molar proportions of *iso*-butyric, *iso*- and *n*-valeric acids in rumen fluid.

5. The effect of CT upon the true and apparent digestion of methionine and cysteine in the small intestine (SI) of sheep fed *L. corniculatus* containing 30 g total CT/kg DM were determined, using sheep prepared with rumen and abomasal cannulae. An indigestible liquid phase marker chromium ethylene diamine tetra acetic acid (Cr-EDTA) was continuously infused into the rumen of all sheep, and PEG was continuous infused into the rumen of half the sheep. The true digestibility of methionine and cysteine in the SI and their absorption sites in the SI were measured from continuous intra-abomasal infusion of plant homogenate from *L.corniculatus* containing ^{35}S-labelled protein. Action of CT substantially reduced the true digestibility of methionine (0.72 *v* 0.88) and cysteine (0.66 *v* 0.81) in the SI, but increased the total amount of plant methionine and cysteine absorbed in the SI due to reduced rumen degradation. Action of CT slowed the digestion of both ^{35}S-methionine and ^{35}S-cysteine in the SI, and increased the flux of both amino acids in the mid and latter thirds of the SI. CT increased abomasal flux (as a proportion of eaten) of total methionine (0.88 *v* 0.76) and total cysteine (0.74 *v* 0.62), and increased absorption of total methionine (0.72 *v* 0.63 g/g eaten) but not total cysteine (0.49 *v* 0.48 g/g eaten) from the SI. Calculated endogenous loss of cysteine at the terminal ileum was greater than for methionine and both appeared to be increased by action of CT.

It was concluded that action of CT in *L. corniculatus* increased wool growth rate in high wool producing sheep and increased milk production in lactating ewes without affecting VFI, thus improving production efficiency. It was deducted that the improved animal production was probably due to the action of CT reducing rumen protein degradation,

increasing non-ammonia nitrogen (NAN) flux into the SI, increasing essential amino acid (EAA) especially methionine absorption from the SI, and increasing the flux of cystine to body synthetic reactions. Further researches are needed to study the effects of CT in the range 10-20 g/kg DM on animal production and nutrient metabolism, and to study the effects of forage CT upon milk production and composition in dairy cows.

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LIST OF ABBREVIATIONS

AA	amino acids
ADF	acid detergent fibre
A-V	arterio-venous
BCT	bound condensed tannins
BF	blood flow
BSA	bovine serum albumin
BW	body weight
CA	metabolite concentrations in arterial blood
cm	centimetre
CP	crude protein
Cr	chromium
Cr-EDTA	chromium ethylenediaminetetra acetic acid
CT	condensed tannin
CV	metabolite concentrations in venous blood
CYS	cystine
CW	carcass weight
D	digestibility
d	day
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
DP	degradable protein
DPM	disintegrate per minute
EAA	essential amino acids
ECT	extractable condensed tannins
Exp	experiment
FCT	fibre bound condensed tannins
F	faecal OM output
FE	faecal energy

FG	fermentation gas energy
Fig	figure
FO	faeces output
FrCT	free condensed tannins
FV	feeding value
g	gram
GE	gross energy
GI	gastro-intestine
GR	a measurement of total soft tissue depth over the 12th rib at a point 11 cm from the carcass midline
h	hours
ha	hectare
H-	hydrogen
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HT	hydrolysable tannins
ID (i.d.)	internal diameter
INAN	increment of NAN flux into the SI
IRL	irreversible loss rate
iu	international units
kBq	kilo-becquerel
kg	kilograms
K_f	efficiency of utilization of ME for fattening
K_g	efficiency of utilization of ME for growth
K_l	efficiency of utilization of ME for lactation
K_m	efficiency of utilization of ME for maintenance
l	litres
LO	lotus
Ltd	limited
LU	lucerne

LWG	liveweight gain
m	metres
mBq	mega-becquerel
ME	metabolisable energy
MET	methionine
meq	milliequivalent
mg	milligram
min	minute
MJ	megajoule
ml	millilitres
mm	millimetres
mmol	millimole
mol	mole
MW	molecular weight
N	nitrogen
Na	sodium
NAN	non-ammonia nitrogen
NaOH	sodium hydroxide
nd (ND)	no determined
NDF	neutral detergent fibre
NEAA	non-essential amino acid
NH_3	ammonia
NV	nutritive value
NZ	New Zealand
OM	organic matter
OMD	organic matter digestibility
OMI	organic matter intake
OPA	orthophthaldehyde
PC	cyanidin
PCSII	Phase Combining System II
PCT	protein bound condensed tannins
PD	Delphinidin
PEG	polyethylene glycol
PITC	pyenylisothiocyanate

P+M	productive processes and maintenance
PRPs	proline-rich proteins
Pty	Company
PVP	Polyvinylpyrrolidone
RCEL	rumen digestion as proportion of total digestion of cellulose
RFC	readily fermentable carbohydrate
RHCEL	rumen digestion as proportion of total digestion of hemicellulose
^{103}Ru-P	^{103}Ru-labelled tris-(1,10-phenanthroline)-ruthenium II chloride
rpm	revolutions per minute
S	sulphur
^{35}S	radioactive isotope of sulphur
SA	specific activity
SAA	sulphur amino acids
SE	standard error
SEM	standard error of mean
SI	small intestine
t	tonne
TCA	trichloroacetic acid
TCT	total condensed tannin
TQ	transfer quotient
UK	United Kingdom
UDP	undegraded protein
UE	urine energy
µl	microlitre
µmol	micromole
USA	United States of America
VFA	volatile fatty acids
VFI	voluntary feed intake
v/v	volume by volume
WGR	wool growth rate
WSC	water soluble carbohydrates

w/v	weight by volume

INTRODUCTION

Because of the specific structure of the ruminant digestive tract, nutrients consumed by ruminants first go into the rumen and are digested by rumen microbes. This process, commonly called "fermentation", accounts for 55-65% of the total apparent organic matter digestion. When ruminants are fed on high quality fresh forages containing high concentrations of protein, such as temperate forages used for ruminant production in New Zealand (NZ), about 70% of the forage protein is degraded in the rumen and only 30% escapes to the small intestine for absorption (Ulyatt *et al* 1975). Of the protein degraded in the rumen, a large proportion is absorbed as ammonia and excreted as urea in the urine. Duodenal protein N flow (undegraded dietary protein (UDP)+microbial protein) is about 65-75% of N intake and 25-33% of the N intake is lost as NH_3 absorbed from the rumen (MacRae & Ulyatt 1974). There also appears to be a shortage of amino acid (AA) supply from such forages for high producing animals, such as fast growing, high wool producing or lactating animals, since supplementing protected protein or abomasal infusion of AA have been shown to improve productivity of these animals (Barry 1980; 1981; Black *et al* 1979; Rogers *et al* 1980; Stobbs *et al* 1977). Therefore, with such high quality fresh forage, a major concern is how to utilize protein efficiently to maximize animal production.

Several treatments have been used to control the degradation of dietary protein in the rumen. These methods consist of the use of coating agents, heating the protein, treatment with vegetable tannins and treatment with formaldehyde or other aldehydes. These methods, however, are not well adapted to the grazing system.

Condensed tannins (CT), naturally occurring plant secondary compounds, have caused great interest in NZ as a practical means of protecting forage protein from degradation in the rumen, because of their capacity to precipitate protein at rumen pH. However, animal productivity has been shown to be reduced by high concentrations of CT (50-100 g extractable CT/kg DM) in forages, depressing digestibility and voluntary feed intake (Barry and Duncan 1984; Barry and Manley 1984; Reed *et al* 1982; Pritchard *et al* 1992). It has been suggested that low

concentrations of CT (20-40 g/kg DM for lotus species) may be beneficial for ruminant animals (Barry 1989; Barry *et al* 1986; Waghorn *et al* 1987). This thesis studies the effects of low CT concentrations in forages upon nutrient digestion, metabolism and upon animal production, using *L. corniculatus* (birdsfoot trefoil), a forage containing about 35 g total CT/kg DM.

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Chapter 1

Review of literature

1.1 INTRODUCTION

Forage is the main feed source of ruminant animals, especially in New Zealand (NZ), where use of pasture by grazing animals is the basis of pastoral agriculture. Fresh forages are grazed by ruminants for 12 months of the year in NZ, with very little supplementation with other feed sources. This review, based on published literature, first defines the feeding value (FV) and factors limiting FV of forages. Secondly, the occurrence of plant condensed tannins (CT), their chemistry and reactivity with various substances is reviewed. Finally, detailed consideration is given to the effects of CT on forage FV, including effects on nutrient digestion and metabolism and on the production of ruminant animals.

1.2 FORAGE IN RUMINANT ANIMAL PRODUCTION

Domestic ruminants make a major contribution to human welfare. They provide 70% of the total animal protein eaten and 10% of the natural fibre used by humans (Minson 1990). Ruminants, with their symbiotic population of rumen microbes and ability to chew partially digested food (rumination), have the capacity to utilize highly fibrous forages that cannot be utilized by humans and other non-herbivore monogastric animals.

In the grazing ecosystem, two kinds of pastoral forage are important for ruminant animals, namely tropical and temperate forages. Animal production from grazing tropical forages is usually lower than when grazing temperate forages. This lower production is mainly caused by the poorer FV of tropical than temperate forages, since high levels of animal production can be achieved in the tropics if grain supplements are fed (Minson 1981). Only temperate forages are fed in NZ and this review will therefore concentrate on temperate forages.

1.3 FORAGE FEEDING VALUE

1.3.1 Definition of FV

Ulyatt (1973) defined forage FV as the animal production response to grazing a

specific forage under a given set of environmental circumstances:

FV = Animal production = f(Intake x Nutritive Value)

Typical measures of FV are liveweight gain (LWG) with growing stock or milk production with dairy cows. Nutritive value (NV) is defined as the concentration of nutrients in a forage or the animal production response (meat, milk and wool production) per unit of feed intake. Thus, FV is a function of both NV and intake (Ulyatt 1973).

Both efficiency of nutrient digestion in the gastro-intestinal (GI) tract of ruminants and the efficiency of utilization of digested nutrients comprise the NV of a forage. The major sites of digestion of ruminants are the stomach (microbial fermentation), the small intestine (SI; animal enzymes) and the large intestine (microbial fermentation). The most significant characteristic of the digestion of ruminants is the rumen microbial fermentation. The rumen accounts for 55-65% of total apparent organic matter (OM) digestion, the SI 25-30% and the large intestine 5-15% (Waghorn & Barry 1987). The sections of the digestive tract where different forage components are digested, and the products of digestion are summarized in Figure 1.1

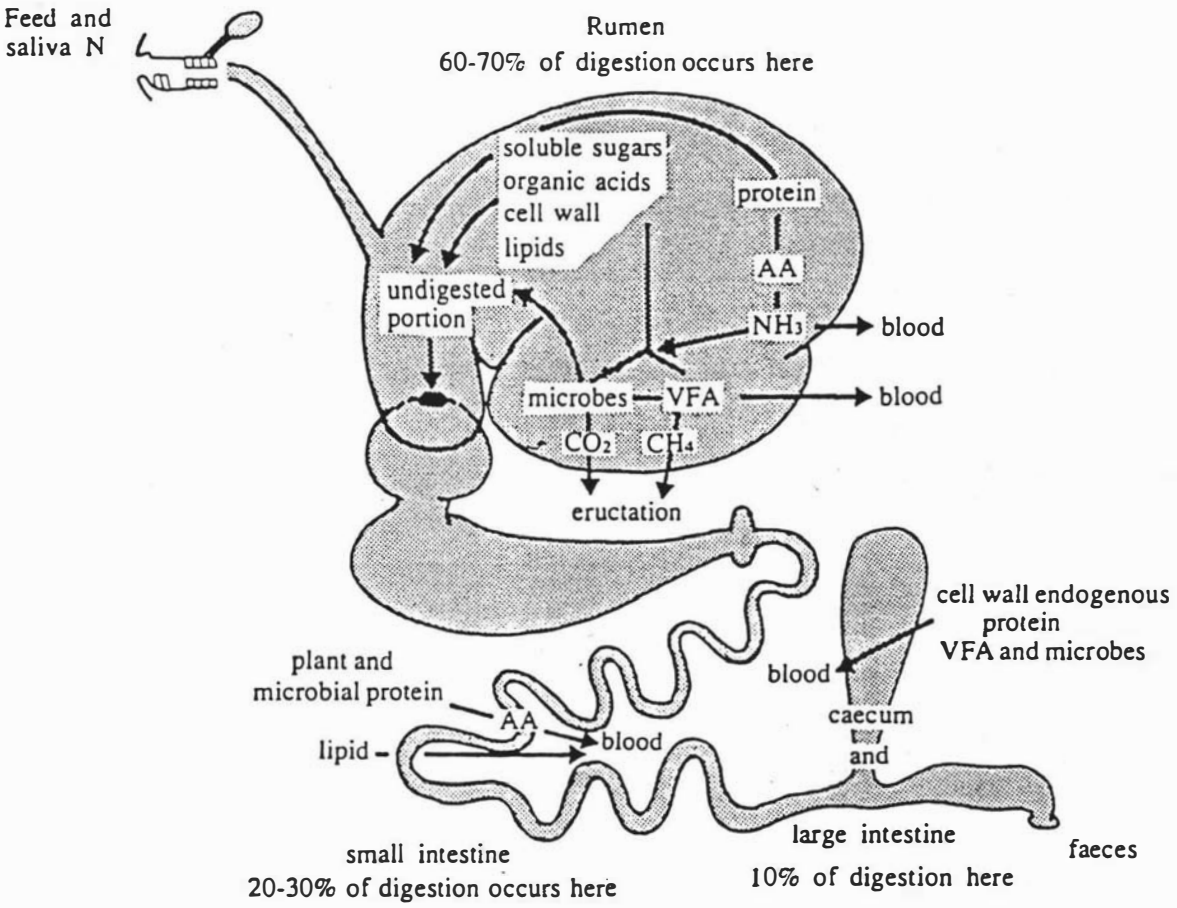

Figure 1.1 The ruminant digestive system and the products of digestion when offered fresh forage (From Waghorn & Barry 1987).

Animals utilize absorbed nutrients for both maintenance and production. The most commonly used index for utilization of digested nutrients is the efficiency of utilization of metabolizable energy (ME). ME is calculated as described below:

ME=GE-FE-UE-FG

Where GE is gross energy (the heat derived from combustion of the dried feed in a bomb calorimeter), FE is energy in faeces, UE is energy in urine and FG is energy in fermentation gases.

The efficiency of utilization of ME is defined as:

$$K = \frac{\Delta \ Energy \ \ retained}{\Delta \ ME \ \ intake}$$

The efficiency of utilization of ME is usually quoted as a coefficient *k*, with a suffix *m*, *f* or *l* depending on whether *k* refers to the use of the feed for maintenance, fattening/growth or lactation.

1.3.2 Methods studying FV in the grazing ecosystem

Ulyatt (1973) suggested two methods could be used to measure FV of a forage: to measure the comparative FV and to measure the components of forage FV. LWG or milk production can be used as indices of comparative FV. Although measurements of comparative FV will rank forages in order of merit, this technique has several important limitations: variation between animals is high so large numbers of animals are required per treatment to measure significant production responses; production responses are generally slow, so that in order to detect differences between forages the experiment must be of relatively long duration. To assess causal factors, voluntary feed intake (VFI) and precise measurements of the components of NV are required.

1.3.2.1 Measuring VFI in the grazing ecosystem

Measurement of VFI of grazing animals can be made for (*a*) individuals using animal techniques, or (*b*) on a group basis by pasture sampling.

1.3.2.1.1 Animal technique

The most common animal technique involves indirect measurement of VFI from *in vitro* determination of forage digestibility (D) of the diet selected and faecal output of grazing animals (FO, kg OM/d). Intake (VFI, kg OM/d) is estimated from:

$$VFI = \frac{FO}{1-D}$$

Faecal output is usually measured either directly by bagging the animal, or indirectly by use of an indigestible marker. The bagging method is precise provided that any harness does not restrict the animal. However, indirect measurement is most commonly made using an indigestible 'marker' such as chromium sesquioxide (Cr_2O_3). Parker *et al* (1989) described a method to estimate VFI using slow release chromium capsules. Daily FO is estimated by:

$$FO = \frac{x}{y}$$

Where, x=Cr_2O_3 release rate (mg/d) and y=Cr_2O_3 concentration in faeces (mg/g OM).

Forage digestibility can be obtained from direct animal measurement (*in vivo*) or estimated from the *in vitro* digestibility of forage samples, using either hand-plucked material to imitate diet selection or collected via an oesophageal fistula. Digestibility can also be estimated indirectly from the relative concentration of an indigestible component in the feed and faeces (Kennedy *et al* 1959; Kotb & Luckey 1972).

1.3.2.1.2 Pasture sampling technique

Mean apparent intake or pasture disappearance for groups of animals can be estimated from the difference between pre- and post-grazing pasture mass determined from mechanically harvested pasture samples. Estimates of DM intake (DMI) are based on:

$$DMI(kg/head/d) = \frac{pre\text{-}grazing\ DM(kg)\ -\ post\text{-}grazing\ DM(kg)}{number\ of\ animal\ grazing\ days}$$

This method is most successful over short grazing intervals (4-5 d) and when pasture growth is minimal (Walters & Evans 1979). Comparisons have shown pasture sampling estimates of intake to be 30-40% lower than those using animal methods (Ulyatt *et al* 1974).

1.3.2.2 Measuring NV of forages

1.3.2.2.1 Measuring apparent digestibility and sites of digestion

Apparent digestibility has long been widely used as an index of NV of forages. It measures the difference between feed intake and faeces output. Apparent digestibility can be measured either by conventional (faeces collection) or by indigestible marker (internal and external marker) methods. However, because nutrients may be digested and absorbed from several parts of the digestive tract, the nutritive value to the animal of a constituent will depend not only on the extent to which it is digested (i.e. its apparent digestibility) but also on the site of digestion.

Two techniques can be applied to determine the site of digestion within the GI tract, both of them measuring digesta flow passing specific points in the GI tract. The first technique involves the exteriorization of digesta flow through re-entrant cannulae so that the flow rate can be estimated directly (MacRae 1975). The second technique, which is described by Faichney (1975), calculates the digesta flow with reference to indigestible markers which are either present in the feed or administrated independently.

The most commonly used method for measuring digesta flow involves infusion of marker(s) at a constant rate, followed by sampling once equilibrium (steady-state) conditions have been achieved. The animals are usually fed at hourly intervals during the measurement. Steady-state conditions exist when marker pools and flows proximal to the sampling point(s) are constant and are reflected in constant concentrations related to the feeding and/or marker dosing patterns (Dove *et al* 1988; Faichney 1980). Digesta flow can then be calculated as:

Digesta flow = (Marker infusion rate)/(Mean marker concentration in digesta)

Faichney (1975) proposed a double-marker method to measure both liquid and solid phase flow, allowing calculation of true digesta flow. The double-marker method is used because digesta consists of two phases, a liquid phase and a particulate or solids phase and the flow rates of these two phases from the rumen are different. Chromium ethylene diamine tetra-acetic acid (Cr-EDTA) is commonly used to mark the liquid phase and ^{103}Ru-labelled tris-(1,10-phenanthroline)-ruthenium II chloride (^{103}Ru-P) and lignin are used to mark the particulate phase.

1.3.2.2.2 Measuring utilization of digested nutrients

1.3.2.2.2.1 Measuring the utilization of energy

Methods used for measuring the efficiency of utilization of ME using calorimetry have been described by Blaxter (1962). The technique measures the efficiency with which mixed nutrients (volatile fatty acid (VFA), protein and fat) absorbed from the GI tract are utilized by the whole animal.

The efficiency of utilization of ME (k) is an incremental measurement which is measured at two intake levels; if these are both below maintenance, or one is below and the other at maintenance, then the technique measures the efficiency of utilization of ME for maintenance (k_m). If both intakes are above maintenance, or one is above and the other at maintenance, the technique measures the efficiency of utilization of ME for fattening/growth (k_f or k_g).

1.3.2.2.2.2 Measuring the utilization of individual nutrients

The utilization of individual nutrients is commonly measured by the arterio-venous (A-V) technique and/or isotope dilution techniques.

The A-V technique is mostly suitable for nutrient utilization by individual organs. It involves measuring the concentration of each nutrient in both arterial and venous blood entering and leaving that organ, and blood flow rate through the organ. Therefore the uptake by the organ can be calculated as:

$$\text{Uptake} = (CA-CV) \times BF$$

Where CA, CV and BF are metabolite concentrations in arterial and venous blood and

the blood flow through the organ. There are several methods of measuring organ blood flow which have been reviewed by Linzell and Annison (1975).

Isotope dilution provides an invaluable method for study of the kinetics of nutrient transfer and turnover in the conscious, undisturbed animal without changing the circulating levels of the nutrient. Two methods are available, namely the single injection or the continuous infusion methods. Trace amounts (mass insignificant in relation to body pool size) of an isotopically labelled substrate are used to label the body pool, and the single injection method is dependent on mathematical analysis of the time course of changes in the specific radioactivity (SA) of the circulating nutrient. In the continuous infusion method, the infusion is continued until plateau SA has been attained, and samples are then taken. This procedure, as generally applied, requires steady state conditions in which body pools remain of constant size while undergoing replacement by an input equal to the rate of outflow. In order to achieve relatively constant levels of blood metabolites, continuous feeding systems, i.e. feeding at intervals of 1 or 2 h is desirable, as this feeding system stabilizes levels of metabolites in the rumen, and the flow of digesta throughout the alimentary tract is more constant.

An important requirement of the isotope dilution technique is that the substrate in the sampled pool must be uniform SA, which implies that substrate leaving the pool must have the same SA as that of the remainder of the pool.

Irreversible loss rate (IRL) of a metabolite is often obtained from isotope dilution measurements and represents kinetics of a nutrient after being absorbed into the blood. In the continuous infusion method it is calculated as follows:

$$\text{IRL (mmol/min)} = I/SA = \text{Tissue synthesis} + \text{Oxidation} = \text{Production rate}$$

Where I is rate of isotope infusion and SA is the specific radioactivity.

If more than one nutrient pool is being considered, then to quantitatively estimate the exchange rate between pools, transfer quotients (TQ) are needed. TQ measured at plateau SA is the proportion of isotope detected in a secondary pool (S), which originated from an infusion into a primary pool (P).

$$TQ = \frac{SA\ of\ pool\ S}{SA\ of\ pool\ P}$$

Nolan *et al* (1976), using these techniques, established a three-pool, open-compartment model for N transaction between rumen, caecum and blood plasma (Figure 1.2)

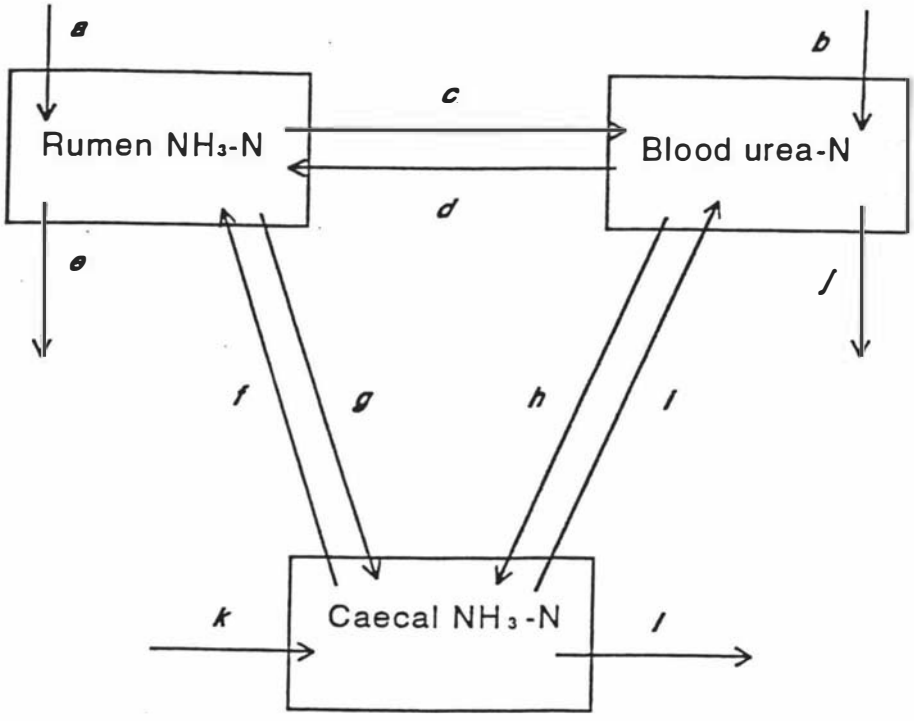

Figure 1.2 A general, three-pool, open-compartment model for nitrogen (N) transactions associated with rumen ammonia, plasma urea and caecal ammonia for sheep. *a, b, c, ..., l* are the rates of flow of N which are a composite of several pathways of transfer. The important components of these multiple pathways are: (*a*) ammonia derived from dietary and endogenous sources; (*b*) urea formed from ammonia derived during degradation of amino acids in the body, but excluding urea derived from microbial amino acids which were derived from rumen ammonia; (*c*) urea formed from ammonia (1) absorbed from the forestomachs, (2) from digestion and metabolism of rumen microbial amino acids; (*d*) rumen ammonia derived from blood urea; (*e*) ammonia leaving the rumen in pathways other than incorporated into blood urea or caecal NH_3; (*g*) caecal ammonia derived from rumen-microbial protein formed from rumen NH_3; (*h*) caecal ammonia derived from blood urea; (*i*) plasma urea formed from absorbed caecal ammonia; (*j*) excretion of urea in the urine; (*k*) caecal ammonia from previously undigested endogenous and other miscellaneous N (dietary N and part of microbial N); (*l*) caecal ammonia-N synthesized into body tissues plus a component of faecal N excretion.

1.3.3 Factors affecting FV of fresh forages

Grasses and legumes are the main forage sources and FV of legumes is generally superior to that of grasses (Table 1.1). FV of annual or short rotation grasses are also generally higher than those of perennial grasses.

Table 1.1 The comparative feeding value (FV) in terms of sheep liveweight gain of some pasture species grown in New Zealand. All values are expressed relative to white clover (Grasslands Huia) and all plants grown under high soil fertility conditions.

	Relative FV	Approx Growth rate (g/d)	Number of studies
Legumes			
White clover,'Grasslands Huia'	100	250	14
Lotus pedunculatus,'Grasslands Maku'	84	210	6
Sainfoin,'Melrose'	84	210	2
Lucerne, 'Wairau'	82	205	10
Red clover, 'Grasslands Hamua'	71	178	5
Red clover, 'Red West'	69	173	2
Red clover, 'Grasslands Pawera'	65	163	4
Grasses			
Italian ryegrass,'Grasslands Paroa'	83	208	1
Short-rotation ryegrass,'Grasslands Manawa'	77	193	11
Timothy, common	67	168	5
Perennial ryegrass,'Grasslands Ariki'	58	145	2
Perennial ryegrass,'Grasslands Ruanui'	52	130	16
Browntop, common spring	52	130	1
Browntop, summer	43	108	1

(From Ulyatt 1981).

1.3.3.1 Factors affecting VFI

VFI accounts for at least 50% of the FV of temperate forages (Ulyatt 1973), therefore any factors affecting VFI will significantly effect FV. In the grazing ecosystem, such as in NZ, VFI is influenced by both nutritional and non-nutritional factors, and non-nutritional factors (pasture structure, pasture mass, pasture allowance and post-grazing pasture mass) are thought to be important factors limiting intake by grazing animals (Poppi *et al* 1987).

The major nutritional factor influencing VFI is the digestibility of the feed selected; as digestibility increases so does intake (Blaxter *et al* 1956). However, the relationship between intake and digestibility is not consistent for all pasture species, with intake of legumes being up to 40% greater than grass and leaf 100% greater than stem when compared at the same digestibility (Cruickshank *et al* 1985; Laredo & Minson 1973; Ulyatt 1971). Therefore, other possible control factors have been investigated, such as physical factors: retention time of digesta in the GI tract and quantity of material in the rumen; and metabolic factors: energy yielding substrates (such as acetic and propionic acids, lipids and amino acids; Faichney 1986; Poppi *et al* 1981).

The important concept is that no one factor (physical or metabolic) adequately explains intake regulation and that a combination of factors, both physical and metabolic, are integrated to control intake.

1.3.3.2 Factors affecting digestion

Apart from animal factors, apparent digestibility is clearly related to forage maturity and there is a general pattern for all plant species: a high apparent digestibility associated with the vegetative state is found in spring and this declines as the plant matures over the summer. The decrease in apparent digestibility with increasing maturity can be explained in terms of changes in plant structure (physical property) and chemical composition. As a plant matures, the proportion of stem increases, the proportions of slowly digested and indigestible chemical constituents (cellulose, hemicellulose and lignin) in the stem also increase, and as a result apparent digestibility declines.

Proteins are the main nitrogenous materials in forages, and consist of fraction 1 leaf

protein, fraction 2 leaf protein, plus small amounts of membrane and enzyme protein and nuclear proteins. Most fresh forages used in NZ agricultural systems contain 12-25% crude protein (CP). With these fresh forages containing high quantities of CP, about 70% is degraded in the rumen and only 30% escapes to the SI for absorption (Ulyatt *et al* 1975). Of the protein degraded in the rumen, a large proportion is absorbed as ammonia and excreted as urea in the urine. Duodenal protein N flow (undegraded protein (UDP)+microbial protein) is about 65-75% of N intake and 25-33% N intake lost as NH_3 absorbed from the rumen (MacRae & Ulyatt 1974). Therefore, with such high quality fresh forage, a major concern is how to utilize protein efficiently to maximize animal production.

1.3.3.3 Factors affecting utilization of digested nutrients

The utilization of the end products of digestion depends both on the type of animal used and on forage quality. K_m is relatively similar between legumes (white clover) and grasses (perennial ryegrass), but k_g is higher for legumes (white clover) than for grass (perennial ryegrass; Table 1.2).

Table 1.2 Efficiency of metabolisable energy (ME) utilization for maintenance (K_m) and gain (K_g) by young sheep (7 month lambs) fed fresh forages in New Zealand.

Feed	K_m	K_g
White clover	0.67	0.51
Ruanui ryegrass	0.62	0.33
50:50 grass/white clover	0.63	0.40

(From Rattray & Joyce 1974).

Season also has a great effect on ME utilization from forage diets, with K_g being higher in spring than in autumn (Table 1.3), whilst K_m is similar between seasons. It has been suggested that the lower soluble carbohydrate content of autumn than spring forages limited microbial protein synthesis in autumn, which caused the lower

K_g of autumn forages (MacRae *et al* 1985).

Table 1.3 Efficiency of metabolisable energy utilization for maintenance (K_m) and gain (K_g) by mature sheep fed autumn (A) and spring (S) harvested forage.

Season	Feed	Apparent digestibility	K_m	K_g
S	Grass	66	0.72	0.44
A		66	0.65	0.33
S	Dried grass	76	0.70	0.45
A		70	0.71	0.34
S	Dried grass	-	-	0.48
A		-	-	0.30
S	Dried grass	77	0.79	0.54
A		66	0.82	0.43
S	Ryegrass/clover	75	0.63	0.40
A		79	0.50	0.25

(From Waghorn & Barry 1987).

1.3.3.4 Abomasal infusion and protected protein studies identifying essential amino acids (EAA) as limiting for high animal production from temperate forages.

The high degradation of fresh forage proteins in the rumen may result in a shortage of protein, especially limiting essential amino acids (EAA), for high producing animals, such as rapidly growing animals and lactating animals. There is ample evidence showing improved animal production from abomasal infusion of protein and protected protein supplementation to such animals fed fresh forage (Barry 1980; 1981; Black

et al 1979; Rogers *et al* 1980; Stobbs *et al* 1977). Therefore, one objective of research in the nutrition of grazing animals is to reduce rumen degradation of forage protein.

Various chemical and physical treatments have been applied to reduce the degradation of dietary protein in the rumen and to increase the fraction that escapes to the intestine. These methods consist of the use of coating agents (such as lipids), heating the protein, treatment with vegetable tannins, formaldehyde or other aldehydes and alkalis (Satter 1986). Other chemical modification, including acetylation and sialic acid substitution, have been used successfully to reduce the degradability of bovine salivary proteins (Nugent *et al* 1983). However, these methods are time-consuming, relatively expensive and not well adapted to grazing systems. Therefore, the protein precipitating property of condensed tannins (CT), naturally occurring compounds, has caused great interest in NZ as a means of reducing rumen degradation of forage proteins and of increasing EAA absorption from the SI.

1.4 STRUCTURE, MEASUREMENT AND REACTIVITY OF FORAGE CONDENSED TANNINS

1.4.1 Structure of plant CT

Tannins are naturally occurring phenolic plant secondary compounds and have been classified into two groups based on their structures: hydrolysable tannins (HT) and condensed tannins (CT). This thesis deals only with CT, as HT rarely occur in temperate forages. Structurally, CT are complexes of polymeric monomers, including flavan-3-ols, flavan-3,4-diols and biflavans. The structures of plant CT are not fully understood because of the complicated structures of the differing precursors. In general, CT from plants are mixtures of several polymers which are condensation products of the flavans described above. It seems highly likely that in any CT several different types of linkages exist simultaneously. The typical linkage between monomers is a carbon-carbon condensation (Reid *et al* 1974), which is shown in Figure 1.3

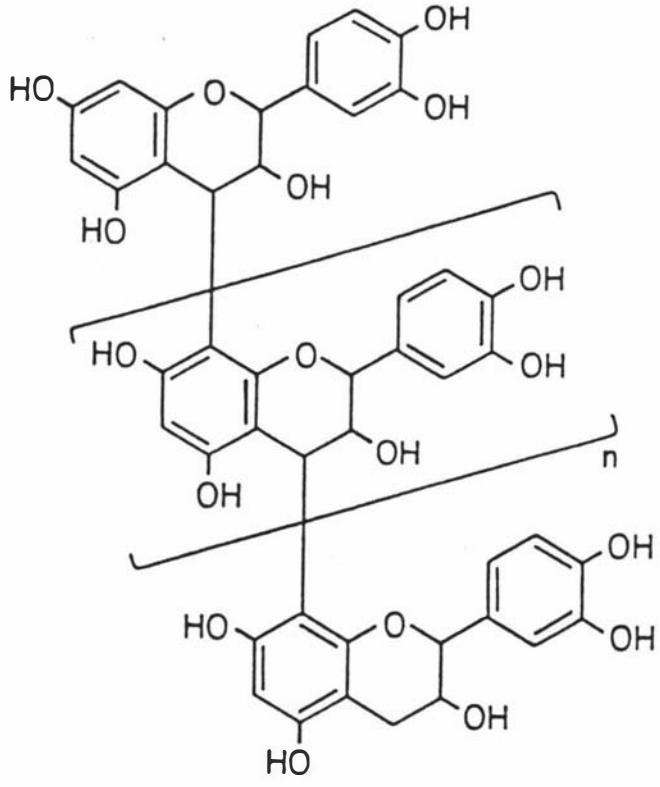

Figure 1.3 A 4,8 linked procyanidin (CT; From Hagerman & Butler 1991).

1.4.2 Analytical methods for measuring plant CT

Estimations of CT content begin with extraction of CT from the plant tissue. Although analysis of fresh forage can provide accurate data, it is inconvenient for large numbers of samples. Therefore, freeze-dried forage is usually used for CT analysis. Aqueous acetone and methanol have been widely used as extraction solvents (Makkar 1989), and aqueous acetone (7:3 V/V acetone:water) is generally recognized as the most efficient for extracting CT from forages (Terrill *et al* 1990). However, a problem with this procedure is that other compounds, such as soluble lipids and non-tannin pigments, are extracted as well and have been suggested to interfere with colour development in the subsequent vanillin-HCl colorimetric method (Terrill *et al* 1992a; Walton *et al* 1983).

Jones *et al* (1976) developed a method which involves adding N_aCl to salt out acetone, washing with petrol to remove fats and pigments, followed by chromatography (Sephadex G 50 and LH 20) to remove contaminants in CT extracts. This method has been used to produce CT standards from plant materials. Although this procedure yields extremely pure CT with a specific molecular weight range, it involves the risk of losing some CT, and also is time consuming and unsuited for large numbers of unknown samples.

Terrill *et al* (1992a) reported a simplified method in which a mixture of acetone:water:diethyl ether (4.7:2.0:3.3 v/v) is used to extract CT and to remove lipids and non-tannin pigments in one step. This technique allows a rapid and gross clean-up of the extract while still permitting quantitative recovery of CT present.

Colorimetric methods are widely used for subsequent CT analysis, mainly due to their specificity and high sensitivity. The most specific colour reactions to measure CT are the vanillin-HCl and butanol-HCl methods. In the acid butanol assay as described Porter *et al* (1986), proanthocyanidins (CT) are depolymerized oxidatively to yield a red anthocyanidin pigment. The yield of anthocyanidin pigments is dependent on reaction conditions as well as the structure of proanthocyanidin, but is roughly proportional to the concentration of total flavonoid groups. Monomeric flavonoids are not detected under the usual assay conditions. The vanillin-HCl assay (Price *et al*

1978) can be used to estimate CT in the presence of HT and other phenolic compounds, but is based on different chemistry, with vanillin reacting with the terminal flavenoid residues in the CT molecule. These methods normally only measure extractable CT (ECT).

Terrill *et al* (1992a), using their developed/modified butanol-HCl procedure, were able to measure total CT which include ECT, protein-bound CT (PCT) and fibre-bound CT (FCT) in forage samples. Their subsequent work showed that the method could not be used to measure bound CT in freeze dried rumen, abomasal and ileal digesta (Terrill *et al* 1994).

1.4.3 The occurrence of CT in plants

In most cases CT are present in the leaves and stems of plants whilst in some plants, such as white clover and red clover, CT occur only in the flower petals (Barry 1989). Table 1.4 lists the concentration of CT in some plants.

Table 1.4 Concentrations (g/kg DM) of extractable condensed tannins (ECT), protein-bound condensed tannins (PCT), fibre-bound condensed tannins (FCT) and total condensed tannins (TCT) in a range of plants.

Forages	ECT	PCT	FCT	TCT	Reference
Hairy canary clover	121	65	1.0	187	Terrill *et al* (1992a)
Prostrate Canary clover	100.0	23.0	3.0	126.0	" " "
Canary Clover	83.0	54.0	6.0	143.0	" " "
Big trefoil	61.0	14.0	1.0	76.0	" " "
Sulla	33.0	9.0	3.0	45.0	" " "
Crownvetch	16.0	13.0	2.0	31.0	" " "
Birdsfoot trefoil	7.0	13.0	1.0	21.0	" " "
Narrow leaf birdsfoot trefoil	2.0	3.0	1.0	6.0	" " "
Sheep's burnet	1.0	1.4	1.0	3.4	" " "
Chicory	1.4	2.6	0.2	4.2	" " "
Yorkshire fog (Wild ecotype)	1.1	0.3	0.4	1.8	" " "
Sainfoin	29.0	nd	nd	nd[1]	Barry & Manley (1986)

1. nd, no determined

CT concentrations in plants can be influenced by many factors. Some of the important factors are:

A. Season: CT concentration in plants has been found to vary seasonally. In *Sericea* species the highest concentrations occurred in midsummer (Cope *et al* 1971; Donnelly 1959). These seasonal changes may reflect the influences of light intensity and temperature on the occurrence of CT. High CT concentration have been linked with high light intensity, high temperature and severe drought.

B. Soil conditions: CT contents in plants vary according to the soil in which the plants grow. Barry and Forss (1983) demonstrated that when *Lotus pedunculatus* was grown in low fertility acid soils without fertilizer application, the CT contents were 80-110 g/kg DM, but when grown in high fertility soils, the CT concentrations were only 20-30 g/kg DM. Application of P and S fertilizers to the low fertility acid soils reduced CT contents to 40-50 g/kg DM. Table 1.5 lists CT concentrations in lotus species grown under high or low soil fertility conditions.

Table 1.5 Concentrations (g/kg DM) of extractable condensed tannins in vegetative lotus species as affected by soil fertility. (All determined by the vanillin-HCl procedure of Broadhurst & Jones 1978).

Authors	Soil fertility	*Lotus pedunculatus* cv Maku	*Lotus corniculatus* cv Empire	*Lotus corniculatus* cv Maitland
John & Lancashire (1981)	H[1]	20.0 (116)[3]	2.5 (99)	14. 5 (114)
Lowther *et al* (1987)	L[2]	94.5	2.8	28.1
Barry & Forss (1983)	H	32.0	-	-
	L	78.0	-	-
Barry & Duncan (1984)	H	45.6 (132)		
	L	105.9 (152)	-	-

1: H, high soil fertility pH > 5.3; Olsen P > 18 g/ml; SO^4-S > 12 µg/g.

2: L, low soil fertility pH < 5.2; Olsen P > 8 g/ml; SO_4-S > 5 µg/g.

3: Lignin (g/kg DM).

C. Stage of maturity: Usually mature leaf and stem contain more CT than young ones. CT and lignin are both produced in plants from the shikimic acid biochemical pathway and share many common intermediates (Swain 1979). Lignin content increases as plants mature. It is therefore no accident that CT in plants increases with maturity and plants containing high levels of CT also tend to be highly lignified (Table 1.5).

D. Leaf to stem ratio: CT content differs between tissues of a plant. Douglas *et al* (1993) reported that in species with total CT higher than 20 g/kg DM (such as *C. varia, H. coronarium* and *L. corniculatus*), total CT concentration in leaf is up to five times higher than in stem. Stitt & Clarke (1941) also showed a similar trend for *Sericea lespedeza*.

1.4.4 Evolutionary role of CT

Feeny (1976) suggested that phenolic compounds in plants served as chemical defence agents through their astringent taste and by inactivating digestive enzymes of their predators. Swain (1979) proposed that plants evolved CT as a defence against invasion by bacteria and fungi, relying on the ability of CT to complex with protein and polysacharides. With time this defence system further evolved as a defence against attack by insects and finally against being eaten by herbivores.

Whilst plants evolved the chemical defence system by producing CT, some observations indicated that successful herbivores have developed mechanisms for overcoming these effects. Rats have been shown to synthesize salivary proline-rich proteins (PRPS) which have high affinity for tannins, in response to feeding tannin-containing diets (Meshansho *et al* 1987). Austin *et al* (1989) reported that browsing deer can produce a tannin-binding protein which is a small glycoprotein containing large amounts of proline, glycine and glutamate/glutamine and not closely related to the proline-rich salivary protein found in rats. Domesticated ruminants (sheep and cattle) do not produce tannin-binding protein in their saliva.

1.4.5 Reaction of condensed tannins with protein

CT-protein reactions have been widely investigated with respect to their chemical nature and to factors influencing the reactivity (Asquith & Butler 1986; Asquith *et al* 1987; Haslam 1974; Oh *et al* 1980). Several types of chemical bond have been proposed for CT-protein complexes (Van Sumere *et al* 1975). These include: hydrogen (H-) bonds, ionic bonds or salt linkages and covalent bonds. Hydrogen bonding is considered to be the most common in CT-protein interactions, but is also the least stable and is affected by several factors.

It has been suggested that the interaction between protein and tannins involves both H-bond formation and hydrophobic interactions (Haslam 1989; Mueller-Harvey *et al* 1988; Oh *et al* 1980), and involves two distinct phases. The first stage is the formation of a hydrophobic pocket or environment. In this process the main force is action of hydrophobic groups on both tannin and protein. The second stage is the reinforcement of protein-tannin interaction by the appropriate development of H-bonds between tannin residues and polar groups (eg, guanidine, amide, peptide, amino, hydroxyl and carboxyl groups). The final result is the formation of a reversible complex between tannin and protein. In this interaction the tannin acts as polydonate ligands acting through several potential sites (phenolic residues) and the protein substrate is potentially a multi-site acceptor molecule.

1.4.5.1 Factors influencing CT-protein interactions

1.4.5.1.1 pH

It is generally believed that CT-protein interactions are largely dependent on pH, which greatly influences the stability of H-bonds. The H-bonds of CT (from sainfoin)-protein (fraction 1 leaf protein) complexes are strong at pH 3.5-7.0 but are weak at pH < 3.0 and pH > 8.0 (Jones & Mangan 1977). The reason why H-bonds are strongest at pH mentioned above may possibly be interpreted by the pH of CT (4.0-5.0) and the isoelectric points (PI) of the protein. Hagerman & Butler (1978) reported precipitation was greatest at a pH within ±1 unit of the PI of the protein.

1.4.5.1.2 CT composition and their molecular size or molecular weight (MW)

Protein precipitating properties of CT differ depending on their composition and

molecular sizes. Jones *et al* (1976) suggested the astringency of the CT was approximately determined by delphinidin (DP) content. Horigome *et al* (1988) showed that for a given CT, the protein-precipitating capacity increased with the increasing in degree of polymerization (Figure 1.4).

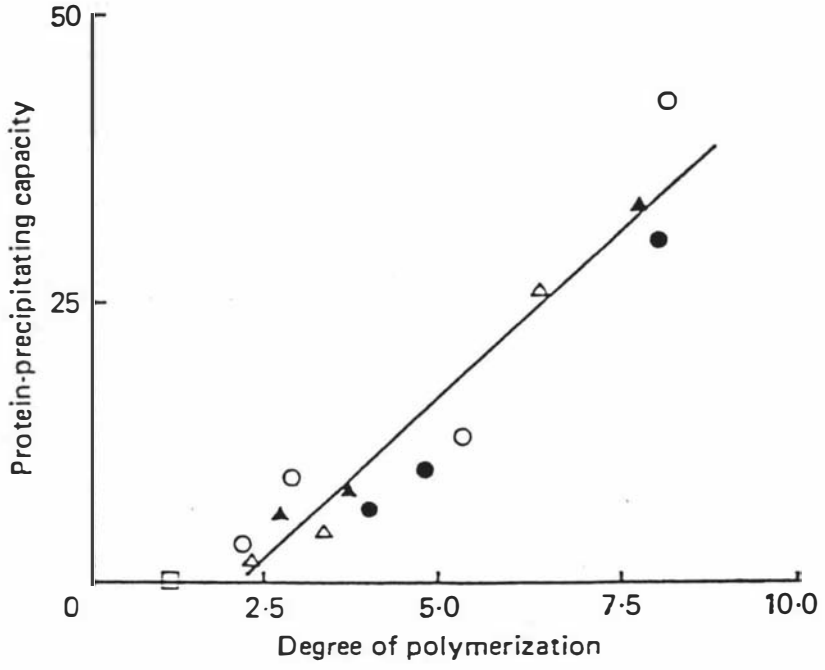

Figure 1.4 Relation between the degree of polymerization and protein-precipitating capacity of CT (r=0.855). Protein-precipitating capacity was determined by measurement of the precipitated bovine serum albumin (BSA) in a mixture of each fractionated CT (5 mg), BSA (20 mg) and 0.067 M-phosphate buffer, pH 7.8. (○) Black locust (*Robinia pseudo-Acacia*) tannins; (●), bush clover (*Lespedeza bicolor*) tannins; (△), wistaria (*Wistaria floribunda*) tannins; (▲), Japanese knotgrass (*Reynoutria japonica*) tannins; (□), catechin (From Horigome *et al* 1988).

1.4.5.1.3 Physical and chemical properties of proteins

Proteins having open, loose conformations, high molecular weight and high contents of proline and other hydrophobic amino acid have a high affinity for CT (Asquith & Butler 1986; Hagerman & Butler 1981).

1.4.5.2 Bound CT and free CT

Barry and Forss (1983) defined CT bound to plant protein after maceration as bound-CT and that still in supernatant after centrifugation as free-CT (FrCT). They suggested that it is FrCT that can react with other sourcesof protein after chewing by animals. This definition can be used to explain nutritional effects of CT in fresh forages eaten by ruminants (Figure 1.5).

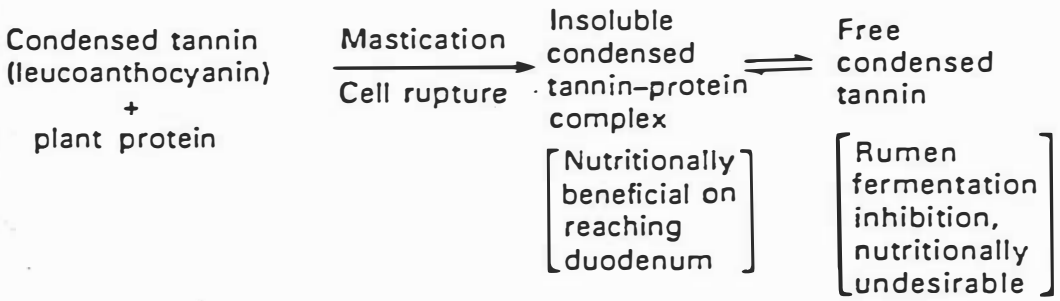

Figure 1.5 Nutritional effects of free condensed tannins (FrCT) and bound condensed tannins (BCT) after rupture of forages. (From Barry & Manley 1986).

1.4.6 Reactions between CT and carbohydrate

CT can also react with carbohydrates (or polysacharides), however the reactions are less well understood than with protein. Observations showed, however, a remarkably similar pattern of affinity to those noted for proteins, in particular the affinity of polyphenols for sites and environments which permit the hydrophobic interactions to develop (Haslam 1989).

1.4.7 Reactions of CT and minerals

In general, CT can react with metal ions, such as Ca^{++}, Fe^{++}, Cu^{++}, Mg^{++}, Sn^{++}, Pb^{++}, Zn^{++}, Hg^{++}, Al^{+++} and Cr^{+++} to form complexes which are insoluble or partly soluble. However, the mechanism of the formation of these complexes is not fully understood.

1.4.8 Reactions of condensed tannins with other compounds

Polyethylene glycol (PEG) and Polyvinylpyrrolidone (PVP) have been widely used to study the interactions between CT and protein, due to their strong reactivity with CT. However, it is PEG that is widely used in studies of the effects of CT in animal nutrition (Barry *et al* 1986a; McNabb *et al* 1993; Oh *et al* 1980; Waghorn *et al* 1987a).

PEG can specifically combine with CT to form CT-PEG complexes. CT-PEG bonds are stronger than CT-protein bounds, and thus adding PEG to diets can be used to prevent CT reacting with plant proteins. This provides a unique way to study the effect of CT without affecting other nutrients in the diet. PEG would seeming not offer strong proton acceptor sites to the tannins, but is instead known to exhibit relatively hydrophobic properties (Oh *et al* 1980).

1.4.9 The potential value of CT in ruminant nutrition

From the previous sections it has been shown that extensive degradation of high quality protein by rumen micro-organisms when the animal is fed fresh forage causes inefficient utilization of diet protein. Plants evolved CT as a defence against invasion by bacteria and fungi, relying on the ability of CT to complex with protein and

polysacharides, and finally against being eaten by herbivores. Although some animals such as rats and deer have developed a system to overcome the effects of tannins, this animal defence system is not found in domesticated sheep and cattle. Therefore, CT can be used as a practical means to improve protein nutrition of grazing sheep and cattle.

1.5 EFFECTS OF CONDENSED TANNINS ON FORAGE FEEDING VALUE FOR GRAZING ANIMALS

1.5.1 The effect of CT on VFI and animal production in ruminants fed fresh forages

1.5.1.1 Effect of CT on animal production

Studies showed that action of high concentrations of CT reduced both body and wool growth rates of sheep (Pritchard *et al* 1988, 1992). Barry (1985) reported oral administration of PEG (75-100 g/d) to sheep grazing *L. pedunculatus* (extractable CT 76-90 g/kg DM) increased LWG by 41-61 g/day and also increased wool growth (Table 1.6), showing that these levels of CT were limiting animal production.

Table 1.6 Liveweight gain (LWG) and wool growth of lambs grazing *Lotus pedunculatus* containing 76-90 g extractable CT/kg DM, with or without oral supplementation of polyethylene glycol (PEG; 75-100 g/d).

	Control	PEG supplemented
Experiment 1:		
LWG (g/d)	125	166
Wool growth (g/d)	8.5	9.5
Experiment 2:		
LWG (g/d)	27	70
Wool growth (mg/100 cm^2)	81	104

(From Barry 1985).

Compared to the effect of high CT levels, the effects of low CT concentrations on animal production is less understood. It is suggested that for the *Lotus* species, levels of extractable CT in the range 20-40 g/kg DM may be beneficial (Barry *et al* 1986a; Barry 1989). Although several studies comparing low CT-containing forages (such as *L. corniculatus*, extractable CT less than 40 g/kg DM) with other non CT-containing forages, such as red clover (*Trifolium pratense*), lucerne (*Medicago sativa)* and perennial ryegrass (*Lolium perenne*) indicated improved animal performance (John & Lancashire 1981; Marten & Jordan 1979; Ulyatt *et al* 1977), the results cannot be considered as solely due to CT, since the forages differed in several other aspects as well as CT concentration. Terrill *et al* (1992b), using oral PEG supplementation to the grazing sheep, showed that the action of CT in sulla (total CT 40-50 g/kg DM) increased LWG and wool growth in the summer season. However, they suggested that once daily PEG supplementation may not completely render all CT inert, and the animal response to the CT may not be fully expressed. Therefore there is a need to assess the effect of low levels CT on animal production in grazing ecosystems.

Purchas and Keogh (1984) found lower carcass fat concentrations in lambs grazing *L. pedunculatus* than those grazing white clover. A similar result was reported by Terrill *et al* (1992b), who found carcass fat concentration reduced by the action of CT in sheep grazing on sulla. This could be dilution of fat concentration by increased N retention associated with feeding fresh forages containing CT, and increased lipolysis may have been mediated by increased secretion of growth hormone (GH, Barry *et al* 1986b). This effect needs to be assessed using other forages containing low levels of CT.

Bloat is a widespread disorder when cattle are fed on certain common legumes such as white clover, red clover, subterranean clover and lucerne (Reid *et al* 1974). The primary cause of bloat is the formation of a stable foam in the reticulo-rumen, with soluble plant protein appearing to be the major foaming agent. CT have been found to be an anti-bloat agent, due to their ability to reduce leaf protein solubility (Jones *et al* 1973; Mangan *et al* 1976; Waghorn & Jones 1989). This may also contribute to the high animal productivity when grazing low CT-containing forages.

1.5.1.2 Effects of CT on VFI

High concentrations of CT (50-100 g extractable/kg DM) have been shown to depress VFI (Barry & Duncan 1984; Chiquette *et al* 1988; Pritchard *et al* 1988; Reed *et al* 1982) of ruminants. Barry & Duncan (1984) showed that reducing extractable CT from 63 to 7 g/kg DM increased ME intake of sheep by 44%. Waghorn *et al* (1990) showed that in sheep fed on forage containing 55 g extractable CT/kg DM, feed intake began to decline after two weeks and was 10% lower after 18 days than that of PEG sheep (CT inactivated). They suggested that this is because the high level of CT over protected protein so that there is insufficient N for rumen microbial growth, resulting in a low rate of fibre digestion in the rumen. These effects of high CT concentrations are consistent with the proposed evolutionary role of plant CT production. However there is limited information on the effect of low levels of CT on the VFI of grazing animals. Terrill *et al* (1992b) reported that CT in sulla (*Hedysarum coronarium*, total CT 40-50 g/kg DM) had no effect on VFI of grazing sheep. This needs to be investigated for other low CT-containing forages, such as *Lotus corniculatus*. Studies are also needed to define accurately the CT level at which VFI begins to decline.

1.5.2 Effects of CT on nutrient digestion and metabolism in ruminant animals fed fresh forages

1.5.2.1 Effects of CT on nutrient digestion

1.5.2.1.1 Effects of CT on N digestion

1.5.2.1.1.1 Effects of CT on N digestion in the rumen

Non ammonia nitrogen (NAN) flowing out of the rumen into the abomasum is positively related to total N intake (Waghorn & Barry 1987; Figure 1.6). However, this relationship differs between herbages. Ruminants consuming CT-containing fresh forages have more NAN leaving the rumen per unit N eaten compared to those consuming non CT-containing fresh forages. This is due to the action of CT reducing protein degradation in the rumen, which is clearly shown by the ammonia concentrations in the rumen fluid of control sheep fed on CT-containing diets being lower than that of PEG-supplemented sheep (Table 1.7).

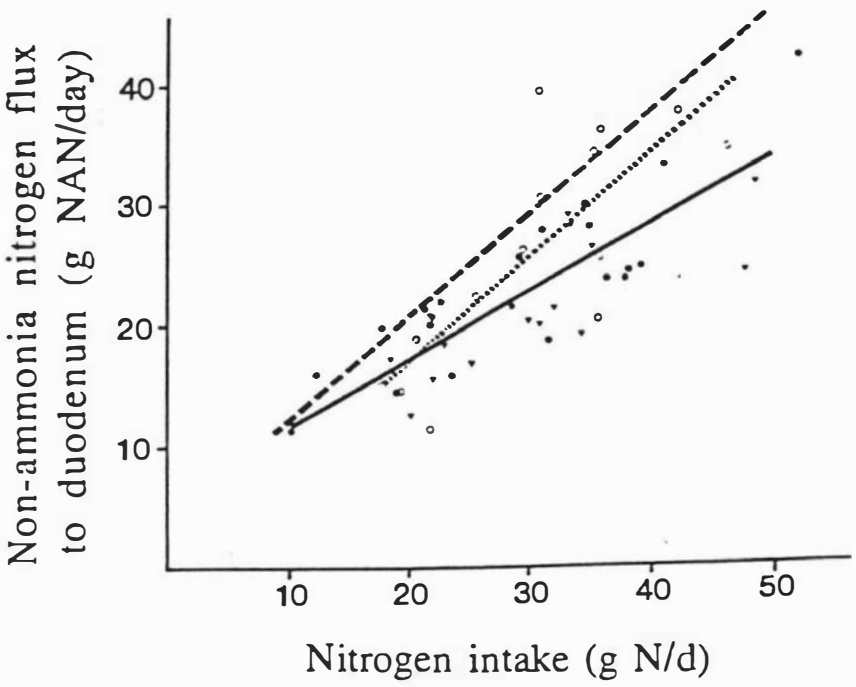

Figure 1.6 Relationship between N intake and non ammonia nitrogen (NAN) passing the duodenum of sheep offered fresh grasses and legumes (▲————▲), dry feeds (●----●) and tannin containing fresh legumes (O.....O; from Waghorn & Barry 1987).

Table 1.7 Concentrations of ammonia (mg NH_3 N/l) in the rumen fluid of sheep fed fresh forages containing different level (g/kg DM) of extractable condensed tannins (ECT), with or without polyethylene glycol (PEG) supplementation.

Forage	ECT concentration	PEG supplementation	Ammonia concentration	Sources
Lotus corniculatus	22	–	367	Waghorn *et al* (1987b)
		+	504	
Lotus pedunculatus	95	–	275	Barry *et al* (1986a)
	45	+	287	
	14	++	389	
" " " " "	50	–	106	McNabb *et al* (1993)
		+	200	
" " " " "	55	–	175	Waghorn *et al* (1990)
		+	485	
Sulla	36	–	153	Terrill *et al* (1992b)
		+	321	

Barry & Manley (1984) established a significant linear relationship between dietary CT concentration and NAN flow per unit total N intake in sheep fed fresh *L. pedunculatus* and *L. corniculatus* (Figure 1.7). Figure 1.7 shows that, for lotus species, NAN flow out of the rumen per unit N eaten increased as ECT concentration increased, and it become unity at the value of about 40 g ECT/kg DM; ie undegraded dietary N+ microbial N flowing at the duodenum equalled the N eaten.

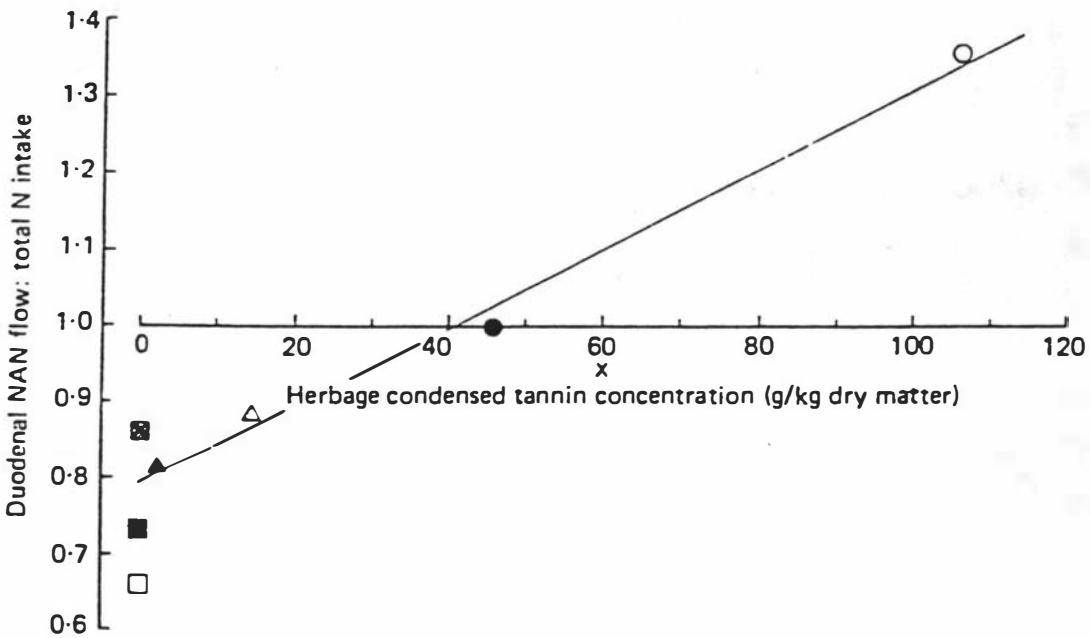

Figure 1.7 Duodenal non-ammonia nitrogen (NAN) flow per unit total N intake as a function of herbage condensed tannin (CT) concentration in sheep fed on *Lotus* species. (○) High CT (106 g extractable CT/kg DM) *Lotus pedunculatus*; (●) low CT (46 g extractable CT/kg DM) *Lotus pedunculatus*; (△) high CT (14.5 g extractable CT/kg DM) *Lotus corniculatus*; (▲) low CT (2.5 g extractable CT/kg DM) *Lotus corniculatus* (John & Lancashire 1981); (⊠) short rotation ryegrass; (□) perennial ryegrass; (■) white clover (MacRae & Ulyatt 1974) and (×) sainfoin (Ulyatt & Egan 1979). (From Barry & Manley 1984).

Waghorn *et al* (1987b) reported that when sheep were fed *L. corniculatus* containing 22 g ECT/Kg DM, the abomasal amino acid (AA) flux increased 26% due to the action of CT. CT especially reduced EAA degradation in the rumen, increasing EAA flow at the abomasum by 50%. However, for the non-EAA (NEAA) only a 14% increase was found. Their information did not include sulphur amino acids (SAA). The reason why CT preferentially increased abomasal flow of EAA relative to NEAA is not clear and requires further study.

McNabb *et al* (1993) found that the action of CT in *L. pedunculatus* (ECT 55 g/kg DM) reduced the proteolysis of forage SAA in the rumen. The effects of CT in reducing rumen SAA degradation could have significance in sheep production, since SAA are considered to be the most limiting AA for wool growth (Reis 1979). These effects need further study using forages containing low concentrations of CT.

1.5.2.1.1.2 Effects of CT on the post-ruminal N digestion

Barry & Manley (1984) observed post rumen apparent digestibility of NAN to be 0.71 and 0.67 with sheep fed *L. pedunculatus* containing 40 g and 106 g ECT/kg DM, respectively. Waghorn *et al* (1994a) reported apparent N digestibility in the SI (as a proportion of abomasal N flow) was 0.61 for control sheep (CT active) and 0.74 for PEG infused sheep (CT inactivated) fed *L. pedunculatus* (ECT 55 g/kg DM).

The above evidence showed that action of CT in plants reduced post ruminal N apparent digestibility. Experiments carried out to study the AA digestibility in the SI also showed the same trend (McNabb *et al* 1993; Waghorn *et al* 1987b, 1994a). However, in terms of the quantitative absorption from the SI, AA, especially EAA, were greatly increased due to the action of CT (Barry & Manley 1984; Barry *et al* 1986a; McNabb *et al* 1993; Waghorn *et al* 1987a; Table 1.8), mainly due to the effect of CT in increasing abomasal flow. Compared to knowledge of the effect of CT on N digestion in the rumen, the effect of CT on the post rumen N digestion is less well known and should be further studied.

Table 1.8 The effect of condensed tannins (22 g extractable CT/kg DM) upon the digestion of amino acids in sheep fed fresh *Lotus corniculatus*.

	Essential [1]		Non-essential [2]	
	Control	PEG	Control	PEG
Intake (g/d)	98.9	98.9	97.9	97.9
Abomasal flow:				
g/d	84.7	55.5	68.6	59.1
proportion intake	0.86	0.56	0.70	0.60
Apparent absorption from small intestine:				
g/d	58.8	36.2	37.4	41.3
proportion abomasal flow	0.67	0.67	0.54	0.67
proportion intake	0.59	0.37	0.38	0.42

(From Waghorn *et al* 1987b).

1. Threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine and lysine.
2. Asparagine, serine, glutamate, proline, glycine and alanine.

1.5.2.1.2 Effect of CT on the digestion of carbohydrate

Digestion of carbohydrate occurs in the rumen by the process of microbial fermentation, from which volatile fatty acids (VFA) are produced. The mechanism of CT-carbohydrate formation is believed to be similar to that of CT-protein, but CT react more strongly with protein than with carbohydrate and the CT-carbohydrate complex is less stable than that of CT-protein. Usually only a small proportion of CT bind with carbohydrate either before or after animal chewing (Terrill *et al* 1992a). Therefore it is reasonable that the interaction between CT and carbohydrate has little influence on the digestion of carbohydrate. The main effects of CT on carbohydrate digestion may be due to CT precipitating enzymes secreted by rumen micro-organisms. Hence

this adverse effect can only occur when CT levels are so high that significant amounts of unbound CT are produced. However, when forages contain low levels of CT, the proportion of unbound CT (i.e. FrCT) after chewing by animals would be small and would have no significant effect on microbial fermentation.

Low concentrations of CT (22 g extractable/kg DM) in *L. corniculatus* have been reported to have no effects on the digestion of both water soluble carbohydrates (WSC) and structural carbohydrates in sheep (Ulyatt & Egan 1979; Waghorn *et al* 1987b). In contrast, the high CT concentration (106 g ECT/kg DM) in *L. pedunculatus* caused a reduction in readily fermentable carbohydrate (RFC; WSC+pectin), structural carbohydrate and lignin digestibility in the rumen, but this was compensated by increased digestibility in the intestine (Barry & Manley 1984; Table 1.9). Studies are needed to determine at what levels the action of CT starts to depress rumen carbohydrate digestion.

Table 1.9 Ruminal and post-ruminal digestion of readily fermentable carbohydrate (RFC), cellulose and hemicellulose in sheep fed *Lotus pedunculatus* differing in extractable condensed tannin (ECT) content.

	Low-CT lotus (46 g ECT/kg DM)			High-CT lotus (106 g ECT/kg DM)		
	RFC	Hemicellulose	Cellulose	RFC	Hemicellulose	Cellulose
Apparent digestibility						
proportion of intake	0.95	0.73	0.78	0.93	0.56	0.63
Ruminal digestion						
proportion of intake	0.80 (0.93)	0.44 (0.58)	0.69 (0.69)	0.78 (0.93)	0.21 (0.42)	0.53 (0.54)
proportion of total digested	0.84	0.61	0.89	0.83	0.38	0.85
Post-ruminal digestion						
proportion of intake	0.15 (0.06)	0.28 (0.15)	0.09 (0.09)	0.16 (0.06)	0.35 (0.14)	0.10 (0.09)

(From Barry & Manley 1984).

Numbers in parentheses are predicted from the equations of Ulyatt & Egan (1979), derived with non-tannin-containing fresh forages.

1.5.2.1.3 Effect of CT on the digestion of minerals

There is limited information on the effect of CT on mineral digestion in ruminant animals. Waghorn *et al* (1987a) reported that in sheep fed on *L. corniculatus* the action of CT (22 g extractable CT/kg DM) reduced absorption of sulphur, potassium and magnesium in the whole GI tract. As total sulphur consists mainly inorganic sulphur and organic sulphur from SAA, and it is latter that is important to the animal. Therefore the effect of CT on SAA digestion needs to be assessed. Waghorn *et al* (1994b) showed that CT (55 g extractable/kg DM) in *L. pedunculatus* reduced rumen degradation and absorption of sulphur and increased net absorption of both phosphorus and zinc, whilst McNabb *et al* (1993) showing that CT at this level reduced SAA degradation in the rumen. Other minerals essential to ruminant animal nutrition have not been investigated yet and research needs to conducted in this area.

1.5.2.2 Effect of CT on the metabolism of digested nutrients and on the endocrine system

There is no information available on the effect of CT on the efficiency of utilization of ME. Since the low animal production associated with high levels of CT is mainly caused by low VFI and low digestibility, any effects on efficiency of utilization of ME would probably not be important. However, with low levels of CT, the improved AA nutrition might increase the efficiency of utilization of ME. Research is required in this area.

McNabb *et al* (1993) reported that CT in *L. pedunculatus* (55 g extractable/kg DM) had no effect upon IRL of methionine from blood plasma but markedly increased the IRL of cystine and reduced the IRL of inorganic sulphate from blood plasma (Table 1.10). They also found that CT at this level reduced the oxidization of both methionine and cystine to inorganic sulphate and increased the proportion of cystine transferred to body synthetic reactions. The effect of CT upon these transactions needs to be studied with forages containing low levels of CT.

Table 1.10 The irreversible loss (IRL; μmol/min) from blood plasma and the proportion of total flux of sulphur amino acids transferred to various processes in sheep fed on *Lotus pedunculatus,* with or without an intraruminal infusion of polyethylene glycol (PEG).

	Control sheep	PEG-infused sheep	SED	Significance
IRL:				
Methionine	20.6	19.9	2.95	NS
Cystine	39.8	22.4	7.36	*
Sulphate	35.9	50.2	4.45	**
Proportion of flux transferred:				
Methionine to cystine	0.57	0.31	0.11	*
Methionine to sulphate	0.04	0.06	0.019	NS
Cystine to sulphate	0.10	0.26	0.053	*
Cystine to P+M	0.91	0.74	0.050	*
Methionine to P+M	0.40	0.62	0.110	†

From McNabb *et al* 1993).

(P+M), Productive processes and maintenance; NS, not significant ($P < 0.10$); * $P < 0.5$; ** $P < 0.01$; † $P < 0.10$.

Nunez-Hernandez *et al*(1991) reported that action of CT (41 g extractable/kg DM) reduced serum urea N concentration. Since the main source of blood urea N is NH_3 absorbed from rumen, it is therefore no coincidence that CT reduced both ammonia concentration in the rumen and urea N concentration in the serum. However, proportions of N produced from metabolism of AA either from body AA turnover or from absorption from the SI also join into the blood urea-N pool and may affect the urea N concentration in the blood. Whether the action of CT affects AA metabolism other than SAA in blood or not is not known. Therefore, there is a need to further study the effects of CT on AA metabolism in the blood. Whether the action of CT

affects partition of absorbed AA to different physiological pathways also needs to be studied.

Barry *et al* (1986b) reported plasma concentrations of GH were positively and linearly related to dietary reactive CT concentration. They suggested that the increase in GH concentration in plasma with increasing reactive CT concentration might be related to the inactivation of gut wall protein by CT. Nunez-Hernandez *et al* (1991) observed the insulin concentration in serum was lower in sheep and goats fed on diets containing CT than those of PEG supplemented sheep. The same trend was observed by Barry *et al* (1986b) but not at significant levels. Both GH and insulin play a important role in nutrient partition and metabolism. Therefore research is needed to study the effect of low levels of dietary CT on the endocrine system.

1.6 CONCLUSION AND NEEDS FOR RESEARCH

1.6.1 Forage is the main feed source for ruminant animals, especially in NZ where use of pasture by grazing animals is the basis of pastoral agriculture. One of the main factors affecting the FV of fresh forage is the extensive degradation of protein in the rumen (70%), with duodenal NAN flow being approximately 65% of the N eaten. The natural occurrence of low levels of CT (total CT 20-40 g/kg DM) in some plants provides a promising means to overcome this problem in sheep and cattle.

1.6.2 CT are polymerised phenolic compounds. Their structures differ between different plants. The occurrence of CT in plants can be influenced by season, soil fertility and stage of plant maturity. Some plants first evolved CT production as a defence against attack by pathogenic bacteria and fungi, which then further evolved as a defence against being eaten by insects and herbivores and to adapt themselves to a given environment. Hence, plants produce CT as a chemical defence system. Some animals (i.e. browsers) have evolved salivary protein to bind CT, but these proteins are absent in domesticated sheep and cattle, meaning that CT can be used to manipulate protein digestion.

1.6.3 The most common methods to measure CT in plants are the vanillin-HCl

and butanol-HCl procedures. A new method which can measure extractable, protein-bound and fibre-bound CT has been developed.

1.6.4 CT react with proteins and carbohydrates by H-bonding. The reactions between CT and proteins are influenced by pH, structure and molecular sizes of CT and protein properties. The reactions are strong at pH 3.5-7.0. However, when pH is < 3.0 and > 8.0, the CT-protein complexes dissociate and proteins are released from the complexes. Reactivity increases with increasing polymerization of the CT. Proteins having open, loose conformations, high molecular weights and high contents of proline and other hydrophobic amino acids have a high affinity for CT.

1.6.5 Action of CT reduced protein degradation in the rumen as shown by reduced rumen ammonia concentration and increased NAN flow out of the rumen per unit total N intake. The apparent absorption of EAA from the SI was increased by the action of low CT concentration. The effectiveness of CT in low levels on protein digestion in the rumen and on the post-ruminal AA digestion needs to be studied.

1.6.6 High levels of extractable CT depressed rumen carbohydrate digestion, whilst low levels of CT had no effect on rumen carbohydrate digestion. Research is needed to determine the CT concentration at which rumen fibre digestion begins to decline.

1.6.7 Action of CT increased IRL of SAA from blood plasma, increased cystine flow to body synthetic reactions and decreased plasma IRL of inorganic sulphate. This effect needs to be assessed with forages containing low levels of CT. Studies also are required to investigate the effects of CT on metabolism of absorbed AA, and on the partition of absorbed AA to various physiological pathways.

1.6.8 Whilst evidence showed that action of CT in high levels depresses VFI, LWG and wool growth, low levels of CT may be beneficial for increasing animal production in grazing ecosystems, due to increasing EAA absorption. However,

there is lack of information on this aspect and research in these areas needs to be conducted.

1.6.9 It is suggested that action of CT at the level of 20-40 g total/kg DM may be beneficial for the nutrition of grazing sheep. At this level the action of CT could reduce protein degradation in the rumen and increase AA absorption from the SI, without depressing fibre digestion. This hypothesis needs to be tested both using indoor metabolism and grazing experiments, with the latter assessing effects of CT upon animal production.

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Chapter 2

The effect of condensed tannins in *Lotus corniculatus* upon plasma metabolism of methionine, cystine and inorganic sulphate by sheep

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2.1 *ABSTRACT*

Fresh *Lotus corniculatus* containing 27 g/kg DM of extractable condensed tannin (CT) and 8 g bound CT/kg DM was fed at hourly intervals to sheep held in metabolism crates, to study the effects of CT upon nutrient digestion and upon metabolism of methionine, cystine and inorganic sulphate in plasma. Polyethylene glycol (PEG) was continuously infused into the rumen of half the sheep to remove the effects of CT. Principal measurements in the two groups were plasma irreversible loss rate (IRL) and interconversions of methionine, cystine and inorganic sulphate using ^{35}S labelling. CT in *Lotus corniculatus* had no effects upon the apparent digestion of cellulose and minerals, slightly depressed DM, OM and hemicellulose digestion and markedly reduced the apparent digestion of nitrogen (N) ($P < 0.01$). The concentration of ammonia and molar proportions of *iso*-butyric acid, *iso*-valeric acid and *n*-valeric acid in rumen fluid were markedly increased by the PEG infusion ($P < 0.01$), whereas total VFA concentration and molar proportions of acetic acid, propionic acid and *n*-butyric acid were not affected. PEG infusion temporarily increased rumen protozoa numbers. CT greatly increased the IRL of plasma cystine (13.1 *vs* 7.0 μmol/min; $P < 0.05$) and reduced IRL of plasma inorganic sulphate (36.8 *vs* 48.1 μmol/min; $P < 0.01$) but had no effect upon methionine IRL. CT increased transulphuration of methionine to cystine (4.37 *vs* 1.24 μmol/min; $P < 0.05$), increased cystine entering the plasma from whole body protein turnover plus absorption from the small intestine (9.34 *vs* 5.75 μmol/min; $P < 0.05$) and increased cystine flux to body synthetic reactions (11.89 *vs* 5.41 μmol/min; $P < 0.05$). CT had no effect upon the proportion of methionine total flux transferred to sulphate (0.05 *vs* 0.06; $P > 0.05$), reduced the proportion of methionine flux transferred to body synthetic reactions (0.68 *vs* 0.86) and markedly reduced the proportion of cystine flux transferred to sulphate (0.09 *vs* 0.27; $P < 0.01$). It was concluded that CT in *Lotus corniculatus* reduced rumen protein degradation and markedly increased utilization of plasma cystine for body synthetic reactions.

KEY WORDS: Condensed tannins, digestibility, sulphur-containing amino acids, inorganic sulphate, sheep

2.2 INTRODUCTION

Condensed tannins (CT) are plant secondary compounds which can react with protein and carbohydrate by hydrogen bonding in a pH-dependent manner (McLeod 1974). Nutritional effects of CT on the ruminant animal are dependent in part upon their concentration in the plant. High levels of CT (50-100 g extractable CT/kg DM) have been demonstrated to depress voluntary feed intake (VFI) and reduce apparent digestibility of DM, OM and fibre (Barry and Duncan 1984; Barry and Manley 1984; Chiquette *et al* 1988; Donnelly and Anthony 1969, 1970; Reed *et al* 1982; Pritchard *et al* 1988). In particular, rumen protein and fibre digestion are depressed. As the CT:protein complex is stable and insoluble at pH 3.5-7.0 but dissociates and releases protein at pH < 3.5 (Jones and Mangan 1977), low levels of CT (20-40 g extractable CT/kg DM) are believed to be beneficial in ruminant diets by reducing protein degradation in the rumen and increasing amino acid absorption from the small intestine, without depressing rumen fibre digestion and VFI (Barry *et al* 1986; Barry 1989; Waghorn *et al* 1987a; 1990).

When *Lotus corniculatus* was fed to sheep, Waghorn *et al* (1987b) found that apparent absorption of essential amino acids (EAA; excluding methionine, cystine and tryptophan) from the small intestine was increased 62% by the CT (22 g extractable / kg DM). Apparent absorption of non essential amino acids (NEAA) was reduced 10%. More recently McNabb *et al* (1993) observed that in sheep fed *Lotus pedunculatus*, the presence of CT (55 g extractable / kg DM) prevented a net loss of methionine and cystine across the rumen, and increased methionine apparent absorption from the small intestine by 27%. CT did not increase apparent absorption of cystine but it did increase both plasma irreversible loss (IRL) of cystine and the amount of cystine incorporated into body synthetic reactions, due to transulphuration of methionine to cystine. In the same study, CT had no effect upon the apparent digestibility of methionine in the small intestine (0.78), but reduced the apparent digestibility of cystine in the small intestine from 0.53 to 0.42, showing that at this concentration CT may be affecting the hydrolysis of proteins in the small intestine and reducing the apparent absorption of some amino acids.

Sulphur-containing amino acids are of particular importance for sheep because both cysteine and methionine are precursors of cystine, which is a major component of

wool protein (Reis 1979). This experiment was designed primarily to determine the effects of CT in *Lotus corniculatus* on plasma IRL of sulphur-containing amino acids, together with transulphuration of methionine to cystine and oxidation to sulphate. The experimental protocol also provided an opportunity to examine effects of CT on rumen VFA concentrations and rumen protozoal numbers when *Lotus corniculatus* was fed.

2.3 MATERIALS AND METHODS

The experiment involved fourteen male castrated Romney sheep aged 18 months which had been fitted previously with rumen cannulae (55 mm ID). They had been grazing out of doors and were brought inside (day 1) and held in indoors metabolism crates for 34 days. The sheep were introduced to *Lotus corniculatus* and allowed a 10 day adjustment period before sampling commenced.

2.3.1 Animals

The sheep weighed 48 kg (SE 1.5) at the beginning of the experiment. All were drenched with anthelmintic to control internal parasites (12 ml; Ivomec, Merk Sharp and Dohme, NZ Ltd.) and treated for external parasites (10 ml; Wipeout, Coopers Animal Health, NZ Ltd.) prior to the experiment commencing. The sheep had been held in metabolism crates previously. Feed intakes were recorded from day 4, and an intraruminal infusion of polyethylene glycol (PEG; MW 3,500) was given (50g/day in 240 ml water) to six animals commencing on day 12 and continuing until day 34 (PEG group). The PEG binds with CT, preventing the CT from binding with protein (Jones and Mangan 1977). The remaining eight animals did not receive an intraruminal infusion and acted as a control group.

2.3.2 Feed

The *Lotus corniculatus* was harvested daily at about 08:00 hours with a sickle bar mower. It was vegetative throughout the trial and was fed hourly from overhead feeders. Feed was offered *ad libitum* for the first 7 days of the experiment and at about 90% of *ad libitum* thereafter. About 40% of the daily feed was placed on the feeders at 09:30 hours and the remainder held at 4°C until 17:00 hours and then placed on the feeders. Refusals were collected each morning and daily DM intakes measured throughout the experimental period. Feed samples were collected at 2 day intervals and held separately at -20 °C for chemical analyses.

2.3.3 Rumen sampling protocols

Metal probes covered in a synthetic fibre (Estal-mono; Swiss Screens; Sydney; 88 µ pore size) were suspended in the rumen, enabling liquor to be collected by gentle suction (20 ml syringe). Rumen liquor was sampled between 13:00 and 15:00 hours commencing on day 11 of the experimental period (prior to PEG infusion) and on days 1, 2, 4, 6, 10, 16 and 22 after the PEG infusion started. The rumen fluid was used to determine rumen NH_3 and VFA concentrations and protozoal numbers.

The rumen fluid used for the determination of NH_3 concentration (20 ml) and VFA concentration (5 ml) was deproteinized immediately after sampling (5 ml of 2.5 *M* H_2SO_4 saturated with $MgSO_4$ for NH_3 samples, and 1 ml of a mixture of metaphosphoric acid and formic acid for VFA samples). VFA protein precipitant comprised 375 g metaphosphoric acid and 500 ml 100% formic acid made up to 2 litres. The deproteinized NH_3 and VFA samples were then centrifuged at 1,600 g and the supernatant fluid was stored at -20 °C. The rumen fluid used to determine protozoal numbers was preserved by addition of formal saline (9 g NaCl and 40 g HCHO/l) and stored at à °C.

2.3.4 Sulphur amino acid kinetics

Six control sheep and six PEG sheep had catheters installed in both jugular veins on day 15 of the experimental period in order to infuse ^{35}S-methionine (day 18), ^{35}S-cysteine (day 21) and $^{35}SO_4$ (day 24). Each isotope was infused continuously for 30 hours. Blood was sampled from the opposite catheter prior to infusion (background) and after 24, 26, 28, 30 hours of infusion to determine the specific radioactivity (SA) of isotope in plasma. The isotopes (Amersham, Australia Pty Ltd, Auckland, New Zealand) were prepared as follows: ^{35}S methionine (203.5 MBq) was added to 3.3 litres sterile saline containing 0.25 *m*mol/l inert L-methionine (BDH) as a carrier; ^{35}S cysteine (199.8 MBq) was added to 3.2 litres sterile saline containing 0.24 *m*mol/l inert L-cysteine (BDH) as a carrier and ^{35}S ammonium sulphate (388.5 MBq) was added to 3.3 litres of sterile saline containing 0.31 *m*mol/l inert ammonium sulphate (BDH) as a carrier. The rate at which methionine , cysteine and inorganic sulphate were infused into each sheep were 0.4144, 0.4846 and 0.9250 MBq/h respectively.

During the methionine and cysteine infusions, about 25 ml blood per sampling time

was collected into syringes with Na-EDTA as an anticoagulant and placed on ice. The blood was centrifuged (3,000 g, 15 minutes) to obtain plasma which was divided into two aliquots. One aliquot (10 ml) was treated with 15 µl of 2-mercaptoethanol to reduce oxidation and centrifuged through a molecular filter (Centriprep-10 membrane filter tubes; molecular weight cut off 10,000; Amican, USA) at 1,900 g to remove large proteins. The filtrate was frozen at-80 °C until analyzed for methionine, cysteine and cystine. A second aliquot (2 ml) was deproteinized with 0.2 ml of 50% (w/v) trichloroacetic acid (TCA) solution, allowed to stand at 0 °C for 15 minutes and was centrifuged at 3,000 g for 20 minutes. The supernatant was retained at -80 °C for sulphate analysis. When $^{35}SO_4$ was infused, only 10 ml blood was collected and the plasma was deproteinized with TCA and held at -80 °C for analysis.

2.3.5 Digestibility

Following the isotope infusions, harnesses were put onto the sheep (day 26) enabling faeces collection bags to be attached for total faeces collection from day 27 to day 34. Faeces were collected, weighed and a sample (10% by weight) was bulked over the 7 day collection period (at -20 °C) for analysis. Feed DM determinations were made in triplicate (95 °C for 24 hours) and the quantity of material refused determined for each sheep. Samples of feeds and refusals were retained for analysis. Faeces DM was determined by drying at 95 °C for 48 hours.

2.3.6 Analytical

2.3.6.1 Feed, feed refusal and faeces

All analyses of feeds, refusals and faeces were made with freeze dried material. Carbohydrate fractions were determined by sequential detergent extraction (Van Soest 1983) and total nitrogen (N) by Kjeldahl digestion. Samples were heated at 550 °C for 16 hours to determine ash content in the DM. Condensed tannins were determined as extractable, protein-bound and fibre-bound fractions using the modified butanol-HCl procedure (Terrill *et al* 1992a). Total sulphur in feed DM was determined after oxidation by NaOBr (Tabatabai and Bremner 1970) and feed samples for sulphate analysis were prepared by adding 1.0 g herbage to 50 ml of 10% HCl, shaking over night and making the extract up to 250 ml. The above prepared samples were analyzed using an Auto-analyzer (Technicon Industrial System, USA) by the modified method of Johnson and Nishita (1952).

2.3.6.2 Rumen fluid

Rumen fluid samples were centrifuged (950 g; 15 min) and the supernatant was used for analysis. Rumen ammonia was determined by distilling samples which had been made weakly alkaline with sodium tetraborate (pH 9.5) into boric acid (10 g /l) and titrated against 0.02 M-HCl using mixed bromocresol green/methyl red indicator (Kjeltec Auto 1030 Analyzer; Sweden). Rumen VFA were determined by capillary gas chromatography (Carlo Erba GC-5380, Italy) using a 15 m x 0.53 mm id FFAP (1.0 µm coat thickness column, Quadrex, USA). The procedure used a split injection mode (≈ 2 : 1 split ratio) and a 5 °C/min temperature programme from 115-145 °C, with hydrogen as carrier gas (pressure 0.5 Kg/cm^2), and a flame ionization detector. External standardisation was used and the data was calculated using a Maxima 820 chromatography workstation (Waters Associates, USA). Protozoa were counted in a 0.1 mm deep haemocytometer chamber, using light microscopy.

2.3.6.3 Plasma samples

All high performance liquid chromatography (HPLC) and radioactivity analyses for methionine, cysteine, cystine and inorganic sulphate commenced immediately after the animal work concluded. Measurement of SA from one control and one PEG sheep showed that SA of methionine, cystine and inorganic sulphate had reached plateau values after 24 hours of infusion. Therefore the plasma samples from 26, 28 and 30 hours were pooled for each sheep. The concentrations and the radioactivities of methionine, cysteine, cystine and inorganic sulphate were then determined in the bulked samples.

The concentrations of methionine, cysteine and cystine were determined by HPLC (Waters Associates, USA), using a reverse phase Pico.Tag column for free amino acids and using the Pico.Tag analytical method for physiological samples (Cohen *et al* 1989). Twenty µl of deproteinized plasma, derivatized with phenylisothiocyanate (PITC), was injected into the column. The amino acids were detected by the fluorescence of their PITC derivatives using a Waters (USA) 490E programmable multiwavelength Detector. The data were calculated using a Maxima 820 chromatography workstation (Waters Associates, USA).

The ^{35}S radioactivity in methionine, cysteine and cystine were determined in a 1 ml aliquot of deproteinized plasma, by HPLC with an ion-exchange column, using the

method described by McNabb *et al* (1993). Radioactivities were counted in a liquid scintillation counter (Wallac 1409, Pharmacia, Finland).

Inorganic sulphate in deproteinized plasma was separated from organic sulphur with Dowex 1-X8 resin (Cl-form, 18-52 mesh size, 1.33 meq/ml binding capacity; BDH; England). Preparation of the resin and the separation procedure were the same as described by McNabb *et el* (1993), except that 3 M HCl instead of 1 M HCl was used to elute inorganic sulphate off the resin. The radioactivity of inorganic sulphate was determined by adding 1 ml of eluent to 10 ml of high efficiency phase combining system (PCS II) and 2.0 ml of glacial acetic acid and using a liquid scintillation counter (Wallac 1409, Pharmacia, Finland). Inorganic sulphate concentration was determined by the automated method of Johnson and Nishita (1952), using an auto-analyzer (Technicon Industrial System, USA). All the standards used for auto-analysis were made up in 3 M HCl which had been treated in an identical fashion to deproteinized plasma samples.

^{35}S-labelled methionine, cysteine and ammonium sulphate infusates were added to deproteinized plasma in order to determine the recovery of ^{35}S-labelled methionine, cysteine and sulphate in each analytical method. Radioactivity of background samples were also determined in an identical fashion.

2.3.7 Calculation of the data and statistical analysis

Comparison between control and PEG treatments was by analysis of variance. Means are presented with the standard error of mean (SEM). DM and OM digestibilities were calculated on a PEG-free basis.

Both concentrations and radioactivity of cysteine and cystine were combined and expressed as cystine. The calculation of SA, IRL and transfer quotient (TQ) used methods described by McNabb *et al* (1993). Background values were deducted in determining the SA for all metabolites. All plasma metabolite concentrations and SA values were corrected for recoveries, determined for each analytical method. The results of sulphur amino acid kinetics are presented as a three pool, compartmentalized model as described by McNabb *et al* (1993) and the amounts of S from inorganic sulphate transferred to methionine and cystine were assumed to be

zero. Total flux was calculated as the summation of all outflows from each pool, and individual outflows then expressed as a proportion of the total flux. Oxidation of methionine and cystine was calculated as the proportion of total flux transferred to inorganic sulphate.

2.4 RESULTS

The DM content of *Lotus corniculatus* was 157 (SE 9.6) g/kg, and the chemical composition of the DM showed little variation throughout the experiment (Table 2.1).

Table 2.1 Chemical composition of the *Lotus corniculatus* (g/kg DM). (Mean values for 12 samples with SE).

Organic Matter	906 ± 5.3
Total Nitrogen	34.8 ± 0.3
NDF [1]	307 ± 8.3
ADF [2]	229 ± 4.9
Hemicellulose	78 ± 1.3
Cellulose	142 ± 6.2
Lignin	80 ± 8.1
Total sulphur	2.01 ± 0.123
Sulphate sulphur	0.43 ± 0.022
Extractable CT [3]	27.1 ± 1.69
Protein-bound CT	6.1 ± 0.27
Fibre-bound CT	1.8 ± 0.12
Total CT	35.0 ± 1.78

1. NDF: Neutral detergent fibre;
2. ADF: Acid detergent fibre;
3. CT: Condensed tannin.

2.4.1 Apparent digestibility

During the digestibility period, the DM intake of the fresh *Lotus corniculatus* of the control sheep (985 g DM/d; SE, 84.5) and the sheep receiving PEG (982 g DM/d; SE, 90.7) was not significantly different ($P > 0.05$). The apparent digestibilities of DM (0.766 *vs* 0.786; SEM 0.0079) and OM (0.774 *vs* 0.797; SEM 0.0084) were lower ($p=0.084$ and 0.060 respectively) and total N (0.721 *vs* 0.795; SEM 0.0124) was significantly lower ($P < 0.01$) for control than for PEG sheep. Hemicellulose apparent digestibility (0.605 *vs* 0.666; SEM 0.0256) also tended to be lower in control sheep than in PEG sheep ($P=0.118$). However, there were no differences between control and PEG sheep in the digestibility of cellulose (0.0.757 *vs* 0.763; SEM 0.0151) and minerals (0.692 *vs* 0.684; SEM 0.0097).

2.4.2 Rumen fluid

2.4.2.1 Ammonia concentration

There were no significant differences in the concentration of NH_3 in rumen liquor between control and PEG-infused sheep ($P > 0.05$; Figure 2.1 **A**) prior to commencement of PEG infusion or after 1 day of intraruminal PEG infusion. However, from day 2 onwards, rumen NH_3 concentration was significantly lower in control sheep than in PEG-infused sheep. Rumen NH_3 concentrations after 4, 6, 10, 16 and 22 days of PEG infusion were also significantly higher ($P < 0.01$) than those prior to PEG infusion commencing, whereas NH_3 concentration remained unchanged for control sheep during the experimental period ($P > 0.05$).

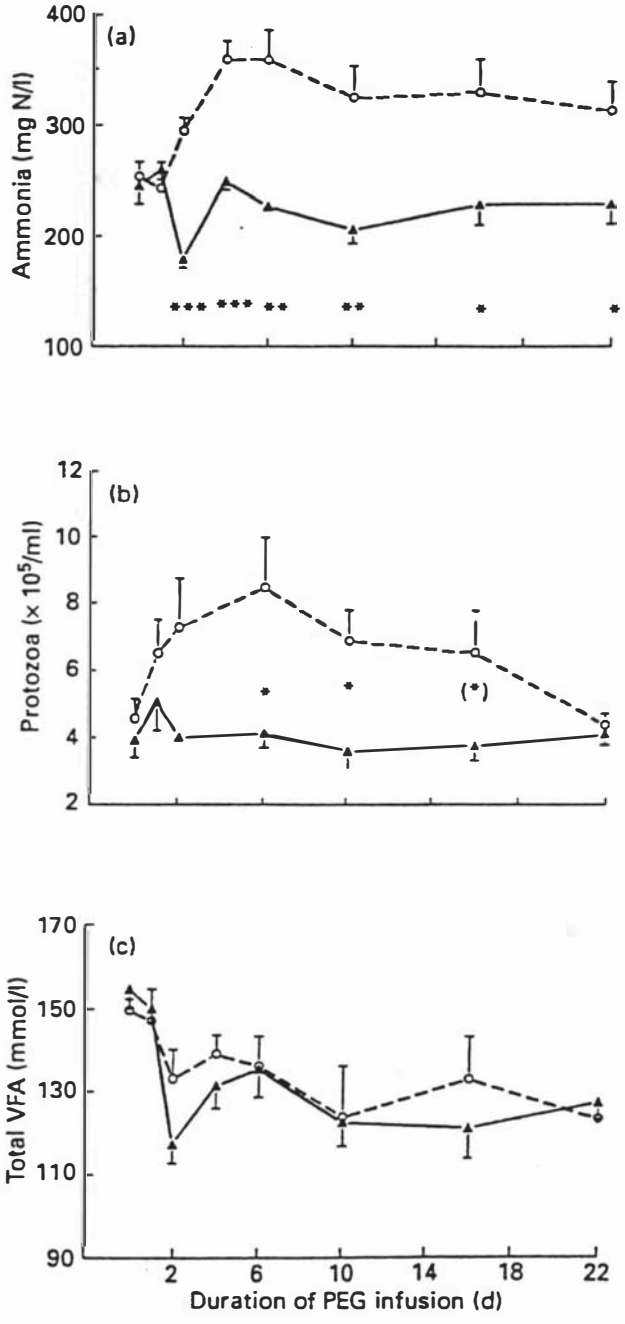

Figure 2.1 The effect of intraruminal infusion of polyethylene glycol (PEG; MW 3,500) upon (A) ammonia concentration (mg N/l), (B) protozoa numbers (x 10^5/*ml*) and (C) total VFA concentration (*m*M/l) in the rumen liquor of sheep fed fresh *Lotus corniculatus* at hourly intervals. Six sheep were fed each diet. ——, control sheep; ....., PEG infused sheep; (*), $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; I, SE.

2.4.2.2 Rumen protozoa

Rumen protozoa numbers were not significantly different between control and PEG sheep prior to and on day 1 of the PEG infusion ($P > 0.05$), but protozoal numbers increased in PEG sheep relative to controls after 6 and 10 days ($P < 0.05$) and 16 days ($P=0.064$) of PEG infusion (Figure 2.1 B). By day 22 the protozoal numbers had declined in PEG sheep and were not significantly different from control sheep ($P > 0.05$).

2.4.2.3 Rumen VFA

Total VFA concentrations (*m*Mol/l rumen fluid) were similar for control (131.6; SE 2.54) and PEG sheep (135.9; SE 2.81) and no significant differences were detected at any sampling time (Figure 2.1 **C**), however, PEG infusion markedly changed molar proportions of some individual VFAs (Figure 2.2).

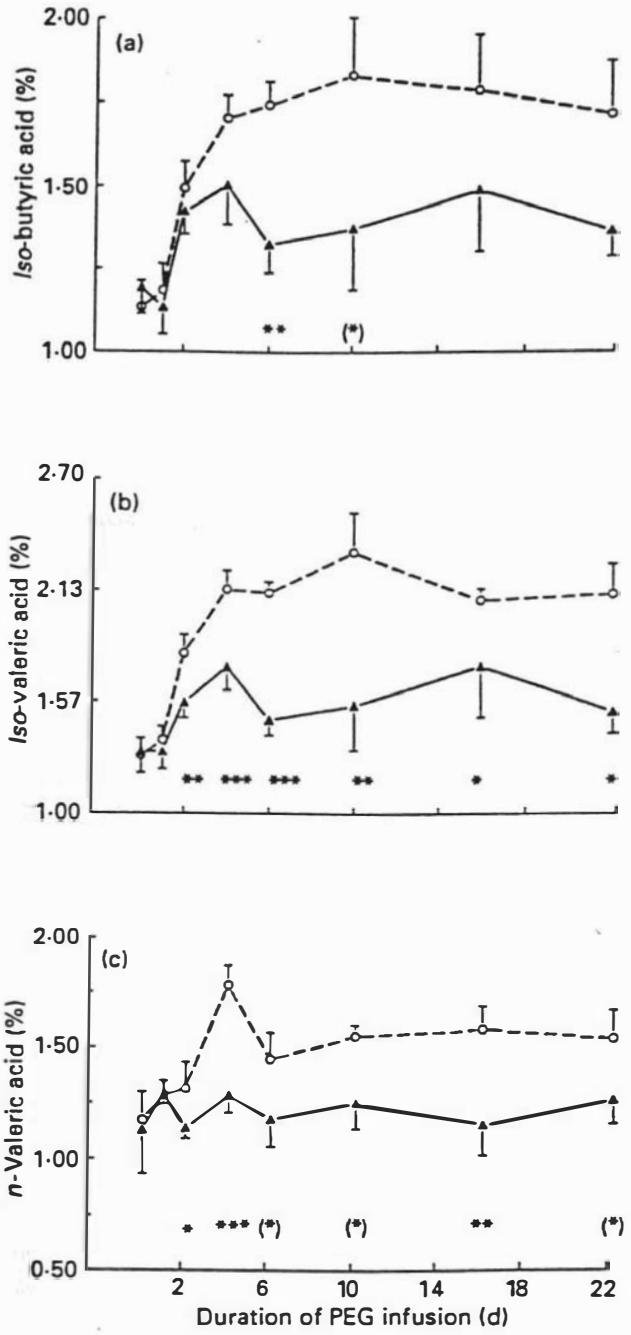

Figure 2.2 The effect of intraruminal infusion of polyethylene glycol (PEG; MW 3,500) upon molar proportion (%) of (A) *iso*-butyric acid, (B) *iso*-valeric acid and (C) *n*-valeric acid in the rumen liquor of sheep fed fresh *Lotus corniculatus* at hourly intervals. Six sheep were fed each diet. ——, control sheep; ....., PEG infused sheep; (*), $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; I, SE.

The molar proportions of acetic acid, propionic acid and *n*-butyric acid were not significantly different between control and PEG-infused sheep. Mean molar proportions of acetic acid, propionic acid and *n*-butyric acid over the entire experimental periods were 0.642 (SE 0.003), 0.220 (SE 0.004) and 0.098 (SE 0.003) for control sheep and 0.634 (SE 0.003), 0.214 (SE 0.004) and 0.104 (SE 0.002) for PEG infused sheep. However, intraruminal infusion of PEG markedly increased the molar proportions of *iso*-butyric acid, *iso*-valeric acid and *n*-valeric acid after 2 days PEG infusion until the conclusion of the experiments ($P < 0.05$; Figure 2.2).

2.4.3 Sulphur amino acid kinetics

The feed intake of the control and the PEG sheep were 989 (SE, 71.4) g DM/d and 983 (SE, 87.6) g DM/d respectively and were not significantly different ($P > 0.05$) during the period when sulphur amino acid kinetics were measured.

2.4.3.1 Methionine

The recovery of both methionine and ^{35}S-methionine infusate added to the deproteinized plasma by HPLC was 0.97 (SE 0.02). The plasma methionine concentration and IRL rates were not significantly different between control and PEG sheep (Table 2.2; Figure 2.3). However, the proportion of methionine total flux transferred to production and maintenance function in control sheep (0.68) was lower than that in PEG sheep (0.86, $P < 0.05$, Table 2.2), whilst the proportion of methionine flux transferred to cystine was much higher for control than for PEG sheep ($P < 0.01$). The proportion of methionine transferred to sulphate (ie oxidation) was very low (0.05 *vs* 0.06; $P > 0.05$) in control and PEG sheep respectively.

Table 2.2 The concentration (μmol/l), irreversible loss rate (μmol/min), transfer quotients and the proportion of total flux flowing to various processes for cystine, methionine and inorganic sulphate in sheep fed fresh *Lotus corniculatus*, with and without an intraruminal infusion of polyethylene glycol (PEG). Mean values with their SEM are for six sheep in each group.

	Control Sheep	PEG-infused Sheep	SEM	Significance
CONCENTRATION (μmol/l)				
Methionine	34.0	35.3	1.61	NS
Cystine	35.1	19.8	2.88	**
Sulphate	1012	1434	82.9	**
TRANSFER QUOTIENT				
Cystine from Methionine	0.38	0.38	0.036	NS
Sulphate from Methionine	0.01	0.01	0.001	NS
Sulphate from Cystine	0.03	0.03	0.003	NS
IRREVERSIBLE LOSS (μmol/min)				
Methionine	16.9	17.0	1.48	NS
Cystine	13.1	7.0	1.44	*
Sulphate	36.8	48.1	2.36	**
PROPORTION OF FLUX TRANSFERRED				
Methionine to Cystine	0.27	0.08	0.039	**
Methionine to Sulphate	0.05	0.06	0.004	NS
Methionine to P + M [1]	0.68	0.86	0.040	*
Cystine to Sulphate	0.09	0.27	0.043	**
Cystine to P + M	0.91	0.73	0.043	**

*: $P < 0.05$; **: $P < 0.01$; NS: $P > 0.05$.

1. P + M: Production and maintenance.

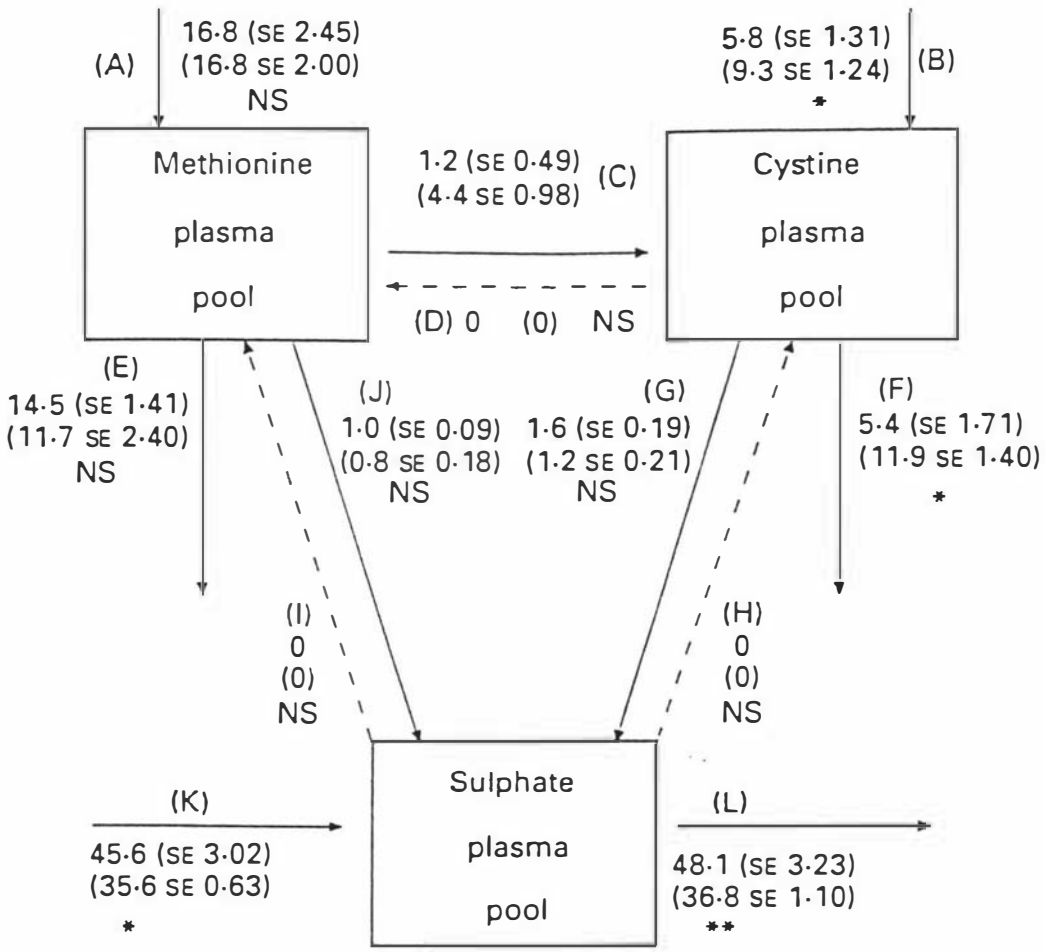
(A)
16·8 (SE 2·45)
(16·8 SE 2·00)
NS
5·8 (SE 1·31)
(9·3 SE 1·24)
*
(B)
Methionine plasma pool
1·2 (SE 0·49)
(4·4 SE 0·98)
(C)
Cystine plasma pool
(D) 0 (0) NS
(E)
14·5 (SE 1·41)
(11·7 SE 2·40)
NS
(J)
1·0 (SE 0·09)
(0·8 SE 0·18)
NS
(G)
1·6 (SE 0·19)
(1·2 SE 0·21)
NS
(F)
5·4 (SE 1·71)
(11·9 SE 1·40)
*
(I)
0
(0)
NS
(H)
0
(0)
NS
Sulphate plasma pool
(K)
45·6 (SE 3·02)
(35·6 SE 0·63)
*
(L)
48·1 (SE 3·23)
(36·8 SE 1·10)
**

Figure 2.3 A general three-pool, compartmentalized model for sulphur amino acid transactions in the post-hepatic plasma of sheep fed on fresh *Lotus corniculatus*, with and without an intraruminal infusion of PEG. Control sheep are represented by numbers in brackets and PEG -infused sheep by open numbers. Mean values are for six sheep in each group with SE. *, $P < 0.05$; **, $P < 0.01$; NS, $p > 0.05$.

Rates of flow (μmol/min) represented by arrows in the model are often composite of several pathways of transfer. The pathways are: **A**. Methionine entering the plasma pool from whole body protein turnover and absorption from the small intestine; **B.** Cystine entering the plasma pool from whole body protein turnover and absorption from the small intestine; **C.** Transulphuration of methionine to cystine; **D.** Conversion of cystine to methionine, which does not occur in mammalian tissue; **E.** Methionine leaving the plasma and being utilised for productivity processes and maintenance; **F.** Cystine leaving the plasma pool and being utilised for productivity processes and maintenance; **G.** Cystine oxidized to sulphate (and carbon dioxide); **H.** Plasma sulphate reassimilated as cystine. This cannot occur directly in mammalian tissue,but sulphate re- entering the rumen via saliva may be absorbed as cystine from microbial protein; **I.** Plasma sulphate reassimilated as methionine. This cannot occur directly in mammalian tissue, but sulphate re- entering the rumen via saliva may be absorbed as methionine from microbial protein; **J.** Methionine oxidized to sulphate (and carbon dioxide); **K.** Sulphate entering the plasma, chiefly from oxidation of sulphide absorbed from the rumen, but also sulphate and oxidation of sulphide absorbed from the intestine; **L.** Sulphate leaving the plasma chiefly in urine, but also recycled directly to the intestines and rumen via saliva.

2.4.3.2 Cystine

The recovery of both cystine and ^{35}S-cysteine infusate added to the deproteinized plasma by HPLC was 0.96 (SE 0.03). Both plasma cystine concentration ($P < 0.01$) and IRL rate ($P < 0.05$) were higher in control sheep than in PEG sheep (Table 2.2). Cystine flux (μmol/min) into the plasma pool from both whole body protein turnover plus absorption from small intestine and from the transulphuration of methionine were significantly higher in control sheep than in PEG sheep ($P < 0.05$; Figure 2.3). The control sheep had higher flux of cystine (μmol/min) to production and maintenance than the PEG sheep ($P < 0.05$) and a higher proportion of the cystine total flux transferred to production and maintenance than for PEG sheep ($P < 0.01$). However the proportion of total cystine flux transferred to sulphate (ie oxidation) was less for control than for PEG sheep ($P < 0.01$).

2.4.3.3 Inorganic sulphate

The recovery from the separation procedure, of both potassium sulphate and ^{35}S-sulphate infusate added to the deproteinized plasma was 0.67 (SE 0.03). Control sheep had a lower plasma inorganic sulphate concentration and IRL rate than PEG sheep ($P < 0.01$; Table 2.2). This translated into less inorganic sulphate both entering the plasma pool from the digestive system and leaving *via* urinary excretion or recycling to the gut in control compared with PEG sheep (Figure 2.3).

2.5 DISCUSSION

The most significant result of this study was the action of CT in increasing the amount of cystine leaving the plasma cystine pool to be used for body synthetic reactions (11.9 *vs* 5.4 μmol/min). This was due to increased entry flux (**B**, 9.3 *vs* 5.8 μmol/min) and increased transulphuration of methionine to cystine (**C**, 4.4 *vs* 1.2 μmol/min). McNabb *et al* (1993) reported similar findings for the CT in *Lotus pedunculatus*, but cystine IRL rate determined in this experiment for control *Lotus corniculatus* (13.1 μmol/min) was lower than that for control *Lotus pedunculatus* (39.8 μmol/min). Lee *et al* (1992) also found cysteine IRL in whole blood to be lower for sheep fed *Lotus corniculatus* than *Lotus pedunculatus* (11 vs 24 μmol/min). This difference may be due to differences between the two *Lotus* species in CT reacting with plant protein and the different seasons when the two experiments were conducted.

Reactivity of CT with protein is probably a function of plant CT concentration, molecular weight and structure. Extractable and total (extractable + bound) CT were 27 and 35 g/kg DM for *Lotus corniculatus (cv Goldie)* in the present study and 55 and 75 g/kg DM for *Lotus pedunculatus* (*cv Maku*; Douglas *et al* 1993; McNabb *et al* 1993; Terrill *et al* 1992a). In addition to the effects of increased concentration, reactivity of CT with protein increases with increasing degree of CT polymerisation, and hence molecular weight (Horigome *et al*, 1988) and with increasing ratio of the CT constituent prodelphinidin (PD) to procyanidin (PC; Jones *et al* 1976). Hence the higher PD : PC ratio and molecular weight of *Lotus pedunculatus* CT (70 : 30; 2900) than for *Lotus corniculatus* CT (27 : 73; 2000; Foo *et al* 1982), together with the higher CT concentration, suggests that reactivity is greater for the CT in *Lotus pedunculatus* than in *Lotus corniculatus*

Wool growth rate has been showed to be seasonal in Romney sheep in New Zealand, with highest rates in spring and summer and lowest rates in winter (Bigham *et al* 1978). The experiment undertaken by McNabb *et al* (1993) was carried out in spring, whereas the present study was conducted in late autumn, when wool growth rate would be declining. Because of its high sulphur content, which is mainly in cystine form (Reis 1965a, 1965b; Hogan 1975), rapid wool growth will create a high demand for cystine. Therefore, compared to the results of McNabb *et al* (1993), the lower flow of cystine to body synthetic reactions in the present study may also be due to lower potential wool growth rates and hence lower cystine requirement. The increased cystine flux to body synthetic reactions due to CT could result in increased wool growth.

Results from a grazing experiment using the same sward and twice daily oral administration of PEG showed that action of CT in *Lotus corniculatus* increased wool growth by 12% ($P < 0.05$; Wang *et al* unpublished). Increased wool growth due to CT has also been reported in sheep grazing sulla (*Hedysarum coronarium;* Terrill *et al* 1992b).

The best indication of methionine and cystine oxidation in plasma is considered to be the proportion of total flux transferred to inorganic sulphate. CT markedly reduced the oxidation for cystine but not methionine, which was already low. The low cystine

oxidation rate (0.09) and high proportion used for production and maintenance function (0.91) in sheep fed *Lotus corniculatus* with CT operating suggests that cystine availability may limit wool growth in sheep selected for high wool production.

The effects of CT are normally assessed through administration of PEG to bind and inactivate CT, and a comparison is made between control and PEG sheep (Barry *et al* 1986; Barry 1989; McNabb *et al* 1993; Terrill *et al* 1992b; Waghorn *et al* 1987a, 1987b). The effects of PEG are assumed to be specific for CT and to affect only protein digestion, without effecting digestion of other nutrients, but this has not been fully investigated. These experiments showed that PEG supplementation increased protein breakdown in the rumen, as indicated by the increased NH_3 concentration. Minor VFAs are produced from deamination of specific AA, with *iso*-butyric acid from valine, *iso*-valeric acid from leucine and *n*-valeric acid from arginine, ornithine, proline, σ-aminovaleric acid or lysine (El-Shazly 1952; Van Soest 1983), of which valine, leucine, arginine and lysine are EAA. Hence the reduced *iso*-butyric acid, *iso*-valeric acid and *n*-valeric acid in control compared to PEG sheep also suggests that CT reduced deamination of these EAA. However, the similarities of total VFA concentration and major VFAs resulting from carbohydrate digestion (acetic acid, propionic acid and *n*-butyric acid) in control and PEG-infused sheep suggest that 35 g total CT/kg in the forage DM does not affect carbohydrate digestion in the rumen.

The present results for rumen protozoa populations are opposite to the results of Terrill *et al* (1992b), who found CT in Sulla (*Hedysarum coronarium*; 29 g extractable CT and 47 g total CT/Kg DM) increased rumen protozoa counts. They explained the effect of CT in increasing protozoa numbers by the requirement of protozoa for insoluble protein and soluble carbohydrates. The rapid increase in protozoal numbers following PEG administration in the present experiment suggests inhibition of protozoa by CT. However, the response was transitory and a component may have been due to effects of PEG *per se*. A long term study is needed to evaluate the effect of CT in *Lotus corniculatus* upon rumen protozoal populations.

Tannins have been reported to inhibit digestive enzyme activities (Ahmed *et al* 1991; Horigome *et al* 1988; Longstaff *et al* 1991a, 1991b; Yuste et al 1992) and to increase endogenous nitrogen excretion in monogastric animals (Price and Butler 1980; Cousins *et al* 1981). The rapid increasing pH along the small intestine may enable

CT-protein complexes to be re-formed to protect protein against being hydrolysed by endogenous enzymes, or the dissociated CT may bind to the gut epithelium and affect AA absorption. All of these could reduce the apparent digestibility of N in the post ruminal digestive tract and may explain the effects of CT in reducing apparent N digestibility in the present study. Research in this area should be conducted to quantify the effects of low dietary CT levels upon post-ruminal protein digestion, and especially upon endogenous nitrogen excretion.

The results of this study show that the lower CT concentration in *Lotus corniculatus* compared with *Lotus pedunculatus* (McNabb *et al* 1993) still effectively increased the amount of cystine flowing to body synthetic reactions, but was probably close to the CT range for depressing rumen fibre digestion. Hence, *Lotus* species with lower content of CT should be evaluated in order to define optimum concentration of CT in this species for increasing the absorption of EAA, increasing the flow of sulphur-containing amino acids to body synthetic reactions and increasing the efficiency of livestock production.

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Chapter 3

Live weight gain and wool production of sheep grazing *Lotus corniculatus* and lucerne (*Medicago sativa)*

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3.1 *ABSTRACT*

Two grazing experiments were conducted to compare the productivity of lactating ewes (Experiment 1) and weaned lambs (Experiment 2) grazing swards of *Lotus corniculatus* (birdsfoot trefoil; *cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and a mixture of lucerne and lotus. Measurements were made of pre and post grazing herbage mass, the composition of the feed on offer and diet selected, and of voluntary feed intake (VFI; Experiment 2 only), body growth and wool growth. From the agronomic measurements, it was concluded that the diet selected was mainly leaf in both experiments. Total condensed tannin (CT) content was 32-57 g/kg DM for lotus, 8-10 g/kg DM for the mixture and was negligible for lucerne (< 2 g/kg DM). In Experiment 1, ewe wool production and lamb live weight gain (LWG) did not differ between forages, but ewe LWG was greater on lotus than on lucerne (251 vs 65 g/d; $P < 0.001$), with the mixture being intermediate (115 g/d). In Experiment 2, VFI (1.76 vs 1.65 kg OM/d; $P < 0.05$), LWG (228 vs 183 g/d; $P < 0.001$), wool production (2.78 vs 2.25 kg; $P < 0.05$) and carcass weight (20.4 vs 17.8 kg; $P < 0.05$) were greater for lambs grazing lotus than lucerne; lambs grazing the mixture had similar VFI (1.63 kg OM/d) to those grazing lucerne, but wool production (2.49 kg) was intermediate between lucerne and lotus lambs. Male lambs showed a greater LWG response to lotus relative to lucerne (+83 g/d; $P < 0.01$) than female lambs (+15 g/d; $P > 0.05$). When adjusted for differences in carcass weight, lotus did not affect carcass fatness (GR 13.1 vs 12.8 mm; $P > 0.05$). It was concluded that *Lotus corniculatus* (*cv.* Grasslands Goldie) supported high levels of sheep productivity, with the responses in wool production and superior growth of male lambs in Experiment 2 suggesting that part of the response may be due to increased protein supply from action of CT in the digestive system.

Key words Condensed tannin; *Lotus corniculatus*; wool growth; body growth.

3.2 INTRODUCTION

In ruminants grazing high quality fresh forages, about 70% of the protein ingested is degraded in the rumen and only 30% escapes to the small intestine for absorption (Waghorn and Barry 1987; Ulyatt *et al.* 1975). Although much of the protein lost to deamination is compensated by microbial protein synthesised, the extensive protein degradation can result in insufficient amino acid absorption for maximizing productivity in young growing animals and lactating animals (Barry 1981; Chrisp *et al.* 1989; Rogers *et al.* 1980).

Condensed tannins (CT) are secondary plant compounds that occur in the leaves and stems of species such as *Lotus pedunculatus, Lotus corniculatus, Onobrychis viciifolia* (sainfoin) and *Hedysarum coronarium* (sulla) (Jones *et al.* 1976; Lowther *et al.* 1987; Barry 1989). Since CT can react with protein at near neutral pH to form CT - protein complexes, but dissociate and release protein at pH < 3.5 (Jones and Mangan 1977), forages containing CT may have an advantage in reducing protein degradation in the rumen and increasing protein absorption from the small intestine.

Nutritional effects of CT in *Lotus* species fed to ruminant animals depend upon the concentration of CT in the forage. High concentrations of CT (50 -100 g extractable/kg DM) have depressed voluntary feed intake (VFI) and reduced apparent digestibility of DM, OM and fibre (Barry and Duncan, 1984; Chiquette *et al.* 1988; Pritchard *et al.* 1988). However, for the *Lotus* species, levels of extractable CT in the range 20 - 40 g/kg DM are thought to be beneficial (Barry 1989; Waghorn *et al.* 1987a). *L. corniculatus* contains a low level of CT (27 g extractable and 35 g total/kg DM; Terrill *et al.* 1992a). Waghorn *et al.* (1987b) reported that CT (22 g extractable/kg DM) in *L. corniculatus* increased essential amino acid (EAA) apparent absorption from the small intestine by 62% and decreased non-EAA (NEAA) apparent absorption by 10% in sheep. Thus, the productivity of sheep grazing *L. corniculatus* needs to be compared with the productivity of sheep grazing a similar legume which does not contain CT.

The objectives of this study were to compare the productivity of lactating ewes and growing lambs when grazing *L. corniculatus*, lucerne (*Medicago sativa*) and a mixed lotus/lucerne sward.

3.3 MATERIALS AND METHODS

3.3.1 Experiment design

Two grazing experiments were conducted at the AgResearch Grasslands Aorangi Research Station in the Manawatu during the 1991/1992 summer. Experiment 1 commenced on 14th of October and finished on 25th of November 1991, lasting 42 days. Experiment 2 began on 13th of December and concluded on 26th of February 1992, lasting for 76 days.

Experiment 1 compared the productivity of lactating ewes and their lambs grazing swards of *L. corniculatus* (CT-containing), lucerne (non-CT containing) and a lotus/lucerne mixture. Experiment 2 compared the productivity of weaned lambs grazing the same swards. The establishment of the lotus/lucerne mixed sward was an attempt to reduce CT level in the feed under grazing conditions.

3.3.2 Forages

L. corniculatus (birdsfoot trefoil; *cv.* Grasslands Goldie), lucerne (*cv.* Grasslands Oranga) and a lotus/lucerne mixture were grazed in both experiments. In Experiment 1, all three swards were in the vegetative growth stage, whilst in Experiment 2, all three swards were mature in places when the experiment started. Therefore, half the areas of all swards were lightly topped at the commencement of Experiment 2 to stimulate vegetative growth. Pre-grazing and post-grazing herbage mass and botanical composition were determined weekly, immediately before and after grazing, by cutting two 0.125 m^2 quadrats per break of each forage to ground level and drying overnight (15 hrs) in a forced-air oven at 80 °C. Pre-grazing herbage samples (feed-offered) were collected weekly to estimate chemical composition, by cutting three samples per break of each forage to ground level and bulking over 2-3 weekly intervals. Three wire mesh cages measuring about 1.4x0.9m were placed in each break immediately before lambs commenced grazing. At the end of grazing that break, the cages were removed and the forage cut and samples taken corresponding to what animals were observed to be eating. These are hence referred to as diet-selected, and were analysed for chemical composition.

3.3.3 Animals

3.3.3.1 Experiment 1

The trial used 60 lactating Romney ewes and their 65 lambs, which were aged 3-7 weeks. The mean initial live weights were 53.0 kg (SE, 0.77) for ewes and 15.5 kg (SE, 0.36) for lambs. The ewes were tagged, drenched with ivermectin (Ivomec, Merk, Sharp and Dohme, NZ Ltd.) to control internal parasites, treated with deltamethrin (Wipeout, Coopers Animal Health NZ) for external parasites, and divided randomly into three groups for lotus, lucerne and lotus/lucerne mixture. All animals were randomly allocated to the three swards, with 20 ewes and 23, 23 and 19 lambs grazing lotus, mixture and lucerne respectively being used to calculate live weight gain.

Live weight gains of both ewes and lambs were measured at two week intervals. Wool growth of ewes was measured from clipping 10 X 10 cm patches to skin level on the right mid-side at the beginning and end of the experiment. In addition, five ewes were selected from each treatment group and about 50 fibres were taken at random from the wool patch. The true length of these fibres was determined by computerised digitometry.

3.3.3.2 Experiment 2

Sixty eight lambs were divided randomly into three groups for grazing on lotus, lucerne and the lotus/lucerne mixture. Mean initial live weight was 27.9 kg (SE, 0.50). Numbers of lambs for lotus, lucerne and the lotus/lucerne mixture were balanced for sex and allocated to treatment on the basis of forage mass and were 20, 24 and 24, respectively. All lambs were treated with ivermectin and deltamethrin to control internal and external parasites at the beginning and mid-point of the experiment.

Live weight was measured at two week intervals using electronic scales. All lambs were shorn at the end of the experiment. Wool samples from mid points of both sides were collected to determine clean wool yield. A dye banding technique (Chapman and Wheeler 1963) was used to estimate fibre length growth and it involved application of dye to a 10 cm strip of wool on the mid side. The fleece was parted and dye applied at the skin with a blunted needle and 5 ml syringe. The dye banding solution was prepared by mixing 0.4 g Durafux Black R dye in 50 ml water and adding 1 ml hydrogen peroxide immediately prior to application. The first application of dye was made on 14th of October, 1991, and on 9th of January, 1992, a sample was removed

to determine the length of the wool. A further band of dye was applied on 9th of January, 1992 so that fibre length could be determined until the conclusion of the experiment.

All female lambs grazing lotus (10) and lucerne (12) were slaughtered at the end of the experiment to measure carcass weight, dressing-out percent and an indirect measurement of carcass fatness (GR). GR is a measurement of total soft tissue depth over the 12th rib at a point 11 cm from the carcass midline (Kirton 1989).

VFI was estimated by using intra-ruminal slow release chromium capsules (Captec NZ Ltd, Auckland) as described by Parker *et al.* (1989). Chromium release rate for different swards was estimated using 8 sheep with rumen fistulae (3 for lotus and lucerne and 2 for the mixture), by using callipers to measure chromium disappearance from capsules suspended in the rumen.

3.3.4 Grazing Management

Feed allowance in Experiment 1 was 6.0 kg green DM/ewe/day, whilst in Experiment 2 it was 3.5 kg green DM/lamb/day when the trial commenced and gradually increased to 5.5 kg green DM/lamb/day as the lambs increased in weight.

Rotational grazing was used in both experiments, by partitioning each sward into breaks using electric fences. Each break was grazed for one week, with the area calculated on the basis of the specified allowance, the number of animals and green herbage mass. In Experiment 1, lambs stayed with their dams throughout the experiment. Each break was back fenced and after grazing the herbage was topped with a tractor operated rotary mower to a 5-10 cm residual height, and then removed from the paddock. This practice facilitated regrowth of vegetative material. Management was governed by the need to provide vegetative high quality forage at all times. Sheep had free access to water in both experiments.

3.3.5 Laboratory analyses

All samples of feed-offered and diet-selected were stored at -20 °C, freeze dried, and ground to pass through a 1 mm diameter sieve prior to laboratory analyses. Extractable CT, protein-bound CT and fibre-bound CT were analyzed using the

method described by Terrill *et al.* (1992a). Total nitrogen (N) was determined by the Kjeldahl method. Organic matter (OM) was measured by ashing herbage samples at 550 °C for 15 hours and *in vitro* digestibility by the enzymic method of Roughan and Holland (1977). Because extractable CT would be solubilized in the initial *in vitro* extraction steps, but is known to be indigestible *in vivo* (Terrill *et al.* 1994), extractable CT (% OM) values were deducted from all *in vitro* OMD determinations. Cell wall constituents were determined by the detergent system of Van Soest (1983). Chromium in faeces was determined by atomic absorption spectroscopy using the method of Costigan and Ellis (1987), and clean wool yield by washing 5 g samples in 300 ml tap water containing 2 ml Triton X-100 (BDH Chemicals Ltd., Poole, England), shaking for 4 min, drying at 60 °C, and weighing after being in a constant humidity room for 48 hours.

3.3.6 Calculation of data and statistical analyses

VFI of OM (OMI) was calculated as:

$$VFI = \frac{F}{1-D} \qquad (1)$$

Where F is faecal OM output which was calculated from the chromium release rate divided by chromium concentration in faeces, and D is the *in vitro* OM digestibility (OMD) from the samples of diet-selected.

One-way analysis of variance was used to assess the effects of forage type (lotus *vs* lucerne *vs* lotus/lucerne mixture). In Experiment 1, initial live weight of ewes was used as a covariate for analysing ewe LWG, and initial live weight of lambs as a covariate for lamb LWG. In Experiment 2, final live weight was used as a covariate for analysing clean fleece weight and carcass weight as a covariate for analysing carcass GR data.

3.4 RESULTS

3.4.1 Botanical composition

For lotus and lucerne swards, the yield of plant stems (t DM/ha) was similar between pre and post grazing herbage, in both experiments ($P > 0.05$; Figures 3.1 and 3.2). However, leaf yields in post grazing herbage were significantly lower than in pre-

grazing herbage ($P < 0.001$). There was no difference in leaf : stem ratio between lotus and lucerne in pre grazing herbage ($P > 0.05$). The percentage of leaf in the pre versus post grazing legume DM mass for lotus was 50.8 *vs* 8.5 ($P < 0.001$) and for lucerne was 51.0 *vs* 9.3 ($P < 0.001$) in Experiment 1. In Experiment 2 the percentage of leaf in lotus was 29.9 *vs* 8.6 ($P < 0.001$) and in lucerne was 39.2 *vs* 11.0 ($P < 0.001$).

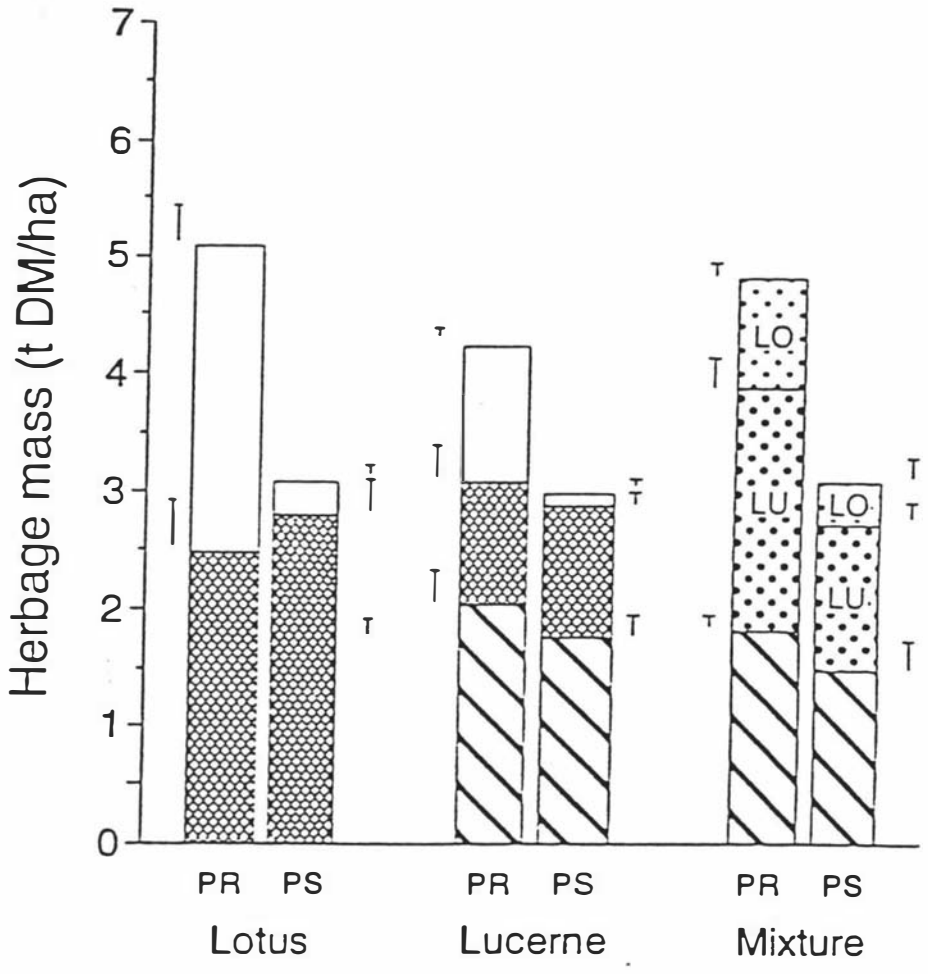

Figure 3.1 Experiment 1. Pre (PR) and post (PS) grazing herbage mass of *Lotus corniculatus* (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga)and a lotus/lucerne mixture. ☐ Leaf only (lotus & lucerne); ▩ Stem only (lotus & lucerne); ▨ Other species. Mean values are for 6 determinations per forage. Vertical bars represent SE. In the case of the mixture, samples were dissected into whole lucerne (LU) and whole lotus (LO) plants; dissection was not conducted into leaf and stem components.

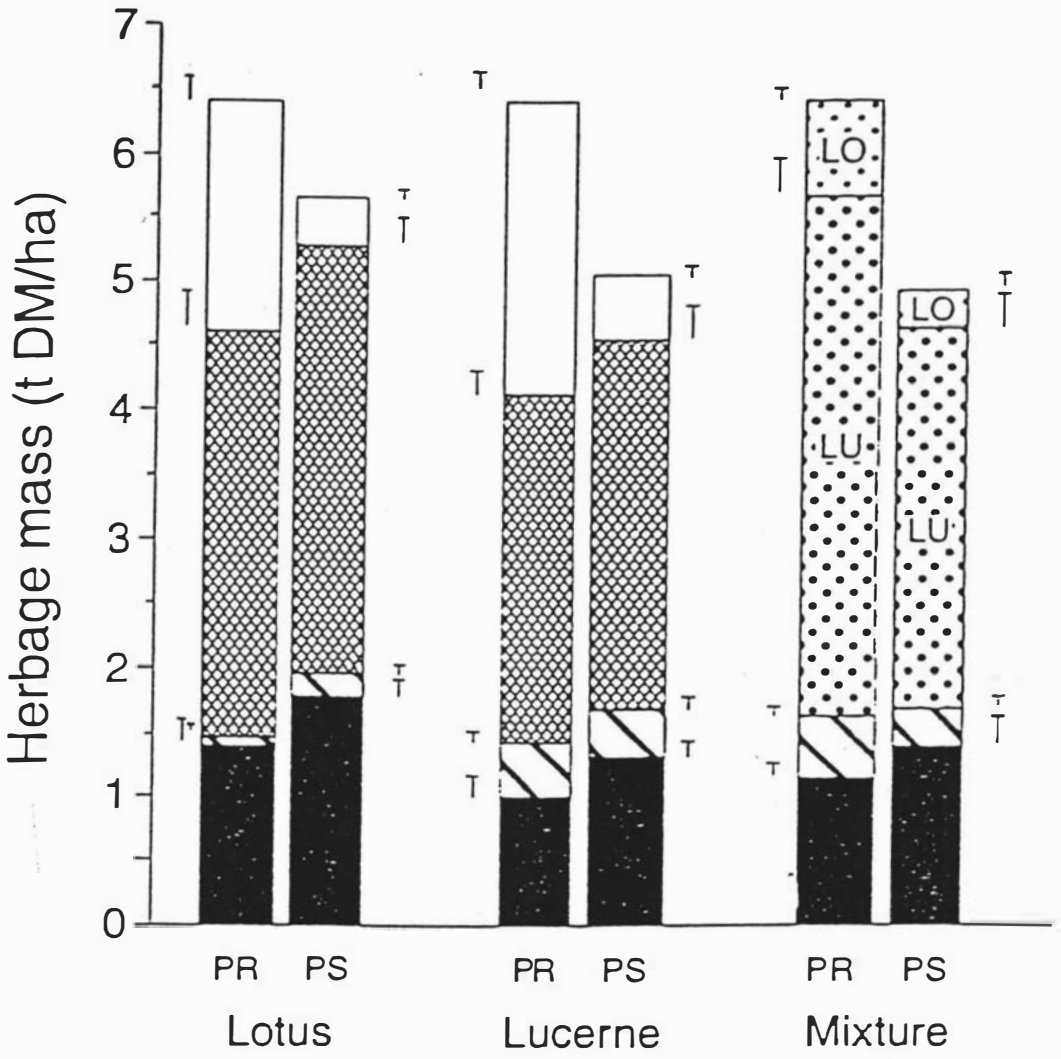

Figure 3.2 Experiment 2. Pre (PR) and post (PS) grazing herbage mass of *Lotus corniculatus (cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and a lotus/lucerne mixture. ☐ Leaf only (lotus & lucerne); ▩ Stem only (lotus & lucerne); ▨ Other species; ■ Dead matter. Mean values are for 12 determinations per forage. Vertical bars represent SE. In the case of the mixture, samples were dissected into whole lucerne (LU) and whole lotus (LO) plants; dissection was not conducted into leaf and stem components.

In both experiments, some non-legume other species were present in the lucerne and mixed swards but were absent in lotus in Experiment 1 (Figures 3.1 and 3.2), with the principal species being perennial ryegrass. However, there were no differences in the amount of other species (t DM/ha) between pre and post grazing for all three swards ($P > 0.05$) in both experiments. In Experiment 2 there was no difference in the yield of dead matter either between the three swards or between pre and post grazing for each sward ($P > 0.05$).

In the mixed sward, lucerne : lotus ratios were 1.00 : 0.57 and 1.00 : 0.28 for pre and post grazing samples in Experiment 1 and 1.00 : 0.17 and 1.00 : 0.10 for pre and post grazing samples in Experiment 2, with the difference being non-significant within each experiment. Measurements of DM disappearance, calculated from pre and post grazing DM mass, indicated that in the mixed sward lotus and lucerne were consumed in approximately equal quantities in Experiment 1, whilst in Experiment 2 lotus was approximately 25% of the diet and lucerne 75% of the diet.

3.4.2 Chemical composition of feed-offered and selected

NDF, ADF, hemicellulose, cellulose and lignin contents were all similar in feed-offered and diet-selected for all three swards in Experiment 1 (Table 3.1), whereas in Experiment 2, all of them were lower in diet-selected than in feed-offered for all three swards ($P < 0.05$; Table 3.2). However, in feed-offered NDF and ADF were not different between swards in both experiments ($P > 0.05$). For diet-selected, NDF was lower in lotus than in lucerne and in the lotus/lucerne mixture ($P < 0.05$) in Experiment 1, but was not different in Experiment 2 ($P > 0.05$). Lotus tended to have a lower hemicellulose and higher lignin content than lucerne in both experiments, with the mixed sward being intermediate. Cellulose content was similar in all three swards. Total N content was similar for lotus and lucerne in Experiment 1, but was lower for lotus in Experiment 2 ($P < 0.05$).

Table 3.1 Experiment 1. Chemical composition (g/kg DM) and *in vitro* organic matter digestibility (% OMD) of *Lotus corniculatus* (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and lotus/lucerne mixture offered and selected by grazing sheep.

		n [1]	LOTUS	MIXTURE	LUCERNE	SEM
Ash	offered	5	99	103	101	2.1
	selected	5	100	105	102	2.6
Total N	offered	5	33	30	31	3.0
	selected	5	49	41	42	1.4
NDF	offered	3	304	390	406	46.3
	selected	3	247	332	315	19.0
ADF	offered	3	230	252	244	25.3
	selected	3	189	219	210	14.8
Hemicellulose	offered	3	74	139	163	20.8
	selected	3	75	113	132	10.7
Cellulose	offered	3	173	201	202	22.0
	selected	3	130	168	162	11.8
Lignin	offered	3	65	51	41	5.1
	selected	3	58	50	45	4.1
OMD	offered	5	78.6	76.0	74.5	2.07
	selected	5	85.1	82.3	79.0	1.07

1. Sample numbers.

Table 3.2 Experiment 2. Chemical composition (g/kg DM) and *in vitro* organic matter digestibility (% OMD) of *Lotus corniculatus* (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and lotus/lucerne mixture offered and selected by grazing sheep.

		n [1]	LOTUS	MIXTURE	LUCERNE	SEM
Ash	offered	6	91.5	103.7	106.7	4.25
	selected	6	100.7	104.7	104.6	2.59
Total N	offered	6	26.2	35.7	32.5	1.75
	selected	6	40.2	47.3	47.2	1.36
NDF	offered	3	446	389	413	25.9
	selected	3	255	238	247	5.2
ADF	offered	3	356	277	286	23.2
	selected	3	205	160	163	1.1
Hemicellulose	offered	3	106	113	126	7.4
	selected	3	73	78	84	5.2
Cellulose	offered	3	240	185	230	15.9
	selected	3	140	128	134	7.7
Lignin	offered	3	99	58	56	5.2
	selected	3	65	32	29	3.8
OMD	offered	6	66.8	72.3	71.5	1.42
	selected	6	81.1	85.7	85.2	1.08

1. Sample numbers.

Organic matter digestibility (OMD) was higher for diet-selected than for feed-offered ($P < 0.05$) in both experiments for lotus and lotus/lucerne mixture (Tables 3.1 and 3.2). For lucerne, diet-selected had higher OMD than feed-offered in Experiment 2, whilst they were similar in Experiment 1. In Experiment 1, OMD was similar for the three swards ($P>0.05$) for feed-offered but lotus had a higher OMD than lucerne ($P < 0.05$) for feed selected. Lotus had lower OMD than the other two swards for both feed on offer and diet-selected in Experiment 2 ($P < 0.05$).

Total CT in lotus in Experiment 1 was 38 g/kg DM, with 70% being in the extractable fraction, whilst in Experiment 2, total CT in lotus was 32 g/kg DM for feed-offered and 57 g/kg DM for diet-selected (Table 3.3). Only trace amounts of CT were detected in lucerne, and 6-10 g CT/kg DM were detected in diet-selected for the lotus/lucerne mixture.

Table 3.3 Concentration of condensed tannin (g/kg DM)in *Lotus corniculatus* (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and lotus/lucerne mixture offered and selected by grazing sheep. Mean values with SE are for 3 samples per forage, for both feed offered and selected.

		LOTUS	MIXTURE	LUCERNE
		Experiment 1		
Extractable	Pooled [1]	25.2 ± 0.63	4.6 ± 1.20	0
Protein - bound	Pooled	11.0 ± 0.36	4.4 ± 0.44	1.3 ± 0.29
Fibre - bound	Pooled	1.4 ± 0.23	0.9 ± 0.46	0.5 ± 0.12
Total	Pooled	37.6 ± 0.54	9.9 ± 0.20	1.8 ± 0.44
		Experiment 2		
Extractable	Offered	20.2 ± 0.69	2.4 ± 0.49	0
	Selected	41.2 ± 0.64	3.2 ± 0.38	0
Protein - bound	Offered	9.7 ± 2.18	5.3 ± 0.61	1.4 ± 0.52
	Selected	14.0 ± 2.04	4.2 ± 1.11	1.2 ± 0.56
Fibre - bound	Offered	1.6 ± 0.45	1.9 ± 0.23	0.2 ± 0.66
	Selected	2.1 ± 0.68	1.2 ± 0.70	0
Total	Offered	31.5 ± 1.80	9.6 ± 0.84	1.6 ± 1.17
	Selected	57.3 ± 0.67	8.6 ± 2.12	1.2 ± 1.26

1. Pooled: offered + selected.

3.4.3 Animal performance

3.4.3.1 Experiment 1

Ewes grazing lotus had a significantly higher LWG than those grazing lucerne ($P < 0.001$; Table 3.4), with the mixture being intermediate ($P < 0.05$). However, there was no difference between the three swards in lamb LWG and wool growth of the ewes ($P > 0.05$). Fibre taken from the mid-side patch had a similar length in ewes grazing lucerne and the mixture, but was longer in ewes grazing lotus ($P < 0.001$).

Table 3.4 Experiment 1. Live weight gain and wool growth of lactating ewes and live weight gain of their lambs when grazing *Lotus corniculatus* (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and lotus/lucerne mixture.

	LOTUS (*n=20*)[1]	MIXTURE (*n=20*)	LUCERNE (*n=20*)	SEM
Live weight gain (g/d):				
Ewes	251	115	59	13.5
Lambs	275 (23)[2]	260 (23)	263 (19)	10.2
Ewes wool growth:				
mg/100cm ^{2}/day	133	130	123	6.6
length (mm) [3]	21.2	20.1	19.6	0.20

1. Numbers of ewes per treatment group.
2. Numbers of lambs per treatment group.
3. Based on 50 fibres from each of 5 ewes in each treatment.

3.4.3.2 Experiment 2

Voluntary intake of organic matter was slightly higher for lambs grazing lotus than those grazing either lucerne or the lotus/lucerne mixture ($P < 0.05$; Table 3.5). There

was no difference between lucerne and the mixed sward ($P > 0.05$).

Live weight gain of lambs grazing lotus was significantly higher than those grazing either lucerne or the lotus/lucerne mixed sward ($P < 0.001$; Table 3.5), with the response to lotus being greater for male than for female lambs ($P < 0.001$). Carcass weight (female only) ($P < 0.05$) and dressing-out percent ($P < 0.01$) were greater for lambs grazing lotus than lucerne but there were no differences in GR measurement when adjusted to equal carcass weight ($P > 0.05$).

Lambs grazing lotus produced more wool than those grazing lucerne ($P < 0.001$). When adjusted to equal final live weight, wool production was still significantly greater for lambs grazing lotus than lucerne ($P < 0.05$). Wool growth of lambs grazing the mixed lotus/lucerne sward was intermediate and was not significantly different from either lotus or lucerne ($P > 0.05$). The increment in wool growth per day can be calculated by subtracting clean fleece growth of lucerne lambs from that of the other groups. Lambs grazing lotus and the mixture grew 7.8 and 4.1 g/d more wool than those grazing lucerne and 3.6 and 1.7 g/d more when adjusted to equal final live weight. However, there was no significant difference ($P > 0.05$) in wool length between the treatment groups as indicated by dye banding measurement (Table 3.5).

Table 3.5 Experiment 2. Voluntary intakes of organic matter, live weight gain (LWG), clean fleece weight, carcass weight and carcass fatness (GR) of lambs grazing Lotus corniculatus (*cv.* Grasslands Goldie), lucerne (*Medicago sativa; cv.* Grasslands Oranga) and lotus/lucerne mixture.

	LOTUS (*n=20*)[1]	MIXTURE (*n=24*)	LUCERNE (*n=24*)	SEM
Organic matter intake				
kg/d	1.76	1.63	1.65	0.04
LWG (g/d)				
Male	265 (10)[1]	207 (12)	186 (12)	10.4
Female	193 (10)	166 (12)	178 (12)	8.2
Pooled	228	186	183	8.2
Carcass weight (kg)	20.4	ND [2]	17.8	0.82
Dressing - out (%)	47.7	ND	44.6	0.62
GR (mm)[3]	13.1	ND	12.8	0.74
Fleece weight (kg)				
Unadjusted	2.78	2.49	2.25	0.091
Adjusted [4]	2.61	2.48	2.40	0.073
Wool length (mm;dye banding)				
14/10/91 - 9/1/92	43.7 (20)	46.2 (24)	44.9 (24)	1.07
14/10/91 - 5/3/92	101.4 (15)	100.3 (18)	97.1 (15)	2.91

1. Numbers of lambs per treatment group.
2. ND: Not determined.
3. Adjusted to equal carcass weight.
4. Adjusted to equal final live weight.

3.5 DISCUSSION

The objective of using *L. corniculatus*, lucerne and a lotus/lucerne mixture was to establish three swards that differed in CT concentration but were similar in other aspects. In both experiments, lotus, lotus/lucerne mixture and lucerne swards had the highest, intermediate and trace amount of CT respectively, and were generally similar in other aspects. Therefore the objective was successfully achieved. The higher lignin content in lotus than in lucerne and the mixture can be explained by CT and lignin both being produced in plants from the shikimic acid biochemical pathway and sharing many common intermediates (Swain 1979).

Grazing *L. corniculatus* significantly increased sheep productive performance compared to grazing lucerne, as judged by ewe and lamb LWG, lamb carcass gain, carcass dressing-out percent and wool growth. Other studies have shown similar results for LWG (Marten *et al.* 1990).

These increases in productivity in sheep fed lotus can result from increased VFI, higher digestibility or improved utilization of digested nutrients. Lambs grazing lotus had a higher VFI than those grazing lucerne in Experiment 2, but the difference was small. As there was a considerable reduction in leaf mass (t DM/ha) following grazing, but no reduction in the mass of stem, other species or dead matter, it is apparent that the diet-selected comprised almost entirely leaf in both experiments. Because *in vitro* OMD was lower for lotus than lucerne in Experiment 2, the greater VFI of lambs grazing lotus can not be explained from higher digestibility. The effect of CT in *L. corniculatus* on VFI needs further study.

It is possible that a component of the superior wool and body growth of lambs grazing lotus in Experiment 2 could be due to better utilization of digested nutrients. CT in *L. corniculatus* and *Lotus pedunculatus* have been shown to reduce protein degradation in the rumen, to increase amino acid absorption from the small intestine (Barry *et al.* 1986; Waghorn *et al.* 1987b) and to increase flux of cystine in blood to productive processes (McNabb *et al.* 1993; Wang *et al.* 1994). This could be responsible for the increased wool and body growth observed in sheep grazing lotus.

The dye banding technique was reasonably satisfactory, but after several months the

dye appeared to become diffused in some sheep, hence the lower number of observations for the total period in Table 3.5.

The greater response in LWG in male than in female lambs grazing lotus suggests that male lambs are more sensitive to protein supply and hence more suitable for future studies of the effects of CT on animal growth. Theoretically, the effect of CT may be best expressed when protein supply is restricting animal production. Results of animal productive performance as well as herbage botanical composition from both experiments all suggested that the feed allowance in the present study might be too high and that protein supply was not very restricting. Therefore experiments with lower feed allowances are needed to study the effect of CT in grazing sheep.

The lack of response in carcass fatness, as measured by GR, contrasts with the results of Purchas & Keogh (1984) and Terrill *et al.* (1992b), who found that grazing sheep on *Lotus pedunculatus* and *Hedysarum coronarium* (sulla), respectively, both reduced carcass fatness. Further experiments are required to study the effect of CT in *L. corniculatus* upon carcass fatness.

CT content of lotus in Experiment 1 and in diet offered in Experiment 2 were similar to other reported values (Waghorn *et al.* 1987a; Terrill *et al.* 1992a), but the value obtained for diet-selected in Experiment 2 was high. This may be due to most of the diet-selected being leaf as *L. corniculatus* leaf contains much higher levels of CT than stem (Douglas *et al.* 1993). Although the swards were predominantly vegetative, some flowers were available, which were readily eaten by the lambs. Lotus flowers contain a high CT concentration (extractable 56 and total 87 g/kg DM; Wang *et al.* unpublished data) and this may also have contributed to the high CT value in diet-selected in Experiment 2.

In conclusion, sheep grazing *L. corniculatus* showed increased productivity compared to sheep grazing lucerne. The greater body and wool growth of sheep grazing *L. corniculatus* than grazing lucerne may be due to the CT in *L. corniculatus*. Results for the mixed sward were intermediate for wool growth and body growth of male lambs, suggesting that low CT concentration may have had an effect in improving protein utilization. In order to define the nutritional effect of CT in *L. corniculatus* upon

ruminant productivity, further experiments are needed involving PEG administration (which binds and inactivates CT; Jones and Mangan 1977) and these should be conducted at lower feed allowance than used in the present study.

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Chapter 4

Effect of condensed tannins upon the performance of lambs grazing *Lotus corniculatus* and lucerne (*Medicago sativa*)

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4.1 *ABSTRACT*

A grazing experiment, conducted for 22 weeks in 1992/93 at Aorangi Research Station, AgResearch Grasslands, Manawatu, New Zealand, compared the productivity of weaned lambs grazing *Lotus corniculatus* (birdsfoot trefoil) and lucerne (*Medicago sativa*). Effects of condensed tannins (CT) in lotus were evaluated by studying the responses of lambs to twice daily oral supplementation with polyethylene glycol (PEG). A rotational grazing system with restricted feed allowance was used. Measurements were made of pre- and post-grazing herbage mass, the composition of the feed on offer and diet selected, voluntary feed intake (VFI), liveweight gain, carcass growth, wool growth and the concentration of metabolites in rumen fluid. For both lotus and lucerne swards, the diet selected was mainly leaf. Lotus contained 34 g total CT/kg dry matter in the diet selected, whilst there were essentially no CT in lucerne. Compared to lambs grazing lucerne, lambs grazing lotus had slightly lower VFI, and higher liveweight gain (LWG), carcass weight gain, carcass dressing-out percent and wool growth. PEG supplementation had no effect on these measurements or upon the composition of rumen fluid in lambs grazing lucerne. However, in lambs grazing lotus, PEG supplementation reduced wool growth (10.9 *v.* 12.1 g/day), slightly reduced LWG (188 *v.* 203 g/day), increased rumen ammonia concentration, and increased the molar proportions of *iso*-butyric, *iso*-valeric and *n*-valeric acids and protozoa numbers in rumen fluid. PEG supplementation did not affect carcass gain, carcass fatness or the molar proportion of rumen acetic, propionic or *n*-butyric acids in lambs grazing lotus. It was concluded that the principal effect of CT in growing lambs grazing lotus was to increase wool growth without affecting VFI, thereby increasing the efficiency of wool production, that the greater rate of carcass gain of lambs grazing lotus than those grazing lucerne was mainly caused by factors other than CT and that CT did not affect the rumen fermentation of carbohydrate to major volatile fatty acids.

4.2 INTRODUCTION

Lotus corniculatus (birdsfoot trefoil) is a perennial forage legume which, although widely distributed throughout the world, is of minor significance in New Zealand (NZ) pastoral agriculture at present. However, it is resistant to drought, tolerant of acidic and poorly drained infertile soils, and has high nutritive value, so that it may possibly play a larger role in NZ agriculture in the future.

L. corniculatus contains condensed tannins (CT) and cultivars of the species have been reported to contain 1.3-39.0 g extractable CT/kg dry matter (Lowther *et al.* 1987). CT are polyphenolic secondary compounds that can react by hydrogen bonding with plant protein in the near neutral pH range to form CT-protein complexes, which are stable and insoluble at pH 3.5-7.0, but dissociate and release protein at pH < 3.5 (Jones & Mangan 1977). Thus CT contained in plants can protect dietary protein against degradation in the rumen and increase amino acid (AA) supply for absorption in the small intestine.

It is now thought that the nutritional role of CT for ruminant animals depends on their concentration, structure and molecular weight in plants (Barry *et al.* 1986; Barry 1989; Waghorn 1990; Waghorn & Shelton 1992; Wang *et al.* 1994). In lotus, its low CT concentration and moderate protein precipitating capacity have been shown, in several indoor metabolism experiments, to reduce dietary protein degradation in the rumen and to increase essential amino acid (EAA) absorption from the small intestine (Waghorn *et al.* 1987*a, b*). The action of CT in lotus has also been shown to increase plasma cystine flux to body synthetic reactions, with little effect on dry matter (DM), organic matter (OM) and fibre apparent digestibility (Lee *et al.* 1992; Waghorn & Shelton 1992; Wang *et al.* 1994). However, the significance of these positive actions of CT in lotus for increasing animal production has not been assessed. Although several studies comparing lotus with other forages not containing CT, such as red clover (*Trifolium pratense*), lucerne (*Medicago sativa)* and perennial ryegrass (*Lolium perenne*) indicated improved animal performance with lotus (Marten & Jordan 1979; Douglas *et al.* 1995), the results could not be conclusively considered unique to CT, since the forages differed in several other factors as well as CT concentration.

Purchas & Keogh (1984) reported that carcass fat content of lambs grazing a CT-

containing legume (*Lotus pedunculatus*) was consistently lower than that of lambs grazing non-CT-containing white clover (*Trifolium repens*). Therefore the effect of CT in *L. corniculatus* upon carcass fatness also needs to be assessed. Objectives of the present study were to evaluate the effects of CT in *L. corniculatus* upon wool growth, body growth, carcass fatness, voluntary feed intake (VFI) and the concentration of rumen metabolites in young grazing sheep under restricted feed allowance. The strategy of low feed allowances was to restrict VFI such that protein supply was limiting animal productivity. Hence the action of CT in *L. corniculatus* in increasing protein supply had maximum opportunity for increasing productivity. Lucerne was included as a non-CT-containing control forage.

4.3 MATERIALS AND METHODS

4.3.1 Experimental design

A grazing trial with 80 weaned lambs was conducted at Aorangi Research Station, AgResearch Grasslands, Manawatu, New Zealand, from late November 1992 until mid-April 1993 (22 weeks).

The experiment was a 2 X 2 factorial design, with two types of forage (*L. corniculatus v.* lucerne)with and without the oral administration of polyethylene glycol (PEG). PEG (MW 3500) was orally administered to half the sheep grazing each forage, whilst the other sheep acted as controls. During the disintegration of plant material, CT bind to PEG in preference to protein (Jones & Mangan 1977; Barry & Manley 1986), and the CT-PEG complex is not dissociated in the digestive tract of the animals. Therefore, comparing control lambs (CT-acting) with lambs given PEG (CT-inactivated) can be used to quantify the effects of CT in sheep grazing *L. corniculatus*, whilst the studies with lucerne can be used to quantify the nutritional effects of PEG independently of CT.

4.3.2 Forages

Pure swards of *L. corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) and lucerne (Medicago sativa; cv. Grasslands Oranga) were grazed by the lambs. The primary growth of both swards was mature in places when the experiment commenced, especially for lotus. Half the area of lotus was lightly topped to remove flowers and to stimulate vegetative growth at the start of the experiment. The second and third

growths of both swards were in the vegetative state and never flowered. Herbage mass and botanical composition were determined weekly, immediately before and after grazing, by cutting 3 x 0.125 m^2 quadrats per sward to ground level and drying for 16 h in a forced-air oven at 80 °C. Herbage samples for determination of pre-grazing herbage quality (feed on offer) were collected weekly by cutting to ground level. Two wire mesh cages measuring about 1.4 x 0.9m were placed in each break immediately before lambs commenced grazing. At the end of grazing that break, the cages were removed and the forage cut and samples taken corresponding to what animals were observed to be eating. These are hence referred to as diet selected, and were analysed for chemical composition.

Flowers were collected from each forage on two occasions and analysed for CT content.

4.3.3 Animals

Eighty male Romney cryptorchid lambs aged 8-10 weeks were used. An extra ten lambs with a mean liveweight of 22.4 kg (S.D. 1.34) were weighed, shorn and slaughtered at the beginning of the experiment as the initial slaughter group. The values of body weight, clean wool weight, carcass weight and carcass fatness were recorded and used to estimate the initial carcass weight and initial clean wool weight of the 80 experimental lambs.

The 80 selected test lambs with an initial weight of 22.8 Kg (S.D. 4.47 kg) were weighed, tagged, drenched with anthelmintic (Ivomec; Merk, Sharp and Dohme, NZ Ltd.) to control internal parasites, treated for external parasites (Wipeout; Coopers Animal Health NZ) and then divided randomly into two groups for lotus and lucerne, with 40 lambs on each sward. Half of the lambs in each sward were selected randomly for oral PEG supplementation (CT-inactivated) and the remainder acted as control lambs (CT-acting). Anthelmintic (Ivomec) was given at monthly intervals.

Liveweight gain (LWG) was estimated from fortnightly weighings. Lambs were shorn after 19 weeks of grazing and samples from both left and right sides were used to determine clean wool yield, wool staple length and fibre diameter. All lambs were slaughtered at the end of the experiment and hot carcass weight and carcass GR

measured. GR is an indirect fat content assessment based on measurement of total soft tissue depth over the 12th rib at a point 11 cm from the midline of the carcass (Kirton 1989).

PEG was administered orally twice daily, at 08.00 and at 18.00 h. PEG was administered as 50% w/v solution, with 40 g PEG/lamb daily at the beginning and increasing to 67 g PEG/lamb by the end of the experiment. The dose of PEG was calculated on the basis of estimated daily VFI and the CT content in lotus, measured in previous studies, with the objective of administering 1.7 g PEG/g CT consumed (Barry & Forss 1983). Lambs grazing lucerne received identical doses of PEG to lambs grazing lotus.

VFI was measured using slow releasing chromium capsules (Nufarm, Auckland, NZ), as described by Parker *et al.* (1989). Chromium capsules were orally administered to each lamb on 1 February 1993. Faeces sampling from the rectum commenced eight days after chromium capsules had been given and continued every two day for two weeks. Faeces samples were bulked for each lamb over the two week sampling period and dried at 80 °C for three days for chromium analysis. Chromium release rates for different treatments were measured by introducing six sheep (three control, three PEG orally supplemented) with rumen cannulae to each sward, and measuring chromium disappearance over 16 day periods from capsules suspended in the rumen.

Rumen fluid was sampled monthly from 20 lambs grazing each sward (10 control lambs, 10 PEG lambs), using a stomach tube. The 10 lambs per treatment were selected at random at each sampling time. Rumen fluid samples for ammonia and volatile fatty acid (VFA) analyses, and protozoa counting, were prepared as described by Wang *et al.* (1994). Where insufficient rumen fluid was obtained for all three analyses, priority was ammonia, followed by protozoa and then VFA.

4.3.4 Grazing Management

Feed allowance for both groups of lambs was 2 kg green DM (total DM-dead DM) per lamb daily, when the experiment commenced, and this was gradually increased to 2.5-3.0 kg green DM as the lambs increased in weight. After shearing at week 19,

feed allowance increased by 30% to compensate for increased heat loss.

Each sward was partitioned into breaks using electric fences. Control and PEG lambs grazed together on each sward. Each break was grazed for 2 days. Areas of breaks were calculated based on number of lambs, herbage mass, days of grazing and feed allowance. Each break was back-fenced and after grazing, it was cut to uniform height (10 cm) and the herbage removed. This management was intended to provide vegetative and high quality forages at all times. The relatively short period of grazing for each break was in order to provide reasonably constant levels of available feed at all times. Lambs had free access to water throughout the experiment.

4.3.5 Laboratory analyses

All samples of feed offered and diet selected were stored at -20 °C, freeze-dried, and ground to pass through a 1 mm diameter sieve, prior to laboratory analyses. Herbage samples were analysed for extractable and bound CT by the method of Terrill *et al.* (1992*a*), total nitrogen (N) by the Kjeldahl method, OM by ashing samples for 16 h at 550 °C, water-soluble carbohydrates and pectin by the extraction procedure of Bailey (1967), cell wall constituents by the detergent system of Van Soest (1983) and *in vitro* digestibility by the enzymic method of Roughan & Holland (1977). Because extractable CT would be dissolved in the initial *in vitro* extraction steps, but are known to be indigestible *in vivo* (Terrill *et al.* 1994), extractable CT (% OM) values were deducted from all *in vitro* OM digestibility (OMD) determinations. Chromium in faeces was determined by the method of Costigan & Ellis (1987). Ammonia, VFA and protozoa in the rumen fluid were measured as described by Wang *et al.* (1994).

Clean wool yield was determined by washing 40 g greasy wool in detergent and dried to a constant weight on a forced air dryer and calculated using the standard 16% regain. Wool staple length was measured by randomly subsampling 15-20 staples then measuring them unstretched along a ruler. Average fibre diameter (AFD) was measured using an Optical Fibre Diameter Analyser (OFDA; Melden Laboratories, Western Australia).

4.3.6 Calculation of data and statistical analyses

Regression equations were established of carcass weight and clean wool weight upon

liveweight, using data from the initial slaughter group. The initial carcass and initial clean wool weights of the 80 experimental animals were then estimated using these equations. Both carcass weight gain and wool growth were then calculated by deducting the predicted initial carcass and clean wool weight from the final estimates. VFI was calculated as:

$$VFI = \frac{F}{1 - D} \qquad (1)$$

Where D is the *in vitro* OM digestibility of the diet selected and F is faeces OM output, which was calculated as capsule chromium release rate divided by chromium concentration in faeces. PEG was deducted in the calculation of faeces OM output.

One way analysis of variance was used to examine differences in herbage chemical composition between lotus and lucerne, and a separate one way analysis of variance was also used to analyse differences between feed offered and diet selected within each plant species. Differences in herbage mass between plant species, and between pre-grazing and post-grazing herbage mass within each species were also analysed using one way analysis of variance. Factorial analysis of variance was used to examine treatment effects in the animal data, with the factors being forage type, PEG and their interaction. Carcass weight (CW) was used as a covariate to analyse carcass GR data.

4.4 RESULTS

4.4.1 Forage composition

4.4.1.1 Botanical composition

Both forage species were in a vegetative state throughout the experiment. Averaged over the 22 week experimental period, lotus and lucerne swards had similar amounts of green DM (5.27 *v.* 5.24 tonnes (t)/ha), stem and dead matter before grazing (Table 4.1). Lotus, however, had a significantly lower leaf content than lucerne ($P < 0.05$). After grazing, leaf mass was zero for both lotus and lucerne, whilst the stem and dead matter contents remained similar to pre-grazing levels.

Table 4.1 Pre-grazing and post-grazing forage mass (tonne dry matter (DM)/ha) of *Lotus corniculatus* (cv. Grasslands Goldie) and lucerne (*Medicago sativa;* cv. Grasslands Oranga). (Mean values with their S.E. are for 20 samples per forage).

Plant component	Grazing	Lotus	Lucerne	S.E.
Leaf	Pre-	1.90	2.40	0.145
	Post-	0	0	0
	S.E.	0.088	0.122	
Stem	Pre-	3.38	2.85	0.322
	Post-	3.06	2.53	0.313
	S.E.	0.387	0.289	
Dead matter	Pre-	1.02	0.85	0.147
	Post-	1.37	1.09	0.167
	S.E	0.152	0.163	
Total	Pre-	5.27	5.24	0.422
	Post-	3.06	2.53	0.313
	S.E.	0.429	0.304	

4.4.1.2 Chemical composition

The lower proportion of leaf DM in lotus compared to lucerne was associated with lower N and pectin contents than lucerne (Tables 4.2 and 4.3) in feed on offer. Lotus contained higher contents of organic matter ($P < 0.05$), soluble carbohydrate ($P < 0.01$) and lignin ($P < 0.001$) in both feed on offer and diet selected. Neutral detergent fibre (NDF) and cellulose concentrations and the ratio of readily fermentable : structural carbohydrate (CHO; (soluble CHO + pectin) : (cellulose + hemicellulose) were similar for both forages. Acid detergent fibre (ADF) was higher and hemicellulose lower ($p < 0.05$) for lotus than lucerne in diet selected, whilst there was

no difference between species in feed on offer. Total CT in lotus was 22 and 34 g/kg DM in feed offered and diet selected respectively, whilst only trace amounts of total CT were detected in lucerne. OMD was slightly lower for lotus than for lucerne, with the difference attaining significance for feed on offer ($P < 0.05$) but not for diet selected.

Table 4.2 Carbohydrate and lignin contents (g/kg dry matter (DM)) in the feed offered and diet selected by sheep grazing *Lotus corniculatus* (cv. Grasslands Goldie) and lucerne (*Medicago sativa;* cv. Grasslands Oranga). (Mean values with their S.E. are for 8 samples per forage).

		Lotus	Lucerne	S.E.
Soluble carbohydrate	Offered	71	58	2.8
	Selected	84	64	3.8
Pectin	Offered	35	46	1.1
	Selected	39	50	1.5
Neutral detergent fibre	Offered	449	423	23.8
	Selected	346	299	15.8
Acid detergent fibre	Offered	350	311	18.4
	Selected	271	210	12.2
Hemicellulose	Offered	99	112	5.8
	Selected	74	89	4.3
Cellulose	Offered	242	243	13.7
	Selected	183	167	10.4
Lignin	Offered	108	69	5.0
	Selected	88	42	2.4
Ready fermentable: Structural carbohydrate [1]	Offered	0.32	0.30	0.022
	Selected	0.49	0.44	0.025

1. (Soluble carbohydrate + pectin) : (Cellulose + hemicellulose).

Table 4.3 Organic matter (OM), total N and condensed tannin (CT) contents (g/kg DM) and *in vitro* OM digestibility (OMD) of feed offered and diet selected by sheep grazing *Lotus corniculatus* (cv. Grasslands Goldie) and lucerne (*Medicago sativa;* cv. Grasslands Oranga). (Mean values with their S.E. are for 8 samples per forage for OM, *in vitro* OMD and total N).

		Lotus	Lucerne	S.E.
Organic matter	Offered	913	899	3.4
	Selected	908	893	3.4
In vitro OMD	Offered	0.65	0.72	0.021
	selected	0.73	0.76	0.014
Total N	Offered	27.7	33.9	1.85
	Selected	31.4	41.8	1.32
Extractable CT	Offered	14.7 (5) [1]	0.5 (4)	0.77
	Selected	24.7	0.3	1.75
Protein bound CT	Offered	6.7	0	0.62
	Selected	7.9	0	0.31
Fibre bound CT	Offered	0.9	0	0.63
	Selected	1.4	0	0.08
Total CT	Offered	22.3	0.5	1.12
	Selected	34.0	0.3	1.82

1. Sample numbers for CT analysis.

For both forages, diet selected was predominately leaf. The selected forage had higher contents of pectin ($P < 0.05$) and the ratio of readily fermentable : structural CHO ($P < 0.001$) than feed on offer. ADF, NDF, hemicellulose, cellulose and lignin concentrations were all lower ($P < 0.01$) in diet selected than feed offered for both lotus and lucerne. There was no difference in OM content between diet selected and feed offered for both forages. For lotus, diet selected had higher soluble CHO content and *in vitro* OMD ($P < 0.05$, 0.01 respectively) than feed on offer, whilst no differences were detected for lucerne. Diet selected had higher N content than feed offered ($P < 0.01$) for lucerne, whilst the N content of diet selected and feed on offer in lotus was not significant.

Total CT concentration in lotus flowers was 88 g/kg DM, with 55, 29 and 3 g/kg DM being extractable, protein-bound and fibre-bound respectively. There were no CT in lucerne flowers.

4.4.2 Initial slaughter group

Carcass weight (CW; kg) and clean wool weight (WW; g) were related to liveweight (LW; kg) by the following regression equations:

$$CW = -2.75 + 0.57LW \qquad (r^2 = 0.957) \qquad (2)$$
$$(SE \quad 0.957 \quad 0.042)$$

$$WW = -52.3 + 33.2LW \qquad (r^2 = 0.763) \qquad (3)$$
$$(SE \quad 147.55 \quad 6.53)$$

4.4.3 Voluntary feed intake

Voluntary organic matter intake (OMI) was slightly lower for lambs grazing lotus than lucerne ($P < 0.05$; Table 4.4). PEG supplementation had no effect on OMI in lambs grazing either lotus or lucerne ($P > 0.05$).

4.4.4 Rates of body and wool growth

Lambs grazing lotus had better performance than lambs grazing lucerne in LWG (196 *v.* 181 g/day; $P < 0.05$), carcass weight gain (77.1 *v.* 65.2 g/day; $P < 0.001$), carcass dressing-out percentage (44.5 *v.* 43.2%; $P < 0.01$) and wool growth (11.5 *v.* 10.5 g/day; $P < 0.05$; Table 4.4). PEG supplementation had no effect upon any of these

measurements in lambs grazing lucerne. However, in lambs grazing lotus, PEG supplementation reduced wool growth ($P < 0.05$) and slightly reduced LWG ($P = 0.07$), without affecting carcassweight gain or carcass fatness. There were no significant differences in wool staple length and average fibre diameter (AFD) between treatments.

Table 4.4 Voluntary feed intake, Live weight gain, carcass gain and wool growth of sheep grazing *Lotus corniculatus* (cv. Grasslands Goldie) and lucerne (*Medicago sativa;* cv. Grasslands Oranga) with or without polyethylene glycol (PEG; MW 3500) oral administration. (Mean values with S.E. are for 19 or 20 animals per group).

	Lotus		Lucerne		S.E.
	Control n=20	PEG supplemented n=20	Control n=20	PEG supplemented n=19	
OM intake (kg/lamb.d)	1.19	1.20	1.32	1.34	0.056
Live weight gain					
(g/day)	203	188	185	178	5.8
Carcass					
Carcass gain (g/d)	78.7	75.2	67.7	62.9	2.87
Dressing out (%)	44.5	44.6	43.3	43.0	0.49
Carcass fatness (GR, mm)*	10.6	9.6	11.0	9.8	0.64
Wool growth (g/day)	12.1	10.9	10.8	10.2	0.39
Staple length (mm)	119	123	118	125	2.6
Fibre diameter(μ)	33.5	32.5	32.9	32.6	0.50

*. Adjusted to equal carcass weight;

The degree of freedom for error is 75.

4.4.5 Rumen metabolites

4.4.5.1 Ammonia concentration

PEG administration increased rumen ammonia concentration at all sampling times ($P < 0.05$; Figure 4.1*a*) for lambs grazing lotus, but had no significant effect upon rumen ammonia concentration at any sampling time in lambs grazing lucerne (Figure 4.1*b*).

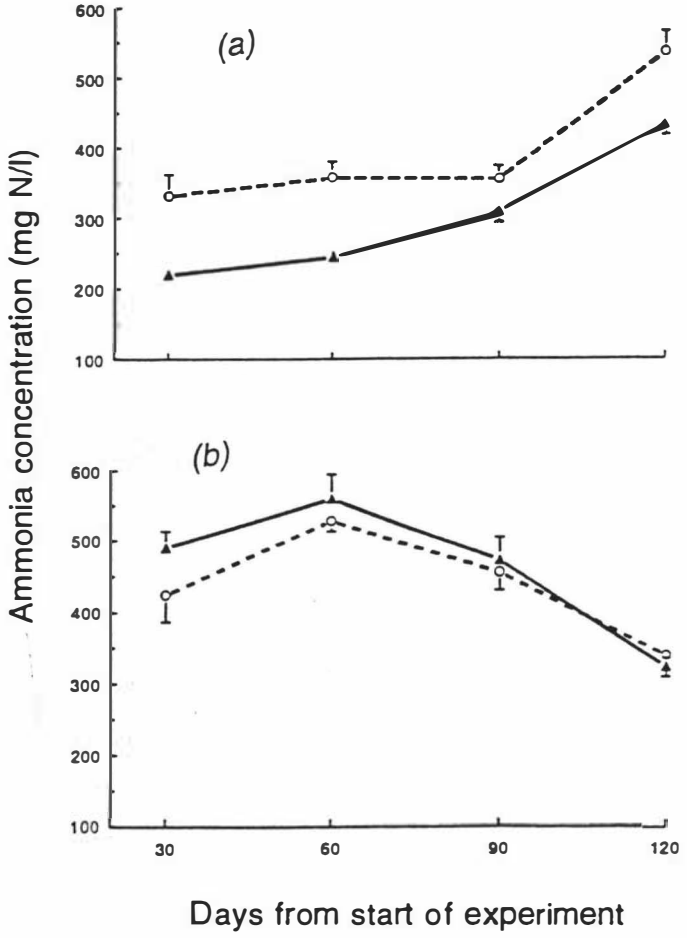

Figure 4.1 Rumen ammonia concentration in lambs grazing (*a*) *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) and (*b*) lucerne (*Medicago sativa;* cv. Grasslands Oranga), with or without twice daily oral administration of polyethylene glycol (PEG; MW 3500). ▲——▲ control lambs; o- - - -o PEG lambs. Mean values are for 6, 10, 10 and 8 control lambs and 10, 10, 10 and 10 PEG supplemented lambs grazing lotus, and 7, 10, 10 and 10 control lambs and 8, 9, 10 and 5 PEG supplemented lambs grazing lucerne after 30, 60, 90 and 120 days of grazing respectively. I S.E.

4.4.5.2 Protozoa numbers

For lambs grazing lotus, PEG supplementation increased protozoa numbers in rumen fluid taken after 30 ($P < 0.1$), 60 ($P < 0.05$) and 120 ($P < 0.1$) days (Figure 4.2*a*). PEG supplementation had no effect on protozoa numbers in lambs grazing lucerne (Figure 4.2*b*).

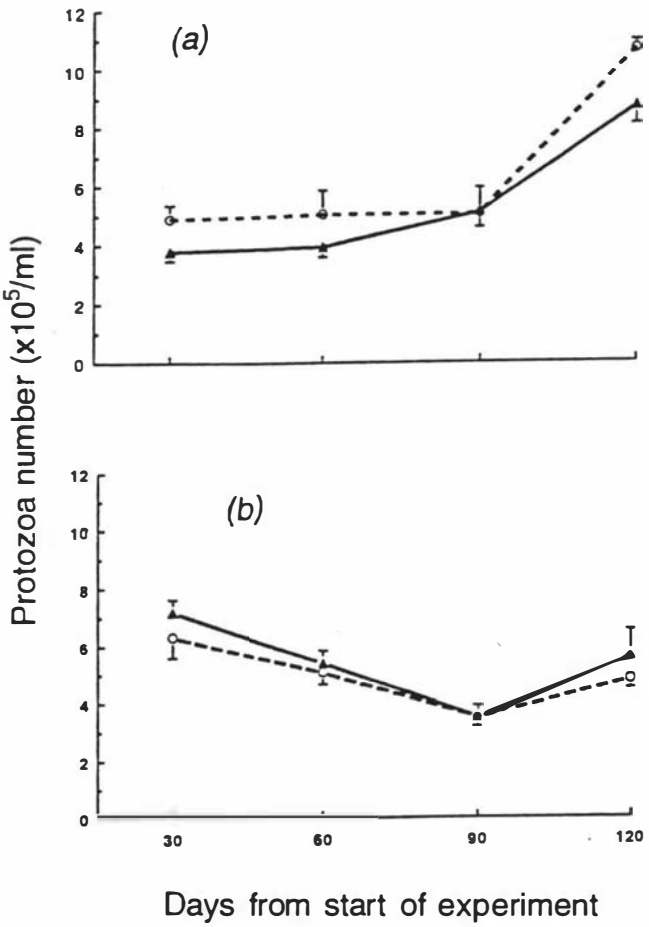

Figure 4.2. Rumen protozoa numbers (x 10 ^{5}/ml) in lambs grazing (*a*) *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) and (*b*) lucerne (*Medicago sativa;* cv. Grasslands Oranga), with or without twice daily oral administration of polyethylene glycol (PEG; MW 3500). ▲——————▲ control lambs; o— — —o PEG lambs. Mean values are for 10, 10, 10 and 8 control lambs and 9, 10, 10 and 9 PEG supplemented lambs grazing lotus, and 10, 9, 10 and 4 control lambs and 9, 10, 10 and 6 PEG supplemented lambs grazing lucerne after 30, 60, 90 and 120 days of grazing respectively. I SE.

4.4.5.3 VFA

Total VFA concentration in rumen fluid was consistently higher for PEG than for control lambs grazing lotus, with the difference attaining significance on day 90 ($P < 0.05$; Figure 4.3), but was unaffected by PEG supplementation in lambs grazing lucerne.

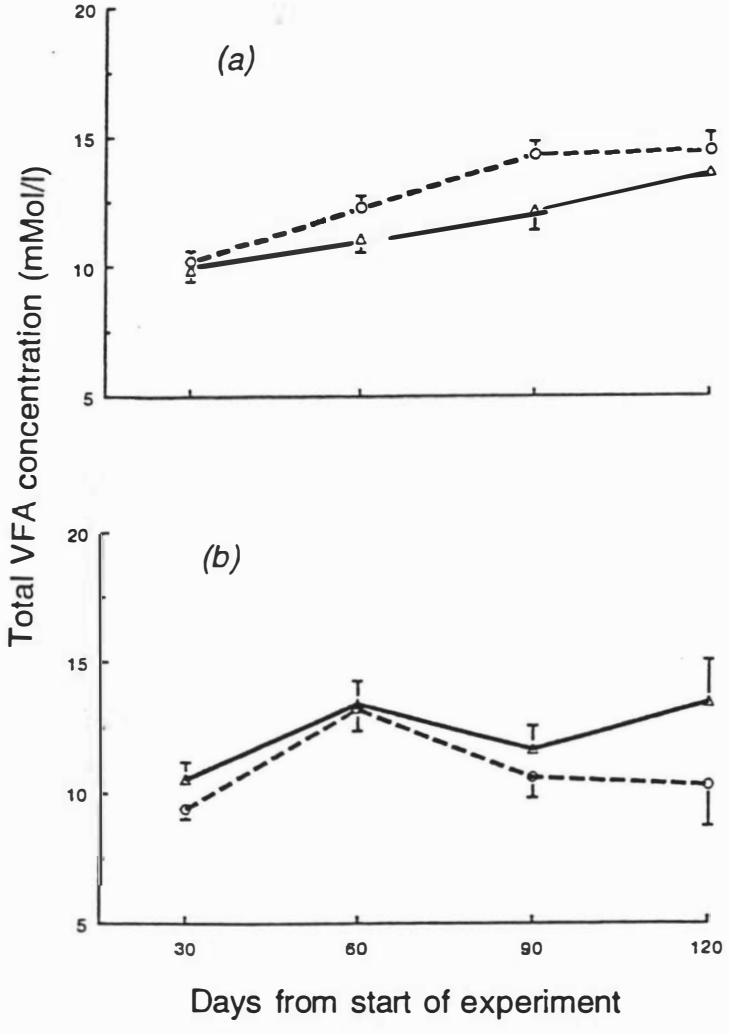

Figure 4.3 Rumen total volatile fatty acids (VFA; *m*Mol/l) in lambs grazing (*a*) *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) and (*b*) lucerne (*Medicago sativa;* cv. Grasslands Oranga), with or without twice daily oral administration of polyethylene glycol (PEG; MW 3500). ▲——————▲ control lambs; o— — — —o PEG lambs. Mean values are for 7, 10, 10 and 6 control lambs and 10, 9, 10 and 7 PEG supplemented lambs grazing lotus, and 8, 8, 9 and 2 control lambs and 9, 8, 10 and 2 PEG supplemented lambs grazing lucerne after 30, 60, 90 and 120 days of grazing respectively. I S.E.

Lambs grazing lotus had a higher acetic acid molar proportion on days 30 ($P < 0.001$), 60 ($P < 0.01$) and 90 ($P < 0.001$) than lambs grazing lucerne (Figure 4.4*a*). Forages had no consistent effect upon the molar proportion of other major VFA (propionic and *n*-butyric acids). The ratio of (acetic acid+2*n*-butyric acid)/propionic acid was higher for lambs grazing lotus than for lambs grazing lucerne on days 60 ($P < 0.1$) and 90 ($P < 0.001$). PEG supplementation had no significant effect on the molar proportions of major VFA in lambs grazing either forage. PEG supplementation increased the molar proportions of *iso*-butyric ($P < 0.10$), *iso*-valeric ($P < 0.01$) and *n*-valeric acids ($P < 0.01$) in lambs grazing lotus (Figure 4.5), but had no effect upon these minor VFA in lambs grazing lucerne.

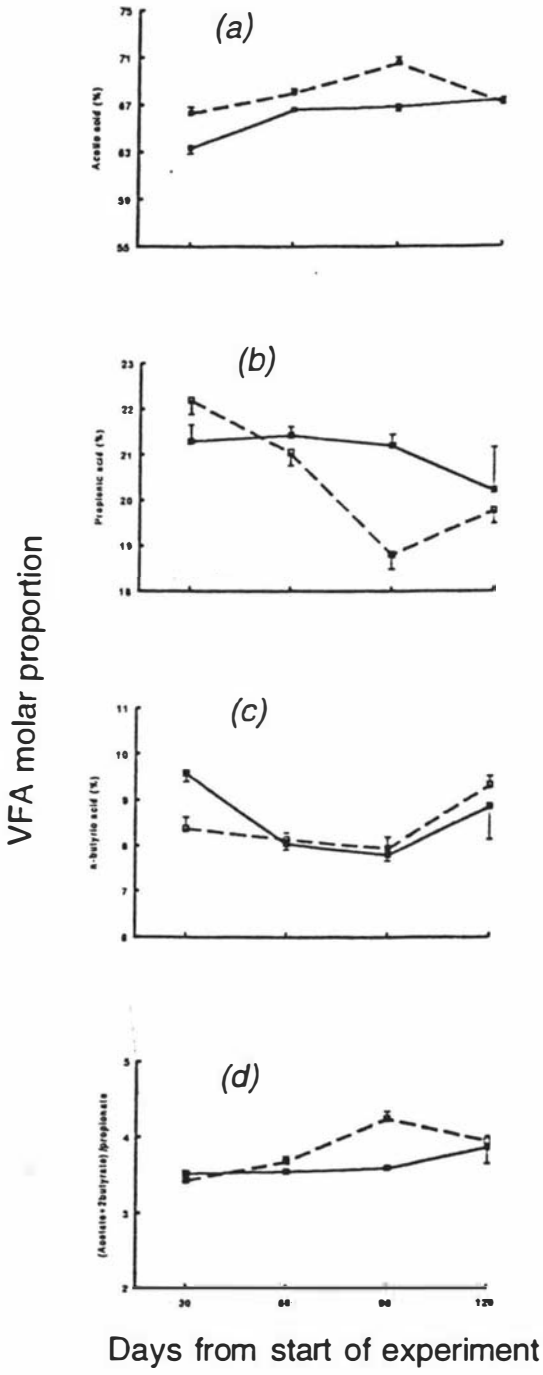

Figure 4.4 Molar proportion (%) of (*a*) Acetic acid, (*b*) propionic acid, (*c*) *n*-butyric acid and of the ratio of (acetic acid + 2*n*-butyric acid):propionic acid in the rumen fluid of lambs grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) □— — — —□ and of lambs grazing lucerne (*Medicago sativa*; cv. Grasslands oranga) ■————————■. Mean values are for 17, 19, 20 and 13 lambs grazing lotus and 17, 16, 19 and 4 lambs grazing lucerne at 30, 60, 90 and 120 days sampling. I SE.

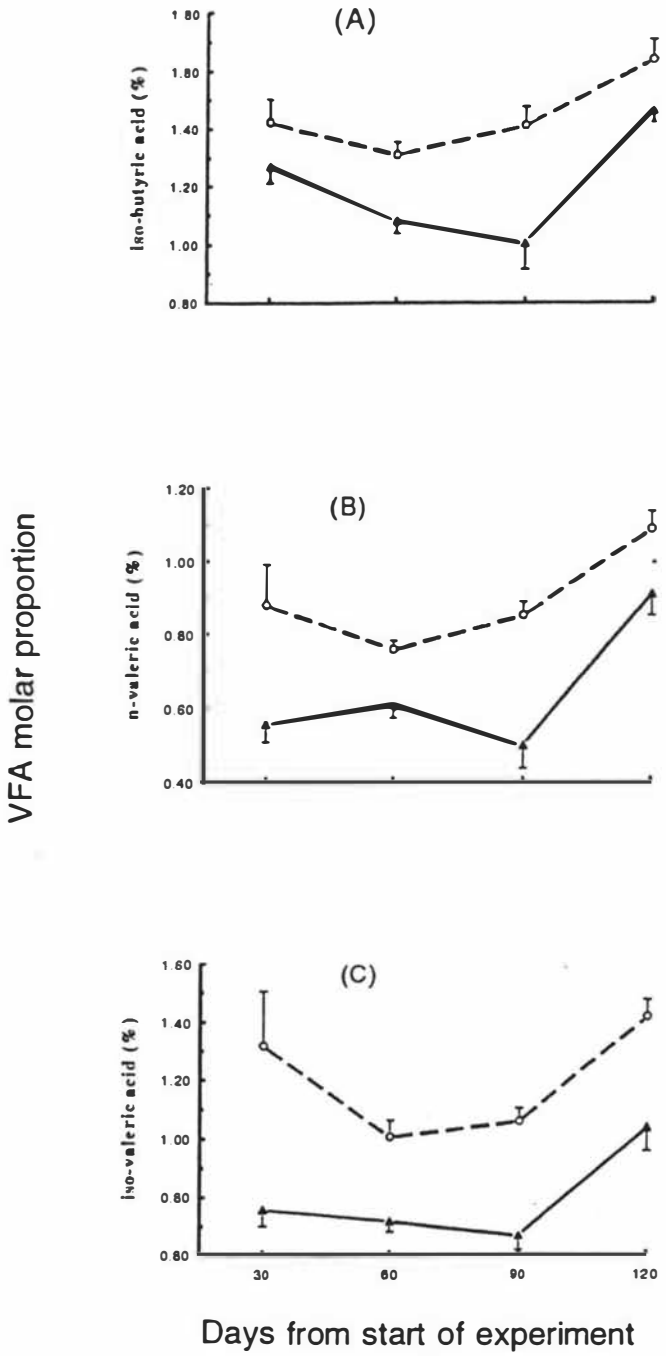

Figure 4.5 Molar proportion (%) of (*a*) *iso*-butyric acid, (*b*) *n*-valeric acid and (*c*) *iso*-valeric acid in lambs grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) with or without twice daily oral administration of polyethylene glycol (PEG; MW 3500). ▲——————▲ control lambs; o– – – – – –o PEG lambs. Means are for 7, 10, 10 and 6 control lambs and 10, 9, 10 and 7 PEG supplemented lambs at 30, 60, 90 and 120 days sampling. I SE.

4.6 DISCUSSION

The main findings of this study were that the action of CT in lotus increased wool growth and improved the efficiency of feed utilization. The lambs grazing lotus had higher rates of carcass gain, higher carcass dressing-out percentage and better efficiency of feed utilization than lambs grazing lucerne.

PEG administration showed that the principal effect of CT in lotus was to increase wool growth, with a minor effect in increasing LWG and no effect on carcass gain, carcass fatness and VFI. The rumen ammonia concentrations in samples obtained by stomach tube suggested that the superior productive performance of control lambs grazing lotus was due to CT reducing protein degradation in the rumen, resulting in more protein absorption from the small intestine. The lower total VFA concentration in control lambs than PEG-supplemented lambs grazing lotus, compared with the similarity of total VFA concentration between control and PEG-supplemented lambs grazing lucerne, also indicated that CT decreased protein degradation and amino acid deamination in the rumen. Minor VFA are produced from deamination of specific AA, with *iso*-butyric acid from valine, *iso*-valeric acid from leucine and *n*-valeric acid from arginine, ornithine, proline, σ-aminovaleric acid or lysine (El-Shazly 1952; Van Soest 1983), of which valine, leucine, arginine and lysine are EAA. Hence the reduced *iso*-butyric, *iso*-valeric and *n*-valeric acids in the rumen fluid of control compared to PEG lambs grazing lotus also suggests that CT reduced deamination of these specific EAA.

Wool has a high sulphur content (2.7-4.2%) mainly as cystine (Reis 1965*a, b*; Hogan 1975), and post-ruminal supplementation with sulphur-containing amino acids (SAA) has been shown to increase wool growth markedly (Reis 1979). Studies with lotus (Wang *et al*. 1994) and with *L. pedunculatus* (McNabb *et al*. 1993) showed that CT reduced SAA degradation in the rumen, increased the irreversible loss (IRL) of cystine from blood plasma and increased the flow of cystine to body synthetic reactions. The increased wool growth in control lambs grazing lotus in the present study is thus probably due to the effect of CT in increasing EAA absorption and cystine availability for body synthetic reactions. Increased wool growth due to CT is also supported by reports of Terrill *et al*. (1992*b*) and Lee *et al*. (1992).

The growth rates of lambs grazing lotus were lower than those in earlier experiments

with lambs fed lotus *ad libitum* (228 g/day; Douglas *et al.* 1994). This indicated that the strategy of restricting protein intake to limit animal productivity under grazing was successful, with CT expressing its action under the feed allowance and grazing management used in the present study, and increasing the productivity of lambs grazing lotus. Although lambs grazing lotus had slightly lower VFI than lambs grazing lucerne, they also had superior body growth, with the small increase in LWG being due to CT, but the superior carcass growth of lambs grazing lotus being independent of CT. The different effects on body growth (i.e. LWG) and carcass growth suggest that action of CT affected non-carcass components in lambs grazing lotus, of which the skin seems most probable in view of its effect on increasing wool growth. The VFI and body growth data suggest that feed conversion efficiency of lambs grazing lotus was higher than for lambs grazing lucerne. High acetic:propionic acid ratios in rumen VFA can be a cause of low efficiency of utilisation of metabolisable energy (ME) for growth, and reduced feed conversion efficiency, due to insufficient NADPH being generated from glucose oxidation to allow synthesis of body fat from acetic acid (Black *et al.* 1987). However, this cannot explain the superior feed conversion to carcass production in lambs grazing lotus in the present study, as the ratio of (acetic acid+2 butyric acid):propionic acid was higher for lambs grazing lotus than for lambs grazing lucerne. The cause of the better feed conversion in lambs grazing lotus is therefore unknown. Because PEG supplementation had no effect on VFI for either lotus or lucerne lambs, the lower VFI of lambs grazing lotus than those of lucerne is most likely to be due to the higher lignin content in lotus than in lucerne.

The higher numbers of protozoa in the rumen fluid of PEG-supplemented lambs grazing lotus than control lambs suggests that CT in lotus had an adverse effect on protozoa growth, and confirms the findings of Wang *et al.* (1994) using continuous infusion of PEG into the rumen for 22 days. The lack of effect of PEG in lambs grazing lucerne suggests that the effect on protozoa is specific to CT and not to PEG *per se*. The reason why CT in lotus reduce protozoa numbers is unknown. It could be an inhibiting effect due to the astringent nature of CT, or an indirect effect, by inhibiting rumen bacterial growth, thus limiting the feed sources for protozoa. This is opposite to the result obtained by Terrill *et al.* (1992*b*), who found CT in sulla (*Hedysarum coronarium*) to increase rumen protozoa numbers, suggesting that CT from different plant species have different effects upon rumen protozoa. Further

studies are needed to evaluate the effects of CT upon the kinetics of growth and death of rumen protozoa.

Once daily oral PEG supplementation to sheep grazing the CT-containing legume sulla (Terrill *et al*. 1992*b*) indicated that this did not eliminate binding of plant proteins to CT in the rumen for a full 24-h period. In the present study, the lack of response to PEG in lambs grazing lucerne that does not contain CT showed that PEG administration *per se* did not affect nutrient digestion and lamb productivity. The responses of lambs grazing lotus to PEG supplementation showed that these were due to the effect of PEG in preventing CT binding to protein. The results showed that the technique of twice daily oral PEG supplementation to lambs was sufficient to get responses, but it is still not known if the response was maximal. Although ammonia concentrations in rumen fluid of PEG sheep were significantly higher than those of control sheep, these samples were taken 5-7 h after oral PEG supplementation, and it is not known what the situation was at other times. Experiments with Yorkshire fog (*Holcus lanatus*; 2 g CT/kg DM) suggested that rumen ammonia concentration was higher for PEG than for control sheep 4 h after oral PEG supplementation, but similar after 8 h (Liu personal communication). Therefore, further experiments are needed to study the technique in order to quantify the maximum effect of CT.

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Chapter 5

The effect of condensed tannins in *Lotus corniculatus* upon lactation performance in ewes

This Chapter has been accepted for publication in *Journal of Agricultural Science, Cambridge* (in press).

5.1 *ABSTRACT*

A grazing experiment was conducted for 8 weeks in the spring/summer of 1993 at Palmerston North, New Zealand, to study the effects of condensed tannins (CT) in *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) upon the lactation performance of ewes rearing twin lambs. Effects of CT were evaluated by studying the responses of ewes to twice daily oral supplementation with polyethylene glycol (PEG; MW 3500), which binds and inactivates CT. A rotational grazing system with restricted feed allowance was used. Measurements were made of pre- and post-grazing herbage mass, the composition of the feed on offer and diet selected, voluntary feed intake (VFI), milk yield and composition, liveweight gain and wool production. The concentration of metabolites in rumen fluid and in blood plasma was also measured. Lotus contained 35.5 g total nitrogen and 44.5 g total CT/kg dry matter in the diet selected, with an *in vitro* digestibility of 73%. At peak lactation (weeks 3 and 4) milk yield and composition was similar for control (CT acting) and PEG supplemented (CT inactivated) ewes , but as lactation progressed the decline in milk production and in the secretion rates of protein and lactose was less for control than for PEG supplemented ewes. In mid and late lactation (weeks 6-11), control ewes secreted more milk (21%), more milk protein (14%) and more lactose (12%) than PEG supplemented ewes. Milk fat percentage was lower for control than for PEG supplemented ewes, but secretion rates of fat were similar for the two groups. VFI, liveweight gain and wool growth were similar for both groups. Plasma urea and glucose concentrations were lower for control than for PEG supplemented ewes, but concentrations of non-esterified fatty acids (NEFA), growth hormone and insulin were similar for the two groups. The concentrations of ammonia and molar proportions of *iso*-butyric, *iso*- and *n*-valeric acids in rumen fluid were lower for control than for PEG supplemented ewes; molar proportions of acetic, propionic and *n*-butyric acids were similar for the two groups. It was concluded that for ewes rearing twin lambs grazing *L. corniculatus*, the action of CT increased milk yield and the secretion rates of protein and lactose without affecting VFI, thereby increasing the efficiency of milk production. The increased milk production did not appear to be mediated by effects on plasma concentrations of growth hormone or insulin.

5.2 INTRODUCTION

Condensed tannins (CT) occur in a restricted range of forage legumes and can bind to plant protein in the rumen to form CT-protein complexes, which reduce microbial degradation of protein to ammonia. The CT-protein complexes dissociate below pH 3.5 (Jones & Mangan 1977), increasing non-ammonia nitrogen (NAN) flux to the intestine (John & Lancashire 1981; Barry *et al.* 1986*a*; Waghorn *et al.* 1987*a*). Waghorn *et al.* (1987*b*) reported that the action of CT in *Lotus corniculatus* increased absorption of essential amino acids (EAA, excluding methionine and cystine) from the small intestine of sheep by 62%. Wang *et al.* (1994) showed that CT in *L. corniculatus* markedly increased cystine irreversible loss rate (IRL) from blood plasma and increased cystine flux to body synthetic reactions. Therefore, CT in suitable levels in plants may provide a practical means of increasing amino acid (AA) absorption and utilization in grazing ruminant animals. The ideal CT concentrations for ruminant animal nutrition has been suggested to be in the range 20-40 g/kg DM (Barry 1989; Waghorn 1990). A previous study with weaned lambs grazing *L. corniculatus* and lucerne showed that the action of CT in lotus slightly increased liveweight gain (LWG) and increased wool growth by 12% (Wang *et al.* 1995). Because extensive ruminal protein degradation (70% for high quality forages; Ulyatt *et al.* 1975) can result in insufficient amino acid absorption to maximize productivity in grazing ruminants, the action of a low concentration of CT in *L. corniculatus* may increase productivity of sheep in other physiological states, such as lactation.

Lactating ewes have a high demand for protein. Theoretically there is a requirement for more rumen undegradable protein as milk yield increases (Ørskov 1982). Supplements of formaldehydrate-treated casein, proteins with low rumen degradability, and increasing protein intakes have increased milk production and milk protein yield in sheep and cattle fed forage diets (Barry 1980; Flores *et al.* 1979; Penning *et al.* 1988; Rogers *et al.* 1980). The increased milk yield was associated with increased quantities of NAN reaching the abomasum (Robinson *et al.* 1979; Rogers *et al.* 1979, 1980).

Objectives of the present study were to evaluate the effect of CT in *L. corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) for increasing lactation performance of ewes

·earing twin lambs.

5.3 MATERIAL AND METHODS

5.3.1 Experimental design

A grazing experiment using lactating ewes rearing twin lambs was conducted at Aorangi Research Station, AgResearch Grasslands, Manawatu, New Zealand (NZ), commencing in mid October 1993, when the lambs were aged 3-4 weeks and continued for 8 weeks.

Twenty eight lactating ewes were grazed on *L. corniculatus,* with half being orally supplemented with polyethylene glycol (PEG; MW 3500) twice daily (PEG supplemented group) and the other animals acting as controls. During disintegration of plant material, CT binds to PEG in preference to protein (Barry & Manley 1986; Jones & Mangan 1977), with the CT-PEG complex not being dissociated in the digestive tract. Therefore, comparing control ewes (CT acting) with PEG supplemented ewes (CT inactivated) enables the effects of CT to be quantified. Previous studies (Wang *et al.* 1995) have shown that PEG had no effect upon protein breakdown or animal production in lambs grazing vegetative lucerne that does not contain CT; hence effects of PEG are specific to binding and inactivating CT. A rotational grazing system was used in the experiment. Effects of CT in *L. corniculatus* upon milk yield, milk composition and rates of body and wool growth were studied.

5.3.2 Forages

Pure swards of vegetative *L. corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie) were grazed by ewes and lambs in the experiment. Herbage mass and botanical composition were determined weekly, immediately before and after grazing, by cutting 3 x 0.125 M^2 quadrats per sward to ground level and drying for 15 h in a forced-air oven at 80 °C. Samples of feed on offer were collected weekly by cutting to ground level. Two wire mesh cages measuring about 1.4x0.9m were placed in each break immediately before the animals commenced grazing. At the end of grazing that break, the cages were removed and the forage cut and samples taken corresponding to what animals were observed to be eating. These are hence referred to as diet selected, and were analysed for chemical composition. All samples of feed on offer and diet selected were stored at -20 °C for analysis.

5.3.3 Animals

Lactating Romney ewes aged 3 to 5 years were used, and were selected from an initial group of 40 ewes that had been synchronized during oestrus and were diagnosed by ultrasound as carrying twin lambs. Mid side wool patches measuring 10x10 cm were clipped on both sides of each ewe before lambing. Lambs were tagged at birth (16 to 22 September 1993) and the ewes were grazed on perennial ryegrass/white clover pasture until the lambs were 3 weeks old. Twenty eight ewes with initial liveweight of 50.9 kg (SD 5.03) were selected from those 40 ewes as the experimental animals on the basis of a narrow range of lambing date and good body condition. Before the experiment commenced, all 28 ewes were machine milked twice, one week apart (weeks 2 and 3 after lambing), in order to accustom ewes to the milking procedure, and to obtain milk parameters to be used as covariates. At the first milking, each ewe was intravenously injected with 4.5 ml strepcin antibiotic (Stochguard Laboratories NZ Limited) to prevent mastitis. All ewes were drenched with anthelmintic (Ivomec, Merk, Sharp and Dohme, NZ Ltd.) during weeks 3 and 7 of lactation to control internal parasites.

The 28 selected ewes plus their twin lambs were divided into two groups based on live weight, milk yield and lambing date, and were introduced to the lotus sward at the commencement of week 4 of lactation. One group of 14 ewes was orally supplemented daily with PEG (CT inactivated) whilst the other group acted as control (CT acting). PEG was administered orally twice daily, at 08.00 and at 16.00 h. The dose of PEG was calculated on the basis of estimated daily voluntary feed intake (VFI) and the CT content in lotus, measured in previous studies, with the objective of administering 1.7 g PEG/g total CT consumed (Barry & Forss 1983). PEG was orally supplemented as 50% w/v solution, with 120 g PEG/ewe being given daily.

5.3.4 Grazing management

Feed allowance was 4.5 kg green DM per ewe per day when the experiment commenced and this was gradually increased to 6.0 kg green DM by week 8 of lactation. VFI was restricted so that protein supply limited animal productivity. Hence the action of CT in lotus in increasing protein supply had maximum opportunity for increasing productivity.

The sward was partitioned into breaks, with all ewes and lambs grazing together. Each break was grazed for 2 days, back-fenced and topped to uniform height (10 cm) after grazing and the herbage removed. The relatively short period of grazing each break was intended to provide reasonably constant levels of available feed at all times. Sheep had free access to water throughout the experiment. The ewes and lambs were transferred to ryegrass/white clover pasture at the conclusion of the experiment (end of week 11 of lactation), for a 5 week post-treatment period; PEG was not orally administered during this time.

5.3.5 Sampling procedures

Milk yield was measured by machine milking at weekly intervals. On the milking day, ewes were brought into the shed at 08.30 h and lambs were held separately. One *IU* oxytocin in 1.0 ml saline (Ethical Agents Ltd, NZ) was injected intravenously to each ewe before milking. The milking lasted for 1 min and any residual milk was removed by hand. After milking, the ewes were allowed access to pasture and water without lambs for 5 h, after which the milking was repeated as previously described to measure the amount of milk produced in this period. Time of milking was exactly recorded for each ewe. Milk produced by each ewe in the afternoon milking was weighed and samples taken for composition.

Liveweight gains (LWG) of ewes and lambs were calculated from fortnightly weighings. Wool samples were taken from 10x10 cm areas on both sides of each ewe at the end of pre-experimental feeding (end of week 3 of lactation), to be used as a covariate. Wool samples were also taken from the same site at the conclusion of the grazing experiment (end of week 11 of lactation) and also 5 weeks after the experiment concluded to determine any post-treatment effects upon wool growth.

Voluntary feed intake (VFI) was measured in all ewes using slow releasing chromium capsules (Nufarm, Auckland, NZ), as described by Parker *et al.* (1989). Chromium capsules were orally administered to each ewe in week 4 of the experiment. Rectal sampling commenced 10 days after chromium capsules had been given and continued every 2 days for 2 weeks. Faeces were bulked for each ewe, dried at 80 °C for 4 days and then ground ready for analysis. Chromium release rates were measured by inserting capsules into the rumen of rumen fistulated sheep (3 control;

3 PEG supplemented) which grazed lotus with the ewes and lambs, and measuring chromium disappearance over a 14 day period.

Rumen fluid samples were taken from all 28 ewes by stomach tube, 2 weeks before the conclusion of the experiment, and were deproteinized for ammonia and volatile fatty acid (VFA) determination as described by Wang *et al.* (1994).

Blood samples (10 ml) were taken weekly from the jugular vein of each ewe using ethylene diamine tetra acetic acid (EDTA)-vacutainers (Becton Dickinson, New Jersey, USA), prior to the oxytocin injection at the morning milking. Blood samples were held on ice and centrifuged at 1800 g for 20 min at 4 °C to obtain plasma. Plasma samples were stored at -20 °C for analysis.

5.3.6 Laboratory analyses

All samples of feed offered and diet selected were freeze-dried and ground through a 1 mm diameter sieve prior to analysis. Herbage samples were analysed for extractable and bound CT by the method of Terrill *et al.* (1992), total nitrogen (N) by the Kjeldahl method, organic matter (OM) by ashing samples overnight at 550 °C, water soluble carbohydrates and pectin by the procedure of Bailey (1967), cell wall constituents by the detergent system of Van Soest (1983) and *in vitro* digestibility by the enzymic method of Roughan & Holland (1977). Because extractable CT would be solubilized in the initial *in vitro* extraction steps, but is known to be indigestible *in vivo* (Terrill *et al.* 1994), extractable CT (% OM) values were deducted from all *in vitro* OMD determinations. Chromium in faeces was determined by the method of Costigan & Ellis (1987). Ammonia and VFA in rumen fluid were measured as described by Wang *et al.* (1994). Milk samples were analysed within 24 h of collection for protein, fat and lactose concentration, using an infra-red milk analyser ('Milko-scan', N. Foss Electric, Denmark).

Plasma growth hormone (GH) and insulin (I) concentrations were determined using the heterologous radioimmunoassay methods described by Flux *et al.* (1984). Intra- and inter-assay coefficients of variation (C.V.) and the minimal detectable concentrations were 9.5%, 16.2% and 0.5 ng/ml for GH and 10.1%, 15.2% and 49.7 pg/ml for I. Plasma concentrations of glucose (Trinder 1969) and urea (Tiffany *et al.*

1972) were measured using a Cobas Fara II autoanalyser (Hoffman LA Roche Ltd, Switzerland). The intra- and inter-assay CV for glucose were 1.6 and 3.6% and for urea were 2.8 and 4.1%. Plasma non-esterified fatty acid (NEFA) concentrations were measured colourimetrically by the method described by McCutcheon & Bauman (1986), with the intra- and inter-assay CV's being 2.4 and 8.6% respectively.

5.3.7 Calculation of data and statistical analyses

VFI was calculated as:

$$VFI = \frac{F}{1 - D} \qquad (1)$$

Where D is *in vitro* OM digestibility (OMD) of the diet selected and F is faeces OM output, which was calculated as capsule chromium release rate (mg/day) divided by chromium concentration (mg/kg OM) in faeces. PEG was deducted in calculation of faeces OM output.

Analysis of variance by repeated measures was used to examine the effects of time and time x PEG interactions on milk yield, milk composition, and milk secretion rates of protein, lactose and fat. One way analysis of variance was used to assess the effect of PEG supplementation on LWG of both ewes and lambs, wool growth and VFI of the ewes and to examine the differences in chemical composition between feed on offer and diet selected. Milk yield, milk constituents and wool growth rate measured prior to the experiment were used as covariates.

5.4 RESULTS

5.4.1 Herbage

5.4.1.1 Forage mass

The lotus sward was in the vegetative growth stage throughout the experiment. Leaf mass was greatly reduced ($P < 0.001$) by grazing (Figure 5.1). There was no difference between pre- and post-grazing herbage for either stem mass or for dead matter, showing that the diet selected comprised green leaf only.

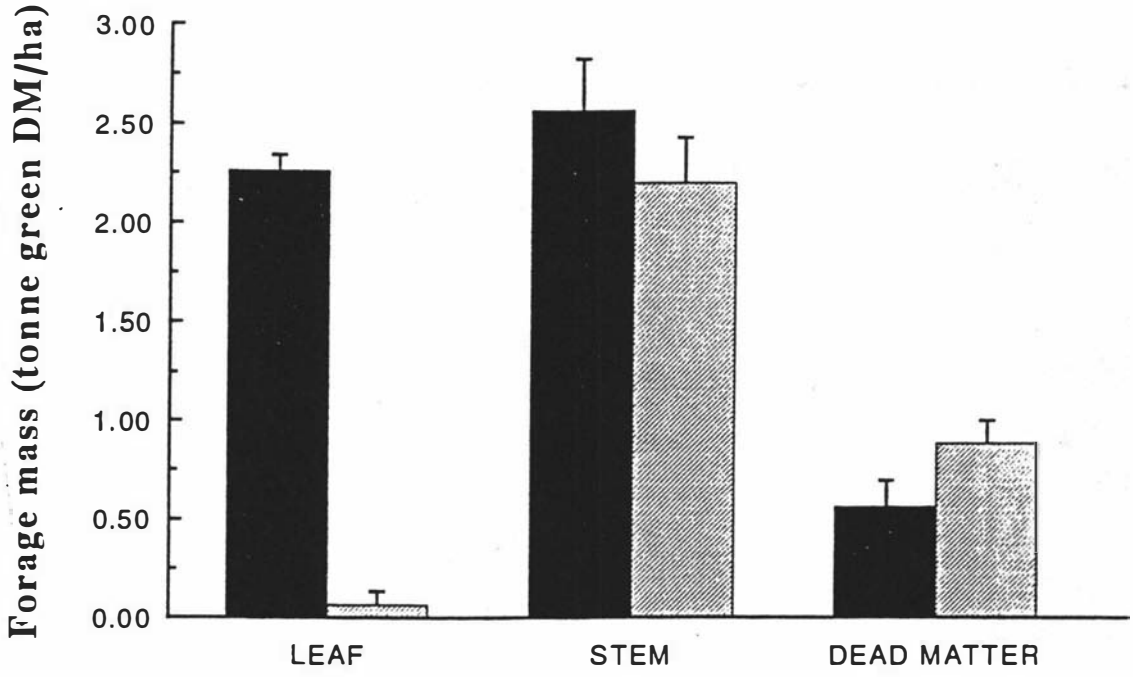

Figure 5.1. Forage mass (kg DM/ha) of *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ■ pre-grazing; ▨ post-grazing. I, S.E. Means are for 8 samples of both pre grazing and post grazing forage.

5.4.1.2 Chemical composition

Feed on offer had an *in vitro* OMD of approximately 68% (Table 5.1) and contained 39.5 g total CT/kg DM. Compared with feed on offer, diet selected had a higher *in vitro* OMD of 73% ($P < 0.05$), a higher ratio of readily fermentable:structural carbohydrate ($P < 0.01$) and contained 45 g CT/kg DM. Acid detergent fibre, Neutral detergent fibre, hemicellulose, cellulose and lignin concentrations were all lower in the diet selected than in the feed on offer ($P < 0.05$).

Table 5.1 Chemical composition (g/kg dry matter (DM)) and *in vitro* organic matter digestibility (% OMD) of feed offered and diet selected by sheep grazing *Lotus corniculatus* (birdsfoot trefoil; *cv.* Grasslands Goldie). Mean values with their standard error (S.E.).

	Number of samples	Feed offered	Diet selected	S.E.
Organic matter (OM)	8	904	907	4.3
Total N	8	32.4	35.5	1.80
Water soluble CHO (a)	4	89	95	6.0
Pectin (a)	4	41	40	1.1
Cellulose (b)	4	192	154	9.3
Hemicellulose (b)	4	83	68	3.2
Ratio (a/b)*	4	0.47	0.62	0.019
Lignin	4	83	65	4.0
Neutral detergent fibre	4	358	286	14.0
Acid detergent fibre	4	275	218	12.6
Extractable CT	4	24.7	29.6	1.14
Protein-bound CT	4	13.1	13.6	1.34
Fibre-bound CT	4	1.8	1.3	0.13
Total CT	4	39.5	44.5	1.94
In vitro OMD	8	67.8	73.3	1.19

*. Readily fermentable carbohydrate/structural carbohydrate.

D.F. for OM, total N and *in vitro* OMD are 14 and for others are 6.

5.4.2 Animal performance

5.4.2.1 Milk yield and milk composition

Control ewes produced more milk than PEG supplemented ewes. The PEG x time interaction (P=0.07) indicated that the milk production response to CT changed as the experiment progressed (Figure 5.2). There was no difference in milk yield between control and PEG supplemented ewes in weeks 4 and 5 of lactation. Thereafter, the control ewes produced more milk than PEG supplemented ewes (21%; $P < 0.05$).

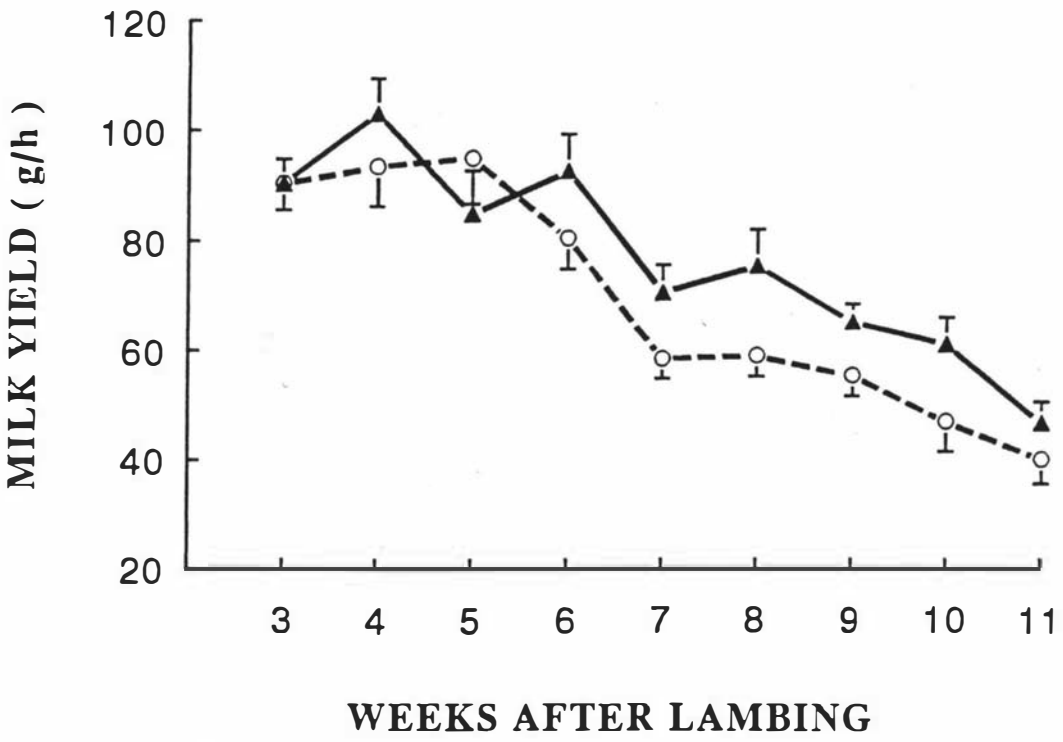

Figure 5.2 Milk yield (g/h) of twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ▲——▲ control ewes; o- - - - -o ewes given twice daily oral administration of polyethylene glycol (PEG; MW 3500); Means are for 14 ewes in each group. I, S.E.

The contents of milk protein, lactose and fat all significantly changed with time ($P < 0.001$; Figure 5.3). Control ewes had a higher lactose content and a lower fat content in their milk than PEG supplemented ewes, with the difference attaining significance for lactose in weeks 5 and 6 ($P < 0.1$) and for fat in week 9 ($P < 0.1$) and weeks 10 and 11 ($P < 0.05$). Milk protein percentage did not differ between control and PEG supplemented ewes.

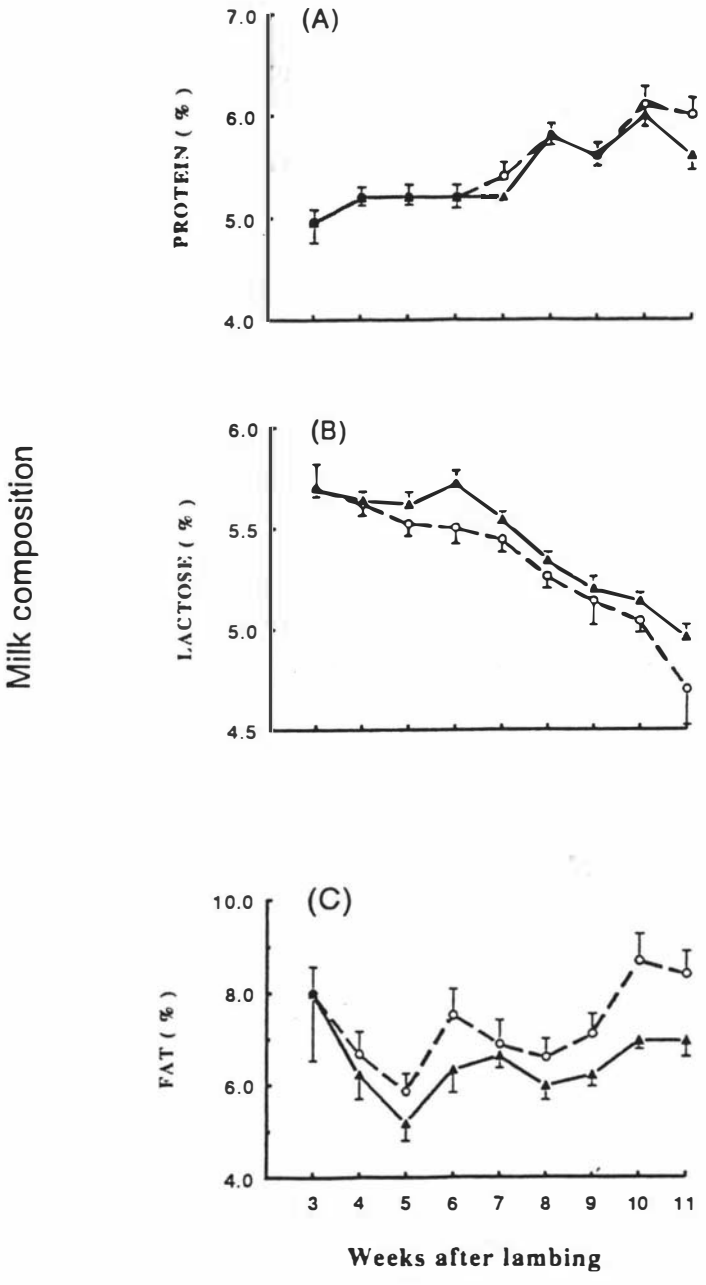

Figure 5.3 Concentrations (%) of (*a*) protein, (*b*)lactose and (*c*) fat in the milk of twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ▲——▲ control ewes; o- - - -o ewes given twice daily oral administration of polyethylene glycol (PEG; MW 3500). Means are for 14 ewes in each group. I, S.E.

The yields (g/h) of milk protein, lactose and fat all declined throughout the experiment (Figure 5.4). The significant PEG x time interactions for protein yield ($P=0.09$) and lactose yield ($P < 0.05$) indicated that yields of these constituents declined slower in control than in PEG ewes. Control ewes produced more milk protein (14%) and lactose (12%) than PEG supplemented ewes from weeks 6-11 of lactation, with the differences attaining significance in weeks 8-9 ($P < 0.1$) for milk protein and in weeks 5-11 ($P < 0.05$) for milk lactose. Control and PEG supplemented ewes produced similar amount of fat in the whole experimental period.

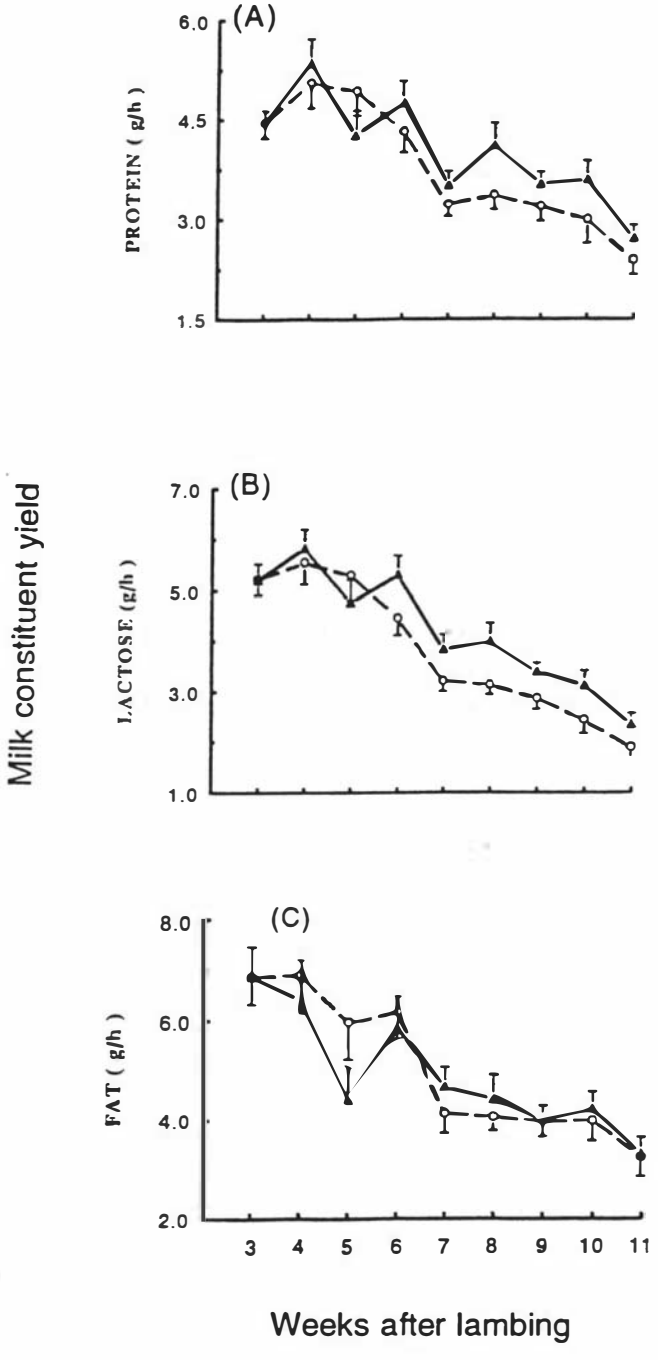

Figure 5.4 Yields (g/h) of (*a*) protein, (*b*) lactose and (*c*) fat in the milk of twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ▲——▲ control ewes; o- - - -o ewes given twice daily oral administration of polyethylene glycol (PEG; MW 3500). Means are for 14 ewes in each group. I, S.E.

5.4.2.2 Intake and liveweight gain

VFI of control ewes and of PEG supplemented ewes were not significantly different ($P > 0.05$; Table 5.2). Control ewes tended to have a higher LWG than PEG supplemented ewes ($p=0.12$). Lambs in both groups had a similar LWG. There were no differences in wool growth between the two groups of ewes in either the experimental or post experimental periods.

Table 5.2 Daily organic matter (OM) intake (kg/ewe.day), liveweight gain (LWG) of ewes and their twin lambs (g/day) and wool growth (mg/100 cm^2.day) of ewes grazing *Lotus corniculatus* (birdsfoot trefoil; *cv.* Grasslands Goldie), with and without twice daily oral administration of polyethylene glycol (PEG; MW 3500). (Mean values are for 14 ewes and 28 lambs in each group).

	Control ewes	PEG supplemented ewes	S.E.	D.F.
OM intake	2.18	2.00	0.128	26
LWG:				
Ewes	67	27	18.1	26
Lambs	231	236	6.4	54
Ewe wool growth:				
Experimental period	84	82	2.6	26
Post-experimental period	180	190	5.5	26

5.4.3 Rumen metabolites

Rumen ammonia concentration was significantly lower in control than in PEG supplemented ewes ($P < 0.001$; Table 5.3). Control and PEG supplemented ewes had similar concentrations of total VFA and similar molar proportions of acetic, propionic and *n*-butyric acids. However, control ewes had slightly lower molar proportions of *iso*-butyric and iso-valeric acids ($P < 0.1$) and significantly lower molar proportion of *n*-valeric acid ($P < 0.05$) than PEG supplemented ewes.

Table 5.3 Concentrations of ammonia (mg N/l) and total volatile fatty acids (VFA; *m*mol/l) and molar proportions (%) of individual VFA's in rumen fluid of ewes grazing *Lotus corniculatus* (birdsfoot trefoil; *cv*. Grasslands Goldie), with and without twice daily oral administration of polyethylene glycol (PEG; MW 3500). (Mean values are for 14 ewes in each group).

	Control ewes	PEG supplemented ewes	S.E.
Ammonia	237.7	483.4	47.44
Total VFA	109.0	117.7	5.90
Acetic acid	67.0	66.4	0.55
Propionic acid	21.3	21.6	0.40
n-butyric acid	8.5	9.1	0.50
iso-butyric acid	1.06	1.17	0.048
n-valeric acid	0.70	0.94	0.066
iso-valeric acid	0.72	0.88	0.067
Acetic acid:propionic acid	3.16	3.10	0.078
(Acetic+2*butyric acid):propionic acid	4.06	4.05	0.114

D.F. for all measurements are 26.

5.4.4 Plasma metabolites and hormones

Plasma concentrations of urea and glucose (Figure 5.5) were consistently lower for control than for PEG supplemented ewes, with the differences attaining significance in weeks 4-10 ($P < 0.05$) for urea and in weeks 8 ($P < 0.1$) and 10 ($P < 0.05$) for glucose. No difference was detected in plasma NEFA concentration between control and PEG supplemented ewes at any stage of the experiment. There were no differences in plasma concentration of GH, I and the GH/I ratio between control and PEG supplemented ewes at any stage of the experiment (Figure 5.6).

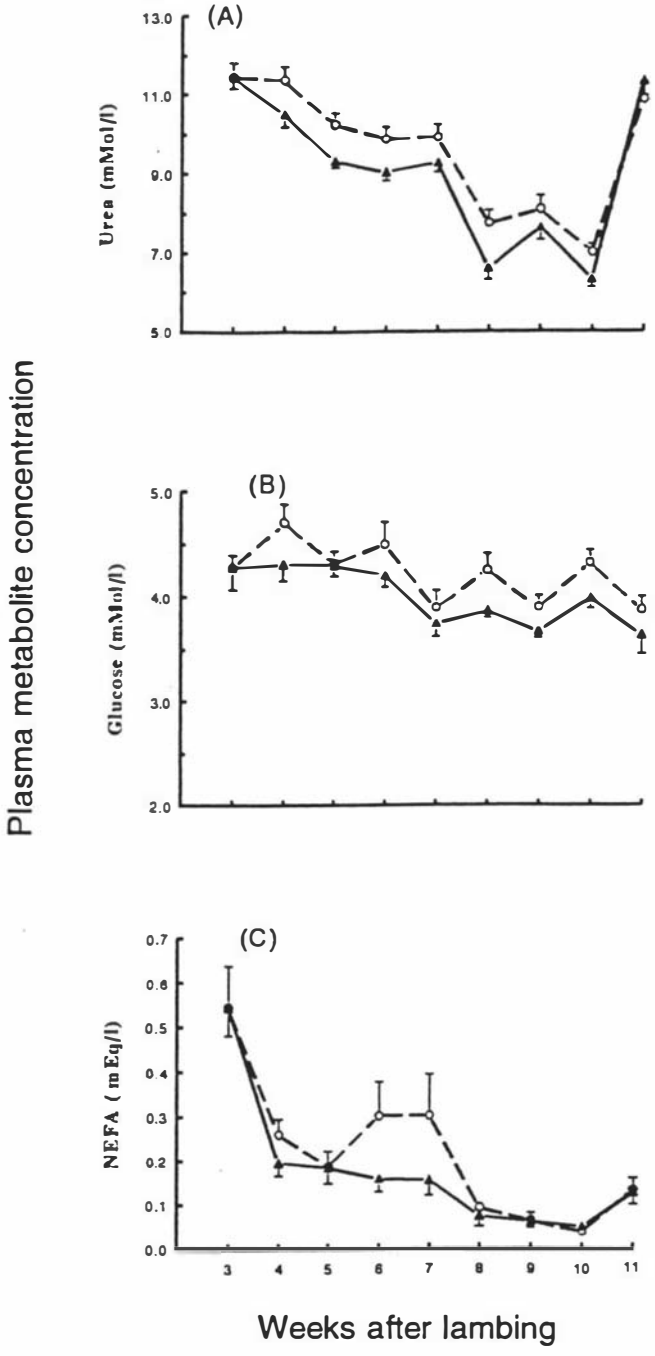

Figure 5.5 Plasma concentrations of (*a*) urea, (*b*) glucose and (*c*) non estified fatty acids (NEFA) in twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ▲——▲ control ewes; o- - - -o ewes given twice daily oral administration of polyethylene glycol (PEG; MW 3500). Means are for 14 ewes in each group. I, S.E.

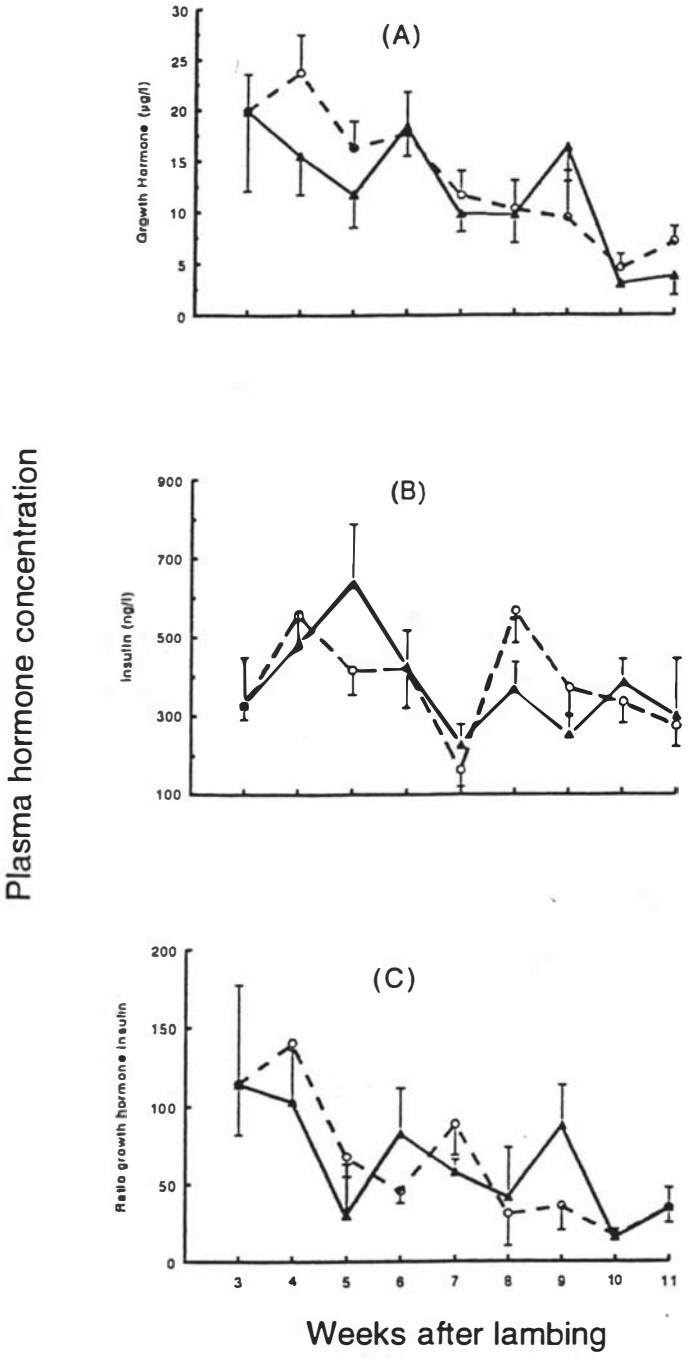

Figure 5.6 Plasma concentrations of (*a*) growth hormone, (*b*) insulin and (*c*) the ratio of growth hormone/insulin for twin-bearing lactating ewes grazing *Lotus corniculatus* (birdsfoot trefoil; cv. Grasslands Goldie). ▲——▲ control ewes; o- - - -o ewes given twice daily oral administration of polyethylene glycol (PEG; MW 3500). Means are for 14 ewes in each group. I, S.E.

5.5 DISCUSSION

The most significant finding in this experiment was the effect of CT in lotus in increasing milk production, increasing lactation persistency and changing milk composition in ewes. The reduced concentration of ammonia and molar proportions of *iso*-butyric and *iso*- and *n*-valeric acids in rumen fluid of control compared with PEG supplemented ewes, together with the lower plasma urea concentration in control ewes suggests that action of CT in lotus reduced protein degradation in the rumen, resulting in more AA (especially EAA) absorption from the small intestine, as shown in previous studies (Waghorn *et al.* 1987*b*; Wang *et al.* 1994). Methionine and lysine are thought to be the most limiting AA's for milk protein synthesis (Schwab & Satter 1974). Leucine, valine, arginine and ornithine have also all been shown to be limiting for milk production in some circumstances (Derrig *et al.* 1974; Spires 1974). The effect of CT in increasing milk protein yield in the present study is probably due to its effect in increasing EAA absorption from the small intestine.

The most interesting finding of this experiment is the action of CT in lotus in increasing milk lactose concentration and the rate of milk lactose secretion. This suggests that the action of CT in lotus markedly increased lactose synthesis in the mammary gland. Up to 71% of milk lactose in sheep is derived directly from blood glucose (Oddy *et al.* 1985), hence the increased milk lactose concentration and output in control ewes in the present study suggests increased uptake of glucose from blood. In ruminants, glucose supply is limited because of extensive carbohydrate fermentation in the rumen, and glucose is synthesised by gluconeogenesis from propionic acid and amino acids. In the present study propionic acid concentration and molar proportion in rumen fluid and VFI were similar between control and PEG supplemented ewes, as also found by Wang *et al.* (1994, 1995) with growing sheep fed lotus, suggesting that propionic acid absorption is likely to be similar between control and PEG supplemented sheep. Therefore, the higher glucose supply needed for lactose synthesis in control ewes was most likely derived by gluconeogenesis from the increased AA absorption induced by CT. Post-ruminal infusion of casein to both dairy cows and sheep has produced increased glucose synthesis from protein (Barry *et al.* 1982; Clark *et al.* 1977). However, even with increased supply of gluconeogenic precursors, the lower plasma glucose concentration of control than PEG supplemented ewes suggests that glucose supply may still not fully meet the

requirement for lactose synthesis in the mammary gland. Increased lactose secretion can be explained by the hypothesis that it is lactose that determines milk volume, that the milk protein response was driven by increased AA availability from the action of CT, and this was accompanied by increased lactose secretion in order to facilitate an increase in milk volume. Whilst diet did not change the circulating concentration of GH and I in this study it is possible that action of CT may nevertheless have changed paracrine factors within the mammary gland (such as an insulin like growth factor or hormone receptor) in such a way as to have increased lactose secretion.

Whilst the action of CT did not change the total amount of fat secreted per hour in the milk, it did substantially reduce milk fat concentration. The latter could be due to simple dilution, caused by action of CT in increasing the secretion rates of protein and lactose. The reduced fat concentration due to the CT could have considerable relevance for the dairy cow industry, where a major industry objective is to lower fat content and to increase protein content.

The restricted feed allowance strategy was successful in terms of response of the animal. The similar LWG and wool growth rate of both groups of ewes but greater milk yield in control ewes, suggests that the priority for nutrient utilization by these lactating ewes was milk production. Since control and PEG supplemented ewes had a similar VFI, the higher milk lactose and milk protein yield for control than for PEG supplemented ewes indicates that action of CT improved efficiency of feed utilization in lactating ewes, as also reported by Wang *et al.* (1995) for growing sheep.

Despite control ewes producing more milk and more milk protein and lactose than PEG supplemented ewes, lamb growth rates were similar for both groups. This is probably because the lambs had free access to the lotus forage and the increased nutrient supply from the milk may have been compensated by eating less forage.

It is well documented that administration of GH can increase milk and milk protein production (Eppard *et al.* 1985; Hart *et al.* 1985). However, increased GH secretion in response to increased protein supply is controversial, as some studies have shown increased plasma GH concentration associated with increased protein supply (Oldham *et al.* 1978, 1982), whilst other studies failed to show a GH response (Gow

et al. 1979; Peel *et al.* 1981). Evidence showed that the galactopoietic responses to GH were normally seen only when plasma GH concentration increased at least 10-20 ng/ml (Eppard *et al.* 1985). In the present study the similar plasma GH concentrations between control and PEG supplemented ewes suggests that the increased milk production due to action of CT was not mediated via effects on plasma GH concentration or by changes in the GH:I ratio. However, because this is the first report on the effect of forage CT upon milk production, and as very high levels of CT (95 g/kg DM) increased plasma GH concentration in sheep fed *L. pedunculatus* (Barry *et al.* 1986*b*), it seems that further study is required in this area.

In conclusion, the action of CT in lotus significantly increased the efficiency of milk production and increased the secretion rates of milk protein (14%) and lactose (12%) in mid and late lactation. The effects of CT upon plasma glucose irreversible loss rate (IRL) and metabolism need to be studied in lactating animals, and the effects of CT upon milk production from dairy cows should be established.

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Chapter 6

Effect of condensed tannins in *Lotus corniculatus* upon the digestion of methionine and cysteine in the small intestine of sheep

This Chapter has been submitted to *Journal of Agricultural Science, Cambridge.*

6.1 *ABSTRACT*

An experiment was conducted at Palmerston North, New Zealand, to determine the effect of condensed tannins (CT) on the true and apparent digestion of methionine and cysteine in the small intestine (SI) of sheep fed fresh *Lotus corniculatus*. The lotus contained about 20 g/kg dry matter (DM) of extractable CT and about 30 g/kg DM of total CT and was fed hourly to sheep in metabolism crates. Four sheep were prepared with rumen and abomasal cannulae which enabled the indigestible liquid phase marker, chromium ethylene diamine tetra acetic acid (Cr-EDTA), to be infused into the rumen to estimate digesta flow. True digestibility of plant methionine and cysteine in the SI and their site of absorption in the SI were determined from ^{35}S-labelled *L. corniculatus* homogenate continuously infused into the abomasum. After 9 h of infusion of the ^{35}S-labelled lotus homogenate the sheep were slaughtered and digesta samples taken at intervals along the small and large intestines. Effect of CT was determined by comparing 2 control sheep (CT acting) with 2 sheep given a continuous intra-ruminal infusion of polyethylene glycol (PEG, MW 3500) to bind and inactivate the CT.

The CT reduced the true digestibility of plant methionine (0.72 *v* 0.88) and cysteine (0.65 *v* 0.81) in the SI relative to sheep receiving PEG. CT also appeared to alter the site of digestion of both ^{35}S-methionine and ^{35}S-cysteine in the SI, and increased the flux of both amino acids in the mid and latter thirds of the SI. CT did not affect the apparent digestibility of methionine but reduced the apparent digestibility of cysteine from 0.77 to 0.66. In control sheep CT increased the abomasal flux (as a proportion of eaten) of total digesta methionine (0.88 *v* 0.76) and total digesta cysteine (0.74 *v* 0.62). The apparent absorption of total methionine was increased by the action of CT (0.72 *v* 0.63 g/g eaten) but was similar for total cysteine (0.49 *v* 0.48 g/g eaten) in both groups. It was concluded that CT reduced the true digestibility of plant methionine and cysteine in the SI. However, it was calculated that action of CT actually increased the total amounts (g/g eaten) of plant methionine and cysteine absorbed from the SI, due to its effect in increasing abomasal flux. The increased absorption of methionine (14%) will benefit livestock production.

6.2 INTRODUCTION

Condensed tannins (CT) are polyphenolic compounds that can react with plant proteins by hydrogen bonding in the near neutral pH range to form CT-protein complexes, which are stable and insoluble at pH 3.5-7.0, but dissociate and release protein at pH < 3.5 (Jones & Mangan 1977). Therefore the presence of CT in forage may provide a practical means of protecting dietary forage protein from degradation in the rumen and enable more plant protein to be absorbed from the SI.

When *Lotus corniculatus* was fed to sheep Waghorn *et al* (1987) found that action of CT (22 g extractable CT/kg dry matter (DM)) increased apparent absorption of essential amino acids (EAA) from the small intestine (SI) by 62% and decreased apparent absorption of non-essential amino acids (NEAA) by 10%. Sulphur-containing amino acids (SAA) were not determined in this study, but Wang *et al* (1994) using ^{35}S labelled SAA showed that the CT in *L. corniculatus* reduced the irreversible loss rate (IRL) of plasma inorganic sulphate, indicating reduced ruminal degradation of SAA, increased plasma cystine IRL and the transulphuration of methionine to the cystine and increased the flow of cystine to body synthetic reactions.

It has been suggested that the nutritional role of CT in ruminant diets depends on the concentration, structure and molecular weight of CT in plants (Barry 1989; Wang *et al* 1994). Studies using sheep grazing *L. corniculatus* showed that action of CT (35 g total CT/ kg DM) increased wool growth in growing lambs and milk production in lactating ewes without affecting voluntary feed intake (Wang *et al* 1995*a, b*). This increased efficiency of feed utilization and improved animal performance can be explained by an increased supply of EAA due to the action of CT.

The effects of CT upon protein digestion, plasma SAA kinetics and animal production in sheep fed *L. corniculatus* have been defined, but little is known about the effect of CT upon the true digestibility of SAA in the SI. McNabb *et al* (1993) reported that CT in *Lotus pedunculatus* fed to sheep (55 g extractable CT/kg DM) increased apparent absorption (g/d) of methionine from the SI by 27% but had no effect on apparent absorption of cystine. The CT did not affect apparent digestibility of methionine in the SI (0.78) but depressed that of cysteine (0.42 v.0.53). In view of the effect of CT in

L. corniculatus upon animal performance and the importance of SAA in wool and milk protein synthesis, it is necessary to know the extent to which the CT affects the digestion and absorption of SAA from the SI.

Objectives of the present experiment were to determine the effect of CT in *L. corniculatus* upon the true and apparent digestibility of methionine and cysteine in the SI of sheep and the sites of digestion along the SI.

6.3 MATERIAL AND METHODS

The experiment was conducted using four sheep fitted with rumen and abomasal cannulae and a single non-fistulated sheep, all of which were fed fresh *L. corniculatus* at hourly intervals. The work was carried out at AgResearch Grasslands, Palmerston North, New Zealand during early 1994. A plant homogenate containing ^{35}S-labelled protein was prepared from *L. corniculatus* which had been fertilized with ^{35}S-labelled inorganic sulphate, and this was infused into the abomasum of fistulated sheep which also received a continuous intra-ruminal infusion of the liquid phase marker chromium ethylene diamine tetra acetic acid (Cr-EDTA). The true digestibility and absorption sites of cysteine and methionine in *L. corniculatus* were then determined from samples of SI digesta taken at slaughter using the technique of Terrill *et al* (1994). Two of the four fistulated sheep were given an intra-ruminal infusion of polyethylene glycol (PEG, MW 3500) to prevent CT from binding with protein (Jones & Mangan 1977). The remaining two animals did not receive an intraruminal infusion of PEG and acted as control animals. Thus the effect of CT on the true digestibility and absorption sites can be determined by comparing control sheep (CT acting) with PEG infused sheep (CT inactivated).

6.3.1 Animals

The Romney wether sheep were aged about 18 months and 4 of the animals were fitted with rumen and abomasal cannulae about one month prior to the commencement of the 26 day experiment. The sheep were housed indoors in metabolism crates and were drenched with anthelmintic to eliminate internal parasites (12 ml; Ivomec, Merk Sharp and Dohme, NZ Ltd.) and treated for external parasites (10 ml; Wipeout, Coopers Animal Health, NZ Ltd.) prior to the experiment commencing. *L. corniculatus* was fed as a sole diet during the experimental period,

with 2 sheep receiving an intraruminal infusion of PEG (60 g/day per sheep in 260 ml water) from day 3 until slaughter. The liquid digesta marker Cr-EDTA (10.5 ml/h containing 470 µg Cr/ml) was infused into the rumen of the sheep from day 17 of the experiment until slaughter by overdose of sodium pentabarbital on days 24 and 26.

6.3.2 Feed

The *L. corniculatus* was vegetative throughout the experiment and was harvested daily at about 08.00 hours with a sickle bar mower. Feed was offered at about 1300 g DM/day in hourly increments from overhead feeders. Refusals were collected and daily DM intakes measured throughout the experimental period. Feed samples were collected and stored at -20 °C for analysis.

6.3.3 Determination of apparent digestibility

Apparent digestibility of dry matter (DM), organic matter(OM), total nitrogen (N) and fibre in the whole digestive tract was determined between days 15-22. Feed refusals and faeces were collected, weighed and subsampled each day and were bulked over the 7 day collection period (at -20 °C) for subsequent analysis. Feed DM determinations were made daily in triplicate (95 °C for 24 hours) and samples of feeds and refusals were retained for analysis. Faeces DM was determined by drying at 95 °C for 48 hours.

Abomasal digesta samples were taken from each sheep at 08.00, 12.00, 16.00, 20.00, 24.00 and 04.00 h over three days beginning day 20. The samples were bulked for determination of Cr concentration to measure flow of total DM, N, non-ammonia nitrogen (NAN), methionine and cysteine.

6.3.4 Preparation of ^{35}S labelled cysteine and methionine in *L. corniculatus*

Tips (30 mm long) of vegetative *L. corniculatus* (birdsfoot trefoil; cv Grassland Goldie) were cut and cultured on a pumice/composted bark media in a glasshouse for 20 days with nutrients supplied in solution (Middleton & Toxopeus 1973). Rooted tips were then transplanted into pure sand media (< 2 mm particle size) in 16 plastic pots (15 cm diameter and height) and allowed to grow for two and half weeks. After trimming to 5 cm height, 370 MBq of $H_2{}^{35}SO_4$ (925-1480 GBq/mg of sulphur; Amershan, England) in 200 ml water containing 0.01 M K_2SO_4 as carrier was evenly

distributed to all 16 pots about 4 weeks prior to cutting. The lotus was grown in an area isolated by clear polycarbonate sheeting, under a regime of 16 h light/8 h dark using sulphur-free nutrient solution until harvest. Sulphur-free nutrient solution was used to ensure the maximum incorporation of ^{35}S into plant proteins as ^{35}S-methionine and ^{35}S-cysteine.

On the morning of days 24 and 26, half of the ^{35}S-labelled plants (about 200 g) were cut above ground level, chopped and homogenized with 3 parts of artificial saliva (McDougall 1948) and filtered through 4 layers of cheese cloth. Unlabelled lotus (about 300 g) was prepared using the same procedure and the two solutions combined and held on ice for infusion into the abomasum. The infusate contained ^{35}S labelled protein (methionine and cysteine) as well as ^{35}S labelled inorganic sulphur. A portion of the infusate was retained for quantifying methionine and cysteine concentrations and determination of specific radioactivity (SA). The percentage of protein ^{35}SAA bound to CT was calculated from HPLC determinations of total ^{35}SAA in the homogenate less ^{35}SAA remaining in the supernatant after centrifugation (20000 g, 30 min).

6.3.5 Determination of digestibility and sites of absorption of methionine and cystine

On day 23 the non-fistulated sheep was slaughtered to establish procedures and to obtain digesta samples for background determination of radioactivity and chromium concentration in intestinal contents. On day 24 two sheep (one control and one PEG) were given a continuous abomasal infusion of plant homogenate (132 ml/h) containing ^{35}S-labelled protein, commencing 08.00 and 09.30 h. After 9 h of infusion they were slaughtered and samples were taken from the small and large intestines. This procedure was repeated with the other two sheep on day 26 of the experiment. The ^{35}S-labelled infusate was maintained on ice and stirred throughout infusion period. Previous work by Terrill *et al* (1994) suggested that 9 h infusion would enable ^{35}S-labelled methionine and cysteine to reach the rectum, and it was assumed that minimal recycling of absorbed ^{35}S into the digestive tract would have occurred in this time.

Digesta samples were obtained from sections of the gastro-intestinal tract (meters

from the pylorus) as follows: 0-1, 1-3, 3-6, 6-9, 9-12, 12-15, 15-18 and 18-21, as well as from the caecum, proximal and spiral colon (Terrill *et al* 1994). In addition, rumen contents were sampled and filtered through 2 layers of cheese cloth, centrifuged at 1000 g for 5 min and then 20000 g for 20 min to obtain bacterial pellets for determination of radioactivity in bacterial protein. All digesta samples were stored at -80 °C prior to determination of cysteine, methionine and Cr concentration.

6.3.6 Analytical

6.3.6.1 Feed, feed refusals and faeces

All samples of feeds, refusals and faeces were freeze dried, ground to pass a 1 mm sieve and were analysed for fibre fractions by sequential detergent extraction (Van Soest 1983), soluble sugar and pectin (Bailey 1967), total N by Kjeldahl digestion and ash by heating at 550 °C for 16 h. CT were determined as extractable, protein-bound and fibre-bound fractions using the modified butanol-HCl procedure (Terrill *et al* 1992).

6.3.6.2 Digesta

Freeze-dried digesta samples (25 mg) were analysed for methionine and cysteine concentration and radioactivity by high performance liquid chromatography (HPLC) with an ion-exchange column (Na^+-form AA analysis column; Waters Associates, USA). The procedure was similar to the method described by McNabb *et al* (1993), involving performic acid oxidation to convert cysteine to cysteic acid and methionine to methionine sulphone, followed by hydrolysis in 6 N HCl and precipitation of inorganic sulphate with $BaCl_2$. Duplicate extracts were analysed independently for quantification of cysteic acid and methionine sulphone and were combined to determine the radioactivity in 200 µl combined hydrolysates. After separation by HPLC, cysteic acid and methionine sulphone peaks were collected into scintillation vials as they flowed from the detector, using a 202 Fraction Collector (Gilson Medical Electronics, WI, USA) with a time programme mode. Radioactivity was determined according to the method described by Wang *et al* (1994), with background values for methionine and cysteine determined from digesta samples obtained from the non-fistulated sheep. Radioactivity of methionine and cysteine in rumen bacterial protein and radioactivity of taurine in SI digesta samples were also measured to determine the extent of ^{35}S recycling into the rumen and the SI. All HPLC and radioactivity

analyses for methionine and cysteine commenced immediately after the animal work concluded.

Methionine, cysteine, ^{35}S-labelled methionine and ^{35}S-labelled cysteine were added to digesta samples from the non-fistulated sheep to determine the recovery in each analytical method. Cr concentration in digesta was analysed by inductively coupled argon plasma spectrometry (ICAPS; Lee 1981).

6.3.7 Calculation of the data and statistical analysis

Both concentration and radioactivity of cysteine and cystine were measured as cysteic acid and expressed as cysteine. Background values were deducted when determining the SA for all metabolites. Amino acid concentrations and SA values were corrected for recoveries in each analytical method.

Total DM flows in the abomasum and sections of the SI were calculated by dividing Cr infusion rate (mg/day) by Cr concentration in digesta (µg/g DM). True digestibility (TD) of plant methionine and cysteine in the SI was calculated using equation 1, with duodenal samples being that from the first metre of the SI and ileal samples from the last three metres of the SI:

$$TD = \frac{\text{KBq/µg Cr (duodenum)} - \text{KBq/µg Cr (ileum)}}{\text{KBq/µg Cr (duodenum)}} \qquad (1)$$

Apparent absorption of total methionine and total cysteine was calculated from flows at the abomasum and ileum, and comprise amino acids from plant, microbial and endogenous sources.

Results are presented as mean values ± range, with the limits of the range above and below the mean indicating individual values for the two animals comprising each mean. DM and OM digestibilities were calculated on a PEG-free basis.

6.4 RESULTS

6.4.1 Composition of diet fed, intake and digestibility

The lotus contained 230 g DM/kg fresh feed over the experimental period. The composition (g/kg DM) was OM 903, total N 30.8, soluble sugar 72, pectin 34, neutral detergent fibre (NDF) 338, acid detergent fibre (ADF) 237, hemicellulose 101, cellulose 161, lignin 76, extractable CT 19.5, protein-bound CT 8.2 and fibre-bound CT 1.5.

Average daily DM intake from day 15-24 was 1071 and 1228 g for the control sheep and 1139 and 740 g for the PEG infused sheep. Apparent digestibility of N was 5% lower for control than for PEG infused sheep (Table 6.1), but apparent digestibility of all other nutrients was similar for the two groups.

Table 6.1 Apparent digestibility of *Lotus corniculatus* determined with sheep, with or without an intraruminal infusion of polyethylene glycol (PEG). Mean values with range are for two sheep in each group.

	Control sheep		**PEG infused sheep**	
Dry matter	0.691	±0.000	0.702	±0.018
Organic matter	0.707	±0.001	0.712	±0.016
Total N	0.712	±0.014	0.765	±0.022
Neutral detergent fibre	0.491	±0.012	0.517	±0.032
Acid detergent fibre	0.463	±0.006	0.514	±0.021
Hemicellulose	0.566	±0.012	0.586	±0.001
Cellulose	0.646	±0.023	0.663	±0.014
Lignin	0.076	±0.133	0.173	±0.001

6.4.2 Methionine and cysteine determination

The recovery by HPLC of both methionine and cysteine added to the digesta was 0.96 (SE 0.018; n=14) and 0.98 (SE 0.013; n=14) respectively, whilst the recoveries of ^{35}S-labelled methionine and cysteine added to the digesta was 0.96 (SE 0.024; n=12) and 0.98 (SE 0.011; n=12) respectively.

Methionine and cysteine concentrations in the homogenate infusate were 16.83 µmol/g DM and 17.78 µmol/g DM respectively and the ^{35}S radioactivity was 2.30 GBq/mol cysteine and 2.12 GBq/mol methionine at the time of infusion. Sixty nine percent of protein ^{35}S-cysteine and ^{35}S-methionine in the infusate was bound to CT and precipitated by centrifugation.

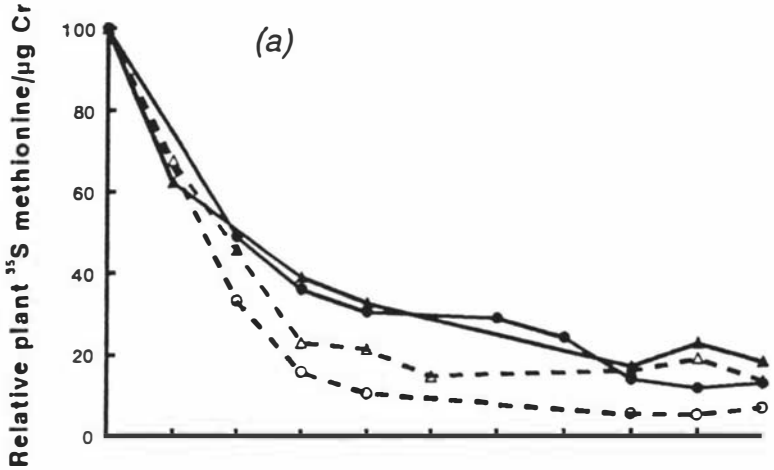
There was no radioactively labelled cysteine or methionine found in rumen bacterial protein, and no radioactivity was found in taurine isolated from digesta samples taken from the SI.

6.4.3 True digestibility of methionine and cysteine in the SI

The true digestibility of ^{35}S-methionine and ^{35}S-cysteine in the SI was about 16% units higher for PEG sheep than for control sheep (Table 6.2). When PEG was given, true digestion was primarily in the first 9 meters of the SI (Figure 6.1), whereas true digestion occurred in all segments of the SI of control sheep. ^{35}S-methionine and ^{35}S-cysteine flux in the mid and latter sections of the SI was higher for control than for PEG infused sheep, indicating a delayed digestion of ^{35}S-methionine and ^{35}S-cysteine in the SI in the presence of CT.

Table 6.2 True digestibility in the small intestine (SI) of ^{35}S-methionine and ^{35}S-cysteine (g/g entering SI) contained in the protein of *Lotus corniculatus* fed to sheep, with or without an intraruminal infusion of polyethylene glycol (PEG). Mean values with range are for two sheep in each group.

	Control sheep		PEG infused sheep	
Methionine	0.72	±0.04	0.88	±0.03
Cysteine	0.65	±0.02	0.81	±0.03


(a)
Relative plant ^{35}S methionine/µg Cr
100
80
60
40
20
0

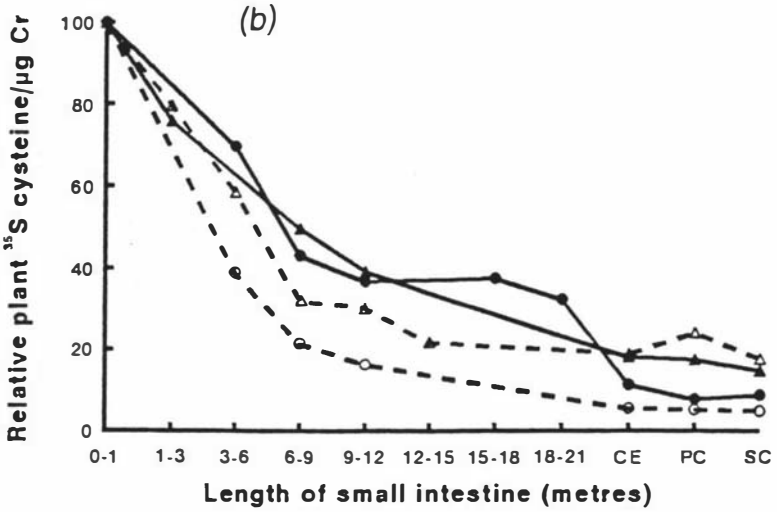

(b)
Relative plant ^{35}S cysteine/µg Cr
100
80
60
40
20
0
0-1
1-3
3-6
6-9
9-12
12-15
15-18
18-21
CE
PC
SC
Length of small intestine (metres)

Figure 6.1 The relative flow of (a) ^{35}S-labelled methionine/μg chromium (Cr) and (b) ^{35}S-labelled cysteine/μg Cr in segments of the small intestine (SI) and large intestine of sheep fed fresh *Lotus corniculatus* at hourly intervals, with (- - -) or without (————) an intraruminal infusion of polyethylene glycol (PEG; MW 3500). Each line represents data for one sheep. The last point for the SI represents the terminal ileum, with length of the SI differing between sheep. All sheep were continuously infused into the rumen with chromium ethylene diamine tetra acetic acid (Cr-EDTA) and were infused into the abomasum for 9 h before slaughter with a plant homogenate from *L. corniculatus* fertilized with ^{35}S inorganic sulphate. All values are expressed relative to the first metre of the SI as 100. CE, caecum; PC, proximal colon; SC, spiral colon.

6.4.4 Abomasal flow and apparent digestibility

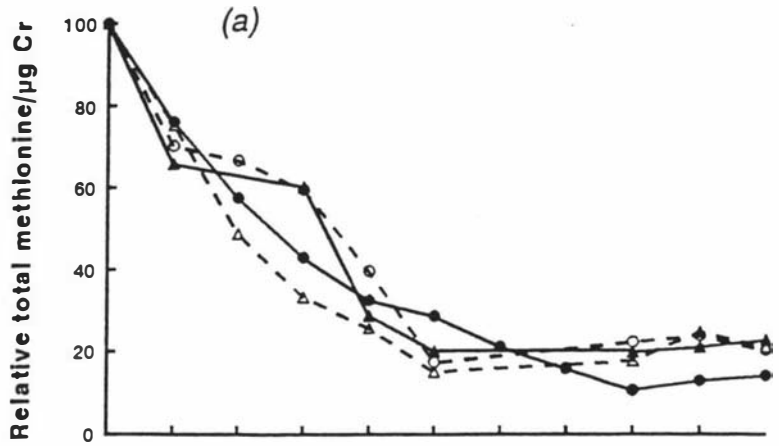
Abomasal flux of total N, non-ammonia nitrogen (NAN), methionine and cysteine per unit eaten was greater for control than for PEG sheep (Table 6.3). Although there were losses of all these constituents across the rumen for both control and PEG infused sheep, the action of CT reduced the extent of loss from the rumen.

Table 6.3 Intake, abomasal flux and digestibility of total nitrogen (N), non-ammonia nitrogen (NAN), methionine and cysteine in sheep fed *Lotus corniculatus*, with or without an intraruminal infusion of polyethylene glycol (PEG). Mean values with range are for two sheep in each group.

	Control sheep		PEG infused sheep	
Nitrogen				
Intake (g/d)	38.4	±2.2	31.6	±6.8
Abomasal total N flux				
g/d	23.6	±1.6	18.0	±3.7
g/g N eaten	0.62	±0.01	0.57	±0.01
Abomasal NAN flux				
g/d	22.4	±1.5	17.2	±3.7
g/g N eaten	0.59	±0.01	0.54	±0.00
Methionine				
Intake (g/d)	2.53	±0.16	2.15	±0.50
Abomasal flux				
g/d	2.23	±0.22	1.62	±0.39
g/g eaten	0.88	±0.04	0.76	±0.02
Apparent digestibility in small intestine (SI)				
g/g entering SI	0.82	±0.02	0.84	±0.01
g/g eaten	0.72	±0.05	0.63	±0.01
Cysteine				
Intake (g/d)	2.71	±0.19	2.37	±0.51
Abomasal flux				
g/d	2.01	±0.10	1.51	±0.46
g/g eaten	0.74	±0.01	0.62	±0.06
Apparent digestion in SI				
g/g entering SI	0.66	±0.02	0.77	±0.01
g/g eaten	0.49	±0.01	0.48	±0.05

Control sheep had a similar apparent digestibility of total (plant, microbial and endogenous) digesta methionine in the SI to that of PEG sheep but the apparent digestibility of total digesta cysteine in the SI was lower than that of PEG sheep (Table 6.3). However, when expressed as g/g eaten, control sheep digested 14% more total methionine in the SI than PEG sheep, with a similar amount of total cysteine digested for both groups. Apparent digestibility in the entire SI was higher for total methionine (0.83) than for total cysteine (0.71).

In both groups the apparent absorption of methionine occurred mainly in the first half of the SI, whereas the apparent absorption of cysteine tended to occur throughout the SI. This was especially true in control sheep where the apparent absorption of cysteine from the SI was lower (0.66) than for PEG sheep (0.77) and a substantial proportion of the cysteine disappeared from the distal portion of the SI (Figure 6.2).


(a)
Relative total methionine/µg Cr
100
80
60
40
20
0

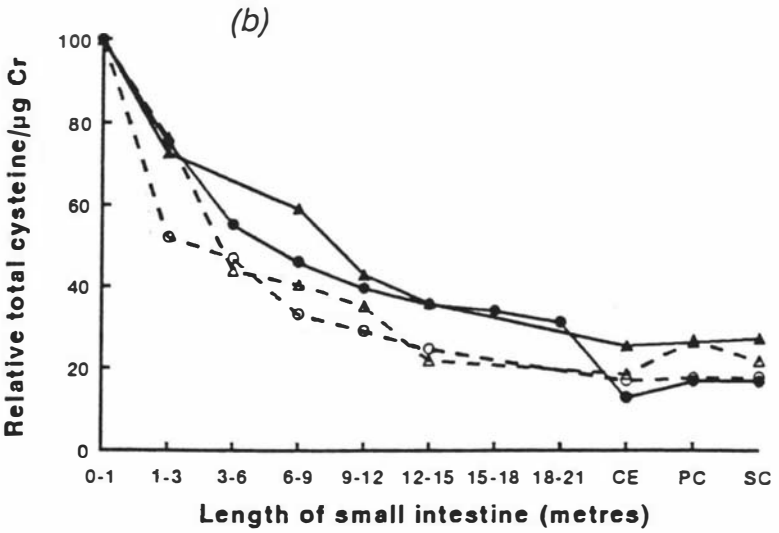

(b)
Relative total cysteine/µg Cr
100
80
60
40
20
0
0-1
1-3
3-6
6-9
9-12
12-15
15-18
18-21
CE
PC
SC
Length of small intestine (metres)

Figure 6.2 The relative flow of (a) total methionine/μg chromium (Cr) and (b) total cysteine/μg Cr in segments of the small intestine (SI) and large intestine of sheep fed fresh *Lotus corniculatus* at hourly intervals, with (- - - - -) or without (————————) an intraruminal infusion of polyethylene glycol (PEG; MW 3500). Each line represents data for one sheep. The last point for the SI represents the terminal ileum, with length of the SI differing between sheep. All sheep were continuously infused into the rumen with chromium ethylene diamine tetra acetic acid (Cr-EDTA) and were infused into the abomasum for 9 h before slaughter with a plant homogenate from *L. corniculatus* fertilized with ^{35}S inorganic sulphate. All values are expressed relative to the first metre of the SI as 100. CE, caecum; PC, proximal colon; SC, spiral colon.

6.4.5 pH values of digesta in the SI

Digesta pH increased more slowly in control sheep than sheep given PEG as it passed down the SI (Figure 6.3). The pH of the digesta for both control and PEG infused sheep attained a maximum value of approximate 8.5 towards the terminal ileum, but this was attained further down the SI for control sheep than for PEG infused sheep.

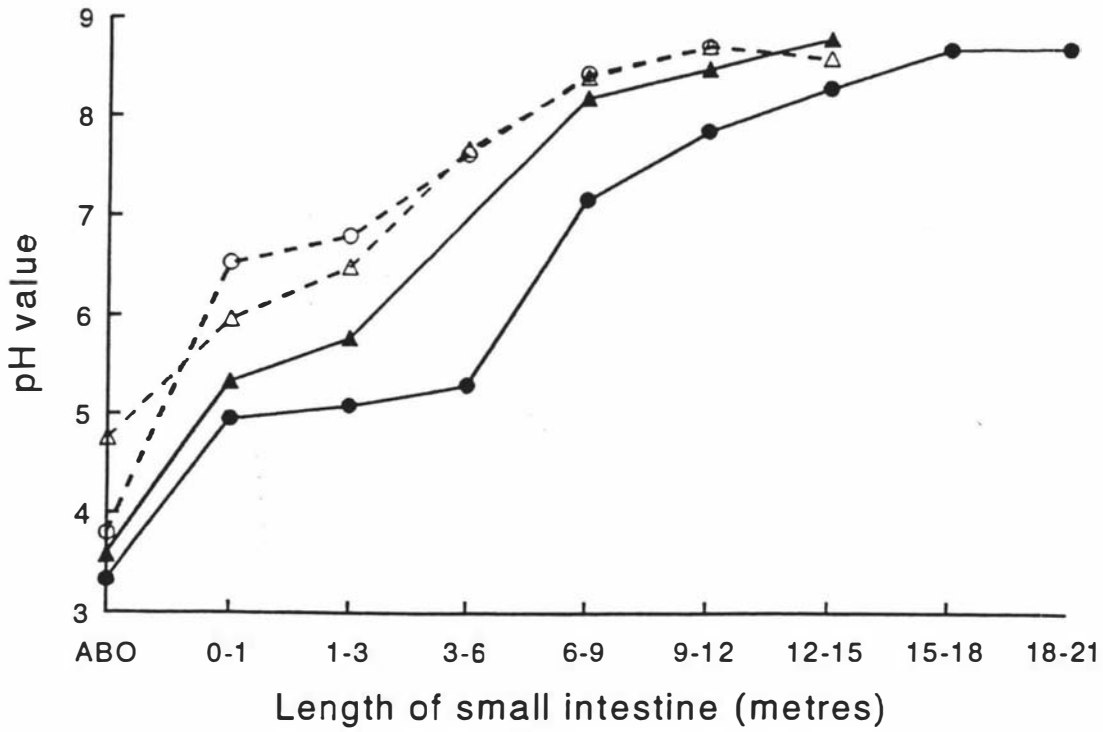


Figure 6.3 pH values in segments of the SI of sheep fed *Lotus corniculatus* at hourly intervals, with (- - - - - -) or without (————————) an intraruminal infusion of polyethylene glycol (PEG; MW 3500). ABO, abomasum.

6.5 DISCUSSION

This experiment successfully determined the true digestibility of plant methionine and cysteine in the SI. The main finding was that the action of CT in *L. corniculatus* depressed the true digestibility of plant methionine and cysteine in the SI and altered the site of digestion, reducing the proportion digested in the proximal part of the SI and increased the proportion digested in the last third of the SI. This suggested that the action of CT slowed the rate of digestion of these amino acids in the SI.

The pH of intestinal contents may have affected the change in site and extent to which methionine and cysteine were digested in the presence of CT and PEG. pH can affect the release of protein from CT:protein complexes and may affect activity of intestinal protease enzymes. Jones & Mangan (1977) found that CT:protein complexes from sainfoin (*Onobrychis viciifolia)* are stable in the pH range 3.5-7.0, but dissociate and release protein at pH values < 3.5 and > 8.0. On this basis, the CT:protein complex in control sheep (CT acting) would be expected to dissociate in the abomasum, but may have re-formed in the proximal part of the SI, where pH values were 1.0-2.0 units lower for control sheep than for PEG sheep (Figure 6.3). In the latter part of the SI the pH increased to over 8.0 units and some CT-protein complexes may again dissociate, allowing protein to be digested. These pH effects could have delayed the release of protein from the CT:protein complex during its passage through the first two thirds of the SI, and slowed the digestion of ^{35}S labelled methionine and cysteine. The results suggested that some of the CT-protein complexes had not dissociated by the terminal ileum. However, fluxes of ^{35}S-methionine and ^{35}S-cysteine in the large intestine were similar for both control and PEG sheep suggesting a further dissociation of the CT-protein complex in control sheep occurred in the large intestine, probably as the result of microbial fermentation.

A pH range of 7 to 8 was reported optimal for most intestinal protease enzymes (Ben-Ghedalia *et al* 1974). The lower pH values in the proximal part of the SI of control sheep than PEG sheep may have also reduced the digestion of methionine and cysteine due to effects upon enzyme activity. CT have been shown to inhibit endogenous enzyme activity (Horigome *et al* 1988; Ahmed *et al* 1991; Longstaff *et al* 1991*a, b*; Yuste *et al* 1992). Depressed enzyme activity *in vivo* may be due to a direct effect of CT binding to digestive enzymes or due to an indirect effect of CT

changing the reaction environment (e.g. intestinal pH).

Action of CT did not effect the apparent digestibility of total digesta methionine but depressed the apparent digestibility of total digesta cysteine entering the SI and increased the abomasal flux of both amino acids. There was a substantial increase in the amount of total methionine but not total cysteine apparently absorbed from the SI. This suggests that the effect of CT on the digestion of total methionine and total cysteine occurred mainly through reduced rumen degradation of both amino acids and reduction of apparent digestibility of cysteine in the SI. Similar results were reported by McNabb *et al* (1993) with CT from *L. pedunculatus* (extractable CT 55 g/kg DM; Table 6.4). Apparent absorption of methionine was similar within both lotus spp but the apparent digestibility and apparent absorption of cysteine in the present study were 24% units and 27% units higher than that of McNabb *et al* (1993). Differences may be due to the effects of concentration, molecular weight and molecular structure of the CT in the two lotus species (Foo *et al* 1982). McNabb *et al* (1993) reported that the increased methionine absorbed from the SI induced by action of CT was converted to cystine. The increased amount of methionine absorbed from the SI in this study, together with the increased IRL of plasma cystine and the increased cystine flux to body synthetic reactions in sheep fed *L. corniculatus* (Wang *et al* 1994) may explain the increased wool growth and milk production in sheep fed *L. corniculatus* attributable to CT (Wang *et al* 1995*a, b*).

Table 6.4 Comparison of the apparent digestibility of total methionine and total cysteine in the small intestine of sheep fed *Lotus corniculatus* and *Lotus pedunculatus*, with or without a continuous intraruminal infusion of polyethylene glycol (PEG).

Author	Diet		Apparent digestion coefficient		Apparent absorption (g/g eaten)	
			Methionine	Cysteine	Methionine	Cysteine
This paper	*L. corniculatus* (20 g extractable CT/kg DM)	Control	0.82	0.66	0.72	0.49
		PEG	0.84	0.77	0.63	0.48
McNabb *et al* (1993)	*L. pedunculatus* (55 g extractable CT/kg DM)	Control	0.77	0.42	0.75	0.42
		PEG	0.79	0.53	0.56	0.38

As ^{35}S was not detected in cysteine or methionine in rumen bacterial protein, or in taurine in the SI, there was no recycling of absorbed ^{35}S into the digestive tract. Thus, disappearance of ^{35}S labelled cysteine and methionine from the SI represented true absorption of cysteine and methionine from the protein in *L. corniculatus*.

The true digestibility of plant methionine and cysteine and the apparent digestibility of total digesta methionine and cysteine in the SI enable the absorption of plant methionine and cysteine from the SI (g/g eaten) to be calculated and an estimate be made of endogenous losses of methionine and cysteine at the terminal ileum, due to the effects of CT (Table 6.5). These values were calculated by first partitioning abomasal NAN flow measured in the present study into microbial, endogenous and undegraded plant components. The microbial component was estimated by applying the ratios of microbial N/total abomasal NAN in the abomasal digesta reported by Waghorn *et al* (1994) for *L. pedunculatus*, with the assumption that they would be similar for sheep fed *L. corniculatus*. An endogenous secretion of 6 g N/day anterior to the SI was assumed (Nolan & MacRae 1976), with the balance of the abomasal NAN flux being undegraded dietary N. Quantities of microbial methionine and cysteine digested in the SI were calculated by multiplying the estimated flow of each amino acid of microbial origin at the abomasum (multiplying microbial protein flow by the proportion of each amino acid in microbial protein) by the true digestibility of microbial methionine (0.74) and cysteine (0.72) from Bird (1972) and Elliott & Little (1977). A similar calculation was conducted to estimate the plant methionine and cysteine digested in the SI, using estimated abomasal plant amino acid flow and the true digestibility determined in this study. Methionine and cysteine flowing at the terminal ileum that was of microbial origin (A) or plant origin (B) was calculated according to Equations 2 and 3. Thus net endogenous loss of methionine and cysteine at the terminal ileum could be calculated according to Equation 4, and represents endogenous secretions that were not re-absorbed in the SI.

A = Abomasal flow of microbial methionine or cysteine x (1-TD) **(2)**

B = Abomasal flow of plant methionine or cysteine x (1-TD) **(3)**

Endogenous flow = Total ileal flow - (A+B) **(4)**

These calculations were carried out to indicate the effect of CT on digestive function.

Although action of CT reduced the true digestibility of plant methionine and cysteine in the SI, it actually increased the total amounts (g/g eaten) of plant methionine and cysteine absorbed from the SI (Table 6.5). These values were similar to those directly measured by McNabb *et al* (1995) for ribulose-1,5-*bis*phosphate carboxylase (Rubisco) protein in sheep fed *L. pedunculatus* (0.27 and 0.04 g digested in the SI per g eaten for control and PEG sheep). Digestibility (ie degradation) in the rumen calculated in this study for control sheep (0.75 methionine; 0.79 cysteine) and PEG sheep (0.88 methionine; 0.90 cysteine) were similar to that of Rubisco protein (0.72 control; 0.96 PEG sheep) measured by McNabb *et al* (1995). Therefore, the increased absorption of plant methionine and cysteine from the SI caused by action of CT was probably due to a reduction in rumen degradation and increased flow of plant protein at the abomasum.

Table 6.5 Calculated absorption of plant methionine and cysteine from the small intestine (SI) and calculated endogenous loss of methionine and cysteine at the terminal ileum of sheep fed *Lotus corniculatus*, with or without an intraruminal infusion of polyethylene glycol (PEG).

	Control sheep	PEG infused sheep
Absorption from SI (plant origin; g/g eaten)		
Methionine	0.18	0.11
Cysteine	0.14	0.08
Endogenous loss (mg/g entering SI)		
Methionine	142	83
Cysteine	358	276

Calculated endogenous loss of cysteine was greater than for methionine at the terminal ileum and this could be one reason that apparent absorption of cysteine was lower than methionine from the SI. Higher amount of endogenous cysteine loss than

methionine may be explained by the high content of cysteine and low content of methionine in mucin secreted into the SI (Forstner & Forstner 1986) in conjunction with the lower re-absorption of endogenous cysteine than that of methionine. It seems that CT increased endogenous flow of both methionine and cysteine at the terminal ileum. Further studies are needed to measure directly the effects of CT upon endogenous secretion.

In conclusion, the action of CT in *L. corniculatus* reduced the true digestibility of both plant methionine and cysteine in the SI and altered their sites of digestion. The CT increased the amount of total digesta methionine absorbed from the SI by 14%, due to reduced rumen degradation and increased abomasal flow. However, CT did not increase the amount of total digesta cysteine absorbed from the SI, because the increased abomasal flow was counteracted by reduced true digestibility in the SI and probably increased endogenous loss.

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Chapter 7

General Discussion

7.1 INTRODUCTION

Condensed tannins (CT) are plant secondary compounds that occur in the leaves and stems of specialized plants, such as *Lotus pedunculatus* (big trefoil), *Lotus corniculatus* (birdsfoot trefoil), *Onobrychis viciifolia* (sainfoin), *Hedysarum coronarium* (sulla) (Jones *et al* 1976). CT have been shown to combine with protein, carbohydrate and other compounds to form complexes. When CT (from sainfoin) reacted with fraction I leaf protein by hydrogen bonding and by hydrophobic reaction, the CT-protein complexes were stable and insoluble at pH 3.5-7.0, but dissociated and released protein at pH < 3.5 and at pH > 8.0 (Jones & Mangan 1977). Nutritional effects of forage CT in ruminant animals probably depends upon its concentration, molecular weight and structure. High concentrations of CT have been shown to depress voluntary feed intake, organic matter digestibility, rumen fibre digestion and animal production (Barry & Duncan 1984; Barry & Manley 1984; Reed *et al* 1982; Pritchard *et al* 1992). However, low CT concentrations (20-40 g/kg DM) have been suggested to have beneficial effects (Barry 1989; Waghorn *et al* 1987*a*). Although several studies have indicated that forages containing low levels of CT (such as *L. corniculatus* and sainfoin) have higher feeding value (FV) and nutritive value (NV) than comparable non CT-containing forages, the forages also differ in other aspects as well as CT concentration, and the effects of low levels of CT *per se* upon nutrient metabolism and animal performance have not been specifically examined. This thesis specifically studied the effect of low concentrations of CT (25-30 g extractable CT (ECT)/kg DM) in *L. corniculatus* upon nutrient metabolism and upon animal performance in grazing sheep.

7.2 THE EFFECT OF CT IN *L. CORNICULATUS* ON NUTRIENT DIGESTION AND METABOLISM

It has been shown in Chapters 3, 4 & 5 that CT in *L. corniculatus* greatly reduced rumen ammonia concentration, reduced rumen degradation of some essential amino acids (EAA), such as sulphur amino acids (SAA), valine, leucine, arginine and lysine, and increased non-ammonia-nitrogen (NAN) outflow from the rumen. These all indicated that the action of CT in *L. corniculatus* reduced rumen degradation of protein.

A comparison of rumen ammonia concentrations in both control (CT acting) and polyethylene glycol (PEG) supplemented (CT inactivated) groups of sheep fed on forages containing a range of ECT is shown in Figure 7.1. Whilst the action of high CT concentrations in *L. pedunculatus* and low CT concentrations in *L. corniculatus* both reduced rumen ammonia concentration, as would be expected, it seems that presence of trace amounts of CT (such as in the grass Yorkshire fog) also reduced rumen ammonia formation. This indicates that a very wide range of CT concentration is effective in reducing forage protein degradation in the rumen of sheep. This relationship can be quantified by plotting NAN flux from the rumen per unit of N eaten (NAN) as a function of dietary ECT concentration (Figure 7.2*a*). The relationship obtained for control sheep (Equation 1) showed that in the absence of CT, abomasal NAN flux was only approximately 0.60 of N intake in ruminants fed fresh forages, which is similar to the conclusion of MacRae & Ulyatt (1974), and that this is linearly increased with increasing CT concentration.

$$NAN=0.596+0.00378\ ECT \quad r=0.87 \qquad (1)$$

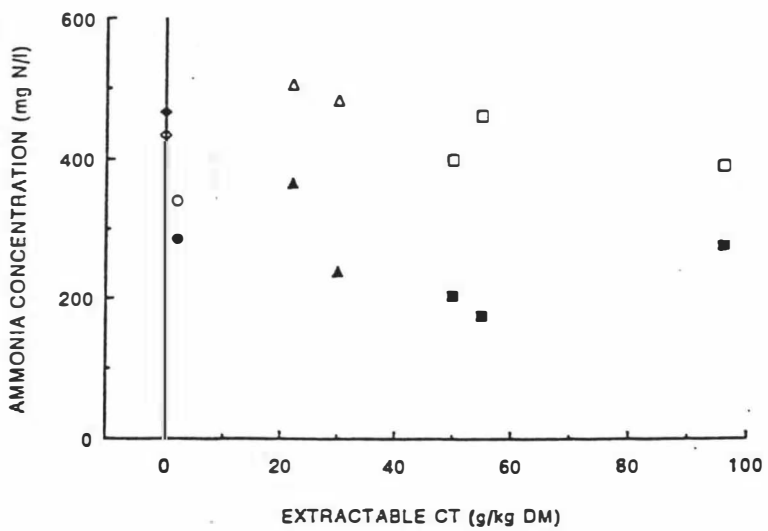

Figure 7.1 The effect of polyethylene glycol (PEG) supplementation on rumen ammonia concentration (mg NH_3 N/l) in sheep fed a range of fresh forages. Solid symbols represent control (i.e. CT acting) groups and open symbols represents PEG supplemented (i.e. CT inactivated) groups. ♦ Lucerne, Chapter 4; ● Yorkshire fog, Liu personal communication; ▲ *L. corniculatus*, Waghorn *et al* 1987a, Chapter 3; ■ *L. pedunculatus*, Barry *et al* 1986, McNabb *et al* 1993, Waghorn *et al* 1994a.

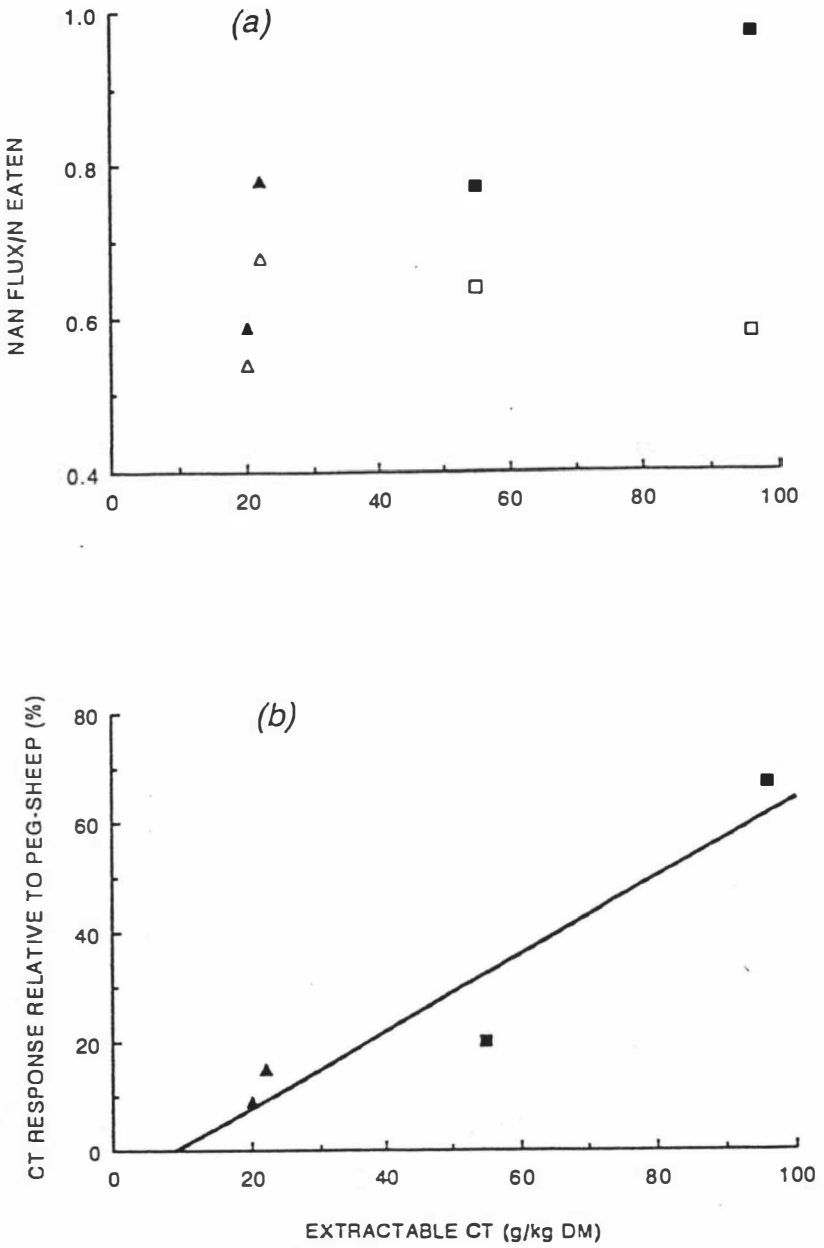

Figure 7.2 The effect of polyethylene glycol (PEG) supplementation upon abomasal non-ammonia nitrogen (NAN) flux in sheep fed lotus species containing different concentrations of extractable condensed tannins (ECT, g/kg DM). (*a*) NAN flux per unit of N eaten and (*b*) response to CT, calculated as NAN flux per unit of N eaten of control sheep (CT acting) relative to PEG sheep (CT inactivated). Solid symbols represent CT acting groups and open symbols represent CT inactivated groups. ▲ *L. corniculatus,* Chapter 6, Waghorn *et al* 1987a; ■ *L. pedunculatus*, Barry *et al* 1986; Waghorn *et al* 1994*a*.

The increment of NAN flux into the SI (INAN; %) due to the action of CT is also linearly related to the CT concentration (g ECT/kg DM; Figure 7.2b). The response (i.e. increment) was calculated as abomasal NAN flux of control sheep minus abomasal NAN flux of PEG sheep, expressed as a proportion of abomasal NAN flux of PEG sheep. Equation 2 shows that NAN flux into the SI per unit N eaten increases only if the concentration of CT is above 9 g ECT/kg DM. Therefore, concentrations of ECT lower than 9 g/kg DM are unlikely to increase animal production. This indicates that a minium level of CT (about 10 g ECT/kg DM) is needed to increase abomasal NAN flux and that above this level the response is linear for lotus CT. It has been shown that CT from several different plant sources were equally effective at reducing rumen degradation of forage proteins (Tanner *et al* 1994). From Figure 7.2 it also seems that CT from *L. corniculatus* and *L. pedunculatus* are equally effective per unit concentration in reducing rumen protein degradation.

$$INAN=-6.24+0.705\ ECT \qquad r=0.95 \qquad (2)$$

As experiment showed that CT reduced proportion of microbial NAN in the total NAN out of the rumen (McNabb *et al* 1993), the increased NAN flux because of the action of CT is due to CT increasing plant NAN flux (i.e. reducing plant protein degradation). The reduced protein degradation in the rumen due to the action of CT can be explained by the formation of CT-protein complexes, which are formed during chewing and not dissociated at the rumen pH (Jones & Mangan 1977), hence preventing or reducing the rate of protein degradation by rumen microorganisms. Whether action of CT in lotus had any effects on rumen bacteria or on extracelluar protease enzyme activity which is responsible for the breakdown of protein was not examined in the present studies. The concentration of CT used in the present study was 20-30 g ECT/kg DM, of which the calculated free CT (i.e. 10% of ECT; Barry & Manley 1986) in the rumen of sheep is about 130-200 μg/ml, which would inhibit proteolytic activity of some strains of bacteria (Jones *et al* 1993). Therefore, the reduced protein degradation in the rumen can probably be further explained by reduced proteolytic activity of some rumen microbes due to action of CT. The reason why CT specifically reduce EAA degradation in the rumen is still not known. It may be due to the reactivity between CT and EAA being stronger than between CT and non-EAA (NEAA), or CT selectively reducing the activity of microbes which

specifically hydrolyse EAA. These need to be further studied.

Unlike the effects upon rumen protein degradation, there was no clear relationship between the apparent post-ruminal digestibility of N and ECT concentration (Figure 7.3). However, there do appear to be differences between sources of CT upon digestion of some amino acids (AA) in the small intestine (SI), and this is shown in Table 7.1 for the effect of CT from *L. corniculatus* and *L. pedunculatus* upon digestion of EAA in sheep. Whilst both CT increased abomasal flux, the CT in *L. pedunculatus* reduced the apparent digestibility of EAA (excluding methionine and cysteine) in the SI, but the CT in *L. corniculatus* had no effect on this aspect (Table 7.1; Waghorn *et al* 1987*a*, 1994*a*). Hence, action of CT increased net absorption of EAA from the SI in sheep fed *L. corniculatus*, whereas with *L. pedunculatus* there was no change. This shows that the effect of CT on post-ruminal digestion is more complicated than on ruminal digestion. The effect of CT on post-ruminal N digestion may be a product of both concentration and reactivity. Apparent absorption of AA from the SI is governed by true digestibility and endogenous losses, and the true digestibility will be affected by the release of AA from CT:protein complex. The reduced EAA apparent digestibility in the SI of sheep fed *L. pedunculatus* may be due to both slow release of EAA from CT:protein complex and to increasing endogenous protein losses. Research in this area is needed.

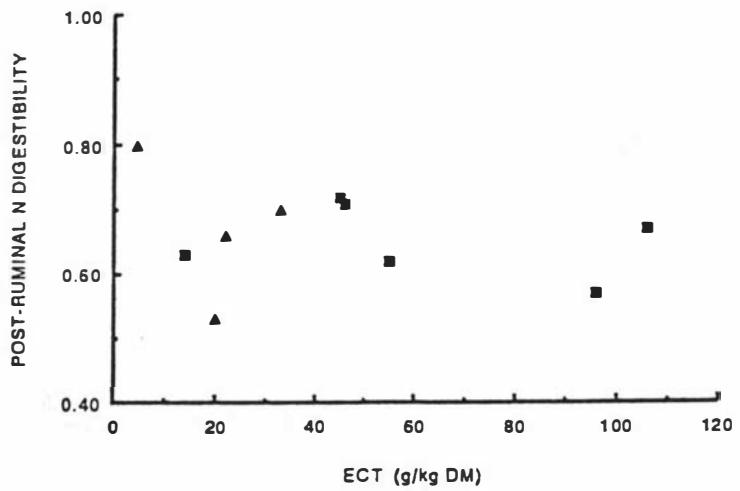

Figure 7.3 The effect of extractable condensed tannin (ECT) concentration upon post-ruminal N digestion in sheep fed *L. corniculatus,* (▲, Chapter 6; Waghorn *et al* 1987*a, b*) and *L. pedunculatus* (■, Barry & Manley 1984; Barry *et al* 1986; Waghorn *et al* 1994*a*).

Table 7.1 The effect of condensed tannins upon the digestion of essential amino acids in sheep fed fresh *L. corniculatus* (22 g extractable CT/kg DM) and *L. pedunculatus* (55 g extractable CT/kg DM).

	L. corniculatus		*L. pedunculatus*	
	Control	PEG	Control	PEG
Intake (g/d)	98.9	98.9	103.2	116.8
Abomasal flow:				
g/d	84.7	55.5	121.1	105.6
proportion intake	0.86	0.56	1.17	0.90
Apparent absorption from small intestine:				
g/d	58.8	36.2	81.4	83.5
proportion abomasal flow	0.67	0.67	0.67	0.79
proportion intake	0.59	0.37	0.79	0.72

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

The digestion of hemicellulose in the rumen was greatly depressed compared to that of cellulose due to action of CT in sheep fed *L. pedunculatus* (Figure 7.4) and rumen digestion as proportion of total digestion of both cellulose (RCEL) and hemicellulose (RHCEL) were negatively related to ECT concentration (g/kg DM), as shown in Equations 3 and 4.

RCEL=0.99-0.0011 ECT (r=0.75) **(3)**

RHCEL=0.88-0.0037 ECT (r=0.78) **(4)**

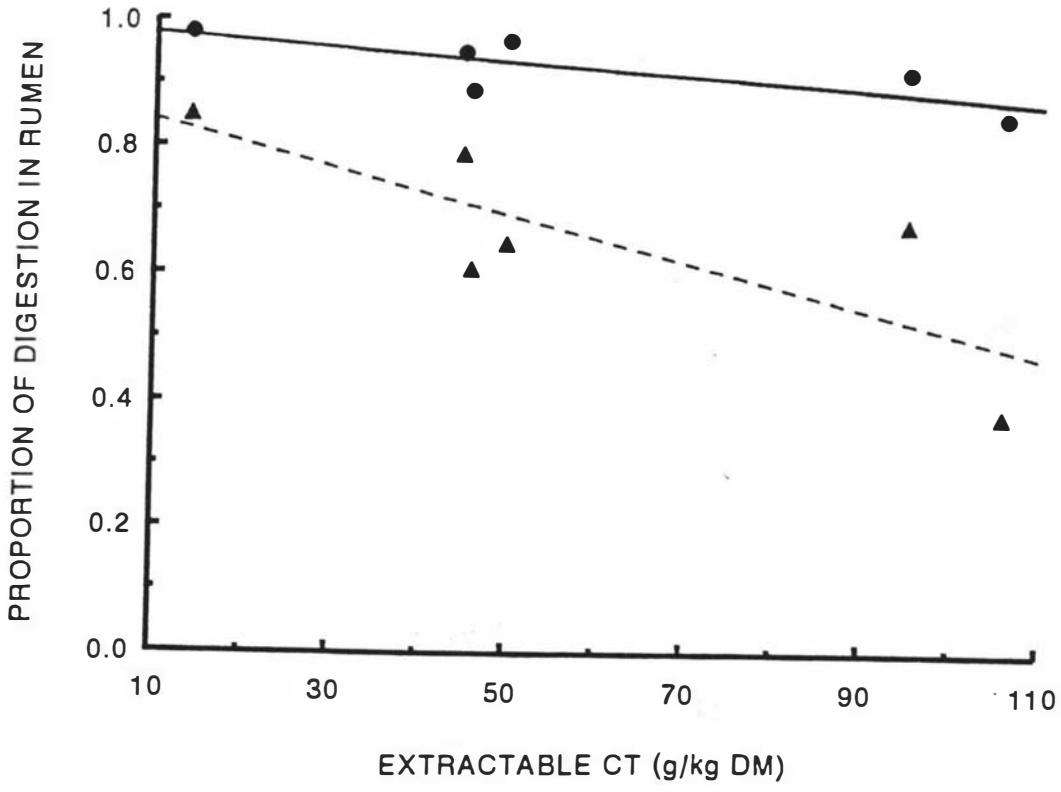

Figure 7.4 The effect of increasing content of extractable condensed tannins (ECT) in *L. pedunculatus* upon digestion of cellulose and hemicellulose in the rumen, expressed as a proportion of total digestion in the gastro-intestinal tract. ●, cellulose; ▲, hemicellulose. (Data are taken from Barry & Manley 1984; Barry *et al* 1986; Waghorn *et al* 1994*b*).

Action of CT in *L. corniculatus* had a similar trend on both cellulose and hemicellulose digestion (Chapters 3 & 6). Generally, these showed that firstly CT-cellulose complexes are readily dissociated in the rumen, allowing rumen microbes to attack cellulose, and secondly that the free CT in the rumen (130-200 μg CT/ml) of sheep had no significant effects on rumen total cellulase activity (McAllister *et al* 1993). However, the reduced hemicellulose and lignin digestion suggested that either the complexes of these two substances and CT are more resistant to attack by rumen microbes, or action of CT selectively depressed the activity of rumen microbes responsible for hydrolysis of hemicellulose and lignin. It is estimated from the results of McAllister *et al* (1993) that cellulolytic activity begins to decline at about 30-40 g ECT/kg DM in sheep fed *L. corniculatus*. As studies in this thesis and in other reports (Barry *et al* 1986) showed that digestion of hemicellulose is more sensitive to CT than cellulose, it is possible that digestion in the rumen begins to decline at a lower concentration of CT for hemicellulose than for cellulose. Chapter 3 showed that ECT at 27 g/kg DM already reduced the digestion of hemicellulose. Therefore, it seems that the optimum amount of ECT in *L. corniculatus* would appear to be around 20 g ECT/kg DM. At this level, CT had no effect on rumen fibre digestion, but substantially reduced protein degradation in the rumen and increased NAN flux to the SI. Research is needed to study the effect of CT in the range of 10-20 g ECT/kg DM on nutrient digestion and metabolism.

Methionine and cysteine were investigated in detail in these studies (Chapter 3 & 6). Action of CT reduced SAA degradation in the rumen, resulting in increased total methionine and total cysteine flow out of the rumen, but reduced true digestibility of plant methionine and cysteine in the SI and reduced overall apparent digestibility of cysteine (but not methionine) in the SI. This shows firstly that CT reduced apparent absorption of cysteine by reducing the release of it from CT:protein complex and increasing endogenous loss, and secondly that the beneficial effects of CT depends both on its effects in reducing forage protein degradation in the rumen and its effects on digestion in the SI (Table 7.1). In the case of methionine the beneficial effects of CT in the rumen more than counteracted the small reduction in apparent digestion in the SI, resulting in increased absorption, whereas in the case of cysteine the beneficial effects of CT in the rumen were cancelled out by the major reduction in digestion in the SI, resulting in little or no increase in absorption. The reason that the

apparent digestibility of cysteine in the SI is lower than that of methionine may be related to the more endogenous loss of cysteine than methionine and the slower release rate of cysteine than methionine from the complexes (Chapter 6). Research is required to study the effect of CT with different reactivity and in different concentrations on releasing SAA and other AA from the CT-protein complexes in the SI.

In two experiments measuring the effects of CT upon rumen protozoa numbers, it was shown that action of CT in *L. corniculatus* reduced rumen protozoa numbers per ml of rumen fluid (Chapter 3 & 4). Leng *et al* (1992) also showed that action of CT in *L. pedunculatus* killed rumen protozoa in *in vitro* culture studies. This showed that CT in lotus species had an adverse effect on rumen protozoa. The reduced number of protozoa in rumen fluid due to the action of CT could partly be responsible for the low ammonia concentration and low breakdown rate of SAA in the CT acting group, as it has been shown that protozoa have much higher proteolytic activity than bacteria and most species deaminate AA and excrete ammonia as an end-product (Nolan 1993). Mixed protozoa are also responsible for appreciable activity of both cysteine and aspartate proteases and they exhibit higher amino peptidase activity than bacteria (Bird *et al* 1990; Ushida *et al* 1984). The reasons why CT in lotus reduced rumen protozoa numbers are not known. Research is needed to study the effect of CT on protozoa kinetics, including re-cycling of N in the rumen, pool size and rumen outflow rate. Rumen protozoa have been recognized to reduce protein/energy ratio in absorbed nutrients in animals fed poor quality (high fibre and low protein content) forage by decreasing the flow of bacterial cells to the SI and digesting the particulate protein in the rumen (Bird 1991). Therefore, the anti-protozoal effects of CT may also be beneficial for animals fed on poor quality forages, such as in tropical countries.

7.3 EFFECT OF CT ON NUTRIENT UTILIZATION

Action of CT in *L. corniculatus* reduced the irreversible loss rate (IRL) of inorganic sulphate in blood plasma (Chapter 3), reflecting reduced breakdown of SAA in the rumen. This is consistent with the finding in Chapter 6 that action of CT in *L. corniculatus* increased both methionine and cysteine flux out of the rumen per unit

of intake. Action of CT increased absorption of methionine but not cysteine from the SI (Chapter 6), yet plasma IRL was unaffected for methionine but increased for cystine (Chapter 3). This is due to transulphuration of methionine to cystine (Chapter 3). Action of CT in *L. pedunculatus* had a similar effect (McNabb *et al* 1993), but cystine IRL was greater than determined in Chapter 3. These suggest firstly that reactivity of CT in *L. pedunculatus* is stronger than CT in *L. corniculatus*, and secondly that cysteine is more limiting than methionine in high wool producing sheep. However, these measurements were made with sheep whose main productivity is wool protein synthesis. With lactating animals, the main productivity is casein protein synthesis, for which AA requirements may differ from wool protein synthesis, and therefore the effect of CT on AA kinetics in the blood plasma may differ from those found in the present studies. Studies in this area may explain why CT had major effects on wool growth and lactation performance but only a minor effect on body growth. Research using the arterio-venous (A-V) technique to study the effects of CT upon nutrient uptake by the mammary gland and the IRL of plasma glucose is also needed.

7.4 EFFECT OF CT ON ANIMAL PRODUCTION

One of the main findings in this study was that the action of CT in *L. corniculatus* increased wool growth (Chapter 4), milk production, milk protein yield and milk lactose yield (Chapter 5), and only had minor effects on LWG (Chapters 4 & 5). The increased animal production can be explained by the action of CT increasing EAA, especially SAA, absorption from the SI (Chapters 3 & 6; Waghorn *et al* 1987a), and increasing plasma cystine flow to body synthetic reactions (Chapter 3), whilst having no effect on VFI and fibre digestion (Chapters 3, 4, 5 & 6). The reason why CT only had minor effects on LWG may be that at these growth stages EAA supply is not a main factor limiting body growth in sheep fed high protein forages (30-35 g N/kg DM). However, the action of CT in *L. corniculatus* increased the amount of EAA which animals require for wool (cystine) and milk protein synthesis. Further research needs to be conducted to study the effects of CT on modifying the nutrient partitioning rate for different physiological pathways.

Wool growth response (WGR) to action of CT depends upon the level of ECT

concentration (Figure 7.5), with positive responses in the range 25-33 g/kg DM. As CT concentration increased above 25 g ECT/kg DM, the positive response due to CT became less, and depressions in wool growth were recorded at very high ECT concentrations. CT at very low concentration (1.6 g ECT/kg DM) had no effect on WGR, probably due to very low CT concentrations (less than 9 g ECT/kg DM) having no effect in increasing abomasal NAN flux. This shows that whilst high CT concentrations depress animal production, very low levels of CT also have no nutritional effect on ruminant animal. The greater decrease in WGR due to high concentration of CT in mulga (80 g ECT/kg DM) than in *L. pedunculatus* (106 g ECT/kg DM) may well reflect differences in molecular weight (MW), structure and reactivity of the CT from these two plants. Beneficial effects of CT in *L. corniculatus* need to be studied in the concentration range 10-20 g ECT/kg DM. Further research is required to study the effects of MW and reactivity of CT and the interaction of reactivity and concentration on animal production, in order to define the most effective type of CT and to determine the optimal plant concentration.

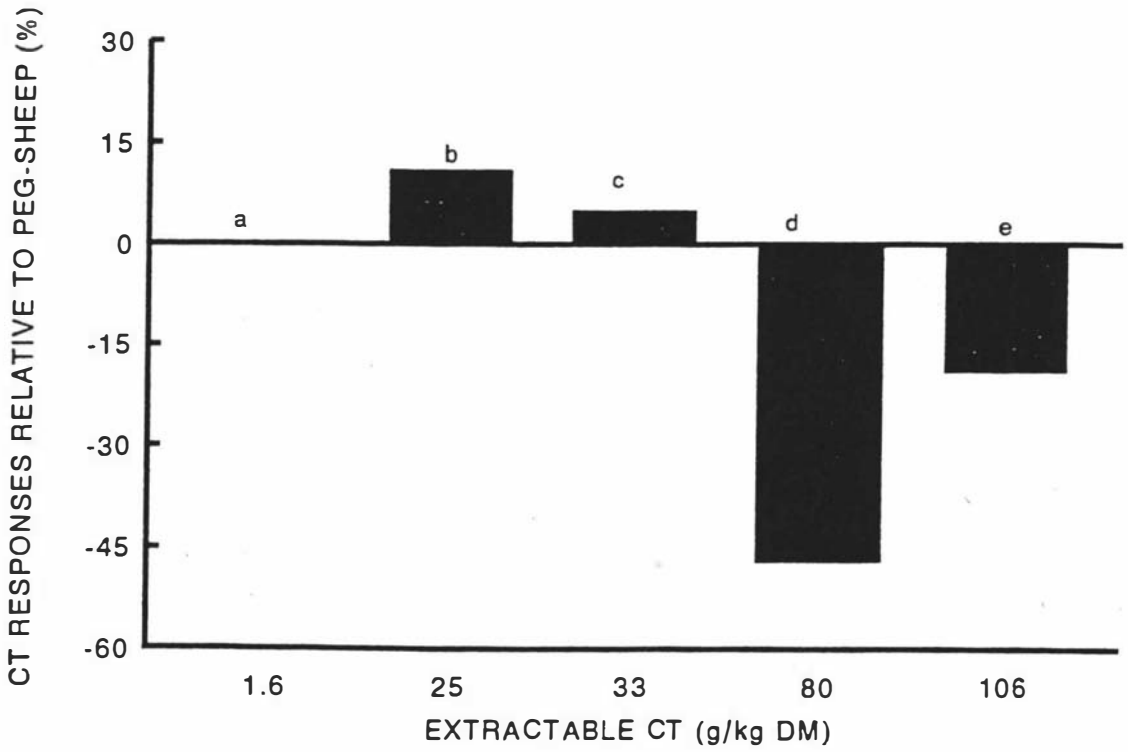

Figure 7.5 The effect of forage CT concentration on wool growth rate (WGR) of grazing sheep, calculated as the WGR response of control sheep (CT acting) relative to PEG supplementation sheep (CT inactivated). *a*, Yorkshire fog (*Holcus lanatus,* Liu personal communication); *b*, Birdsfoot trefoil (*Lotus corniculatus*, Chapter 4); *c*, Sulla (*Hedysarum coronarium*, Terrill *et al* 1992); *d*, Mulga (*Acacia aneura*, Pritchard *et al* 1992); *e*, Big trefoil (*Lotus pedunculatus*, Barry 1985).

7.5 ROLE OF PEG AND THE RESTRICTED FEED ALLOWANCE IN STUDYING EFFECTS OF CT

In both the grazing and indoor metabolism experiments, PEG (MW 3,500) was either given orally twice daily (grazing experiments; Chapter 4 & 5) or continuously infused into the rumen (indoor metabolism experiments; Chapter 3 & 6) to define the effects of CT. PEG has been shown to displace CT from CT-protein complexes at the ratio of 1.7 g PEG/g CT and this ratio of PEG completely binds available CT, preventing CT from binding to protein and releasing protein from CT-protein complexes in the rumen (Jones & Mangan 1977; Barry & Forss 1983). This thesis demonstrated that the administration of PEG to sheep either by twice daily oral supplementation or by continuous intraruminal infusion at 1.7 g PEG/g CT was successful for deducting the effects of CT. Rumen ammonia concentration was greater for PEG than for control sheep 4 h after dosing but was similar 8 h after PEG supplementation in control and PEG sheep grazing the grass Yorkshire fog (Liu personal communication); hence there may be a possibility of underestimating the animal response to CT in the present field experiments. Therefore, to accurately quantify the effects of CT on animal production, techniques need to be developed to deliver PEG continuously in grazing sheep. Neither nutrient metabolism nor animal performance was affected by PEG supplementation in sheep grazing lucerne (*Medicago sativa*), a non CT-containing legume, showing firstly that PEG specifically combined with CT and secondly that PEG *per se* did not affect nutrient digestion and lamb productivity.

In grazing experiments measuring effects of CT on animal production, a strategy of restricted feed allowance was used so that protein intake limited animal productivity. It was successful in terms of the responses of the animals. However, care should be taken to distribute feed equally in the duration of grazing. Therefore it is necessary to shift animals more frequently from break to break than the normal weekly grazing shift.

7.6 TOWARDS THE PRACTICAL APPLICATION OF CT IN TEMPERATE FORAGES

L. pedunculatus grows well on acid (pH 4.4-4.8), low fertility tussock grassland soils

in NZ and is used in steep hill country development programmes that have very low levels of fertilizer input. However, under these conditions, the CT concentration is very high (80-110 g ECT/kg DM). Although growing in high fertility soils can reduce CT content, *L. pedunculatus* competes poorly with other plants such as white clover, and does not persist under these conditions. *L. corniculatus* grows well in medium fertility soils (pH 5.0-5.5) and may well find application in NZ in low cost development programmes in medium fertility soils. *L. corniculatus* is also a poor competitor with other plants in high fertility soil conditions. Therefore there are limitations in the application of these two plants in NZ agriculture. The beneficial effect of CT in *L. corniculatus* and *L. pedunculatus* in high fertility grazing ecosystems may be best expressed through Genetic Engineering programmes, to transfer DNA which is responsible for CT production into leaves and stems of white clover and ryegrass that have wide application in NZ grazing ecosystems.

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Appendix

The extraction of radiolabelled inorganic sulphate from blood plasma

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ABSTRACT

A study was conducted into factors governing the efficiency of the ion exchange method for extracting ^{35}S-labelled inorganic sulphate (SO_4^{2-}) from blood plasma, using Dowex'1 - X8 ion exchange resin. The study compared effects of trichloroacetic acid (TCA) strength as protein precipitant, different HCl strengths as resin eluent, sodium citrate/HCl (SC/HCl) vs HCl as eluents, and evaluated ultrafiltrated (UF) plasma upon the adsorption and recovery of added $^{35}SO_4^{2-}$. Both adsorption and release of $^{35}SO_4^{2-}$ from the resin were inhibited by the presence of TCA, and HCl was not as effective as 1M SC/2M HCl in releasing $^{35}SO_4^{2-}$ adsorbed to resin. The rates of $^{35}SO_4^{2-}$ adsorbed onto resin and recovered were markedly increased by using UF plasma and 1M SC/2M HCl as eluent, with the values being 96.3±0.11% and 91.1±0.39% respectively where 1 g resin was used. Therefore the use of UF for deproteinising and 1M SC/2M HCl as eluent are recommended for extracting $^{35}SO_4^{2-}$ from blood plasma when Dowex'1-X8 resin is used as the ion exchanger.

Key words: Inorganic sulphate, extraction, plasma, eluent.

INTRODUCTION

In isotope dilution experiments involving measuring the irreversible loss rate (IRL) of inorganic sulphate from blood plasma and the oxidation of methionine and cystine to inorganic sulphate, it is necessary to isolate inorganic sulphate from blood plasma, and to measure both its radioactivity (disintegrations per minute; DPM mg^{-1} S) and concentration, in order to calculate specific radioactivity (SA). Such an experiment was described by McNabb *et al* (1993), involving measuring the effect of plant condensed tannins (CT) upon the IRL and interconversions of plasma methionine, cystine and inorganic sulphate, using ^{35}S labelling, in sheep fed the legume *Lotus pedunculatus*.

Methionine and cystine in deproteinised plasma can be separated by high performance liquid chromatography (HPLC) to determine both concentration and radioactivity (McNabb *et al* 1993). Several methods have been developed to determine inorganic sulphate (Bird and Fountain 1970; Johnson and Nishita 1952). However, because these measure inorganic sulphate in the presence of organic sulphur (ie inorganic and organic sulphur are not separated before measurement), they could not be used in the above mentioned labelling experiment. McNabb *et al* (1993) described a method using ion exchange resin to extract inorganic sulphate from blood plasma, but the recovery in this method was low (70%).

In the present investigation, factors affecting efficiency of the ion exchange resin method for extracting inorganic sulphate from blood plasma were studied, and a new procedure developed which greatly increased recovery (91%). Developments included a new procedure for deproteinising plasma and a more effective eluent for releasing inorganic sulphate from the resin.

METHODS

The ion exchange method (McNabb *et al* 1993) consists of adding a strong basic exchange resin (Dowex'1-X8) in the Cl^- form (1 g) to trichloroacetic acid (TCA) deproteinised plasma (3 ml), with SO_4^{2-} being adsorbed onto the resin. This is then washed to remove the deproteinising agent (TCA), amino acids and any other organic sulphur compounds. SO_4^{2-} is then eluted off the resin by shaking in 1.0M HCl (5 ml) for 16 hours at room temperature, decanting and determining SO_4^{2-} radioactivity and

concentration. The present study investigated using different deproteinising methods (TCA vs ultrafiltration) and different kinds of eluents for increasing both the adsorption of $^{35}SO_4^{2-}$ onto the resin and recovery of $^{35}SO_4^{2-}$ after elution. All studies were done using $^{35}SO_4^{2-}$, which was added to plasma after deproteinisation.

Blood samples were withdrawn from a sheep using a syringe containing heparin, and were then centrifuged at 3,000 g for 15 min to obtain plasma.

TCA procedure: One ml 3.1 M TCA (50% w/v) and 10 ml plasma were added to a 25 ml centrifuge tube. After vortexing, the tube was left at 4 °C for 15 min and then centrifuged at 3,000 g for 20 min. The supernatant (TCA-precipitated plasma; TCA final concentration 0.28M; 4.5% w/v) was decanted for SO_4^{2-} extraction.

Ultrafiltration (UF) procedure: In this procedure, Centriprep-10 concentrators (Amicon, Beverly, MA, USA; 50 ml; membrane MW cutoff 10,000) were used. Plasma was added to the concentrator and was centrifuged at 1,900 g for 30 minutes. The ultrafiltrated plasma (UF plasma) was decanted for SO_4^{2-} extraction.

Resin preparation and extraction procedure were the same as described by McNabb *et al* (1993). ^{35}S labelled SO_4^{2-} was used to determine adsorption and recovery. A known radioactivity (8.44 KBq) of $^{35}SO_4^{2-}$ was added to the deproteinised plasma of each replicate (for both 1 and 3 ml plasma), mixed and stood at room temperature for 5 min prior to the extraction by ion exchange resin. After extraction of $^{35}SO_4^{2-}$ by resin and washing resin with eluent, radioactivity (DPM) in the eluent was counted in a liquid scintillation counter. Rate of $^{35}SO_4^{2-}$ adsorbed onto resin and recovery from the whole procedure represented $^{35}SO_4^{2-}$ extraction efficiency.

A Wallac 1409 (Pharmacia, Finland) liquid scintillation counter was used to count the radioactivity (DPM) of $^{35}SO_4^{2-}$. Ten ml of high efficiency phase combining liquid scintillant (PCSII; Amersham Corporation) and 2 ml of acetic acid were added to each of the scintillation vials, followed by adding 1 ml eluent from the resin. After thoroughly mixing, vials were put into the scintillation counter using the quench curve which had already been set up.

$^{35}SO_4^{2-}$ adsorbed onto the resin was calculated by subtracting radioactivity in extracted solution from added radioactivity. Recovery from the whole procedure was expressed as eluent radioactivity as a percentage of ^{35}S added. Means are presented with standard error (SE). Comparison of extraction efficiency between treatments was done by factorial analysis of variance.

RESULTS

Experiment 1

Two volumes of TCA-precipitated plasma (1 and 3 *ml*) were extracted and 4 concentrations of HCl (1, 3, 4 and 5 M) were used as eluent (5.0 ml). One g of Dowex'1-X8 was used as the ion exchange resin. Each treatment had 4 replicates. With 1 ml TCA-precipitated plasma, 89.4% (SE 0.79) of added $^{35}SO_4^{2-}$ was adsorbed onto the resin, compared with only 70.4% (SE 0.31) for 3 ml of TCA-precipitated plasma ($P < 0.01$; Figure 1). Increasing eluent (HCl) concentration from 1 to 5 M significantly improved recovery of $^{35}SO_4^{2-}$ from both 1 ml and 3 ml TCA-precipitated plasma ($P < 0.01$), with most of the increase occurring between 1 M and 3 M. Increasing HCl strength above 3 M did not result in significant increases for both 1 and 3 ml TCA-precipitated plasma. When 3 M HCl was used as eluent, recoveries of $^{35}SO_4^{2-}$ in 1 ml and 3 ml TCA-precipitated plasma were 67.3% (SE 0.52) and 49.3% (SE 0.65) respectively ($P < 0.001$).

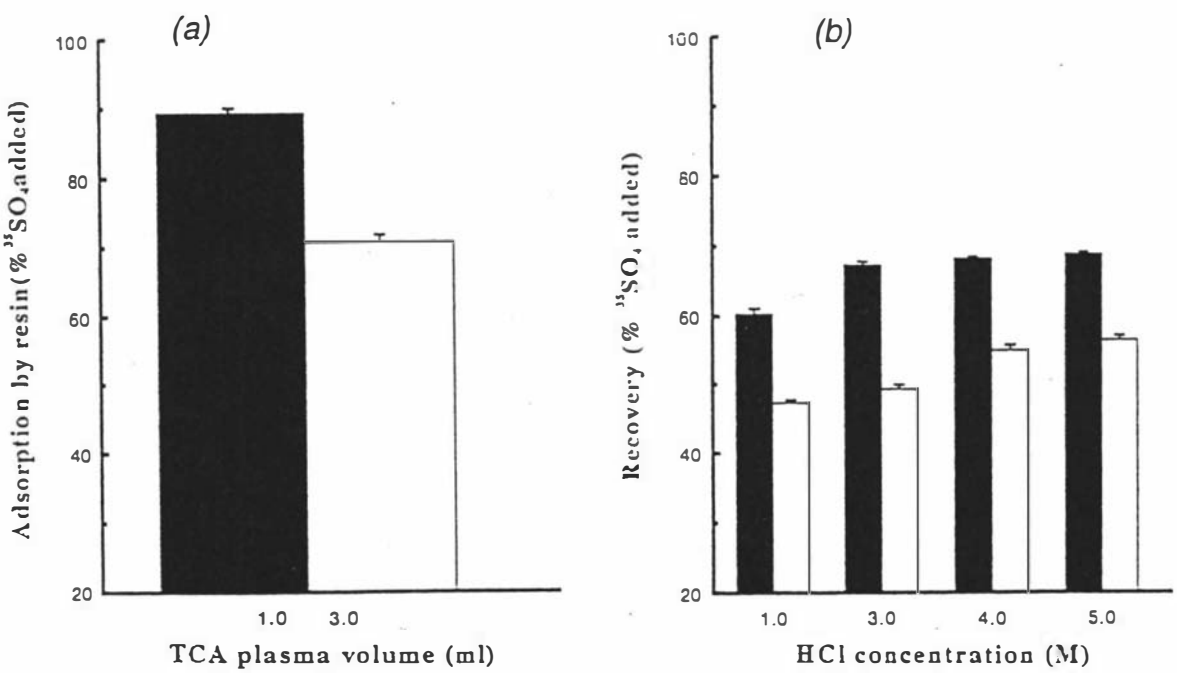

Figure 1. Experiment 1. The effect of plasma volume upon adsorption (*a*) and plasma volume and HCl concentration upon recovery (*b*) of $^{35}SO_4^{2-}$ added to TCA-precipitated sheep plasma. Each treatment had 4 replicates. One g of resin (Dowex'1 - X8) was used in all determinations. (■) 1 ml plasma; (□) 3 ml plasma; I SE.

Experiment 2

This experiment was designed to determine the effect of TCA upon the SO_4^{2-} extracting efficiency in 0.1 M NaCl/2.0 mM K_2SO_4 buffer which simulated the ionic strength of deproteinised plasma. Concentrations of TCA in the buffer were 0, 0.04 M and 0.12 M. One g resin and 5.0 ml of 3 M HCl were used in all series. Twelve replicates were used for each treatment. Increasing TCA concentration from zero to 0.12 M decreased rate of $^{35}SO_4^{2-}$ adsorbed onto resin from 98.9% (SE 0.22) to 86.5% (SE 2.62; $P < 0.01$) and decreased recovery from 72.8% (SE 0.24) to 57.2% (SE 0.85; $P < 0.01$).

Experiment 3

Effect of 1 M SC/2 M HCl *vs* 2 M HCl (6.0 ml) as eluents and two amounts of resin (0.5 *vs* 1.0 g) were examined in this study, each with 4 replicates. Three *ml* of UF plasma was extracted. Increasing resin amount from 0.5 to 1.0 g increased the rate of $^{35}SO_4^{2-}$ adsorption ($P < 0.001$; Table 1), but markedly decreased recovery when 2 M HCl used as eluent ($P < 0.01$). No difference was observed in recovery between 0.5 and 1.0 g resin when 1 M SC/2 M HCL used as eluent ($P > 0.05$). The recovery of $^{35}SO_4^{2-}$, when using 1 M SC/2 M HCl, was significantly higher than using 2 M HCl as eluent for either 0.5 g or 1.0 g of resin ($P < 0.001$).

Table 1. Comparison of adsorption and recovery from Dowex'1-X8 resin of $^{35}SO_4^{2-}$ added to ultrafiltrated sheep blood plasma (3 ml). The eluents used to release resin-bound $^{35}SO_4^{2-}$ were 1 M sodium citrate/2 M HCl (1 M SC/2 M HCl) and 2 M HCl. Mean values with their SE in brackets are for 8 replicates for adsorption rate and 4 replicates for recovery.

Resin (g)	0.5		1.0	
Eluents	1 M SC/2 M HCl	2 M HCl	1 M SC/2 M HCl	2 M HCl
Adsorbed onto resin (% of $^{35}SO_4$ added)	93.6 (0.10)		96.4 (0.11)	
Recovery from resin (% of $^{35}SO_4$ added)	91.6 (0.23)	84.6 (0.26)	91.1 (0.39)	74.2 (0.20)

Experiment 4

The recoveries of ^{35}S-labelled methionine and cysteine from the extraction procedure were also determined. A known radioactivity (4.07 KBq of methionine or cysteine) was added separately to 3 ml TCA-precipitated plasma and 3 ml UF plasma. After being extracted by resin and washed with 5.0 ml of 3 M HCl (TCA plasma) or 5.0 ml of 1 M SC/2 M HCl (UF plasma), the radioactivity was counted in the scintillation counter. Each isotope had 3 replicates for each type of plasma. No radioactivity was detected in eluent from the resin using either ^{35}S-methionine or ^{35}S-cysteine added to TCA-precipitated or UF plasma.

DISCUSSION

The high and consistent recovery of $^{35}SO_4^{2-}$ obtained using UF plasma and using 1 M SC/2 M HCL as eluent (91%) represents considerable improvement in the ion exchange method over using TCA-precipitated plasma and 1 M HCl as eluent (70%). This was due to the higher adsorption of $^{35}SO_4^{2-}$ onto the resin using UF plasma compared with TCA-precipitated plasma and the stronger ability of SC/HCl to release $^{35}SO_4^{2-}$ from the resin compared with HCl alone.

TCA is a commonly used protein precipitant. It is negatively charged (pKa=0.63) and

the pH of a 0.1 M aqueous solution is 1.2 (Budavari and O'Neil 1989). Because of its poly-valencies, negatively charged TCA may be a strong competitor with other anions to be exchanged with resin, and this is evident for SO_4^{2-} in Experiment 2. The lower values for both adsorption and recovery for 3 ml compared with 1 ml of TCA-precipitated plasma in Experiment 1 and the reduced values of adsorption and recovery of $^{35}SO_4^{2-}$ in buffers in Experiment 2 illustrated that the presence of residual TCA after protein precipitation inhibits SO_4^{2-} adsorbed onto and released from the resin. As UF plasma does not contain this inhibitor, this explains the higher extraction efficiency of SO_4^{2-} from UF plasma than from TCA-precipitated plasma.

According to Kunin and Myers (1947), some common anion affinities for the basic exchangers are: $SO_4^{2-} > CrO_4^{2-} >$ citrate > tartrate > $NO^{3-} > AsO_4^{3-} > PO_4^{3-} > MoO_4^{2-}$ > acetate > $I^- > Br^- > Cl^- > F^-$. This series shows that the affinity of Cl^- is much less than that of SO_4^{2-}. Therefore HCl used as eluent may not be powerful enough to release all SO_4^{2-} from the resin, and even when the concentration of HCl was increased to 5 M or when UF plasma was used, there was still a proportion of adsorbed SO_4^{2-} which was not released from the resin. The improved recovery of SO_4^{2-} with SC/HCl can be explained by citrate having a higher affinity for the resin than Cl^-. When 1 M sodium citrate aqueous solution alone was used as eluent the PCSII gel became cloudy and unstable, whereas when 1 M SC/2 M HCl was used as eluent the PCSII gel was clear and stable. Therefore 1 M SC/2 M HCl solution was used in Experiment 3.

In conclusion, when using Dowex'1 - X8 as ion exchanger to extract and recover SO_4^{2-} from blood plasma, TCA is not a suitable protein precipitant due to its inhibition of SO_4^{2-} exchange with the resin, and HCl alone is not a completely effective eluent for releasing all bound SO_4^{2-} from the resin. The procedure recommended involves adding 3 ml UF plasma to 1.0 g Dowex'1 - X8 resin and using 6 ml 1 M SC/2 M HCl as eluent. This is effective for giving high and constant recovery of SO_4^{2-} from blood plasma. The method is especially applicable to ^{35}S labelling experiments, and carries a safety factor, as similar results were also obtained using 0.5 g resin.

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