

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

STUDIES ON THE FOAMING PROPERTIES OF PROTEINS

The role of soluble leaf proteins and other  
surfactants in the persistence  
of bloat foams.

A thesis presented in partial fulfilment of the  
requirements for the degree of Doctor of Philosophy  
in Biochemistry at Massey University.

William Thomas Jones

- 1971 -

## CONTENTS

### ACKNOWLEDGEMENTS

### ABSTRACT

Page

### SECTION I

CHAPTER 1	<u>INTRODUCTION</u>	1
1.1	<u>The soluble proteins present in the leaves of higher plants.</u>	2
1.2	<u>Bloat History</u>	
	(a) Toxic Factors	4
	(b) Retention of Gas in the Rumen	6
	1. The excessive gas production theory.	6
	2. The excessive consumption of dense feed.	6
	3. The foam theory.	6
1.3	<u>The Complex Nature of the Bloat Syndrome</u>	8
	(a) Materials implicated as the foaming agent responsible for the bloat foam.	11
	1. Saponins	11
	2. Pectin and Pectin methyl esterase	11
	3. The role of saliva	13
	4. The role of calcium and magnesium	14
	5. The role of bacteria and protozoa	16
	6. The role of plant proteins	17
	(b) The antifoaming agents and foam inhibitors associated with the bloat syndrome.	20
	1. The plant lipids	20
	2. Plant tannins and polyphenolics	21
1.4	<u>Foams</u>	
	(a) Factors determining foam stability	
	1. The rate of drainage	24
	2. Diffusion across the liquid lamellae	25

3.	The thickness of the electrical double layer	25
4.	Surface viscosity and critical yield stress	25
5.	The restoring properties of surface tension	26
(b)	Protein foams	27

CHAPTER 2	<u>THE AIMS OF THE PRESENT RESEARCH INVESTIGATION.</u>	29
-----------	--	----

SECTION 2

EXPERIMENTAL METHODS AND RESULTS

CHAPTER 1	<u>EXPERIMENTAL METHODS</u>	32
1.1	<u>Analytical Methods</u>	32
(a)	Protein analyses	32
1.	Determination of soluble protein nitrogen	32
2.	Determination of Fraction 1 content of protein mixtures	33
(i)	Acrylamide gel electrophoresis	33
(ii)	Analytical ultracentrifugation	35
3.	Determination of Fraction 2 proteins	37
4.	Amino acid analyses	37
(b)	<u>Carbohydrate Analyses</u>	
1.	Total hexose	37
2.	Pectin	38
3.	Sialic acid in salivary secretions	38
4.	Free sialic acid	38
5.	Starch	39
6.	Hydrolysis of carbohydrate and chromatography of the sugars	39

(c)	<u>Determination of calcium</u>	40
	1. Total soluble calcium	40
	2. Calcium bound to macromolecules	40
(d)	<u>Determination of lipids</u>	41
	1. Total lipids	41
	2. Thin layer chromatography of lipids	41
1.2	<u>Preparative Methods</u>	42
(a)	<u>The isolation and fractionation of the soluble leaf proteins</u>	42
	1. Preliminary investigations to obtain leaf proteins free of polyphenol oxidation products	43
	(i) Alcohol extraction	43
	(ii) Absorption of phenolics on Polyclar AT	44
	(iii) Inhibition of the phenoloxidase enzyme	45
	2. Fractionation of the soluble leaf proteins	46
	(i) Salt precipitation	47
	(ii) Preparative ultracentrifugation	49
	(iii) Molecular sieve chromatography	50
	3. The isolation and fractionation of the soluble leaf proteins of white and red clovers	51
(b)	<u>Preparation of the salivary secretions</u>	54
	1. Salivary mucoprotein	54
	2. Oesophageal mucin	56
(c)	<u>Protozoal proteins</u>	57
(d)	<u>The isolation and fractionation of the polar lipids of red clover</u>	58
	1. Extraction of total lipids	58
	2. Separation of the acidic lipids	59
	3. Separation of the lipids	60

1.2	(e) <u>Isolation of the protein precipitating agents (tannins) from Lotus pedunculatus Cav.</u>	61
1.3	<u>Methods of studying foams</u>	64
	(a) <u>The measurement of protein foam parameters</u>	64
	1. The modified Mangan apparatus	64
	2. The Laby apparatus	67
	3. Reproducibility of foam measurements	70
CHAPTER 2	<u>THE SOLUBLE PROTEINS OF BLOAT AND NON-BLOAT PROVOKING LEGUME SPECIES, AND THE FOAMING PROPERTIES OF THE EXTRACTS DERIVED FROM THEM</u>	73
2.1	Detection of protein precipitants in legumes	74
2.2	The soluble leaf proteins of the legume forages used in New Zealand	75
2.3	A survey of the legume forages which do and do not cause bloat	76
2.4	Examination of the <u>Trifolium</u> species for tannins	81
2.5	The occurrence of tannins in the <u>Lotus</u> species	84
	<u>Summary</u>	84
CHAPTER 3	<u>THE FOAMING PROPERTIES OF THE SOLUBLE LEAF PROTEINS OF RED AND WHITE CLOVER, THE HOLOTRICH PROTOZOAL PROTEINS AND THE SALIVARY SECRETIONS</u>	87
3.1	The foaming properties of the soluble leaf proteins	87
	(a) <u>Studies with the Mangan apparatus</u>	87
	1. The effect of protein concentration on foam strength	87
	2. The effect of bubble size on foam strength	88
	3. The influence of temperature on foam strength	90
	4. The effect of pH on foam strength	92

3.1 (b)	<u>Studies using the Laby apparatus</u>	94
1.	The effect of protein concentration on the foam parameters	94
2.	The relation between bubble size and compressive strength	95
3.	Examination of compressive strength of foams at an early stage in their formation.	98
4.	The effect of temperature on the compressive strength and stress relaxation of protein foams	100
5.	The effect of pH on the foam properties of plant leaf proteins	100
6.	Relationship between the compressive strength of Fraction 1 protein foams and the amount of surface denatured protein in a fixed volume of foam	103
7.	Fraction 2 protein surface denatured on foaming their solutions over the pH range 3.5 to 7.5.	106
8.	Solubility of Fraction 1 and Fraction 2 proteins at various pH's	106
(c)	<u>The action of plant phenol/phenoloxidase on plant proteins and its effect on the foams generated from them.</u>	111
1.	Analyses on acrylamide gel electrophoresis of plant proteins which have and have not reacted with the phenol/phenoloxidase system	112
2.	The influence of the phenoloxidase interaction with plant proteins on the foam parameters of foams derived from them	115
3.2	<u>The foaming properties of protozoal proteins and salivary secretions</u>	116
(a)	The effect of concentration on the foam properties of the salivary secretions and protozoal proteins	117
(b)	The effect of pH on the foaming properties of the salivary secretions and protozoal proteins.	120
	<u>Summary</u>	120

CHAPTER 4	<u>STUDIES ON RUMEN FOAMS AND THE SURFACTANTS PRESENT IN RUMEN LIQUOR OF BLOATING RUMINANTS</u>	124
4.1	The foaming properties of rumen liquor	125
4.2	The effect of pH on the foam parameters of rumen foams	126
4.3	The effect of temperature on the foam parameters of rumen foams	129
4.4	Attempts to change the characteristics of rumen foams by addition of Fraction 1 protein to the rumen liquor	131
4.5	The surface active materials in rumen liquor	132
	(a) The concentration of Fraction 1 protein in rumen liquor	132
	(b) Calcium levels in rumen liquor	134
	(c) Foam fractionation experiments	134
	(d) Examination of the soluble foaming agents in rumen liquor	137
	<u>Summary</u>	142
CHAPTER 5	<u>THE INTERACTION BETWEEN THE SOLUBLE LEAF PROTEINS AND OTHER SURFACTANTS INVOLVED IN THE BLOAT SYNDROME</u>	145
5.1	The influence of calcium on the compressive strength of protein foams	146
5.2	The effect of polygalacturonic acid on plant protein foams	150
5.3	The effect of the salivary secretions on Fraction 1 protein foams	154
5.4	The effect of tannins	162
5.5	The antifoaming properties of the polar lipids of red clover	164
	<u>Summary</u>	171
	<u>SECTION 3</u>	
	<u>DISCUSSION</u>	174
	The properties of leaf protein foams	177
	The properties of protozoal protein and the	

salivary foams	179
The relation of the protein foams with bloat	180
Interactions between Fraction 4 protein and other surfactants	183
(a) Lipids	184
(b) Pectin	185
(c) The salivary secretions	185
The surfactants in the rumen of bloating animals	189
The role of tannins in the bloat syndrome	191
Concluding Remarks	194

References

Appendix

LIST OF TABLES AND FIGURES

Page

FIG. 1	The Bloat Syndrome	10
FIG. 1 (2.1)	Elution of the soluble leaf proteins of red clover on Sephadex G-75 dextran gel.	48
FIG. 1 (2.2)	The fractionation of soluble leaf proteins of white clover.	55
FIG. 1 (2.5)	Fractionation of the polar lipids of red clover ( <u>Trifolium pratense L.</u> )	62
FIG. 1 (3.1a)	The Mangan and Laby apparatus	65
FIG. 1 (3.1b)	The Falling weight assembly used in the Mangan apparatus.	65a
FIG. 2 (2)	The soluble proteins extractable from the leaves of the four main legume pastures in New Zealand.	77
TABLE 2 (3.1)	The properties of bloating and non-bloating legume forages.	79
TABLE 2 (4.1)	The presence of condensed tannins in selected Trifolium species.	82
FIG. 2 (4.1)	Acrylamide electrophoretic analyses of the soluble proteins extracted from the leaves of some of the Trifolium species.	83
TABLE 2 (5.1)	The tannin content of Lotus species.	85
FIG. 3(1.1)	The effect of protein concentration on the foam strength of the soluble plant protein foams.	89
FIG. 3 (1.2)	The effect of bubble size on foam strength.	90
FIG. 3 (1.3)	The influence of temperature on the foam strength of protein foams.	91
FIG. 3 (1.4)	The effect of pH on the foam strength of soluble leaf proteins of white clover.	93
FIG. 3 (1.5)	The effect of protein concentration on the foam parameters of soluble leaf protein foams.	96
FIG. 3 (1.6)	The effect of bubble size on the compressive strength of Fraction 1 protein foams.	97

LIST OF TABLES AND FIGURES, CONTD..

Page

FIG. 3 (1.7)	The compressive strength of foams at an early stage in their formation.	99
FIG. 3 (1.8)	The effect of temperature on the compressive strength of protein foams.	101
TABLE 3 (1.1)	The effect of temperature on stress relaxation of leaf protein foams.	102
FIG. 3 (1.9)	The effect of pH on the foam properties of the soluble leaf proteins.	104
FIG. 3 (1.10)	The relationship between the amount of surface denatured Fraction 1 protein in a fixed volume of foam at different pH's.	105
FIG. 3 (1.11)	Microdensitometer tracings of proteins separated by acrylamide gel electrophoresis showing Fraction 2 proteins denatured on foaming at various pH's.	107
FIG. 3 (1.12)	The effect of pH on the concentration of leaf proteins in solution.	109
FIG. 3 (1.13)	The action of phenol/phenoloxidase system on the soluble leaf proteins.	114
FIG. 3 (1. a)	The effect of concentration on the compressive strength of holotrich protozoal protein and salivary mucoprotein foams.	118
FIG. 3 (2.1.b.)	The effect of protein concentration on the stress relaxation of holotrich protozoal protein and salivary mucoprotein foams.	119
FIG. 3 (2.2.a.)	The effect of pH on the compressive strength of holotrich protozoal protein and salivary mucoprotein foams.	121
FIG. 3 (2.2.b.)	The effect of pH on the stress relaxation of holotrich protozoal protein and salivary mucoprotein foams.	122
FIG. 4 (2. a.)	The effect of pH on the compressive strength of foams derived from rumen liquor.	127
FIG. 4 (2. b.)	The effect of pH on the stress relaxation of <u>in vitro</u> rumen foams.	128
FIG. 4 (3)	The effect of temperature on the compressive strength rumen foams.	130

Dr R.T.J. Clarke for helpful discussions concerning various aspects of the bloat work.

Mr M. Greenwood for growing all the plants examined in this work.

Mr P. Pearce for amino acid analyses;

Dr N. Grace for calcium analyses;

Mr B. Henderson for technical assistance during part of the work;

Miss Cynthia Owen, Librarian at D.S.I.R., Palmerston North.

Miss Margaret Soulsby for taking the photographs.

Mr P. Mintoft of the Electrical Workshop for making the electric circuitry required for the Laby apparatus.

I am grateful to have had the opportunity to discuss my ideas with Dr J. M. McArthur (Canadian Department of Agriculture, Summerland, B.C.) during his visit to Palmerston North and to have had the opportunity to meet Dr R. H. Laby (C.S.I.R.O., Melbourne Australia) and to gain experience in using his foaming apparatus prior to my starting this research.

Finally I should like to express my thanks to my wife for her patience and assistance during the past three years.

FIG. 4 (5. c.)	Thin layer chromatography of the neutral lipids that concentrate in the <u>in vitro</u> foams derived from rumen liquor.	136
FIG. 4 (5. d. 1.)	An ultracentrifugation of the proteins in rumen liquor.	139
FIG. 4 (5. d. 2.)	Analytical ultracentrifugation of the major protein in rumen liquor of bloating animals.	141
FIG. 5 (1. a.)	The effect of calcium on the compressive strength of Fraction 1 and Fraction 2 leaf protein.	147
FIG. 5 (1. b.)	The effect of pH on the interaction between calcium and Fraction 1 protein as shown by its effect on compressive strength of the foams.	149
FIG. 5 (2. a.)	The effect of polygalacturonic acid on the compressive strength of fraction 1 protein foams.	151
FIG. 5 (2. b.)	The effect of polygalacturonic acid on the stress relaxation of Fraction 1 protein foams.	152
FIG. 5 (2. c.)	The effect of polygalacturonic acid on the foam retention volume of Fraction 1 protein foams.	153
FIG. 5 (3. a.)	The effect of salivary secretions on the compressive strength of Fraction 1 protein foams.	156
FIG. 5 (3. b.)	The effect of bovine salivary secretions on the stress relaxation of Fraction 1 protein foams.	157
FIG. 5 (3. c.)	The effect of bovine salivary secretions on the foam retention volume of Fraction 1 protein foams.	158
TABLE 5 (5. 1.)	The effect of salivary mucoprotein on the amount of Fraction 1 protein denatured on foaming Fraction 1 protein/salivary mucoprotein mixture.	160
FIG. 5 (4)	The effect of tannins on the compressive strength of protein foams.	163
FIG. 5 (5. a.)	The fractionation of the polar lipids of red clover ( <u>Trifolium pratense L.</u> ).	166

FIG. 5 (5. b.)	The effect of polar lipids on the compressive strength of Fraction 1 protein foams.	167
FIG. 5 (5. c.)	The effect of lipids on the stress relaxation of Fraction 1 protein foams.	168
FIG. 5 (5. d.)	The effect of lipids on the foam retention volume of Fraction 1 protein foams.	169

## ACKNOWLEDGEMENTS

I wish to express my appreciation to the Department of Scientific and Industrial Research for allowing me to carry out this work at the Applied Biochemistry Division, Palmerston North, and for preparing the final copies of this thesis.

I should like to thank Dr G. W. Butler, Director of the Applied Biochemistry Division, for creating the opportunity for me to carry out this work, and for his encouragement during the course of the project.

I should like to thank my supervisors, Dr J. W. Lyttleton (Applied Biochemistry Division, D.S.I.R., Palmerston North) for his supervision of the research work, for many helpful discussions and for his supervision during the writing of the thesis; Professor G. Malcolm (Professor of Physical Chemistry at the Biochemistry Department, Massey University, Palmerston North) for his supervision and helpful suggestions during the writing of this thesis.

I am grateful to Dr R. W. Bailey, my Section Leader at Applied Biochemistry Division, for his continual interest shown in the work, his willingness to assist in any way he could, for frequent discussions, and for helpful comments concerning the work and writing up of the thesis.

I should also like to thank the following members of the D.S.I.R. Staff at Palmerston North:

Dr C.S.W. Reid for making available the animals for the bloat studies and for supplying samples of the salivary secretions.

Dr R.T.J. Clarke for helpful discussions concerning various aspects of the bloat work.

Mr M. Greenwood for growing all the plants examined in this work.

Mr P. Pearce for amino acid analyses;

Dr N. Grace for calcium analyses;

Mr B. Henderson for technical assistance during part of the work;

Miss Cynthia Owen, Librarian at D.S.I.R., Palmerston North.

Miss Margaret Soulsby for taking the photographs.

Mr P. Mintoft of the Electrical Workshop for making the electric circuitry required for the Laby apparatus.

I am grateful to have had the opportunity to discuss my ideas with Dr J. M. McArthur (Canadian Department of Agriculture, Summerland, B.C.) during his visit to Palmerston North and to have had the opportunity to meet Dr R. H. Laby (C.S.I.R.O., Melbourne Australia) and to gain experience in using his foaming apparatus prior to my starting this research.

Finally I should like to express my thanks to my wife for her patience and assistance during the past three years.

ABSTRACT

Methods were developed for the isolation of the soluble leaf proteins in as pure a form as possible and free of any phenoloxidase products. This protein material was separated into two fractions (Fraction 1 and Fraction 2 proteins). A detailed study of the foaming properties of these soluble protein fractions was made so that the conditions necessary for the production of stable foams from these solutions could be evaluated.

The nature of the foams derived from bovine salivary secretions and the soluble proteins of the holotrich protozoa were also examined. The foams derived from the leaf and protozoal proteins were rigid and of high stability only when the foams were of high compressive strength. In contrast the salivary secretions produced foams of low compressive strength but high persistence. For protozoal proteins and Fraction 1 protein of white clover and red clover the optimum pH for foam production was close to pH 5.8 to 5.9 and for the plant Fraction 2 proteins in the range 5.1 to 5.4. The foams derived from bovine salivary mucoprotein was unaffected by changes in pH over the range 3.5 to 7.5.

The foams generated in vitro from rumen liquor were of low compressive strength but extremely high persistence, and their properties were very different from those of the foams generated from either the plant or protozoal proteins except that they showed maximum foam persistence in a similar pH range.

The concentration of Fraction 1 protein in the rumen liquor was below the minimum concentration required to produce stable Fraction 1 protein foams. Of this low concentration only 24%

was surface denatured in production of these very stable rumen foams. The significance of this result is discussed. Apart from the low level of Fraction 1 protein, other low molecular weight proteins together with a major component containing carbohydrate as well as protein, were observed on analysing the rumen liquor by acrylamide gel electrophoresis and cellulose acetate electrophoresis. This major component resembled salivary mucoprotein in its schlieren profile in an analytical ultracentrifuge. This material was isolated by preparative ultracentrifugation and some of its properties examined. It was not precipitated by trichloroacetic acid, unlike the protozoal and plant proteins, but was precipitated by 60% ammonium sulphate, 80% ethanol, and an equal volume of 1% cetavlon. The antibody to this material gave a positive precipitin reaction with the salivary mucoprotein, the sensitivity of which could be increased by incubating the salivary mucoprotein with neuraminidase, an enzyme which removes the sialic acid from the mucoprotein molecule. The significance of these findings in relation to other work is discussed.

The action of various surfactants that have been implicated in the bloat syndrome on the foaming properties of Fraction 1 protein foams was examined. Thus calcium was found to increase the rigidity of Fraction 1 protein foams, slightly increase the rigidity of Fraction 2 protein foams at high calcium concentrations only, but was without effect on salivary mucoprotein foams.

Sodium polygalacturonate increased the persistence of Fraction 1 protein foams at concentration greater than 0.04% w/v.

Two salivary secretions were examined for their effect on Fraction 1 protein foams. The first of these was bovine salivary

mucoprotein, which whilst increasing the persistence of the foam, decreased its rigidity. Foams of maximum persistence were produced from solutions containing Fraction 1 protein/mucoprotein in the ratio 2/1, w/w. The second salivary secretion examined was the oesophageal mucin. This material did not produce stable foams by itself, but was an extremely effective stabilizing agent of Fraction 1 protein foams.

The most effective antifoaming agent of the polar lipids of red clover examined in this thesis, was phosphatidyl choline which at a concentration of  $50 \mu\text{g ml}^{-1}$  completely inhibited the production of Fraction 1 protein foams.

Addition of mucoprotein to lipid/Fraction 1 protein mixtures which would not support stable foams, resulted in production of extremely persistent foams. Both Fraction 1 protein and mucoprotein were essential for the formation of these foams which resembled the properties of the foams generated in vitro from rumen liquor.

From this study it appeared that neither the plant nor the protozoal proteins by themselves could account for the properties of the rumen foams. The properties of the rumen foams could be reproduced by generating foams from mixtures of Fraction 1 protein/plant lipid and salivary mucoprotein.

The soluble proteins and the foaming properties of extracts of bloat and non-bloat provoking legume pastures were examined. It was found that the temperate non-bloating legumes contained condensed tannins which precipitated the soluble leaf proteins and thus by removing the plant foaming agents from solution inhibited foam production from these extracts. These

tannins were isolated from Lotus pedunculatus Cav., and were shown to form insoluble complexes, not only with the soluble leaf proteins, but with protozoal proteins, salivary mucoprotein, and the protein present in rumen liquor.

Tannins were common in the Lotus species, but of the Trifolium species examined, they were found only in Trifolium arvense L. The significance of incorporation of tannins into bloating pastures in an attempt to eliminate bloat is discussed.

The non-bloating tropical legumes, apart from the Desmodium species, did not contain tannins but were lower in soluble leaf protein, and the bloat potential could be correlated with the compressive strength of the foams derived from extracts of these plants.