DIGESTION AND METABOLISM OF SULPHUR CONTAINING AMINO ACIDS IN SHEEP FED FRESH FORAGE DIETS.

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DEDICATION.

To April Shannon, without whose constant love and support, this thesis would not have been possible.

ABSTRACT

Sulphur amino acids (SAA) are important in the sheep industry because they appear in many instances, to limit wool protein synthesis. Experiments were carried out to study two methods with the potential to increase the absorption of SAA from the small intestine of sheep fed fresh forage diets. The first experiment evaluated the effects of condensed tannins (CT) in *Lotus pedunculatus* upon the following; (**1**) Rumen-S metabolism, and (**2**) the digestion of methionine and cystine. (**3**) The metabolism of plasma SAA and inorganic sulphate, and (**4**) The solubility and (**5**) degradation of protein in the rumen. The second experiment identified proteins that contained a high proportion of SAA, and assessed their potential for expression in the leaves of forage legumes using genetic engineering techniques. Two aspects were measured; (**6**) Rates of rumen degradation of ovalbumin and sunflower albumin 8 (SF8) proteins *in vitro*, and (**7**) The expression of SF8 protein from sunflower seeds in the leaves of transgenic tobacco.

The nutritional consequences of CT in *Lotus pedunculatus* were assessed by infusing polyethylene glycol (PEG), into the rumen of one group of sheep (PEG sheep; CT not operating), whilst a separate group of sheep received an infusion of water (CONTROL sheep; CT operating). PEG selectively binds CT, preventing CT from binding plant proteins in the rumen, so that CT effects on digestion could be evaluated. Polyethylene glycol was also added to *in vitro* incubations as required.

The principal results were;

(**1**) Condensed tannins had a major effect on rumen sulphur (S) metabolism. The irreversible loss rate (IRL) of reducible-S (total non-protein S in rumen fluid, measured as sulphate after performic acid oxidation of rumen fluid) from the rumen was lower ($P<0.001$) in control (0.84gS/d) than PEG (2.49gS/d) sheep. This was due in part to a higher ($P<0.001$) flux of methionine (2.75 and 2.09g/d) and cystine (3.33 and 2.52g/d) through the abomasum in control than PEG sheep. There was no net loss of dietary methionine or cystine from the rumen in control sheep, whilst 29% of methionine ($P<0.001$) and 28% of cystine ($P<0.001$) intake disappeared from the rumen in PEG sheep. The proportion of microbial-non-ammonia-nitrogen (NAN) in whole rumen digesta-NAN was lower ($P<0.001$) in control (0.44) than PEG (0.71) sheep, although it was calculated that the rumen microbial-NAN pool size was similar in control (2.9g) and PEG (3.1g) sheep. These observations suggest CT reduced the degradation of forage protein and SAA in the rumen.

(**2**) The apparent absorption of methionine from the small intestine was higher (27%; $P<0.001$) in control (2.11g/d) than PEG (1.66g/d) sheep, but the apparent absorption of cystine from the small intestine was similar (4%; $P>0.05$) for control (1.40g/d) and PEG

(1.34g/d) sheep. The apparent digestibility of methionine in the small intestine was similar (0.78) for both groups, whilst for cystine, it was lower ($P<0.01$) in control (0.42) than PEG (0.53) sheep. The increased absorption of methionine from the small intestine with CT was due to an increased flux from the rumen, whereas the digestibility of cystine in the small intestine may have been lower as a consequence of binding to CT complexes.

(3) Condensed tannin had a major effect on plasma SAA fluxes, especially cystine. Plasma cystine concentration and IRL were higher ($P<0.001$) in control (41.7μmol/l and 39.8μmol/min) than PEG (27.5μmol/l and 22.4μmol/min) sheep. Condensed tannins resulted in a 79% increase in the transulphuration of methionine to cystine (11.7 and 6.5μmol/min; $P<0.05$) and a decrease ($P<0.05$) in the oxidation of cystine (3.33 and 5.2μmol/min) and methionine (0.2μmol/min and 1.2μmol/min) to sulphate in control compared to PEG sheep. The net effect of CT was to increase ($P<0.05$) the flux of plasma cystine to productive processes and maintenance by 110% in control (36.5μmol/min) compared to PEG (17.4μmol/min) sheep. This represented 91% of total plasma cystine flux in control sheep, compared to only 74% of total cystine flux in PEG sheep ($P<0.05$). Since wool growth is generally accepted as the major productive process utilising plasma cystine, these results indicate that a major effect of CT in the diet would probably be to increase wool growth. The IRL of plasma methionine was similar in control (20.5μmol/min) and PEG (19.9μmol/min) sheep, whilst the IRL of plasma sulphate was lower ($P<0.01$) in control (35.9μmol/min) than PEG (50.1μmol/min) sheep.

(4) The rate of protein solubilization in the rumen was studied by measuring the loss of N, corrected for microbial-NAN contamination (true), from fresh minced (FM) and freeze-dried and ground (FD) *Lotus pedunculatus*, incubated in polyester-fibre bags suspended in the rumen of control and PEG sheep fed *Lotus pedunculatus*. Freshly minced *Lotus* was chosen as one treatment because it more closely represented the effects of chewing on cell rupture and CT release than was likely with freeze drying. Mincing resulted in a much greater initial N loss (47%) than freeze drying (14%). The true loss of N from FD *Lotus* was higher in PEG than control sheep at 2, 4, 6.5, 11 and 24 hours of incubation, whilst with FM *Lotus*, N losses were similar. Microbial-NAN adhering to FD residues was higher in PEG than control sheep at 2, 4, 6.5 and 11 hours, but was similar at 24 hours of incubation. However, microbial-NAN adhering to FM Lotus was higher in PEG compared to control sheep, only at 6.5 and 24 hours. These observations suggest that CT reduced protein solubility and microbial colonization of FD *Lotus* to a much greater extent than FM *Lotus*.

(5) The rate of protein degradation in the rumen was studied *in vitro* by incubating FM and FD *Lotus pedunculatus* in rumen fluid, with and without PEG, and using sodium-dodecyl-

sulphate gel electrophoresis (SDS-PAGE). After 4 hours of incubation protein from FD and FM *Lotus* was clearly degraded when PEG was present, whilst after 8 hours of incubation it was essentially undetectable in both incubations. In contrast, after 8 hours, leaf protein from FM and FD *Lotus* was still readily detectable in incubations without PEG. Therefore, CT substantially reduced the rate at which soluble protein was degraded by rumen microorganisms but had little effect on the rate at which it was solubilized, particularly when minced.

(6) The rate of degradation of SF8 protein was compared to the degradation of the LSU and small subunit (SSU) of Fraction 1 leaf, vicilin and ovalbumin proteins using *in vitro* incubations and SDS-PAGE. The SF8 protein had a rate of proteolysis of $0.23h^{-1}$ and a half-life of 3.0 hours, but the principal degradation product of SF8, which had a half-life of 69. hours, was extremely resistant to rumen degradation. Proteolysis of the LSU of Fraction 1 leaf protein was resolved into two components. The first product had a degradation rate of $0.06h^{-1}$ and a half-life of 11.6 hours, whilst the second component of proteolysis, which occurred from 12 hours onwards, had a degradation rate of $0.45h^{-1}$ and a half-life of 4.6 hours. The proteolysis of the SSU of Fraction 1 leaf protein had a degradation rate of $0.04h^{-1}$ and a half-life of 17.3 hours. Ovalbumin was not degraded during the initial 16 hours of incubation, but was then degraded at a rate of $0.08h^{-1}$, with a half-life of 8.7 hours. Vicilin had a rate of proteolysis of $4.3h^{-1}$ and a half-life of about 10 min. Both SF8 protein and ovalbumin were found to be more resistant to rumen proteolysis than the LSU of Fraction 1 leaf protein, but different mechanisms were involved in conveying resistance. Therefore it is worthwhile to introduce expression of genes coding for these proteins into the leaves of important agricultural legumes, using genetic engineering techniques, with a view to increasing the availability of SAA for sheep.

(7) The gene for SF8 is normally expressed only in seeds. Therefore a SF8 cDNA clone was genetically engineered for expression in the leaves of tobacco plants and inserted into a gene delivery system in *Agrobacterium tumefaciens* and transferred to tobacco. Transcription of the SF8 synthetic (chimeric) gene occurred in the leaves of transformed tobacco, with the level of SF8 mRNA varing over a 100 fold range, but in the highest expressor, it represented 14% of the SF8 mRNA level found in sunflower seeds. However, SF8 protein was not detected in the leaves of transformed tobacco. Consequently, the level of SF8 in the leaves of transgenic tobacco must have been less than 0.03% of total leaf protein. The highest SF8 mRNA expressor contained nine times more SF8 mRNA than an ovalbumin-transformed tobacco contained ovalbumin mRNA. As the ovalbumin transformed tobacco produced 0.01% of its leaf protein as ovalbumin, there is at least sufficient SF8 mRNA to support up to nine times that level of protein expression. As this level of SF8 protein expression would be detectable using sensitive immunological

procedures, it seems that either SF8 mRNA is not translatable in tobacco plants or SF8 protein is very unstable in tobacco leaves. Translatability of SF8 mRNA was tested in *E. coli* using an expression vector, pJLA602 without success. If SF8 protein was unstable in tobacco leaves, then SF8, which is a seed storage protein, should be stably accumulated in tobacco seeds. The expression of the SF8 chimeric gene was monitored in tobacco seeds, and again the results were negative. It would appear that the SF8 cDNA coding region was untranslatable so that DNA sequencing of the SF8 chimeric gene will be necessary to correct the DNA sequence by oligonucleotide-directed, site specific mutagenesis.

(8) Both CT and proteins, resistant to degradation in the rumen and with a high proportion of SAA, have the potential to increase the absorption of SAA from the small intestine in sheep grazing fresh forages. However, further research is required to examine; (a) the effectiveness of lower dietary CT concentrations than were examined in the present studies, on increasing SAA absorption and metabolism, and (b) what level of foreign gene expression is required in transgenic legumes to stimulate wool growth.

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LIST OF ABREVIATIONS

AA	amino acids
ATG	methionine codon.
ATP	adenosine triphosphate.
BamHI/EcoRI/HindIII	restriction endonuclease enzymes.
bp	base-pair.
BSA	bovine serum albumin
BW	body weight.
CaMV	cauliflower mosiac virus.
cDNA	complementary DNA.
CNBr	cyanogen-bromide.
cpm	counts per minute.
^{51}Cr-EDTA	^{51}Chromium ethylenediaminetetra acetic acid.
C.S.I.R.O.	Commonwealth Scientific and Industrial Research Organisation.
CT	condensed tannin.
cys	cystine.
d	day.
dCTP	deoxycytosine triphosphate.
dH_2O	distilled water.
dig%	digestibility.
DM	dry matter.
DNA	deoxyribonucleic acid.
D.S.I.R.	Department of Scientific and Industrial Research.
EAA	essential amino acids.
E. coli	*Escherichia coli*.
EDTA	ethylenediamine tetraacetic acid.
F1	Fraction 1 leaf protein.
FCT	free condensed tannin.
FD	freeze dried and ground
FM	fresh minced
FOR	fractional outflow rate.
FV	feeding value.
g	gram.
h	hours.
HCl	hydochloric acid.
HPLC	high performance liquid chromotography.
H_2S	sulphide.
ICP-ES	inductively coupled argon plasma emission spectrometry.

ID	internal diameter.
IgG	immunoglobulin.
IRL	irreversible loss rate.
iu	international units.
kb	kilobases.
kDa	kilodaltons.
KDEL	lys-asp-glu-leu polypeptide.
kg	kilograms.
kPa	kilopascals.
l	litres.
LSU	large subunit.
Ltd	Limited.
LWG	liveweight gain.
m	metres.
mA	milliampere.
mCi	millicurrie.
MCS	multiple cloning sites.
ME	metabolisable energy.
met	methionine.
meq	milliequivalents.
mg	milligram.
min	minute.
MJ	megajoule.
ml	millilitres.
mm	millimetres.
mol	moles.
mRNA	messenger RNA.
MW	molecular weight.
N	nitrogen.
Na	sodium.
NAN	non-ammonia-nitrogen.
NaOH	sodium hydroxide.
NC	nitrocellulose.
NEAA	non-essential amino acids.
NH_3	ammonia.
nm	nanometre
NOS	nopaline synthase.
NV	nutritive value.
NZ	New Zealand.

OM	organic matter.
OMI	organic matter intake.
OPA	orthophthaldehyde.
^{32}P	radioactive isotope of phosphate.
PA1	pea albumin 1.
PC	cyanidin.
PCS II	Phase Combining System II.
PD	delphinidin.
PEG	polyethylene glycol.
P+M	productive processes and maintenance.
psi	pounds per square inch.
Pty	Company.
pWM1	plasmid Warren McNabb 1.
RNA	ribonucleic acid.
rpm	revolutions per minute.
^{103}Ru-phenanthroline	tris(1,10-phenanthroline) ^{103}Ruthenium (II) chloride.
S	sulphur.
^{35}S	radioactive isotope of sulphur.
SA	specific activity.
SAA	sulphur amino acids.
SD	standard deviation.
SDS	sodium-dodecyl-sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis.
SE	standard error.
SED	standard deviation of the difference.
SF8	sunflower albumin 8.
SI	small intestine.
SSU	small subunit.
TCA	trichloroacetic acid.
TCT	total condensed tannin.
TQ	transfer quotient.
tRNA	transfer RNA
UK	United Kingdom.
µl	microlitre.
µmol	micromole.
VFI	voluntary feed intake
vol	volume.
v/v	volume by volume.

$W^{0.75}$	metabolic liveweight.
WSC	water soluble carbohydrate.
w/v	weight by volume.
yr	year.

1. REVIEW OF LITERATURE

In 1989 the average fleeceweight per sheep (greasy) in New Zealand was 4.6kg, which represented a total wool clip of 341,000 tonnes. This generated $1795.9m in export earnings, representing 12% of New Zealand's total export earnings (NZ Official Yearbook, 1990), which demonstrates the importance of wool production to the New Zealand economy.

The sulphur amino acids (SAA), methionine and cysteine, have been identified as the limiting amino acids for wool growth in sheep fed roughage diets (Reis, 1979). This thesis examines two potential methods for increasing the flow of SAA from the rumen to the small intestine for absorption. Although, SAA are also necessary for body growth and milk production, this thesis will concentrate on the SAA requirements for wool growth.

1.1 SULPHUR METABOLISM.

1.1.1 THE SULPHUR AMINO ACIDS.

The structural formulae of methionine, cysteine and cystine are detailed in Fig. 1. Methionine can be converted to cysteine by transulphuration, whilst cysteine is nonenzymatically oxidised to cystine in the presence of oxygen and cations. Both methionine and cystine are synthesized from homocysteine, whilst the cystine found in proteins is formed by oxidation of cysteine residues, with the resultant formation of disulphide bonds after the incorporation of cysteine into the polypeptide chain (Meister, 1965). Cysteine cannot be used to synthesize methionine in mammalian tissue (Meister, 1965).

For amino acid analysis of proteins, cystine and cysteine are oxidised to cysteic acid, and methionine is oxidised to methionine sulfone, by performic acid oxidation (Fig. 2). Cysteic acid and methionine sulfone are more stable than cystine, cysteine and methionine (Meister, 1965).

1.1.2 DIETARY SULPHUR.

The S and N content of some commonly utilised ruminant feeds are given in Table 1.

In forage plants most of the sulphur (S) is in the form of protein, with an average nitrogen (N):sulphur (N:S) ratio of 15:1 (Dijkshoarn and Van Wijk, 1967). However, the N:S ratio can vary from 4:1 to 55:1 (Stewart and Porter, 1969). A N:S ratio of 10 to 15:1 is believed to be optimal for maximum microbial utilisation of N in the rumen of sheep and cattle (Bird, 1972 and 1974).

Fig. 1: The structural formulae of the sulphur amino acids (Meister, 1965).

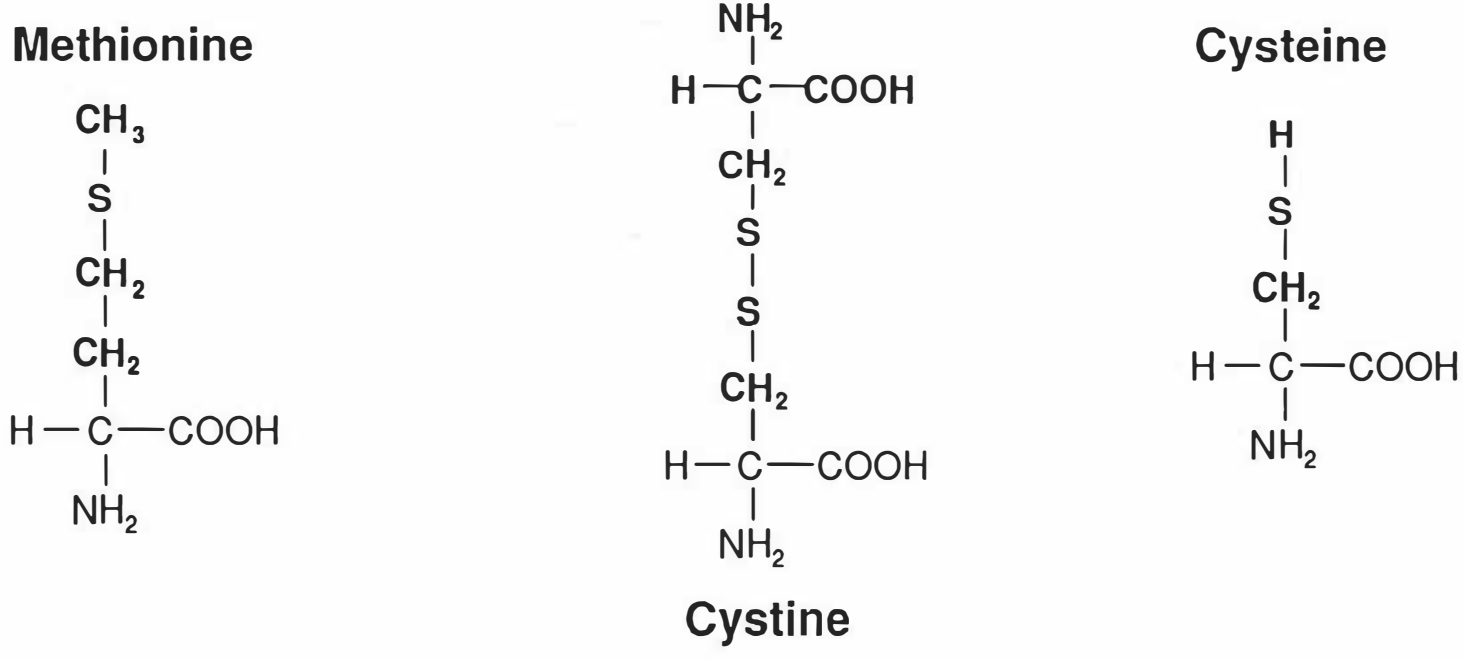


Fig. 2: Performic acid oxidation of sulphur amino acids in proteins to cysteic acid and methionine sulphone for amino acid analysis (Meister, 1965).

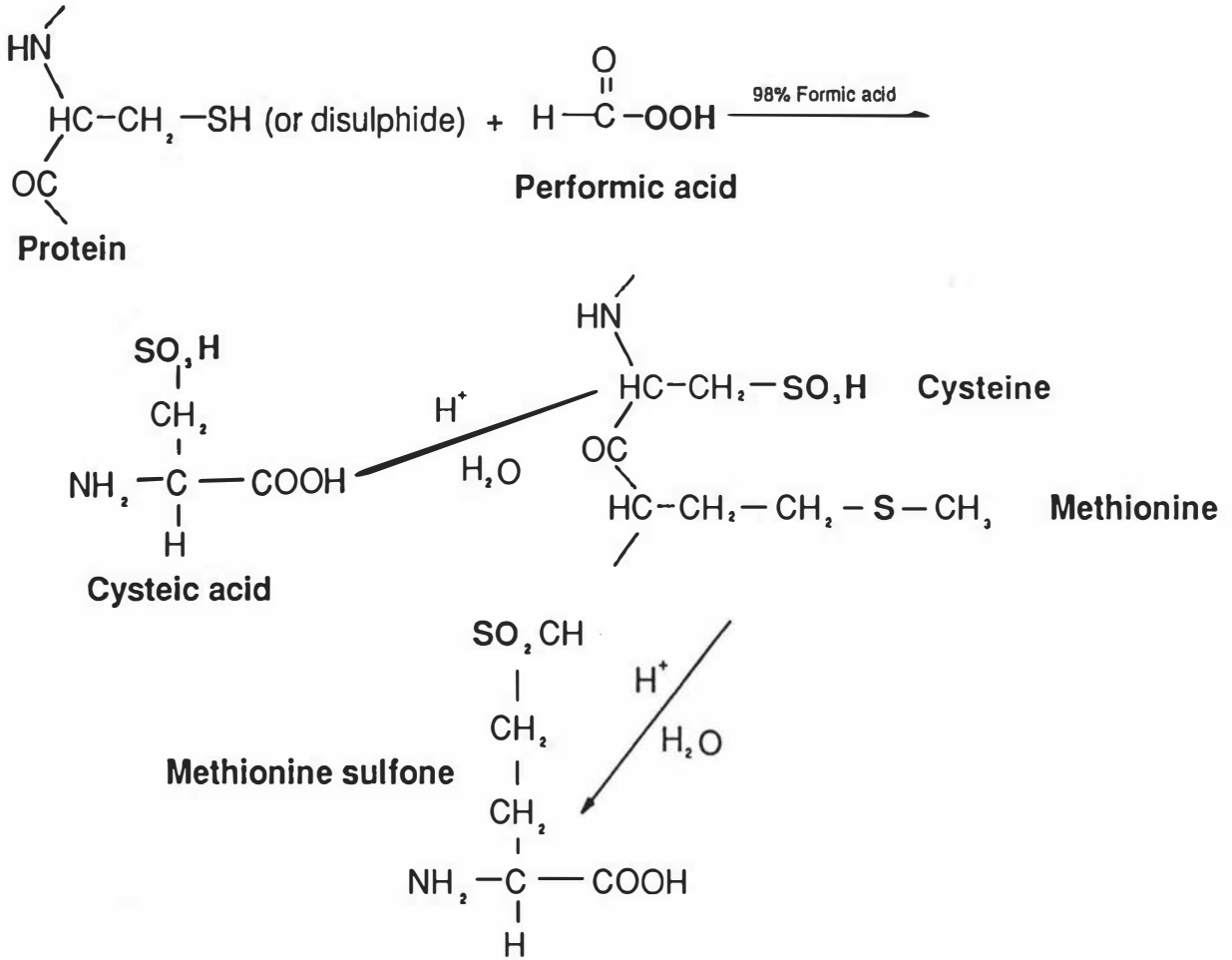


Table 1: The concentration of sulphur and nitrogen in some commonly used forage-based diets in New Zealand and Australia.

AUTHOR	DIET	NITROEN (gN/kgDM)	SULPHUR (gS/kgDM)	N:S Ratio
Kennedy and Milligan (1978)	Brome Grass Pellets	19.6	1.44	13.6:1
	Lucerne Pellets	26.3	2.14	12.3:1
Rees et al., (1974)	Pangola Grass	13.0	0.90	14:1
Kennedy and Siebert, (1972b)	Spear Grass	3.9	0.80	4.9:1
Barry, (1981)	RG:WC Pasture *	29.1	3.00	9.7:1
Waghorn et al., (1990)	Perennial Ryegrass	37.0	3.40	10.9:1
	Red Clover	35.0	1.90	18.4:1
	Lucerne	33.0	3.20	10.3:1
	Lucerne Chaff	21.0	2.30	9.1:1
	Meadow Hay	17.0	2.20	7.1:1

(*) Perennial ryegrass:white clover pasture.

1.1.3 METABOLISM OF SULPHUR IN THE GASTROINTESTINAL TRACT.

Sulphur metabolism can be divided into two systems, ruminal and post-ruminal digestion.

1.1.3.1 RUMINAL DIGESTION.

The metabolism of sulphur in the rumen is summarised in Fig. 3, and can be considered as four major transactions:

(1) Protein-S from the ingested diet which escapes rumen degradation and passes into the duodenum. Kennedy and Milligan (1978) reported that in sheep fed a brome grass diet (19.6gN/kgDM, 1.44gS/kgDM), 81% of ingested organic-S, and on a lucerne diet (26.3gN/kgDM, 2.14gS/kgDM), 45% of ingested organic-S flowed into the abomasum undegraded. The remaining ingested organic-S entered the amino acid and peptide pool and was either utilised by microbes directly or reduced to sulphide

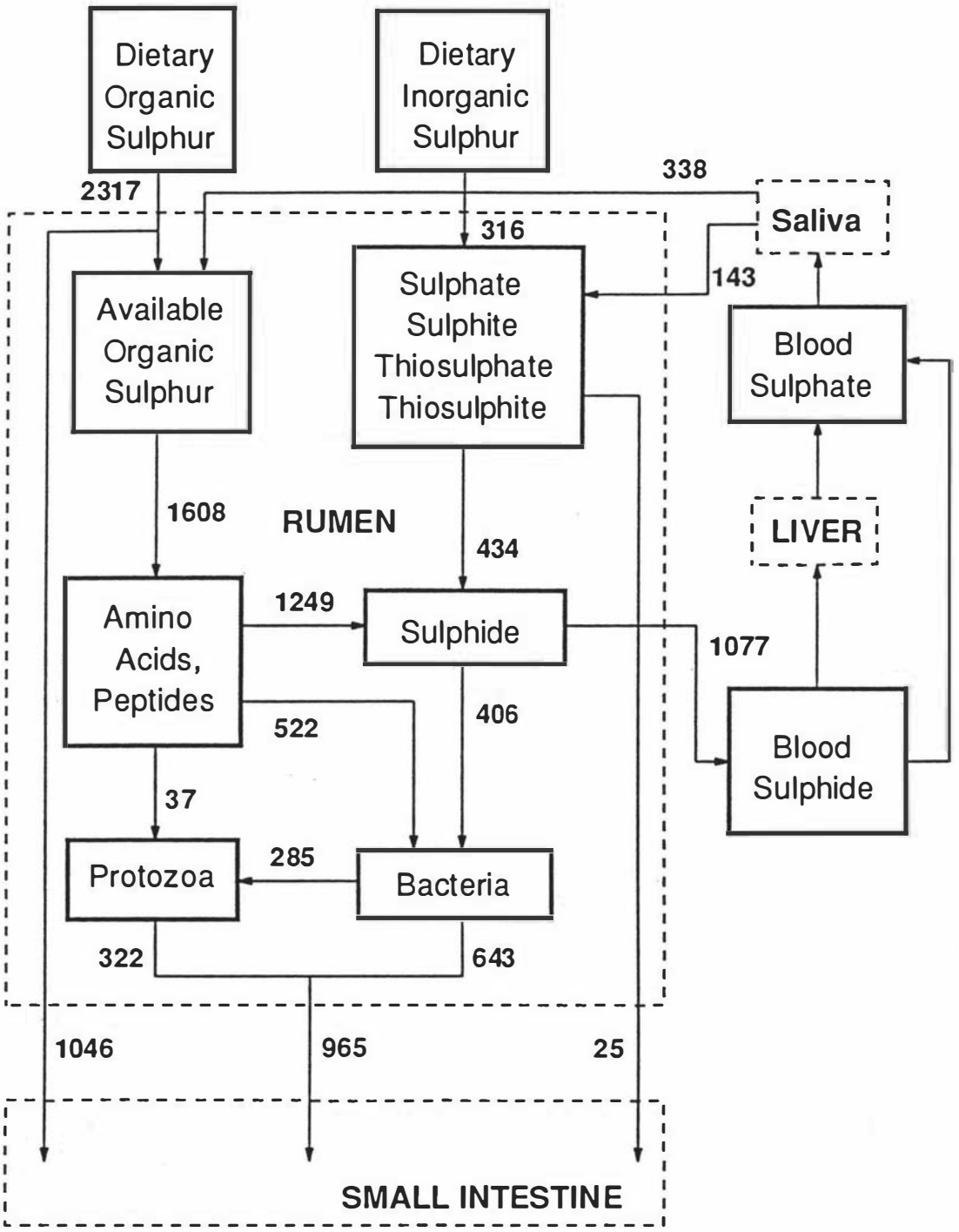
(2) Protein-S from the ingested diet which is degraded to polypeptides and amino acids, and used for microbial protein synthesis. Kennedy and Milligan (1978) reported that with a lucerne diet, sulphide, and free amino acids and peptides contributed respectively, 45 and 55% of the S for microbial protein synthesis, whilst for a Brome grass diet it was 52% and 48% respectively. Similarly, Gawthorne and Nader (1976) suggested rumen bacteria derived 53-57% of their protein-S from rumen sulphide.

(3) Dietary inorganic-S, polypeptides and amino acids, which are reduced to sulphide, may be either, absorbed directly from the rumen or converted by rumen microbes into protein. Three categories of rumen microorganisms are able to reduce inorganic and organic-S to sulphide. They include:

(a) Dissimilatory sulphate reducers (10^2-10^8 bacteria/ml rumen fluid; Coleman, 1960) which produce large quantities of sulphide (Bird and Moir, 1971). (b) Assimilatory sulphate reducers which only reduce sufficient sulphate to meet their own nutritional requirements. (c) Bacteria that are unable to supply their own nutritional requirements for reduced S (Bray and Till, 1975).

Rumen sulphide concentration is dependent on intake and sulphur content of the diet, but a range of 0.6-288μgS/ml has been reported (Bray and Till, 1975). The minimum rumen concentration of sulphide required to support maximum bacterial growth has been reported

Fig.3: A model of sulphur metabolism, showing the movement of S (mgS/d) between pools in the rumen and blood of sheep fed lucerne (Kennedy and Milligan, 1978).



as 1mgS/l (Bray and Till, 1975) and 3.8mgS/l (Kandylis, 1984). This can be compared with a minimum ammonia (NH_3-N) concentration of 50mgN/l required to support maximum rates of rumen microbial synthesis (Satter and Roffler, 1976). The optimal N:S ratio reported by Bird (1972 and 1974) was 10 to 15:1, which suggests 3.8mgS/l maybe the minimum concentration required.

Sulphide absorption from the rumen is concentration dependent, with a half-life in the rumen of 10-15 min (Bray, 1969). In solution, the dissociation of hydrogen sulphide can be represented by:

$$H_2S \rightleftharpoons HS^- + H^+$$
$$HS^- \rightleftharpoons H^+ + S^{2-}$$

Undissociated hydrogen sulphide (half-life of 8.2 min) is absorbed from the rumen more rapidly than the sulphydryl ion (half-life of 29.4 min; Bray, 1969). This is similar to ammonia, which exists in the rumen as either ammonia or the ammonium ion, with ammonia being absorbed more rapidly than ammonium (Hogan, 1961). Therefore, rumen pH of 6.0-7.0 favours retention of ammonia relative to sulphide, as 99% of rumen ammonia would be present as the ammonium ion (half-life of 150 min; Hogan, 1961) while 35-95% of rumen sulphide would be present as hydrogen sulphide (half-life of 8.2 min). Kennedy and Milligan (1978) reported that 35% of total-S entering the rumen, was absorbed from the rumen as sulphide in sheep fed a lucerne diet, whilst only 10% was absorbed with a brome grass diet.

(4) The outflow of microbial protein into the duodenum. The flow of digesta into the abomasum of sheep is generally 10-16 l/d/kgOMI (Bird and Thornton, 1972; Grovum and Williams, 1973; Weston and Hogan, 1971), so that in diets that provide 0.6-6.6gS and have N:S ratios between 6.5 and 35:1, 1-1.5gS/d will flow into the abomasum. This S comprises; inorganic-S (3-5%), sulphide (0.4-2.8%), ester-sulphates (3%) and neutral-S (92%). Neutral-S was 68% protein-S (Bird and Hume, 1971; Bird and Moir, 1972). Kennedy and Milligan (1978) reported that in sheep fed a brome grass diet, 88% of ingested total-S flowed from the rumen, of which 23% was bacterial-S and 65% was undegraded plant-S, whilst with a lucerne diet, 65% of ingested total-S flowed from the rumen of which 31% was bacterial-S and 51% was undegraded plant-S.

1.1.3.2 POST-RUMINAL DIGESTION.

1.1.3.2.1 The Digestibility of Sulphur in the Small Intestine.

Moir (1970) reported that the digestibility of total-S in the small intestine of sheep fed oat hulls (600gDM/d) was 36-44%. The digestibility of bacterial-S was 31-71% (Bird, 1972; Judson *et al.*, 1975) and protein-S was 26.3-41.3% (Doyle and Moir, 1980) in sheep fed lucerne based diets (500-1000gDM/d). Armstrong and Hutton (1974) reported that the true small intestine digestibility of cystine was 31-57% and methionine was 64-88% in sheep fed on a range of forage diets.

1.1.3.2.2 The Absorption of Sulphur Amino Acids.

Egan *et al.* (1984) reported that on wheaten hay diets fed to sheep (800gDM/d), the apparent absorption from the small intestine was 29mg methionine/h and 17mg cysteine/h (Table 2), although very little experimental data is available concerning the absorption of SAA from the small intestine. Guerino and Baumrucker (1987) reported that the most active site for methionine absorption was the mid-ileum whilst the proximal jejunum was the least active site. Methionine absorption occurred by both, Na-dependent and Na-independent transport systems, whilst physiological concentrations of the competitive inhibitors, lysine, aminobutyric acid and cycloleucine did not inhibit methionine uptake (Guerino and Baumrucker, 1987).

1.1.4 Nitrogen and Sulphur Retention.

As both N and S will be deposited in the body as protein, it seems reasonable that their rates of retention should be related. Bray and Till (1975) suggested the following mathematical relationship between S and N retention for sheep fed a range of roughage diets:

$$N = 10.37S - 0.038 \qquad r = 0.952$$

1.1.5 RECYCLING OF SULPHUR.

Sulphide and sulphate are recycled in similar fashion to the ammonia-urea system originally described by McDonald (1948). Sulphide absorbed from the rumen and intestines is oxidised to sulphate in the blood and liver. Plasma sulphate becomes distributed in the

Table 2: Absorption from the small intestine and plasma irreversible loss of methionine and cysteine in sheep fed wheaten chaff and lucerne hay diets (Egan et al., 1984).

	WHEATEN HAY		LUCERNE HAY	
	mg/h	g/d	mg/h	g/d
ABSORPTION FROM SMALL INTESTINE:				
METHIONINE	29	0.67	65	1.56
CYSTEINE	17	0.41	27	0.65
PLASMA IRREVERSIBLE LOSS:				
METHIONINE	91	2.18	129	3.10
CYSTEINE	35	0.84	37	0.89
FLOW OF IRREVERSIBLE LOSS TO WOOL: *				
METHIONINE	20	0.48	28	0.67
CYSTEINE	6	0.14	6	0.14

(*) Assumes that 21.5% of methionine and 16.5% of cysteine IRL flows to wool (Downes et al., 1970).

extracellular fluid, from which it can be recycled to the large intestine or rumen via the saliva or excreted in the urine (Bray, 1969).

1.1.5.1 Rumen Recycling.

Saliva provides the majority of recycled sulphur, and in sheep accounts for between 70 to 180mgS/d (Bray.1964; Bray and Till, 1975), although up to 500mgS/d can be recycled in this fashion (Moir, 1970; Kennedy and Milligan, 1978). Nolan and Leng (1972) reported that 30% of NH_3-N incorporated into microbial protein was recycled to the rumen NH_3 pool. Walker and Nader (1968 and 1975) were unable to demonstrate similar recycling of bacterial-S to the sulphide pool. In sheep fed diets providing low concentrations of S (less than 1.3gS/kgDM), there are direct benefits for microbial protein synthesis from S recycling in the rumen (Bray and Till, 1975).

1.1.5.2 Intestinal Recycling.

The bile-pancreatic secretions in sheep contributed 142-245mgS/d (Bird, 1972; Bird and Thornton, 1972) and plasma proteins contributed 80mgS/d (Campbell *et al.*, 1961). Mucosal cell turnover was 2-7mgS/d/kgBW in sheep, with the gut wall containing 70mgS/kgBW, but the proportion entering the gut was unknown (Till *et al.*, 1973).

1.1.6 EXCRETION OF SULPHUR.

The excretion of S in urine and faeces will depend on the intake and the sulphur content of the diet.

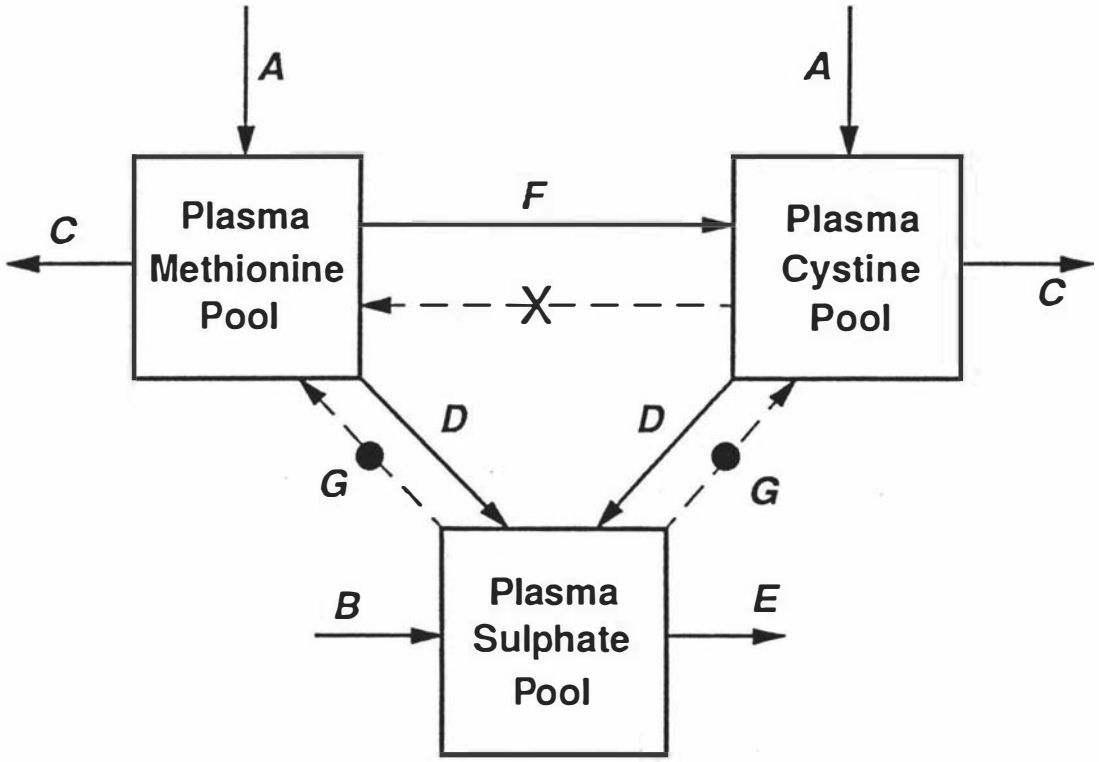
Downes *et al.* (1970) reported that in sheep fed wheaten/lucerne hay (800gDM/d), 10% and 8-13% of the S in abomasally infused methionine appeared in the urine and faeces respectively. Bird and Moir (1972) reported similar figures, although with ruminal infusions of methionine, 30 and 39% of the S appeared in the faeces and urine respectively. The higher values indicate the extent to which methionine was degraded in the rumen.

1.1.7 METABOLISM OF SULPHUR IN THE BLOOD PLASMA.

The interconversion of SAA and their catabolism are summarised in Fig. 4.

(1) The inflowing arrows, (A) represent cystine and methionine entering the plasma from the digestive tract and whole body protein turnover.

Fig. 4: A compartmentalised three pool model indicating the exchanges between plasma methionine, cystine and sulphate. Arrows indicate the direction of flow.



X, In mammalian tissue, the conversion of cystine to methionine is not possible.

● In mammalian tissue, the synthesis of sulphur amino acids from inorganic sulphate is not possible. However, in ruminants, plasma sulphate can return to the rumen and be converted to cystine or methionine by bacteria.

(2) The inflowing arrow, (B) represents sulphate entering the plasma, principally from sulphide absorbed from the rumen.

(3) The outflowing arrows, (C) represent cystine and methionine leaving the plasma for use in productive processes (eg...body and wool growth) and maintenance of biochemical processes. The rate at which components leave the plasma and flow to productive processes, catabolism and maintenance without returning to the plasma within the time course of an experiment is defined as the irreversible loss rate (IRL is usually expressed as μmol/h).

(4) The arrows, (D) represent the oxidation of plasma SAA to sulphate.

(5) The outflowing arrow, (E) represents sulphate leaving the plasma and either re-entering the digestive tract, principally in the saliva, or being excreted in the urine.

(6) The arrow (F) represents the transulphuration of methionine to cysteine. Cysteine is readily exchangeable with cystine. Cystine cannot be transulphurated to methionine in mammalian tissue (denoted X in Fig. 4).

(7) The arrows, (G) represent recycling of plasma sulphate to plasma SAA. Mammalian tissues can not convert sulphate directly to plasma SAA. Plasma sulphate-S that is recycled to plasma SAA, must first return to the rumen and be incorporated into microbial protein.

1.1.7.1 Interconversion of Sulphur Amino Acids.

The transulphuration and transamination of the sulphur amino acids in mammalian tissue is outlined in Fig. 5. In sheep fed either wheaten or lucerne hay 15-30% of plasma methionine was transulphurated to produce 25-30% of plasma cysteine (Egan *et al.*, 1984). Between 20 and 80% of plasma methionine can be catabolised to carbon dioxide and sulphate from low and high methionine intakes (Egan *et al.*, 1984).

The majority of transulphuration occurs in the liver and kidneys, with lower levels of activity in the pancreas, intestinal mucosa and muscle, whilst no transulphuration occurs in the wool follicle (Benevenga *et al.*, 1983) although, Downes *et al.* (1964) reported there was considerable activity in the skin.

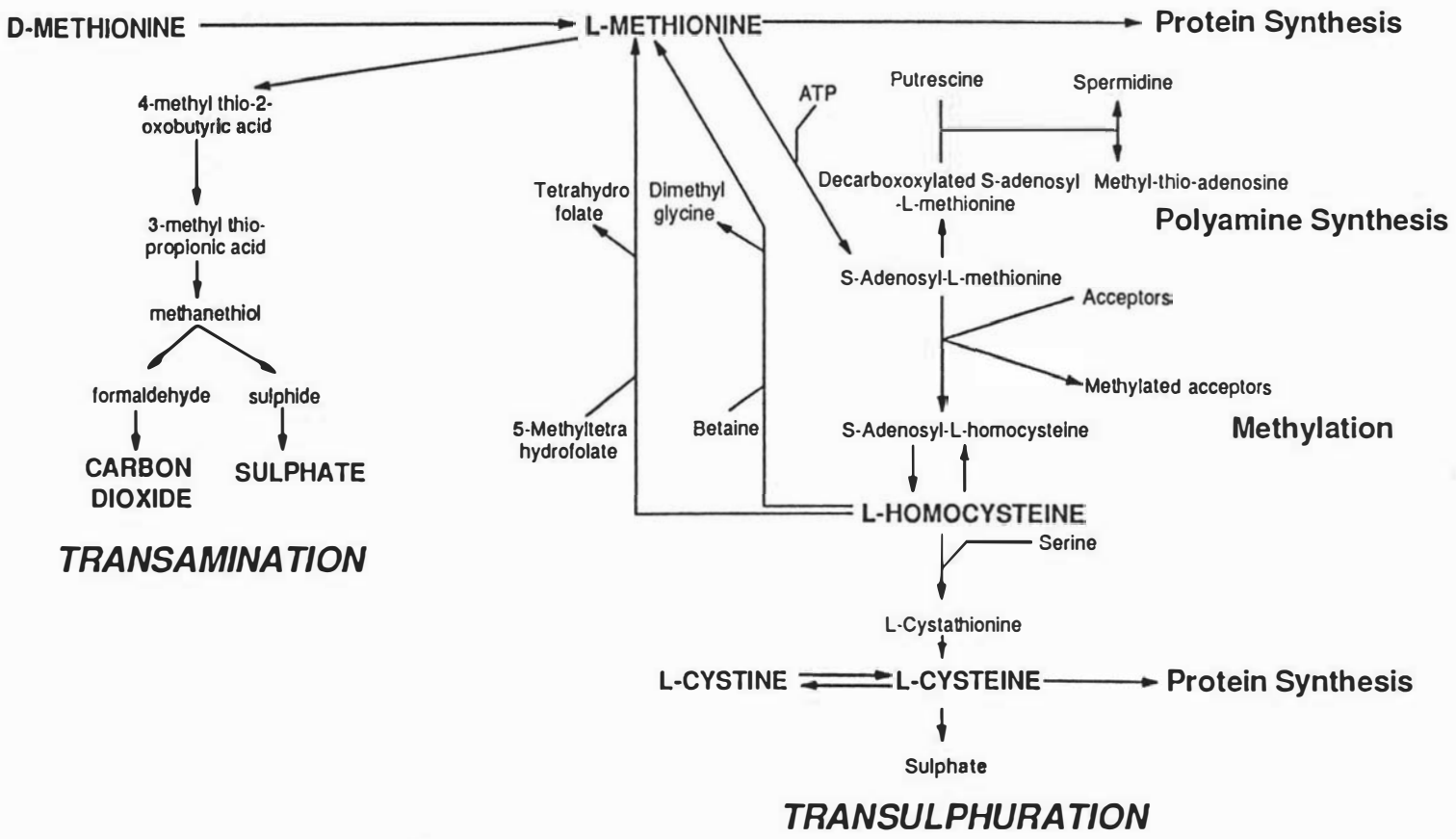


Fig. 5: Transulphuration and transamination of sulphur amino acids in mammalian tissue (Hemsley and Reis, 1984).

1.1.7.2 Irreversible Loss of Sulphur Amino Acids.

Egan *et al.* (1984) reported that the irreversible loss (IRL) of methionine and cysteine from the plasma of sheep was 91mg/h and 35mg/h for a wheaten hay diet (800gDM/d) and 129mg/h and 37mg/h for a lucerne hay diet (800gDM/d; Table 2). For both diets it would appear that insufficient methionine and cysteine were absorbed from the small intestine to meet the demands placed on the plasma SAA pools. The difference between absorption and plasma IRL would have to be supplied by whole body protein turnover. Buttery *et al.* (1975) reported that whole body protein turnover releases 130mg cysteine/h, but Williams (1973) reported that only a small proportion of the released cysteine enters the plasma pool because of recycling in body tissues. To met the difference between the cysteine absorbed from the small intestine and the cysteine irreversibly lost from the plasma reported by Egan *et al.* (1984), 14% of whole body protein cysteine turnover would need to enter the plasma cysteine pool.

1.1.8 WOOL.

1.1.8.1 An Introduction to Wool.

Clean dry wool is almost completely protein. The growth of a wool fibre is a continuous process, commencing with division of cells in the bulb region at the base of the follicle, followed by growth and migration of the cells. During growth, protein synthesis occurs and the process ends with keratinization and cell death. Keratinization is the oxidation of thiol groups from cysteine to form the disulphide bonds of cystine (Chapman, 1979).

Wool proteins are characterised by high sulphur content, with clean, dry wool containing 2.7-4.2% sulphur (Reis 1965a,b). The sulphur is present as cystine (measured as 8-15% half-cysteine residues) and 3% methionine (Reis and Schinckel, 1964). Harris *et al.* (1989) suggested that a significant proportion of amino acids used in whole body protein synthesis are supplied as peptides, so the presence of methionine in wool suggests that the wool follicle, although unable to use methionine directly (Hemsley and Reis, 1984), is able to incorporate methionine supplied as polypeptides. Wool is also rich in glutamic acid, serine and glycine (Marshall and Gillespie, 1977).

Soluble proteins from wool can be fractionated into:

(1) Low sulphur proteins which contain little cystine, all the methionine and most of the lysine and represent 66% of wool protein (Marshall and Gillespie, 1977).

(2) High sulphur proteins which are rich in cystine, proline and serine (Marshall and Gillespie, 1977) and represent 18-35% of wool protein (Broad *et al.*, 1970).

(3) High-tyrosine proteins which contain little cystine but are rich in tyrosine and glycine (Gillespie and Frenkel, 1972) and represent 1-12% of wool protein.

1.1.8.2 Wool Growth.

Wool growth depends on the number of wool follicles, the efficiency of conversion of feed to wool, competition from other productive processes and the protein absorbed from the small intestine.

1.1.8.2.1 Follicle Number.

The maximum number of wool follicles is genetically determined and varies with breed, but the maximum number of productive follicles ($12\text{-}62 \times 10^6$ per sheep; Black and Reis, 1979) is not often achieved due to nutritional stress (Corbett, 1979). Nutritional stress in the gestating ewe can lead to permanent reduction in the number of follicles in the developing foetus, although some compensation may occur through an increase in production per follicle (Schinckel and Short, 1961).

1.1.8.2.2 Efficiency of Feed Conversion to Wool.

Williams (1973) used Merino sheep selected for high and low fleeceweight and reported that at any given feed intake, high fleeceweight lines produced more wool, had a lower cystine plasma pool size and a higher incorporation of infused [^{35}S] cystine into wool. Thomson *et al.* (1989) suggested the principal difference for the higher voluntary feed intake of fleeceweight (F+) selected Romney sheep compared to unselected controls, fed lucerne chaff, was an increase in the particulate matter fractional outflow rate (h^{-1}; FOR) from the rumen to the abomasum. It was suggested that there was a corresponding increase in the fluid FOR. An increased fluid FOR is associated with an increased outflow of microbial protein from the rumen (Harrison *et al.*, 1975 and 1976). The combination of increased particulate matter and microbial protein outflow to the abomasum may be responsible for the observed differences between high and low fleeceweight lines.

Walker and Norton (1971) and Black *et al.* (1973) reported that provided adequate dietary energy is provided for survival, wool growth was dependent on the flow of protein to the small intestine.

1.1.8.2.3 Competition between Productive Processes.

The growth of the foetus may reduce wool growth of the dam by 30% during gestation (Langlands, 1977) with the reduction being greater for twin than single lambs. Changes in intake during pregnancy are small, so the change represents repartitioning of nutrients from wool growth to gestation (Corbett, 1979).

Lactation generally reduces annual fleece weight by 5-8% and somewhat more if lactation is prolonged (Corbett, 1979).

The annual cycle of reproduction reduces fleeceweight production by 10-14% and efficiency of its production to 60% of that of non-breeding ewes (Corbett, 1979).

1.1.8.3 NUTRITIONAL STIMULATION OF WOOL GROWTH.

1.1.8.3.1 Dietary Supplements of Sulphur Amino Acids.

Dietary supplements of protein and SAA are either ineffective or inefficient at stimulating wool growth unless the diet is deficient in sulphur relative to energy for microbial growth. Doyle and Bird (1975) demonstrated small increases in wool growth with large oral methionine supplements of 15.4g/d. Hemsley *et al.* (1973) reported small increases in wool growth after oral supplementation of sheep with casein. However, when the casein was protected from rumen degradation by formaldehyde treatment, the increase in wool growth was substantially greater. Barry and Manley (1985) suggested that 29% of orally supplemented methionine was washed out of the rumen into the abomasum, therefore, the variability of wool growth responses to orally administered methionine may be due to variation in the extent with which the fluid FOR exceeds the rate of degradation of methionine in the rumen.

1.1.8.3.2 Abomasal, Duodenal and Parental Supplements.

Responses to post-ruminal supplementation of SAA are much greater and more repeatable than oral supplements of SAA. On a range of roughage diets (800gDM/d to *ad lib.*) fed to sheep, the increase in wool growth was 50-100% compared to similarly fed sheep not receiving methionine or cystine infusions (Reis, 1979; Table 3).

In sheep eating a dried roughage diet, providing 1-1.5gSAA/d, maximum responses in wool growth were seen with abomasal infusions of 1-2g/d of methionine and 8g/d of cystine or

Table 3: The effect of post-ruminal SAA supplementation on wool growth in sheep fed forage diets. The wool growth response indicates the percentage increase in wool growth after the treatment.

Author	DIET	ROUTE	AMINO ACID	RATE OF SAA (g/d)	WOOL GROWTH		RESPONSE (%)
					BEFORE	AFTER	
Dove and Robards,(1974)	Lucerne Chaff (720gDM/d)	A	M	2.6	9.1*	12.4*	36
	Wheat Chaff (900gDM/d)	A	M	2.6	7.6*	9.3*	22
Radcliffe et al., (1985)	Lucerne Hay (800gDM/d)	A	Ce	3.9	9.2*	13.4*	46
Williams et al., (1972)	Lucerne/Wheaten Hay (800gDM/d)	A	C	2.0	9.3*	13.1*	41
	Lucerne/Wheaten Hay (800gDM/d)	A	M	2.5	9.3*	14.4*	55
Downes et al., (1970)	Lucerne/Wheaten Hay (800gDM/d)	IV	Ce	2.0	0.3#	0.4#	34
		IV	M	2.5	0.2#	0.2#	33
		IP	Ce	2.0	0.2#	0.3#	83
		IP	M	2.5	0.3#	0.4#	56
Reis et al., (1973)	Lucerne/Wheaten Hay (800gDM/d)	IV	C	2.0	0.14#	0.18#	28
		A	C	2.0	0.14#	0.18#	29
		IV	Ce	2.0	0.14#	0.19#	35
		A	Ce	2.0	0.14#	0.17#	24
Robards, (1972)	Lucerne/Oat Chaff (ad lib.)	A	M	2.6	7.4*	10.2*	37
Reis, (1967)	Lucerne/Wheaten Hay (800gDM/d)	A	M	2.5	4.1*	5.9*	104

(A) Abomasum, (IV) Intravenous, (IP) Intraperitoneal, (M) Methionine,

(Ce) Cysteine, (C) Cystine, (*) g/d and (#) mm^3/d

cysteine, whilst large infusions of methionine (6-10g/d) depressed wool growth (Reis *et al.*, 1973a).

Reis *et al.* (1973) reported quantitatively similar responses to equimolar post-ruminal infusions of methionine (2.5g/d), cystine and cysteine (2g/d) in sheep fed lucerne/wheaten hay (800gDM d).

Both D and L-forms of methionine were equally effective in stimulating wool growth. The liver contains a D-amino acid oxidaze which is able to catalyse the conversion of D- to L-methionine, whilst the wool follicle utilises L-cysteine only. (Bender and Krebs, 1950).

Intravenous and intraperitoneal infusions of methionine and cystine are effective at stimulating wool growth in sheep given a range of roughage diets, with the level of response being similar to that for abomasal infusions (Downes *et al.*, 1970a).

The administration of no other, single amino acid stimulated wool growth in sheep fed roughage diets, however the ratio of amino acids absorbed from the small intestine did. Gelatin, which is deficient in several essential amino acids (EAA), failed to stimulate wool growth when given abomasally, even when supplemented with methionine and cysteine (Reis and Schinckel, 1964). Similarly, Zein which is deficient in lysine, depressed wool growth but this effect was overcome by addition of lysine (Reis and Tunks, 1976).

This section has identified methionine and cysteine as the limiting amino acids for wool growth. Two methods were studied which have the potential for increasing the flow of sulphur amino acids to the small intestine. The first is the inclusion of condensed tannins in the diet, whilst the second is the use of genetic engineering to express the genes coding for proteins, which contain a high proportion of SAA, in the stems and leaves of important pastoral legumes.

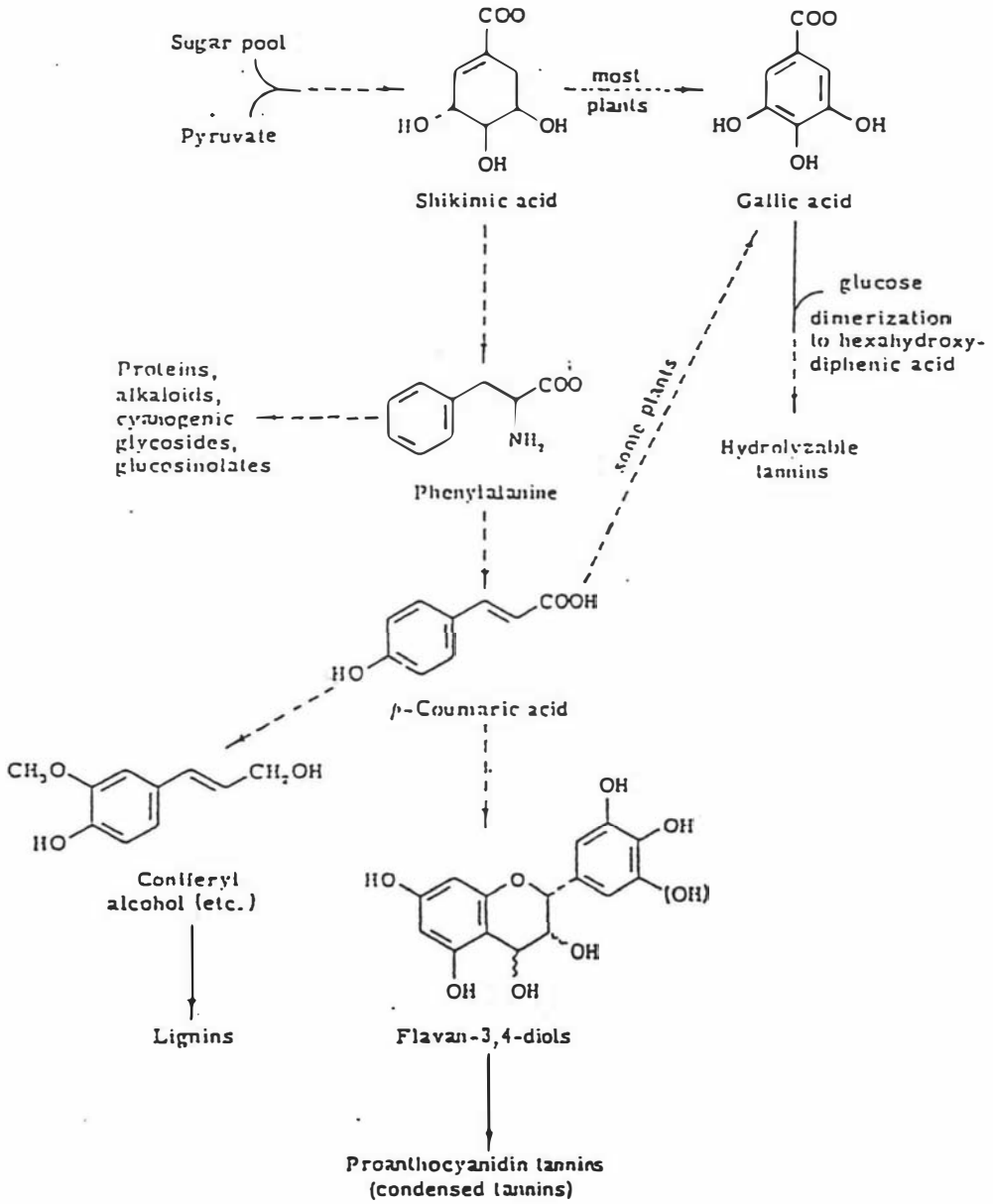
1.2 TANNINS.

Tannins can be chemically classified into two groups; the hydrolysable tannins and the condensed tannins (CT).

1.2.2 HYDROLYSABLE TANNINS.

The biosynthesis of hydrolysable tannins are shown in Fig. 6. Hydrolysable tannins have a carbohydrate core, which serves as a polyalcohol to which phenolic carboxylic acids bind by ester linkages (McLeod, 1974). Hydrolysis yields simple phenolic carboxylic acids, such

Fig. 6: Biosynthetic origins of lignin, condensed and hydrolysable tannins in plants (Swain, 1979).



as gallic acid (McLeod, 1974). Hydrolysable tannins are found in fruit pods and plant galls (McLeod, 1974) and have not been detected in grasses and legumes in New Zealand (Barry and Blaney, 1987). Hydrolysable tannins bind to protein at pH 3-4 but to a continuously decreasing extent above pH 5.0 (Loomis and Battaile, 1966). The inability of hydrolysable tannins to bind proteins at higher pH values (>5.0) suggest they won't have the same nutritional benefit as condensed tannins and will not be considered in this thesis.

1.2.3 CONDENSED TANNINS.

Condensed tannins occur in the leaves and stems of a restricted range of agricultural plants including sainfoin (*Onobrychis viciifolia*), birdsfoot trefoil (*Lotus corniculatus*), *Lotus pedunculatus*, crown vetch (*Coronilla varia*), dock (*Rumex obtusifolius*), sulla (*Hedysarum coronarium*) and the flower petals of white and red clover (*Trifolium repens* and *pratense*). They have not been detected in the leaves and stems of lucerne or temperate grasses (Jones *et al.*, 1976; Tables 4 and 5).

1.2.3.1 Biosynthesis of Condensed Tannins.

The biosynthesis of CT are shown in Fig. 6. Condensed tannins have no carbohydrate core and are products of the shikimic acid biosynthetic pathway (McLeod, 1974). Condensed tannins are produced from leucoanthocyanins (flavan-3,4-diols) and a second flavonoid, catechin (hydroxy-flavan-3-ol; Swain, 1979). Condensation of monomeric flavanols, such as delphinidin and catechin, produces complex polymers known as proanthocyanidins or condensed tannins (Swain, 1979).

Heating proanthocyanidins in dilute mineral acid breaks the polymer structure into its constitutive, strongly coloured monomeric flavanols, cyanidin and delphinidin (Mangan, 1988). Flavanols are hydroxylated, which enables CT to form extensive hydrogen bonds with oppositely charged reactive groups.

Jones *et al.* (1976) reported that CT from sainfoin (*Onobrychis viciifolia*) had a MW of 17-28000 which constituted 57-94 repeated flavanol monomers while in Grasslands "Maku" Lotus (*Lotus pedunculatus*) the MW was 6-7000 with 23-24 repeated monomers. However, Foo and Porter (1980) reported that the MW of CT from sainfoin was 3800, while for *Lotus pedunculatus* is was 6000. They attributed the anomaly to the ability of sainfoin CT polymers to self-associate in aqueous solution.

Table 4: Extractable condensed tannin concentration in some forage legume and browse species. (All determinations by the vanillin-HCL procedure of Broadhurst and Jones, 1978).

SPECIES	AUTHOR	CONDENSED TANNIN (g/kgDM)	LIGNIN (g/kgDM)	NITROGEN (g/kgDM)
SAINFOIN	Barry and Manley, 1986	29	ND	ND
	Ulyatt and Egan, 1979	ND	48	34
SULLA				
Spring *	Terrill unpublished	29	ND	22
Autumn *	Deadman unpublished	33	ND	33
WHITE CLOVER	Ulyatt and Egan, 1979	ND	23	ND
TREE WILLOW **	McCabe and Barry, 1988	29	182	18
OSIER WILLOW **	McCabe and Barry 1988	66	197	24
CANARY CLOVER #	Terrill unpublished	83	ND	ND
EUCALYPTUS *	Foley and Hume, 1987	200	98	16
MULGA **	Pritchard, Martin and Stocks, unpublished	65	231	17

(ND) Not determined (*) Butanol-HCL method

(**) leaves + stems tips up to 5mm dia (#) leaves only

Table 5: Extractable condensed tannin concentration (g/kgDM) of Lotus species grown under high (H) and low (L) soil fertility. Tannin concentration determined by the vanillin-HCL method of Broadhurst and Jones (1978).

AUTHOR	SOIL FERTILITY	Lotus pedunculatus cv. MAKU	Lotus corniculatus cv. MAITLAND	Lotus corniculatus cv. EMPIRE
John and Lancashire 1981	H	20	15	3
Lowther et al., 1987	L	95	28	3
Barry and Forss, 1983	H	32	NA	NA
	L	78	NA	NA
Barry and Duncan, 1984	H	46	NA	NA
	L	106	NA	NA

(H), soil pH 5.5-6.5, adequate P and S status; (L), soil pH 4.5-5.2, deficient in both P and S.
NA, Not applicable.

1.2.3.2 The Evolutionary Significance of Condensed Tannins.

Swain (1979) proposed that plants evolved CT production as a defence against invasion by bacteria and fungi, relying on the ability of CT to complex with proteins and polysaccharides to provide protection. It is possible that plants then evolved the ability to synthesize CT at concentrations in plant tissues high enough to dissuade predation by insects and herbivores. The concentration required to reduce predation by herbivores is probably 40-60gCT/kgDM (Barry, 1989), although sheep will eat *Lotus pedunculatus* leaves containing 80gCT/kgDM (Waghorn *et al.*, 1989). Most CT producing species are able to produce CT concentrations of 40gCT/kgDM or greater (Table 4 and 5).

Condensed tannins and lignin are both produced from the shikimic acid pathway (Fig. 6; Swain, 1979; Van Soest, 1982). Plants with high CT concentration can also be highly lignified (Table 4). Lignification is common to all plants, but CT are produced by only a few species, therefore CT may have evolved from a change in lignin biosynthesis. In low fertility soils, the prevalent habitat of CT producing species, the ability to produce CT at concentrations sufficient to reduce predation, would have conferred a survival advantage over plants unable to produce CT. Such an advantage would have provided positive selection pressure for the maintenance of genes coding for CT biosynthesis, in the plant's gene pool.

1.2.3.3 Factors Affecting CT Concentration in Plants.

The concentration of CT in various plants is affected by soil fertility, genotype and physiology, although environmental factors effecting CT biosynthesis are poorly understood. The effect of soil fertility on CT concentration is summarised in Table 5. In low soil fertility (pH 5.2), concentrations of CT in *Lotus corniculatus* (1.3-39gCT/kgDM) were lower than in *Lotus pedunculatus* (58-98gCT/kgDM), whilst the concentration in semi-erect *Lotus corniculatus* (1.3-8.4gCT/kgDM) was lower than in erect *Lotus corniculatus* (11.6-39gCT/kgDM; Lowther *et al.*, 1987).

1.2.3.4 Properties of Condensed Tannins.

1.2.3.4.1 Action of Condensed Tannin.

Condensed tannins react and form complexes by hydrogen bonding with both carbohydrates and proteins although at neutral pH they form stronger bonds with protein (McLeod, 1974). The strength of hydrogen bonding is both pH dependent and reversible. In

homogenised preparations of sainfoin, the CT bound and precipitated Fraction 1 leaf (F1) protein at pH 4.0-7.0 but the precipitate dissociated at pH values below 3.5 (Jones and Mangan, 1977). Therefore, it can be expected that at pH values common in the rumen (pH 5.5-7), stable complexes will form between CT and protein, which will dissociate under the acidic conditions of the abomasum (pH 1.3-3.5), releasing the proteins for enzymic digestion in the small intestine.

1.2.3.4.2 Condensed Tannin and Polyethylene Glycol.

Polyethylene glycol (PEG; MW 3350) preferentially binds with CT preventing CT reacting with protein or carbohydrate and displacing CT from CT:protein/carbohydrate complexes (Jones and Mangan, 1977; Barry and Manley, 1986). If PEG is included with a CT-containing forage the CT will still be present, but will be rendered unreactive in the rumen because of the preferential nature of the binding between CT and PEG. This provides a unique way of studying the effect of presence or absence of reactive CT without affecting the nutritive composition of the diet. Barry and Forss (1983) reported that for CT from *Lotus pedunculatus*, 1.5-2.0gPEG/gCT was required to render the CT unreactive.

1.2.3.4.3 Reactivity.

The reactivity of CT can be defined as the ability of CT to precipitate protein per unit weight. Jones *et al.* (1976) and Bate-Smith (1973) defined the reactivity of CT as astringency, which was determined by the colormetric estimation of haemoglobin remaining in solution after reaction of CT with the proteins of haemolysed blood.

The reactivity of CT is a function of two components:

(1) Molecular weight.

(2) Delphinidin content.

Jones *et al.* (1976) suggested the reactivity of high MW CT isolated from species such as sainfoin (MW 17-28000) is lower than for low MW CT isolated from species such as *Lotus* (MW 6-7000). In contrast, Horigome *et al.* (1988) reported that as the degree of polymerisation and therefore MW of CT increased, so did the reactivity. There are two conflicting explanations for the observation that, CT from sainfoin is less reactive than CT from *Lotus pedunculatus* and this contentious issue has yet to be resolved.

Jones *et al.* (1976) reported that as the delphinidin:cyanidin (PD:PC) ratio increased so did the reactivity. It would appear that when considering the reactivity of CT of largely differing MW, MW will be the primary determinant of reactivity, while when considering the reactivity of CT of similar MW, PD content will be the primary determinant of reactivity.

1.2.3.4.4 Interrelationship between Total and Free Condensed Tannin.

Lotus species have a total CT (TCT) content, of which a part can be termed free CT (FCT). Free CT is that proportion of TCT unable to be bound by plant protein in mascerates of fresh *Lotus* (Barry and Manley, 1986). The relationship between TCT and FCT in *Lotus* species is a two component system (Fig 7). For TCT concentrations up to 90g/kgDM the relationship is:

$$FCT\ (g/kgDM) = 0.15+0.099TCT \qquad r=0.856, \qquad P<0.001$$

For concentrations of TCT greater than 90g/kgDM the relationship is:

$$FCT\ (g/kgDM) = -61.3+0.73TCT \qquad r=0.797, \qquad P<0.10$$

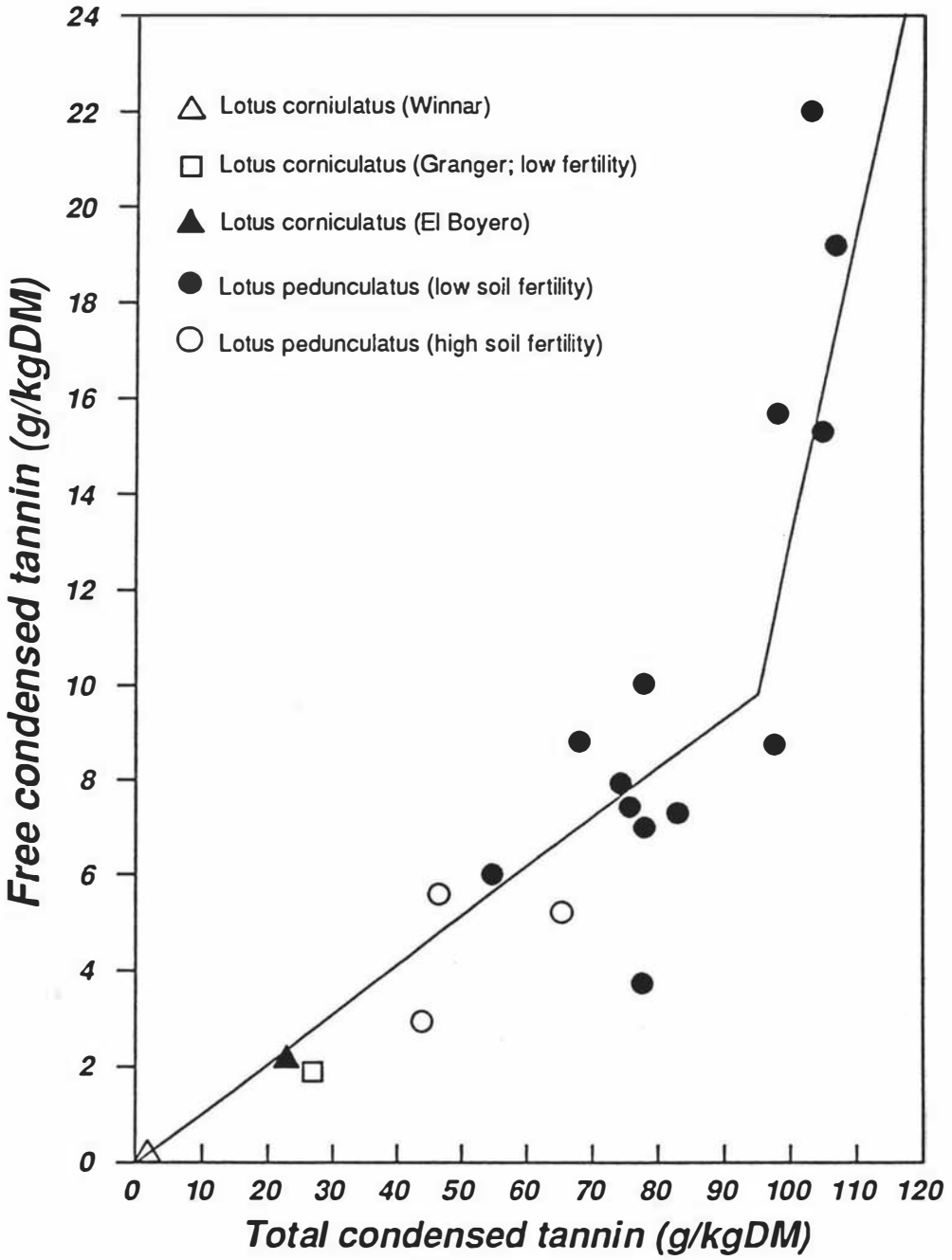
With TCT concentrations greater than 90g/kgDM the insoluble protein:CT complexes are nearing saturation so that the majority of increment increases in TCT appear as FCT (Barry and Manley, 1986). It has been proposed that the proportion of TCT able to bind to protein is nutritionally beneficial, preventing rumen degradation of plant proteins. However, FCT is nutritionally detrimental as it may be able to complex structural carbohydrate and bacterial enzymes in the rumen, reducing fibre digestion (Barry and Manley, 1986).

1.2.3.5 DETERMINATION OF CONDENSED TANNIN CONCENTRATION.

The presence of CT in plant tissue is determined by their conversion to cyanidins by hot mineral acid solutions. The red pigmentation of cyanidins, which have an absorbance maximum at 550nm, allows colormetric determination of CT concentration (Broadhurst and Jones, 1978).

The most widely used hot mineral acid solutions are n-butanol:conc-HCL in the ratio of 95:5 v/v (Swain and Hillis, 1959; Bate-Smith, 1954 and 1973) and vanillin:conc-HCL in the ratio 1:12.5 v/v (Broadhurst and Jones, 1978).

Fig. 7: Free condensed tannin concentration as a function of total condensed tannin in mascerates of fresh lotus species (Barry and Manley, 1986).



Total plant CT is defined by W.T. Jones (personal communication) as acetone extractable-CT + protein-bound CT (SDS-mercaptoethanol extractable) + structural carbohydrate-bound (residue) CT.

The vanillin-HCL (Broadhurst and Jones, 1978) and butanol-HCL (Swain and Hillis, 1959; Bate-Smith, 1954 and 1973) methods utilise an acetone extraction, which only measures the extractable-CT. The method of T.N.Terrill (personal communication), in addition to acetone, utilises sodium-dodecyl-sulphate (1% w/v):mercaptoethanol (5% v/v) to extract protein-bound CT, whilst structural carbohydrate-bound CT is measured in the residue remaining after both extractions.

Barry and Manley (1986) described TCT and FCT, which are not to be confused with the total and extractable-CT described above. Barry and Manley (1986) used an acetone extraction only, so the TCT described by Barry and Manley (1986) is only the total extractable-CT. The relationship between free, protein and residue-bound CT in plant tissue is not fully understood, but in general, acetone-extractable CT represents 75% of total CT in freeze-dried plant tissue (T.N.Terril, personal communication).

The butanol-HCL (Ribereau-Gayon, 1972) and vanillin-HCL (Swain and Hillis, 1959) methods can lack reproducibility of CT yield. Therefore, it is vital to standardize the method of CT quantification chosen. Due to variation in the reactivity of CT from different species it is important to use a CT standard for colormetric determination, prepared from the plant being studied. Lowther *et al.* (1987) reported a LSD of 17gCT/kgDM for CT concentrations from a range of plant species using a standardised vanilin-HCL method. This suggests caution should be exercised when comparing tannin concentrations which differ by less than 1.7% of the plant DM.

1.2.3.6 THE EFFECTS OF CT ON DIGESTION AND ANIMAL PRODUCTION.

1.2.3.6.1 Herbage Feeding Value.

Ulyatt (1973) defined herbage feeding value (FV) as the animal production potential of a herbage under a given set of environmental circumstances and described it mathematically as;

$$FV = \text{Animal Production} = f(\text{Intake} \times \text{Nutritive Value})$$

Ulyatt (1973) defined nutritive value (NV) as equal to utilisation, so whereas FV is the animal response to eating a herbage, NV is the response per unit intake. Condensed tannins

have their effect on FV by influencing both I and NV. The effect of CT on NV is mediated through the effect of CT on both protein and structural carbohydrate digestion.

1.2.3.6.2 Voluntary Feed Intake.

Barry and Duncan (1984) demonstrated that a reduction in reactive CT from 63gCT/kgDM to 7gCT/kgDM by spraying *Lotus pedunculatus* herbage with PEG (2g/gCT) prior to feeding increased voluntary metabolisable energy (ME) intake by 44%. Similarly, Foley and Hume (1987) found that in brushtailed possums fed Eucalyptus leaves containing 200gCT/kgDM, PEG treatment increased voluntary ME intake by 67%. In both cases the effect was mediated through increased DM intake and fibre digestion. These results show that CT concentrations at or above 63gCT/kgDM depress voluntary feed intake, but there is little evidence to accurately define the CT concentration at which voluntary intake begins to decline in ruminants fed CT-containing forages.

1.2.3.6.3 Readily Fermentable Carbohydrate.

Ulyatt and Egan (1979) reported that 90% of water soluble carbohydrate (WSC) and 95-100% of pectin were digested in the rumen when sheep were fed fresh ryegrass and white clover of a high digestibilty (0.7-0.8).

In a comparison between low and high CT *Lotus pedunculatus* (46 and 106g/kgDM respectively), high CT caused a small reduction in WSC and pectin digestion in the rumen, but this was compensated for by an increased digestion in the small intestine (Barry and Manley, 1984).

1.2.3.6.4 Structural Carbohydrate and Lignin.

The structural carbohydrates, cellulose and hemicellulose together with lignin, make up the fibre component of plants. Ulyatt and MacRae (1974) reported that the digestibility of hemicellulose and cellulose was 76-90% and 87-92%. whilst 90 and 82% of digestion took place in the rumen, when sheep were fed fresh ryegrass and white clover of a high digestibility (0.7-0.8).

Low concentrations of CT (22g/kgDM) in *Lotus corniculatus* (Waghorn *et al.*, 1987) had no effect upon rumen fibre digestion. Increasing the CT concentration from 14g/kgDM to 95g/kgDM lowered the proportion of hemicellulose, cellulose and lignin digestion taking place in the rumen, with the reduction being greatest for lignin (Barry *et al.*, 1986). However, there was a corresponding increase in post-ruminal digestion of the three

fractions, with the increase being smallest for lignin. Therefore, increasing the dietary concentration of CT had no effect on the overall apparent digestibility of hemicellulose and cellulose but lowered the overall apparent digestibility of lignin (Barry *et al.*, 1986).

1.2.3.6.5 NITROGEN.

The effect of CT on nitrogen metabolism can be divided into two systems, ruminal and post-ruminal digestion.

1.2.3.6.5.1 Nitrogen Digestion in the Rumen.

A model of nitrogen metabolism in the rumen is presented in Fig. 8. Nitrogen metabolism in the rumen can be divided into five major transactions;

(1) Protein-N from the ingested diet which escapes rumen degradation and passes into the abomasum.

(2) Protein-N from the ingested diet and non-urea-N from the saliva which is degraded to polypeptides and amino acids. Rumen microbes are able to utilise the polypeptides and amino acids for synthesizing microbial protein.

(3) The reduction of amino acids, peptides and urea from saliva to ammonia by rumen bacteria and the utilisation of ammonia for synthesis of bacterial protein.

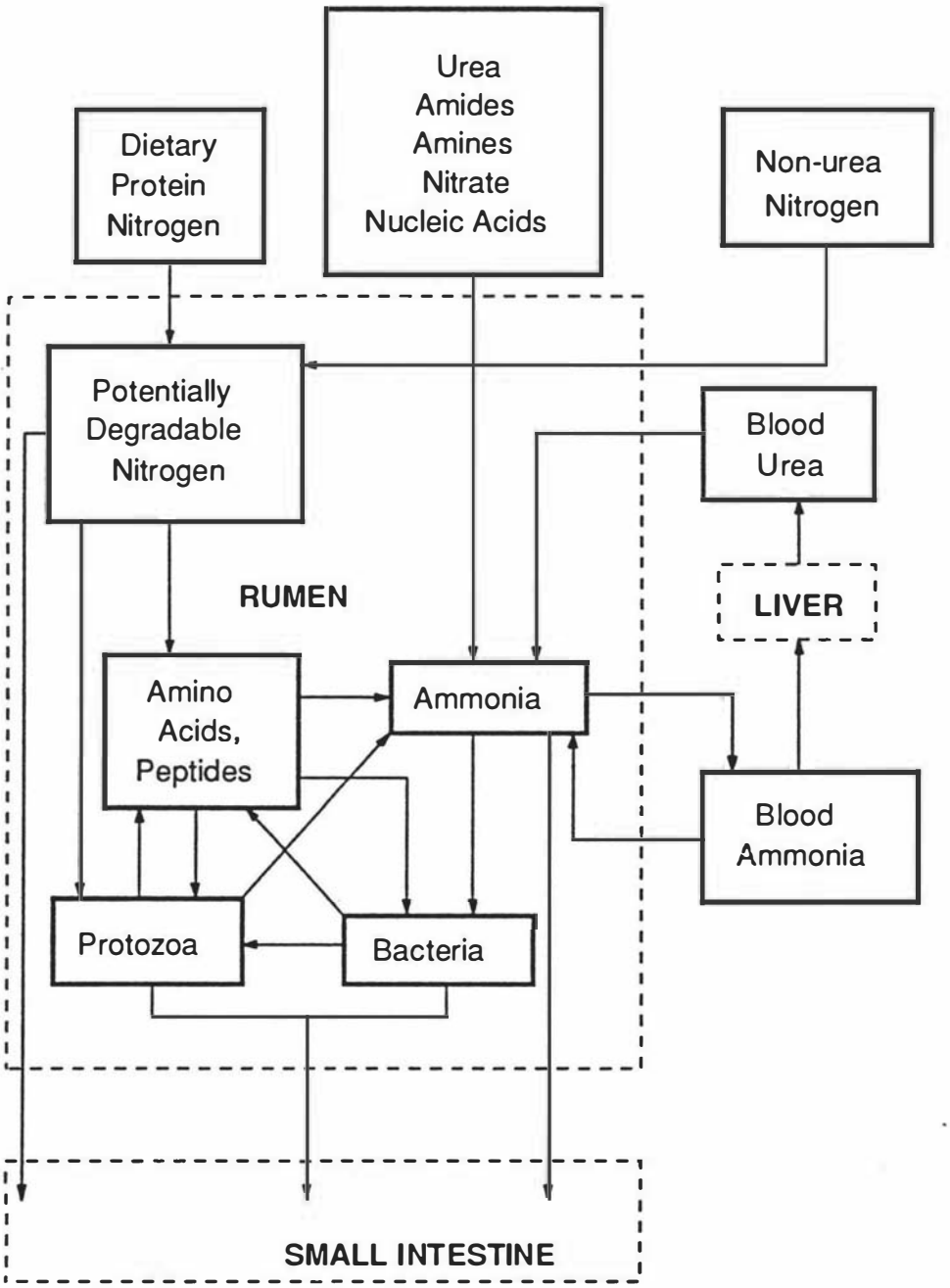
(4) The absorption of ammonia across the rumen wall into the blood and its conversion to urea by the liver. Urea is either excreted in the urine or returned to the rumen via the saliva.

(5) The outflow of microbial protein from the rumen into the abomasum.

In ruminants fed high quality forage diets, duodenal N flow is only 65-75% of N intake (MacRae and Ulyatt, 1974). The large loss of N across the rumen increases with increasing N intake (Ulyatt and Egan, 1979) and is due to the degradation of soluble protein (degradability 70%) to NH_3 (Ulyatt *et al.*, 1975) exceeding the capacity of rumen microorganisms to synthesize protein from NH_3. The result is that 25-33% of the N eaten is absorbed as NH_3 across the rumen wall (Beever and Siddons, 1986).

The presence of CT in the diet decreases rumen NH_3 concentration. Waghorn *et al.* (1987a) reported that in sheep eating *Lotus corniculatus* (22gCT/kgDM), intraruminal infusion of PEG increased rumen NH_3 concentration from 367mg/l to 504mg/l. Similarly, Barry *et al.*

Fig. 8: A model of the metabolism of nitrogen in the rumen (Leng and Nolan, 1984).



(1986) reported that when feeding sheep *Lotus pedunculatus* at three CT concentrations (95, 45 and 14g/kgDM) the rumen NH_3 concentrations were 275, 287 and 389 mg/l respectively. The addition of docks (17gCT/kgDM) to a lucerne diet being fed to cows, such that the final CT concentration in the diet was 0.17g/kgDM, decreased rumen soluble protein N from 71mgN/l to 48mgN/l (Waghorn and Jones, 1989). Proteins must be in solution before bacterial proteases can act efficiently (Hungate, 1966) and since ammonia is the main degradation product of soluble protein, these results suggest that the presence of CT in the diet reduces the degradation of protein to NH_3 in the rumen.

Tanner *et al.* (1990) reported that casein was completely degraded within 15min in an *in vitro* rumen assay while the large subunit (LSU) of Fraction 1 leaf (F1) protein was completely degraded after 1 hour. Addition of CT isolated from sainfoin to the *in vitro* assay at a CT:protein ratio of 1:2 resulted in casein and the LSU of F1 protein still being detectable after 2 hours of incubation indicating that the presence of CT prevented the degradation of the LSU of F1 protein.

1.2.3.6.5.2 Post-Ruminal Nitrogen Digestion.

The non-ammonia-nitrogen (NAN) flowing out of the rumen, into the abomasum consists mainly of undegraded plant and microbial protein. Figure 9 summarises the NAN:N intake flow out of the rumen for a number of commonly fed fresh forages.

Linear relationships have been established for *Lotus pedunculatus* and *Lotus corniculatus* which show that increasing CT concentration increases the ratio of NAN:N intake flowing out of the rumen. They have been summarised mathematically;

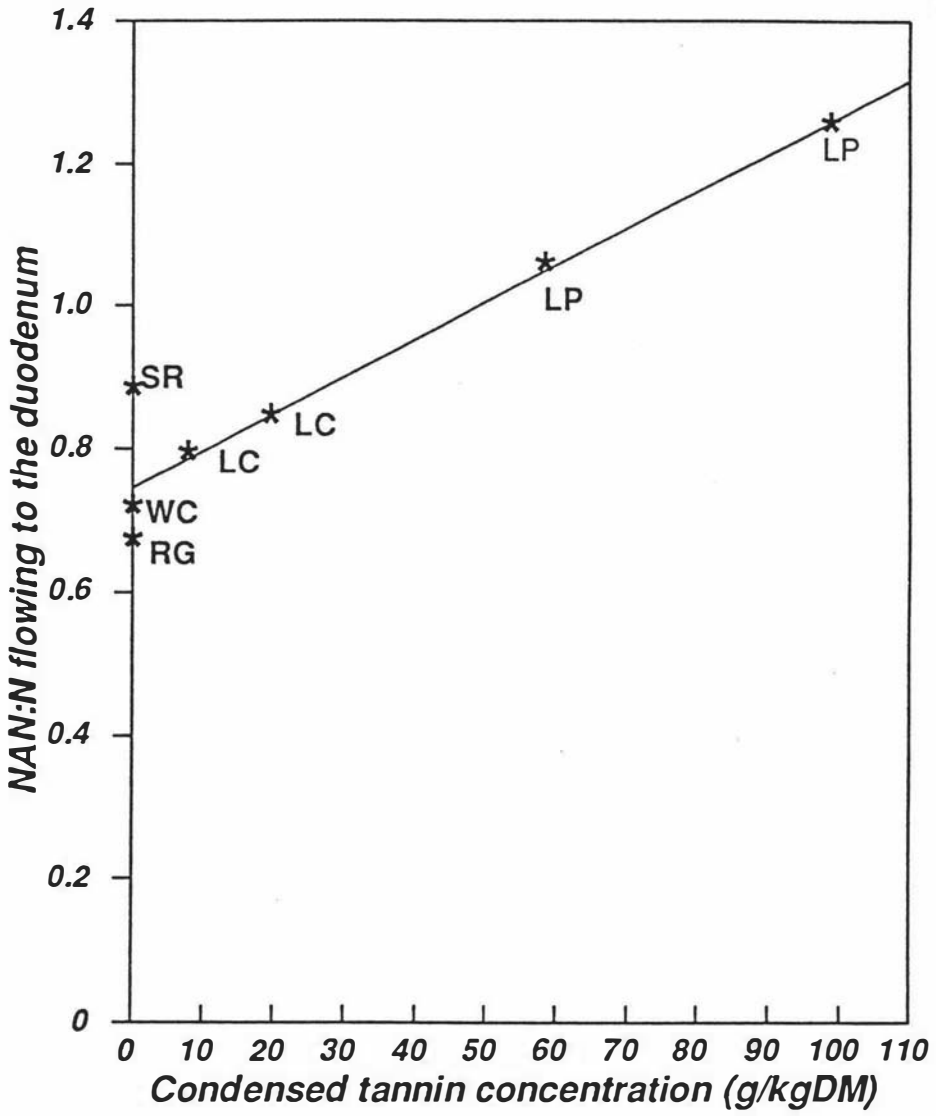
NAN:N=0.71+0.0045CT $r=0.66$ $P<0.05$ (Waghorn *et al.*, 1987).

NAN:N=0.54+0.0047CT $r=0.92$ $P<0.01$ (Barry *et al.*, 1986).

These equations apply to data gathered from experiments with *Lotus corniculatus* (5-30gCT/kgDM) and *pedunculatus* (14-95gCT/kgDM) and indicate that for Lotus CT, each increase in CT concentration of 10g/kgDM, increases NAN:N flow out of the rumen by 0.046 units, whilst at CT concentrations of 64-98g/kgDM the NAN flow out of the rumen equalled the N intake. The relationship between NAN flow out of the rumen and CT concentration in the diet will vary according to the reactivity of the CT being ingested.

Very little information is available concerning the effect of CT on the flow of microbial-N out of the rumen. Waghorn *et al.* (1987) reported that sheep eating *Lotus corniculatus* (cv

Fig. 9: Duodenal non-ammonia nitrogen (NAN) flow per unit of total-N intake in sheep fed Lotus pedunculatus, Lotus corniculatus short rotation ryegrass, perennial ryegrass and white clover (Barry et al., 1986).



References: Lotus pedunculatus (LP; Barry et al., 1986); Lotus corniculatus (LC; John and Lancashire, 1981); White clover (WC), short rotation (SR) and perennial (RG) ryegrass (MacRae and Ulyatt, 1974).

Empire; 4.6gCT/kgDM) had a higher flow of microbial-N out of the rumen than sheep eating *Lotus corniculatus* (cv Maitland; 32gCT/kgDM).

Waghorn *et al.* (1987) reported that the essential amino acids (EAA) for sheep were threonine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine, whilst the non-essential amino acids (NEAA) were asparagine, serine, glutamate, proline, glycine and alanine. In sheep fed *Lotus corniculatus* (22gCT/kgDM), the presence of CT increased the flow of EAA to the abomasum by 50% and NEAA by 15%. The apparent absorption of EAA from the small intestine was increased by 62%, whilst the apparent absorption of NEAA was decreased by 10% (Table 6). The reason why CT are able to preferentially effect amino acid absorption is unknown. The SAA were not measured in this study.

1.2.3.6.6 SULPHUR.

The effect of feeding *Lotus corniculatus* (22gCT/kgDM) on total-S absorption from the small intestine in sheep is summarised in Table 7. Waghorn *et al* (1987) reported that abomasal flux of total-S was increased from 2.73g/d to 3.38g/d and S absorption from the small intestine was increased from 0.94g/d to 1.38g/d in sheep eating *Lotus corniculatus* (22gCT/kgDM). No other experimental data are available which investigates the effect of CT on sulphur and sulphur amino acid metabolism in the ruminant, although since CT increased abomasal flux and post-ruminal absorption of total-S to a greater extent than total-N in the studies of Waghorn *et al.* (1987) it is probable that CT has a similar effect on SAA as it does on other EAA.

1.2.3.6.7 ANIMAL PRODUCTION.

Table 8 describes the FV and LWG for some commonly used forage species in New Zealand.

High concentrations of CT (75-90g/kgDM) depress both VFI and fibre digestion, so it is not surprising that daily PEG administration increased LWG in lambs grazing *Lotus pedunculatus* (75-90gCT/kgDM) by 50g/d (Barry, 1985). Low concentrations of CT do not depress VFI or fibre digestion (Waghorn et al., 1987), so growth rates should be relatively high.

Low concentrations of *Lotus* CT (10-30g/kgDM) also reduced carcass fatness in growing lambs (Purchas and Keogh, 1984).

Table 6: Amino acid (AA) flow (g/d) through the digestive tract of sheep fed Lotus corniculatus (22gCT/kgDM) with and without PEG infusion (Waghorn et al., 1987).

	ESSENTIAL AMINO ACIDS		NON-ESSENTIAL AMINO ACIDS	
	CONTROL	PEG	CONTROL	PEG
AA INTAKE (g/d)	98.9	98.9	97.9	97.9
ABOMASAL FLUX (g/d)	84.7	55.5	68.6	59.1
APPARENT ABSORPTION FROM SI				
(g/d)	58.8	36.2	37.4	41.3
proportion intake	0.59	0.37	0.38	0.42

Table 7: Comparison of apparent absorption of sulphur and nitrogen in sheep fed Lotus corniculatus (22gCT/kgDM; Waghorn et al., 1987).

	CONTROL SHEEP	PEG INFUSED SHEEP
TOTAL N INTAKE (g/d)	37.8	37.8
ABOMASAL TOTAL N		
(g/d)	33.4	29.8
proportion intake	0.88	0.79
TOTAL-S INTAKE (g/d)	3.7	3.7
ABOMASAL TOTAL-S		
(g/d)	3.4	2.7
proportion intake	0.92	0.74
POST-RUMEN S ABSORPTION		
(g/d)	1.4	0.9
proportion intake	0.38	0.26

Table 8: The comparative feeding value in terms of sheep LWG of some pasture species grown in New Zealand (Ulyatt, 1981). All values relative to white clover. (From Barry, 1989)

SPECIES	RELATIVE FEEDING VALUE	GROWTH RATE g/d
WHITE CLOVER 'Grasslands Huia'	100	250
LOTUS PEDUNCULATUS 'Grasslands Maku'		
ND **	84	210
75-90gCT/kgDM *	ND	85
SAINFOIN 'Melrose'		
ND **	84	210
LUCERNE 'Wairau'	82	205
ITALIAN RYEGRASS 'Grasslands Paroa'	83	208
PERENNIAL RYEGRASS 'Grasslands Ruanui'	52	130

* Barry, 1985

** Not determined

Work presently being done at Massey University with sulla (*Hedysarum coronarium*), a CT containing legume, suggests wool growth is increased by CT. Sheep grazing sulla (48gCT/kgDM) produced 117mg wool/100cm²/d while sheep grazing sulla and receiving daily drenches of PEG produced 105mg wool/100cm²/d (T.N.Terrill *et al.*, personal communication).

1.2.3.6.8 FEEDING VALUE AND CT; SOME CONCLUSIONS.

Ulyatt (1973) suggested that the NV of a herbage is often divided into two components;

(1) Apparent digestibility (Dig %) of the herbage:

$$\text{Dig \%} = 100 \times \frac{(\text{Intake - Faeces})}{\text{Intake}}$$

(2) The efficiency of utilisation of digested nutrients for maintenance and production.

Table 9 describes comparisons between sainfoin, low CT *Lotus*, and herbage without CT which were fed to sheep at similar levels of intake. The observations, except source 8 refer specifically to low CT concentrations (upto 34g/kgDM), which did not reduce voluntary feed intake (Waghorn *et al.*, 1989). Although, the apparent digestibility of N in the CT-containing feeds were 2-25% lower than equivalent CT-free herbage, N-retention or apparent N absorption from the small intestine was always higher. Therefore, the high NV of the CT-containing herbages, sainfoin and Grasslands 'Maku' Lotus, reported by Ulyatt (1981) would appear not to be due to changes in apparent digestibility, but rather to the effect low concentrations of CT (upto 34g/kgDM) has on improving the site of digestion of N and the biological value of the products of digestion, available for utilisation. At high concentrations of CT (>60g/kgDM), the potential benefits to animal production of improved NV are negated by reduced VFI and fibre digestion.

1.3 HIGH SULPHUR PROTEINS FOR USE IN PLANT GENETIC ENGINEERING TO IMPROVE NUTRITIVE VALUE.

Improving NV of forage legumes by expressing foreign genes coding for proteins, which contain a high proportion of SAA, in leaves and stems, using genetic engineering techniques relies on five criteria;

(1) The identification of suitable proteins.

Table 9: The nutritive value of sainfoin and effect of CT in Lotus on nitrogen retention and digestion relative to CT-free lotus and legumes fed to sheep at similar levels of intake (Waghorn et al., 1989).

PLANT COMPARISON	RESPONSE TO CT (%)		Change in N digestibility (%)	Source
Sainfoin v. W.C.	+18.4	Protein:Energy Abs *	-10	1
Sainfoin v. W.C.	+16.7	N digest in SI **	-8.4	2
E Sainfoin v. W.C.	+124	N retained	-11.9	3
L Sainfoin v. W.C.	+19	N retained	-17.9	3
Sainfoin v. Lucerne	+56	N digest in SI **	-25	4
Lotus, Mait v. Emp ~	+130	N retained	-6.5	5
Spring, Mait v. Emp	+29	N retained	-12	6
Autumn, Mait v. Emp	+340	N retained	-2.1	6
Mait v. Mait+PEG #	+62	EAA absorbed	-10.2	7
Maku v. Maku+PEG	+42	N retained	-18.5	8

* An increase in the ratio of protein absorbed:non protein energy absorbed.

** An increase in nitrogen digestion in the small intestine.

~ Empire has low CT content (1.5% in DM) and Maitland has 3.5% CT in DM.

PEG removes the effect of tannin.

Abbrevations: (L) late, (E) early, (WC) white clover, (Mait) Maitland, (Emp) Empire, (EAA) essential amino acids, (N) nitrogen, (SI) small intestine.

References: (1) Ulyatt et al., 1976, (2) Reid et al., 1974, (3) Egan and Ulyatt, 1980, (4) Thomson et al., 1971, (5) John and Lancashire, 1981, (6) Waghorn et al., 1987, (7) Waghorn et al., 1987, (8) Waghorn unpublished.

(2) The isolation and characterisation of genes that code for the proteins.

(3) A requirement for the protein to be resistant to degradation in the rumen, so that it may pass undegraded, into the abomasum.

(4) The mechanism which confers resistance to rumen degradation must be reversible in the small intestine of the ruminant, so that the AA in the protein can be released for absorption.

(5) High levels of foreign gene expression in transgenic plants needs to be achieved if foreign proteins are to have any nutritional benefit.

Four proteins that fit the first four criteria have been identified and are summarised in Table 10.

1.3.1 Ovalbumin.

Ovalbumin is the most abundant protein in chicken egg white, comprising 54% of the total protein (Gilbert, 1971). A complex glycoprotein, ovalbumin contains an amino-terminal acetyl group, carbohydrate residues and two phosphate residues (Lai *et al.*, 1980). Although, ovalbumin contains all the EAA required by the developing egg, its embryonic function is not fully understood (Gilbert, 1971). Ovalbumin is synthesized in the cells of the oviduct. The transcriptional product of the ovalbumin gene is 7.5 kilobases (kb) in length and consists of 8 introns (5.5kb) and 7 exons (2.0kb). Mature ovalbumin mRNA is produced after post-translational splicing to remove the introns (Lai *et al.*, 1980). A cDNA clone which codes for the entire primary ovalbumin translation product has been isolated and sequenced (T.J.Higgins, personal communication). Expression of the ovalbumin gene in mice (Lai *et al.*, 1980) and tobacco (T.J.Higgins, personal communication) results in a mature protein of MW, 41000. The reduction in MW compared to natural ovalbumin (MW 43000) is due to the inability of transgenic plants and animals to post-translationally glycosalate ovalbumin.

1.3.2 Thionins.

Endospermic thionins are synthesized on membrane-bound polysomes. After post-translational processing they appear to remain associated with the endoplasmic reticulum (Bohlmann and Apel, 1987), representing a large fraction of seed protein, although their exact biological function in the endosperm is unknown (Bohlmann and Apel, 1987). Recently, Reimann-Phillip *et al.* (1989) identified thionins which were either, specific to the leaf-cell wall or the leaf-cell vacuole. The relationship between endospermic and leaf-

Table 10: The molecular weight and sulphur amino acid content of the high sulphur proteins for which genomic or cDNA sequences have been isolated.

	MW	TOTAL AMINO ACID RESIDUES	METHIONINE RESIDUES	CYSTEINE RESIDUES	SAA as a % of TOTAL AMINO ACIDS
OVALBUMIN (1)	43000	354 *	16 ** (4.5)	5 ** (1.5)	6
THIONIN (2)	5000	46	0	8 (17.0)	17
PEA ALBUMIN 1 (3)	11000	104	1 (1.0)	10 (10.0)	11
SF8 (4)	11000	103	16 (15.5)	8 (7.5)	23

(1) Gilbert (1971); (2) Rodriquez-Palenzula (1988); (3) Higgins et al. (1986);

(4) Kortt et al. (1990); (*) Bull (1941a); (**) Fevold (1951).

SF8, sunflower albumin 8; (parenthses) % of the total amino acid residues; SAA,sulphur amino acids.

specific thionins is unknown, although they are coded for by separate genes (Reimann-Phillip *et al.*, 1989).

The thionins are small, strongly basic proteins which are rich in lysine as well as cysteine. Thionins occur in many dicotyledonous and monocotyledonous plants (Table 11). Bohlmann and Apel (1987) reported that the amino acid sequence homology of the thionins summarised in Table 11 was 65%, whilst conservation of cysteine number and position was almost 100%.

Endospermic thionins are synthesized as a large precursor of MW 15000, which undergoes two processing steps to yield mature thionin (MW 5000):

(1) Co-translational excision of a signal peptide.

(2) Post-translational elimination of a C-terminal acidic polypeptide (Rodriguez-Palenzuela *et al.*, 1988). This results in a functional thionin product, although the nature of the cleavage or the function of the C-terminal polypeptide is unknown (Bohlmann and Apel, 1987).

Reimann-Phillip *et al.* (1989) reported that hordothionin mRNA was present in barley seedlings grown in the dark, but the mRNA declined upon exposure of the seedlings to light. However, accumulated hordothionin protein was stable. Hordothionin mRNA reaccumulation could be stimulated by exposing the seedlings to stress or pathogenic attack. In general, thionins at concentrations as low as 5×10^{-4}mol/l (Bohlmann *et al.*, 1988) are toxic to yeast, bacteria, fungi and to mammals if administered intraperitonially or intravenously (Wada and Buchanan, 1981). These observations suggest that the biological role of thionins is to protect plants against pathogenic attack.

1.3.3 Seed Albumins.

The seed storage proteins of dicotyledonous plants contain two major protein classes, globulins and albumins, which can be separated by their solubility;

(1) The globulins are characterised by high levels of arginine, glutamine and asparagine. They are soluble in high-salt buffers and have sedimentation coefficients of 7S and 12S. (Higgins, 1984).

(2) The albumins are a more diverse group of water soluble proteins characterised by high levels of cysteine, and which have a sedimentation coefficient of 2S. Although, some 2S

Table 11: Plant species that contain thionins, and the common names of the thionins detected in those plant species.

Author	PLANT SPECIES	THIONINS DETECTED
Samuelsson et al. (1968)	European Mistletoe	Viscotoxin A3
		Viscotoxin B
		Viscotoxin A2
Vernon et al. (1985)	Pyrularia pubera	Pyrulaia
Ozaki et al. (1980)	Wheat	β-purothionin
		α_1-purothionin
		α_2-purothionin
Rodriguez-Palenzuela et al. (1988)	Barley	β-hordothionin
		α-hordothionin
Reimann-Philipp et al. (1989)	Barley	Leaf vacuole specific thionin
		Leaf cell wall specific thionin
Bekes and Lasztity (1981)	Oats	β-avenothionin
		α-avenothionin

proteins have only a storage role, other 2S proteins have biological activity such as proteases and amylase inhibitors. The nutritional value of seeds is highly correlated with their albumin content, due to the relatively high level of EAA in albumins (Bajaj *et al.*, 1971).

1.3.3.1 Pea Albumin 1.

Albumins in pea seeds contain two low molecular weight seed storage proteins of MW 6000 and 4000, which contribute 10% of the total seed protein, 50% of the seed SAA, and are thought to act as a source of SAA for the developing seedling (Higgins *et al.*, 1986).

The processing of pea albumin 1 (PA1) protein is summarised in Fig. 10. The MW 6000 and the MW 4000 proteins are coded for by separate regions on the same mRNA. The initial translation product is a preprotein, from which a signal peptide is removed, co-translationally. The resultant proprotein, PA1 (MW 11000) is then cleaved post-translationally to yield the mature form of PA1, PA1a (MW 6000) and PA1b (MW 4000; Higgins *et al.*, 1986).

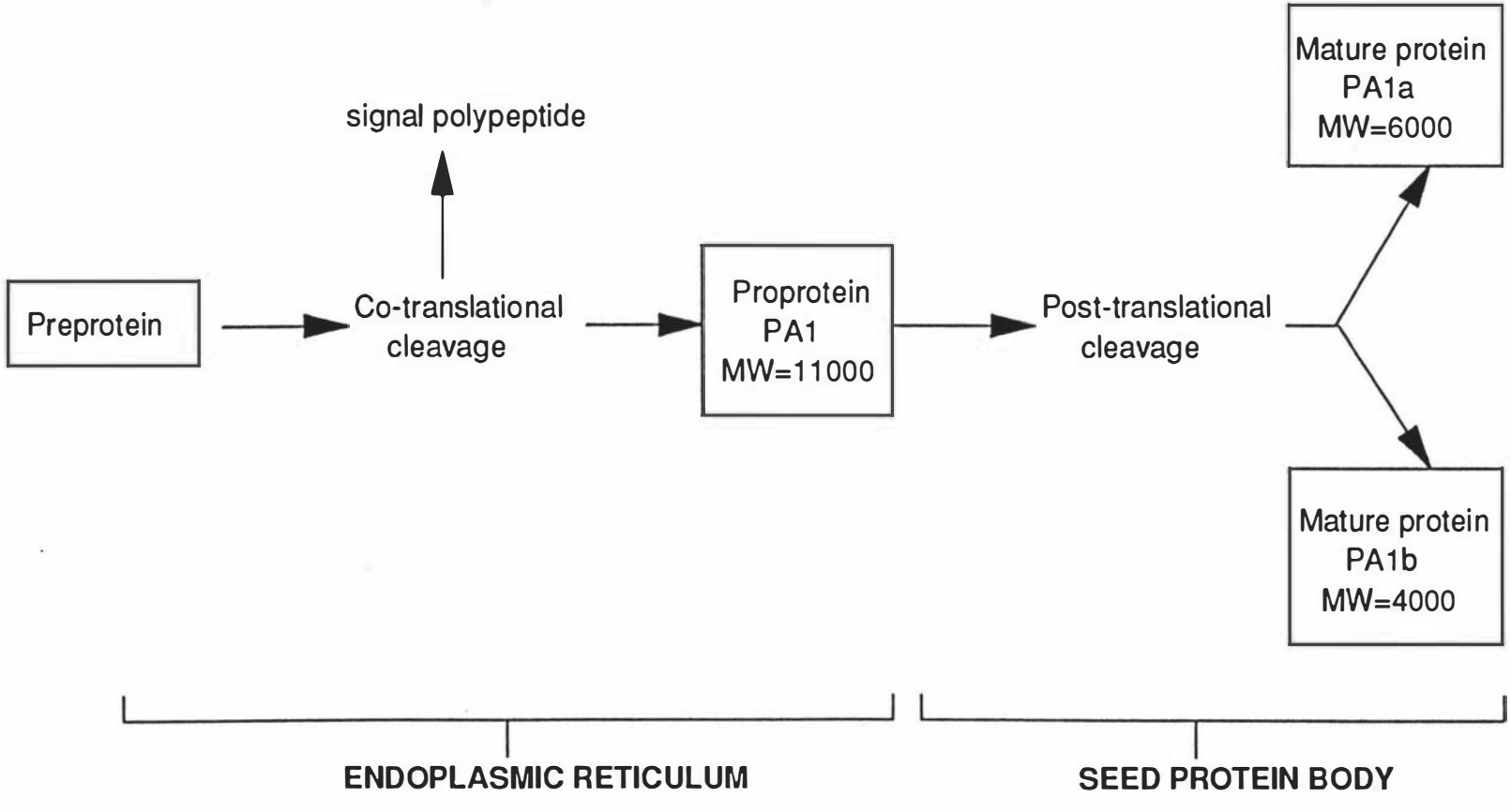
The major pea seed storage proteins, legumin and vicilin, are synthesized as preproteins on the rough endoplasmic reticulum. They are then transported to the seed protein bodies, where post-translational cleavage to yield the mature proteins and storage occurs (Higgins *et al.*, 1986). It has been observed that in pea seeds, PA1a is also associated with the endoplasmic reticulum and is deposited in protein bodies, so it is likely that PA1 is processed in similar fashion to legumin and vicilin (Higgins *et al.*, 1986).

1.3.3.2 Sunflower Albumin 8.

Sunflower seed 2S proteins contain at least eight distinct proteins, two of which are sulphur rich (Kortt *et al.*, 1990). The amino acid sequence and the sequence of a cDNA clone which codes for the entire primary translation product of the sunflower 2S protein, sunflower albumin 8 (SF8) has been determined (Kortt *et al.*, 1990). Like PA1, SF8 is a seed storage protein which is thought to be a source of SAA for the germinating seedling (Kortt *et al.*, 1990).

The SF8 protein is synthesized as a precursor of 141 amino acids, with a hydrophobic signal sequence of 25 amino acids and a hydrophobic prosequence of 13 amino acids. These are both cleaved post-translationally to yield mature SF8, which is a single polypeptide chain of 103 amino acids (Kortt *et al.*, 1990).

Fig. 10: The processing of pea albumin 1 protein in pea seeds.



The 2S seed storage proteins from castor bean (Sharief and Li, 1982), rapeseed (Ericson *et al.*, 1986), mustard seed (Menendaz-Arias, 1988) and brazil nut (Ampe *et al.*, 1986) all show similarities to SF8, particularly in the position and number of cysteine residues (Kortt *et al.*, 1990). There is very little experimental data available concerning SF8 although, a partial cDNA clone coding for SF8 has been constructed (Lilley *et al.*, 1989) and was used in this thesis to construct a chimeric gene for the expression of SF8 in the leaves of transgenic tobacco.

1.3.4 Protein Degradation in the Rumen.

The degradation of plant protein in the rumen follows the sequence;

Protein > Large Polypeptides > Small Polypeptides > Amino Acids > Ammonia

The proteolytic activity in the rumen is almost entirely associated with bacterial cells, with cell free rumen fluid and protozoa having very little proteolytic activity (Nugent and Mangan, 1981). Nugent *et al.* (1983) suggested that the rate of proteolysis of a protein in the rumen was a function of;

(1) Total protein concentration in the rumen, with a higher concentration favouring faster proteolysis.

(2) The degree of competitive inhibition of bacterial proteases by proteins resistant to degradation.

Nugent and Mangan (1981) suggested the production of NH_3 from amino acids was comparatively fast and that the rate limiting step in protein degradation was the initial formation of large polypeptides. Degradation of protein to peptides and amino acids in the rumen is catalysed by proteases and peptidases which are located on the surface of the bacteria (Blackburn, 1968). However, initial breakdown of protein in the rumen is correlated with protein solubility, as proteins must be in solution for bacterial proteases to work efficiently (Hungate, 1966).

1.3.4.1 Degradation of High Sulphur Proteins and Casein in the Rumen.

There is very little experimental data available examining the rumen degradation of proteins containing a high proportion of SAA. However, in an *in vitro* rumen assay using rumen fluid from a sheep fed chaffed lucerne hay, ovalbumin, PA1 (Spencer *et al.*, 1988) and SF8 (D.Spencer, personal communication) were still detectable after 8 hours of

incubation, whilst casein (Spencer *et al.*, 1988) was undetectable after 1 hour of incubation. Mangan (1972) reported that the half-life of casein in the rumen of cows eating a hay/concentrate diet was in the range of 5.6-12.3 min whilst ovalbumin had a half-life of 180 min. Mangan (1972) suggested it was unlikely that any casein would have survived rumen degradation and flowed intact, into the abomasum, whilst ovalbumin was likely to flow undegraded into the abomasum. Therefore, transgenic plants expressing ovalbumin have more potential for improving animal production than transgenic plants expressing casein.

1.3.4.2. Degradation of Individual Amino Acids.

Varvikko (1986) reported that residues of barley and barley-straw remaining in nylon bags after suspension of the nylon bags in the rumen of a cow eating a grass silage/hay diet (4600gDM/d), had a higher EAA:NEAA ratio than prior to suspension in the rumen. The slower degradation of EAA compared to NEAA appeared to be related to the source of the protein, as nylon bag residues from rapeseed meal and ryegrass had unchanged EAA:NEAA ratios. Branched-chain amino acids tended to increase in all feed residues, suggesting they are more resistant to rumen degradation (Varvikko, 1986).

Interestingly, the amino acids which were least resistant to rumen degradation varied depending on the protein source; glutamic acid in rapeseed, methionine, alanine and glycine in barley, arginine and alanine in ryegrass and methionine, asparagine and tyrosine in barley straw were all degraded faster than the protein as a whole (Varvikko, 1986). However, the relationship between protein source and the rate of amino acid degradation in the rumen has not been resolved.

1.3.4.3 DISULPHIDE BONDS AND PROTEIN STABILITY.

Disulphide bonding occurs in a polypeptide chain between two cysteine residues. The reaction involves the free SH groups from cysteine only, the S group of methionine being protected by a CH_3 group is unable to form a disulphide bond.

Matsumura *et al.* (1989) constructed mutants of phage T4 lysozyme, normally a disulphide free protein, which contained either 1,2 or 3 disulphide bonds. He reported that as the number of disulphide bonds increased, the melting temperature of the protein increased. As melting temperature is an index of protein stability, these results suggest that the presence of disulphide bonds increased protein stability. Treatment of mutant proteins to destroy disulphide bonds, reversed the trend. Nugent *et al.* (1983) reported that breaking the disulphide bonds in bovine serum albumin (BSA) with dithiothreitol, converted the protein

from being highly resistant to rumen degradation, to one which was readily degraded in the rumen. Therefore, the relatively high cysteine content of ovalbumin, PA1, and SF8 may be responsible for their resistance to rumen degradation.

1.3.4.4 RUMEN DEGRADATION MEASURED BY THE NYLON BAG TECHNIQUE.

Mehrez and Orskov (1977) proposed a method by which the degradability of dietary protein could be measured. The method involved suspending nylon bags containing the test diet in the rumen for varying lengths of time. The rate of degradation of nitrogen or protein was equated to the rate of loss of protein or nitrogen from the nylon bag over time.

Estimates of the effective degradation of a protein can be calculated from an equation proposed by Orskov and McDonald (1979).

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

where:

p = the effective degradation of the protein.
a = the instantly degradable fraction of the protein.
b = the proportion of the protein degraded over time (t) at a constant rate (c).
(a+b) = the potential degradability.

1.3.4.5 DEGRADATION VERSUS SOLUBILITY.

It has generally been assumed that proteins which disappear from a feed incubated in the rumen in a synthetic-fibre bag have been completely degraded (Ganev *et al.*, 1979).

Nugent and Mangan (1981) demonstrated that it was possible to study degradation of individual proteins within a mixture of proteins during their incubation in the rumen, by SDS-polyacrylamide gel electrophoresis. This application is possible because the proteins of rumen microflora are extremely heterogeneous and do not interfere with detection of added plant proteins (Spencer *et al.*, 1988). Using this approach, Nugent and Mangan, (1981), Nugent *et al.* (1983) and Spencer *et al.*, (1988) demonstrated that the loss of protein or nitrogen from nylon bags suspended in the rumen was not necessarily correlated with the rate of protein degradation in the rumen. It would appear that the loss of nitrogen or protein from nylon bags suspended in the rumen is more correctly equated to the rate of

solubilisation of plant protein in rumen fluid, and that the rate of solubilisation is not necessarily a good measure of the rate of degradation of the individual protein components that constitute total plant protein.

1.3.5 GENETIC ENGINEERING.

1.3.5.1 GENETIC ENGINEERING: AN INTRODUCTION.

Amino acids cannot bind to DNA molecules, so a cell cannot produce a protein molecule directly from DNA, whilst there is less risk of damage to a cell's single DNA molecule if it is not directly used as a template for protein synthesis. Therefore, an intermediate molecule, messenger RNA (mRNA) is copied from DNA and then used repeatedly for protein synthesis (Fig. 11).

1.3.5.1.1 The Genetic Code.

A DNA molecule consists of two deoxyribose-phosphate chains, which are coiled about one another to form a double-stranded helix. The chains follow the outer edge of the molecule and the nucleotide bases adenine (A), thymine (T), guanine (G) and cytosine (C) are in a helical array in the central core. The nucleotide bases from one strand are linked to nucleotide bases from the other strand by hydrogen-bonding to form the purine-to-pyrimidine base pairs; A:T and G:C.

The sequence of amino acids in a polypeptide chain is ultimately coded for by the sequence of nucleotide bases in DNA. However, there are only four nucleotide bases but 20 amino acids. Therefore, triplets of nucleotides or codons specify each amino acid eg... TGC specifies cysteine while AAG specifies lysine. The collection of codons that specify all the amino acids are known as the genetic code. The genetic code is nonoverlapping, therefore each codon specifies only one amino acid.

1.3.5.1.2 The Gene.

A segment of DNA molecule specifying a complete polypeptide chain is defined as a gene. Genes normally remain on chromosomes and do not directly serve as templates for protein biosynthesis, which takes place on the ribosomes. Instead, mRNA which contains the complementary codon sequence to the DNA, serves as the template, thus providing the genetic information specifying the sequence of amino acids during protein biosynthesis. Figure 12 describes a chimeric gene, which differs from a natural gene in that it has been constructed in a laboratory using genetic engineering techniques. A chimeric gene, once

Fig. 11: The interrelationship between DNA, RNA and protein synthesis.

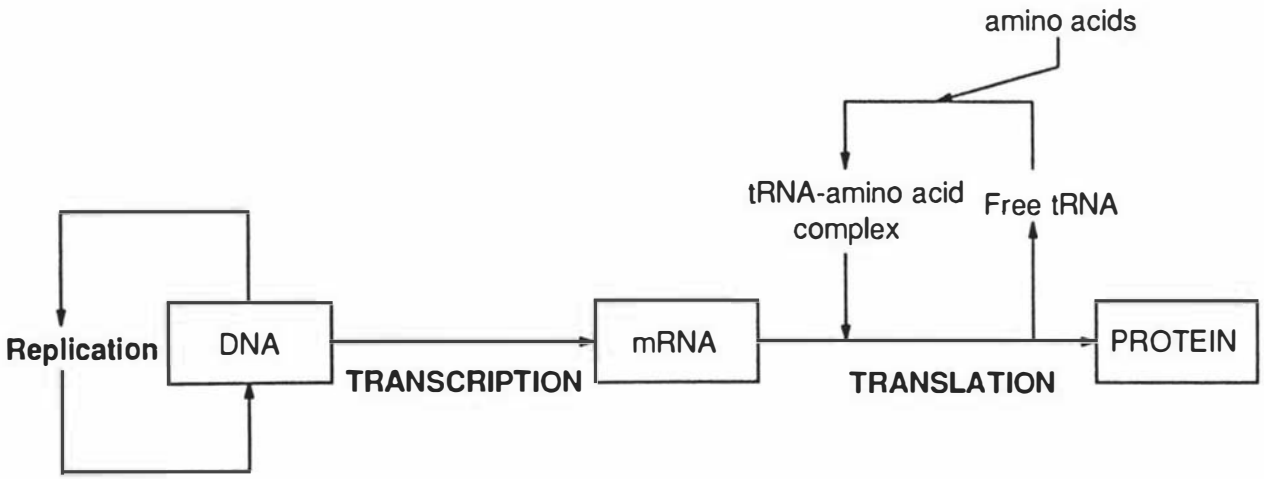
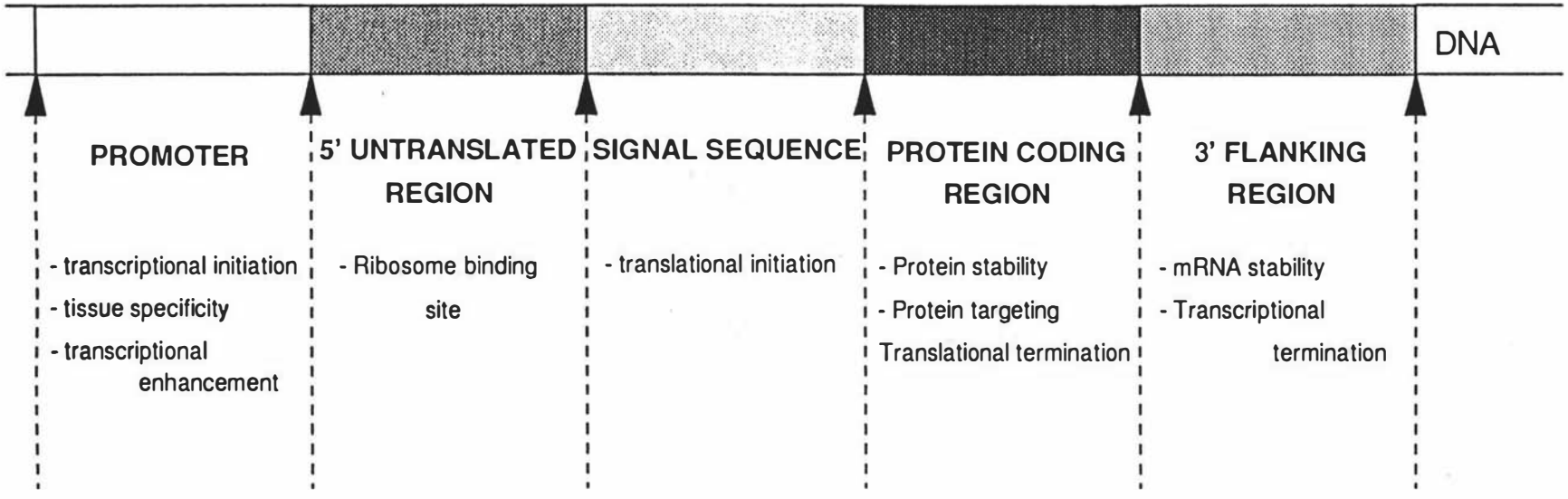


Fig. 12: A chimeric gene showing the components required for construction and the probable role each component has in successful expression of the coding region in a transgenic host.



incorporated into a host organism, relies on the organism to transcribe mRNA before the gene product can be translated.

1.3.5.1.3 Transfer of Chimeric Genes to Plants.

The most common method of transferring foreign genes into plant cells is the use of the Ti (tumour-inducing) plasmid from the bacteria *Agrobacterium tumefacians*.

The soil bacteria *Agrobacterium tumefacians* is responsible for the plant disease crown gall, which is a true plant tumour, causing prolific cell growth. Crown gall can occur in most dicotyledonous plants, but can not occur in monocotyledonous plants. The cells of the tumour synthesize opines, which are unusual amino acid derivatives not found in normal plant tissue. The bacteria utilise opines as their sole source of carbon and nitrogen. Opine synthesis is a property conferred upon the plant cells by the invading bacteria, but continued synthesis of opines by plant cells does not require the continued presence of the bacteria, suggesting the bacteria transfer a genetic element capable of coding for synthesis of opines.

Agrobacterium tumefacians contains extrachromosomal DNA in the form of the Ti-plasmid, which is essential for the bacterial virulence, but is not found in plant tumour cells. Instead, a small, specific segment of the Ti-plasmid is found integrated into the plant nuclear DNA. This DNA segment is called T-DNA (transferred DNA).

In general:

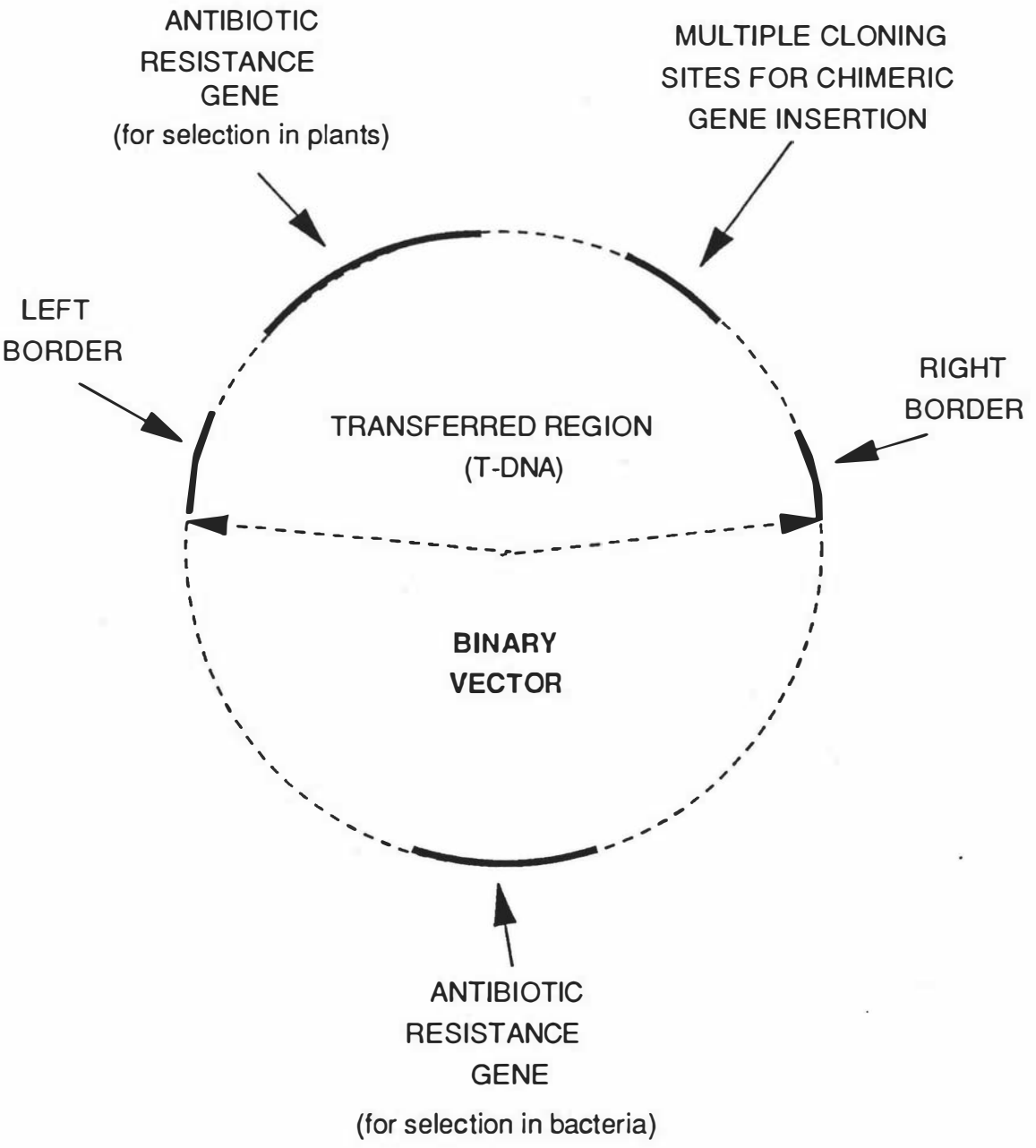
(1) Integration of the T-DNA can occur at many random sites in the plant nuclear DNA.

(2) T-DNA contains 6-8 genes which affect tumour morphology and opine synthesis.

(3) The Ti-plasmid contains sequences at either end of the T-DNA (the left and right border), and are all that is required for integration of the T-DNA into the plant nuclear DNA.

The Ti-plasmid is a natural vector for genetically engineering plant cells because it can transfer its T-DNA from the bacterium to the plant genome. Unfortunately wild-type Ti-plasmid is not suitable as an experimental gene vector, as transfer of foreign DNA to plant cells would be accompanied by transformation of cells to tumorous growth. This problem resulted in the development of binary vectors which in essence, are disarmed or inactivated Ti-plasmids (Fig. 13).

Fig. 19: A schematic representation of a binary vector. The DNA is indicated as a circle.



Binary vectors:

(1) Replicate in *Agrobacterium tumefacians*.

(2) Contain a plant selectable marker gene within the T-DNA, which usually confers resistance to an antibiotic and is used to indicate successful transformation.

(3) Contains the Ti-plasmid sequences required for integration of the T-DNA into the plants genomic DNA.

(4) Contains a polylinker within the T-DNA, which is a short sequence of DNA into which chimeric genes can be inserted.

1.3.5.1.4 Regeneration of Plants Transformed with a Binary Vector.

As wounding is required for crown gall tumour growth in nature, plant cell transformation is performed by innoculation of wounded plant tissue with *Agrobacterium tumefacians* containing a binary vector.

The presence, within the binary vector of sequences required for transfer of T-DNA, results in the incorporation of the chimeric and plant selectable marker genes into the plant's genomic DNA. Subsequent shoots which grow from infected plant tissue and are able to survive and proliferate on the selectable marker usually contain the chimeric gene.

1.3.5.2 FOREIGN GENE EXPRESSION IN PLANTS.

Table 12 summarises a number of examples of foreign genes that have been transferred into plants. Stable genetic transformation is now routinely obtained with the following economically important plants: tomato, potato, cotton, white clover, lucerne, flax, rape, tobacco, *Lotus* sp., Douglas fir and poplar. However, despite this progress, regeneration of soybean, cereals and temperate grasses is not yet possible as these species are not able to be transformed by *Agrobacterium tumefacians* and are difficult to regenerate by presently available tissue culture techniques (Weising *et al.*, 1988).

1.3.5.3 THE LEVEL OF EXPRESSION OF FOREIGN GENES IN PLANTS.

The level of expression of a foreign gene in plants would appear to depend upon the source of the gene and the plant tissue in which the gene is to be expressed. The levels of

Table 12: Examples of genes from animals, yeast, plant viruses, bacteria and plants which have been transferred to either tobacco, tomato or petunia plants by Agrobacterium tumefacians mediated transformation.

AUTHOR	ORIGIN OF GENE	TRANSFORMED SPECIES
	ANIMAL	
Ow et al. (1986)	Firefly Luciferase Gene	Tobacco *
Hunt et al. (1987)	Human Growth Hormone Gene	Tobacco *
	YEAST	
Barton et al. (1983)	Aldehyde Dehydrogenase Gene	Tobacco #
	PLANT VIRUS	
Bevan et al. (1985)	Tobacco Mosiac Virus Coat Protein Gene	Tobacco
Turner et al. (1987)	Lucerne Mosiac Virus Coat Protein Gene	Tobacco and Tomato
	BACTERIA	
Della Cioppa et al. (1987)	EPSP Synthase Gene (1)	Tobacco
	PLANT	
Lawton et al. (1987)	Soybean B-conglycinin Gene	Petunia
Keith and Chua (1986)	Wheat rbcS Gene (SSU) (2)	Tobacco *
Hilder et al. (1987)	Cowpea CpT1 Gene (3)	Tobacco

(*) Transient expression only

(#) No expression (1) Enolpyruvylshikimate-3-phosphate

(2) Ribulose-1,5-bisphosphate carboxylase

(3) Cowpea trypsin inhibitor

expression of foreign seed storage genes in the seeds of transformed plants, although generally lower than in the native seed, can be as high as 2-8% of total seed protein (Sengupta-Gopalan *et al.*, 1985; Altenbach *et al.*, 1989; Vandekerckhove *et al.*, 1989), whereas the level of expression of foreign genes in the leaves of transformed plants is very low and generally in the range of 0.001-0.1% of total leaf protein (Weising *et al.*, 1988).

Low levels of expression of herbicide and insect resistance genes in the leaves of transformed plants gives acceptable levels of plant protection (Weising *et al.*, 1988; Schulz *et al.*, 1990). However, it has been estimated that the level of expression of genes coding for proteins containing a high proportion of SAA, in the leaves of lucerne would need to be at least 5% of total leaf protein in order to have any positive benefits on animal production (T.J. Higgins, personal communication).

In contrast to the generally low level of expression of foreign genes in the leaves of their new hosts, there are four examples of foreign genes showing relatively high levels of expression in the leaves of transgenic plant hosts.

(1) Glutamine synthatase (GS) is an enzyme which plays a key role in the detoxification of ammonia in plants, and therefore its overproduction is of potential use where herbicides are being used which inhibit GS activity (Eckes *et al.*, 1989). A leaf-specific GS from lucerne has been engineered for expression in the leaves of tobacco. The foreign GS gene was expressed at a level of 5% of total tobacco leaf protein, whilst tobacco plants expressing the GS gene at that level, were 20-fold more tolerant of the GS inhibiting herbicide, L-phosphinothricin (Eckes *et al.*, 1989).

(2) The gene coding for vicilin, a pea seed protein, when engineered for expression in the leaves of tobacco, expressed vicilin at a level of 0.01% of total leaf protein (T.J. Higgins, personal communication). Munro and Pelham (1987) identified an amino acid sequence (lys-asp-glu-leu), which occurred at the carboxy terminus of some proteins and appeared to be responsible for targeting those proteins for retention in the endoplasmic reticulum. When the before mentioned vicilin gene was modified to include the endoplasmic reticulum targeting sequence, vicilin was expressed in the leaves of tobacco at a level of 1% of total leaf protein (T.J. Higgins, personal communication).

(3) When the seed-specific cowpea trypsin inhibitor gene (CpT1), was engineered for expression in the leaves of tobacco, the trypsin inhibitor was expressed at a level of 1% of total leaf protein. Expression of the CpT1 gene enhanced plant resistance to insect pests (Hilder *et al.*, 1987).

(4) When mouse antibody cDNA's derived from hybridoma mRNA, were engineered for leaf expression, functional antibodies were expressed at a level of 1.3% of total leaf protein in tobacco (Hiatt *et al.*, 1989).

These examples of relatively high levels of foreign gene expression in the leaves of a transgenic host suggest it is worthwhile pursuing high levels of expression of genes coding for proteins containing a proportion of SAA, in the leaves of agriculturally important legumes, such as lucerne and white clover.

However, there is no experimental data available to indicate whether 5% of total leaf protein is the level of expression required to achieve significant responses in animal production, particularly wool growth. There is a need for experiments to determine the relationship between the level of expression of high sulphur protein genes in lucerne and the animal production response to grazing that lucerne, in an effort to establish exactly what level of expression is required. In the case of PA1, SF8 and thionin, a suitable source of large quantities of pure protein for orally or intraruminally supplementing grazing animals is not available. However, ovalbumin is more readily obtainable and thus a more likely test protein for use in experiments to determine the ideal level of transgenic protein expression.

2. The effect of condensed tannins in *Lotus pedunculatus* on rumen-S metabolism, and the digestion of methionine and cystine in sheep.

2.1 INTRODUCTION.

In ruminants fed high quality forage diets, duodenal N flow is only 65-75% of N intake (MacRae and Ulyatt, 1974). The large loss of N across the rumen is due to the rapid degradation (70%) of soluble plant protein to ammonia, exceeding the rate of microbial ammonia incorporation (Ulyatt *et al.*, 1975). Consequently, 25-33% of the N eaten is absorbed as NH_3 across the rumen wall (Beever and Siddons, 1986).

Condensed tannins (CT) occur in the leaves and stems of some forage legumes, but are generally absent from grasses used in temperate agriculture (Barry, 1989). Damage to plant cells, for example, during chewing, enables CT to bind to proteins and form stable complexes in the pH range 3.5-7.0. The complexes dissociate at pH <3.5 (Jones and Mangan, 1977), so that plant protein should be bound to CT and protected from microbial degradation in the rumen (pH 5.5-7.0), but released in the abomasum (pH 1.3-3.0), enabling subsequent absorption of amino acids in the small intestine.

Dietary CT reduces rumen ammonia concentration and increases non-ammonia-nitrogen (NAN) outflow from the rumen in sheep fed *Lotus* species (Barry *et al.*, 1986; Waghorn *et al.*, 1987), suggesting CT reduced forage protein degradation. In sheep fed *Lotus corniculatus*, the presence of CT (22g/kgDM), resulted in a 62% increase in apparent absorption of essential amino acids (EAA) and a 10% decrease in the apparent absorption of nonessential amino acids (NEAA) from the small intestine (Waghorn *et al.*, 1987).

However, the effects of dietary CT on the degradation of sulphur amino acids (SAA) to sulphide in the rumen, and the absorption of cystine and methionine from the small intestine of sheep has not been measured. Similarly, the effect on microbial protein synthesis of reduced rumen availability of polypeptides, amino acids and ammonia in sheep fed CT-containing forages is not well documented.

The three objectives of the present study were to determine if the presence of CT in *Lotus pedunculatus* fed to sheep affected the following criteria;

(1) The digestion in the rumen, and absorption from the small intestine, of cystine and methionine (Experiment 1).

(2) The irreversible loss rate (IRL) of rumen reducible-S, which is a degradation product of SAA (Experiment 2).

(3) The contribution of microbial-NAN to total rumen-NAN (Experiment 2).

2.2 METHODOLOGY.

2.2.1 EXPERIMENTAL DESIGN.

Two experiments were conducted with sheep fed the CT-containing legume, *Lotus pedunculatus* (cv Grasslands "Maku"). In experiment one the sites of methionine and cystine digestion were determined using digesta fluxes calculated from an intraruminal infusion of two indigestable markers (Chromium-EDTA and Ruthenium-phenanthroline). The protocol for experiment one is outlined in Fig. 1, with the experiment being conducted in early summer (Sept-Dec), 1987.

In experiment two the proportion of microbial-NAN in whole rumen digesta-NAN and the IRL of reducible-S from the rumen were determined from an intraruminal infusion of [^{35}S] inorganic sulphate. The protocol for experiment two is outlined in Fig. 2, with the experiment being conducted in early summer (Sept-Nov), 1988.

In both experiments, one group of sheep (six in experiment 1; seven in experiment two; PEG sheep) received an intraruminal infusion of polyethylene glycol (PEG), while the remaining group of sheep (eight in experiment one; seven in experiment two; CONTROL sheep) received an intraruminal infusion of water. Polyethylene glycol (MW 3500) preferentially binds with CT, preventing CT reacting with protein (Jones and Mangan, 1977; Barry and Manley, 1986). Therefore, comparing control with PEG sheep provides a means of quantifying the effects of CT upon rumen digestion.

2.2.2 FEED.

In both experiments, *Lotus peduculatus* was harvested daily at 08.00 hours from a vegetative stand (300-400mm high), with a sickle-bar mower. Immediately after harvest it was further cut into 50mm lengths with a chaff cutter to facilitate hourly feeding. One-third of the daily allowance was placed on belt feeders by 10.00 hours, and the remaining two-thirds stored at 4°C until 16.00 hours, when it was then placed on the belt feeders.

2.2.2.1 Experiment One.

The *Lotus* was grown at Aorangi Research Station, D.S.I.R. Grasslands Division, Palmerston North in medium-high fertility soil. The *Lotus pedunculatus* (2.97gS/kgDM, 38.6gN/kgDM, 55gCT/kgDM) was fed for a total of 30 days, commencing on day 29, and

was offered *ad libitum* for the first seven days. Thereafter, it was fed at a daily allowance of 1400gDM/sheep/day.

2.2.2.2 Experiment Two.

The *Lotus* was grown at D.S.I.R., Grasslands Division, Palmerston North in medium-high fertility soil. The *Lotus pedunculatus* (34.0gN/kgDM, 50gCT/kgDM) was fed for a total of 27 days commencing on day 8, at a daily allowance of 900gDM/sheep/d.

2.2.3 POLYETHYLENE GLYCOL INFUSION.

In experiment one, PEG (MW 3500; Union Carbide, USA) was continuously infused into the rumen at a rate of 100g/d (in 340ml of water), from day 36 until slaughter, whilst in experiment two, PEG (MW 3500; 100g/d in 330ml of water) was continuously infused into the rumen from day 15 until day 35.

2.2.4 ANIMALS.

Castrated male sheep were used in both experiments and were housed in metabolism crates. Water was not provided but salt-lick (Dominion Salt (NZ) Ltd) was freely available. On day 1 all sheep were drenched with anthelmintic to control internal parasites (12ml; Ivomec, Merck Sharp and Dohme (NZ) Ltd.) and treated for lice (10ml; Wipeout, Coopers Animal Health (NZ) Ltd.).

Fourteen, 15 month-old Romney sheep, mean liveweight 43.9$\pm$3.0kg (SD), fitted with rumen (55mm ID) and abomasal (10mm ID) cannula about one month prior to the experiment commencing, were used in experiment one. A separate group of fourteen, 30 month-old Romney sheep, mean liveweight 55.0$\pm$2.1 kg (SD), with rumen cannula (95mmID) were used in experiment two.

2.2.5 INFUSATES AND INFUSION.

All radiochemicals were obtained from Amersham (UK) Pty Ltd.

2.2.5.1 Experiment One.

Digesta flow through the intestines was measured using a double (solid and liquid phase) marker system. The liquid marker was ^{51}Chromium ethylenediaminetetra acetic acid (^{51}Cr-EDTA) prepared from 10mCi of $^{51}CrCl_3.6H_2O$ (100-400mCi/mg) and 14.2g of $CrCl_3.6H_2O$

according to the method described by Binnerts *et al.* (1968). The solid phase marker was tris(1,10-phenanthroline) ^{103}Ruthenium (II) chloride (^{103}Ru-phenanthroline) prepared from 4mCi of $^{103}RuCl_3$ and 1.0g of $RuCl_3$ according to the method described by Tan *et al.* (1971).

Sheep received a continuous intraruminal infusion (230ml/d) containing 50μCi of ^{51}Cr-EDTA and 9μCi of ^{103}Ru-phenanthroline from day 53 until 5 min before slaughter on day 59 or 60.

2.2.5.2 Experiment Two.

An intraruminal infusion of [^{35}S] inorganic sulphate was used to determine the IRL of reducible-S from the rumen and the proportion of microbial-NAN in whole rumen digesta-NAN. Most inorganic-sulphate (about 98%) in the rumen is reduced to sulphide, with sulphide being the intermediate for most rumen inorganic-S transactions (Kennedy and Milligan, 1978). Therefore, the IRL of reducible-S represents the IRL of sulphide from the rumen. [^{35}S] Sodium sulphate (25 mCi; 25-40 Ci/mg) was added to 25 litres of water with 53 g of inert sodium sulphate (BDH; 15mmol/l final concentration) as a carrier. [^{35}S] Inorganic sulphate was infused at 0.2mCi/d (in 230ml containing 0.5g of inert sodium sulphate) from day 28 to day 35.

2.2.6 SAMPLING PROCEDURES.

In both experiments a 20ml sample of strained rumen fluid was collected from each sheep prior to, and 3 days after PEG infusion commenced and was used to determine rumen NH_3 concentration.

2.2.6.1 Experiment One.

2.2.6.1.1 Rates of Digesta Flow.

Sampling of abomasal digesta (50-100ml) occurred at 6-8 hour intervals during days 55 to 58. Sheep were slaughtered on day 59 or 60 by intravenous administration of sodium pentabarbital (May and Baker (NZ) Ltd) and a midline incision made in the abdomen. The terminal ileum was located and sectioned. Ileal digesta was gently 'milked' from the last 4m of the terminal ileum within 2 min of death. The samples of abomasal and ileal digesta were used to determine ^{51}Cr and ^{103}Ru specific activity for the calculation of digesta flow rates.

2.2.6.1.2 Methionine and Cystine Concentration in Whole Digesta.

Abomasal and ileal whole digesta samples were collected as above. Whole rumen digesta samples where collected at the same time as abomasal samples. Feed offered and refused was subsampled daily. The concentration of cystine (measured as cysteic acid) and methionine (measured as methionine sulfone) was determined in feed, whole rumen digesta and abomasal and ileal samples once abomasal and ileal samples were physically reconstituted (Faichney, 1975).

2.2.6.2 Experiment Two.

2.2.6.2.1 Reducible-Sulphur Irreversible Loss Rate.

Rumen fluid was sampled at 10.00 and 16.00 hours on day 35 and processed according to the method of Kennedy *et al.* (1975) to determine the IRL of reducible-S from the rumen. Strained rumen fluid (5ml) was immediately treated with 1ml of hydrogen peroxide (100vol) in a tube containing 25mg of mercuric chloride as a preservative, to oxidise all inorganic sulphur to sulphate. The mixture was allowed to stand for 30 min at room temperature before addition of 2ml of 20% TCA to precipitate protein. The mixture was allowed to stand at 0°C for 10 min before centrifugation at 3000g for 15 min. The protein-free supernatant was stored at -20°C for determination of radioactivity and inorganic sulphate concentration.

2.2.6.2.2 Proportion of Microbial-NAN in Whole Rumen Digesta-NAN.

Rumen whole digesta was sampled at 10.00 and 16.00 hours on day 35 and processed according to the method of Mathers and Miller (1980) to determine the proportion of microbial-NAN in whole digesta-NAN. Rumen whole digesta (200g) was stored at -20°C, whilst strained rumen fluid (100ml) was centrifuged at 1000g for 1 min to precipitate feed particles. The supernatant was decanted into a second tube and centrifuged at 20000g for 20 min to precipitate a microbial pellet. The radioactivity and NAN concentration in rumen whole digesta and microbial pellet samples were determined.

2.2.7 SAMPLE ANALYSIS.

2.2.7.1 Rumen Ammonia.

Rumen NH_3 was determined by the method of Williams and Twine (1967) using an autoanalyser (Technicon Industrial Systems, USA).

2.2.7.2 Experiment One.

2.2.7.2.1 ^{51}Chromium and ^{103}Ruthenium.

Flow of digesta through the abomasum and ileum was determined by the method described by Faichney (1975), which requires ^{51}Cr and ^{103}Ru activity to be determined in whole digesta and supernatant (29000g centrifugation) fractions. ^{51}Cr and ^{103}Ru activity in the supernatant and whole digesta was determined using a gamma counter (Packard, USA).

2.2.7.2.2 Methionine and Cystine Concentration in Digesta and Feed Samples.

Freeze-dried digesta and feed samples (10mg) were placed in vacuum hydrolysis tubes and oxidised with performic acid (5.0ml for 16 hours at 4°C) to convert cystine and methionine to cysteic acid and methionine sulfone. Oxidation was stopped by the addition of 0.75ml of 45% hydrobromic acid and the contents of the flasks were concentrated by evaporation under reduced pressure at 40-50°C to dryness. Then 20ml of 6.8M HCl was added to the flasks and the samples hydrolysed at 110°C for 22 hours. The hydrolysates were filtered through Whatman No. 1 paper and concentrated by rotary evaporation under reduced pressure at 40-50°C to dryness. The hydrolysates were transferred to 25ml volumetric flasks using three 5ml aliquots of 0.2M sodium citrate/nitric acid buffer (pH 2.2; $7.5x10^{-5}$% pentachlorophenol), and made up to 25ml. Samples were then passed through 0.45µm cellulose acetate filters (Micro Filtration Systems, USA) and stored at -20°C.

Cysteic acid and methionine sulfone in digesta and feed samples were separated by ion exchange chromotography on a high performance liquid chromotograph (HPLC; Waters Associates, USA), using a sodium form amino acid analysis column (Waters Associates, USA) maintained at 62°C.

Over the initial 20 min of amino acid separation there was a linear change from 100% 0.2M sodium citrate/nitric acid buffer (pH 3.03; $7.5x10^{-5}$% w/v pentachlorophenol) to 100% 0.2M sodium nitrate/borate buffer (pH 9.8; $7.5x10^{-3}$% w/v EDTA). This was followed by a further 20 min of separation by sodium nitrate/borate buffer, pH 9.8 and then equilibration of the HPLC with sodium citrate/nitric acid buffer, pH 3.03 for 25 min. The combined buffer flow was 0.4ml/min, giving a column pressure of 1000 to 1500 psi (6900-10400 kPa).

Cysteic acid and methionine sulfone peaks were detected by the fluorescence of O-phthaldehyde derivatives of the amino acids, formed by a post-column reaction of the

column eluate with orthophthaldehyde (OPA) using a Waters (USA) 420AC Fluorescence Detector (338nm excitation and 425nm emission wavelength). The OPA solution comprised of 800mg OPA in 10ml methanol with 400 µl mercaptoethanol and 400 µl 30% Brij-35 (polyoxyethylene lauryl ether) made up to 1000ml with 0.5M borate buffer, pH 10.6 and was pumped at 0.4ml/min.

All solutions for use in the HPLC were filtered through a 0.2µm cellulose acetate (aqueous) or polytetrafluoroethylene (non-aqueous) filter (Micro Filtration Systems, USA) and saturated with oxygen-free nitrogen gas.

The concentration of cysteic acid and methionine sulfone were determined after injection of 40 µl of hydrolysate into the HPLC, by comparison of areas under peaks calculated by a Waters (USA) 730 Data Module integrator, with an external standard (equimolar (2.5mol/l) cysteic acid, methionine sulfone and norleucine). The external standard was used for 48 hours before being discarded. Cysteic acid and methionine sulfone concentration were expressed as cystine and methionine

The recovery by the HPLC, of cysteic acid and methionine sulfone added to feed hydrolysate samples was 0.98±0.03 (SD; cysteic acid) and 0.97±0.04 (SD; methionine sulfone).

2.2.7.3 Experiment Two.

2.2.7.3.1 Reducible-Sulphur Irreversible Loss Rate from the Rumen.

In rumen fluid treated with hydrogen peroxide, the [^{35}S] label was present as both microbial protein-S and inorganic sulphate, whilst plant protein-S was unlabelled. Both microbial and plant protein-S were removed by precipitation with 20% TCA, leaving inorganic sulphate in the protein-free supernatant. [^{35}S] Radioactivity in the protein-free supernatant, was determined by adding 1ml of supernatant to 10ml of PCS II (Phase Combining System II; Amersham (Australia) Pty Ltd.), which was then counted in a scintillation counter (Beckman LS3801, USA). Samples were corrected for quenching using Automatic External Standardization, utilising a spiked quench curve where samples containing known activities of the relevant infusate, were quenched with chloroform. The supernatant was acidified with an equal volume of 4M HCl and the concentration of inorganic sulphate was determined by inductively coupled argon plasma emission spectrometry (ICP-ES; Lee, 1983).

2.2.7.3.2 The Proportion of Microbial-NAN in Whole Rumen Digesta-NAN.

Microbial pellets and rumen whole digesta samples were processed according to the method of Mathers and Miller (1980). Freeze-dried samples were oxidised with performic acid (as described previously) to convert inorganic [^{35}S] to ^{35}sulphate and methionine and cystine to methionine sulfone and cysteic acid respectively. Following performic acid oxidation, samples were acid hydrolysed (as described previously). Inorganic sulphate was removed as insoluble $BaSO_4$ by adding the hydrolysates (10ml) to 1.5 ml of saturated $BaCl_2$. The tube contents were throughly mixed and allowed to stand at room temperature for 15 min to precipitate $Ba^{35}SO_4$, before being centrifuged at 1000g for 15 min.

The radioactivity of [^{35}S] labelled sulphur amino acids in the sulphate-free supernatant samples were determined by adding 1ml of the supernatant to 10ml of PCS II for scintillation counting as previously described. The total-N and NH_3-N concentration in the original whole rumen digesta samples and the total-N concentration in sulphate-free rumen whole digesta and microbial supernatants were determined by a micro-Kjeldahl procedure (Tecator Kjeltec Auto 1030 Analyser; Tecator AB, Sweden). It was assumed that total-N present in microbial samples was NAN. Multiplication of ^{35}S:N (digesta) by N:NAN (digesta) gave an estimate of ^{35}S:NAN (digesta).

2.2.8 CALCULATION OF DATA AND STATISTICAL ANALYSIS.

Means are presented with the standard deviation of the difference (SED) or the standard error (SE) as appropriate. Comparison between control and PEG treatments was done by analysis of variance.

2.2.8.1 Rates of Digesta Flow in Experiment One.

The rate of digesta flow was determined so that the flux of methionine and cystine through the intestines could be calculated. It was assumed that ruthenium was bound to solids as the phenanthroline complex, whilst Cr-EDTA remained in the liquid phase, so that their concentration in post-ruminal digesta indicated the flux of solids and liquids at the point of sampling. The method of Faichney (1975) was used to determine fluxes.

The flux of true digesta is calculated from equation 1.

$$R=(Ru_D-Cr_D/Cr_F-Ru_F) \qquad (1)$$

where; R= the reconstitution factor (eg...a positive R means supernatant was flowing through the tract more rapidly than particulate matter so that true digesta can be obtained by adding supernatant to sampled digesta).

Ru_D= ^{103}Ru/g abomasal whole digesta
Cr_D= ^{51}Cr/g abomasal whole digesta
Ru_F= ^{103}Ru/g abomasal supernatant
Cr_F= ^{51}Cr/g abomasal supernatant

Concentration of markers in True Digesta (TD) can be calculated from equation 2.

$$Cr_{TD} = (Cr_D+R.Cr_F)/(1+R) = Ru_{TD} = (Ru_D+R.Ru_F)/(1+R) \qquad (2)$$

Therefore, the flux of TD can be calculated from equation 3.

$$\text{Flux of TD} = \frac{1}{Cr_{TD}} = \frac{1}{Ru_{TD}} \qquad (3)$$

Similarly, True digesta DM% can be calculated from equation 4.

True DM% = Whole digesta DM% + (R x Supernatant DM%)/(1+R)(4)

Therefore, True DM flux can be calculated from equation 5.

True DM Flux = True Digesta Flux x True DM% (5)

2.2.8.2 Rates of Methionine and Cystine Flow in Experiment One.

Methionine and cystine flux down the digestive tract was calculated from equation 6.

SAA Flux (g/d) = Digesta SAA Conc (g/gDM) x True Digesta Flux (g/d) (6)

2.2.8.3 Reducible-Sulphur Irreversible Loss Rate from the Rumen in Experiment Two.

It was assumed that after the eight days of [^{35}S] inorganic sulphate infusion the rumen inorganic-S pool had attained plateau values of specific radioactivity (Kennedy *et al.*, 1975; Mathers and Miller, 1980).

Specific Activity (SA) of rumen reducible-S at plateau was calculated from equation 7.

$$SA\ (dpm/gS) = \frac{\text{Radioactivity of sampled pool (dpm/ml)}}{\text{Concentration of sampled pool (gS/ml)}} \quad (7)$$

Irreversible Loss Rate (IRL) measured at plateau SA, is the rate at which rumen reducible-S leaves the rumen pool and does not return within the time course of the experiment, and was calculated from equation 8.

$$IRL\ (gS/d) = \frac{\text{Infusion rate into pool (dpm/d)}}{\text{SA of pool (dpm/gS)}} \quad (8)$$

2.2.8.4 The Proportion of Microbial-NAN in Whole Rumen Digesta-NAN in Experiment Two.

The proportion of microbial-NAN in whole rumen digesta-NAN was calculated from equation 9. (Mathers and Miller, 1980).

$$\frac{^{35}\text{S:NAN (digesta)}}{^{35}\text{S:NAN (microbial)}} \quad (9)$$

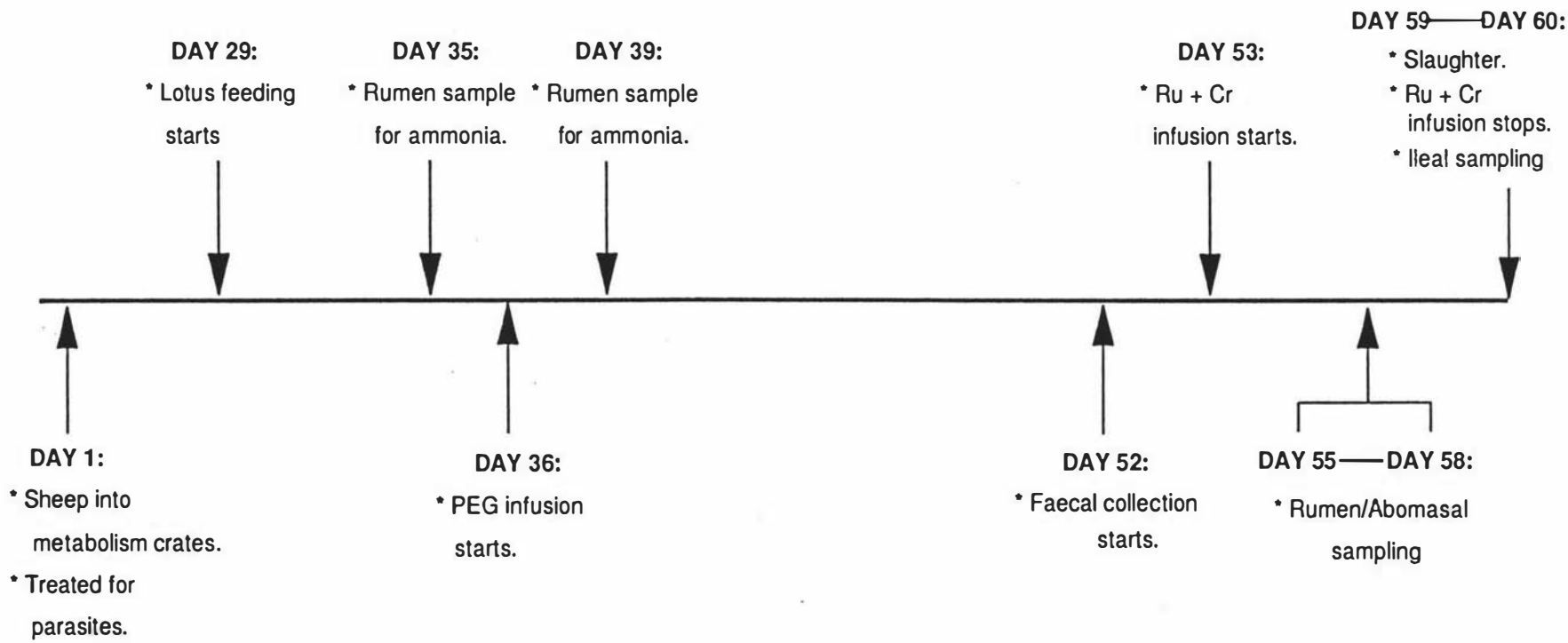


Fig. 1: A diagramatic representation of the protocol for experiment one.

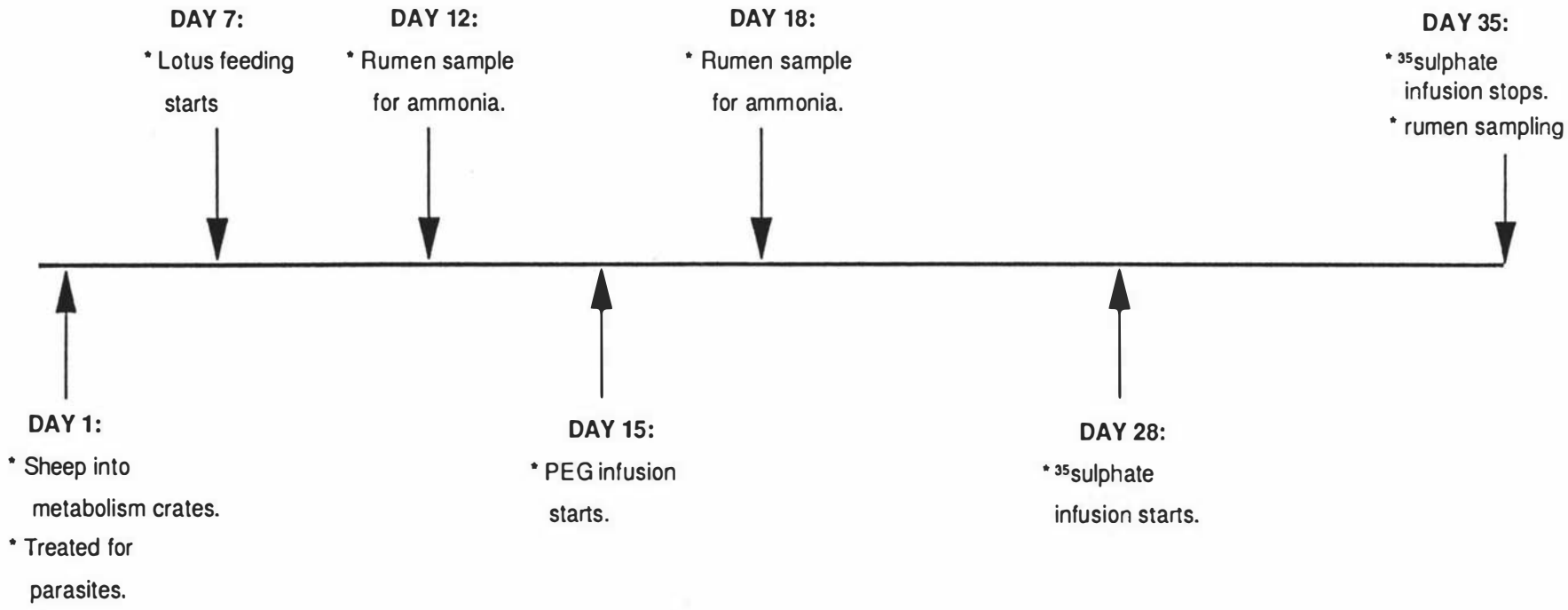


Fig. 2: A diagramatic representation of the protocol for experiment two.

2.3 RESULTS.

2.3.1 EXPERIMENT ONE.

2.3.1.1 Intake.

The DM intakes of control (1212gDM/d) and PEG (1296gDM/d; 43.1, SED) sheep fed fresh *Lotus pedunculatus* were not significantly different (P>0.05). Total-S intake (3.6 and 3.9g/d; 0.13, SED) and total-N intake (43.5 and 47.6 gN/d; 0.90, SED) were also similar (P>0.05) for control and PEG sheep.

2.3.1.2 Rumen Ammonia Concentration.

Rumen NH_3 concentration before PEG infusion was similar (P>0.05) in control (111.2$\pm$6.56 mgNH_3-N/l; SE) and PEG (134.7$\pm$8.50 mgNH_3-N/l; SE) sheep. However, after 3 days of PEG infusion, rumen NH_3 concentration increased to 199.9$\pm$10.46 mgNH_3-N/l (SE; P<0.001), whereas in the control sheep it remained unchanged (P>0.05; 105.9$\pm$6.99 mgNH_3-N/l).

2.3.1.3 The Digestion of Methionine and Cystine in the Gastro-Intestinal Tract.

The intakes of methionine (2.81 and 2.95g/d; Table 1) and cystine (3.33 and 3.52g/d; Table 2) were similar (P>0.05) for control and PEG sheep. The abomasal flux of methionine (2.75 and 2.09g/d) and of cystine (3.33 and 2.52g/d) were both higher (P<0.001) in control than PEG sheep. There was essentially, no loss of methionine or cystine from the rumen in control sheep, whilst 29% of methionine intake (P<0.001) and 28% of cystine intake (P<0.001) disappeared from the rumen in PEG sheep. Although, the ileal flux of methionine (0.63 and 0.43g/d) and cystine (1.93 and 1.18 g/d) were higher (P<0.001) in control than PEG sheep, the apparent absorption of methionine from the small intestine was 27% higher (P<0.001) in control (2.11g/d) than PEG (1.66g/d) sheep, whereas the apparent absorption of cystine from the small intestine was similar (P>0.05) for control (1.40g/d) and PEG (1.34g/d) sheep. The apparent digestibility of methionine in the small intestine was similar (P>0.05) for control (0.77) and PEG (0.79) sheep. In contrast, the apparent digestibility of cystine in the small intestine was lower (P<0.01) in control (0.42) than in PEG (0.53) sheep.

2.3.2 EXPERIMENT TWO.

2.3.2.1 Intake.

The DM intakes of control (818gDM/d) and PEG (893gDM/d; 47.2,SED) sheep fed fresh *Lotus pedunculatus* were not significantly different (P>0.05). Total-N intake was also similar (P>0.05) for control (27.8gN/d) and PEG (30.4gN/d; 0.30, SED) sheep.

2.3.2.2 Rumen Ammonia Concentration.

Rumen NH_3 concentration before PEG infusion was higher (P<0.05) in the control (186±12.28 mgNH_3-N/l; SE) than PEG (153±10.98 mgNH_3-N/l; SE;) sheep. However, after 3 days of PEG infusion, rumen NH_3 concentration increased to 398±19.3 mgNH_3-N/l (SE; P<0.001), whereas in the control group it remained unchanged (P>0.05; 204±18.0 mgNH_3-N/l).

2.3.2.3 Rumen Reducible-Sulphur Irreversible Loss Rate.

The concentration of reducible-S in rumen fluid was similar (P>0.05) for control (26.8mgS/l) and PEG (31.1mgS/l) sheep (Table 3). However, the IRL of reducible-S from the rumen was considerably lower (P<0.001) in control (0.84gS/d) than PEG (2.49gS/d) sheep.

2.3.2.4 The Proportion of Microbial-NAN in Rumen Whole Digesta-NAN.

The concentration of total-NAN in rumen whole digesta was higher (P<0.001) in control (18.9mg/gDM) than PEG (12.3mg/gDM) sheep, but similar (P>0.05) for the microbial fraction in control (26.8 mg/gDM) and PEG (28.0mg/gDM) sheep. The proportion of microbial NAN in rumen whole digesta NAN was lower (P<0.001) in control (0.44) than PEG (0.71) sheep (Table 4).

Table 1: The intake and digestion of methionine by sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG during experiment one.

(Mean values with SED are for six animals per treatment)

METHIONINE	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
INTAKE (g/d)	2.81	2.95	0.082	NS
RUMEN POOL (g)	1.52	1.19	0.094	**
ABOMASAL FLUX:				
(g/d)	2.75	2.09	0.063	***
(g/g eaten)	0.98	0.71	0.018	***
ILEAL FLUX:				
(g/d)	0.63	0.43	0.043	***
(g/g eaten)	0.22	0.15	0.016	***
APPARENT ABSORPTION FROM SMALL INTESTINE				
(g/d)	2.11	1.66	0.023	***
(g/g eaten)	0.75	0.56	0.017	***
(g/g entering SI)	0.77	0.79	0.011	NS

NS, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001

SI, Small intestine

Table 2: The intake and digestion of cystine by sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG during experiment one.

(Mean values with SED are for six animals per treatment)

CYSTINE	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
INTAKE (g/d)	3.33	3.52	0.102	NS
RUMEN POOL (g)	1.31	1.00	0.095	**
ABOMASAL FLUX:				
(g/d)	3.33	2.52	0.181	***
(g/g eaten)	1.00	0.72	0.096	***
ILEAL FLUX:				
(g/d)	1.93	1.18	0.131	***
(g/g eaten)	0.58	0.34	0.032	***
APPARENT ABSORPTION FROM SMALL INTESTINE				
(g/d)	1.40	1.34	0.114	NS
(g/g eaten)	0.42	0.38	0.048	NS
(g/g entering SI)	0.42	0.53	0.031	**

NS, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001

SI, Small intestine

Table 3: Rumen-reducible sulphur concentration and irreversible loss rate in sheep fed Lotus pedunculatus, with and without an intraruminal of PEG during experiment two.

(Mean values with SED are for six animals per treatment)

RUMEN-REDUCIBLE S	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
Concentration (mgS/l rumen fluid)	26.8	31.1	3.10	NS
IRL: (gS/d)	0.84	2.49	0.261	***

NS, P>0.05; ***, P<0.001
IRL, Irreversible loss rate

Table 4: The proportion of microbial NAN in total rumen digesta NAN in sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG during experiment two.

(Mean values with SED are for six animals per treatment)

	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
RUMEN WHOLE DIGESTA				
Total N (mg/gDM)	19.6	14.0	0.77	***
Total NAN (mg/gDM)	18.9	12.3	0.76	***
NAN:N	0.9	0.9	0.01	NS
SA (dpm/gNAN)	15.0	10.4	1.17	**
RUMEN MICROBIAL				
Total NAN (mg/gDM)	26.8	28.0	1.56	NS
SA (dpm/gNAN)	34.5	14.6	1.97	***
Proportion microbial NAN in digesta NAN	0.44	0.71	0.052	***

NS, P>0.05; **, P<0.01; ***, P<0.001

N, Nitrogen; NAN, Non-ammonia-nitrogen; SA, Specific activity

2.4 DISCUSSION.

Polyethylene glycol (1.7g/g CT) displaces CT from CT-protein complexes (Jones and Mangan, 1977) and completely binds available CT (Barry and Forss, 1983). Consequently, when PEG binds CT in the rumen, preventing it from binding to protein, plant protein can be degraded to rumen NH_3. Therefore, the increase in rumen NH_3 concentration after 3 days of intraruminal PEG infusion, indicates that in the present study PEG infusions effectively rendered CT unreactive in the rumen in both experiments.

Dietary CT lowered ($P<0.001$; Table 4) the proportion of microbial-NAN in whole rumen digesta-NAN in control (CT operating) compared to PEG (CT not operating) sheep, suggesting CT had an effect on rumen microbial protein synthesis. However, when the rumen microbial-NAN pool size is calculated, using the assumptions outlined in Table 5, it would appear to be similar in control and PEG sheep. The data do not indicate the relative contribution of microbial and plant-NAN for the two treatments, to outflow from the rumen and absorption from the small intestine. However, sheep eating *Lotus corniculatus* (cv Empire; 4.6gCT/kgDM) had a higher flux of microbial-N out of the rumen than sheep eating *Lotus corniculatus* (cv Maitland; 32gCT/kgDM; Waghorn *et al.*, 1987). The effect of dietary CT concentration upon rumen microbial protein synthesis and rumen outflow therefore, requires further research.

The non-microbial-NAN pool in the rumen, which consists mainly of undegraded plant protein, was calculated to be larger in control than PEG sheep (Table 5). In the present study, there was a net loss of 30% of dietary methionine and cystine ($P<0.001$) from the rumen in PEG sheep. Similarly, 25-35% of N-intake is lost from the rumen in sheep fed fresh pasture diets (MacRae and Ulyatt, 1974). In contrast, there was no net loss of dietary methionine and cystine across the rumen in control sheep (Tables 1 and 2). Since the rumen microbial-NAN pool size was similar between control and PEG sheep, these results suggest that CT reduced rumen proteolysis of plant protein. Tanner *et al.* (1990) also reported that CT from sainfoin (*Onibrychis viciifola*) reduced the proteolysis of Fraction 1 leaf protein in control compared to PEG *in vitro* rumen incubations.

The IRL of reducible-S from the rumen was lower ($P<0.001$) in control than PEG sheep, when measured in rumen fluid as [^{35}S] inorganic sulphate. However, Kennedy and Milligan (1978) reported that inorganic-S was reduced to sulphide in the rumen and that sulphide was the intermediate for all rumen inorganic-S transactions. Therefore, the reducible-S IRL determined in the present study represents sulphide IRL from the rumen. Kennedy and Milligan (1978) reported that abomasal flux of inorganic-S was 0.5-1% of abomasal total-S flux. Therefore, the majority of rumen sulphide flux would be absorbed across the rumen

wall, appearing as inorganic sulphate in the blood (Bray, 1969) or utilised in the rumen for bacterial protein synthesis (Kennedy and Milligan, 1978).

The ability of dietary CT to lower rumen reducible-S IRL in control sheep probably contributed to the lower entry rate ($P<0.05$) of inorganic sulphate into plasma in control sheep compared to PEG sheep fed *Lotus pedunculatus* (55gCT/kgDM; Chapter 3). The majority of recycled sulphide enters the rumen as inorganic sulphate or protein-S in saliva (Kennedy *et al.*, 1975). However, unlike ammonia, recycling of microbial protein to sulphide in the rumen has not been detected (Walker and Nader, 1968; 1975). Consequently, reduction of plant protein-S and inorganic-S in the rumen is the major source of rumen sulphide (Kennedy *et al.*, 1975; Kennedy and Milligan, 1978; Kandylis, 1984). Therefore, the lower reducible-S IRL in control compared to PEG sheep was probably due to reduced rumen proteolysis and degradation of sulphur amino acids from plant protein.

Whilst the abomasal flux of both methionine and cystine were 32% higher ($P<0.001$) in control than PEG sheep, only the apparent absorption of methionine from the small intestine was increased ($P<0.001$) in control sheep. Dietary CT depressed ($P<0.001$) the apparent digestibility of cystine in the small intestine of control sheep. Ashes *et al.* (1984) reported that feeding formaldehyde-treated casein to sheep, increased the abomasal flux of NAN. However, increasing the formaldehyde treatment to increase protection of casein, reduced the apparent digestibility of lysine, cysteine and tyrosine in the small intestine. This was probably due to the increasing strength of the interaction between formaldehyde and these amino acids with increasing protein protection. Jones and Mangan (1977) suggested that protein-CT complexes formed at pH common in the rumen (5.5-7.0), dissociated at pH common in the abomasum (<3.5). However, in the present study cystine may not have dissociated from such complexes in the abomasum as readily as methionine, due to its ability to form free reactive (SH) groups, which may interact more strongly with CT. Waghorn *et al.* (1987) demonstrated a greater increase (62%; $P<0.01$) in EAA absorption from the small intestine in sheep fed *Lotus corniculatus* (22gCT/kgDM) than was reported for methionine (27%; $P<0.001$) and cystine (4.5%; $P>0.05$) in control sheep fed *Lotus pedunculatus* (55gCT/kgDM) in the present study. Therefore, further research is necessary to define the CT concentration in the diet at which the detrimental effects on cystine digestion, due to over-protection by CT, may be avoided. Lower dietary CT concentrations than used here may be more nutritionally desirable. Consequently, it may be possible to further improve the absorption of methionine and cystine from the small intestine in sheep fed CT-containing forages.

Table 5: Microbial-NAN and non-microbial-NAN rumen pools in sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG during experiment two. (Data are calculated from mean values).

RUMEN POOL SIZE (g)	CONTROL SHEEP	PEG INFUSED SHEEP
DRY MATTER (*)	352	277
TOTAL-NAN (+)	6.6	4.3
MICROBIAL-NAN (#)	2.9	3.1
NON-MICROBIAL-NAN	3.7	1.3

(*), Rumen dry matter pool (g) determined in experiment one, adjusted to the DM intake of sheep in experiment two.
(+), Rumen total-NAN concentration of 18.88g/kgDM (control) and 12.34g/kgDM (PEG; Table 4; Experiment 2).
(#), The proportion of microbial-NAN in rumen whole digesta-NAN was 0.44 (control) and 0.71 (PEG; Table 4; Experiment 2).

2.5 CONCLUSIONS.

2.5.1 Two experiments were conducted with sheep fed fresh *Lotus pedunculatus* (55gCT/kgDM, experiment one; 50gCT/kgDM, experiment two). Each experiment comprised a control group of sheep (CT operating) and a group of sheep receiving a continuous intraruminal infusion of PEG (100g/d) to bind and inactivate CT. In experiment one methionine and cystine digestion (g/d) were determined, whilst in experiment two the proportion of microbial-NAN in whole rumen digesta-NAN and the IRL of reducible-S (gS/d) from the rumen were determined.

2.5.2 The intakes (g/d) of all constituents (DM, N, S, methionine and cystine) were similar ($P>0.05$) in experiment one and two.

2.5.3 In experiment one, the abomasal flux (g/d) of both cystine and methionine was higher ($P<0.001$) in control than PEG sheep. There was a net loss of 30% of dietary cystine and methionine from the rumen in PEG sheep, whereas in control sheep there was no net loss of dietary methionine and cystine from the rumen.

2.5.4 The apparent absorption (g/d) of methionine from the small intestine was higher ($P<0.001$) in control than PEG sheep, but both groups had a similar ($P>0.05$) apparent absorption of cystine from the small intestine. The apparent digestibility of cystine entering the small intestine was lower ($P<0.01$) in control (0.42) than PEG (0.53) sheep, whereas the apparent digestibility of methionine in the small intestine was similar (0.78; $P>0.05$) for both groups. This suggests that cystine may not dissociate from CT-protein complexes in the abomasum as readily as methionine, due to its ability to form free reactive (SH) groups, which may interact more strongly with CT.

2.5.5 In experiment two, the IRL of reducible-S, which was measured as inorganic sulphate, from the rumen was lower ($P<0.001$) in control (0.84gS/d) than PEG (2.49gS/d) sheep. As proteolysis of forage protein is a major source of rumen sulphide, this indicates that CT reduced both the proteolysis of forage protein and degradation of SAA to inorganic sulphide in the rumen.

2.5.6 The proportion of microbial-NAN in whole rumen digesta-NAN was lower ($P<0.001$) in control (0.44) than PEG (0.71) sheep. It was calculated that the rumen microbial-NAN pool size (g) was similar in both groups of sheep, whereas the rumen non-microbial-NAN pool size (g), which is mainly undegraded plant protein, was substantially higher in control than PEG sheep, suggesting CT reduced rumen proteolysis of forage protein.

2.5.7 The results of the present study suggest that CT reduced rumen proteolysis of forage protein and also the degradation of SAA to inorganic sulphide, resulting in an increased flux of methionine and cystine to the abomasum. However, detrimental effects of CT on cystine digestion in the small intestine negated the advantage of increased abomasal flux of cystine.

2.5.8 Further research needs to be undertaken to determine if lower forage CT concentrations have the potential to increase the absorption of methionine and cystine from the small intestine to a greater extent than reported in the present study.

3. Metabolism of plasma sulphur amino acids and inorganic sulphate in sheep fed the condensed-tannin containing legume, *Lotus pedunculatus.*

3.1 INTRODUCTION.

In ruminants fed fresh forage diets, abomasal infusions of protein and dietary supplementation with protein protected from ruminal degradation, have shown that absorption of protein from the small intestine limits wool growth (Reis and Schinkel, 1963; Ferguson *et al.*, 1967), milk production (Stobbs *et al.*, 1977; Flores *et al.*, 1979; Rogers *et al.*, 1979) and liveweight gain (Barry, 1981). In particular, post-ruminal supplementaion with sulphur amino acids (SAA) has been effective in markedly increasing wool growth (Reis, 1979).

Condensed tannins (CT) occur in a restricted range of forage legumes, but are generally absent from grasses used in temperate agriculture (Barry, 1989). Condensed tannins bind to proteins to form stable complexes in the pH range 3.5-7.0, but the complexes dissociate at pH <3.5 (Jones and Mangan, 1977). Therefore, plant protein should be bound to CT and protected from microbial degradation in the rumen (pH 5.5-7.0) and released in the abomasum (pH 1.3-3.0), enabling subsequent absorption of amino acids in the small intestine. Waghorn *et al.* (1987) reported that in sheep fed *Lotus corniculatus*, the presence of CT (22g/kgDM), increased the apparent absorption from the small intestine of essential amino acids (EAA) by 62%, whilst the apparent absorption of non-essential amino acids (NEAA) was decreased by 10%. Similarly, in the previous chapter it was reported that sheep fed *Lotus pedunculatus*, containing CT (55g/kgDM), had a 23% increase in apparent absorption of methionine from the small intestine. These results suggest that legumes containing CT may provide a practical means by which the absorption of SAA from the small intestine can be increased in ruminants grazing fresh forage diets.

The objective of the present experiment was to measure the effect of CT in *Lotus pedunculatus*, on the metabolism of SAA in the post-hepatic blood of sheep. Radio-isotopic tracer studies utilising [^{35}S] labelling were used to examine: (1) the quantities of methionine and cystine entering the plasma pool, (2) the interconversion of methionine to cystine, (3) the oxidation of methionine and cystine to inorganic sulphate (4) the rates of utilisation of methionine and cystine for body synthetic reactions. Results are presented as a compartmentalized three-pool model, similar to that proposed by Nolan *et al.* (1976) for studying nitrogen transactions in the rumen.

3.2 METHODOLOGY.

3.2.1 EXPERIMENTAL DESIGN.

The experimental protocol is outlined in Fig. 1, with the experiment being conducted in early summer (Sept-Dec), 1987.

Fourteen sheep fitted with rumen and abomasal cannula were fed lucerne hay (1200gDM/d) from day 1 until day 28, after which they were fed *Lotus pedunculatus*. One group of six sheep (PEG sheep) received an intraruminal infusion of polyethylene glycol (PEG), while the remaining group of eight sheep (CONTROL sheep) received an intraruminal infusion of water. Polyethylene glycol (MW 3500) preferentially binds with CT preventing CT from reacting with protein (Jones and Mangan, 1977; Barry and Manley, 1986), thus providing a means of quantifying the effects of CT upon rumen digestion.

The rates of plasma irreversible loss (IRL) and transfer quotients(TQ) for methionine, cystine and inorganic sulphate were determined from infusions of [^{35}S] labelled methionine, cysteine and inorganic sulphate. These measurements were made after sheep had been fed *Lotus pedunculatus* for 15 days.

3.2.2 FEED.

The *Lotus pedunculatus* (cv Grasslands "Maku") was grown at Aorangi Research Station, D.S.I.R. Grasslands Division, Palmerston North in medium-high fertility soil. The *Lotus pedunculatus* (2.97gS/kgDM, 38.6gN/kgDM, 55gCT/kgDM) was harvested daily at 08.00 hours from a vegetative stand (300-400mm high), using a sickle-bar mower. Immediately after harvest it was further cut into 50mm lengths with a chaff cutter to facilitate hourly feeding. One-third of the daily allowance was placed on belt feeders by 10.00 hours, and the remaining two-thirds stored at 4°C until 16.00 hours, when it was then placed on the belt feeders. The *Lotus* was fed for a total of 30 days commencing on day 29 and was offered *ad libitum* for the first seven days and thereafter at a daily allowance of 1400gDM/sheep/d. Table 1 summarises the cystine and methionine intakes and apparent digestibilities for the PEG and control sheep.

3.2.3 POLYETHYLENE GLYCOL INFUSION.

Polyethylene glycol (MW 3500; 100g/d in 340ml water) was continuously infused into the rumen from day 36 until slaughter on day 59 or 60.

3.2.4 ANIMALS.

Sheep were maintained in metabolism crates for the experiment. Fourteen, 15 month-old Romney castrated male sheep, mean liveweight 43.9±3.0kg (SD), were fitted with rumen (55mm ID) and abomasal (10mm ID) cannula about one month prior to the experiment commencing. On day 42, two days prior to the start of isotope infusion, teflon jugular catheters (1.7mm ID) were inserted into both jugular veins. Catheters were flushed daily with heparinised (100iu/ml) saline (9g/l) to maintain patency. Water was not provided but salt-lick (Dominion Salt (NZ) Ltd) was freely available. On day 1 all sheep were drenched with anthelmintic to control internal parasites (12ml; Ivomec, Merck Sharp and Dohme (NZ) Ltd.) and treated for lice (10ml; Wipeout, Coopers Animal Health (NZ) Ltd.).

3.2.5 INFUSATES AND INFUSION.

Radiochemicals were obtained from Amersham (UK) Pty Ltd. [^{35}S] Methionine (5mCi) was added to 3.7 litres of sterile saline (9g/l) containing 140mg of inert L-methionine (BDH; 0.25 mmol/l final concentration) as a carrier, whist [^{35}S] cysteine (5mCi) was added to 3.5 litres of sterile saline (9g/l) containing 130mg of inert L-cysteine (BDH; 0.24mmol/l final concentration) as a carrier. [^{35}S] Ammonium sulphate (5mCi) was added to 3.2 litres of sterile saline (9g/l) containing 130mg of inert ammonium sulphate (BDH; 0.31 mmol/l final concentration) as a carrier. Rates of infusion and infusate [^{35}S] concentration are summarised in Table 2.

Radioactive [^{35}S] methionine, cysteine and sulphate were infused for 30 hours each into the right jugular catheter with the infusions commencing on day 44 (methionine), day 46 (cysteine) and day 49 (sulphate). Blood was sampled from the left jugular catheter prior to infusion and after 22, 24, 26, 28 and 30 hours of infusion on day 45 (methionine), day 47 (cysteine) and day 50 (sulphate).

3.2.6 SAMPLING PROCEDURE.

3.2.6.1 Sulphur Amino Acids and Inorganic Sulphate.

Blood (10ml) for cystine and methionine analysis was collected into heparinized syringes. Blood samples were then centrifuged at 3000g for 15 min. Plasma was drawn off and 2ml was added to 0.2ml of 50% w/v sulphosalicylic acid and left to stand at 0°C for 10 min to precipitate protein before being centrifuged at 3000g for 15 min. The supernatant was decanted and passed through a 0.45µm cellulose acetate filter (Micro Filtration Systems,

USA) to remove all precipitated protein before storage at -20°C. For inorganic sulphate analysis, a further 2ml of plasma was processed in an identical fashion except 50% w/v sulphosalicylic acid was replaced with 50% w/v trichloroacetic acid.

3.2.6.2 Rumen Ammonia.

A 20ml sample of strained rumen fluid was collected from each sheep on day 35 and again on day 39 and was used to determine rumen NH_3 concentration prior to, and 3 days after PEG infusion commenced.

3.2.7 SAMPLE ANALYSIS.

Once it was established that each infusion was at plateau specific radioactivity by 22 hours (Fig. 2), the 26, 28 and 30 hours samples were bulked for determining the concentration and radioactivity of methionine, cystine and sulphate in plasma.

3.2.7.1 Methionine and Cystine.

Amino acids were separated by ion exchange chromotography on a high performance liquid chromotograph (HPLC; Waters Associates, USA), using a sodium form amino acid analysis column (Waters Associates, USA) maintained at 62°C.

Over the initial 48 min of amino acid separation there was a linear change from 100% 0.2M sodium citrate/nitric acid buffer (pH 3.1; $7.5x10^{-5}$% w/v pentachlorophenol) to 100% 0.2M sodium nitrate/borate buffer (pH 9.6; $7.5X10^{-3}$% w/v EDTA). This was followed by a further 27 min of separation by sodium nitrate/borate buffer, pH 9.6 and then equilibration of the HPLC with sodium citrate/nitric acid buffer, pH 3.1 for 35 min. The combined buffer flow was 0.4ml/min, giving a column pressure of 1000 to 1500 psi (6900-10400 kPa).

Amino acid peaks were detected by the fluorescence of ortho-phthaldehyde derivatives of the amino acids, formed by a post-column reaction of the column eluate with orthophthaldehyde (OPA) using a Waters (USA) 420AC Fluorescence Detector (338nm excitation and 425nm emission wavelength). The OPA solution comprised of 800mg OPA in 10ml methanol with 400µl mercaptoethanol and 400µl 30% Brij-35 (polyoxyethylene lauryl ether) made up to 1000ml with 0.5M borate buffer, pH 10.6 and was pumped at 0.4ml/min.

All solutions for use in the HPLC were filtered through a 0.2µm cellulose acetate (aqueous) or polytetrafluoroethylene (non-aqueous) filter (Micro Filtration Systems, USA) and saturated with oxygen-free nitrogen gas.

The concentration of methionine and cystine were determined after injection of 40µl of protein-free plasma into the HPLC, by comparison of areas under peaks, calculated by a Waters (USA) 730 Data Module integrator, with an external standard (equimolar (2.5mol/l) Amino Acid Hydrolysate (Pierce Chemical Company, USA) with equimolar (2.5mol/l) norleucine (BDH) added). The external standard was used for 48 hours before being discarded.

For determining [^{35}S] activity of methionine and cystine, 2ml protein-free plasma was injected into the HPLC and eluate samples (1ml) containing the relevant peak were collected into scintillation vials as they flowed from the detector. To this was added 8mls of PCS II scintillation fluid (Phase Combining System II; Amersham (Australia) Pty Ltd.) and 2mls of glacial acetic acid. The acetic acid prevented phase separation and its addition resulted in the formation of a clear, stable gel. Vials were counted in a scintillation counter (Beckman LS3801, USA). Samples were corrected for quenching using Automatic External Standardization, utilising a spiked quench curve where samples containing known activities of the relevant infusate, were quenched with chloroform.

The recovery by the HPLC of both methionine and [^{35}S] methionine, and cystine and [^{35}S] cysteine, added to protein-free plasma was 0.98 ± 0.03 (SD; methionine) and 0.95 ± 0.04 (SD; cysteine).

3.2.7.2 Inorganic Sulphate.

Inorganic sulphate in protein-free plasma was separated from organic-S with Dowex 1-X8 resin (BDH; Cl- form, 18-52 mesh size, 1.33 meq/ml binding capacity), which is a strong-base anion exchanger capable of binding inorganic sulphate.

Dowex 1-X8 contains benzyltrimethyl ammonium side chains which can degrade to yield free NH_3. Ammonia interferes with the colormetric determination of sulphate, so the resin was washed before use by shaking in 1M NaOH for 16 hours at room temperature to remove free NH_3, followed by shaking in deionised water three times for 4 hours at room temperature and finally regeneration of the resin by shaking in 1M HCl for 16 hours at room temperature.

Protein-free plasma (3ml), washed Dowex 1-X8 (1g) and deionised water (7ml) were shaken for 16 hours at room temperature. The supernatant, which contains [^{35}S] labelled amino acids was discarded, whilst the resin, which has inorganic sulphate bound to it, was washed three times in 5ml of deionised water to remove all residual protein-free plasma. Inorganic sulphate was eluted off the resin by shaking the resin in 5ml of 1M HCl for 16 hours at room temperature. The HCl, which contained the inorganic sulphate was decanted and 1ml was added to 10ml PCS II for scintillation counting as previously described.

Inorganic sulphate concentration was determined by the automated method of Johnson and Nashida (1952), using an Autoanalyser (Technicon Industrial Systems, USA) as follows. Plasma inorganic sulphate was determined colormetrically by reducing inorganic sulphate in the HCl samples to sulphide, which was absorbed in zinc acetate (0.23mol/l)/sodium acetate (0.15mol/l) and then reacted with p-aminodimethylaniline to produce methylene blue. All standards used for autoanalysis were made up in 1M HCl which had been treated in an identical fashion to the protein-free plasma samples.

The recovery by Dowex 1-X8 resin of both potassium sulphate and [^{35}S] inorganic sulphate, added to protein-free plasma was 0.70$\pm$0.05 (SD). There was no detectable recovery by Dowex-1X8 resin, of either inert methionine or cystine and [^{35}S] methionine or [^{35}S] cysteine added to protein-free plasma.

The [^{35}S] sulphate infusate was treated in an identical fashion to protein-free plasma and had a similar recovery to plasma sulphate; hence for calculation of SA, IRL and TQ no correction for recovery was made, but plasma sulphate concentration was corrected for recovery.

3.2.7.3 Rumen Ammonia.

Rumen NH_3 was determined by the method of Williams and Twine (1967) using an autoanalyser (Technicon Industrial Systems, USA).

3.2.8 CALCULATION OF DATA AND STATISTICAL ANALYSIS.

Means are presented with the standard error of the difference (SED) or the standard error (SE) as appropriate. Comparison between control and PEG treatments was done by analysis of variance.

3.2.8.1 Specific Radioactivity (SA) of plasma cystine, methionine or sulphate was calculated from the following equation.

$$\text{SA (dpm/}\mu\text{mol)} = \frac{\text{Radioactivity of sampled pool (dpm/ml)}}{\text{Concentration of sampled pool (}\mu\text{mol/ml)}}$$

3.2.8.2 Irreversible Loss Rate (IRL) measured at plateau SA, is the rate at which plasma cystine, methionine or sulphate, leaves the sampled pool and does not return within the time course of the experiment.

$$\text{IRL (}\mu\text{mol/min)} = \frac{\text{Infusion rate into pool (dpm/min)}}{\text{SA of pool (dpm/}\mu\text{mol)}}$$

3.2.8.3 Transfer Quotient(TQ) measured at plateau SA is the proportion of radioactive label detected in a secondary pool (B), which originated from an infusion into a primary pool (A).

$$\text{TQ} = \frac{\text{SA of pool B (dpm/}\mu\text{mol)}}{\text{SA of pool A (dpm/}\mu\text{mol)}}$$

3.2.8.4 Total Flux measured at plateau SA, is the combined total of all outflows (μmol/min) from a plasma pool to all processes. When calculating the mathematical model, all outflows were assumed to equal all inflows, therefore total flux represents the total flow through a pool.

The results of this experiment are presented as a three pool, compartmentalized model similar to that proposed by Nolan *et al.* (1976) for N transactions in the rumen. To calculate the various flows within the model, methionine IRL and the proportion of cystine and inorganic sulphate derived from methionine (TQ) were calculated from the [^{35}S] methionine infusion. The procedure was repeated for infusions of [^{35}S]-labelled cysteine and sulphate. The IRL and TQ were used in mathematical equations proposed by Nolan *et al.* (1976) to solve the three pool model.

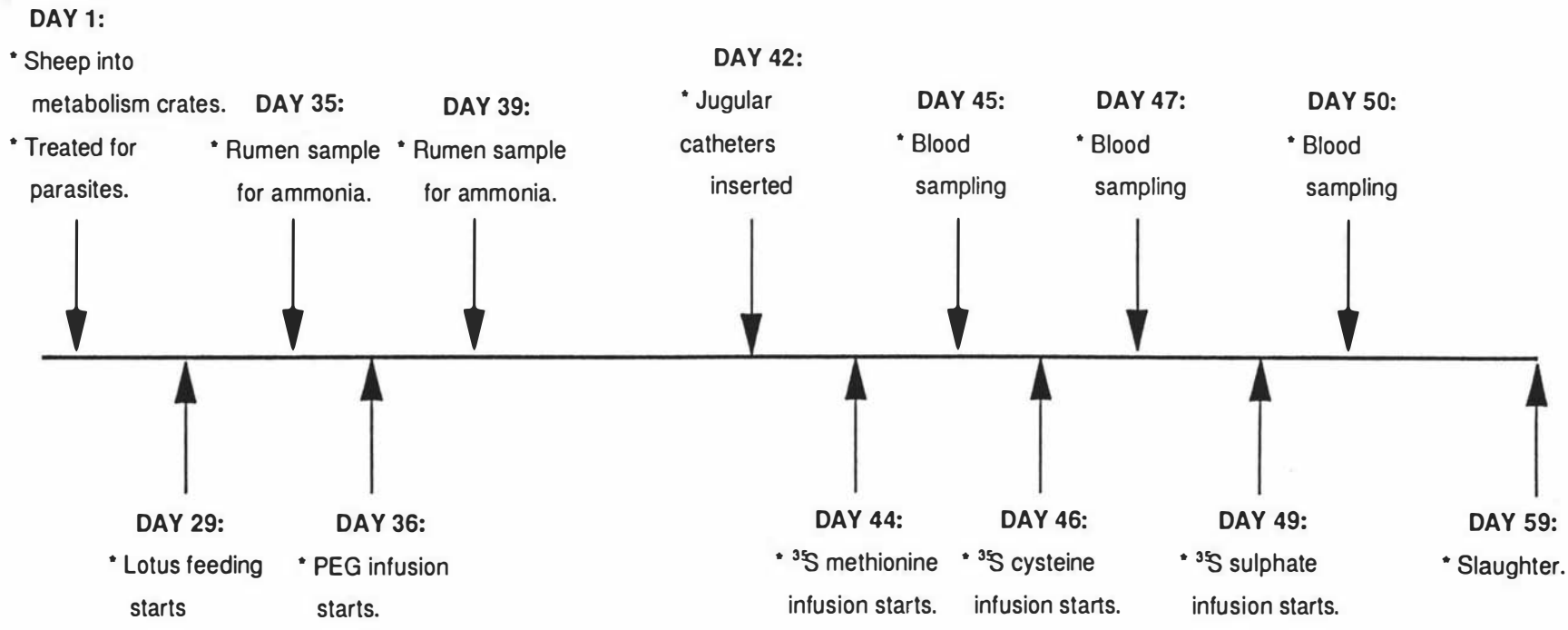


Fig. 1: A diagramatic representation of the experimental protocol.

Table 1: Intakes and apparent digestiblities of methionine, cystine and dry matter for sheep fed Lotus pedunculatus. Data are from chapter 2.

	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
INTAKE (g/d)				
Dry Matter	1,212	1,296	43.3	NS
Total Sulphur	3.6	3.9	0.13	NS
Methionine	3.1	3.2	0.10	NS
Cystine	3.3	3.5	0.08	NS
APPARENT DIGESTIBILITY				
Dry Matter	0.68	0.71	0.504	NS
Methionine #	0.77	0.79	0.011	NS
Cystine #	0.42	0.53	0.031	**

NS, P>0.05; ***, P<0.001; **, P<0.01.

$\frac{\text{Disappearance in small intestine}}{\text{Entry into small intestine}}$

Fig. 2: The specific activity of plasma methionine, cystine and sulphate after 30 hours of continuous infusion of either ^{35}S methionine, cysteine or sulphate into the right jugular of sheep fed Lotus pedunculatus.

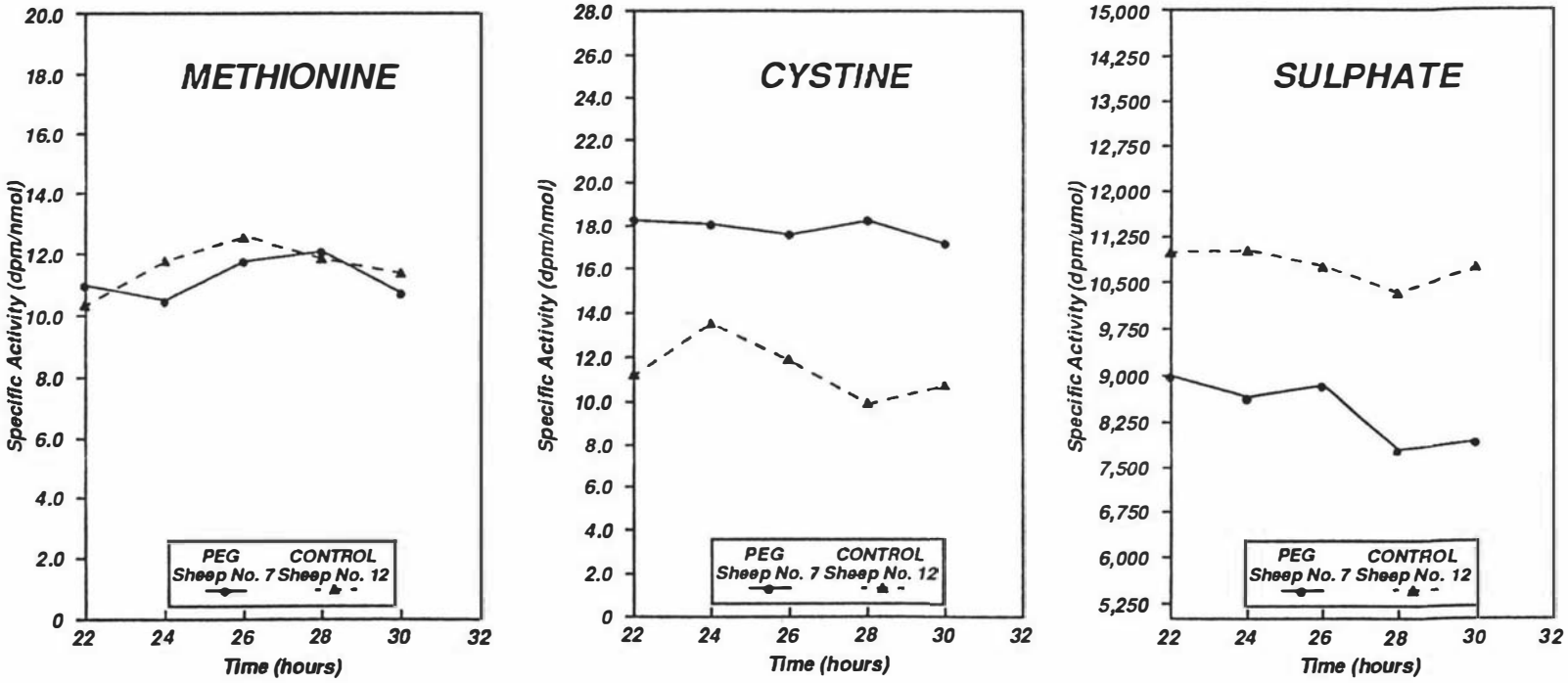


Table 2: The methionine, cysteine and sulphate infusate concentration on the day of infusion, and the rates of infusion into the jugular vein of sheep fed Lotus pedunculatus.

Infusate	Activity (mCi/mmol)	[^{35}S] Infusate Concentration (uCi/ml)	Rate of Infusion	
			ml/h	uCi/h
METHIONINE	77.8	1.35	5.5	7.40
CYSTEINE	51.4	1.18	7.6	8.95
SULPHATE	10.0	1.59	7.6	12.11

3.3 RESULTS.

3.3.1 Rumen Ammonia Concentration.

Rumen NH_3 concentration before PEG infusion was similar ($P>0.05$) in the control (111.2±6.56 $mgNH_3$-N/l; SE) and PEG (134.7±8.50 $mgNH_3$-N/l; SE) sheep. However, after 3 days of PEG infusion, rumen NH_3 concentration increased to 199.9±10.46 $mgNH_3$-N/l ($P<0.001$), whereas in the control group it remained unchanged ($P>0.05$; 105.9±6.99 $mgNH_3$-N/l).

The effect of changes in sulphur amino acid digestion (see Chapter 2), bought about by the presence of reactive CT (55g/kgDM) in *Lotus pedunculatus* fed to sheep, appeared to principally effect plasma transactions involving cystine and sulphate.

3.3.2 Methionine.

Neither plasma methionine concentration or IRL (Table 3) in control sheep (17.5µmol/l and 20.5µmol/min respectively) and PEG sheep (15.4µmol/l and 19.9µmol/min respectively) differed significantly ($P>0.05$). However, in control sheep, methionine had a lower rate of oxidation to sulphate (0.2 and 1.2 µmol/min; $P<0.05$; Fig. 3) and tended to have a lower flux to productive processes and maintenance (8.2 and 13.6 µmol/min; $P<0.1$; Fig. 3) than in PEG sheep.

3.3.3 Cystine.

Plasma cystine concentration was 52% higher in control sheep than in PEG sheep (41.7 and 27.5 µmol/l; $P<0.001$; Table 3). The IRL of cystine from plasma was 78% higher in control than PEG sheep (39.8 and 22.4 µmol/min; $P<0.05$; Table 3). Figure 3 shows that in control sheep the increased plasma IRL of cystine was associated with:

(1) A 76% increase in the entry rate of cystine (28.1 and 16.0 µmol/min; $P<0.05$) into plasma from the combined processes of whole body protein turnover and absorption from the small intestine.

(2) A 79% increase in the transulphuration of methionine to cystine (11.7 and 6.5 µmol/min; $P<0.05$).

(3) A 36% decrease in the oxidation of cystine to sulphate (3.3 and 5.2 µmol/min; $P<0.05$).

These changes in plasma cystine metabolism in control sheep resulted in a 110% increase in the cystine flux to productive processes and maintenance (36.5 and 17.4 μmol/min; $P<0.05$), which represents 91% of total cystine flux, compared to only 74% of total cystine flux in PEG sheep ($P<0.05$; Table 4).

3.3.4 Sulphate.

Plasma sulphate concentration (Table 3) in control sheep (2.0mmol/l) and PEG sheep (2.0mmol/l) was not significantly different ($P>0.05$). However, the IRL of sulphate from plasma was decreased by 40% in control compared to PEG sheep (35.9 and 50.1 μmol/min; $P<0.01$; Table 3) and was associated with (Fig. 3):

(1) A 26% decrease in sulphate entering the plasma from sources other than oxidation of methionine and cystine (31.9 and 43.2 μmol/min; $P<0.05$; Fig. 3).

(2) Lower rates of cystine ($P<0.05$) and methionine ($P<0.05$) oxidation to sulphate.

Table 3: The concentration (umol/l), irreversible loss rate (umol/min) and transfer quotients of plasma sulphur amino acids and sulphate in sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG.

(Mean values with their SED are for six animals per treatment)

	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
CONCENTRATION (umoles/l)				
Taurine	8.7	16.0	2.50	*
Cystine	41.7	27.5	3.80	***
Methionine	17.5	15.4	2.50	NS
Sulphate	1981	1990	1.10	NS
TRANSFER QUOTIENT				
Cys from Met	0.29	0.30	0.04	NS
Sulphate from Met	0.02	0.03	0.01	NS
Sulphate from Cys	0.09	0.11	0.01	NS
IRREVERSIBLE LOSS (umoles/min)				
Methionine	20.5	19.9	2.95	NS
Cystine	39.8	22.4	7.36	*
Sulphate	35.9	50.2	4.45	**

The transfer quotients for cystine from sulphate, methionine from sulphate and methionine from cystine are not presented, but were all determined to be zero within the time course of the experiment.

NS, $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Fig. 3: A general three-pool, compartmentalized model for sulphur amino acid transactions in post-hepatic plasma of sheep fed, *Lotus pedunculatus*, with and without an intraruminal infusion of PEG. Control sheep are represented by numbers in parenthesis. Rates of flow (μmol/min) represented by arrows in the model are often a composite of several pathways of transfer. The pathways are:

(A) Methionine entering the plasma from whole body protein turnover and absorption from the small intestine.

(B) Cystine entering the plasma 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 productive processes and maintenance.

(F) Cystine leaving the plasma and being utilised for productive processes and maintenance.

(G) Cystine oxidised 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 reabsorbed as cystine from microbial protein.

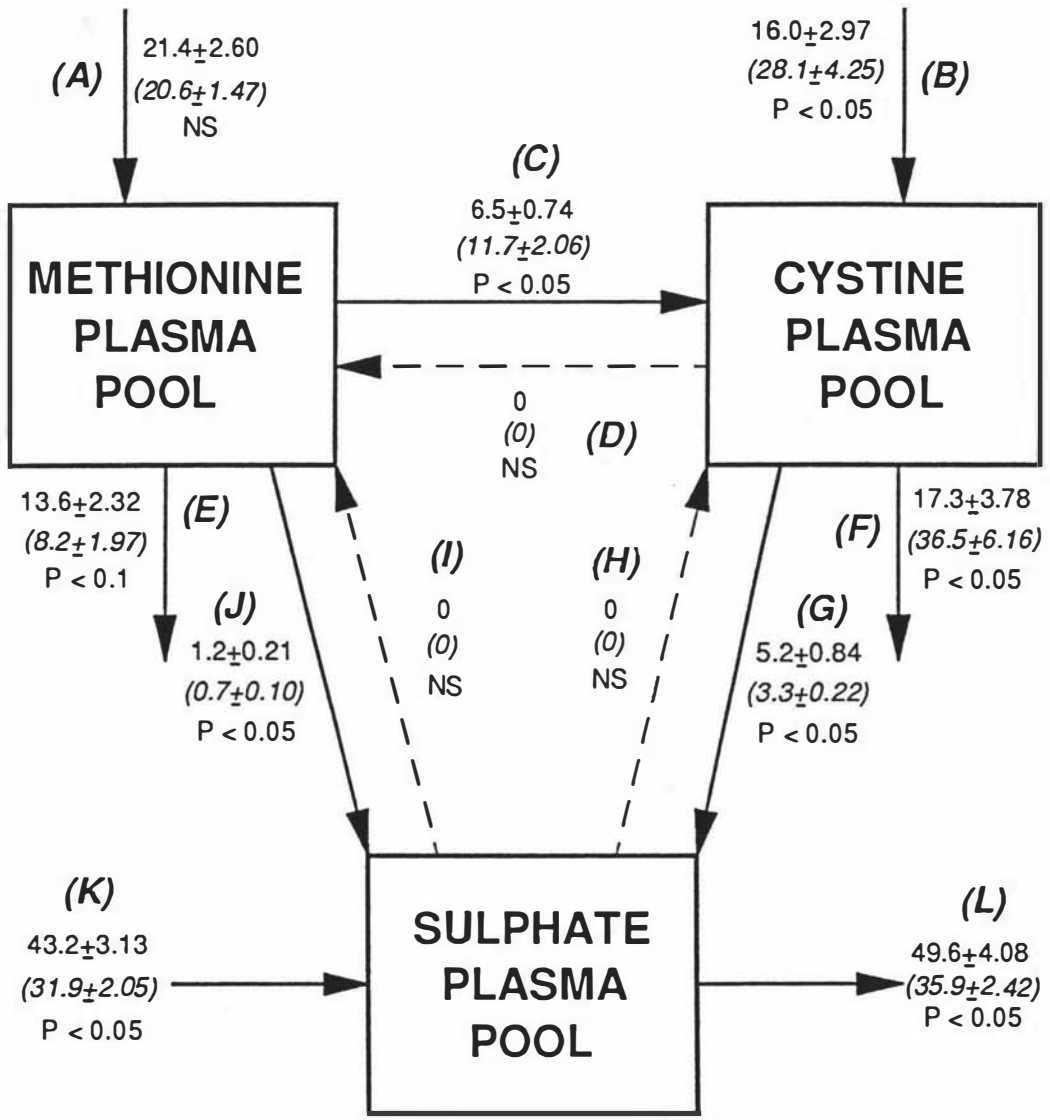
(I) Plasma sulphate reassimilated as methionine. This cannot occur directly in mammalian tissue, but sulphate re-entering the rumen via saliva may be reabsorbed as methionine from microbial protein.

(J) Methionine oxidised to sulphate (and carbon dioxide).

(K) Sulphate entering the plasma chiefly as sulphide absorbed from the rumen, but also sulphide and sulphate absorbed from the intestine.

(L) Sulphate leaving the plasma chiefly in urine, but also recycled directly to the intestines and rumen via saliva.

Fig. 3.



NS, Not significant

PEG Infused Sheep
(CONTROL Sheep)
Flows = umoles/min

Table 4: The total flux (umol/min) of methionine, cystine and sulphate and the proportion of total flux flowing from methionine to cystine, and from methionine and cystine to productive processes and maintenance, in sheep fed Lotus pedunculatus with and without an intraruminal infusion of PEG.

(Mean values with their SED are for six animals per treatment)

	CONTROL SHEEP	PEG INFUSED SHEEP	SED	SIGNIFICANCE OF DIFFERENCE
FLUX (umoles/min)				
Methionine	20.6	21.3	2.85	NS
Cystine	39.8	22.5	7.44	*
Sulphate	35.9	49.6	4.47	*
PROPORTION OF FLUX				
Met to Cys	0.57	0.31	0.110	*
Met to Sulphate	0.04	0.06	0.019	NS
Cys to Sulphate	0.10	0.26	0.053	*
Cys to P + M	0.91	0.74	0.050	*
Met to P + M	0.40	0.62	0.110	(*)

(P + M) Productive processes and maintenance

NS, P>0.1; (*), P<0.10; *, P<0.05.

5.4 DISCUSSION.

Polyethylene glycol (1.7g/g CT) displaces CT from CT-protein complexes (Jones and Mangan, 1977) and completely binds available CT (Barry and Forss, 1983). Consequently, when PEG binds CT in the rumen, preventing it from binding to dietary protein, plant protein can be degraded to rumen NH_3. Therefore, the increase in rumen NH_3 concentration after 3 days of intraruminal PEG infusion, indicates that in the present study PEG infusions effectively rendered CT unreactive in the rumen.

In sheep fed *Lotus pedunculatus*, the presence of CT (55g/kgDM) had a major effect on increasing the entry rate of cystine into plasma, the flux of cystine to productive processes and maintenance and the conversion of plasma methionine to cystine. This has potentially significant benefits for productive processes such as wool growth.

The lower IRL of plasma sulphate in control sheep (ie...with the CT operating) compared to PEG sheep was a result of a lower rate of oxidation of plasma methionine ($P<0.05$) and cystine ($P<0.05$), and a lower rate of sulphate entry ($P<0.05$) into plasma from sources other than the oxidation of SAA. The lower sulphate entry rate into plasma in control sheep was a direct effect of a lower ($P<0.001$) absorption of sulphide from the rumen (represented by rumen reducible-S IRL; Table 5), which is the main source of sulphate entry into plasma (Fig. 3; Kennedy *et al.*, 1975; Kennedy and Milligan, 1978). These observations indicate that the presence of reactive CT reduced rumen protein degradation and the reduction of SAA to sulphide in the rumen of control, compared to PEG sheep. The changes in sulphate metabolism (Table 5) in control sheep compared to PEG sheep resulted in less sulphur being excreted in urine ($P<0.05$) and recycled to the digestive tract and tissues ($P<0.05$).

The recycling of plasma sulphate to the digestive tract and tissues in sheep fed *Lotus pedunculatus* (this study; Table 5) was lower than for sheep with a similar plasma sulphate IRL, but fed lucerne pellets (Kennedy and Milligan, 1978). However, in sheep fed *Lotus pedunculatus* a higher proportion of plasma sulphate was excreted in the urine than in sheep fed lucerne pellets (Kennedy and Milligan, 1978; Table 5). The difference in the partitioning of plasma sulphate IRL between recycling and excretion in the urine was probably due to more extensive degradation of protein in the rumen of sheep fed fresh *Lotus pedunculatus* than sheep fed lucerne pellets.

The plasma methionine and cystine IRL reported in this study are higher than published data (Table 6) although, this was probably due to the higher S-intake of sheep in this study. In control compared to PEG sheep there was a 41% reduction in the rate of plasma methionine oxidation and a 79% increase in the rate of transulphuration of methionine to

cystine in post-hepatic blood. Together with those changes, there was a 36% decrease in plasma cystine oxidation ($P<0.05$) and a 76% increase in cystine entering the plasma from the digestive tract and whole body protein turnover ($P<0.05$). In digestion studies, control sheep fed *Lotus pedunculatus* containing 55gCT/kgDM, had a higher apparent absorption of methionine from the small intestine (+23%; $P<0.001$), whilst cystine apparent absorption was not significantly (+4%; $P>0.05$) affected (Chapter 2). In contrast, in control sheep the entry rate of methionine into post-hepatic blood was not significantly different, whilst the entry rate of cystine was increased ($P<0.05$), compared to PEG sheep. Radcliffe and Egan (1978) reported that the liver was a major site of transulphuration, so it is probable that the increased methionine absorbed from the small intestine in control sheep, was transulphurated in the liver and appeared in post-hepatic blood as cystine.

In control sheep, decreased SAA oxidation, increased transulphuration of methionine to cystine and increased cystine entry rate, resulted in a 110% increase in plasma cystine flux to maintenance and productive processes ($P<0.05$) compared to PEG sheep. This suggests that there was a higher metabolic requirement for cystine than methionine in control compared to PEG sheep.

Waghorn *et al.* (1987) reported that the presence of CT in *Lotus corniculatus* (22gCT/kgDM) increased apparent absorption of EAA from the small intestine by 62%. This effect of CT was probably responsible for the increased N-retention in sheep fed CT-containing forages compared to sheep fed comparable forages which did not contain CT (Egan and Ulyatt, 1980; John and Lancashire, 1981; Waghorn *et al.*, 1987). A possible consequence of the increased protein deposition, would be reduced oxidation of limiting amino acids such as that reported for SAA in the present study.

The sulphur content of clean dry wool is 2.7-4.2% (Reis 1965 a, b) and consists of 26 residues of cysteine for every one residue of methionine (Marshall and Gillespie, 1977). This suggests wool growth is a productive process with a very high demand for cysteine. The increased flux of cystine to productive processes and maintenance in control sheep could therefore be expected to result in an increased rate of wool growth. It has recently been demonstrated that the wool growth of control sheep grazing sulla (*Hedysarum coronarium*) containing CT (48g/kgDM) was 12% higher than that of similar sheep grazing sulla but given a daily drench of PEG (T.N.Terrill *et al.*, unpublished).

Presumably, when methionine absorption from the small intestine (3g/d) exceeds the transulphuration capacity of the liver (Pisulewski and Buttery, 1985), the increased methionine entry into plasma would also stimulate the rate of transulphuration of methionine to cystine in tissues. Although, wool growth apparently requires less

methionine than cysteine, this would explain why post-ruminal infusions of methionine, cystine or cysteine (2-3g/d) are equally effective at increasing wool growth (Reis, 1979).

The high cysteine content of wool and the well documented observation that wool growth responds to post-ruminal supplementation of cystine or cysteine (Reis, 1979) suggests cysteine is a limiting amino acid for wool growth. The apparent flux of sulphur away from methionine, to plasma cystine and the increased flux of plasma cystine to productive processes and maintenance reported in this study for control sheep, also supports this suggestion. The presence of CT in forage diets would appear to provide one practical means of increasing SAA absorption from the small intestine, and the subsequent flux of cystine to productive processes and maintenance. It has been well documented that increasing the dietary CT concentration from 5 to 95gCT/kgDM increases non-ammonia-nitrogen (NAN) flow to the small intestine in sheep fed *Lotus* species (Barry *et al.*, 1986; Waghorn *et al.*, 1989). However, in control sheep fed *Lotus corniculatus* (22gCT/kgDM), Waghorn *et al.* (1987) demonstrated a greater increase (62%; $P<0.01$) in EAA absorption from the small intestine than was reported for methionine (27%; $P<0.001$) and cystine (4.5%; $P>0.05$) in control sheep fed *Lotus pedunculatus* (55gCT/kgDM; Chapter 2). Therefore, further research is necessary to better define the relationship between CT concentration in the diet, absorption from the small intestine and changes in plasma SAA metabolism, particularly at lower CT concentrations, as it may be possible to further improve the flux of cystine to productive processes and maintenance. The dietary concentration of CT needed to maximise these processes needs to be defined.

Table 5: A comparison of the rumen reducible-S and plasma sulphate irreversible loss rates, urine total-S and recycling of plasma sulphate to the digestive tract and tissues in sheep fed fresh Lotus pedunculatus or lucerne pellets.

	DIET	Sulphur Intake (gS/d)	Rumen Reduceable-S IRL (gS/d)	Plasma Sulphate IRL (gS/d)	Urine Total-S (gS/d)	RECYCLING		
						(gS/d)	(% of IRL)	(% of S-intake)
WMcNabb (this study)	Fresh Lotus Pedunculatus							
	CONTROL	3.6	0.8	1.7	1.3	0.3	0.2	0.1
	PEG	3.9	2.7	2.3	1.7	0.6	0.3	0.2
Kennedy and Milligan (1978)	Lucerne Pellets							
	HIGH	3.5	1.7 *	2.0	0.8	1.2	0.6	0.3
	LOW	1.8	1.2 *	1.6	0.4	1.1	0.7	0.6

* Rumen sulphide irreversible loss rate

Table 6: A comparison of plasma sulphur amino acid metabolism in sheep fed a range of diets.

AUTHOR	DIET	Sulphur concentration (gS/kgDM)	Dry Matter Intake ($g/kgW^{0.75}/d$)	IRL (umol/min)		Cys from meth (umol/min)	Oxidation (umol/min)	
				Cystine	Methionine		Cystine	Methionine
WMcNabb (this study)	Fresh Lotus Pedunculatus							
	CONTROL	3.0	71	40	20	12	3	0.7
	PEG	3.0	76	22	20	7	5	1.2
Egan et al. (1984)	Lucerne Hay *	3.2	52	2.6	14	0.4	ND	2.9
	Wheaten Hay	ND	52	2.4	10	0.2	ND	5
Gill and Ulyatt (1979)	Silage + IP methionine (1g/d)	ND	43	ND	13	ND	ND	2.5
	Formaldehyde-treated silage	ND	38	ND	11	ND	ND	1.7
	Silage	ND	41	ND	9	ND	ND	1.3

IP; intraperitoneal ND; not determined * Egan et al., (1975)

3.5 CONCLUSIONS.

3.5.1 The plasma IRL (μmol/min) were determined for cystine, methionine and sulphate in sheep fed *Lotus pedunculatus* (55gCT/kgDM). Transfer quotients between these three metabolites were also determined. This was undertaken to determine if changes in the digestion of methionine and cystine, caused by the presence of CT (55g/kgDM) in *Lotus pedunculatus* fed to sheep (chapter 2), altered the metabolism of the three metabolites in post-hepatic blood.

3.5.2 Results are presented as a three pool compartmentalized model where arrows indicate the flows (μmol/min) between the cystine, methionine and sulphate plasma pools in control and PEG sheep.

3.5.3 The cystine and methionine intakes (g/d) were similar for both control and PEG sheep. The rate of absorption (g/d) of cystine from the small intestine was similar for both control and PEG sheep, whilst control sheep absorbed more methionine from the small intestine ($P<0.001$).

3.5.4 The total plasma flux (μmol/min) of methionine was similar for both control and PEG sheep, but control sheep had a higher total flux of plasma cystine ($P<0.05$) and a lower total flux of plasma sulphate ($P<0.05$) than PEG sheep.

3.5.5 The three pool model showed that the cystine entry rate (μmol/min) into plasma from the combined processes of absorption from the small intestine and whole body protein turnover was higher ($P<0.05$) in control than in PEG sheep. Methionine entry rate was similar for both groups. The entry rate into plasma of sulphate, absorbed chiefly as sulphide and sulphate from the digestive tract, was lower in control than in PEG sheep ($P<0.05$).

3.5.6 The rate of oxidation (μmol/min) of methionine ($P<0.05$) and cystine ($P<0.05$) to sulphate was lower in control than in PEG sheep.

3.5.7 The rate of transulphuration (μmol/min) of methionine to cystine was higher in control than in PEG sheep ($P<0.05$).

3.5.8 The result of these changes in plasma SAA metabolism, was a higher flux (μmol/min) of cystine ($P<0.05$)) and a tendancy for a lower flux of methionine ($P<0.1$) to productive processes and maintenance in control compared to PEG sheep. Plasma sulphate (μmol/min) excreted in urine and recycled to the digestive tract and tissues was lower in control than PEG sheep ($P<0.05$).

3.5.9 Since wool growth is generally accepted as the major productive process utilising plasma cystine, and as the flux of cystine to productive processes was increased in control sheep fed *Lotus pedunculatus* (55g/kgDM; this study), it is probable that one of the major effects of CT in the diet would be to increase wool growth.

4. The effect of condensed tannins in *Lotus pedunculatus* upon the solubility and degradation of protein in the rumen.

4.1 INTRODUCTION.

With most ruminant diets, a major proportion of ingested protein is degraded in the rumen, and the released dietary-nitrogen (N) is either converted into microbial protein or absorbed across the rumen wall as ammonia (Nolan, 1975; Leng and Nolan, 1984). Condensed tannins (CT), which occur in a restricted range of forage legumes (Barry, 1989), reduce rumen ammonia concentration and increase non-ammonia-nitrogen (NAN) outflow from the rumen (Barry *et al.*, 1986; Waghorn *et al.*, 1987). Similarly, Tanner *et al.* (1990) reported that CT from sainfoin (*Onibrychis viciifola*) reduced the *in vitro* rate of proteolysis of Fraction 1 leaf protein when added to *in vitro* rumen incubations. Consequently, dietary CT are thought to reduce forage protein degradation in the rumen.

The loss or solubilization of total-N from synthetic-fibre bags immersed in rumen fluid *in vitro* and *in vivo* has had wide acceptance as an index of N and protein degradability in the rumen (Mehrez and Orskov, 1977; Crooker *et al.*, 1978; Mathers and Aitchison, 1981). However, Stern and Satter (1984) compared protein solubility in a mineral buffer solution with *in vivo* rumen protein degradation, measured as disappearance from dacron-fibre bags, and obtained a correlation coefficient of only 0.26. Similarly, Spencer *et al.* (1988) reported that individual pea seed proteins were relatively resistant to rumen degradation, despite the almost complete loss of total pea seed-N from synthetic-fibre bags suspended in rumen fluid *in vitro*. Protein solubilization is an important prerequiste for rumen degradation (Hungate, 1966), however the rates of solubilization and degradation of forage protein in the rumen are not always similar. Therefore, the solubilization of total-N from synthetic-fibre bags suspended in the rumen may not necessarily be a good index of forage protein degradation in the rumen.

One method for studying the degradation of Fraction 1 leaf protein incubated in rumen fluid, both *in vitro* and *in vivo*, is sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Nugent and Mangan, 1981: Nugent *et al.*, 1983), which forms the basis of some work described in the present study. The objectives of the present investigation with CT from *Lotus pedunculatus*, were to study:

(1) The effect of CT upon rumen protein solubility by measuring the rate of dry matter (DM) and total-N loss from polyester-fibre bags, suspended in the rumen of sheep.

(2) The effect of CT upon rumen degradation (ie...proteolysis) of total leaf protein in an *in vitro* rumen incubation.

4.2 METHODOLOGY.

4.2.1 EXPERIMENTAL DESIGN.

The experimental protocol is outlined in Fig. 1, with the experiment being conducted in early summer (Sept-Dec), 1988, using fourteen sheep fed *Lotus pedunculatus* (cv Grasslands "Maku"). One group of six sheep (PEG sheep) received an intraruminal infusion of polyethylene glycol (PEG), while the remaining group of eight sheep (CONTROL sheep) received an intraruminal infusion of water. Polyethylene glycol (MW 3500) preferentially binds with CT, preventing CT reacting with protein (Jones and Mangan, 1977; Barry and Manley, 1986), therefore comparing control with PEG sheep provides a means of quantifying the effects of CT upon rumen digestion.

Six control and PEG sheep were used to measure the effect of CT upon the rate of DM and total-N loss (solubilization) from polyester-fibre bags containing *Lotus pedunculatus*, suspended in the rumen. Nitrogen disappearance from plant material was corrected for microbial-N contamination using a [^{35}S] inorganic sulphate method for labelling rumen microbial protein.

The effect of CT upon the rate of degradation of total leaf protein from *Lotus pedunculatus* was studied in an *in vitro* incubation, using rumen fluid obtained from the two control sheep not used in the polyester-fibre bag experiment, with and without PEG added.

4.2.2 FEED.

All sheep were offered fresh ryegrass pasture (900gDM/d) from day 1 until day 7, after which *Lotus pedunculatus* (34.0gN/kgDM, 50gCT/kgDM) feeding commenced. The *Lotus* was grown at D.S.I.R., Grasslands Division, Palmerston North in medium-high fertility soil, and was fed for a total of 28 days at a daily allowance of 900gDM/sheep/d. The *Lotus* was harvested daily at 08.00 hours from a vegetative stand (300-400mm high), using a sickle-bar mower. Immediately after harvest, it was further cut into 50mm lengths with a chaff cutter to facilitate hourly feeding. One-third of the daily allowance was placed on belt feeders by 10.00 hours, and the remaining two-thirds stored at 4°C until 16.00 hours, when it was then placed on the belt feeders.

4.2.3 ANIMALS.

Fourteen, 30 month-old Romney castrated male sheep, mean liveweight 55.0±2.1 kg (SD), with rumen cannula (95mmID) were maintained in metabolism crates for the present study. Water was not provided but salt-lick (Dominion Salt (NZ) Ltd) was freely available. On day 1 all sheep were drenched with anthelmintic to control internal parasites (12ml; Ivomec, Merck Sharp and Dohme (NZ) Ltd.) and treated for lice (10ml; Wipeout, Coopers Animal Health (NZ) Ltd.). A continuous intraruminal infusion of PEG (MW 3500; Union Carbide, USA; 100g/d in 330ml water) was given from day 15 until day 35.

4.2.4 INFUSATE AND INFUSION.

A continuous infusion of [^{35}S] inorganic sulphate into the rumen was used to label rumen microorganisms, so that polyester-fibre bag residues could be corrected for microbial-N contamination. [^{35}S] Sodium sulphate (25mCi; 25-40 Ci/mg; Amersham (UK) Pty Ltd) was added to 25 litres of water with 53g of inert sodium sulphate (BDH; 15mmol/l final concentration) as a carrier. [^{35}S] Inorganic sulphate was continuously infused into the rumen at 0.2mCi/d (in 230ml with 0.5g of inert sodium sulphate) from day 28 until day 35.

4.2.5 SAMPLING PROCEDURES.

4.2.5.1 Rumen Ammonia Concentration.

A 20ml sample of strained rumen fluid was collected from each sheep prior to, and 3 days after PEG infusion commenced and was used to determine rumen NH_3 concentration.

4.2.5.2 Rumen *In vivo* Polyester-Fibre Bag Incubations.

Pre-weighed polyester-fibre bags (37μm pore size; Estal Mono; Swiss Screens (Aust) Pty Ltd), containing either freshly minced (FM) or freeze-dried and ground (FD; 1mm sieve size) leaves and stems of *Lotus pedunculatus* (5gDM per bag), were suspended in the rumen of control and PEG sheep. The FM *Lotus* was minced about 1 hour prior to placement in the rumen. Freshly minced *Lotus* was chosen as one treatment because it more closely represented the effects of chewing on cell rupture and CT release than was likely with freeze drying. On each of days 31 to 34, five polyester-fibre bags containing either FM or FD *Lotus* were suspended in the rumen of each sheep so that each feed preparation was incubated on two separate occasions in each sheep (Fig. 1). The bags were removed from the rumen at 2, 4, 6.5, 11 and 24 hours after the start of incubation. Plant residues in

polyester-fibre bags were washed throughly in distilled water and weighed before plant residues were freeze dried, ground (1mm sieve size) and stored at -20°C. The microbial-N contamination, DM and total-N concentration in FM and FD *Lotus* residues were determined.

4.2.5.3 Rumen Sampling for Microbial-NAN Contamination of Polyester-Fibre Bags.

Calculation of microbial-NAN contamination of plant residues required the specific activity of rumen microbial-NAN to be determined. This was done with whole rumen digesta samples taken from each sheep at 10.00 and 16.00 hours on day 35 and strained through muslin cloth. Strained rumen fluid (100mls) was then centrifuged at 1000g for 1 min to precipitate feed particles. The supernatant was decanted into a second tube and centrifuged at 20000g for 20 min to precipitate a microbial pellet. After freeze drying, microbial pellets were bulked within sampling times and treatment, and radioactivity and total-NAN concentration in the microbial pellets were determined.

4.2.5.4 *In vitro* Rumen Incubations.

The *in vitro* rumen incubation was performed as given in stage 1 of the method described by Tilley and Terry (1963). After overnight fasting, on days 28 and 30 rumen fluid was collected at 09.00 hours from control sheep and strained through muslin cloth into a Dewar flask flushed with CO_2 gas and maintained at 39°C. Either FM (7.0g) or FD (0.9g) *Lotus pedunculatus* were added to four volumetric flasks (250ml) containing 60ml of artificial saliva, pH 6.8 (McDougall, 1948), which was saturated with CO_2 gas and held at 39°C. Polyethylene glycol (70mg; MW 3500) was added to two of the volumetric flasks, one for each feed preparation. Rumen fluid (15ml) was then added to each flask, which was again flushed with CO_2 gas. All flasks were fitted with bunsen valves and shaken at 39°C for 24 hours. From each *in vitro* rumen incubation, 10ml aliquots for subsequent protein analysis were removed at 2, 4, 6 and 8 hours after the start of the incubation and frozen immediately in ethanol/solidified CO_2 and stored at -20°C. After each sampling, volumetric flasks were flushed with CO_2 gas.

4.2.6 SAMPLE ANALYSIS.

4.2.6.1 Rumen Ammonia and Total-NAN in Polyester-Fibre Bag Residues.

Rumen NH_3 concentration was determined by the method of Williams and Twine (1967), whilst plant residue-N was determined by kjeldahl digestion and autoanalysis (Technicon Industrial Systems, USA).

4.2.6.2 Microbial Contamination of Polyester-Fibre Bag Residues.

Microbial pellets and polyester-fibre bag residue samples were processed according to the method of Mathers and Miller (1980). Freeze-dried microbial and residue samples (300mg) were placed in 100ml round-bottom Quickfit flasks and oxidised with performic acid (20.0ml for 16 hours at 4°C) to convert cystine, methionine and inorganic total-S to cysteic acid, methionine sulfone and inorganic sulphate, respectively. Oxidation was stopped by the addition of 3.0ml of 45% hydrobromic acid and the contents of the flasks were concentrated by evaporation under reduced pressure at 40-50°C to dryness. Then 20ml of 6.8M HCl was added to the flasks and the samples hydrolysed under reflux for 22 hours. The hydrolysates were filtered through Whatman No. 1 paper and concentrated by rotary evaporation under reduced pressure at 40-50°C to dryness. The hydrolysates were transferred to 10ml volumetric flasks using three 3ml aliquots of distilled water, and made up to 10ml. Inorganic sulphate not incorporated in microbial protein was removed as insoluble $BaSO_4$ by adding the hydrolysates (10ml) to 1.5ml of saturated $BaCl_2$. The tube contents were throughly mixed and allowed to stand at room temperature for 15 min to precipitate $Ba^{35}SO_4$, before being centrifuged at 1000g for 15 min. Samples were passed through 0.45µm cellulose acetate filters (Micro Filtration Systems, USA) and stored at -20°C. The radioactivity and total-N concentration were determined in sulphate-free, polyester-bag residue and microbial supernatants.

The radioactivity of [^{35}S] labelled sulphur amino acids in the sulphate-free supernatant was determined by adding 1ml of the supernatant to 10ml of PCS II (Phase Combining System II; Amersham (Australia) Pty Ltd.), which was then counted in a scintillation counter (Beckman LS3801, USA). Samples were corrected for quenching using Automatic External Standardization, utilising a spiked quench curve where samples containing known activities of the relevant infusate, were quenched with chloroform. The total-N and NH_3-N concentration in the original polyester-fibre bag residues and the total-N concentration in sulphate-free, polyester-fibre bag residue and microbial supernatants were determined by a micro-Kjeldahl procedure (Tecator Kjeltec Auto 1030 Analyser; Tecator AB, Sweden). It was assumed that total-N present in microbial samples was NAN. Multiplication of ^{35}S:N (residue) by N:NAN (residue) gave an estimate of ^{35}S:NAN (residue).

4.2.6.3 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis.

Proteins in aliquots taken from the *in vitro* incubation of FM and FD *Lotus* were analysed using SDS-PAGE. Aliquots were freeze-dried, ground (1mm sieve size) and stored at -20°C. Freeze-dried *in vitro* incubation samples (10mg) containing either FM or FD *Lotus*

were resuspended in 0.5ml of protein digestion buffer (see Appendix 10) and heated at 95°C for 15 min to relax the tertiary structure of the protein. After heating, the proteins in 100µl of the resultant slurry were fractionated by SDS-PAGE according to the method of Spencer *et al.* (1988). Electrophoresis was carried out for 20 hours at a current of 14 milliamps (mA). After SDS-PAGE, the large subunit (LSU) of Fraction 1 leaf protein was detected by staining gels with Coomassie Brilliant Blue R (see Appendix 16) for 1 hour and destaining in destain (see Appendix 17). Stained gels were photographed.

4.2.7 CALCULATION OF DATA AND STATISTICAL ANALYSIS.

Protein disappearance (solubilization) was calculated from polyester-fibre bags residues after correction for microbial-NAN contamination. Contamination was calculated from the ^{35}S:NAN ratio in plant residues relative to the ^{35}S:NAN ratio in rumen microbes using the method described by Mathers and Aitchison (1981; equation 1) It was assumed that after three days of [^{35}S] inorganic sulphate infusion, the rumen inorganic-S pool had attained plateau values of specific radioactivity (Kennedy et al., 1975; Mathers and Miller, 1980), at which time polyester-fibre bags were suspended in the rumen. Measures of degradation were qualitative, but are clearly indicated in the photographs of SDS-PAGE gels.

The proportion of microbial-N contamination of polyester-fibre bag residues was calculated from equation 1. (Mathers and Aitchison, 1981).

$$\frac{^{35}\text{S:NAN (residue)}}{^{35}\text{S:NAN (microbial)}} \qquad (1)$$

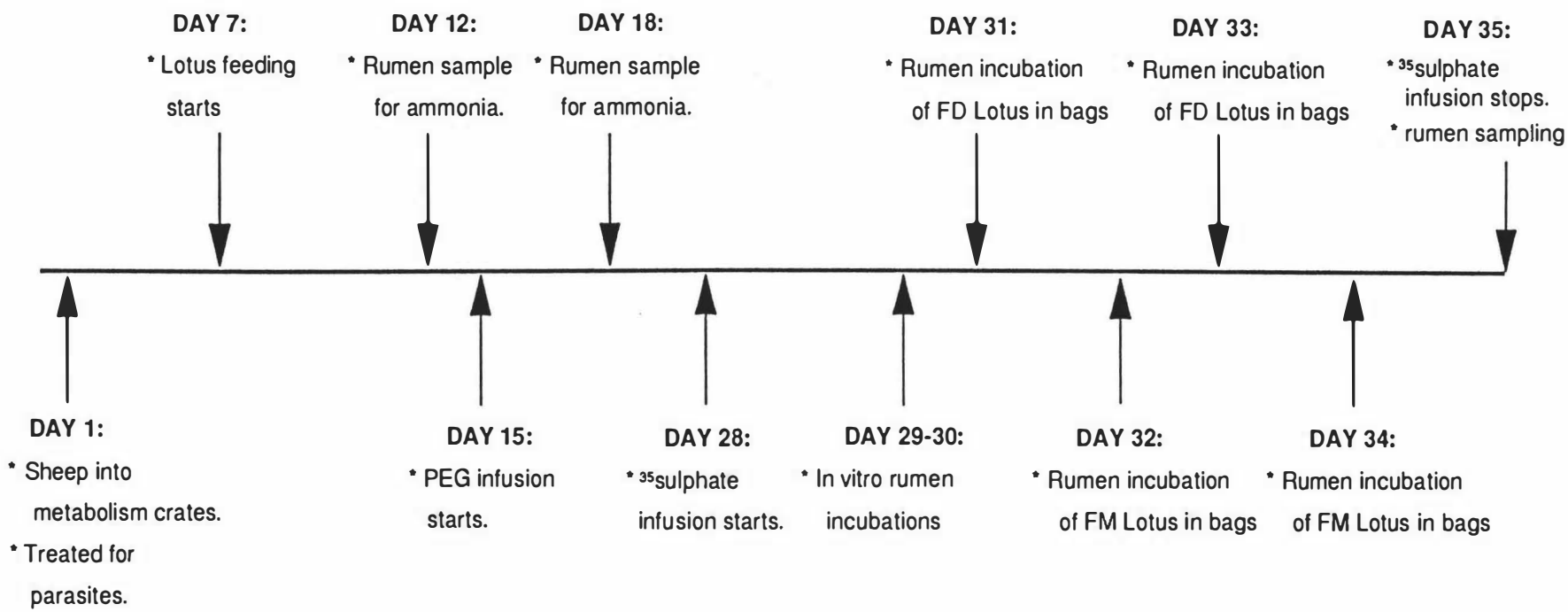


Fig. 1: A diagramatic representation of the protocol for experiment two.

(FD, Freeze dried; FM, Fresh minced)

4.3 RESULTS.

4.3.1 Rumen Ammonia Concentration.

Rumen NH_3 concentration before PEG infusion was higher ($P<0.05$) in the control (186 ± 12.28 mgNH_3-N/l; SE) than PEG (153 ± 10.98 mgNH_3-N/l; SE;) sheep. However, after 3 days of PEG infusion, rumen NH_3 concentration increased to 398 ± 19.3 mgNH_3-N/l (SE; $P<0.001$), whereas in the control group it remained unchanged ($P>0.05$; 204 ± 18.0 mgNH_3-N/l).

4.3.2 Dry Matter Loss from Polyester-Fibre Bag.

Dry matter losses from polyester-fibre bags after 2, 4, 6.5, 11 and 24 hours of incubation in the rumen are shown in Fig. 2. Whereas, 42% of DM in polyester-fibre bags containing FM *Lotus* was soluble at 0 hours, only 23% of DM from FD *Lotus* was soluble at 0 hours. Throughout the 24 hour incubation period, the loss of DM from nylon bags was higher ($P<0.001$) for FM than FD *Lotus*. However, within both FM and FD *Lotus*, there was no difference ($P>0.05$) in the loss of DM between control and PEG sheep over the 24 hours of incubation.

4.3.3 Nitrogen Loss from Polyester-Fibre Bags.

Apparent N and true N, corrected for microbial-NAN contamination, losses from polyester-fibre bags after 2, 4, 6.5, 11 and 24 hours of incubation in the rumen are shown in Figs. 3 and 4. The apparent and true losses of N increased over the 24 hours of incubation. Whereas, 47% of N in polyester-fibre bags containing FM *Lotus* was soluble at 0 hours, only 14% of N from FD *Lotus* was soluble at 0 hours. The true loss of N from polyester-fibre bags containing FM *Lotus* was similar ($P>0.05$) in control and PEG sheep at 2, 4, 6.5 and 24 hours of incubation, but higher ($P<0.001$) in PEG than control sheep at 11 hours of incubation. In contrast, the true loss of N from polyester-fibre bags containing FD *Lotus* was higher in PEG than control sheep at 2 ($P<0.01$), 4 ($P<0.05$), 6.5 ($P<0.01$), 11 ($P<0.01$) and 24 ($P<0.05$) hours of incubation.

4.3.4 Microbial-NAN Contamination of Polyester-Fibre Bag Residues.

Estimates of total microbial-NAN (mg) in polyester-fibre bag residues after 2, 4, 6.5, 11 and 24 hours of incubation in the rumen are given in Fig. 5. The extent of microbial-NAN contamination of FM *Lotus* in the rumen of control and PEG sheep increased until plateau

was reached after 11 hours of incubation. Microbial-NAN in FM residues was higher in PEG than control sheep at 6.5 ($P<0.001$) and 24 ($P<0.05$) hours, but was similar ($P>0.05$) at 2, 4 and 11 hours of incubation. In contrast, the extent to which microbial-NAN contaminated of FD *Lotus* residues in control and PEG sheep, increased over the 24 hours of incubation. Microbial-NAN in FD residues was higher in PEG than control sheep at 2 ($P<0.01$), 4 ($P<0.001$), 6.5 ($P<0.001$) and 11 ($P<0.001$) hours, but was similar ($P>0.05$) after 24 hours of incubation.

4.3.5 The *In vitro* Degradation of Fraction 1 Leaf Protein in Rumen Fluid.

The degradation of the large subunit (LSU) of Fraction 1 leaf protein from *Lotus pedunculatus* during incubation in rumen fluid was studied using SDS-PAGE (Fig. 6a and 6b). When FM and FD *Lotus* were incubated separately in rumen fluid *in vitro*, with and without PEG, the LSU of Fraction 1 leaf protein was readily detectable in the zero-time sample. However, after 4 hours of incubation with PEG, the LSU of Fraction 1 leaf protein from both the FD and FM *Lotus* was clearly degraded, whilst after 8 hours of incubation, it was, essentially, undetectable in both incubations. In contrast, after 8 hours, the LSU of Fraction 1 leaf protein from FM and FD *Lotus* was still readily detectable in incubations without PEG.

Fig. 2: Disappearance of dry matter (DM) from fresh-minced and freeze-dried Lotus pedunculatus during incubation in polyester-fibre bags suspended in the rumen of sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG.

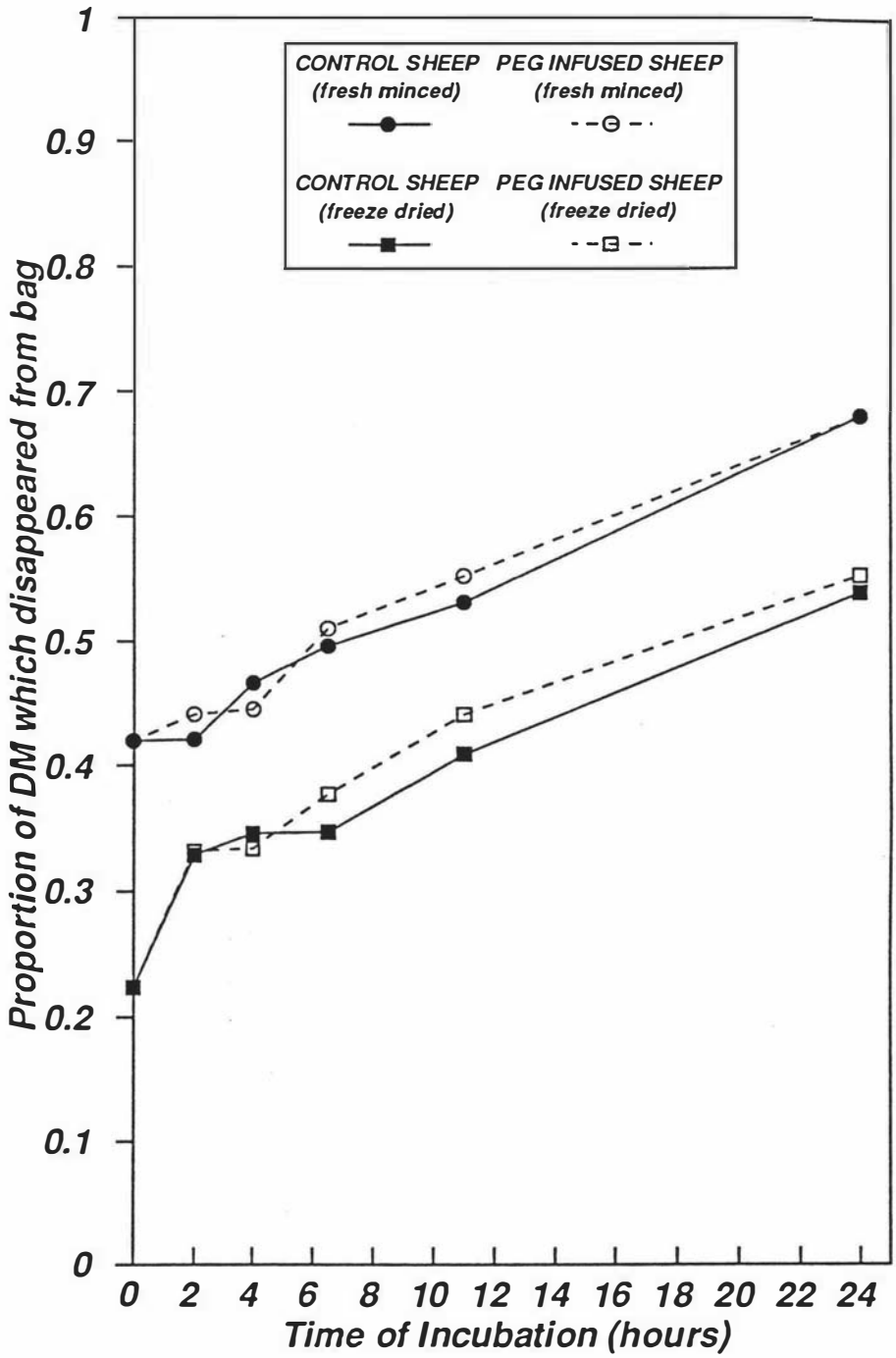


Fig. 3: Disappearance of nitrogen (apparent) and nitrogen corrected for microbial-NAN contamination (true) from fresh-minced Lotus pedunculatus during incubation in polyester-fibre bags suspended in the rumen of sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG.

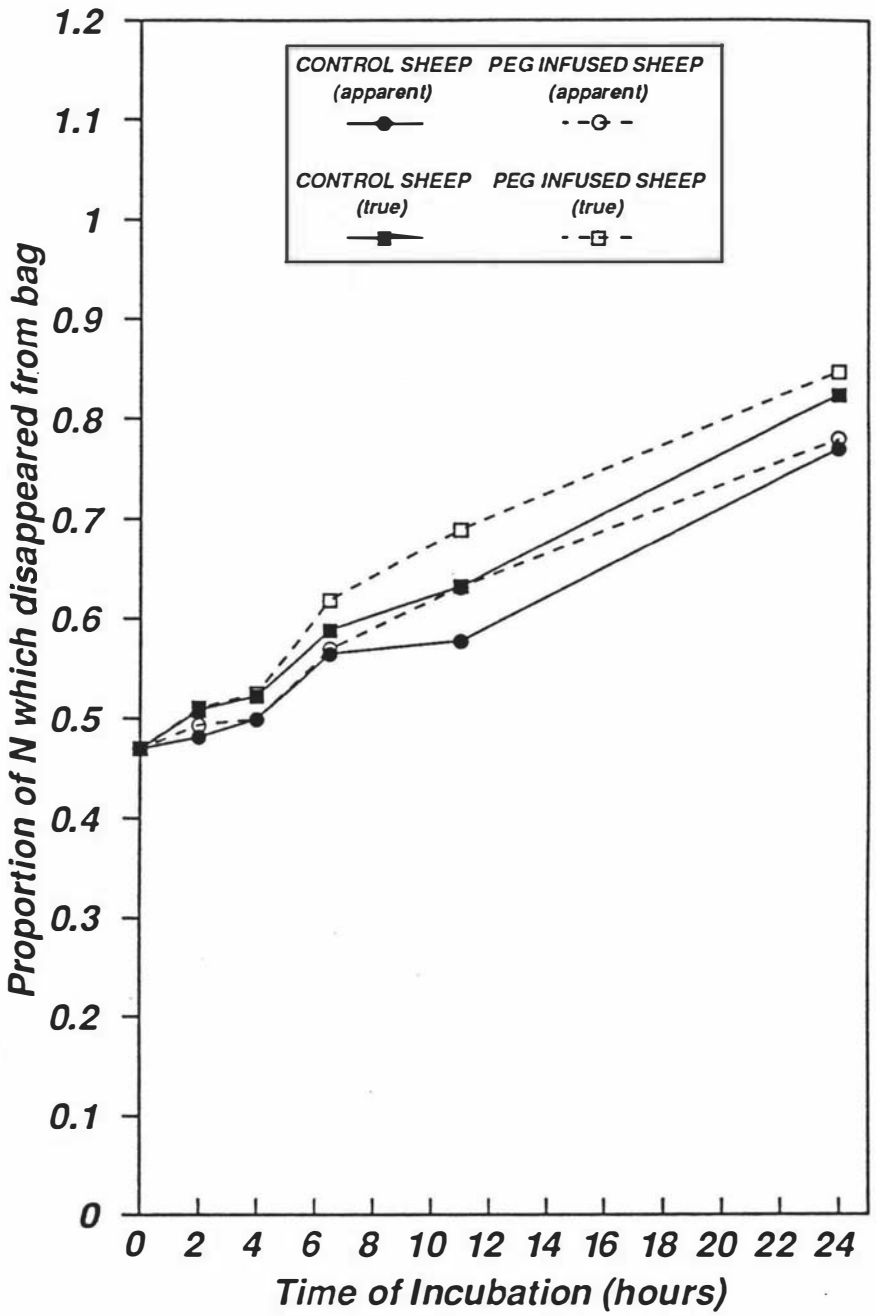


Fig. 4: Disappearance of nitrogen (apparent) and nitrogen corrected for microbial-NAN contamination (true) from freeze-dried Lotus pedunculatus during incubation in polyester-fibre bags suspended in the rumen of sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG.

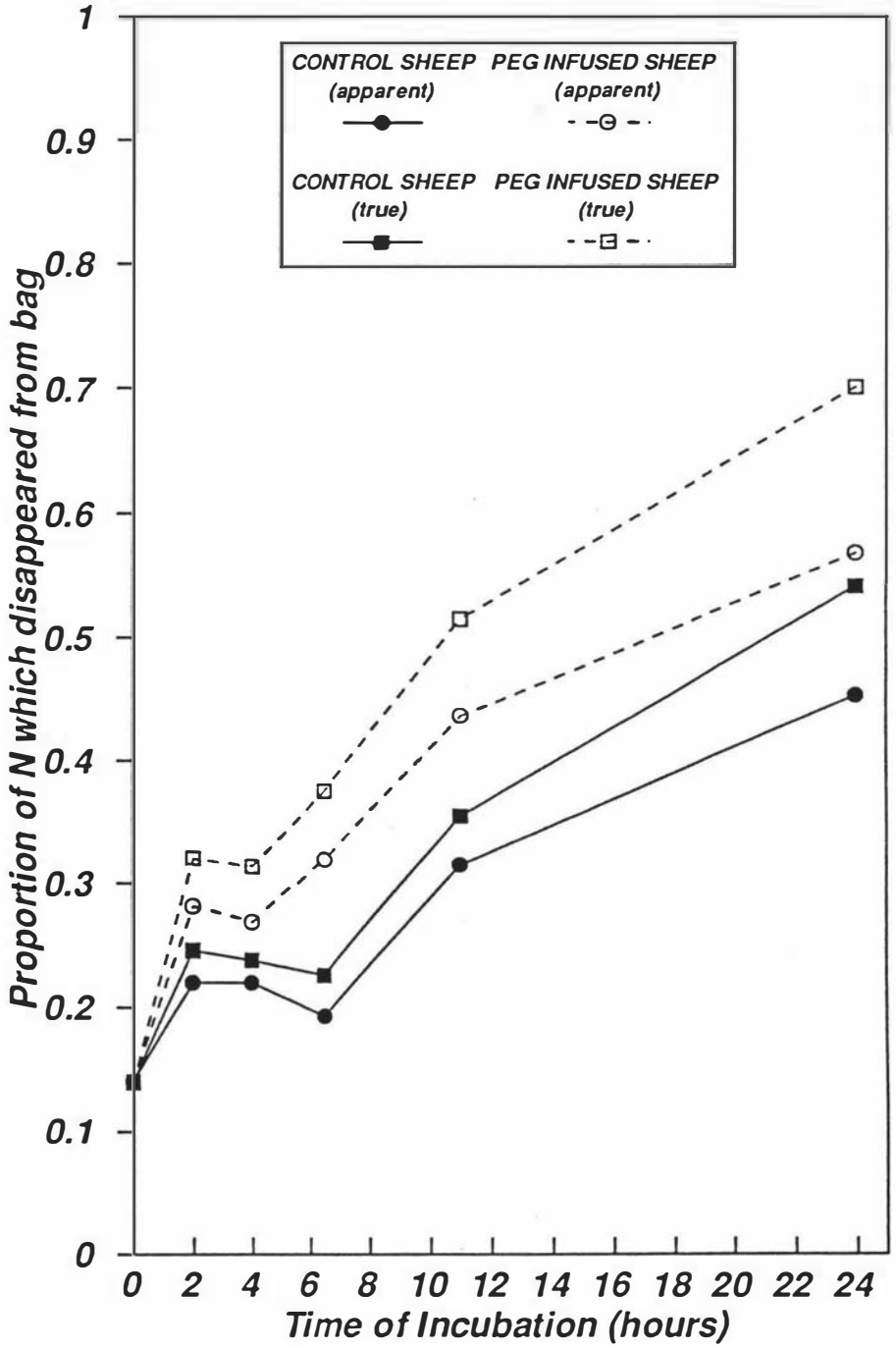


Fig. 5: Total microbial-NAN in polyester-fibre bag residues after incubation of fresh-minced and freeze-dried Lotus pedunculatus in the rumen of sheep fed Lotus pedunculatus, with and without an intraruminal infusion of PEG.

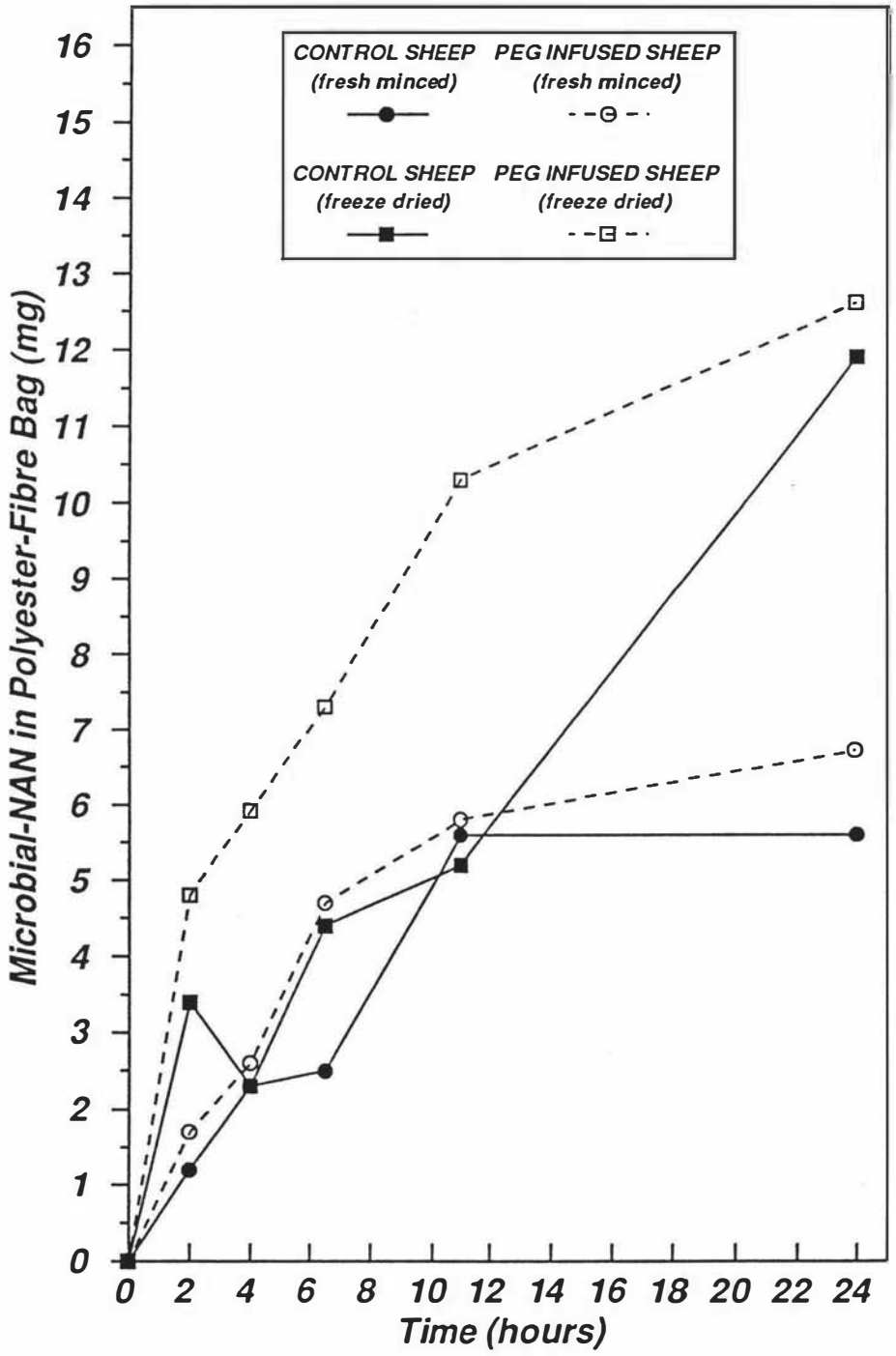
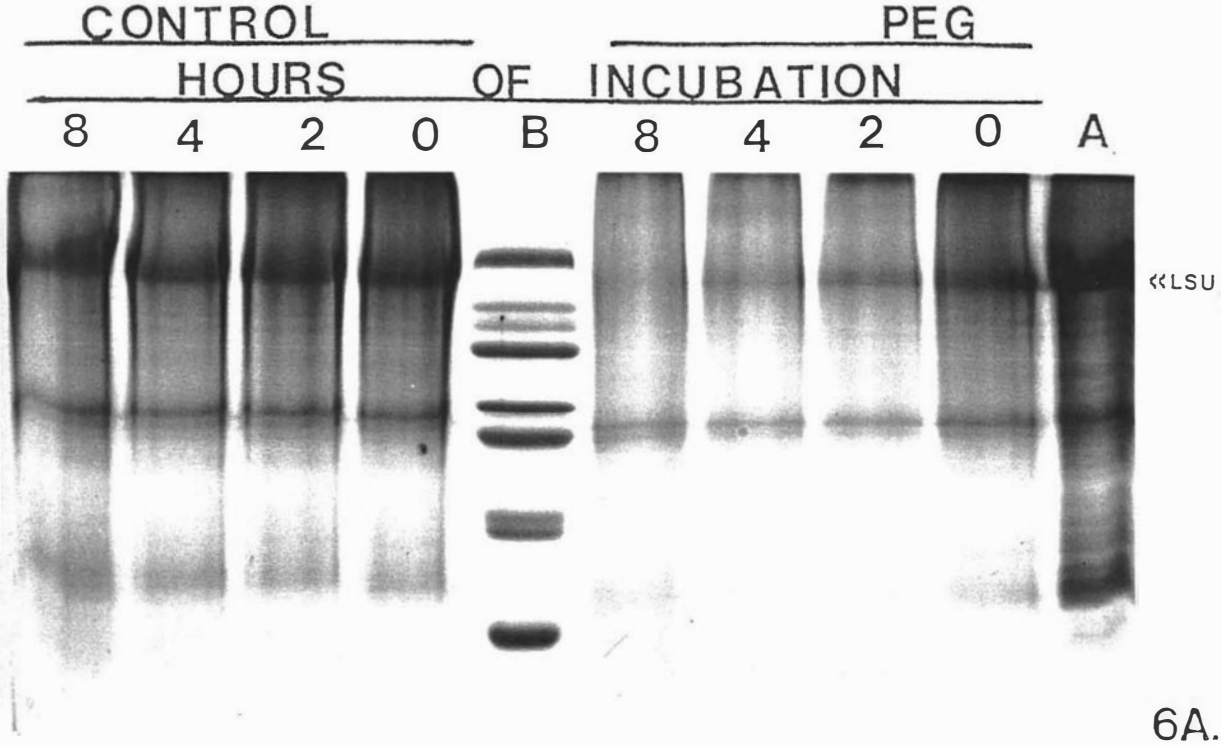
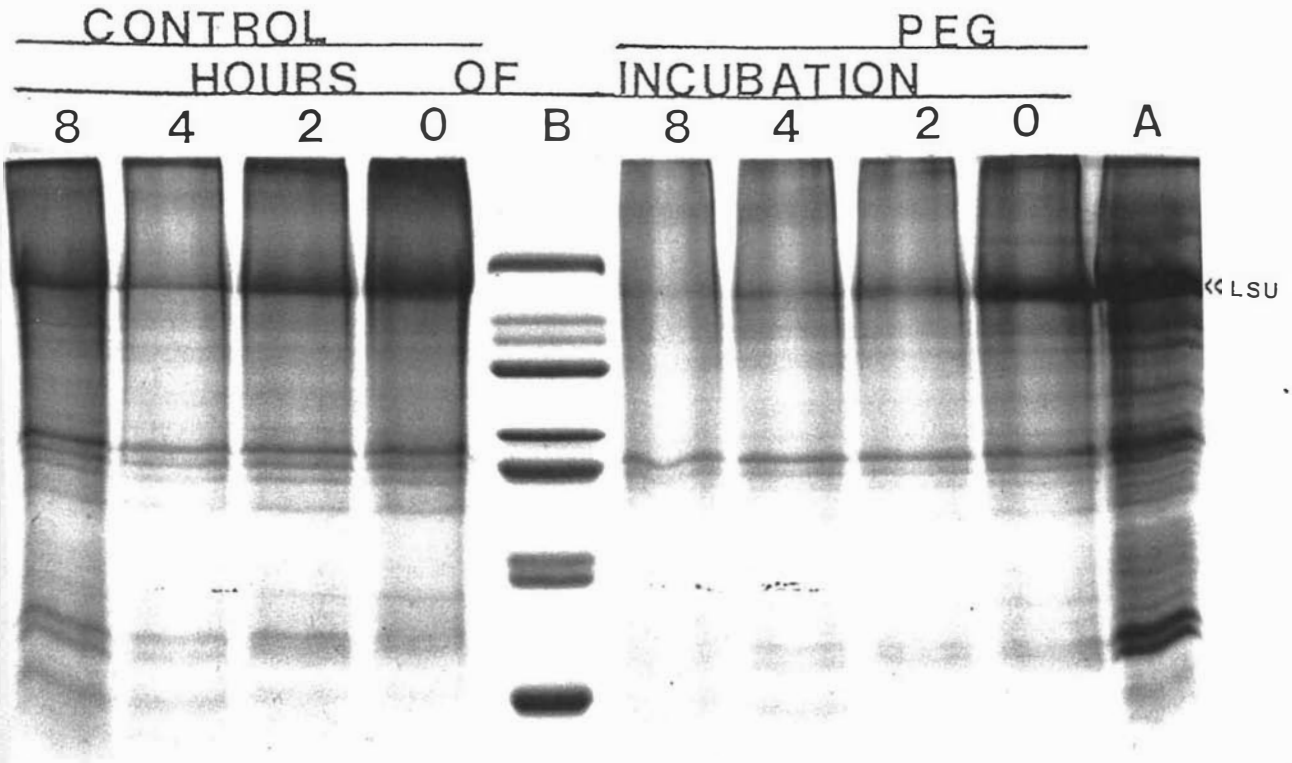


Fig. 6: Fractionation of the proteins of rumen fluid and fresh-minced (FM; Fig. 6a) or freeze-dried and ground (FD; Fig. 6b) *Lotus pedunculatus* (50gCT/kgDM) by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The degradation of the large subunit (LSU) of Fraction 1 leaf protein during incubation in rumen fluid in vitro, with and without PEG added. (A) A sample of FM (Fig. 6a) or FD (Fig. 6b) *Lotus* that has not been incubated in strained rumen fluid. (B) Molecular weight markers.



6A.



6B.

4.4 DISCUSSION.

Polyethylene glycol (1.7g/g CT) displaces CT from CT-protein complexes (Jones and Mangan, 1977) and completely binds available CT (Barry and Forss, 1983). Consequently, when PEG binds CT in the rumen, preventing it from binding to dietary protein, plant protein can be degraded to NH_3 by rumen microorganisms. Therefore, the increase in rumen NH_3 concentration after 3 days of intraruminal PEG infusion, indicates that in the present study PEG infusions effectively rendered CT unreactive in the rumen.

The degradation of plant protein in the rumen follows the sequence:

Insoluble protein > Soluble protein > Large polypeptides > Small polypeptides > Amino acids > Ammonia.

The increase in rumen NH_3 concentration after 3 days of PEG infusion suggests that CT prevented the degradation of forage protein to NH_3. However, the objective of the present study was to ascertain where in the protein degradation sequence (outlined above), CT had an effect on preventing forage protein degradation. The initial solubilization of plant protein in the rumen was studied by measuring the loss of plant-N from polyester-fibre bags suspended in the rumen. The degradation of soluble protein was studied by using SDS-PAGE to measure the *in vitro* degradation of Fraction 1 leaf protein in rumen incubations.

The method of sample preparation had a major effect on N solubility. Mincing resulted in 47% of N being soluble before incubation in the rumen, compared to only 14% of N from FD *Lotus*. These differences were maintained throughout incubation, with 83% of N lost from FM *Lotus* in control and PEG sheep, and 54% (control) and 70% (PEG) of N lost from FD *Lotus*. The difference in the solubilization of N from FD compared to FM *Lotus* was probably due to freeze-drying reducing the initial solubility of N.

The difference in solubilization of N and the microbial-NAN contamination of FM and FD *Lotus* tended to be greater in PEG than control sheep, with the differences being small with FM *Lotus*, but greater with FD *Lotus* over the 24 hours of incubation. Upon disintegration of plant material, such as during chewing, CT bind, firstly to protein, followed by carbohydrate, to form stable complexes at pH common in the rumen (5.5-7.0; Jones and Mangan, 1977). Costerton *et al.* (1978) suggested that rumen bacteria adhere to plant tissue by forming polar bonds between bacterial polysaccharides and negatively charged plant polysaccharides. Consequently, the ability of CT to bind to plant protein at rumen pH may

have disrupted polar bonding, reducing microbial colonization of *Lotus* in control compared to PEG sheep.

Mathers and Aitchson (1981) reported that the true loss of N was greater from lucerne than sprat-meal, incubated in synthetic-fibre bags in the rumen. Associated with this change, was an 8-fold increase in microbial contamination of lucerne compared to sprat-meal. Wallace (1985) suggested that the adsorption of bacteria onto protein was an integral part of protein degradation in the rumen. The extent of N loss from FM and FD *Lotus* corresponded to the extent of microbial colonisation of both feed preparations, in control and PEG sheep. Therefore, the effect of CT in reducing microbial colonization may have been responsible for the small and large difference in the solubilization of N from FM and FD *Lotus* respectively.

The extent to which CT reduced Fraction 1 leaf protein degradation appeared to be similar for both FM and FD *Lotus*. Therefore, once protein was solubilized, it appeared to be degraded in a similar manner, regardless of whether it was derived from fresh or dried *Lotus*. The addition of PEG to the *in vitro* incubations increased the degradation of Fraction 1 leaf protein from *Lotus pedunculatus*. Similarly, Tanner *et al.* (1990) reported that the addition of CT from sainfoin (*Onobrychis viciifola*) reduced the *in vitro* degradation of Fraction 1 leaf protein in similar incubations to those reported in the present study. The ability of CT to bind protein, forming insoluble complexes at rumen pH (5.5-7.0; Jones and Mangan, 1977) may prevent microbial degradation of protein in the rumen. This feature of CT may have been responsible for the reduction in degradation of Fraction 1 leaf protein in control incubations.

Condensed tannins reduced both solubilization and degradation of protein from FD *Lotus*. In contrast, CT had little effect on the solubilization of protein, but reduced the degradation of soluble protein from FM *Lotus*. Minced *Lotus* was included in the present study in an attempt to examine the effect of CT upon protein solubilization and degradation in the rumen of sheep fed fresh CT-containing forages. Therefore, with fresh forage diets, CT probably has little effect on the rate of protein solubilization, but reduces the degradation of soluble protein in the rumen. However, further experiments, similar to those outlined in Chapter 5, are needed to quantify the extent to which protein degradation was reduced by CT.

Spencer *et al.* (1988) suggested that the loss of N from synthetic-fibre bags suspended in the rumen measured the solubilization of plant protein, and that the rates of protein solubilization and degradation in the rumen were not necessarily similar. In the present

study, the solubilization of N from polyester-fibre bags, particularly with fresh *Lotus*, would not have been a good index of the effect of CT on protein degradation in the rumen.

4.5 CONCLUSIONS.

4.5.1 The loss of DM, N (apparent) and N corrected for microbial-NAN contamination (true), were measured for fresh-minced (FM) and freeze-dried and ground (FD) *Lotus pedunculatus* (50gCT/kgDM) incubated in polyester-fibre bags suspended in the rumen of control (CT operating) and PEG (CT not operating) sheep fed *Lotus pedunculatus* (50gCT/kgDM).

4.5.2 Freeze-drying diets is a common preparation for incubation in synthetic-fibre bags, whilst FM *Lotus* was included in an attempt to simulate the loss of DM and N from chewed fresh forages.

4.5.3 The lower true loss of N from FD compared to FM *Lotus* was probably due to freeze-drying reducing the initial solubility of N. Whereas, 47% of N in the polyester-fibre bags containing FM *Lotus* was instantly soluble, only 14% of N was instantly soluble in FD *Lotus*.

4.5.4 The true loss of N from FM and FD *Lotus* suspended in polyester-fibre bags in the rumen tended to be greater in PEG than control sheep. The difference was small with FM *Lotus*, only attaining significance at 11 hours of incubation, whilst the difference was much greater with FD *Lotus*, and was apparent over the 24 hours of incubation.

4.5.5 There was greater microbial-NAN colonization of FD than FM *Lotus* over the 24 hours of incubation. The higher N content of FD compared to FM residues, may have encouraged greater microbial colonization during incubation in the rumen.

4.5.6 Microbial-NAN contamination of undegraded residues tended to be greater in PEG than in control sheep. The difference was small with FM *Lotus*, only attaining significance at 6.5 and 24 hours of incubation, whilst the difference was much greater with FD *Lotus* over the 24 hours of incubation. Consequently, the ability of CT to prevent microbial colonization to a small and large extent respectively, in FM and FD *Lotus*, may have been responsible for the small and large difference in true loss of N in PEG compared to control sheep.

4.5.7 The degradation of the LSU of Fraction 1 leaf protein from *Lotus pedunculatus* (50gCT/kgDM) during incubation in rumen fluid *in vitro*, with and without PEG was

studied using SDS-PAGE. The addition of PEG (1.4g) to *in vitro* incubations increased the degradation of Fraction 1 leaf protein with both FM and FD *Lotus*. The ability of CT to bind protein, forming insoluble CT-protein complexes, at rumen pH (5.5-7.0) may be responsible for the reduction in degradation of Fraction 1 leaf protein in control incubations.

4.5.8 The extent to which CT reduced Fraction 1 leaf protein degradation appeared to be similar for both FM and FD *Lotus*. Therefore, once protein was solubilized, it was degraded in a similar fashion regardless of whether it was derived from fresh or dried *Lotus*.

4.5.9 It was concluded that, with FM Lotus, CT had little effect upon the rate at which protein was solubilized, but substantially reduced the rate at which soluble protein was degraded by rumen microorganisms. In contrast, CT reduced the rates of both solubilization and degradation of protein in FD *Lotus*.

5. The *in vitro* rates of rumen proteolysis of Fraction 1 leaf, vicilin, ovalbumin and sunflower albumin 8 proteins.

5.1 INTRODUCTION.

In ruminants fed high quality forage diets, duodenal nitrogen (N) flow is only 65-75% of N intake (MacRae and Ulyatt, 1974). The large loss of N from the rumen increases with increasing N intake (Ulyatt and Egan, 1979), and is due to the rapid degradation (70%) of soluble plant protein to ammonia, exceeding microbial utilisation of ammonia (Ulyatt *et al.*, 1975).

Ruminant production from forage diets appears to be limited by amino acid absorption from the small intestine (Tamminga, 1981; Hogan, 1982). Wool growth in particular, has been shown to respond to post-ruminal supplementation with sulphur amino acids (SAA; Reis, 1979). Therefore, an ideal dietary protein for ruminant production would contain a high proportion of SAA and be relatively resistant to rumen degradation. The use of genetic engineering to express genes coding for high SAA proteins in the leaves and stems of legumes commonly used in forage grazing systems may provide potential benefits for ruminant animal production.

Individual proteins differ in their susceptibility to degradation in the rumen. Qualitative and semi-quantitative studies suggested Fraction 1 (18S) leaf protein from lucerne (*Medicago sativa*) was completely degraded in the rumen (Nugent and Mangan, 1981), whilst bovine serum albumin, bovine submaxillary protein (Nugent *et al.*, 1983) and ovalbumin (Mangan, 1972) were relatively resistant to rumen degradation.

The sunflower seed (*Helianthus annuus*) 2S fraction contains proteins resistant to rumen degradation (D.Spencer, unpublished). This fraction contains at least eight distinct proteins, of which two have a high SAA content (Kortt *et al.*, 1990). The gene coding for one of these proteins, sunflower albumin 8 (SF8; 15.5% methionine; 7.5% cysteine), has been characterised (Kortt *et al.*, 1990) and genetically engineered for expression in the leaves and stems of a transgenic host (see chapter 6).

The establishment of the relative degradability of proteins in the rumen is an important pre-requisite for genetic engineering studies. The degradation rate of individual proteins incubated in rumen fluid both *in vivo* and *in vitro*, has been studied using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Nugent and Mangan, 1981; Nugent *et al.*, 1983; Spencer *et al.*, 1988). The objective of the present experiment was to develop a quantitative technique for studying the rate of SF8 protein degradation in an *in vitro* rumen incubation using SDS-PAGE, and to compare this with degradation rates for a range of soluble proteins differing in SAA content. The proteins selected were the large

(LSU) and small (SSU) subunit of Fraction 1 leaf protein from lucerne, vicilin protein from pea (*Pisum sativum*) seeds and ovalbumin from chicken egg white.

5.2 METHODOLOGY.

The rate of rumen proteolysis of five proteins, the LSU and SSU of Fraction 1 leaf protein, SF8, vicilin and ovalbumin were studied using single *in vitro* rumen incubations to measure the degradation of each protein. Each *in vitro* rumen was incubated for 36 hours. The objective of a genetic engineering programme described in chapter 6, is to express the genomic or cDNA coding regions for proteins containing a high proportion of SAA, such as SF8 and ovalbumin, in the leaves of plants commonly used in agricultural production systems, such as lucerne and subterranean clover. Therefore, ground fresh lucerne leaves were included in the *in vitro* rumen incubations of SF8, vicilin and ovalbumin.

5.2.1 PROTEIN EXTRACTION.

The source and SAA composition of proteins studied in the experiment are summarised in Table 1.

Total protein was extracted from sunflower (cv. Hysun 30) and pea (cv. Greenfeast) seeds by grinding in 0.02M N-Tis(hydroxymethyl)-methyl-2-aminoethane sulphonic acid, pH 7.8 according to the method of Spencer *et al.* (1988). Sunflower seed total protein extract was used as a source of SF8, whilst total pea seed protein extract was used as a source of vicilin for *in vitro* rumen incubations. The ovalbumin used in the *in vitro* incubations was supplied by BDH, Australia. The LSU and SSU of Fraction 1 leaf protein used in the *in vitro* rumen incubations was supplied by the addition of ground fresh lucerne leaves.

5.2.2 THE PREPARATION OF RUMEN FLUID.

A Border Leicester-Merino castrated male sheep fitted with a rumen cannula (55mm ID) was maintained on a diet of pelleted lucerne hay (1200gDM/d), offered daily at 10.00 hours. Rumen fluid was collected at 08.00 hours, after overnight fasting, and strained through muslin cloth into a Dewar flask flushed with CO_2 gas and maintained at 39°C. After collection, rumen fluid was again flushed with CO_2 gas and used immediately for *in vitro* incubations.

5.2.3 *IN VITRO* INCUBATION IN RUMEN FLUID.

The *in vitro* incubations were performed as given in stage 1 of the method described by Tilley and Terry (1963). Fresh lucerne leaves (4g), were ground to a slurry in a mortar with 40ml of freshly prepared artificial saliva pH 6.8 (McDougall, 1948), which was saturated with CO_2 gas and warmed to 39°C. This slurry was then added to each of four volumetric flasks (200ml). Rumen fluid (10ml) was added to each flask, which was then flushed with CO_2 gas and 2.5 mg of either total pea seed protein extract, total sunflower seed protein extract or ovalbumin was added. The fourth flask was used to study Fraction 1 leaf protein from lucerne leaves. All flasks were fitted with bunsen valves and shaken (200 rpm) at 39°C for 36 hours.

5.2.4 SAMPLING PROCEDURE.

Aliquots (1ml) were removed from each flask at 0, 0.5, 1, 2, 4, 8, 12, 16, 24 and 36 hours after the start of incubation and used for protein analysis. After each sample, volumetric flasks were flushed with CO_2 gas. Aliquots (1ml) were added to Epindorf tubes containing 50µl of 20% sodium-dodecyl-sulphate (SDS) and 50µl of 20% Triton X-100, to prevent protein precipitating out of solution upon freezing and subsequent thawing. Aliquots taken from the *in vitro* rumen incubation containing ground lucerne leaves only, were used for the analysis of the LSU and SSU of Fraction 1 leaf protein. These aliquots were frozen immediately in dry ice and stored at -20°C. Aliquots taken from *in vitro* rumen incubations containing ground lucerne leaves and either ovalbumin or pea or sunflower seed total protein extract were used for the analysis of ovalbumin, vicilin or SF8 protein. These aliquots where centrifuged at 3000g for 3 min and the supernatant transferred to another Epindorf tube and frozen immediately on dry ice and stored at -20°C.

5.2.5 SAMPLE PREPARATION FOR PROTEIN ANALYSIS BY SDS-PAGE.

5.2.5.1 Fraction 1 Leaf Protein.

Aliquots (1ml) taken from the *in vitro* incubation of fresh lucerne only were added to acetone (4ml) and total protein was precipitated at 0°C for 15 min. The residue, which contained both precipitated protein and whole plant material, was resuspended in 1ml of protein digestion buffer (see Appendix 10) and 100µl of the slurry was fractionated by SDS-PAGE.

5.2.5.2 Sunflower Albumin 8.

Aliquots (1ml) taken from the *in vitro* incubation of ground lucerne leaves and sunflower seed total protein extract were added to 2ml of methanol containing 6% glacial acetic acid (v/v) and protein precipitated at 0°C for 1 hour. Sunflower albumin 8 protein is methanol soluble, and thus was present in the supernatant, whilst all other proteins were precipitated. (see Fig. 6; Chapter 6). The supernatant (2ml) was added to acetone (8ml) and protein precipitated at 0°C for 15 min. The precipitate containing SF8 protein, was resuspended in 50µl of protein digestion buffer (see Appendix 10).

5.2.5.3 Vicilin and Ovalbumin Protein.

Vicilin and ovalbumin protein were extracted from the appropriate *in vitro* incubation samples (1ml) by immunocoupling to cyanogen-bromide (CNBr) Sepharose beads (50-150µ particle size; BDH, Australia). Swollen CNBr-Sepharose beads were first coupled to either vicilin or ovalbumin protein, followed by coupling of either sheep anti-vicilin or sheep anti-ovalbumin primary antibodies (IgG). Any remaining protein binding sites were blocked with glycine. Glycine does not block the IgG vicilin or ovalbumin binding sites, as these sites are specific for vicilin or ovalbumin protein and only bind these proteins by antigen-IgG specific interactions.

Immunocoupling was then carried out by adding 25µl of the appropriate CNBr-Sepharose-IgG beads to the *in vitro* rumen incubation samples (1ml) and shaking the samples for 1 hour at room temperature. The CNBr-Sepharose-IgG beads, to which ovalbumin or vicilin protein were coupled, were then resuspended in 50µl of protein digestion buffer (see Appendix 10).

5.2.6 SDS-PAGE, COOMASSIE STAINING AND WESTERN BLOT ANALYSIS.

All proteins resuspended in digestion buffer were fractionated by SDS-PAGE according to the method described by Spencer *et al.* (1988), after heating at 95°C for 15 min to relax the tertiary structure of the protein. Electrophoresis was carried out for 20 hours at a current of 14 milliamps (mA).

After SDS-PAGE, the large subunit (LSU) and small subunit (SSU) of Fraction 1 Leaf protein were detected by staining gels with Coomassie Brilliant Blue R (see Appendix 16) for 1 hour followed by destaining in destain (see Appendix 17) for 16 hours.

Ovalbumin, vicilin and SF8 protein were detected by Western Blot analysis. The primary and secondary antibodies for detecting each protein are summarised in Table 2. As the CNBr-Sepharose-IgG beads were prepared using sheep antibodies, the use of CNBr-Sepharose-IgG beads in Western Blot analysis requires a primary antibody, from a species other than the sheep. During Western Blot analysis, sheep anti-vicilin or anti-ovalbumin primary IgG would detect both vicilin or ovalbumin protein and sheep antibodies released from the CNBr-Sepharose-IgG beads during SDS-PAGE. However, rabbit anti-vicilin and anti-ovalbumin IgG detects only vicilin or ovalbumin protein.

Separated proteins were electroblotted onto nitrocellulose (NC) membranes (Schleicher and Schuell, West Germany) using a semi-dry blotter (BioRad, Australia) run at 200mA for 90 min.

The NC membranes were washed in TBS buffer, pH 7.4; 5% skim milk powder (w/v; see Appendix 11) for 4 hours at room temperature to block all protein binding sites. After blocking, the NC membranes were washed three times for 5 min in TTBS buffer, pH 7.4 (see Appendix 12), at room temperature to remove excess milk protein. The NC membranes were then sealed in plastic bags containing 5ml of TTBS buffer, pH 7.4; 1% skim milk powder (w/v) and the primary antibody, and incubated for 2 hours at room temperature. The NC membranes were then washed three times for 5 min in TTBS buffer, pH 7.4 to remove excess primary antibody not bound to the complementary protein. The NC membranes were then sealed in plastic bags containing 5ml of TTBS buffer, pH 7.4; 1% skim milk powder (w/v) and the appropriate dilution of secondary antibody conjugated to alkaline phosphatase (BioRad, Australia). After incubation for 1 hour at room temperature, the NC membranes were washed three times for 5 min in TTBS buffer, pH 7.4 and once for 5 min in TBS buffer, pH 7.4 to remove excess secondary antibody not bound to primary antibody. The protein bands were then detected by colorimetric reaction of alkaline phosphatase (bound to protein on the NC by the specific interaction between antigen and antibody) with the substrates p-nitro-blue-tetrazolium chloride (NBT) and 5-bromo-4-chlor-3-indolyl-phosphate (BCIP; BioRad, Australia; see Appendix 13) in the presence of Mg^{2+} ions.

5.2.7 QUANTIFICATION OF WESTERN BLOTS.

Developed Western blots and Coomassie Blue stained gels were photographed, and protein bands on the negatives were scanned using a double beam recording microdensitometer (Joyce-Loebl, England). The relative intensity of each protein band was determined by cutting out and weighing the paper scans of each peak. Therefore, the intensity of each protein band is presented as a peak weight (mg).

5.2.8 CALCULATION OF DATA.

The disappearance of protein, represented by changes in peak weight (mg), were plotted against time of incubation and curves representing either one or two components fitted. When proteolysis in the *in vitro* incubation obeyed first order kinetics, then a single exponential component (equation 1; Leng, 1982) was used to fit the data;

$$Y = A_e^{-kt}, \qquad (1)$$

Where;
t = time, in hours (h).
Y = peak weight (mg) at time t.
A = peak weight (mg) at time zero.
k = the fractional degradation rate of the protein (h^{-1})

The fractional degradation rate of the protein can be calculated from equation 2.

$$\log_e(Y/A) = -kt \qquad (2)$$

When proteolysis in the *in vitro* incubation occurred as two components, then two exponential equations (1), one for each component, were used to fit the data.

The proteolysis of MW 12100 SF8 protein in the *in vitro* incubation was associated with the appearance of a MW 8000 polypeptide. The observed peak weights for the MW 8000 polypeptide were a result of the difference between the rate of appearance and the rate of degradation of the MW 8000 polypeptide in the *in vitro* incubation. After 16 hours, the MW 12100 SF8 protein was no longer detectable in the *in vitro* incubation. Consequently, the rate of appearance of the MW 8000 polypeptide from 16 hours onwards was assumed to be zero. An exponential equation (1) was used to fit the data for the MW 8000 polypeptide from 16 hours onwards (A2; Fig. 3). A2 was assumed to represent the rate of degradation of the MW 8000 polypeptide in the *in vitro* incubation. The actual peak weights (mg) over the initial 16 hours of incubation were deducted from peak weights calculated for the initial 16 hours of incubation, from A2. These predicted peak weights (mg) were used to derive a second exponential equation (1), which was assumed to represent the rate of appearance of the MW 8000 polypeptide in the *in vitro* incubation (A1; Fig. 3)

The half-life ($t_{1/2}$; hours) of each protein or each component was calculated from equation 3 (Leng *et al.*, 1981).

$$t_{1/2}\,(h) = \frac{\log_e 0.5}{k} = \frac{0.693}{k} \qquad (3)$$

Table 1: Characteristics of the proteins used in the study of in vitro rumen degradation rates.

Protein	Source	MW	Number of Amino Acid Residues	Methionine Residues (% total)	Cysteine Residues (% total)
Fraction 1 leaf Protein (1)	Lucerne				
LSU		54000 *	491	1.6	1.5
SSU		16000 *	145	1.9	1.6
Sunflower Albumin 8 (2)	Sunflower Seeds	12100	103	15.5	7.5
Vicilin (3)	Pea Seeds	50000	430	0	0
Ovalbumin (4)	Chicken Egg White	43000	354	1.5	4.5

(1), Moon and Thompson (1969); (2), Kortt et al. (1990); (3), Higgins et al. (1988); (4), Gilbert (1971);

*, Kawashima and Wildman (1970); LSU, Large Subunit; SSU, Small Subunit

Table 2: Primary and secondary antibodies used during the detection of vicilin, ovalbumin or sunflower albumin 8 protein in rumen fluid by Western Blotting analysis.

PROTEIN	PRIMARY ANTIBODY		SECONDARY ANTIBODY	
	Source	Dilution	Source	Dilution
Sunflower Albumin 8	Mouse anti-SF8	0.064	Rabbit anti-mouse (BioRad, Australia)	0.0003
Vicilin	Rabbit anti-vicilin	0.002	Goat anti-rabbit (Promega, Australia)	0.0001
Ovalbumin	Rabbit anti-ovalbumin	0.002	Goat anti-rabbit (Promega, Australia)	0.0001

5.3 RESULTS.

The *in vitro* rumen degradation of the LSU and SSU of Fraction 1 leaf protein, SF8, vicilin and ovalbumin were monitored using SDS-PAGE. Microdensitometry was used to convert the density of protein bands on Western blots or Coomassie Blue stained gels into peak areas. Peak areas were weighed and the disappearance of each protein, represented by peak weight (mg), were plotted against time of incubation. Exponential equations were derived according to the line of best fit for the one or two components of proteolysis and used to calculate the rates of proteolysis and half-lives for each protein in the *in vitro* incubation.

When strained rumen fluid from a sheep fed pelleted lucerne hay (1200gDM/d) was fractionated by SDS-PAGE, no major distinctive polypeptide components were detected by either Coomassie Blue staining or Western Blot analysis. Similarly, when CNBr-Sepharose beads bound with either sheep anti-vicilin or sheep anti-ovalbumin IgG, were fractionated by SDS-PAGE, no major distinctive polypeptides were detected by Western Blot analysis. Against this background, proteins added to the *in vitro* rumen incubations were readily detectable.

5.3.1 The Large and Small Subunit of Fraction 1 Leaf Protein.

Proteolysis of the LSU of Fraction 1 leaf protein in the *in vitro* rumen incubation occurred as two components (Fig. 1). The first component, which occurred during the initial 12 hours of incubation had a rate of proteolysis of $0.06h^{-1}$ and a half-life of 11.6 hours (Table 3), whilst the second component, which occurred from 12 hours onwards had a rate of proteolysis of $0.45h^{-1}$ and a half-life of 4.6 hours (Table 3). In contrast, the proteolysis of the SSU of Fraction 1 leaf protein (Fig. 2) in the *in vitro* rumen incubation followed first order kinetics and had a rate of proteolysis of $0.04h^{-1}$ and a half-life of 17.3 hours (Table 3).

5.3.2 Sunflower Albumin 8.

The rate of proteolysis of SF8 protein in the *in vitro* rumen incubation is shown in Fig. 3. The 12100 MW SF8 protein had a rate of proteolysis of $0.23h^{-1}$ and a half-life of 3 hours (First component; Table 3). Disappearance of the 12100 MW SF8 protein coincided with the appearance of an 8000 MW polypeptide, which was also detected by the antibody used in the Western Blot analysis to detect the MW 12100 SF8 protein.

Since the MW 8000 polypeptide was a product of the proteolysis of the MW 12100 SF8 protein, it was assumed that after 16 hours, when the SF8 protein was no longer detectable

in the *in vitro* rumen incubation, that changes in the MW 8000 polypeptide were due to proteolysis. The exponential equation (A2) was calculated using peak weights from 16 hours onwards and therefore represents the rate of proteolysis of the MW 8000 polypeptide. The exponential equation (A1) was calculated using peak weights during the initial 16 hours of incubation, which were corrected for the rate of proteolysis (A2), and thus represents the rate of appearance of the MW 8000 polypeptide in the *in vitro* rumen incubation.

The rate of appearance of the MW 8000 polypeptide ($0.09h^{-1}$), was much faster than its rate of proteolysis ($0.01h^{-1}$), with the latter representing a half-life in the *in vitro* rumen incubation of 69.3 hours.

5.3.3 Vicilin.

The proteolysis of vicilin (Fig. 4) in the *in vitro* rumen incubation followed first order kinetics and had a rate of proteolysis of $4.3h^{-1}$ and a half-life of about 10 min (Table 3).

5.3.4 Ovalbumin.

Proteolysis of ovalbumin during *in vitro* rumen incubation also occurred as two components (Fig. 5). However, the first component, which ocurred during the initial 16 hours of incubation was essentially, not degraded in the *in vitro* rumen incubation (Table 3). The rate of proteolysis of ovalbumin from 16 hours onwards was $0.08h^{-1}$, with the second component having a half-life of 8.7 hours (Table 3).

Fig. 1: The proteolysis of the large subunit of Fraction 1 leaf protein from lucerne leaves in an in vitro rumen incubation.

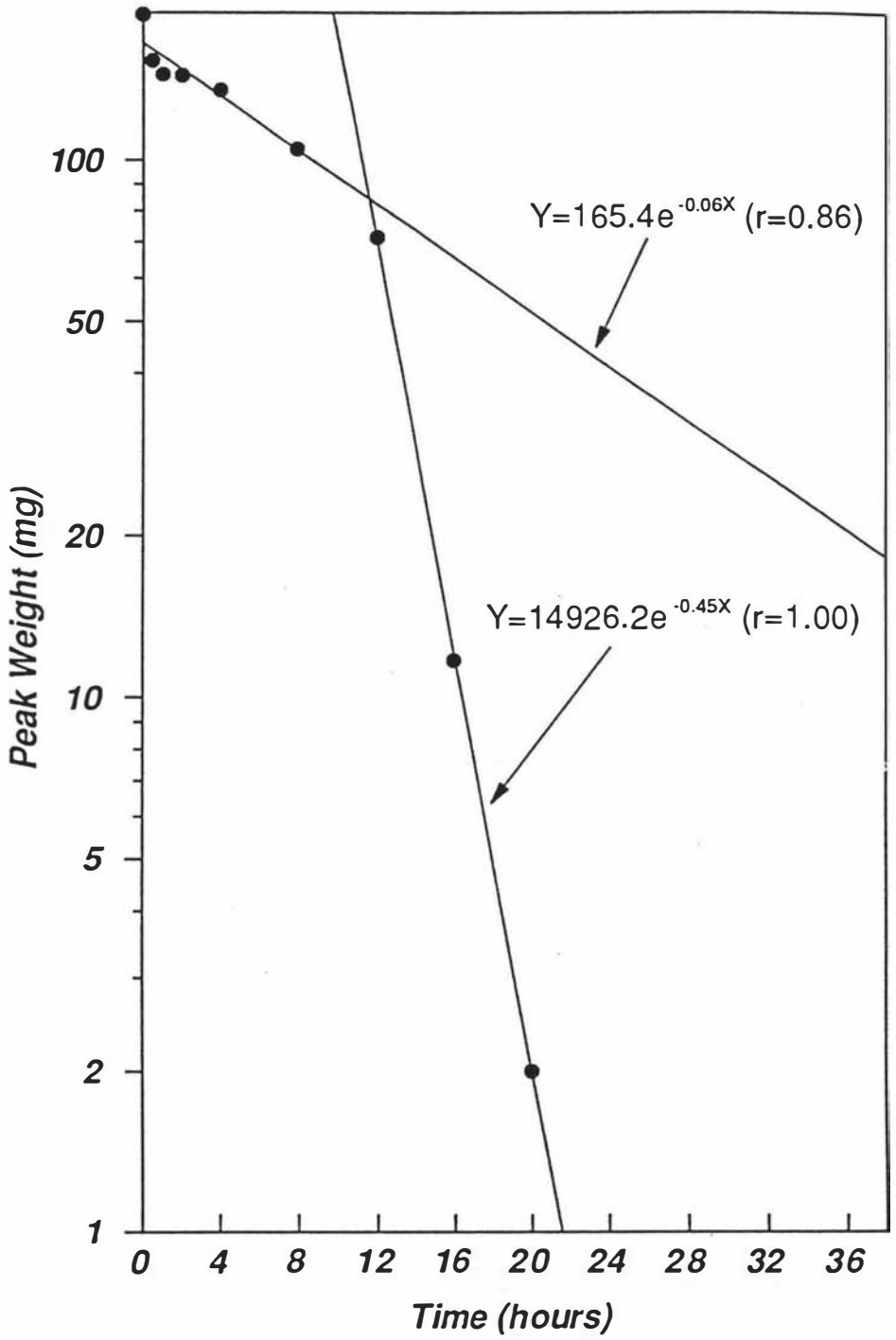


Fig. 2: The proteolysis of the small subunit of Fraction 1 leaf protein from lucerne leaves in an <u>in vitro</u> rumen incubation.

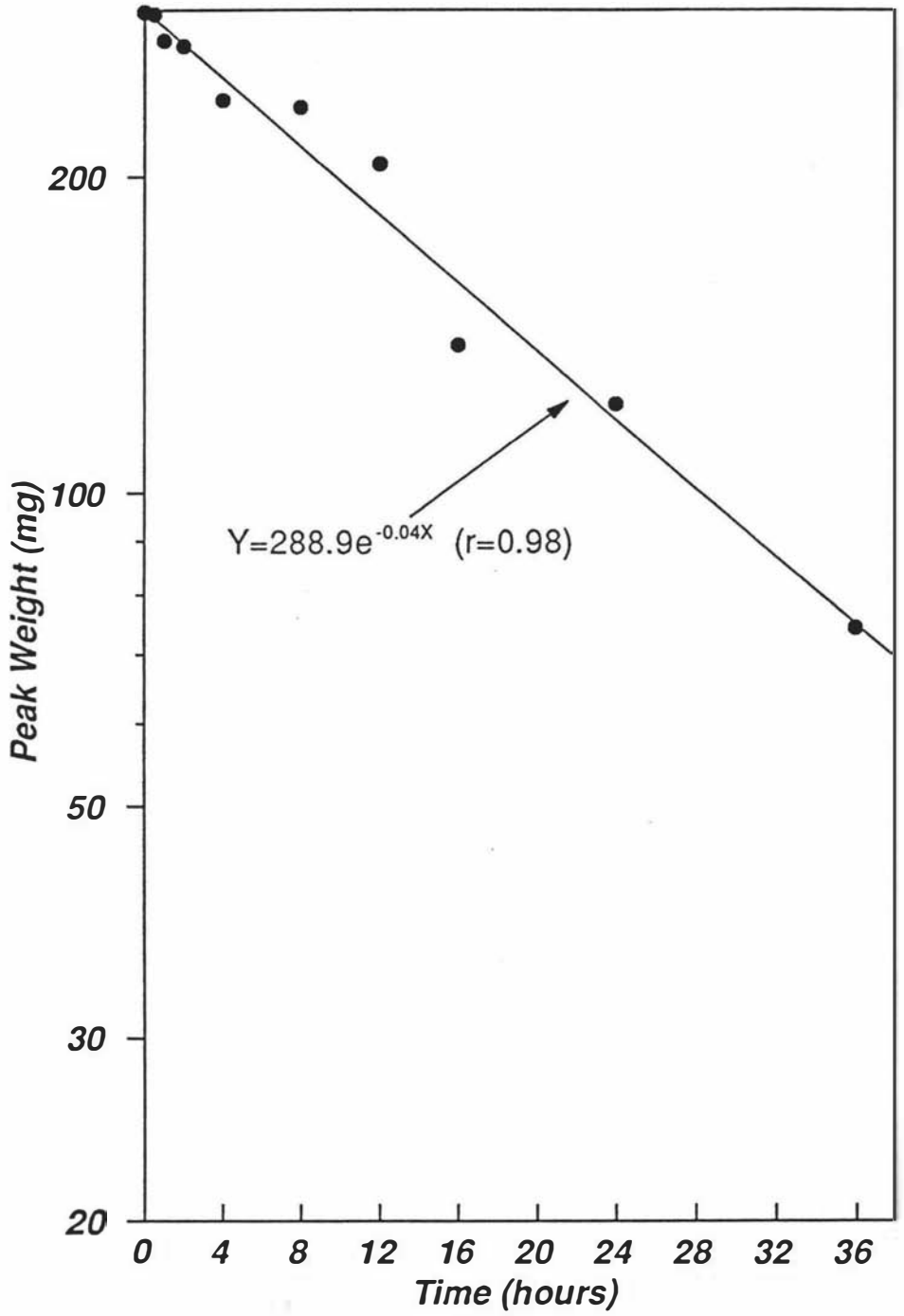


Fig. 3: The proteolysis of sunflower albumin 8 protein in an in vitro rumen incubation after the addition of 2.5mg of sunflower seed total protein extract. The 8kDa polypeptide product of SF8 proteolysis has two components; a rate of appearance (A1) and a rate of proteolysis (A2).

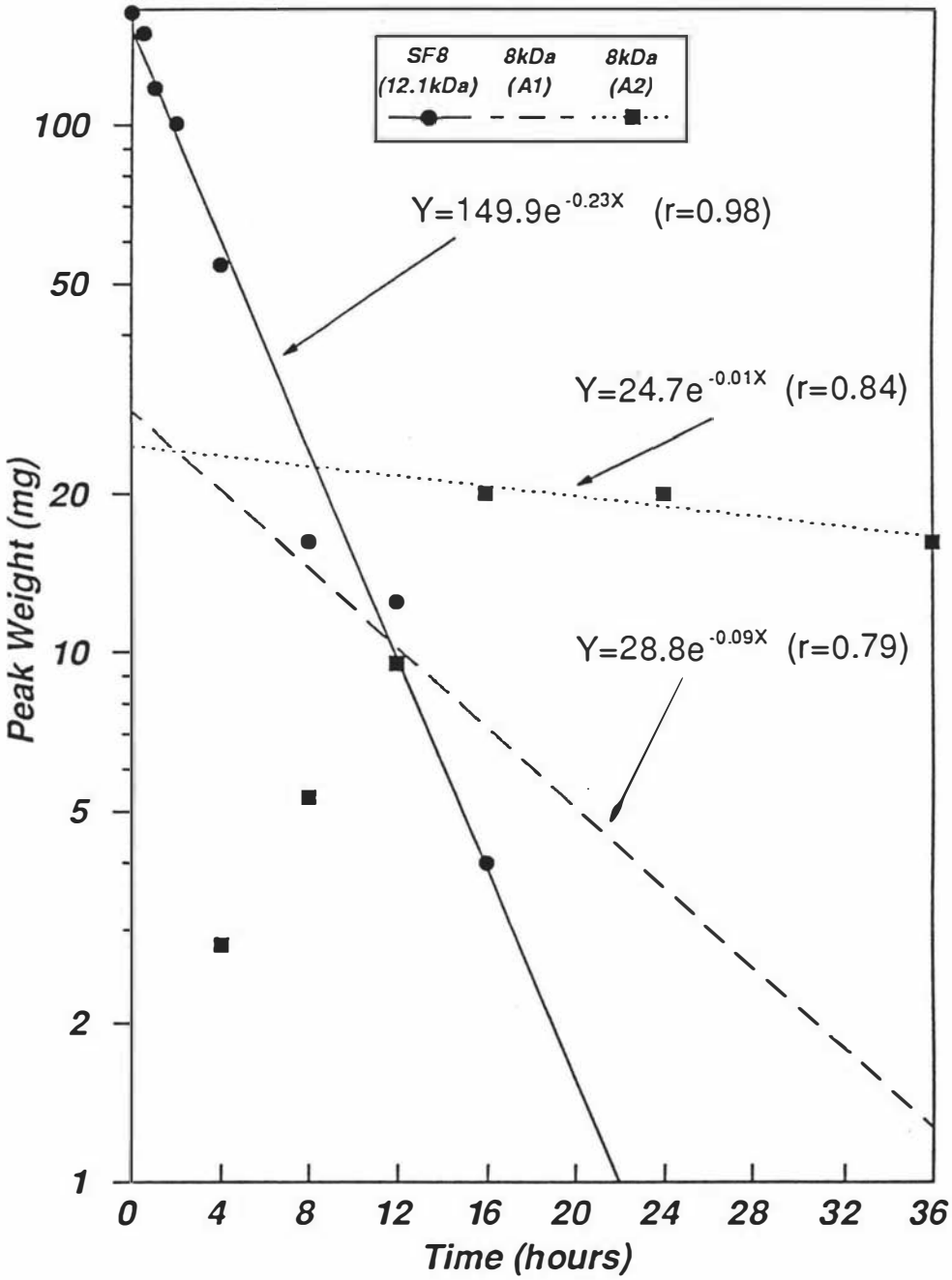


Fig. 4: The proteolysis of vicilin protein from pea seeds in an in vitro rumen incubation.

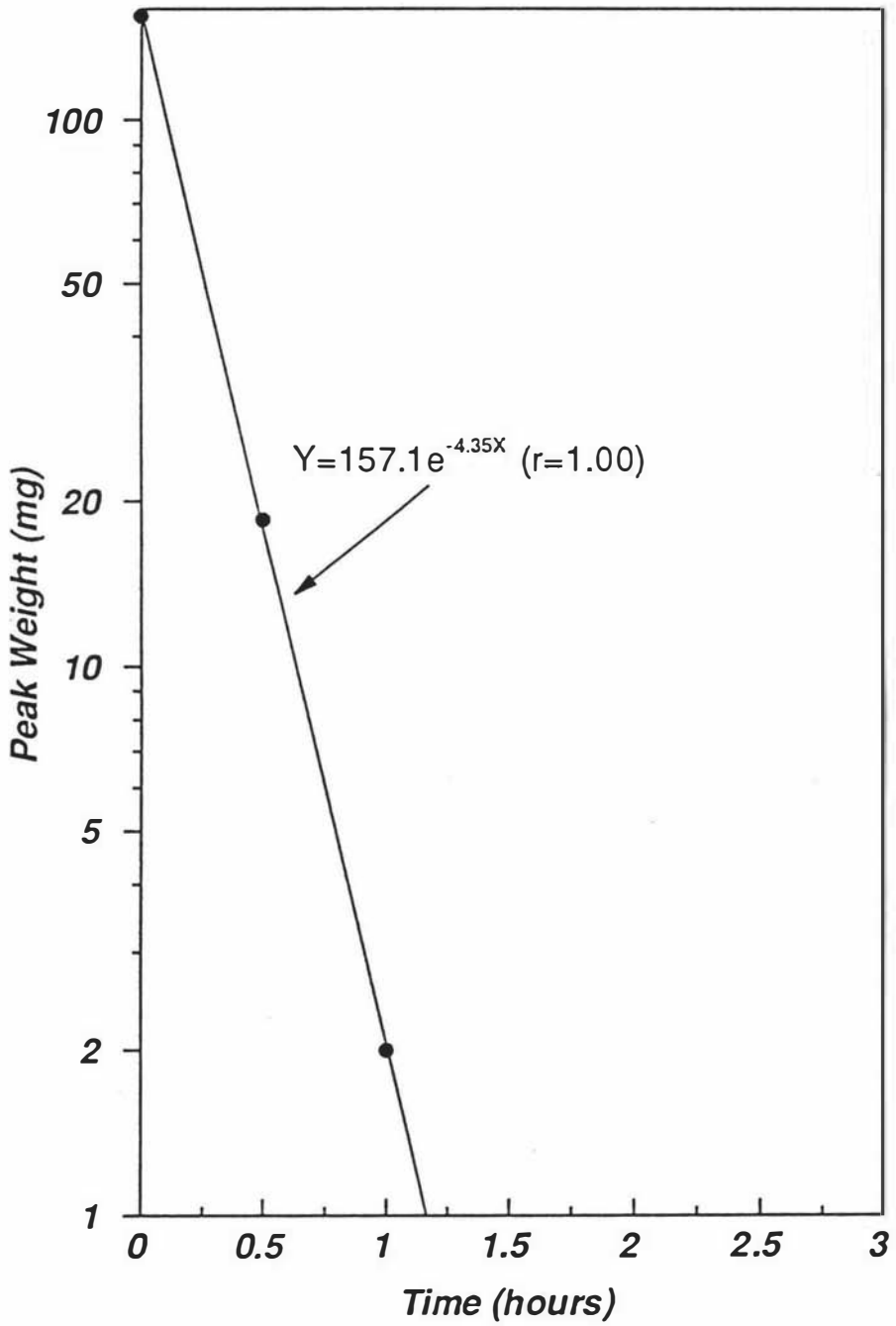


Fig. 5: The proteolysis of ovalbumin protein from from chicken egg whites in an in vitro rumen incubation.

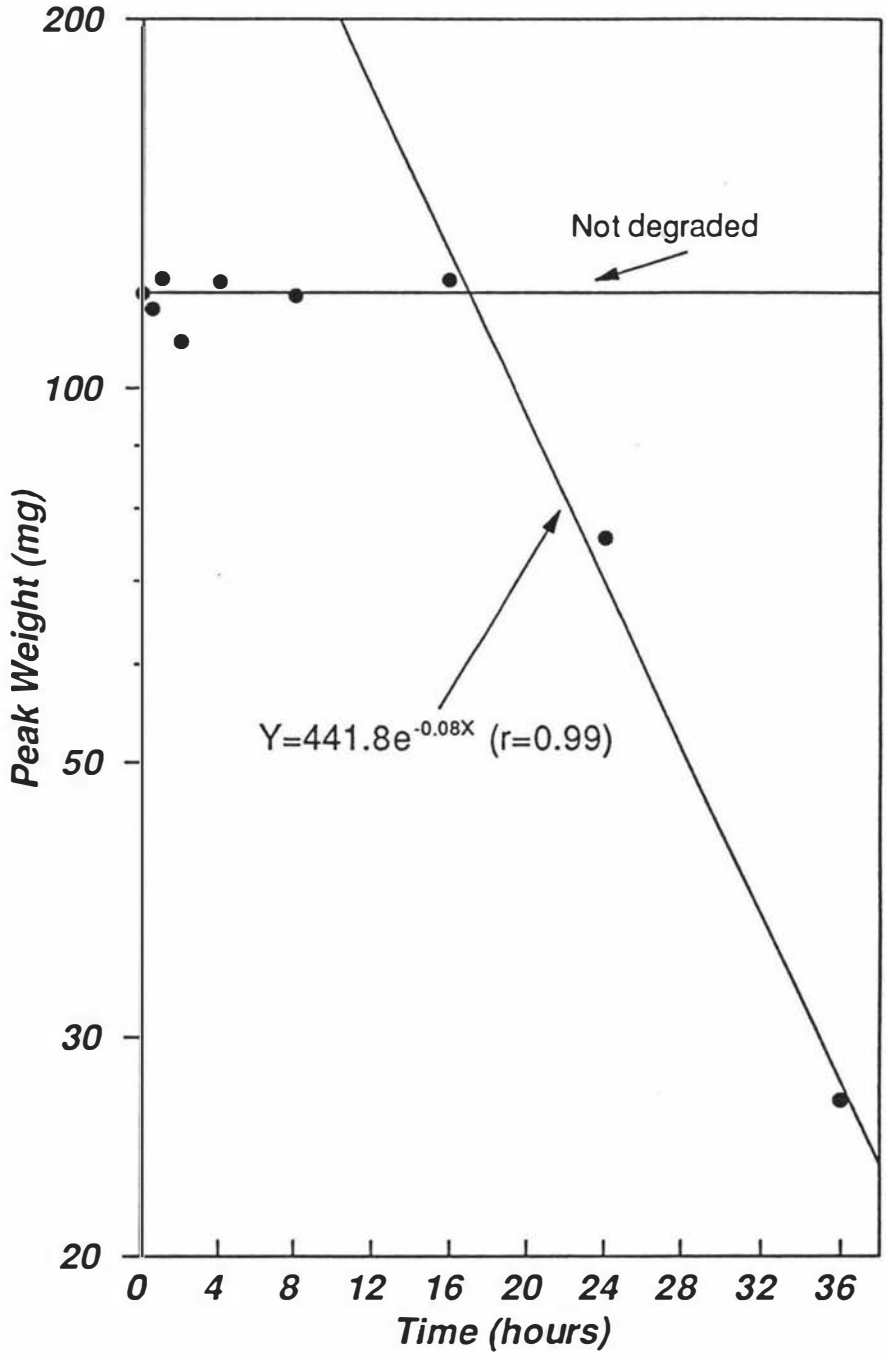


Table 3: The delay time (h) and half-lives (h) of the large and small subunit of Fraction 1 leaf protein, vicilin, ovalbumin and sunflower albumin 8 proteins in an in vitro rumen incubation.

(Data for each protein are from one rumen incubation)

PROTEIN	DELAY TIME (hours)	HALF-LIFE (hours)	
		First Component	Second Component
COOMASSIE STAINING			
Fraction 1 Leaf Protein			
LSU	none	11.6	4.6
SSU	none	17.3	NA
WESTERN BLOTTING			
Sunflower Albumin 8	none	3.0	69.3
Vicilin	none	0.16	NA
Ovalbumin	16	NA	8.7

NA, not applicable; LSU, Large Subunit; SSU, Small Subunit

5.4 DISCUSSION.

Our current research is aimed at using genetic engineering techniques to introduce genes coding for proteins containing high proportions of SAA, such as SF8 and ovalbumin, into pasture species such as lucerne and subterranean clover. Since wool growth in sheep is limited by the supply of SAA (Reis, 1979), it is thought that ingestion of forages containing rumen-resistant, high SAA protein will lead to increased wool growth. Consequently, as lucerne is a target species, all *in vitro* rumen incubations included ground fresh lucerne.

Nugent and Mangan (1981) reported that the proteolysis of Fraction 1 leaf protein in the rumen of sheep fed a hay/concentrate diet occurred as two components, similar to the proteolysis of Fraction 1 leaf protein observed in the present study. However, they reported that the rate of proteolysis of both components was twenty times faster than the rates of proteolysis reported in the present study. This is probably a reflection of the difference between using an *in vivo* and *in vitro* system to study the rate of proteolysis of Fraction 1 leaf protein. Nugent *et al.* (1983) reported that both Fraction 1 leaf protein and bovine serum albumin were degraded more slowly *in vitro* than *in vivo*.

The proteolysis of MW 12100 SF8 protein in the *in vitro* rumen incubation followed first order kinetics, with a shorter half-life (3.0 hours) than that of either component of the LSU of Fraction 1 leaf protein (11.6 hours and 4.6 hours; Table 3). This represents a rate of degradation only exceeded by vicilin. However, the proteolysis of the MW 12100 SF8 protein was associated with the appearance of a MW 8000 polypeptide, which was extremely resistant to rumen proteolysis (half-life of 69.3 hours). Therefore, although SF8 protein was not resistant to rumen proteolysis, a polypeptide product of its degradation was very resistant to further degradation.

The transfer quotient (TQ) or proportion of MW 8000 polypeptide originating from the degradation of MW 12100 SF8 protein can be calculated from equation 4 (Leng, 1982).

$$TQ = \frac{\text{Area under MW 8000 polypeptide curve, A1 (Fig. 5)}}{\text{Area under MW 12100 SF8 protein degradation curve}} \quad (4)$$

After 16 hours the MW 12100 SF8 protein was undetectable in the *in vitro* rumen incubation, therefore no further MW 8000 polypeptide could have been produced. Thus only the area between 0 and 16 hours (Fig. 3) was considered, with the areas being corrected for differences in MW. The calculated TQ of 1.0 suggests that the total MW 12100 SF8 protein that disappeared equalled the total MW 8000 polypeptide that appeared. Presumably a single polypeptide of MW 4100 or a number of polypeptides equivalent in total to MW 4100 were also produced.

In contrast to SF8 protein, ovalbumin was essentially not degraded at all in the *in vitro* rumen incubation during the initial 16 hours of incubation, after which its rate of proteolysis was similar to that of the LSU of Fraction 1 leaf protein. Sunflower albumin 8, ovalbumin and the SSU of Fraction 1 leaf protein were more resistant to rumen degradation than vicilin or the LSU of Fraction 1 leaf protein. However, the difference in the way each protein was degraded suggests different mechanisms were responsible for the relative resistance of each protein to rumen proteolysis.

Mangan (1972) and Nugent *et al.* (1983) reported that ovalbumin was relatively resistant to rumen proteolysis, but that resistance could be reversed by treating the protein chemically, to break disulphide bonds. The lack of disulphide bonding in vicilin may contribute to its relatively rapid proteolysis in the *in vitro* rumen incubation. However, disulphide bonding is not the only factor determining resistance to rumen protein degradation. The SSU of Fraction 1 leaf protein, which has no disulphide bonds (Kawashima and Wildman, 1970), was also relatively resistant to rumen proteolysis (half-life of 17.3 hours). In contrast, both SF8 (Kortt *et al.*, 1990) and ovalbumin (Gilbert, 1971) contain disulphide bonds, yet two different mechanisms appear to be responsible for their resistance to rumen proteolysis. Presumably the tertiary structure of the proteins are involved, although further research is required to develop an understanding of possible interactions between protein structure and rumen proteolysis.

Although, in the present study, both SF8 and ovalbumin protein were more resistant to rumen proteolysis than the LSU of Fraction 1 leaf protein, it is difficult to know if the relative resistance of these proteins determined *in vitro* would be sufficient to survive rumen proteolysis *in vivo*. The response of sheep to post-ruminal supplementation of SAA (Reis, 1979) suggests sheep grazing legumes modified to express high SAA proteins, such as ovalbumin and SF8 in leaf tissue, should produce more wool, provided the absorption of SAA from the small intestine is increased. To date, the release of amino acids from proteins such as ovalbumin and SF8, in the small intestine of ruminants has not been studied. Experiments need to be conducted to determine if oral administration of high SAA proteins, selected *in vitro* for relative resistance to rumen proteolysis, will stimulate wool growth in grazing sheep.

The quantitative techniques developed in this study provide a useful method for the initial screening of proteins for resistance to rumen degradation. They may also be useful for examining mechanisms by which rumen proteolysis of plant protein is reduced, eg...condensed tannins (Waghorn *et al.*, 1987; Waghorn and Jones, 1989; Tanner *et al.*, 1990). The results presented in the present study suggest that genes coding for the

expression of SF8 and ovalbumin protein may have potential for use in genetic engineering programmes, where the objective is to increase the nutritive value of leaves in legumes used extensively in ruminant animal production systems.

5.5 CONCLUSIONS.

5.5.1 Laboratory systems were developed, based upon *in vitro* rumen incubations, to examine the rate of proteolysis of SF8 protein from sunflower seeds, vicilin protein from pea seeds, ovalbumin protein from chicken egg white and Fraction 1 leaf protein from lucerne leaves.

5.5.2 Proteins were analysed, after fractionation by SDS-PAGE, by either Western Blotting using protein specific antibodies (SF8, vicilin and ovalbumin protein) or by Coomassie Blue staining (Fraction 1 leaf protein).

5.5.3 Proteolysis of the LSU of Fraction 1 leaf protein occurred as two components. The first component which occurred during the initial 12 hours of incubation, was relatively resistant to proteolysis, whilst the second component, which occurred from 12 hours onwards, was rapidly degraded. The proteolysis of the SSU of Fraction 1 leaf protein followed first order kinetics, with the SSU being relatively resistant to proteolysis in the *in vitro* rumen incubation.

5.5.4 Proteolysis of the MW 12100 SF8 protein followed first order kinetics and was extremely rapid. However, a degradation product of the MW 12100 SF8 protein, a MW 8000 polypeptide, was extremely resistant to rumen degradation. The rate of disappearance of the MW 12100 SF8 protein was similar to the rate of appearance of the MW 8000 polypeptide.

5.5.5 The proteolysis of vicilin followed first order kinetics, with vicilin being rapidly degraded in the *in vitro* rumen incubation.

5.5.6 Proteolysis of ovalbumin protein also occurred as two components. However, unlike the LSU of Fraction 1 leaf protein, the first component, which occurred during the initial 16 hours of incubation, was essentially not degraded. The second component, which occurred from 16 hours onwards, had a rate of proteolysis similar to that of the LSU of Fraction 1 leaf protein.

5.5.7 It was concluded that both SF8 protein and ovalbumin were resistant to rumen proteolysis due to different mechanisms, that a high proportion of SAA (particularly

disulphide linkages) reduces rumen degradability, but that the tertiary structure of the protein was also important. Therefore introducing genes coding for the expression of foreign proteins, containing a high proportion of SAA, but with low rumen degradability, using genetic engineering techniques is likely to improve forage nutritive value.

6. The expression of a sunflower seed storage gene in the leaves of tobacco (*Nicotiana tobacum L.*).

6.1 INTRODUCTION.

In ruminants fed fresh forage diets, abomasal infusions of protein and dietary supplementation with protein protected from ruminal degradation, have shown that absorption of protein from the small intestine limits wool growth (Reis and Schinkel, 1963; Ferguson *et al.*, 1967), milk production (Stobbs *et al.*, 1977; Flores *et al.*, 1979; Rogers *et al.*, 1979) and liveweight gain (Barry, 1981). In particular, post-ruminal supplementation with sulphur amino acids (SAA) has been effective in markedly increasing wool growth (Reis, 1979). Therefore, an ideal dietary protein for ruminant production, particularly wool growth, should contain a high proportion of SAA and be relatively resistant to rumen degradation.

Recent developments in the procedures of genetic engineering have resulted in the expression of foreign genes in plants. Stable gene transfer (transformation) is now routinely obtained with the following crop and pasture plants: tomato, potato, cotton, flax, rape, white clover, lucerne and *Lotus* (Weising *et al.*, 1988). These developments prompted the present research, the aim of which was to introduce genes coding for proteins containing a high proportion of SAA, and which are relatively resistant to rumen degradation, into pasture species. Pasture plants expressing such proteins may have potential benefits for ruminant production, particularly wool growth.

A number of proteins that would be potentially useful in this respect have been identified and their cDNA or genomic sequences isolated. Ovalbumin, from chicken egg white contains 6.5% SAA (Gilbert, 1971), whilst the seed proteins, pea albumin 1 and sunflower albumin 8 (SF8) contain respectively 11% SAA (Higgins *et al.*, 1989) and 25% SAA (Kortt *et al.*, 1990). All are relatively resistant to rumen degradation (D.Spencer *et al.*, 1988; D.Spencer *et al.*, unpublished; Chapter 5). Sunflower albumin 8 was chosen for the present project because of its very high SAA content and low rumen degradability.

Sunflower albumin 8, a seed storage protein, consists of a single polypeptide chain of 103 amino acids (12.1kDa), containing 16 residues of methionine and 8 residues of cysteine. It is synthesized as a precursor of 141 residues with a hydrophobic signal sequence (25 residues), which is removed co-translationally, followed by a hydrophobic prosequence (13 residues), which is cleaved post-translationally (Kortt *et al.*, 1990). A cDNA has been isolated which contains the signal peptide and prosequence, together with the coding region and 160 base-pairs of the 3' untranslated region (Lilley *et al.*, 1989). Since the gene for SF8 is expressed only in seeds we have engineered the SF8 cDNA clone for expression in the leaves of a transgenic host (*Nicotina tabacum* cv Wisconsin 38) using the constitutive (Benfey *et al.*, 1989) cauliflower mosaic virus (CaMV) 35S promoter (Franck *et al.*, 1980).

The reconstructed gene was inserted into a gene delivery system in *Agrobacterium tumefaciens* and transferred to tobacco. Expression of the synthetic (chimeric) gene was monitored at the mRNA and protein level.

6.2 METHODOLOGY.

6.2.1 RECONSTRUCTION OF THE SF8 cDNA CLONE AS A CHIMERIC GENE.

A SF8 cDNA clone, pSF16 was not complete at the 5' end and contained no sequences beyond the poly(A) addition sites at the 3' end. Therefore a promoter, a 5' untranslated sequence, and a signal sequence had to be provided at the 5' end, whilst a gene termination sequence had to be provided at the 3' end of the cDNA.

The plasmid pCW36, contains the 5' upstream untranslated region (map position -414 to 43), the signal sequence (map position 44 to 132) and the remainder of the coding region from the vicilin pea seed gene (map positions as described by Higgins *et al.*, 1988 where position 44 is the A in the ATG start codon; Fig. 1). This plasmid was digested with the restriction endonuclease AccI, blunt-ended with DNA polymerase I Klenow fragment and digested with HindIII. This yielded a 0.48 kilobase (kb) fragment containing part of the vicilin 5' upstream region and 22 base pairs of the DNA sequence corresponding to the vicilin signal sequence, which were ligated into pUC19 to yield the plasmid pVPL-1 (Fig. 1; D.Llewellyn, unpublished).

A second plasmid, pAK2 similar to pCW36, but containing only part of the vicilin gene, had a BamHI site introduced at a position corresponding to the carboxy terminal end of the vicilin signal sequence, by oligonucleotide-directed-site-specific mutagenesis (Fig. 1; A.Kermode, unpublished). Digestion of pAK2 with HindIII yields a 0.28kb fragment containing 67 bp of the DNA sequence corresponding to the vicilin signal sequence and 0.21kb of the vicilin coding region. Ligation of this fragment into pVPL-1 yields the plasmid pVPLSP-7 (Fig. 1; D.Llewellyn, unpublished). The plasmid, pVPLSP-7 is pCW36 reconstructed to contain the BamHI recognition site at the position corresponding to the start of the mature vicilin polypeptide.

The plasmid pJ35SN (Fig. 2) contains the CaMV 35S promoter (map position 7025 to 7437 as described by Franck *et al.*, 1980), and the 3' termination region of the nopaline synthase (NOS) gene from *Agrobacterium tumefaciens* (Bevan *et al.*, 1983), separated by multiple cloning sites (MCS). This plant gene expression cassette was constructed so that the strong plant promoter from CaMV could be placed upstream of any coding region using the MCS,

whilst the 3' end of the NOS gene provides poly(A) addition signals and gene termination sequences (D.Llwellyn and J.C.Walker unpublished).

Digestion of pVPLSP-7 with ClaI, followed by the attachment of a BglII linker and digestion with BglII and BamHI yields a 0.13kB fragment containing the vicilin 5' untranslated region and the DNA sequence corresponding to the vicilin signal sequence. Ligation into pJ35SN yielded p35SSPN (Fig. 2).The plasmid, p35SSPN contains the CaMV 35S promoter (Franck *et al.*, 1980), the vicilin 5' untranslated region, DNA for the vicilin signal sequence (Higgins *et al.*, 1988) and the NOS termination sequence (Bevan *et al.*, 1983).

The plasmid, pSF16 contains the cDNA encoding SF8 (map position 22 to 609 as described by Kortt *et al.*, 1990 where map position 22 is the A of the ATG start codon and 136 and 456 are the start and stop of the mature SF8 protein). Oligonucleotide-directed-site-specific mutagenesis was used to create a BamHI recognition site at the 3' end of the SF8 prosequence (G.G.Lilley, 1989). Digestion with BamHI and EcoRI yields a 0.48kb fragment containing the SF8 coding region and 3' untranslated region, up to the poly(A) addition signals (Fig. 2). Ligation into p35SSPN yields pGL1 (G.G.Lilley, unpublished; Fig. 2) which contains the chimeric gene encoding SF8, driven by a CaMV promoter and terminated by the NOS termination region.

The plasmid pGL1 was digested with SalI and StuI and ligated into the binary vector pBIN19 (Bevan, 1984; Fig. 3) to create the plasmid WM1, which was introduced into *E. coli* (strain HB101) by high voltage electroporation (Maniatis *et al.*, 1982). The binary vector, pWM1 contains the bacterial neomycin phosphotransferase gene, whose gene product confers on plant cells, resistance to the toxic antibiotic, kanamycin (Bevan, 1984). Thus, resistance to kanamycin was used as a selectable marker.

6.2.2 TRANSFER OF THE CHIMERIC SF8 GENE INTO PLANTS.

The chimeric SF8 gene in the binary shuttle vector (pWM1) was transferred to *Agrobacterium tumefaciens* (strain AGL1) by tri-parental mating using the mobilizing plasmid pRK2013 (Ditta *et al.*, 1980).

Agrobacterium colonies were grown at 28°C for 48 hours on LBMG agar (see Appendix 1) containing the antibiotics, rifampicin (25mg/l) and kanamycin (50mg/l). A single colony was selected and grown at 28°C for 48 hours in 10ml of LBMG broth containing the antibiotics, rifampicin (25mg/l) and kanamycin (50mg/l). Total DNA was prepared from

the *Agrobacterium* culture (Dhaese *et al.*, 1979) and the presence of the chimeric gene verified by Southern blot analysis (Southern, 1975; Fig. 5).

After confirming that the gene was present and intact, an *Agrobacterium* culture containing WM1 was grown at 28°C for 48 hours in 10ml of LBMG broth containing the antibiotics, rifampicin (25mg/l) and kanamycin (50mg/l). The cells were pelleted and washed twice with 5ml of MSO broth (see Appendix 2) and then resuspended in 5ml of MSO broth for use in leaf infection.

Leaf pieces (1.0cm^2) obtained from aseptic tobacco plantlets (*Nicotiana tobacum* cv Wisconsin 38) were infected by incubation in the *Agrobacterium* culture at room temperature for 5 min (Horsch *et al.*, 1985). After inoculation, leaf pieces were incubated on MSO agar without antibiotics, at 28°C for two days. Leaf pieces were then transferred to MS9 agar (see Appendix 3) containing the antibiotics, cefotaxime (300mg/l) and kanamycin (100mg/l), and incubated at 28°C to form callus. Shoots that developed from the callus were transferred to MSO agar containing the antibiotics, cefotaxime (300mg/l) and kanamycin (100mg/l) and allowed to develop roots over the next fourteen days.

Plantlets were transferred into soil (20cm pots with CII and osmocote) and grown in the glasshouse at 26/18°C day/night. Plants from the glasshouse (4-6 weeks old) or plantlets in tissue culture pots were used for RNA and protein analysis.

6.2.3 PREPARATION OF ^{32}P LABELLED PROBES.

The cDNA plasmid, pSF16, was digested with BamHI and EcoRI to yield a 0.48kB fragment containing the SF8 cDNA coding region. The fragment was electroeluted out of agarose gel using a BT-100 Biotrap (Schleicher and Schuell, West Germany) and was labelled with α-^{32}P-dCTP using an oligonucleotide labelling kit (Bresatec, Australia). Probes with specific activities of 2.1×10^8cpm/μg were routinely prepared. Probes were used for detecting SF8 DNA and mRNA in Southern and Northern Blot analysis respectively.

6.2.4 RNA ISOLATION AND ANALYSIS BY NORTHERN BLOTTING.

Northern blot analysis was used to test for the presence of SF8 mRNA in the leaves of tobacco plants transformed with pWM1. Frozen leaf tissue (1g) was ground in liquid nitrogen and extracted with 500μl of extraction buffer:phenol (1:1; see Appendix 4), which had been heated to 80°C. The mixture was centrifuged at 13000g for 5 min. The supernatant was drawn off and 0.25vol of 10M $LiCl_2$ was added before the mixture was left at 0°C for 18 hours to precipitate RNA. After centrifugation at 13000g for 20 min, the RNA

pellet was redissolved in 250µl of sterilized H_2O and 28µl of 3.3M sodium acetate, pH 6.1 was added. After the addition of 560µl of ethanol, the mixture was left at -20°C for 1 hour to precipitate RNA (Verwoerd *et al.*, 1989). The RNA pellet was redissolved in 30µl of sterilized H_2O and the RNA concentration was measured spectrophotometrically (Varian Series 634) assuming that 1 OD unit @ 260nm represents 40µgRNA/ml, using a 1cm path length.

Total RNA (5µg) was analysed using Northern blot analysis after fractionation of RNA on a 1.2% agarose gel containing 5% deionized formaldehyde (Maniatis *et al.*, 1982). RNA was blotted for 18 hours onto a nylon membrane (Hybond N, Amersham (Aus) Ltd), which was presoaked in warm 20x SSC (see Appendix 5). RNA was bonded to the membrane by UV irradiation (12cm, 15watts, 2 min; Khandjian, 1986). The nylon was treated with 0.1ml/cm^2 hybridisation solution (see Appendix 6) for 4 hours at 42°C (Khandjian, 1987) before addition of a ^{32}P labelled probe (see below) and hybridisation for 18 hours at 42°C. Blots were washed three times for 10 min in 2x SSC at room temperature, two times for 20 min in 2x SSC, 0.1% sodium dodecyl sulphate, 0.1% sodium pyrophosphate at 68°C and two times for 20 min in 0.1x SSC, 0.1% sodium dodecyl sulphate, 0.1% sodium pyrophosphate at 60°C.

Blots were exposed to X-ray film at -80°C using Hi-Plus (Du Pont) intensifying screens.

6.2.5 PURIFICATION OF MONOCLONAL ANTIBODIES.

Cell culture supernatants (50ml) from seven mouse hybridomas producing anti-SF8 monoclonal antibodies (D.Hewish, CSIRO, Division of Biomolecular Engineering, Melbourne) were purified on a 2ml column of Protein G Sepharose Fast Flow (Pharmacia; Australia), which had a protein binding capacity of 40mg and a flow rate of 18ml/min. The column was washed three times with 10ml of 20mM phosphate buffer, pH 7.0 (see Appendix 7) to remove all albumins before globulins were eluted with 5ml of 0.1M glycine-HCL buffer, pH 2.6 (see Appendix 8). Eluted fractions of 1ml were collected and the appearance of protein in the fractions was monitored by absorbance at 280nm (Fig. 6). Fractions containing globulin protein were bulked and the pH adjusted to 7.0 with 1M Tris-HCL, pH 9.6. Sodium azide was added to a final concentration of 0.02% w/v before storage at 4°C.

6.2.6 PROTEIN ISOLATION AND ANALYSIS BY WESTERN BLOTTING.

Protein immunoblot (Western) analysis was used to test for the presence of SF8 protein in the leaves of tobacco transformed with pWM1. Fresh tobacco leaves (1-5g) were ground in

a mortar in 3 vol of protein extraction buffer (see Appendix 9) at room temperature. Total protein was extracted from sunflower (cv. Hysun 30) seeds by grinding in 0.02M N-Tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid, pH 7.8 according to the method described by Spencer *et al.* (1988). The extracts were centrifuged at 10000g for 10 min and then the supernatant were drawn off. Total protein in the supernatant was estimated using the method of Bradford (1976). Bovine serum albumin (B grade Calbiochem, Australia) was used as a standard.

The SF8 protein was purified from total protein by adding protein extracts to 2 vol of methanol containing 0.6% acetic acid, and discarding the protein precipitated at 0°C after 1 hour. Sunflower albumin 8 is methanol-soluble and was thus present in the supernatant, whilst most other proteins were precipitated. The methanol supernatant was added to 4 vol of acetone and the methanol-soluble protein was precipitated by standing at 0°C for 15 min.

The precipitate was resuspended in 50µl of protein digestion buffer (see Appendix 10) and incubated at 95°C for 15 min. The slurry was fractionated on a sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE; 12-25% acrylamide gradient). Electrophoresis was carried out for 20 hours at 14 milliamps (mA). The separated proteins were electroblotted onto a nitrocellulose (NC) membrane (Schleicher and Schuell, West Germany) using a semi-dry blotter (BioRad, Australia) run at 200mA for 90 min.

The NC membrane was then washed in TBS buffer, pH 7.4; 5% skim milk powder (w/v; see Appendix 11) for 4 hours at room temperature to block all protein binding sites. After blocking, the NC membrane was washed three times for 5 min in TTBS buffer, pH 7.4 (see Appendix 12) at room temperature to remove excess milk protein. The NC membrane was then sealed in a plastic bag containing 5ml of TTBS buffer, pH 7.4; 1% skim milk powder (w/v) and 300µl (7µg) of SF8-specific monoclonal primary antibodies and incubated for 2 hours at room temperature. The NC membrane was then washed three times for 5 min in TTBS buffer, pH 7.4 to remove excess primary antibody not bound to the SF8 protein. The NC membrane was then sealed in a plastic bag containing 5ml of TTBS buffer, pH 7.4; 1% skim milk powder (w/v) and 1.7µl of goat anti-mouse secondary antibody conjugated to alkaline phosphatase (BioRad, Australia). After incubation for 1 hour at room temperature, the NC membrane was washed three times for 5 min in TTBS buffer, pH 7.4 and once for 5 min in TBS buffer, pH 7.4 to remove excess secondary antibody not bound to primary antibody. The SF8 protein was then detected by colorimetric reaction of alkaline phosphatase (bound to protein-primary antibody complexes on the NC membrane) with the substrates p-nitro-blue-tetrazolium chloride (NBT) and 5-bromo-4-chlor-3-indolyl-phosphate (BCIP; BioRad, Australia; see Appendix 13) in the presence of Mg^{2+} ions.

6.2.7 EXPRESSION AND ANALYSIS OF SF8 IN *E. COLI*.

The translatability of the SF8 cDNA coding region was tested by ligating the vicilin signal sequence and SF8 cDNA coding region from pGL1 into the bacterial expression vector, pJLA602 and transcribing and translating the resultant plasmid (pWM2) in *E. coli*. The vector, pJLA602 contains the bacteriophage l promoters P_R and P_L, the ribosome binding site from the *E. coli* atpE gene (spanning the region from -50 bp to the ATG start codon), the cIts857 gene, and the bacteriophage fd-transcription terminator (McCarthy *et al.*, 1985, 1986). Transcription of any gene is initiated by the P_L and P_R promoters, whilst transcription and translation are temperature shift induced (28°C to 42°C; McCarthy *et al.*, 1985).

The plasmid pGL1 (described previously) was digested with NcoI and EcoRI. A 0.57kb fragment containing the vicilin signal sequence (Higgins *et al.*, 1988) and the SF8 cDNA coding region (Lilley *et al.*, 1989; Kortt *et al.*, 1990) was isolated from an agarose gel. This fragment was cloned into pJLA602, which had been opened with NcoI and EcoRI. After ligation, *E. coli* (strain DH5α) was transformed with the ligation products by high voltage electroporation. Plasmid DNA was prepared (Holmes and Quigley, 1981) from clones selected by colony hybridization using the SF8 cDNA coding region as a probe and checked by endonuclease restriction mapping. The resultant plasmid, WM2 (Fig. 4), was used to prepare plasmid DNA (Holmes and Quigley, 1981), which was transferred into *E. coli* (strain R1180), which lacks the ATP-dependent *lon* protease (Maurizi *et al.*, 1985) and the heat-induced htpR protease (Cooper and Ruettinger, 1975).

Colonies of *E. coli* (strain R1180) containing either pWM2 or the pJLA602 vector were grown at 28°C in 30ml of TB broth (see Appendix 14) containing the antibiotics, tetracycline (12.5mg/l) and ampicillin (100mg/l) to a concentration of 10^8 cells/ml. The cells were then transferred to 42°C and grown for a further 90 min to induce transcription and translation.

Cells were incubated at 0°C for 20 min in 3 vol of lysis buffer (see Appendix 15) containing 50μM phenyl methyl sulfonyl fluoride and 100μg/ml of lysozyme. The cells were then transferred to 37°C and deoxycholic acid was added to a final concentration of 15% w/v. Incubation continued until the suspension was difficult to stir, at which stage deoxyribonuclease was added to a final concentration of 7μg/ml. The suspension was incubated at room temperature for 30 min and then centrifuged at 10000g for 10 min. Protein in this supernatant was precipitated at 0°C for 1 hour after the addition of 2 vol of methanol containing 0.6% acetic acid (as described previously). Methanol-soluble protein was fractionated on a SDS-polyacrylamide gel by electrophoresis. The pellet remaining

after lysozyme treatment was resuspended in 9 vol of lysis buffer containing 0.5% Triton X-100 and 10mM EDTA and incubated at room temperature for 5 min. This suspension was centrifuged at 10000g for 10 min and the protein in the supernatant was precipitated with 2 vol methanol containing 0.6% acetic acid at 0°C for 1 hour (as described previously). Methanol-soluble proteins were fractionated on a SDS-polyacrylamide gel by electrophoresis.

The residual pellet was resuspended in 50µl of protein digestion buffer and any remaining proteins were fractionated on a SDS-polyacrylamide gel by electrophoresis.

6.2.8 QUANTIFICATION OF NORTHERN AND WESTERN BLOTS.

Northern (RNA) and Western (protein) blots were photographed and negatives were scanned at 3 separate locations using a double beam recording microdensitometer (Joyce-Loebl, England). The relative intensity of each band was determined by cutting out and weighing paper scans of each band.

Fig. 1: Construction of a plasmid containing the vicilin 5' untranslated region and the vicilin signal sequence.

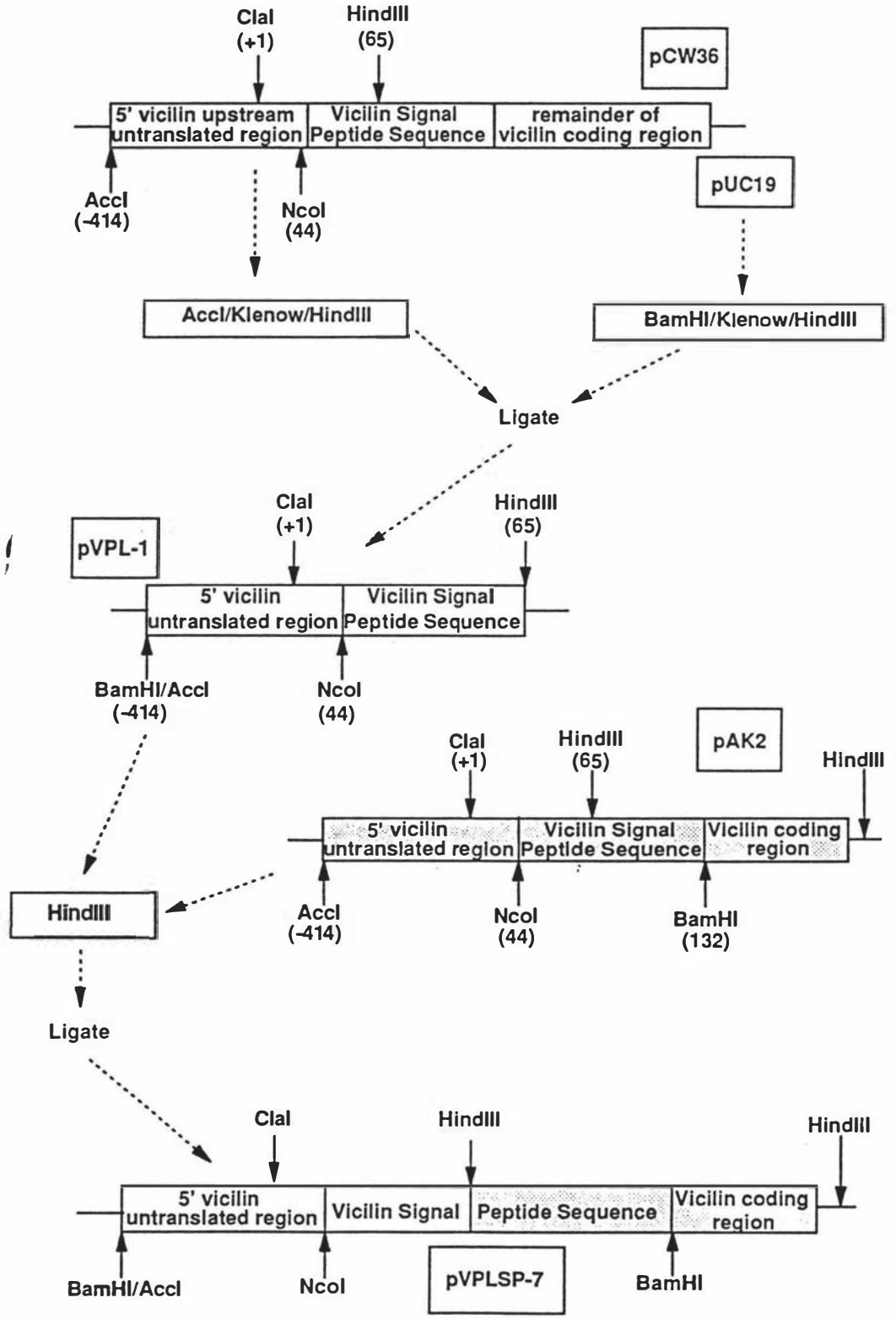


Fig. 2: Reconstruction of the SF8 chimeric gene using the CaMV 35S promoter, the vicilin 5' untranslated region, the vicilin signal sequence, the SF8 cDNA clone and NOS 3' termination sequence.

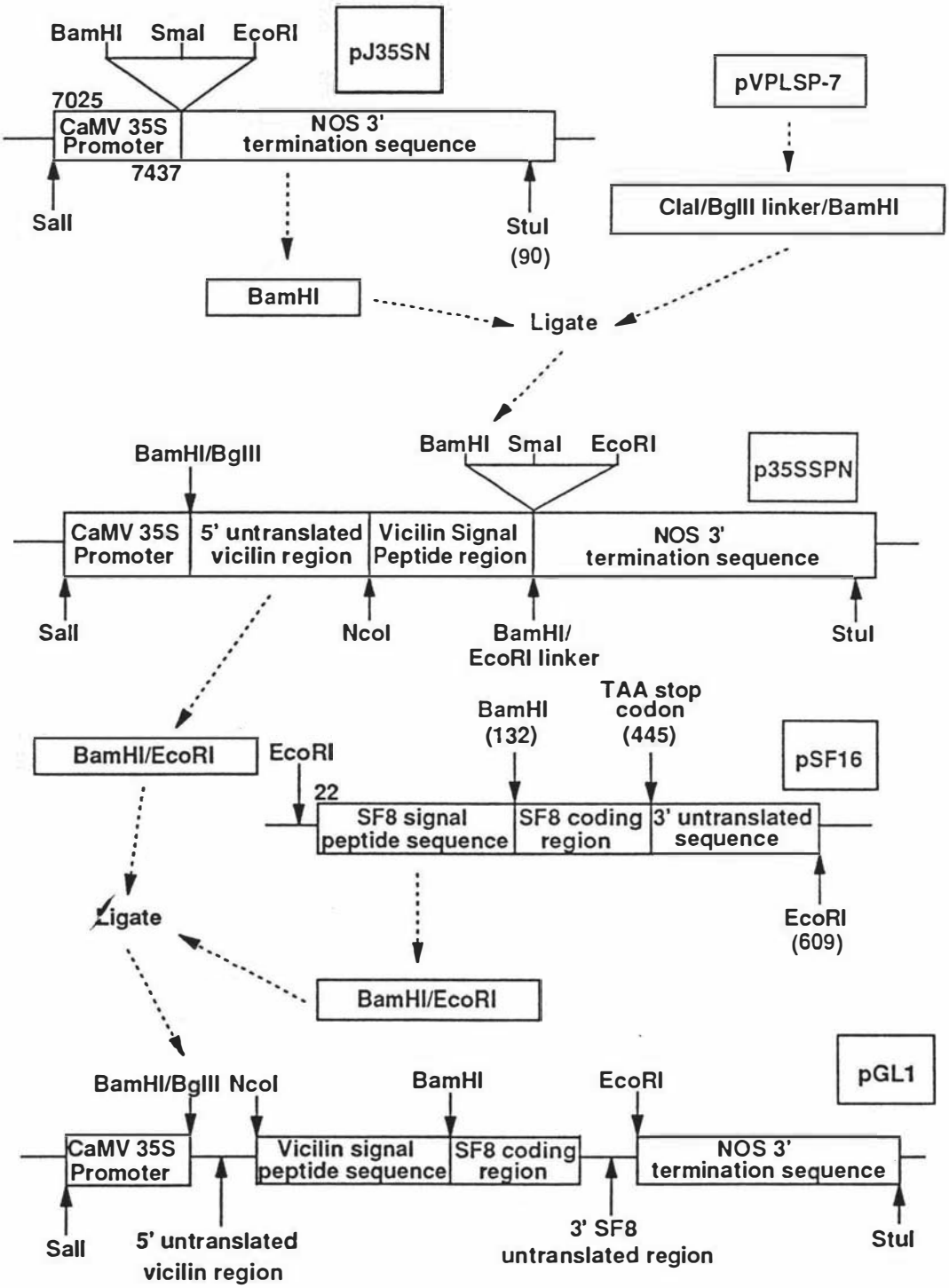


Fig. 3: Construction of the recombinant binary vector WM1, for the SF8 chimeric gene (pGL1) to plants.

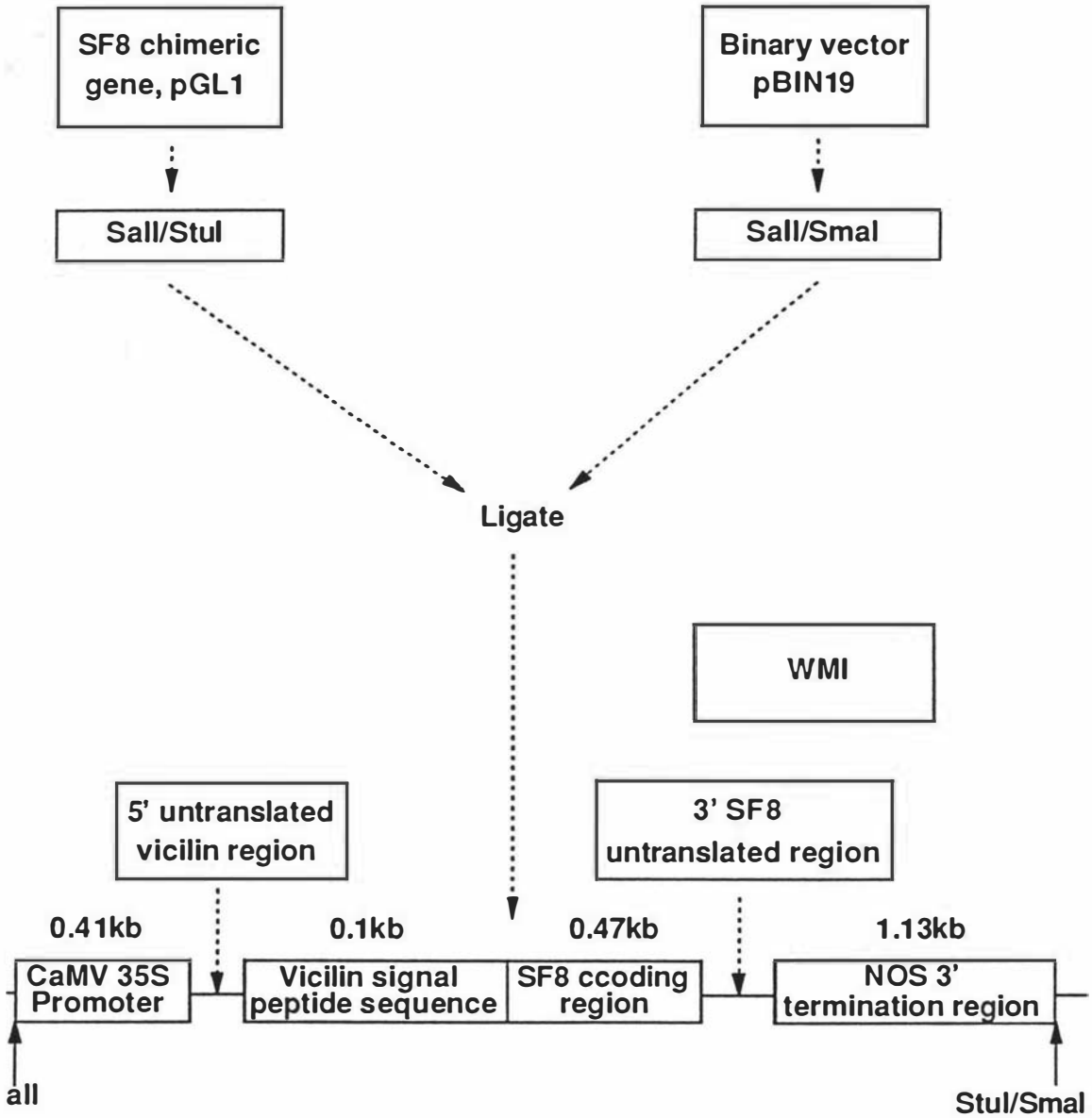


Fig. 4: Construction of WM2, a vector for the expression of SF8 in E. coli.

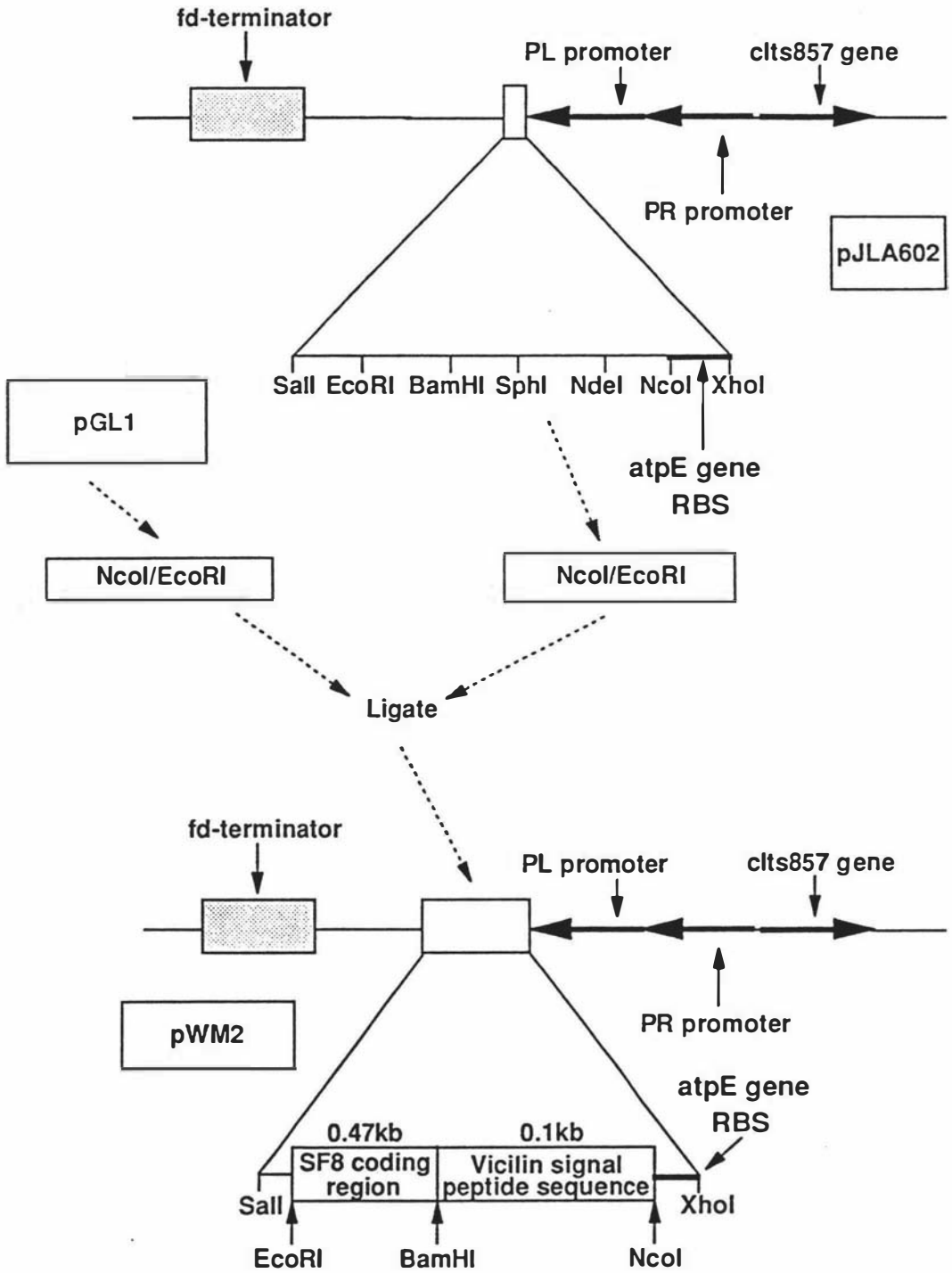


Fig. 5: Southern blot analysis of DNA from *Agrobacterium tumefaciens* harbouring the SF8 chimeric gene. The recombinant binary vector pWM1, containing the chimeric SF8 gene was transferred into *Agrobacterium tumefaciens* (AGL 1) using the mobilizing plasmid pRK2013 by tri-parental mating. Total DNA was prepared from the *Agrobacterium* culture and digested with EcoRI and EcoRI and BamHI. The presence of the chimeric gene was verified by Southern blotting using a ^{32}P-labelled insert from pSF16. Hybridisation signals of the expected size were detected in the EcoRI digested (12.5kb) and BamHI plus EcoRI digested (0.47kb) *Agrobacterium* DNA.

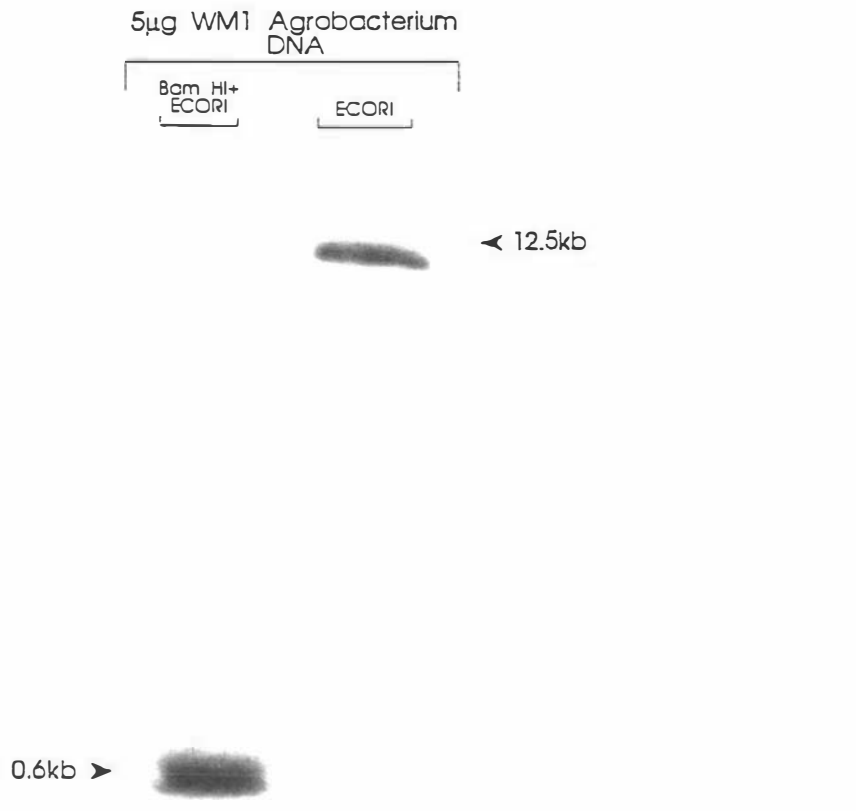
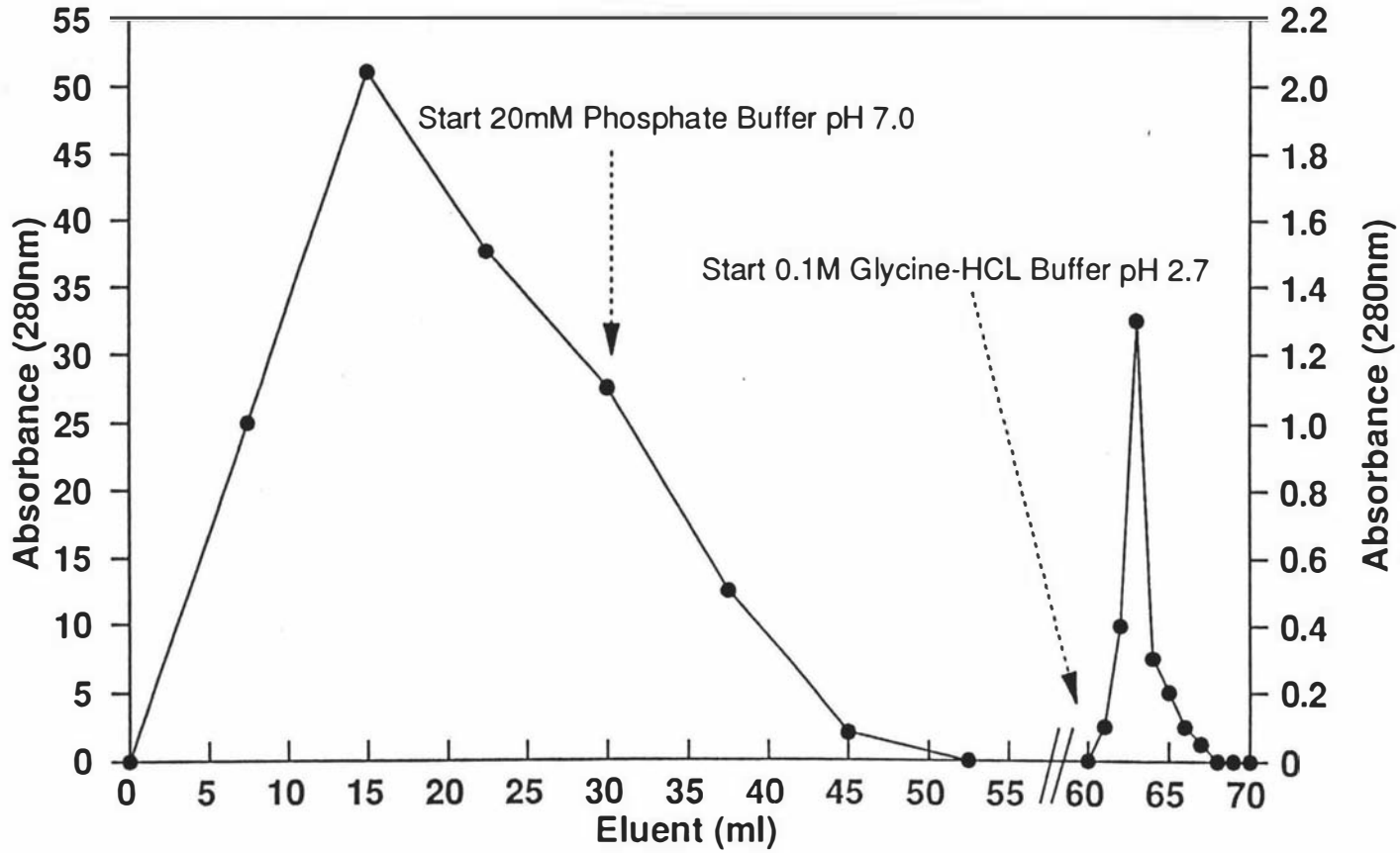


Fig. 6: Purification of mouse monoclonal antibodies using Protein G Sepharose Arrows indicate commencement of buffer flow. The first peak (left y scale) are plasma albumins, whilst the second peak (right y scale) are plasma globulins.



6.3 RESULTS.

6.3.1 Detection of SF8 mRNA in Transformed Tobacco Plants.

A chimeric gene WM1, containing the cDNA encoding SF8 was modified for expression in plant leaves together with a gene for a selectable marker (kanamycin resistance). These genes were introduced into tobacco by an *Agrobacterium tumefaciens* binary vector transformation system. Kanamycin-resistant plants were regenerated from infected leaf pieces and eight plants were analysed for SF8 mRNA by Northern blot analysis (Fig. 7). No hybridization signal could be detected with RNA from untransformed tobacco whilst RNA from transgenic tobacco and sunflower seeds showed a clear hybridisation signal, indicating the presence of SF8 mRNA (530bp) in transgenic tobacco. In the leaves of the eight transformed tobacco plants, the level of SF8 RNA varied over a 100 fold range, but in the highest expressor, it represented 14% of the SF8 mRNA level found in sunflower seeds (Table 1).

6.3.2 Detection of SF8 Protein in Transformed Tobacco.

Western blot analysis was used to test for the presence of SF8 protein. The 12.1kDa SF8 protein was detected in sunflower seeds when 50µg of total seed protein was analysed (Fig. 8). Since the SF8 protein is soluble in methanol (Fig. 8), this property was used as a means of purifying the protein.

However, SF 8 protein could not be detected in the leaves of transgenic tobacco (Figs. 9a and b), even when 35mg of total leaf protein from the highest mRNA expressor (WM1-8) was used. The minimum loading of SF8 protein required to give a detectable signal in Western Blot analysis is 10µg. Therefore, the level of SF8 in the leaves of transgenic tobacco was less than 0.03% of total leaf protein.

6.3.3 Comparison of SF8 mRNA and Ovalbumin mRNA in Transgenic Plants.

Since it is common to find low levels of expression of foreign protein in transgenic plants (in the range of 0.01% and lower) we wished to ascertain whether the level of mRNA for SF8 was much lower than for other transgenic messages. This could account for our inability to detect the SF8 protein. For this analysis we used tobacco plants which were expressing ovalbumin at a level of 0.01% of total leaf protein (T.J.Higgins, unpublished). We isolated RNA from the leaves of one of these plants as well as from the plant which had the highest level of SF8 mRNA (WM1-8; see Fig. 7). These two RNA populations were fractionated on formaldehyde gels and a Northern blot analysis (Fig. 10) was performed

using equal amounts ($9.0x10^7$cpm) of SF8 and ovalbumin hybridisation probes labelled to the same specific activity ($2.0x10^8$cpm/μg).

Assuming that at maximum, mRNA is 1% of total RNA then 50ng of mRNA from ovalbumin and SF8 transformed tobacco was fractionated in this way. Assuming that ovalbumin and SF8 mRNA is at maximum, 0.01% of total mRNA then 0.5pg of SF8 and ovalbumin mRNA was fractionated in this way, in which case the 450ng of each α-^{32}P-dCTP probe used for hybridisation, would not limit signal intensity. Therefore, the intensity of a hybridisation signal seen in Northern blot analysis was a function of the total amount of relevant mRNA present, as the specific activity (cpm/μg DNA) of the two probes was similar. The level of SF8 mRNA in the highest expressor (WM1-8) was 9 times that seen for ovalbumin mRNA in ovalbumin transformed tobacco (Table 1). This indicates that there was sufficient template to produce 0.1% of total leaf protein as SF8, if translation or protein stability were not limiting.

6.3.4 Is the mRNA from the Chimeric SF8 Gene Translatable?

The failure to detect SF8 protein in transgenic tobacco prompted the need to test the translatability of transgenic tobacco leaf RNA. The SF8 cDNA coding region was transcribed and translated in a bacterial expression system using the vector pJLA602. The plasmid WM2, is the pJLA602 vector with the vicilin signal sequence and SF8 coding region inserted. Both the pJLA602 vector and pWM2 in *E. coli* (strain R1180) were grown to a concentration of 10^8cells/ml at 28°C before heat induction of transcription and translation at 42°C for 90 min. The bacterial cultures were lysed and methanol-soluble proteins in the lysate fractionated on a SDS-polyacrylamide gel by electrophoresis. The remaining bacterial pellet was resuspended in lysis buffer, 0.5% Triton X-100, 10mM EDTA to elute any remaining membrane-bound proteins. Methanol-soluble proteins in the Triton X-100/EDTA supernatant and pellet were fractionated on a SDS-polyacrylamide gel by gel electrophoresis.

Western blot analysis of *in vivo* translated products failed to detect SF8 protein in any of the above described fractions (Fig. 11). The experiment was repeated, except heat induction at 42°C was carried out for 16 hours, but again no SF8 protein was detected.

Fig. 7: Nortern blot analysis of RNA from sunflower seeds and tobacco leaves. RNA from WM1 transformed tobacco (5μg) and sunflower seeds (0.1μg) was fractionated on a 1.2% agarose gel containing 5% deionised formaldehyde. Northern blot analysis using a ^{32}P-labelled SF8 probe detected SF8 mRNA in WM1 transformed tobacco RNA and in sunflower seed RNA but did not detect any hybridisation signal in untransformed tobacco RNA. The numbers over the tracks refer to individual plants which were tested for the presence of SF8 mRNA.

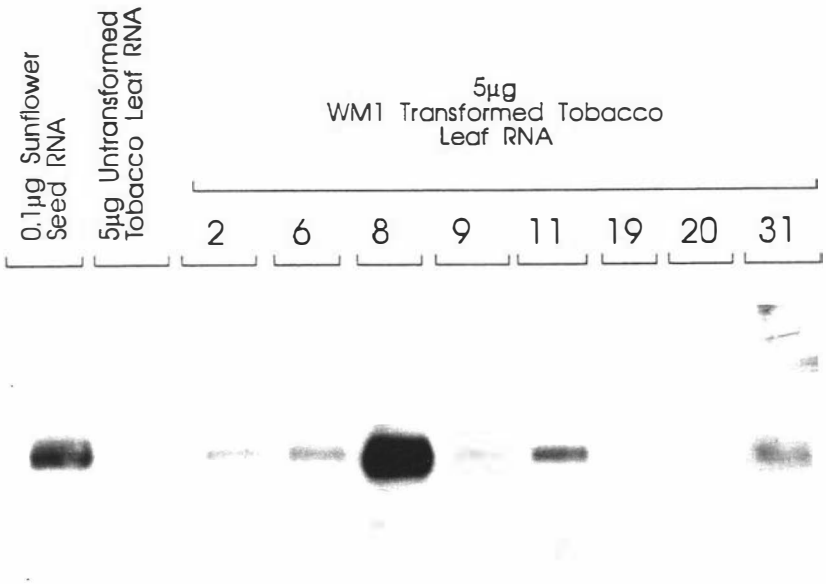


Table 1: The levels of SF8 mRNA in WM1 transformed tobacco and ovalbumin mRNA in ovalbumin transformed tobacco relative to SF8 mRNA in total sunflower seed RNA.

Source	SF8 or ovalbumin mRNA levels (Arbitrary units)
Sunflower seed total RNA	1,000
Transgenic tobacco leaf RNA (ovalbumin)	15
Transgenic tobacco leaf RNA (SF8)	
Plant	
2	2
6	3
8	140
9	1
11	6
19	1
20	0
31	1

SF8, sunflower albumin 8 protein

Fig. 8: Western blot analysis of SF8 purified by methanolic extraction. A total protein extract (50μg) from sunflower seeds was purified using methanol, acetic acid and acetone as described in the materials and methods. Methanol-soluble and methanol-insoluble protein was fractionated on a SDS-polyacrylamide gel by electrophoresis. Western blotting analysis, using mouse monoclonal antibodies produced against SF8 protein, detected SF8 protein (12.1kDa) in sunflower seed total protein and in the methanol-soluble fraction of sunflower seed protein.

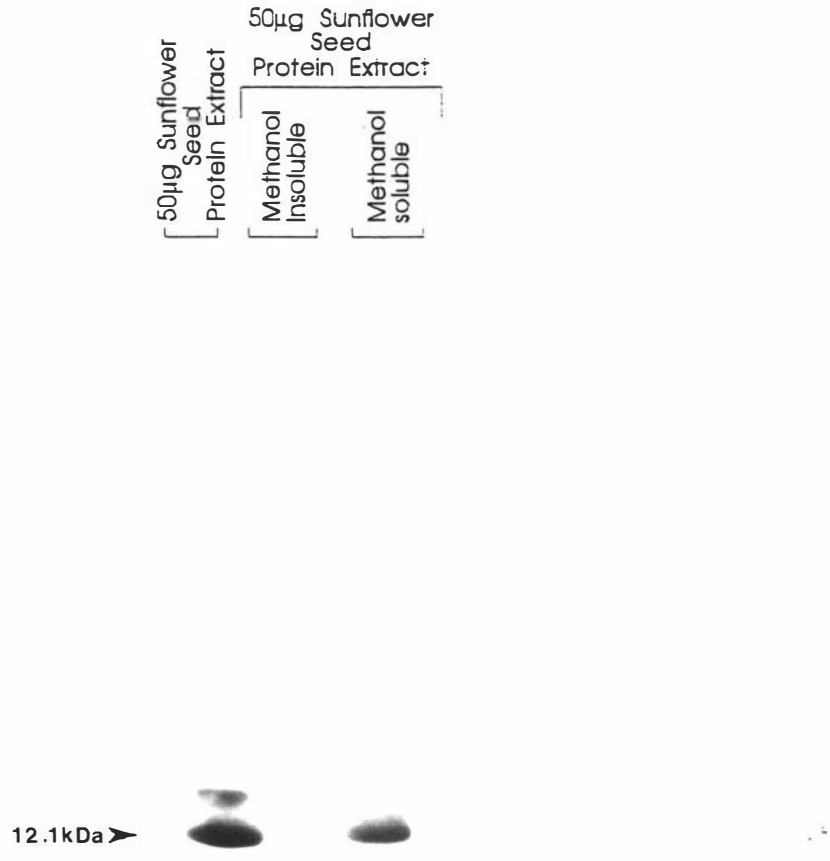


Fig. 9a: Western (immunoblot) blot analysis of leaf protein in transgenic plants. Total leaf protein was extracted from eight WM1-transformed tobacco plants and the methanol soluble protein was purified from 4mg of total protein. The methanol-soluble protein was fractionated on a SDS-polyacrylamide gel by electrophoresis. Monoclonal antibodies were used to detect SF8 protein. The numbers over the tracks refer to individual plants which were tested for the presence of SF8 protein.

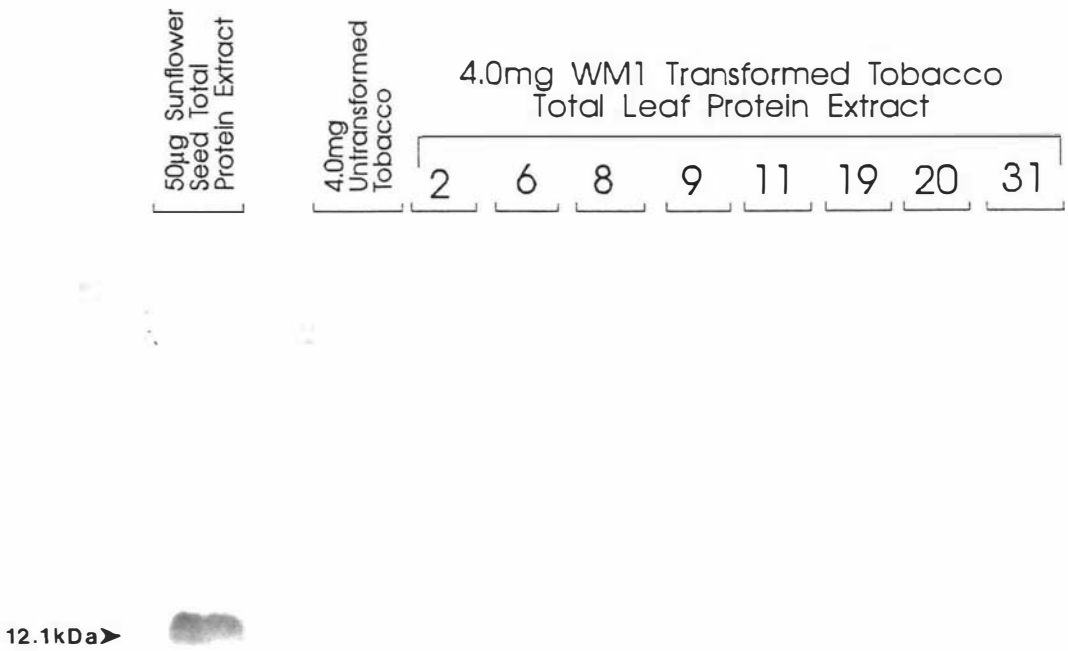


Fig. 9b: Since plant 8 had the highest level of SF8 mRNA, the methanol-soluble protein from 15 and 35mg of total leaf protein was fractionated on a SDS-polyacrylamide gel by elecrophoresis. As a control, the methanol-soluble protein from 22mg of total leaf protein from untransformed tobacco was included on the gel. Western blotting analysis was carried out using SF8-monoclonal antibodies.

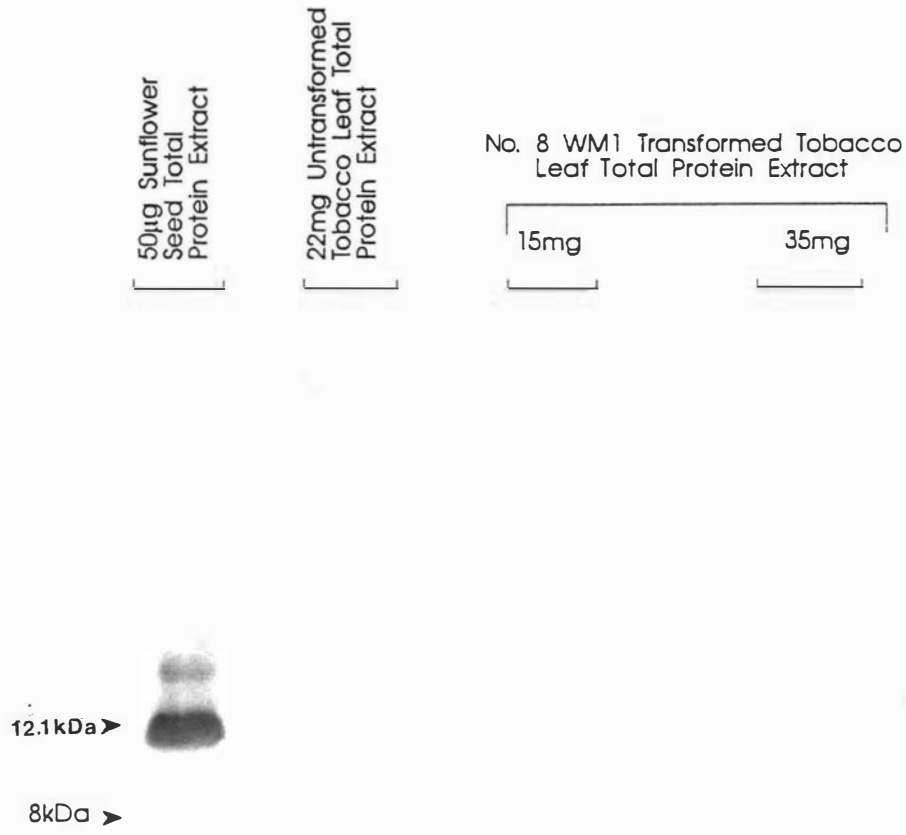


Fig. 10: Northern blot analysis of RNA from transgenic tobacco expressing either the SF8 or ovalbumin gene. Tobacco RNA (5μg) from either control or transformed tobacco was fractionated by electrophoresis on a 1.2% agarose gel. The blot was hybridized simultaneously with $9.0X10^7$cpm of an ovalbumin ($2.0X10^8$cpm/μgDNA) and a SF8 ($2.1X10^8$cpm/μgDNA) probe.

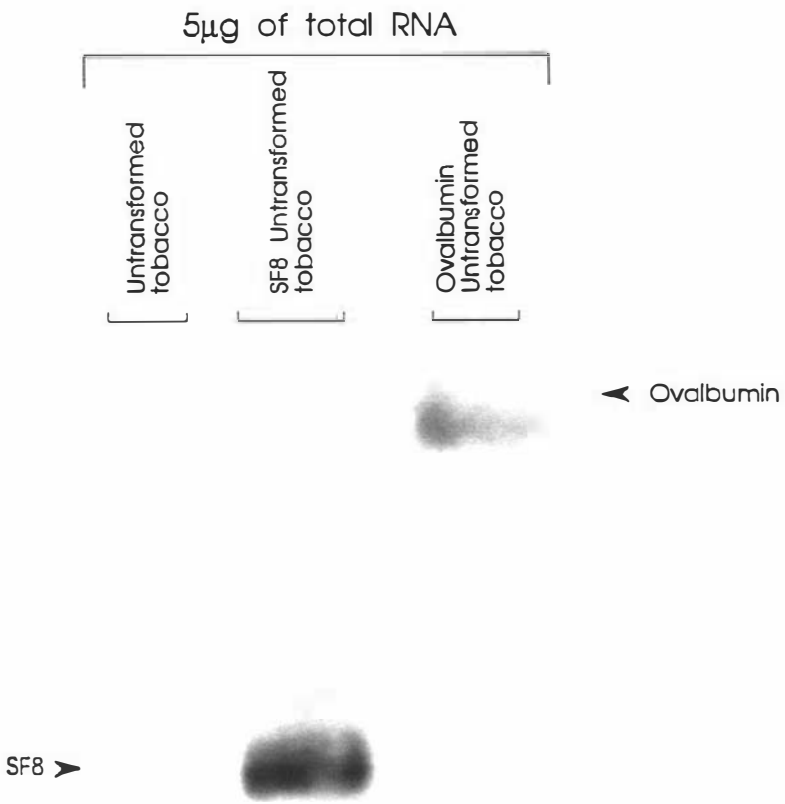
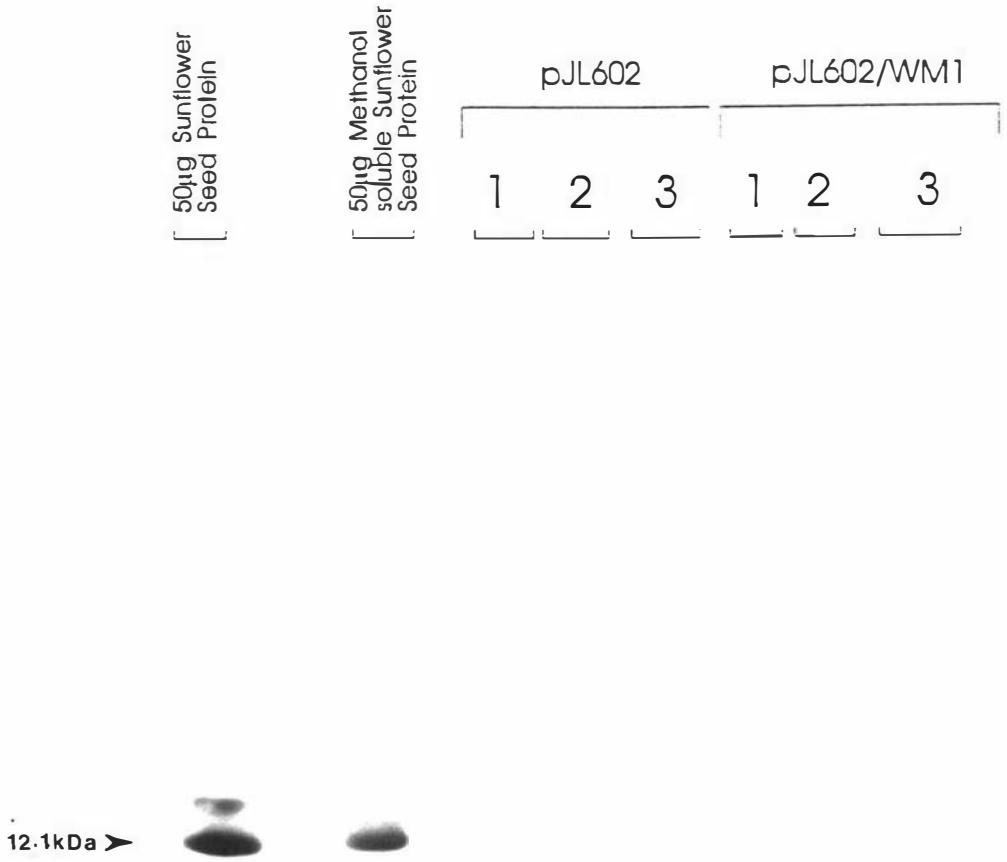


Fig. 11: Western blot analysis of proteins synthesized in an *E. coli* strain carrying the WM2 plasmid. Bacterial proteins were fractionated on a SDS-polyacrylamide gel by electrophoresis and monoclonal antibodies were used to assay for SF8. pJL602 represents the total protein from cells with the vector alone while pJL602/WM1 represents the protein from cells with pWM2. (1) is the methanol-soluble fraction of total protein in the supernatant after lysozyme lysis. (2) is the methanol-soluble fraction of total protein in the supernatant after resuspension of the lysozyme lysis pellet in Triton X-100. (3) is the protein in the pellet remaining after resuspension in Triton X-100. A band corresponding to SF8 can be seen in the control sunflower seed total protein and the protein purified using methanol.



;.4 DISCUSSION.

; chimeric gene in pWM1, containing the cDNA coding region of SF8 was reconstructed nd stably introduced into tobacco together with a selectable marker gene encoding anamycin resistance, using an *Agrobacterium tumefaciens* binary vector transfer system.

he three main criteria for stable introduction of a foreign gene into a transgenic host tudied are: the incorporation of foreign DNA into the host plant's genomic DNA, the anscription of foreign DNA to produce mRNA and the translation of that mRNA to roduce protein.

he binary plasmid, pWM1 carried both the chimeric gene and a selectable marker gene ncoding kanamycin resistance. Selection of plantlets able to grow and proliferate on media ontaining kanamycin (100mg/l) was taken as an indication of incorporation of the himeric gene into the tobacco genome. Only plantlets able to grow and proliferate on anamycin were examined for transcription and translation of WM1.

Northern analysis of tobacco transformed with pWM1 detected an RNA species that was bsent from untransformed tobacco, and identical in size to the SF8 mRNA from sunflower eeds. Therefore, transcription of the chimeric gene WM1 occurred in the leaves of WM1 ransformed tobacco.

However, sensitive immunological procedures failed to detect SF8 protein in the leaves of obacco transformed with pWM1. Similarly, SF8 protein was not detected in the methanol-oluble fraction of 35mg of total leaf protein from WM1-8, the plant with the highest SF8 nRNA level. In Western blot analysis, a maximum of 10µg of SF8 protein was easily letected. Consequently, the level of expression of the chimeric gene in pWM1, is more han 700-fold lower in tobacco leaves than in sunflower seeds.

Expression of the genes encoding the proteins, ovalbumin, pea albumin 1 and vicilin in ransgenic tobacco are generally in the order of 0.001-0.1% of total leaf protein T.J.Higgins, personal communication). Similar levels have been reported for expression of other foreign genes in transgenic tobacco (Vaeck *et al.*, 1987; Robert *et al.*, 1989; Yang *et al.*, 1989). However, plant 8 in the WM1 series reported here, contained nine times more SF8 mRNA than an ovalbumin-transformed tobacco contained ovalbumin mRNA. As the ovalbumin transformed tobacco reported in this study produced 0.01% of its leaf protein as ovalbumin, we can assume that there is at least sufficient SF8 mRNA in plant 8 to support up to nine times that level of protein expression. Therefore, the methanol-soluble fraction from 35mg of total leaf protein from plant 8 should have been sufficient to detect SF8

protein by Western blotting. Two possible explanations for the lack of detectable SF8 protein are that, SF8 mRNA is not translatable in tobacco or SF8 protein is very unstable in tobacco leaves.

Translatability of SF8 mRNA was tested in *E. coli* using an expression vector, pJLA602. Schauder *et al.* (1987) reported that DNA coding regions incorporated into the plasmid pJLA602 have been transcribed and translated in *E. coli* and the resultant protein detected by Coomassie staining or [^{35}S] methionine labelling. The chimeric SF8 coding region was incorporated into the plasmid, pJLA602 and the synthesis of SF8 assayed by Western blotting without success.

This may indicate that the SF8 cDNA coding region is untranslatable in *E. coli* or that there is a frameshift or stop codon in the cloned DNA. McCarthy *et al.* (1985) reported variable levels of expression of *E. coli* c, b and d genes, whilst Schauder *et al.* (1987) reported variable levels of protein expression of *E. coli* atpA, sucC and, sucD, human interferon b and interleukin 2 genes using pJLA vectors. McCarthy *et al.* (1985; 1986) suggested that the variation in the levels of expression of foreign genes was not correlated with codon usage, but to date no one has tried to express a plant gene for a high sulphur protein in *E. coli*. Ikemura (1981) suggested *E. coli* genes contain codons corresponding to major tRNA species and are almost completely devoid of codons corresponding to minor tRNA species. The cysteine tRNA and to a lesser extent, the methionine tRNA, have a very low abundance and frequency of use in *E. coli* (Ikemura, 1981). Given the high sulphur amino acid content (7.5% cysteine and 15.5% methionine) of SF8 (Kortt *et al.*, 1990) the translatability of the SF8 cDNA coding region in *E. coli* may be limited by the availability of cystienyl and methioninyl tRNAs.

Total RNA from WM1-transformed tobacco (1μg) was also translated in a cell-free rabbit reticulocyte lysate in the presence of [^{35}S] methionine. However, the results of this experiment did not allow us to conclude whether the SF8 mRNA was translatable *in vitro* because conditions for purification of the *in vitro* translation products were not optimised in time for inclusion in this thesis. Further experiments are needed to resolve this problem. A future experiment could make use of the opal vectors, pSP72 or pSP73 (Bresatec; Australia), constructed by Krieg and Melton (1987). These vectors contain the promoters for the SP6 and T7 RNA polymerases. The 5' untranslated region from vicilin, the signal peptide sequence from vicilin and the SF8 cDNA coding region could be ligated into these vectors. Using either SP6 or T7 RNA polymerase, large amounts of transcript could be produced *in vitro* for cell free translation.

For technical reasons, these two experiments failed to confirm or deny the translatability of SF8 mRNA. Therefore, DNA sequencing of pWM1 should be done to confirm the integrity of the SF8 chimeric cDNA sequence. In the chimeric gene pWM1, deletion or insertion of a single base pair in the DNA sequence encoding SF8 would be sufficient to disrupt the reading frame required for the successful synthesis of mature SF8. Such a deletion may have occurred during cloning of the SF8 cDNA. Lilley *et al.* (1989) used oligonucleotide-directed-site-specific mutagenesis to create a BamHI site at map position 132 (map position specified by Kortt *et al.*, (1990) where position 22 is the A in the ATG start codon) in the SF8 cDNA coding region used in the construction of pWM1. It is at this site that an insertion or deletion is most likely to have occurred. Endonuclease restriction mapping indicates a BamHI site does exist at or near the intended position. Figure 12 shows that a deletion of the C in the codon for isoleucine (ATC) would result in a functional BamHI recognition site (GGATCC) as the next codon, which specifies the N-terminal amino acid of SF8, is proline (CCC). The resultant cDNA sequence would now code for an entirely different polypeptide. In this case, hybridisation to a ^{32}P labelled SF8 probe would still be possible but detection of SF8 protein would not.

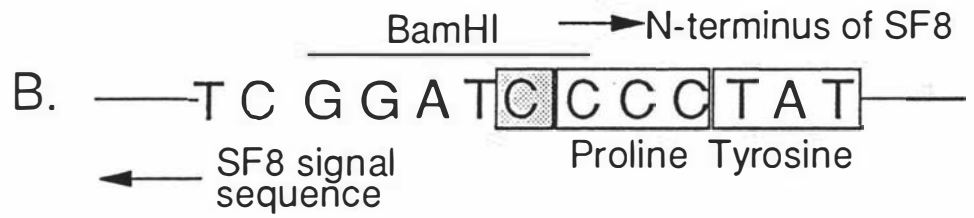
The remaining possibility, should the SF8 cDNA sequence prove to be correct, is that SF8 protein is being synthesized but significant accumulation is being prevented because SF8 protein is very unstable in tobacco leaves. Very little experimental evidence is available on the stability or otherwise of foreign proteins expressed in tobacco leaves. [^{35}S] Methionine pulse-chase experiments indicate that foreign proteins can be relatively unstable when expressed in tobacco leaves (T.J.Higgins and R.Khan, personal communication).

The CaMV 35S promoter (map position 7025 to 7437 as described by Franck *et al.*, 1980) is highly active in most plant organs once integrated into the plant genome. (Kay *et al.*, 1987 and Sanders *et al.*, 1987). Benfey *et al.* (1989) identified two domains in the CaMV promoter; one that principally confers root expression, whilst the other confers expression in all other tissues. The CaMV 35S promoter used in the chimeric gene WM1 has both domains. Therefore, the CaMV 35S promoter used in chimeric gene WM1 should drive expression of the SF8 cDNA chimeric gene in tobacco seeds. Transgenic tobacco transformed with WM1 were grown for seed. If the problem was instability of SF8 protein in tobacco leaves, SF8, which is a seed storage protein, may be stably accumulated in tobacco seeds. The expression of the chimeric gene, WM1 in tobacco seeds was monitored by Western blot analysis and again the results were negative (R.Khan, personal communication). This suggests that SF8 protein was undetectable in pWM1 transformed tobacco leaves because the SF8 cDNA coding region used in pWM1, was untranslatable.

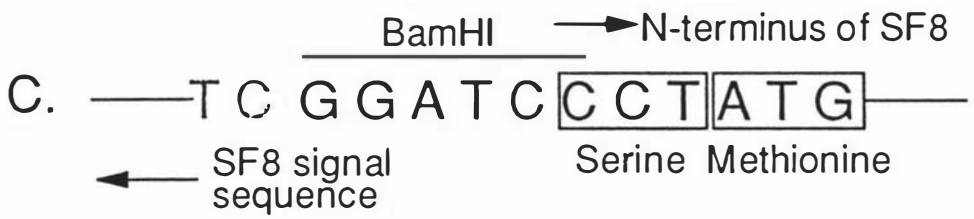
The combination of the three experiments: DNA sequencing of the SF8 cDNA coding region used in the chimeric gene WM1, translation of SF8 mRNA, synthesised *in vitro* by viral RNA polymerases, in a rabbit reticulocyte lysate and pulse-chase labelling of SF8 protein in leaves will enable us to determine whether the apparent lack of translation of the chimeric gene WM1 in transgenic tobacco leaves and seeds is due to either the untranslatibility of SF8 mRNA or the inherent instability of SF8 protein in transgenic tobacco leaves and seeds.

Fig. 12: Theoretical consequences of a deletion in the SF8 cDNA during oligonucleotide-directed-site-specific mutagenesis.
An example of a C base deletion and the resultant change in the amino acid sequence of SF8 is shown.

The SF8 cDNA sequence adjacent to bp 132 as reported by Kortt et al., (1990). Lines under DNA bases indicate bases changed by mutagenesis.



The SF8 cDNA sequence adjacent to bp 132 after mutagenesis (Lilley et al., 1989). A BamHI site has been created at the N-terminus of SF8. For the next example the C in the shaded box is assumed to be have been deleted during mutagenesis.



The deletion of the C has changed the amino acid sequence of SF8 but has not destroyed the BamHI site.
It should be noted that deletion of any C or G either side of the AT in the BamHI site would have a similar effect.

6.5 CONCLUSIONS.

6.5.1 The gene for SF8, a protein with a high proportion of SAA, is expressed only in seeds. The SF8 cDNA clone was genetically engineered for expression in the leaves of tobacco. The reconstructed gene (WM1) was inserted into a gene delivery system (pWM1) in *Agrobacterium tumefaciens* and transferred to tobacco. Expression of the chimeric gene was monitored at the mRNA and protein level.

6.5.2 The SF8 cDNA was not complete at the 5' end and contained no sequences beyond the poly(A) addition sites at the 3' end. Therefore, the CaMV 35S promoter, the vicilin 5' untranslated sequence, and the vicilin signal sequence were provided at the 5' end of a chimeric gene, whilst the NOS gene termination sequence was provided at the 3' end of the cDNA.

6.5.3 Transcription of the chimeric gene WM1 occurred in the leaves of WM1 transformed tobacco, with the level of SF8 mRNA varing over a 100 fold range, but in the highest expressor, it represented 14% of the SF8 mRNA level found in sunflower seeds.

6.5.4 Sensitive immunological procedures (Western blot analysis) failed to detect SF8 protein in the leaves of tobacco transformed with pWM1. The minimum loading of SF8 protein required to give a detectable signal in Western Blot analysis was 10μg. Therefore, the level of SF8 in the leaves of transgenic tobacco was less than 0.03% of total leaf protein.

6.5.5 The highest SF8 mRNA expresser contained nine times more SF8 mRNA than an ovalbumin-transformed tobacco contained ovalbumin mRNA. As the ovalbumin transformed tobacco produced 0.01% of its leaf protein as ovalbumin, there is at least sufficient SF8 mRNA to support up to nine times that level of protein expression. This level of expression would be detectable using sensitive immunological procedures suggesting that either SF8 mRNA is not translatable in tobacco or SF8 protein is very unstable in tobacco leaves.

6.5.6 Translatability of SF8 mRNA was tested in *E. coli* using an expression vector, pJLA602. The chimeric SF8 coding region was incorporated into the plasmid, pJLA602 and the synthesis of SF8 assayed by Western blotting without success. This may indicate that the SF8 cDNA coding region is untranslatable in *E. coli* or that there is a frameshift or top codon in the cloned DNA.

6.5.7 If the problem was instability of SF8 protein in tobacco leaves, SF8, which is a seed storage protein, may be stably accumulated in tobacco seeds. The expression of the chimeric gene, WM1 in tobacco seeds was monitored by Western blot analysis and again the results were negative. This suggests that SF8 protein was undetectable in pWM1 transformed tobacco leaves because the SF8 cDNA coding region used in pWM1, was untranslatable.

6.5.8 DNA sequencing of pWM1 should be done to confirm the integrity of the SF8 chimeric cDNA sequence. In the chimeric gene pWM1, deletion or insertion of a single base pair in the DNA sequence encoding SF8 would be sufficient to disrupt the reading frame required for the successful synthesis of mature SF8

6.5.9 The combination of the three experiments: DNA sequencing of the SF8 cDNA coding region used in the chimeric gene WM1, translation of SF8 mRNA, synthesised *in vitro* by viral RNA polymerases, in a rabbit reticulocyte lysate and pulse-chase labelling of SF8 protein in leaves will enable us to determine whether the apparent lack of translation of the chimeric gene WM1 in transgenic tobacco leaves and seeds is due to either the untranslatibility of SF8 mRNA or the inherent instability of SF8 protein in transgenic tobacco leaves and seeds.

7. GENERAL DISCUSSION AND CONCLUSION

7.1 INTRODUCTION.

In ruminants fed fresh forage diets, abomasal infusions of protein, and dietary protein supplements protected from ruminal degradation have increased wool growth (Reis and Schinkel, 1963; Ferguson *et al.*, 1967), milk production (Stobbs *et al.*, 1977; Flores *et al.*, 1979; Rogers *et al.*, 1979) and liveweight gain (Barry, 1981). In particular, post-ruminal supplementation with sulphur amino acids (SAA) has increased wool growth, so that methionine and cysteine are probably the limiting amino acids for wool growth in sheep fed roughage diets (Reis, 1979). This thesis examined two approaches with the potential to increase the absorption of SAA from the small intestine. The first method examined the effects of condensed (CT) in forages fed to sheep. The second method examined the use of genetic engineering to express genes which code for proteins containing a high proportion of SAA, in important pastoral legumes.

7.2 CONDENSED TANNINS.

7.2.1 Introduction.

Condensed tannins occur in some forage legumes, but are generally absent from grasses used in temperate agriculture (Barry, 1989). Condensed tannins bind to proteins to form stable complexes in the pH range 3.5-7.0, but the complexes dissociate at pH <3.5 (Jones and Mangan, 1977). Therefore, plant protein should be bound to CT and be protected from microbial degradation in the rumen (pH 5.5-7.0), but be released in the abomasum (pH 1.3-3.0), enabling subsequent absorption of amino acids in the small intestine.

7.2.2 The Effect of Polyethylene Glycol.

In each experiment, one group of sheep (PEG sheep) received an intraruminal infusion of polyethylene glycol (PEG), while the remaining group of sheep (CONTROL sheep) received an intraruminal infusion of water. Polyethylene glycol (1.7g/g CT) displaces CT from CT-protein complexes (Jones and Mangan, 1977) and completely binds available CT (Barry and Forss, 1983), preventing it from binding to protein in the rumen. Consequently, protein can be degraded to NH_3 by rumen microorganisms. Therefore, comparing control to PEG sheep provided a means of quantifying the effects of CT on rumen digestion without changing the nutrient composition of the diet offerred.

7.2.3 The Effect of Condensed Tannins on Rumen Digestion.

Dietary CT reduced rumen ammonia concentration (Barry *et al.*, 1986; Waghorn *et al.*, 1987), rumen soluble protein concentration (Waghorn and Jones, 1989) and increased non-ammonia-nitrogen (NAN) outflow from the rumen in sheep fed *Lotus* species (Barry *et al.*, 1986; Waghorn *et al.*, 1987). Tanner *et al.* (1990) reported that CT from sainfoin (*Onobrychis viciifolia*) reduced the rate of Fraction 1 leaf protein degradation in rumen fluid *in vitro*. Therefore, the evidence suggests CT reduces forage protein degradation in the rumen.

The degradation of plant protein in the rumen is likely to follow, at least part of the sequence:

Insoluble protein > Soluble protein > Large polypeptides > Small polypeptides > Amino acids > Ammonia and sulphide.

The effect of CT upon rumen protein solubilization was studied by measuring the rate of nitrogen (N) loss from *Lotus pedunculatus* incubated in polyester-fibre bags, suspended in the rumen of sheep fed *Lotus pedunculatus*, with and without an intraruminal PEG infusion. The effect of CT upon protein degradation was studied qualitatively, by examining the degradation of leaf protein from *Lotus pedunculatus*, in rumen fluid *in vitro*. Condensed tannins had little effect on the solubilization of N from fresh minced (FM) *Lotus*, but reduced the extent to which N was solubilized from freeze dried (FD) *Lotus*. Wallace (1985) suggested that the adsorption of bacteria to plant protein was an integral part of protein degradation in the rumen. Therefore, the ability of CT to prevent microbial colonization of FD *Lotus* in control sheep may have been responsible for the reduced solubilization of N. In contrast, the extent to which CT reduced the rate of protein degradation appeared to be similar for both FM and FD *Lotus*. Therefore, once protein was solubilized, the rate of degradation was similar, regardless of whether it was derived from FM or FD *Lotus*. Consequently, with fresh forage diets, CT probably has little effect on the rate of protein solubilization, but reduces the degradation of soluble protein in the rumen. It is this effect that was probably responsible for the increased outflow of NAN from the rumen of sheep fed *Lotus* species (Barry *et al.*, 1986; Waghorn *et al.*, 1987).

When sheep were fed *Lotus pedunculatus*, the loss of dietary methionine and cystine from the rumen were insignificant, but infusion of PEG increased the loss of both amino acids to 30% of intake. The majority of feed-S is reduced to sulphide in the rumen (Kennedy *et al.*, 1975; Kennedy and Milligan, 1978; Kandylis, 1984), which is an intermediate for most

rumen inorganic-S transactions. The irreversible loss rate (IRL) of reducible-S was greater when PEG was given, than in control sheep, confirming the effect of CT in preventing plant SAA degradation in the rumen.

The majority of the sulphide in the rumen is either absorbed across the rumen wall, appearing as inorganic sulphate in the blood (Bray, 1969) or utilised in the rumen for bacterial protein synthesis (Kennedy and Milligan, 1978). Consequently, the ability of dietary CT to lower the IRL of reducible-S probably contributed to the lower entry rate of inorganic-sulphate into plasma in control compared to PEG sheep.

Although, CT reduced microbial colonization of fresh and dried *Lotus* to a small and large extent respectively, it was calculated that CT did not alter the size of the rumen microbial-NAN pool. Similarly, Beever and Siddons (1986) reported that CT from sainfoin did not inhibit microbial-protein synthesis in the rumen or microbial-protein outflow from the rumen. In contrast, Waghorn *et al.* (1987), reported that sheep eating *Lotus corniculatus* (cv Empire; 4.6gCT/kgDM) had a higher flow of microbial-N to the duodenum than sheep eating *Lotus corniculatus* (cv Maitland; 32gCT/kgDM). Consequently, CT probably do not effect the rumen microbial-pool size, but may reduce microbial-protein outflow from the rumen. Sheep grazing the CT-containing legume, sulla (*Hedysarum coronarium*), had a higher rumen protozoal concentration than sheep grazing sulla, but receiving orally administered PEG (T.H.Terrill, personal communication). Therefore, although CT may reduce the rumen bacterial-protein pool, the rumen microbial-protein pool may be unchanged due to an increase in protozoal-protein. Microbial-protein outflow from the rumen may be reduced because protozoa are preferentially retained in the rumen (Leng, 1984). The effect of CT upon rumen bacterial and protozoal dynamics is poorly understood and requires further research.

7.2.4 Post-Ruminal Digestion.

Although, the abomasal flux of both methionine and cystine were higher in control than PEG sheep, the apparent absorption of methionine from the small intestine was only increased for methionine. In contrast to methionine, dietary CT depressed the apparent digestibility of cystine in the small intestine of control sheep. A low apparent absorption of cystine was also reported by Ashes *et al.* (1984) in sheep fed formaldehyde treated casein. This was probably due to the increasing strength of the bonding between formaldehyde and cystine with increasing protein protection. Therefore, in the present study cystine may not have dissociated from CT-protein complexes in the abomasum as readily as methionine, due to its free SH groups bonding more strongly with CT. Waghorn *et al.* (1987) also reported that CT depressed the apparent digestibility of non-essential amino acids (NEAA)

in the small intestine, whilst having no effect on the apparent digestibility of essential amino acids (EAA; Table 1). The monomeric subunits (flavanols) of CT are hydroxylated, and thus able to form hydrogen bonds. The majority of amino acids which had reduced apparent digestibilites in the small intestine, also have reactive groups able to form hydrogen bonds. Therefore, the formation of strong hydrogen bonds between CT and some amino acids maybe responsible for the reduced apparent digestibility of these amino acids in the small intestine. However, other interactions may also be involved since a number of EAA can also form hydrogen bonds, but are not effected to the same extent as NEAA. The ability of CT to depress amino acid absorption from the small intestine clearly follows the distribution of amino acids between EAA and NEAA (Table 1). Generally, NEAA have small, simple reactive groups, whilst EAA have large, complex reactive groups. Therefore, the strength of the interaction between CT and amino acids may be effected by a combination of their net charge, the spatial arrangement, and the size of the reactive groups of individual amino acids. Further research is necessary to determine the mechanism by which the apparent digestibility of NEAA in the small intestine is selectively depressed in feeds containing CT.

Waghorn *et al.* (1987) demonstrated a greater increase in EAA absorption from the small intestine in sheep fed *Lotus corniculatus* (22gCT/kgDM) than was reported for methionine and cystine in control sheep fed *Lotus pedunculatus* (55gCT/kgDM) in the present study. Further research is necessary to define the CT concentration in the diet at which the detrimental effects on cystine digestion, probably due to over-protection by CT, may be avoided. Lower dietary CT concentrations than used in the present study may be more nutritionally desirable. Consequently, it may be possible to further improve the absorption of methionine and cystine from the small intestine in sheep fed CT-containing forages.

7.2.5 Sulphur Amino Acid Metabolism in Plasma.

Condensed tannin increased the apparent absorption of methionine from the small intestine, whilst cystine apparent absorption was not significantly affected. In contrast, the entry rate of methionine into post-hepatic blood was not significantly different, whilst the entry rate of cystine was increased in control, compared to PEG sheep. This was probably due to transulphuration in the liver (Radcliffe and Egan, 1978), so that the increased methionine absorbed from the small intestine in control sheep appeared in post-hepatic blood as cystine.

In control sheep there was a decrease in methionine and cystine oxidation, an increase in transulphuration of methionine to cystine and an increased cystine entry from whole body turnover and digestion, so that the plasma cystine flux to maintenance and productive

Table 1: The apparent digestibility of amino acids in the small intestine of sheep fed fresh Lotus peduncultus or corniculatus, with and without an intraruminal infusion of PEG.

AUTHOR	DIET	TANNIN CONCENTRATION (g/kgDM)	AMINO ACID	APPARENT DIGESTIBILITY		LEVEL OF SIGNIFICANCE
				CONTROL SHEEP	PEG SHEEP	
W.McNabb, (present study)	LP	50	cystine	0.42	0.53	**
			methionine	0.77	0.79	NS
Waghorn et al. (1987)	LC	22	NEAA			
			alanine	0.60	0.70	NS
			asparagine	0.48	0.63	***
			glutamate	0.60	0.71	**
			glycine	0.63	0.65	NS
			proline	0.43	0.62	*
			serine	0.50	0.70	**
			EAA			
			arginine	ND	0.81	-
			histidine	0.71	0.66	NS
			isoleucine	0.68	0.60	NS
			leucine	0.68	0.70	NS
			lysine	0.74	0.69	NS
			phenylalanine	0.77	0.64	*
			threonine	0.62	0.60	NS
			tyrosine	0.78	0.69	NS
			valine	0.62	0.56	NS

LP, Lotus pedunculatus; LC, Lotus corniculatus; NEAA, non-essential amino acids; EAA, essential amino acids; ND, not determined.

processes was higher than for PEG sheep. This suggests that there was a higher metabolic requirement for cystine than methionine in the sheep used in the present study. The increased flux of cystine to productive processes and maintenance in control sheep could be expected to result in an increased rate of wool growth. It has recently been demonstrated that the wool growth of sheep grazing sulla (*Hedysarum coronarium*) was higher than that of similar sheep grazing sulla, but given a daily drench of PEG to inactivate the CT (T.H.Terrill *et al.*, unpublished).

7.3 GENETIC ENGINEERING.

7.3.1 Introduction.

Responses in wool growth to post-ruminal supplements of SAA suggests an ideal dietary protein for wool growth should contain a high proportion of SAA, and be relatively resistant to rumen degradation. Therefore, expressing genes coding for such proteins in forage legumes, using genetic engineering techniques, may have potential benefits for wool production. A number of proteins that would be potentially useful in this respect have been identified and their cDNA or genomic sequences isolated. Ovalbumin, from chicken egg white contains 6.5% SAA (Gilbert, 1972), and the seed proteins, pea albumin 1 and sunflower albumin 8 (SF8), contain respectively 11% SAA (Higgins *et al.*, 1989) and 25% SAA (Kortt *et al.*, 1990). Sunflower albumin 8 was chosen for the present project because of its very high SAA content.

7.3.2 The Resistance of SF8 to Rumen Degradation.

It is important to establish the relative resistance of SF8 to rumen degradation before embarking on genetic engineering studies. Therefore, the rate of SF8 protein degradation in an *in vitro* rumen incubation was studied and compared with the degradation rates of the large (LSU) and small (SSU) subunit of Fraction 1 leaf protein, and ovalbumin protein.

Proteolysis of SF8 protein in an *in vitro* rumen incubation followed first order kinetics, with a shorter half-life than that of either component of the LSU of Fraction 1 leaf protein. However, the degradation of SF8 protein was associated with the appearance of a polypeptide, which was resistant to rumen degradation. Sunflower albumin 8, ovalbumin and the SSU of Fraction 1 leaf protein were more resistant to rumen degradation than the LSU of Fraction 1 leaf protein. Mangan (1972) and Nugent *et al.* (1983) reported that ovalbumin was relatively resistant to rumen proteolysis, but that resistance could be reversed by treating the protein chemically, to break disulphide bonds. However, disulphide bonding is not the only factor determining resistance to rumen protein degradation, since

the SSU of Fraction 1 leaf protein, which contains no disulphide bonds (Kawashima and Wildman, 1970), was relatively resistant to rumen proteolysis. In contrast, both SF8 (Kortt *et al.*, 1990) and ovalbumin (Gilbert, 1971) contain disulphide bonds, yet two different mechanisms appeared to be responsible for their resistance to rumen proteolysis. However, the relative resistance of SF8 protein to rumen degradation suggests that it should be benefical to express SF8 protein in the leaves of important agricultural legumes using genetic engineering techniques. However experiments need to be conducted to determine if oral administration of proteins containing a high proportion of SAA, but with a low rumen degradability, will stimulate wool growth in sheep fed forages.

7.3.3 Expression of the Seed Protein, SF8, in the Leaves of a Transgenic Host.

A cDNA has been isolated, which contains the signal peptide and prosequence, together with the coding region and 160 base-pairs of the 3' untranslated region of SF8 (Lilley *et al.*, 1989). Since the gene for SF8 is expressed only in seeds, the SF8 cDNA clone had to be genetically engineered for expression in the leaves of tobacco. The reconstructed gene was inserted into a gene delivery system in *Agrobacterium tumefaciens* and transferred to tobacco.

In the leaves of SF8 transformed tobacco plants, the level of SF8 mRNA varied over a 100 fold range, but in the highest expressor, it represented 14% of the SF8 mRNA level found in sunflower seeds. Although, transcription of the SF8 chimeric gene occurred in the leaves of transgenic tobacco, SF8 protein could not be detected with immunological techniques. Therefore, the level of expression of the SF8 chimeric gene in transgenic tobacco was less than 0.03% of total leaf protein. One transformed tobacco plant, contained nine times more SF8 mRNA than an ovalbumin-transformed tobacco contained ovalbumin mRNA. As the ovalbumin transformed tobacco produced 0.01% of its leaf protein as ovalbumin, we can assume that there is at least sufficient SF8 mRNA in the SF8 transformed tobacco plant to support up to nine times that level of protein expression. Such a level of SF8 protein expression in the leaves of tobacco would have been detectable by Western Blot analysis. Two possible explanations for the lack of detectable SF8 protein are that, the SF8 mRNA produced was not translatable in tobacco or SF8 protein is very unstable in tobacco leaves.

Translatability of SF8 mRNA was tested in *E. coli* using an expression vector, pJLA602. The chimeric SF8 coding region was incorporated into the plasmid, pJLA602 and the synthesis of SF8 assayed by Western blotting without success. However, if the problem was instability of SF8 protein in tobacco leaves, then SF8, which is a seed storage protein, would be stably accumulated in tobacco seeds. The expression of the SF8 chimeric gene in tobacco seeds was monitored by Western blot analysis and again the results were negative.

This suggests that SF8 protein was undetectable in tobacco leaves because the SF8 cDNA coding region was untranslatable. Therefore, DNA sequencing should be done to confirm the integrity of the SF8 chimeric cDNA sequence. A deletion or insertion of a single base pair in the SF8 cDNA sequence would be sufficient to disrupt the reading frame required for the successful synthesis of mature SF8 protein. A deletion or insertion in the SF8 cDNA coding region could easily be repaired using oligonucleotide-directed, site-specific mutagenesis.

7.3.4 What Level of SF8 Protein Expression is Required?

The level of foreign gene expression in plants depends upon the source of the gene and the plant tissue in which the gene is to be expressed. The level of expression of foreign seed protein genes in the seeds of transformed plants, although generally lower than in the native seed, can be as high as 2-8% of total seed protein (Sengupta-Gopalan *et al.*, 1985; Altenbach *et al.*, 1989; Vandekerckhove *et al.*, 1989), whereas the level of expression of foreign genes in the leaves of transformed plants is very low and generally in the range of 0.001-0.1% of total leaf protein (Weising *et al.*, 1988). Low levels of expression of herbicide and insect resistance genes in the leaves of transformed plants gives acceptable levels of plant protection (Weising *et al.*, 1988; Schulz *et al.*, 1990). However, it has been estimated that the level of expression of high-sulphur protein genes in the leaves of lucerne would need to be at least 5% of total leaf protein in order to have any positive benefits on ruminant production.

7.3.5 Can We Achieve High Levels of Expression?

In contrast to the generally low level of expression of foreign genes in the leaves of transgenic hosts, there are four examples of foreign genes showing relatively high levels of expression in the leaves of transgenic plants.

(1) A leaf-specific glutamine synthatase (GS) gene from lucerne has been engineered for expression in the leaves of tobacco. The foreign GS gene was expressed at a level of 5% of total tobacco leaf protein (Eckes *et al.*, 1989).

(2) The gene coding for vicilin, a pea seed protein, when engineered for expression in the leaves of tobacco, expressed vicilin at a level of 0.01% of total leaf protein (T.J. Higgins, personal communication). Munro and Pelham (1987) identified an amino acid sequence (lys-asp-glu-leu), which occurred at the carboxy terminus of some proteins and appeared to be responsible for targeting those proteins for retention in the endoplasmic reticulum. When the before mentioned vicilin gene was modified to include the endoplasmic

reticulum targeting sequence, vicilin was expressed in the leaves of tobacco at a level of 1% of total leaf protein (T.J. Higgins, personal communication).

(3) When the seed-specific cowpea trypsin inhibitor gene, was engineered for expression in the leaves of tobacco, the trypsin inhibitor was expressed at a level of 1% of total leaf protein in transgenic tobacco (Hilder *et al.*, 1987).

(4) When mouse antibody cDNA's derived from hybridoma mRNA, were engineered for leaf expression, functional antibodies were expressed at a level of 1.3% of total leaf protein in transgenic tobacco (Hiatt *et al.*, 1989).

These examples of relatively high levels of foreign gene expression in the leaves of a transgenic host suggest it is worthwhile pursuing high levels of expression of high-sulphur protein genes in the leaves of agriculturally important legumes, such as lucerne and white clover. However, there are no experimental data available to indicate whether 5% of total leaf protein is the level of expression required to achieve significant responses in animal production, particularly wool growth. Although, no transgenic plants expressing proteins containing high proportions of SAA are presently available, sheep grazing lucerne could be supplemented with a protein containing a high proportion of SAA, such as ovalbumin and wool growth recorded to determine what level of chimeric gene expression is required for transgenic legumes to have a place in agriculture.

7.4 CONCLUSIONS.

The objective of the present study was to examine two approaches with the potential to increase SAA absorption from the small intestine. Feeding the CT-containing legume, *Lotus pedunculatus*, increased the absorption of methionine from the small intestine, although lower dietary CT concentrations than those in the present study, may effect a greater increase in the absorption of SAA from the small intestine. However, the small increase in methionine absorption from the small intestine did result in a substantial increase in plasma cystine available for productive processes and maintenance so that CT should be effective in promoting wool growth.

Unfortunately, most CT-containing legumes are adapted for survival in poor environments, particularly low soil fertility, and perform relatively poorly in mixed swards grown under medium-high soil fertility. To fully utilise the benefits that CT offers for animal production, genes coding for CT biosynthesis need to be genetically engineered so that they are expressed in the leaves of important agricultural legumes, such as white clover.

The expression of genes coding for proteins containing a high proportion of SAA, and which are relatively resistant to rumen degradation, legumes should provide benefits for animal production, particularly wool growth. Although, expression of the SF8 chimeric gene was probably inhibited by poor translation, DNA sequencing of the SF8 chimeric gene would enable identification of the problem, which could then be readily corrected by oligonucleotide-directed, site-specific mutagenesis. High levels of foreign gene expression in transgenic plants has been demonstrated, so that pursuing high levels of expression of genes coding for proteins containing a high proportion of SAA, such as SF8 is worthwhile.

8. BIBLIOGRAPHY

Altenbach, S.B., Pearson, K.W., Meeker, G., Staraci, L.C. and Sun, S.S.M. (1989). Plant Molecular Biology **13**: 513.

Ampe, C., van Damme, J., de Castro, L.A.B., Sampaio, M.J.A.M., van Montagu, M. and Vandekerckhore, J. (1986). European Journal of Biochemistry **159**: 597.

Ashes, J.R., Mangan, J.L. and Sidhu, G.S. (1984). British Journal of Nutrition **52**: 239.

Bajaj, S., Mickelsen, O., Lillevik, H.A., Barker, L.R., Bergen, W.G. and Gill, J.L. (1971). Crop Science **11**: 813.

Barry, T.N. (1981). British Journal of Nutrition **46**: 521.

Barry, T.N. (1985). British Journal of Nutrition **54**: 211.

Barry, T.N. (1989). 153. In *The Roles of Protozoa and Fungi in Ruminant Digestion*. Edited by J.V.Nolan, R.A.Leng and D.I.Demeyer. University of New England Publishing Unit, Australia.

Barry, T.N. and Blaney, B.J. (1987). 91. In *The Nutrition of Herbivores*. Edited by J.B.Hacker and J.H.Ternouth. Academic Press, Australia.

Barry, T.N. and Duncan, S.J. (1984). British Journal of Nutrition **51**: 485.

Barry, T.N. and Forss, D.A. (1983). Journal of Food, Science and Agriculture **34**: 1047.

Barry, T.N. and Manley, T.R. (1985). British Journal of Nutrition **54**: 753.

Barry, T.N. and Manley, T.R. (1986). Journal of Science, Food and Agriculture **37**: 248.

Barry, T.N., Manley, T.R. and Duncan, S.J. (1986). British Journal of Nutrition **55**: 123.

Barton, K.A., Binns, A.N., Matzke, A.J.M. and Chilton, M.D. (1983). Cell **32**: 1033.

Bate-Smith, E.C. (1954). Advances in Food Research **5**: 261.

Bate-Smith, E.C. (1954). Food **23**: 124.

Bate-Smith, E.C. (1973). Phytochemistry **12**: 907.

Beever, D.E. and Siddons, R.C. (1986). 479. In *Control of Digestion and Metabolism in Ruminants*. Edited by L.P.Milligan, W.L.Grovum and A.Dobson. Prentice Hall, New Jersey.

Bekes, F. and Lasztity, R. (1981). Cereal Chemistry **58**: 360.

Bender, A.E. and Krebs, H.A. (1950). Biochemistry Journal **46**: 210.

Benevenga, N.J., Radcliffe, B.C. and Egan, A.R. (1983). Australian Journal of Biological Science **36**: 475.

Benfey, P.N., Ren, L. and Chua, N.H. (1989). EMBO **8**: 2195.

Bevan, M. (1984). Nucleic Acids Research **12**: 8711.

Bevan, M., Barnes, W.M. and Chilton, M.D. (1983). Nucleic Acids Research **11**: 369.

Bevan, M.W., Mason, S.E. and Goelet, P. (1985). EMBO **4**: 1921.

Binnerts, W.T., van't Klooster, A.Th. and Frens, A.M. (1968). Veterinary Record **82**: 470.

Bird, P.R. (1972). Australian Journal of Biological Science **25**: 185.

Bird, P.R. (1972). Australian Journal of Biological Science **25**: 195.

Bird, P.R. (1972). Australian Journal of Biological Science **25**: 817.

Bird, P.R. (1972). Australian Journal of Biological Science **25**: 1073.

Bird, P.R. (1972). Australian Journal of Biological Science **25**: 1087.

Bird, P.R. (1974). Australian Journal of Agricultural Research **25**: 631.

Bird, P.R. and Hume, I.D. (1971). Australian Journal of Agricultural Research **22**: 443.

Bird, P.R. and Moir, R.J. (1971). Australian Journal of Biological Science **24**: 1319.

Bird, P.R. and Moir, R.J. (1972). Australian Journal of Biological Science **25**: 835.

Bird, P.R. and Thorton, R.F. (1972). Australian Journal of Biological Science **25**: 1299.

Black, J.L. and Reis, P.J. (1979). 269. In *Physiological and Environmental Limitations to Wool Growth.* Edited by J.L.Black and P.J.Reis. University of New England Publishing Unit, Australia.

Black, J.L., Robards, G.E. and Thomas, R. (1973). Australian Journal of Agricultural Research **24**: 399.

Blackburn, T.H. (1968). Journal of General Microbiology **53**: 27.

Bohlmann, H. and Apel, K. (1987). Molecular and General Genetics **207**: 446.

Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V. and Apel, K. (1988). EMBO **7**: 1559.

Bradford, M. (1976). Analytical Biochemistry **72**: 248.

Bray, A.C. (1964). Proceedings of the Australian Society of Animal Production **5**: 336.

Bray, A.C. (1969). Australian Journal of Agricultural Research **20**: 725.

Bray, A.C. (1969). Australian Journal of Agricultural Research **20**: 739.

Bray, A.C. (1969). Australian Journal of Agricultural Research **20**: 749.

Bray, A.C. and Till, A.R. (1975). 243. *In Digestion and Metabolism in the Ruminant.* Edited by I.W. McDonald and A.C.I. Warner. University of New England Publishing Unit, Australia.

Broad, A., Gillespie, J.M. and Reis, P.J. (1970). Australian Journal of Biological Science **23**: 149.

Broadhurst, R.B. and Jones, W.T. (1978). Journal of Science, Food and Agriculture **29**: 788.

Bull, H.B. (1941a). Journal of Biological Chemistry **137**: 143.

Buttery, P.J., Beckerton, A., Mitchell, R.M., Davis, K. and Annison, E.F. (1975). Proceedings of the Nutrition Society **34**: 91-92A.

Campbell, R.M., Cuthbertson, D.P., Machie, W., McFarlane, A.S., Phillipson, A.T. and Sudsaneh, S. (1961). Journal of Physiology **158**: 113.

Chapman, R.E. (1979). 167. In *Haar und Haarkranheiten.* Edited by C.E.Orfavos. Gustav Fischer Verlag, Stutgard.

Coleman, G.S. (1960). Journal of General Microbiology **22**: 423.

Cooper, S. and Ruettinger, T. (1975). Molecular and General Genetics **139**: 167.

Corbett, J.L. (1979). 79. In *Physiological and Environmental Limitations to Wool Growth.* Edited by J.L.Black and P.J.Reis. University of New England Publishing Unit, Australia.

Costerton, J.W., Geesey, G.G. and Cheng, K. (1978). Science in America **238**: 86.

Crooker, B.A., Sniffen, C.J., Hoover, W.H. and Johnson, L.L. (1978). Journal of Dairy Science **61**: 437.

Della Cioppa, G., Bauer, S.C., Taylor, M.L., Rochester, D.E. and Klein, B.K. (1987). Biotechnology **5**: 579.

Dhaese, P., De Greve, H., Decraemer, H., Schell, J. and van Montagu, M. (1979). Nucleic Acids Research **7**: 1837.

Dijkshoorn, W. and van Wijk, A.L. (1967). Plant and Soil **36**: 129.

Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980). Proceedings of the National Academy of Science; U.S.A. **77**: 7347.

Dove, H. and Robards, G.E. (1974). Australian Journal of Agricultural Research **25**: 945.

Downes, A.M., Reis, P.J., Sharry, L.F. and Tunks, D.A. (1970). Australian Journal of Biological Science **23**: 1077.

Downes, A.M., Sharry, L.F. anf Till, A.R. (1964). Australian Journal of Biological Science **17**: 945.

Doyle, P.T. and Bird, P.R. (1975). Australian Journal of Agricultural Research **26**: 337.

Doyle, P.T. and Moir, R.J. (1980). Australian Journal of Biological Science **33**: 303.

Eckes, P., Schmitt, P., Daub, W. and Wengenmayer, F. (1989). Molecular and General Genetics **217**: 263.

Egan, A.R., Benevenga, N.J. and Radcliffe, B.C. (1984). 241. In *Ruminant Physiology: Concepts and Consequences*. Edited by S.K.Baker, J.M.Gawthorne and J.B.Mackintosh and D.B.Purser. University of New England Publishing Unit, Australia.

Egan, A.R. and Ulyatt, M.J. (1980). Journal of Agricultural Science; Cambridge **94**: 47.

Egan, A.R., Walker, D.J., Nader, C.J. and Storer, G. (1975). Australian Journal of Agricultural Research **26**: 909.

Ericson, M.L., Rodin, J., Lenman, M., Glimelius, K., Josefsson, L.G. and Rask, L. (1986). Journal of Biological Chemistry **261**: 14576.

Faichney, G.J. (1975). 227. In *Digestion and Metabolism in the Ruminant*. Edited by I.W.McDonald and A.C.I.Warner. University of New England Publishing Unit, Australia.

Ferguson, K.A., Hemsley, J.A. and Reis, P.J. (1967). Australian Journal of Science **30**: 215.

Fevold, H.L. (1951). Advances in Protein Chemistry **6**: 187.

Flores, J.F., Stobbs, T.H. and Minson, D.J. (1979). Journal of Agricultural Science; Cambridge **92**: 351.

Foley, W.J. and Hume, I.D. (1987). Journal of Comparative Physiology **157**: 67.

Foo, L.Y. and Porter, L.J. (1980). Phytochemistry **19**: 1747.

Franck, A., Guilley, H., Jonard, G., Richards, K. and Hirth, L. (1980). Cell **21**: 285.

Ganev, G., Orskov, E.R. and Smart, R. (1979). Journal of Agricultural Science; Cambridge **93**: 651.

Gawthorne, J.M. and Nader, C.J. (1976). British Journal of Nutrition **35**: 11.

Gilbert, A.B. (1971). 1291. In *Physiology and Biochemistry of the Domestic Fowl.* Edited by D.J.Bell and B.M.Freeman. Academic Press, London.

Gill, M. and Ulyatt, M.J. (1979). British Journal of Nutrition **41**: 605.

Gillispie, J.M. and Frenkel, M.J. (1972). 2: 265. In *Proceedings of the Fifth International Wool Textile Research Conference, Aachen.*

Grovum, W.L. and Williams, V.J. (1973). British Journal of Nutrition **29**: 73.

Guerino, F. and Baumrucker, C.R. (1987). Journal of Animal Science **65**: 619.

Guerino, F. and Baumrucker, C.R. (1987). Journal of Animal Science **65**: 630.

Harris, P.M., Dellow, D.W. and Sinclair, B.R. (1989). Australian Journal of Agricultural Research **40**: 879.

Harrison, D.G., Beever, D.E., Thomson, D.J. and Osbourn, D.F. (1975). Journal of Agricultural Science **85**: 93.

Harrison, D.G., Beever, D.E., Thomson, D.J. and Osbourn, D.F. (1976). Journal of Science and Food in Agriculture **27**: 617.

Hemsley, J.A. and Reis, P.J. (1984). 253. In *Ruminant Physiology: Concepts and Consequences.* Edited by S.K.Baker, J.M.Gawthorne and J.B.Mackintosh and D.B.Purser. University of New England Publishing Unit, Australia.

Hemsley, J.A., Reis, P.J. and Downes, A.M. (1973). Australian Journal of Biological Science **26**: 961.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989). Nature **342**: 76.

Higgins, T.J.V. (1984). Annual Reviews in Plant Physiology **35**: 191.

Higgins, T.J.V., Chandler, P.M., Randell, P.J., Spencer, D., Beach, L.R., Blagrove, R.J., Kortt, A.A. and Inglis, A.S. (1986). Journal of Biological Chemistry **261**: 11124.

Higgins, T.J.V., Newbigin, E.J., Spenser, D., Llewellyn, D.J. and Craig, S. (1988). Plant Molecular Biology **11**: 683.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Baker, R.F. and Boulter, D. (1987). Nature **300**: 160.

Hogan, J.P. (1961). Australian Journal of Biological Science **14**: 448.

Hogan, J.P. (1982). 245. In *Nutritional Limits to Animal Production from Pastures*. Edited by J.B.Hacker. Commonwealth Agricultural Bureaux, Farnham Royal.

Holmes, D.S. and Quigley, M. (1981). Analytical Biochemistry **114**: 193.

Horigome, T., Kumar, R. and Okamoto, K. (1988). British Journal of Nutrition **60**: 275.

Horsch, R.B., Fry, J.E., Hoffmanl, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985). Science **227**: 1229.

Hungate, R.E. (1966). In *The Rumen and its Microbes*. Academic Press, London and New York.

Hunt, A.G., Chu, N.M, Odell, J.T., Nagy, F. and Chua, N.H. (1987). Plant Molecular Biology **8**: 23.

Ikemura, T. (1981). Journal of Molecular Biology **146**: 1.

Ikemura, T. (1981). Journal of Molecular Biology **146**: 389.

John, A. and Lancashire, J.A. (1981). Proceedings of the New Zealand Grasslands Association **42**: 152.

Johnson, C.M. and Nashida, H. (1952). Analytical Chemistry **24**: 736.

Jones, W.T., Broadhurst, R.B. and Lyttleton, J.W. (1976). Phytochemistry **15**: 1407.

Jones, W.T. and Mangan, J.L. (1977). Journal of Food, Science and Agriculture **28**: 126.

Judson, G.J., Abdelsamie, R. and Bird, P.R. (1975). Australian Journal of Agricultural Research **26**: 743.

Kandylis, K. (1984). Livestock Production Science **11**: 611.

Kawashima, N. and Wildman, S.G. (1970). Annual Reviews in Plant Physiology 325.

Kay, R., Chan, A., Daly, M. and McPherson, J. (1987). Science **236**: 1299.

Keith, B. and Chua, N.H. (1986). EMBO **5**: 2419.

Kennedy, P.M. and Milligan, L.P. (1978). British Journal of Nutrition 39: 65.

Kennedy, P.M. and Siebert, B.D. (1972). Australian Journal of Agricultural Research **24**: 143.

Kennedy, P.M., Williams, E.R. and Siebert, B.D. (1975). Australian Journal of Biological Science **28**: 31.

Khandjian, E.W. (1986). Molecular Biology Reports **115**: 107.

Khandjian, E.W. (1987). Biotechnology **5**: 165.

Kortt, A.A., Caldwell, J.B., Lilley, G.G., Higgins, T.J. and Spencer, D. (1990). In press Journal of Biochemistry.

Krieg, P.A. and Melton, D.A. (1987). Methods in Enzymology **155**: 397.

Lai, E.C., Woo, S.L.C., Bordelon-Rieser, M.E., Fraser, T.H. and O'Malley, B.W. (1980). Proceedings of the National Academy of Science; USA **77**: 244.

Langlands, J.P. (1977). Australian Journal of Agricultural Research **28**: 133.

Lawton, M.A., Tierney, M.A., Nakamura, I., Anderson, E. and Komeda, Y. (1987). Plant Molecular Biology **9**: 315.

Lecomte, J.T.J., Jones, B.L. and Llinas, M. (1982). Biochemistry **21**: 4843.

Lee, J. (1983). In *Technical Report No. 3* Palmerston North, New Zealand: Applied Biochemistry Division, D.S.I.R.

Leng, R.A. (1982). British Journal of Nutrition **48**: 399.

Leng, R.A. (1984). 161. In *Ruminant Physiology: Concepts and Consequences.* Edited by S.K.Baker, J.M.Gawthorne and J.B.Mackintosh and D.B.Purser. University of New England Publishing Unit, Australia.

Leng, R.A. and Nolan, J.V. (1984). Journal of Dairy Science **67**: 1072.

Lewis, D. (1954). Biochemistry Journal **56**: 391.

Lilley, G.G., Caldwell, J.B., and Kortt, A.A. (1989). 497. In *Vegetable Protein Utilisation in Human Foods and Animal Feedstuffs*. Proceedings of World Conference of the American Oil Chemists Society. Edited by T.H.Applewhite. Kraft Incorporated, New York.

Loomis, W.D. and Battoile, J. (1966). Phytochemistry **5**: 423.

Lowther, W.L., Manley, T.R. and Barry, T.N. (1987). New Zealand Journal of Agricultural Research **30**: 23.

McCabe, S. and Barry, T.N. (1988). Journal of Agricultural Science; Cambridge **111**: 1.

McCarthy, J.E.G., Schairer, H.U. and Sebald, W. (1985). EMBO **4**: 519.

McCarthy, J.E.G., Sebald, W., Gross, G. and Lammers, R. (1986). Gene **41**: 201.

McDonald, I.W. (1948). Biochemistry Journal **42**: 584.

McDougall, E.I. (1948). Biochemistry Journal **43**: 99.

McLeod, N.M. (1974). Nutrition Abstract Reviews **44**: 803.

MacRae, J.C. and Ulyatt, M.J. (1974). Journal of Agricultural Science; Cambridge **82**: 309.

Mangan, J.L. (1972). British Journal of Nutrition **27**: 261.

Mangan, J.L. (1988). Nutrition Research Reviews **1**: 209.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). In *Molecular Cloning: A Laboratory Manual*. Cold Spring Laboratory. Cold Spring Harbour, New York.

Marshall, R.C. and Gillespie, J.M. (1977). Australian Journal of Biological Science **30**: 389.

Mathers, J.C. and Aitchison, E.M. (1981). Journal of Agricultural Science; Cambridge **96**: 691.

Mathers, J.C. and Miller, E.L. (1980). British Journal of Nutrition **43**: 503.

Matsumura, M., Signor, G. and Mathews, B.W. (1989). Nature **342**: 291.

Maurizi, M.A., Trisler, P. and Gottesman, S. (1985). Journal of Bacteriology **164**: 1124.

Mehrez, A.Z. and Orskov, E.R. (1977). Journal of Agricultural Science; Cambridge **88**: 645.

Meister, A. (1965). 757. In *Biochemistry of Amino Acids Volume II*. Academic Press, London.

Menendaz-Arias, L., Moneo, I., Dominguez, J. and Rodriguez, R. (1988). European Journal of Biochemistry **177**: 159.

Moir, R.J. (1970). 165. In *Sulphur in Nutrition*. Edited by O.H.Muth and J.E.Oldfield. AVI Publishing Co., Westport.

Moon, K.E. and Thompson, O.P. (1969). Australian Journal of Biological Science **22**: 463.

Munro, S. and Pelham, H.R.B. (1987). Cell **48**: 899.

Nolan, J.V. (1975). 416. In *Digestion and Metabolism in the Ruminant*. Edited by I.W.McDonald and A.C.I.Warner. University of New England Publishing Unit, Australia.

Nolan, J.V. and Leng, R.A. (1972). British Journal of Nutrition **27**: 177.

Nolan, J.V., Norton, B.W. and Leng, R.A. (1976). British Journal of Nutrition **35**: 127.

Nugent, J.H.A., Jones, W.T., Jordan, D.J. and Mangan, J.L. (1983). British Journal of Nutrition 357.

Nugent, J.H.A. and Mangan, J.L. (1981). British Journal of Nutrition **46**: 39.

Orskov, E.R. and McDonald, I. (1979). Journal of Agricultural Science; Cambridge **92**: 499.

Ow, D.W., Wood, K.V., DeLuca, M., DeWet, J.R., Helinski, D.R. and Howell, S.H. (1986). Science **234**: 856.

Ozaki, Y., Wada, K., Hase, T., Matsubara, H., Nakanishi, T. and Yoshizumi, H. (1980). Journal of Biochemistry **87**: 549.

Pisulewski, P.M. and Buttery, P.J. (1985). British Journal of Nutrition **54**: 121-129.

Purchas, R.W. and Keogh, R.G. (1984). Proceedings of the New Zealand Society of Animal Production **44**: 219.

Radcliffe, B.C. and Egan, A.R. (1978). Australian Journal of Biological Science **31**: 105.

Radcliffe, B.C., Hynd, P.I., Benevenga, N.J. and Egan, A.R. (1985). Australian Journal of Agricultural Research **36**: 709.

Reid, C.S.W., Ulyatt, M.J. and Wilson, J.M. (1974). Proceedings of the New Zealand Society of Animal Production **34**: 82.

Reimann-Philipp, U., Behnke, S., Batschauer, A., Schafer, E. and Apel, K. (1989). European Journal of Biochemistry **182**: 283.

Reimann-Philipp, U., Schrader, G., Martinoia, E., Barkholt, V. and Apel, K. (1989). Journal of Biological Chemistry **264**: 8978.

Reis, P.J. (1965a) 365. In *Biology of the Skin and Hair Growth.* Edited by A.G.Lyne and B.F.Short. Angus and Robertson, Sydney.

Reis, P.J. (1965b). Australian Journal of Biological Science **18**: 671.

Reis, P.J. (1967). Australian Journal of Biological Science **20**: 809.

Reis, P.J. (1979). 223 In *Physiological and Environmental Limitations to Wool Growth.* Edited by J.L.Black and P.J.Reis. University of New England Publishing Unit, Australia.

Reis, P.J. and Schinckel, P.G. (1963). Australian Journal of Biological Science **16**: 218.

Reis, P.J. and Schinckel, P.G. (1964). Australian Journal of Biological Science **17**: 532.

Reis, P.J. and Tunks, D.A. (1976). Journal of Agricultural Research **86**: 475.

Reis, P.J., Tunks, D.A. and Downes, A.M. (1973a). Australian Journal of Biological Science **26**: 249.

Ribereau-Gayon, P. (1972). 192. In *Plant Phenolics.* Oliver and Boyd, Edinburgh.

Robards, G.E. (1972). Australian Journal of Agricultural Research **22**: 261.

Robert, L.S., Van Montagu, M. and Leemans, J. (1989). EMBO **6**: 2513.

Rodriguez-Palenzuela, P., Pintor-Toro, J., Carbonero, P. and Garcia-Olmedo, F. (1988). Gene **70**: 271.

Rogers, G.L., Bryant, A.M. and McLeay, L.M. (1979). New Zealand Journal of Agricultural Research **22**: 533.

Samuelsson, G., Seger, L. and Olson, T. (1968). *Acta Chemica Scandinarica* **22**: 2624.

Sanders, P.R., Winter, J.A., Barnason, A.R., Rogers, S.G. and Fraley, R.T. (1987). Nucleic Acids Research **4**: 1543.

Satter, L.P. and Roffher, R.E. (1976). 119. In *Tracer Studies on Non-Protein-Nitrogen for Ruminants III.* International Atomic Energy Agency.

Schauder, B., Blocker, H., Frank, R. and McCarthy, J.E.G. (1987). Gene **52**: 279.

Schinckel, P.G. and Short, B.F. (1961). Australian Journal of Agricultural Research **12**: 176.

Schulz, A., Wengenmayer, F. and Goodman, H.M. (1990). Critical Reviews in Plant Sciences **9**: 1.

Sengupta-Gopalan, C., Reichert, N.A., Barker, R.F., Hall, T.C. and Kemp, J.D. (1985) Proceedings of the National Academy of Science; USA **82**: 3320.

Sharief, F. and Li, S.S. (1982). Journal of Biological Chemistry **257**: 14753.

Southern, E. (1975). Journal of Molecular Biology **98**: 503.

Spencer, D., Higgins, T.J.V., Freer, M., Dove, H. and Coombe, J.B. (1988). British Journal of Nutrition **60**: 261.

Stern, M.D. and Satter, L.D. (1984). Journal of Animal Science **58**: 714.

Stewart, B.A. and Porter, L.K. (1969). Agronomy Journal **61**: 267.

Stobbs, T.H., Minson, D.J. and McLeod, M.N. (1979). Journal of Agricultural Science; Cambridge **89**: 137.

Swain, A. (1979). 657. In *Herbivores; Their Interaction with Secondary Plant Metabolites* Edited by G.A.Rosenthal and D.H.Janzen. Academic Press, London and New York.

Swain, T. and Hillis, W.E. (1959). Journal of Science, Food and Agriculture **10**: 63.

Tamminga, S. (1981). 122. Ph.D. Thesis, Wageningen.

Tan, T.N., Weston, R.H. and Hogan, J.P. (1971). International Journal of Applied Radiation and Isotopes **22**: 301.

Tanner, G.J., Moore, A.E. and Larkin, P.J. (1990). In press British Journal of Nutrition.

Thomson, B.C., Dellow, D.W. and Barry, T.N. (1989). Australian Journal of Agricultural Research **40**: 1065.

Thomson, D.J., Beever, D.E., Harrison, D.G., Hill, I.W. and Osbourn, D.F. (1971). Proceedings of the Nutrition Society **30**: 14A.

Till, A.R., May, P.F. and Cumming, M.J. (1973). 9. In *First Australasian Conference on Heat and Mass Transfer*. Monash University, Melbourne.

Tilley, J.M.A. and Terry, R.A. (1963). Journal of the British Grasslands Society **18**: 104.

Tumer, N.E., O,Connell, K.M., Nelson, R.S., Sanders, P.R. and Beachy, R.N. (1987). EMBO **6**: 1181.

Ulyatt, M.J. (1973). 131. In *Chemistry and Biochemistry of Herbage*. Edited by G.W.Butler and R.W.Bailey. Academic Press, London.

Ulyatt, M.J. (1981). New Zealand Journal of Agricultural Science **15**: 200.

Ulyatt, M.J. and Egan, A.R. (1979). Journal of Agricultural Science; Cambridge **92**: 605.

Ulyatt, M.J., Lancashire, J.A. and Jones, W.T. (1976). Proceedings of the New Zealand Grasslands Association **38**: 107.

Ulyatt, M.J. and MacRae, J.C. (1974). Journal of Agricultural Science; Cambridge **82**: 295.

Ulyatt, M.J., MacRae, J.C., Clarke, R.T.J. and Pearce, P.D. (1975). Journal of Agricultural Science; Cambridge **84**: 453.

Vaeck, M., Reynaerts, A., Hofle, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. and Leemans, J. (1987). Nature **328**: 33.

Vandekerckhove, J., Van Damme, J., Van Lijsebettens, M., Botterman, J., De Block, M., Vandewiele, M., De Clercq, A., Leemans, J., Van Montagu, M. and Krebbers, E. (1989). Biotechnology **7**: 929.

Van Soest, P.J. (1982). In *Nutitional Ecology of the Ruminant*. O. & B. Books, Corvallis, Oregon.

Varvikko, T. (1986). British Journal of Nutrition **56**: 131.

Vernon, L.P., Evett, G.E., Zeikus, R.D. and Gray, W.R. (1985). Archieves of Biochemistry and Biophysics **238**: 18.

Verwoerd T.C.V., Dekker, B.M.M. and Hoekema, A. (1989). Nucleic Acids Research **17**: 2362.

Wada, K. and Buchanan, B.B. (1981). FEBS **124**: 237.

Waghorn, G.C., John, A., Jones, W.T. and Shelton, I.D. (1987). Proceedings of the New Zealand Society of Animal Production **47**: 25.

Waghorn, G.C. and Jones, W.T. (1989). New Zealand Journal of Agricultural Research **32**: 227.

Waghorn, G.C., Ulyatt, M.J., John, A. and Fisher, M.T. (1987). British Journal of Nutrition **57**: 115.

Waghorn, G.C., Shelton, I.D. and Sinclair, B.R. (1990). New Zealand Journal of Agricultural Research **33**: 257.

Waghorn, G.C., Jones, W.T., Shelton, I.D. and McNabb, W.C. (1989). Proceedings of the New Zealand Grasslands Association **51**: 171.

Walker, D.J. and Nader, C.J. (1968). Applied Microbiology **16**: 1124.

Walker, D.J. and Nader, C.J. (1975). Australian Journal of Agricultural Research **26**: 689.

Walker, D.M. and Norton, B.W. (1971). British Journal of Nutrition **26**: 15.

Wallace, R.J. (1985). British Journal of Nutrition **53**: 399.

Weising, K., Schell, J. and Kahl, G. (1988). Annual Reveiws in Genetics **22**: 421.

Weston, R.H. and Hogan, J.P. (1971). Australian Journal of Agricultural Research **22**: 139.

Williams, A.J. (1973). Australian Journal of Biological Science **26**: 465.

Williams, A.J., Robards, G.E. and Saville, D.G. (1972). Australian Journal of Biological Science **25**: 1269.

Williams, C.H. and Twine, J.R. (1967). 119. In *C.S.I.R.O. Technical Paper No. 24.* Melbourne C.S.I.R.O..

Yang, M.S., Espinoza, N.O., Nagpala, P.G., Dodds, J.H., White, F.F., Thompson, R.D. and Flaveell, R.B. (1989). The Plant Cell **1**: 569.

9. APPENDICES

Appendix 1: LBMG

Bactotryptone	10g/l
Bacto yeast extract	5g/l
NaCl	10g/l

Adjust to pH 7.5 and autoclave. Add MG salts in a ratio 1:1. For agar add 40g/l bactoagar prior to autoclaving.

1.1 MG Salts

Mannitol	10g/l
KH_2PO_4	0.5g/l
Glutamic acid	2.3g/l
NaCL	0.2g/l
$MgSO_4.7H_2O$	0.2g/l

Adjust to pH 7.0 and autoclave.

Appendix 2: MSO

Ms-Fe	5mls/l
20x Macronutrients	50mls/l
MS-EDTA	5mls/l
1000x Micronutrients	1ml/l
Sucrose	20g/l
Myo-inositol	100mg/l
100x Vitamin solution	10mls/l

Adjust pH to 5.8-6.0. Autoclave. For agar add 8g/l bactoagar prior to autoclaving.

2.1 100x Vitamin Solution

Nicotinic acid	5mg/100mls
Pyridoxine-HCL	5mg/100mls
Thiamine-HCL	1mg/100mls
Glycine	20mg/100mls

Filter sterilize (22μm filter size).

2.2 20x Macronutrients

NH_4NO_3	33g/l
$CaCl_2.2H_2O$	8.8g/l
KNO_3	38g/l
$MgSO_4.7H_20$	7.4g/l
KH_2PO_4	3.4g/l

2.3 1000x Micronutrients

H_3BO_3	6.22g/l
$MnSO_4.4H_2O$	22.3g/l
$ZnSO_4.7H_2O$	8.6g/l
KI	0.83g/l
$NaMoO.2H_2O$	250mg/l
$CuSO_4.5H_20$	25mg/l
$CoCl_2.6H_20$	25mg/l

Appendix 3: MS9

To a litre of MSO add:

Benzylaminopurine (0.1mg/ml)	10mls
Indoleacetic acid (1mg/ml)	0.5mls

Add after autoclaving MSO.

Appendix 4: RNA Extraction Buffer

LiCl	0.1M
Tris-HCL, pH8	100mM
Na-EDTA	10mM
SDS	1%

Autoclave. Mix 1:1 with TE equilibrated phenol and heat to 80°C before use.

4.1 TE Buffer

Tris-HCL	10mM
Na-EDTA	10mM

Adjust pH to 8.0.

Appendix 5: 20x SSC

NaCl	3M
sodium citrate	0.3M

Adjust pH to 7.0.

Appendix 6: Khandjian's Hybridizing Solution

Tris-HCL, pH 7.5	50mM
NaCl	1M
Formamide (deionized)	50%
100x Denhardt's solution	20mls/200mls
Dextran sulphate	10%
SDS	1%
Sodium pyrophosphate	0.1%
Herring Sperm DNA	100mg/ml

6.1 100x Denhardt's Solution

Ficoll	2g/100mls
Polyvinylpyrrolidone	2g/100mls
BSA	2g/100mls

Filter sterilize (22µm filter size).

Appendix 7: 20mM Phosphate Buffer, pH 7.0

0.2M $NaH_2PO_4.2H_2O$	39.0mls
0.2M $Na_2HPO_4.12H_2O$	61.0mls

Dilute to 200mls.

Appendix 8: 0.1M Glycine-HCL, pH 2.6

0.2M Glycine	50mls
0.2M HCL	24.2mls

Dilute to 200mls.

Appendix 9: Protein Extraction Buffer

TES, pH 7.8	0.1M
NaCl	0.2M
Na-EDTA	1mM

To 5mls add 25ml PMSF and 100ml mercaptaethanol prior to use.

TES - N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid

PMSF - phenyl methyl sulfonyl fluoride

Appendix 10: Protein Digestion Buffer

Tris-HCL, pH 6.7	0.125M
SDS	2%
Glycerol	10%
Bromophenol blue	0.003%

To 1ml add 50ml mercaptaethanol prior to use.

Appendix 11: TBS

NaCl	29.3g/l
1M Tris-HCL, pH 7.4	20mls/l

Appendix 12: TTBS

TBS + 0.05% Tween 20

Appendix 13: BCIP/NBT Colour Developing System (BioRad)

BCIP = 5-Brom-4-chlor-3-indolyl-phosphate
25mg/ml in 100% dimethylformamide

NBT = p-Nitro Blue Tetrazolium Chloride
50mg/ml in 70% dimethylformamide

Add 0.6% of each to alkaline phosphatase buffer prior to use.

13.1 Alkaline Phosphatase Buffer

$NaHCO_3$ Buffer, pH 9.6	0.1M
$MgCl_2$	1.0mM

13.2 0.1M Carbonate Buffer pH 9.6

0.2M Na_2CO_3	16mls
0.2M $NaHCO_3$	34mls

Dilute to 200mls.

Appendix 14: TB

Bactotryptone	12g
Bacto-yeast extract	24g
Glycerol	4mls

Dilute to 900mls and autoclave. Add 100mls Potassium phosphate buffer.

14.1 Potassium Phosphate Buffer

KH_2PO_4	0.17M
K_2HPO_4	0.72M

Autoclave.

Appendix 15: Lysis Buffer

Tris-HCL, pH 8	50mM
Na-EDTA	1mM
NaCl	100mM

Autoclave.

Appendix 16: Coomassie Blue

Coomassie Brilliant Blue R 42660	0.5%
Ethanol	40%
Acetic Acid	25%
dH_2O	35%

Appendix 17: Destain

Acetic Acid	700mls
Ethanol	2500mls
dH_2O	6800mls