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

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Science at Massey University, Palmerston North, New Zealand.

WARREN CHARLES McNABB

1990

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.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS.

I am indebted to the New Zealand Wool Board for funding both, my research at Massey University and my transfer to the C.S.I.R.O., Division of Plant Industry, Canberra for twelve months. I wish to thank the William Georgetti Scholarship Trust, Sir Leonard Condell Trust, U.G.C. Scholarship Committee, C. Alma Baker Trust and Massey University for financial support throughout the course of my study.

I am indebted to Massey University, Palmerston North, D.S.I.R., Division of Biotechnology, Palmerston North (now Grasslands Division) and the C.S.I.R.O., Division of Plant Industry, Canberra for the considerable research facilities made available to me.

I am forever indebted to my supervisors and good friends, Professor T.N. Barry (Department of Animal Science, Massey University, Palmerston North), Dr. G.C. Waghorn (D.S.I.R., Grasslands Division, Palmerston North) and Dr. T.J. Higgins (C.S.I.R.O., Division of Plant Industry, Canberra) for there advice, guidance and encouragement during the whole course of my study.

I wish to acknowledge the considerable help and support provided by;

* Miss R. Watson (Massey University, Animal Science Department), Miss K. Newell and Mrs L. Hogie (C.S.I.R.O., Division of Plant Industry) for their friendship and skilled technical assistance.

* Mr D. Shelton (D.S.I.R., Grasslands Division) for his friendship, skilled technical assistance and expertise with the sheep work.

* Dr. R. Patchell and Mr H. Voon (Massey University, Poultry Research Centre-now Monogastric Nutrition Centre) for their help with the HPLC.

* Dr. R. Tillman (Massey University, Soil Science Department) for his help with scintillation counting.

* The Fertiliser and Lime Research Centre (Massey University), Dr. M. Hedley (Massey University, Soil Science Department) and Mr K. Sakadevan (Fertiliser and Lime Research Centre) for their help with the sulphate analysis.

* Dr. D. Spencer (C.S.I.R.O., Division of Plant Industry) for his support upon my arrival in Canberra and assistance with the sunflower albumin 8 project.

* Dr. R. Khan (C.S.I.R.O., Division of Industry) for his analysis of tobacco seeds.

* Dr. D. Garrick (Massey University, Animal Science Department) and Dr. P. Waterhouse (C.S.I.R.O., Division of Plant Industry) for their help with computer facilities.

* The Graphics Department, C.S.I.R.O., Division of Plant Industry for their presentation of photographs.

* Dr. J. Lee and Mr P. Chin (D.S.I.R., Grasslands Division) for the analysis of sulphur by ICP-ES.

* Dr. W. Jones (D.S.I.R., Division of Fruit and Trees) for his help with condensed tannin analysis and SDS-PAGE.

* Dr. G.G. Lilley (C.S.I.R.O., Division of Biomolecular Engineering, Melbourne) for the SF8 cDNA.

* Dr. D. Hewish (C.S.I.R.O., Division of Biomolecular Engineering) for the anti-SF8 monoclonal antibodies.

I wish to acknowledge the help of several colleagues at the C.S.I.R.O., Division of Plant Industry; Roger, Diana, Alison, Christina and Audrey.

I would finally like to acknowledge the great debt I owe my mother, father and brothers for their love and support over the years of my study.

TABLE OF CONTENTS

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.