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

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

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Dr N. Grace for calcium analyses;

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

Miss Margaret Soulsby for taking the photographs.

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

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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 <u>in vitro</u> 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 trichloracetic 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 phosphatydyl choline which at a concentration of 50 $\mu g\ ml^{-1}$ 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.

SECTION 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

This thesis is mainly concerned with a study of the properties of foams that can be generated from solutions of plant leaf proteins, and the possible relationship of these foams to the disorder in cattle known as bloat. Of the leaf proteins, only the fraction which passes into solution on rupture of the leaf cells is likely to be of immediate importance in the production of foam. The literature review which follows is concerned primarily with the physico-chemical studies on plant leaf proteins, the history and present day views as to the cause of bloat, the materials that have been suggested as effecting the occurrence of bloat, and finally the theory of surface chemistry of films and foams, and the factors involved in the production of persistent foams.

LITERATURE REVIEW

1.1 The soluble proteins present in the leaves of higher plants were classified by Singer et al. (1952), primarily on the basis of molecular size, into two fractions. Analytical ultracentrifugation showed that the two fractions were common to most higher plants. The higher molecular weight fraction was called Fraction 1 protein and the lower molecular weight fraction was termed Fraction 2 protein.

Fraction 1 protein accounts for up to 50% of the total soluble leaf protein, and ultracentrifugation and gel electrophoresis has shown it to be a homogenous protein (Wrigly et al. 1966). It exists as the major protein in the chloroplasts of green leaves (Lyttleton and Ts'o, 1958). It has been associated with the enzyme ribulose diphosphate carboxylase, the enzyme responsible for carbon dioxide fixation in the photosynthetic metabolic pathway in higher plants (Mayaudon, 1957; Trown, 1965). Fraction 1 protein has been isolated from a number of plants by preparative ultracentrifugation (Eggman, et al. 1953), and more recently by gel filtration chromatography on dextran gels (Sephadex G 200) from rice leaves (Mendiola and Akazawa, 1964) and from spinach (Pon, 1967; Trown, 1965). Its molecular weight is between 500,000 - 600,000 (Trown, 1965; Pon, 1967). The above mentioned workers have also examined its amino acid composition.

It has recently been shown to be made up of two different subunits (Rutner and Lane, 1967; Moon and Thompson, 1968) of molecular weights approximately 54,000 and 16,000. Kawashima (1969) has shown the larger subunit to have the same amino acid composition when prepared from spinach or tobacco leaves. The lower molecular weight subunit differed in composition for the two plant preparations.

It can be seen therefore that Fraction 1 protein has been examined fairly extensively. An excellent review of the physico-chemistry of Fraction 1 protein has recently been published (Kawashima and Wildman, 1970).

Fraction 2 protein is not in fact a single protein but a mixture of all the other proteins and enzymes present in the leaves of higher plants. They range in molecular weight from 10,000 - 300,000 and have not been studied as extensively as Fraction 1 protein.

This thesis is mainly concerned with a study of the properties of foams which can be produced from solutions of plant leaf proteins, and the modification of these properties that occurs when various other materials are added to the foaming system. Although the study was made in order to produce basic information about these foams, it was carried out in full awareness of the fact that the results might be helpful in understanding the nature of the foam which, when generated in the rumen of cattle grazing rich pasture, gives rise to the disorder known as bloat.

Because the importance of this disorder was largely responsible for the choice of the subject of this thesis, and because the planning of many of the experiments was influenced by their possible relationship to the bloat syndrome, the next section of the literature review will deal with some of the theories that have been put forward to account for this apparently simple but in fact, very complex disorder.

1.2 Bloat history

Bloat, hove or tympanites has been known to occur for over 1000 years. Ancient Roman writings of 60 A.D. describe the condition and its treatment (Tribe, 1947). Bloat in ruminants is characterized by distension of the forestomachs following interference with the normal elimination of gas produced by the microbial digestion in these organs. Over the last 200 years, bloat has been associated with ruminants feeding on lush growing pastures containing a high percentage of legumes such as clovers or lucerne.

Many hypotheses have been put forward to explain the ailment, and these theories have fallen into two groups.

(a) Toxic factors

These theories state that the forage being digested contains toxic principles which are released into the rumen and are capable of paralysing the muscles responsible for eructation of the gaseous products of rumen fermentation. The materials that have been suggested as

being responsible include cyanide, hydrogen sulphide, and saponins.

Cyanide

It has been known for many years that certain legumes, including those which may cause bloat, contain cyanogenic glucosides which are readily hydrolysed to give hydrogen cyanide. This led Evans (1949) to suggest that this was the toxic principle involved in bloat. However Quin and Clarke (1945) administered enough potassium cyanide to inhibit rumen motility and showed no impairment in the animals' ability to eructate carbon dioxide introduced through a rumen fistula.

Dougherty and Cello (1953) showed that Birdsfoot trefoil (Lotus corniculatus L.), a legume not associated with bloat, contained higher levels of cyanogenic glucosides than the legumes which cause bloat. Furthermore Johns (1954) obtained frequent bloat with cattle grazing red clover (Trifolium pratense L.) a legume which does not contain cyanogenic glucosides. This evidence tended to discredit the idea of cyanide being the cause of bloat.

Saponins are known to have the ability to inhibit muscular motility and cause death. Jackson (1957) and Lindahl (1954) isolated saponins from alfalfa (Medicago sativa L.) and proposed that these materials were responsible for bloat.

The main arguments against the toxic factor theory are:

1. Bloat has been shown to occur although the muscles responsible for eructation were functioning (Johns, 1954).

2. Inhibition of eructation does not result in true bloat (Parsons, 1952).

(b) Retention of gas in the rumen

These theories state that the gases produced in the rumen are retained there.

1. The excessive gas production theory

Veetch (1937) postulated that bloat occured when animals were fed succulent legumes which resulted in gas production in excess of that which the animal could eructate. However Cole, et al. (1942) and Quin (1943) showed that the cattle could eructate more gas than was produced in the rumen even by the richest legume feeds.

2. The excessive consumption of dense feed

This theory states that bloat is due to an obstruction of the cardialorifice by an ingesta of dense feed which prevents elimination of gas from the rumen. This theory was however easily discredited since bloat has been produced by drenching animals with legume extracts and therefore in the absence of solid ingesta (Fergusson and Terry, 1955; Lindhahl, 1954; Newbold, 1954).

3. The foam theory

As early as 1937 McLandish claimed that bloat was caused not by excessive gas production but by retention of the gas in a froth which the animals could not eructate.

Olsen (1942) believed that bloat arose as a result of the rumen fermentation gases being trapped in a saponin foam. Quin (1943) stated that bloat was nearly always associated with the presence of foam in the rumen, and that the foam could impede the escape of gas from the rumen.

Although these early workers had postulated that foam was the cause of bloat, the theory did not find wide acceptance until the early 1950's. Johns (1954) reported that neither rumen movement nor the eructation mechanism were inhibited during the onset of bloat, and that the only factor which was consistently correlated with bloat occurrence was the presence of foam in the rumen. He critically examined the theories which had been proposed, and the conditions under which bloat occured with cattle feeding red clover in the New Zealand environment. All his evidence pointed to the trapping of rumen gases in a stable foam being the cause of bloat.

Johns concluded as follows:

1. Antifoaming agents were the only drench materials that consistently relieved the bloating condition.
2. Eructation always was observed to take place when the animals were becoming bloated.
3. Bloat was not relieved by a stomach tube. The tube became blocked with solids which were intimately mixed with a frothy mass.
4. When the rumen was punctured in acutely bloated animals, a certain amount of free gas was released and then the puncturing needle became blocked with

a frothy mass.

5. The rumen contents of a cow that had died of bloat was full of a solid foam i.e. the foam was held by grass and clover stalks that gave it a supporting structure. It did not ooze out but behaved as a solid mass. Johns believed that bloat was due to retention of gas within the rumen digesta by foaming of the rumen contents supported by the mass of plant material.

Reid and Johns (1957) and Reid (1960) published their foam hypothesis. Most research workers in the bloat field have accepted the theory which holds foam to be the cause for bloat occurrence (Bartley, 1967; McArthur, 1964; Stifel, 1968a, 1968b; Conrad, 1961)

1.3 The complex nature of the bloat syndrome

With the wide acceptance of the foam theory of bloat came a surge of research into the identification of surfactant materials that could have been involved in the bloat syndrome. Any plant or animal material which would either produce a stable viscous foam, or increase the viscosity of the rumen contents thereby increasing the persistence of the foam, has been suggested as a possible surfactant responsible for the bloat foam. Similarly any material known to inhibit foam production or to decrease the persistence of foams has been implicated as an antifoaming agent in the bloat syndrome. Fig. 1 shows the materials and factors that have thus been implicated in bloat.

FIGURE 1

The Bloat Syndrome

The circle represents the bloating condition when a stable foam is present in the rumen. Arrows pointing inwards to the circle represent materials and factors which can stabilize rumen foams. Arrows pointing away from the circle indicate antifoaming agents, and arrows pointing in both directions indicate reversible factors involved in bloat.

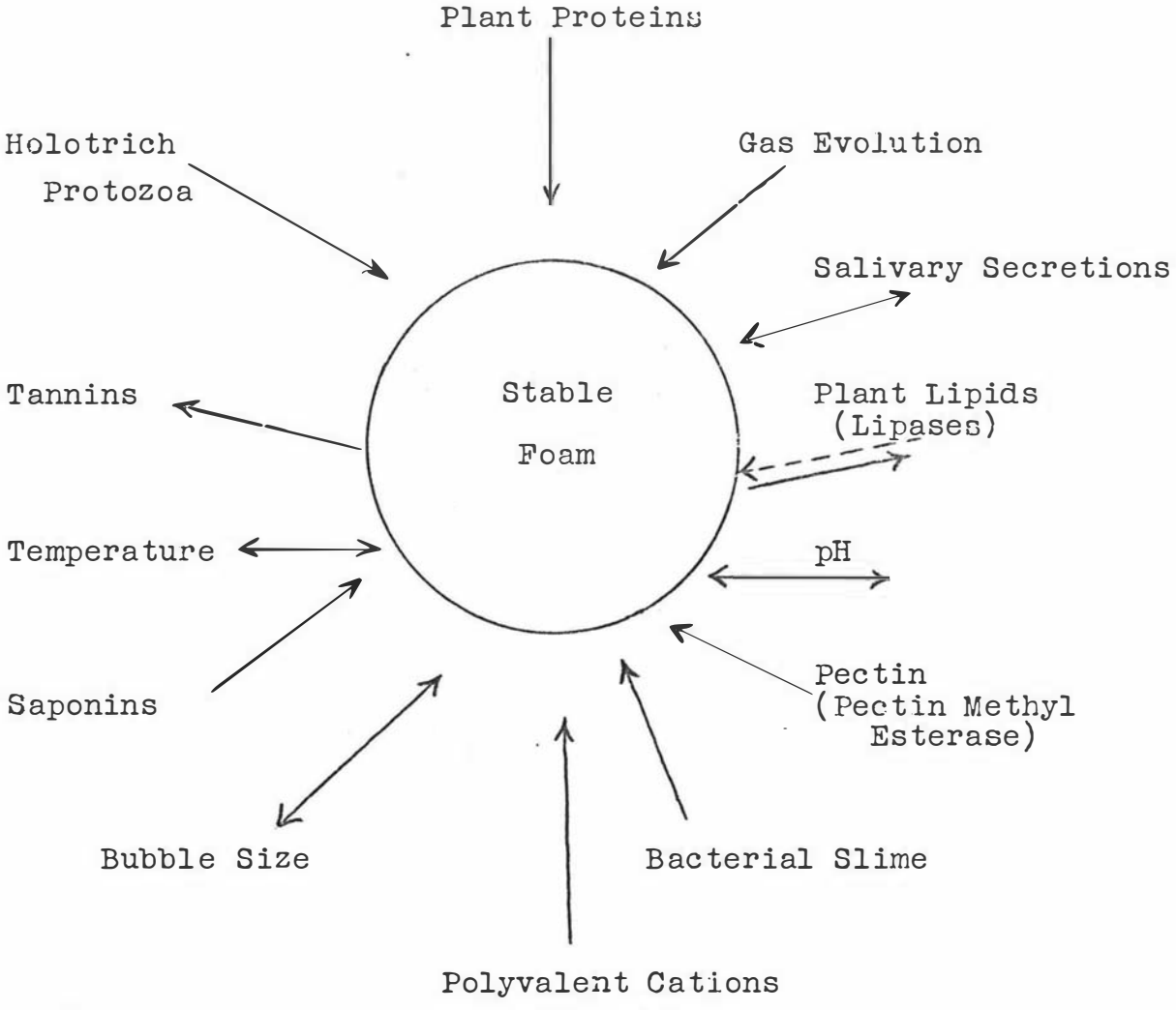


FIG. 1 THE BLOAT SYNDROME

1.3 (a) Materials Implicated as the foaming agent responsible for the bloat foam.

1. Saponins

McLandish (1937) suggested that the alfalfa saponins, which were capable of producing stable viscous foams, could retain the gaseous products of rumen fermentation in these foams giving rise to bloat. Coulson (1960) suggested that saponins could be responsible for either foam production or inhibition of eructation by muscle paralysis. Lindhahl et al. 1954, however, were unable to produce frothy bloat by drenching animals with alfalfa saponins. Mangan (1959) showed that clover saponins had a pH maximum for foam strength at pH 4.5. The pH maximum for foams generated from rumen liquor occured at a higher pH of 5.5 - 6.0.

A modification of the role of saponins was suggested by Gutierrez et al. (1958). They showed that when saponins were incubated with rumen bacteria and protozoa a heavy froth was produced from degradation of the saponins and production of a viscous bacterial slime.

2. Pectin and pectin methyl esterase.

Conrad et al. (1958) claimed that the plant pectins were responsible for high gas production in the rumen and were also capable of causing stable foams similar to those found in pasture bloat. In 1961 Conrad et al. claimed that the highest incidence of bloat corresponded to the highest concentrations of pectic substances in ladino clover and alfalfa. Pressey et al. (1963), however, found

no correlation between pectin levels and bloat frequency.

Head (1959) claimed to have isolated the foaming agent from rumen liquor and shown it to be carbohydrate. Hydrolysis of this material yielded the sugars associated with pectin and hemicellulose. The New Zealand workers believed pectin itself was not the foaming agent, but by increasing the viscosity of the rumen liquor it could act as a stabilizing agent (Wright, 1961). Wright also suggested that pectins could act as foam inhibitors by decreasing the availability of calcium and sodium, which are required to stabilize protein and saponin foams.

Nichols et al. (1966), in several papers, implicated the activity of pectin methyl esterase (PME) as the important factor responsible for bloat. This enzyme hydrolyses the methyl pectins to give pectic acid and methanol. The pectic acid in the presence of calcium or similar cations can increase the viscosity of rumen fluid by producing calcium pectate gels, which trap rumen gases in a stable viscous foam. These authors demonstrated that the PME activity of saline extracts of alfalfa was significantly higher on bloating days than on non-bloating days, e.g. Nichols and Deese, (1966).

Other workers at Wisconsin University indicated that PME activity was greater in young alfalfa than in mature alfalfa.

3. The role of saliva in the bloat syndrome.

Saliva has been suggested as being a factor involved in bloat though its role has been controversial. Johns (1958) considered that the saliva secretions could either assist in preventing bloat by buffering a fall in pH of the rumen or increase bloat severity by adding carbon dioxide to the system, thus assisting in foam formation. He believed that the mucoprotein present in the saliva helped to form a stable viscous foam. Phillipson and Reid (1958) found that pressure in the rumen increased both the flow and mucoprotein concentration in the submaxillary saliva. Their results suggested that this additional flow in response to pressure could cause increased bloat severity.

In contrast, Weiss (1953) concluded in his results that when succulent legume forage was eaten, frothy bloat resulted from insufficient salivation. Roughage in the form of hay or mature legume led to an increase in salivary flow, resulting in a watery, less viscous ingesta which would not support a stable foam. Mendall and Boda (1960) observed that non-bloaters secreted more saliva than bloat susceptible animals. Bartley (1958) stated that identical twins bloated less with watery ingesta than with thick viscid ingesta. Van Horn and Bartley (1961) indicated that addition of saliva to frothy ingesta increased gas release thus destabilizing the foam, an observation which supported the theory of Weiss. They postulated that since bloat results from the presence of foam - promoters, in an absence of an antifoaming agent, the salivary mucin may be this

antifoaming agent. Bartley and Yavada (1961) confirmed the antifoaming properties of salivary mucin and plant mucilages by showing that these compounds could both inhibit and destabilize foams produced from alfalfa saponins.

Mishra et al. (1967) showed that the ruminant saliva contained aerobic bacteria capable of degrading the salivary mucin. Also they isolated from the rumen of these animals aerobic bacteria which utilized saliva as a sole source of nutrients, and showed that these bacteria were present at a higher concentration in the rumen of bloat susceptible animals than in the less susceptible animals. They concluded that bloat may result when the mucin content of the rumen is lowered either by reduced salivation or by the destructive action of mucinolytic bacteria. In a later paper, Mishra, et al. (1969) isolated anaerobic bacteria capable of breaking down mucin and suggested that some of these bacteria were implicated in bloat as slime producers.

4. The role of calcium and magnesium in the bloat syndrome.

The relationship between calcium, magnesium and other cations has been shown by early workers to influence the severity of bloat. Smith (1962) found that calcium and magnesium, when applied to the forage or given as a drench prior to the animals grazing alfalfa, increased the bloat severity. Furthermore drenching the animals with calcium or magnesium chelating agents such as ethylene diamine

tetra-acetic acid, which decrease the availability of the cations, reduced bloat severity. Warner (1962) showed that when alfalfa was sprayed with calcium carbonate, bloat severity increased. Reid et al. (1961) showed that addition of slaked lime to the animals' drinking water also increased the severity of bloat. Jackson et al. (1962) and Smith (1963) did not find any correlation between calcium content of alfalfa plants and bloat severity. Stifel (1968b) observed a positive correlation between the magnesium content of alfalfa chloroplasts and bloat occurence, but a negative correlation between calcium level and bloat. Miltimore et al. (1970) found no correlation between bloat and magnesium content of alfalfa, but significant correlation between bloat and nickel, zinc, and calcium levels.

Mangan et al. (1959) showed that sodium and calcium were essential for the development of strong protein and saponin foams. Precipitation of calcium from solutions of alfalfa saponins reduced the foam production to zero. Harris and Sebba (1965) showed that nickel and zinc became concentrated in the foams derived from alfalfa extracts. Phelps (1962) observed that the amount of ammonium magnesium phosphate isolated from animals with frothy ingesta was three times as great as that produced from non-frothy ingesta.

Stifel (1968a) showed that the calcium and magnesium binding to the chloroplastic proteins of alfalfa, was highly correlated to the bloat potential of the forage. The difference in binding was explained as due to a

difference in amino acid composition of the protein.

5. The role of bacteria and protozoa in the bloat syndrome.

Fermentation gases are produced by rumen bacteria and protozoa, as a result of breakdown of the fermentable plant substrates. Antibiotics such as penicillin have been used as bloat preventatives. Their action has been explained by Mangan (1959) as occuring by destruction of the bacteria which hydrolyse the plant lipids. Bartley (1967) attributed antibiotic action to the destruction of mucinolytic bacteria which degrade the antifoaming salivary mucin. Gutierrez (1958) and Hungate (1955) proposed that bacteria attacking ingested legumes high in carbohydrates produce bacterial slimes which increase the viscosity of the rumen liquor, thus stabilizing the rumen foam.

Holotrich protozoa have been implicated in the bloat syndrome (Clarke, 1965a). He showed that the ciliate rumen protozoa population had a marked diurnal variation which was mainly due to changes in the holotrich population. Holotrich protozoa have a metabolic peculiarity whereby they are unable, in the presence of excess substrate, to stop synthesizing reserve polysaccharide which they do until they burst. The fall in holotrich population is therefore attributable to loss through bursting. This drop is apparent at the time at which bloat develops. Clarke suggested that the protozoal contents, containing proteins and nucleic acids, could have considerable foam stabilizing properties when released into the rumen from

the lysed cells.

Clarke (1965b) showed a relationship between bloat severity and dry weight of protozoa in the rumen liquor sample taken prior to feeding. He showed that the increase in weight of protozoa was not due to an increase in protozoal numbers but to an increase in the weight of the protozoal content, i.e. bloat was more severe when protozoa were very full of polysaccharide and in a state in which they were likely to burst releasing their contents into the rumen.

Clarke (1966) used an anti-protozoal agent, 1,2-dimethyl-5-nitro-imidazole, which prevented bloat in cattle feeding on red clover. This agent completely destroyed the holotrich protozoal population and caused a reduction in fermentation.

Clarke (1969) showed this drug could reduce and ultimately eliminate protozoal holotrich population and in general reduce substantially the incidence and severity of bloat in both stall-fed non-lactating and grazing lactating cows. On a few occasions mild bloat was observed although the rumen was free of holotrich protozoa.

6. <u>The role of plant proteins in bloat</u>.

Bartley and Bassette (1961) analysed rumen foam and found that the major component of the foam was protein, the crude protein in the foam amounting to 63%. The main sources of soluble protein in the rumen are: (1) salivary mucoprotein, (2) protozoal protein, and (3) soluble plant protein. (1) and (2) have already been discussed earlier

in the introduction.

Johns (1954, 1956) postulated that plant protein or its degradation products could be responsible for the foam involved in bloat. This was questioned (Fergusson and Terry, 1955) on the basis of whether the protein would be released fast enough from the ingested forage to be a significant factor, and therefore the authors favoured the theory that the protein responsible for the foam was present in the rumen liquor prior to feeding. Reid et al. (1962) and Bryant (1964) measured the rate and extent of release of soluble plant proteins into the rumen by collecting, through a fistula, the boli of chewed forage as they fell from the cardia into the rumen. They found extensive liberation of soluble cytoplasmic protein had resulted during mastication of the forage by the cattle. The release of soluble protein was rapid enough to account for the early onset of bloat.

Mangan (1959) found that foams generated in vitro from bloating rumen liquor and solutions of the total soluble leaf proteins of red clover, had maximum foam strength in the pH range 5.4 - 6.0, whereas saponins and submaxillary mucoprotein produced foams of maximum foam strengths at lower or higher pH ranges respectively. He stated that the plant proteins must be important factors in the aetiology of bloat. This was substantiated by Boda (1958).

McArthur et al. (1964) isolated from alfalfa leaves a protein fraction with strong foaming properties in the pH

range where bloat occurs. Ultracentrifugation identified this fraction as Fraction 1 protein which has a sedimentation coefficient of 18 S. He stated that the other alfalfa proteins were not surface denatured.

Jones and Lyttleton (1969) showed that in the case of white and red clovers both Fraction 1 and the major Fraction 2 proteins stabilized viscous foams and were surface denatured on foaming, and therefore if plant proteins were the foaming agents responsible for bloat, both fractions would be involved. Bloat has been reported on Amaranthus (Johns, 1962). This species contains only 10% of the Fraction 1 protein content of the clovers. Amaranthus species have a different photosynthetic pathway and this accounts for the low level of Fraction 1 protein in this plant (Hatch and Johnson, 1968).

Stifel (1969) claimed a diurnal variation in the concentration of the chloroplastic protein which could be correlated with maximum bloat occurrence. His results were also claimed to show a significant correlation between the Fraction 1 content of alfalfa chloroplasts, its amino acid composition, and its ability to bind divalent cations with the bloat potential of the forage.

McArthur and Miltimore (1969) showed that bloat was not associated with plants containing less than 1.84% Fraction 1 protein dry weight. Jones and Lyttleton (1970a, 1970b) showed that the temperate legumes not associated with bloat contained tannins which precipitated the soluble proteins of these legumes so that the proteins were not

available for foaming.

1.3 (b) The antifoaming agents and foam inhibiting substances associated with the bloat syndrome.

1. The Plant Lipids

Johns (1954) stated that bloat arose as a result of the normal balance of antifoaming agents and foaming agents in the rumen becoming upset, favouring production of a viscous foam. The search for the antifoaming agents present in the rumen led to an examination of the plant lipids because of the association of fats and vegetable oils with the prevention and cure of bloat (Reid and Johns, 1957; Blake et al. 1957). Mangan (1959) observed that sedimentation of the particulate material in rumen liquor increased the strength of the foams that could be generated in vitro from the rumen liquor. The sediment contained bacteria, protozoa and chloroplastic fragments with their associated lipids. Addition of the chloroplastic fragments to the protein solutions decreased the foam strength of the foams that could be generated. However, if the chloroplastic fragments were defatted by extracting them with lipid solvents, and the resultant fragments added to protein solutions and foamed, the foams so derived were of higher foam strength. This was taken as indicating the antifoaming properties of the plant lipids.

The polar lipids constitute about 60% of the total chloroplastic lipids. These are made up of the phospho-

lipids (phosphatydyl choline, phosphatydyl ethanolamine, phosphatydyl inisitol, phophatydyl glycerol) the sulpholipid, and mono- and di-galactosyl diglyceride (Weenink, 1961). Bailey (1964) did not find any correlation between the level of lipid bound sugars and the bloat potential of the forage. Bailey and Russell (1966) showed the sulpholipid of red clover to be an extremely surface active material which lowered the surface tension of water by 36 dynes cm^{-1} at 0.013% $^{W}/_{V}$ concentration. This is comparible to the surface tension reduction produced by some of the "pluronic"* type detergents which are very effective bloat preventatives.

Stifel (1968b) found that the total leaf and chloroplastic lipids increased with decrease in bloat potential of the forage, and his results indicated a preferential synthesis of chloroplastic phospholipids in the non-bloating forage. He suggested that apart from their anti-foaming properties the phospholipids and fatty acids could destabilize protein films by competing for the divalent cations which are necessary for production of stable protein foams.

In contrast, Miltimore et al. (1970) showed that the lipids of alfalfa increased with bloat potential of the alfalfa.

2. Plant tannins and polyphenolics.

Kendall (1966) observed that extracts of non-bloating legumes produced less foam than extracts of bloat prevoking legumes. He was able to produce large foam volumes from these non-bloating forages by adding

* see appendix

to the leaf extracts soluble polyvinylpyrrolidone (PVP) an agent known to complex tannins and phenolics (Anderson, 1968). He was also able to decrease the volume of foam produced from bloating legume extracts by addition of a commercial tannin preparation to the extracts. He postulated that the non-bloating legumes contained tannins which interacted with the protein-buffer system in such a way as to inhibit foam production. The nature of this interaction was not explained, nor were tannins isolated from the plants which he examined.

Cope et al. (1966) isolated from Lespedeza cuneata a leucoanthocyanin polymer which could inhibit plant enzymes and questioned whether this could inhibit the pectin methyl esterase and so prevent bloat when cattle are fed this pasture.

Hutton and Coote (1966) examined a series of non-bloating tropical legumes for tannins. Tannins were found only in the Desmodium species and therefore the absence of bloat when cattle foraged these pastures could not be explained as being due to the presence of tannins in these plants.

Jeffers et al. (1966) showed a negative correlation between the polyphenolic content of alfalfa plants and its bloat potency. However, Miltimore et al. (1970) indicated that there was a trend for the 'tannin' content of alfalfa plants to increase as the bloat frequency increased. Red

clover (Trifolium pratense L.) has a highly active phenoloxidase system and is high in polyphenolics. The phenoloxidase enzyme converts phenolics into quinones which can inhibit enzymes by interaction with the active centres on the protein molecule, yet this system does not prevent bloat on red clover (Jones and Lyttleton, 1969b).

From the above literature review the complexity of the bloat syndrome can be appreciated. Although the evidence for the soluble plant proteins being involved is substantial (Mangan, 1959; Reid et al. 1952; McArthur and Miltimore, 1964; McArthur et al. 1969; Miltimore et al. 1970; Stifel, 1968a, b; Jones and Lyttleton, 1969a, 1970a, 1970b) it is unlikely that bloat can be explained solely in terms of them, because all the surfactants will contribute, in some degree, to the structure and nature of the films which make up the rumen bloat foam.

The research in this thesis is partially aimed at determining the conditions under which persistent foams are produced from various materials, and as stable foams are believed to be responsible for bloat, a review of the factors thought to be involved in stable foam production is presented in the following section.

1.4 Foams

Foams are dispersions of gas in liquid phases, most of the phase being gas, with liquid in thin sheets called

lamellae between the gas bubbles. The formation of a foam is achieved by agitating the liquid and gas phases in the presence of a stabilizing agent. Because the processes involved in stable foam production are very complex, early studies on the nature of films which make up the foam were studied with single films either spread on to the surface of the liquid phase, or produced when molecules diffused from solutions of the surfactant to the air water or oil water interface (Cheeseman, 1952). The surface films could be classified roughly into two types:

(a) Films of high surface viscosity, such as those produced from solutions of proteins or saponins.

(b) Films of low surface tension, such as those produced from soaps, which have low surface viscosity.

(a) Factors determining foam stability.

Foams can never be thermodynamically stable, since once the lamellae are ruptured, they must break into drops with a lower surface free energy, and therefore a decreased total free energy for the system (Davies and Rideal 1961). Although foams from pure liquids and gases are highly unstable, suitable surface active agents can stabilize a foam almost indefinitely. The following section discusses some of the factors which govern the persistence of foams.

1. The rate of drainage

This is the rate at which liquid drains from a foam whose lamellae are not breaking. It is the settling out of

surplus water from the foam. As drainage occurs, the thickness of the liquid lamellae decrease. If the lamellae are thinned to about 50 Å, molecular forces can cause rupture. Therefore any factor which will delay the thinning of lamellae, should increase the persistence of the foam. Increasing the viscosity of the bulk solution or of the surface film will have this effect (section 5.3 p. 161).

2. Diffusion across the liquid lamellae.

In a foam there will be a tendency for the small bubbles to decrease in size and the large bubbles to increase in size. This transfer of gas is caused by the excess pressure inside the small bubbles over the large neighbouring bubbles. The mechanism of transfer is by dissolution in and diffusion through the liquid lamellae. By decreasing the permeability of the surface lining, by such means as increasing its viscosity, greater persistence of the foam will occur.

3. The thickness of the electrical double layer.

If a film lies between two charged monolayers, it may resist further thinning because further removal of liquid would bring the charged surfaces closer together, which would set up a high osmotic pressure in the lamellae by accumulating many counterions. The energy barrier set up to local thinning is often sufficient to stabilize the foam against appreciable shocks.

4. Surface viscosity and critical yield stress.

A monolayer offers viscous resistance to shear

stress in the plane of the surface, in a manner comparable with liquids which are retarded in flow by viscous forces. In addition to reducing drainage rate, surface viscosity can cushion the thin liquid films against shocks. The highest foam stability is associated with high surface viscosity (Brown, 1953). For protein stabilized foams, foam stabilities and surface viscosity pass through a maximum at the same pH, indicating that the mechanical properties of surface films are of considerable importance in determining foam stability. The critical yield stress, measured as the non-Newtonian component of surface viscosity, is the stress required to cause flow of the monolayer. The greater this value the more rigid will be the film and the greater will be the force required to break the film. Proteins and saponins give films of high critical yield stress (Davies and Rideal, 1961).

5. The restoring properties of surface tension.

If a shock suddenly extends a local area of the lamellae, a surface pressure gradient from the rest of the film to the thinned area will occur, and the monolayer tends to spread back into the extended region. The monolayer takes with it a layer of water and so opposes the thinning that occurred (Marangoni, 1871; Gibbs, 1878). High surface viscosity will delay the transport of water back to the thinned region and thus surface tension and surface viscosity oppose each other. A simple test to show which effect is operating, consists of dropping small solid projectiles through the foam. If a hole is left behind, only viscosity is important since viscosity can

only retard motion and not reverse its effect (Davies and Rideal, 1961). Excellent reviews on the topics of foam stability are given by De Vries (1958, 4 - 5), Bikerman (1953); Davies and Rideal (1961).

(b) Protein Foams

Many protein molecules, on diffusing to the air/liquid interface, become insoluble in the liquid. This process is termed surface denaturation, and occurs as a result of a change in the three dimensional structure of the protein, the molecule unfolding as the forces holding the molecule in a compact globular form give way to those causing spreading in the surface (Davies, 1953).

Cumper and Alexander (1950) postulated that the processes involved in the production of protein films occured in three stages:

(1) The diffusion of the molecules to the surface.

(2) The unfolding or denaturation of the molecules at the surface.

(3) Coagulation of the denatured protein and its subsequent removal from the surface film.

Insoluble protein foams may be regarded as containing films made up of a coagulum, below which is a completely unfolded protein film at the interface and underlying layers of protein in various degrees of unfolding.

These authors observed that such protein films show visco-elastic properties (Cumper and Alexander 1950, 1951). The protein films they studied had a maximum surface viscosity at pH values close to the iso-electric points

of the proteins. High ionic strength and high temperature decreased surface viscosity as a result of increasing the rate of coagulation of the denatured protein.

The plant proteins produce rigid, 'solid' foams (Mangan, 1959; Jones and Lyttleton, 1969a; Laby, 1970) and undoubtedly surface viscosity is an important factor responsible for their mechanical properties.

CHAPTER 2

The aim of the present research investigation

Although many substances have been suggested as the cause of bloat (Chapter 1, Section 1 of this thesis), very few studies have been made on the foams derived from them. The majority of the substances have been suggested because they produce viscous aqueous solutions. The major research on foam parameters of foams derived from surfactants likely to be involved in bloat was carried out by Mangan (1959), Laby (1970), and Jones and Lyttleton (1969). Before behaviour of a mixed system such as is found in the bloating foam can be understood, the nature of the foam characteristics of the main foaming agents need to be fully investigated.

The volume of evidence suggesting soluble leaf protein to be the main surfactant responsible for the bloat foam is so substantial (Section 1, Chapter 1), that a closer study of the conditions necessary for the production of stable foams from both fractions of the soluble leaf proteins was warranted. The objectives of this thesis were therefore to define the conditions for and the characteristics of stable plant protein foams, to determine the effect which other surfactants of plant and animal sources have on these foams, and if possible to correlate these findings with those derived from the study of foams produced from bloaty rumen

liquor. The specific objectives of this research were as follows:

(a) To establish the conditions necessary for the production of stable foams from the soluble leaf protein fractions.

(b) To examine the foam properties of the salivary secretions and the holotrich protozoal proteins.

(c) To examine the interaction of other surfactants, that have been implicated in the bloat syndrome, with Fraction 1 protein by studying the nature of the foams generated from the mixed systems.

(d) To examine the foam properties of rumen liquor, and to attempt to isolate from it the main surfactants responsible for foam production.

(e) To explain the reasons for the different bloat potential of different legume forage species.

SECTION 2

EXPERIMENTAL METHODS AND RESULTS

CHAPTER 1

EXPERIMENTAL METHODS

1.1 Analytical Methods

1.1 (a) Protein Analyses

1. Determination of soluble protein nitrogen

Equal volumes of protein solution and 10% trichloracetic acid (TCA) were mixed in glass centrifuge tubes, allowed to stand at $0^{o}C$ for 20 minutes and centrifuged at 16,000g for 20 minutes to collect the precipitated protein. Salivary proteins were precipitated by addition of absolute ethanol to a concentration of 80% ethanol.

The precipitated protein was washed twice by resuspension in 5% TCA or 80% ethanol for plant proteins and salivary proteins respectively, followed by centrifugation. After draining, the protein pellet was digested with 0.4 ml of concentrated sulphuric acid containing 100g of potassium sulphate and 1g of selenium per litre of acid. The protein was digested until the solution became clear, and then made up to 5 ml with distilled water. The concentration of

ammonia in this solution was determined using either the Conway (1947) method or the phenol hypochlorite method using a Technichon autoanalyser.

2. Determination of Fraction 1 content of protein mixtures.

(i) Acrylamide gel electrophoresis.

Soluble proteins were examined with acrylamide gel electrophoresis in the thin slab gel system of Reid and Bieleski (1968). The electrophoresis buffer was a 0.1M Tris/0.1M glycine solution of pH 8.5.

To prepare the gel, 15 ml of the electrophoresis buffer and 15 ml of a 15% acrylamide solution containing 0.3% bis-acrylamide*, were mixed and deaerated to remove dissolved oxygen. 0.3 ml of a 10% $^{W}/_{V}$ Temed* solution and 0.3 ml of 10% aqueous ammonium persulphate were added and the mixture poured into the gel mould and overlayered with water.

Protein samples were made 10% in sucrose and layered into the gel pockets with a precision microsyringe Electrophoresis was carried out as described by Reid and Bieleski (1968).

Proteins were located in the gels after electrophoresis with the following stains:

1. Coumassie blue*. The gel was stained for 15 minutes in a 0.25% solution $^{W}/_{V}$ of coumassie blue in water/methanol/acetic acid, 68/25/7 v/v/v. After staining, the excess dye was removed from the gel by

* see appendix

destaining in three 30 minute washes of 100 ml of the same solvent, and then overnight in 1 litre of the solvent.

2. Amido black*. Gels were stained in a 0.05% solution of Amido black in methanol/acetic acid/water 5/1/5 v/v/v for 30 minutes. Gels were destained by successive washings of the gel against the staining solvent until the background of the gel was colourless.

3. Procion blue*. Gels were stained with a 0.5%^{w}/v solution of Procion blue MRS in water/acetic acid/methanol 5/1/5 and destained against running water until the background colour had faded.

For qualitative work Coumassie blue was the only dye used because it was approximately five times as sensitive as the other dyes. The gels were either photographed or scanned on a microdensitometer* to give a record of the protein separation.

The relation between intensity of staining and the amount of Fraction 1 applied to the gel in 25mm^3 of solution, was determined for the three protein stains. Coumassie blue was approximately five times as sensitive a protein as Amido black, which was more sensitive than Procion blue.

Gels containing 1, 2, 4, 6, 8 and 10; 5, 10, 20, 30, 40, and 50; and 50, 100, 150, 175, 200 and 225 µg of Fraction 1 protein in 25mm^3 of solution were stained with

* see appendix

each of the three stains, and the intensity of staining was measured as the area under the curve on a Joyce Loebl microdensitometer*.

Coumassie blue stained Fraction 1 protein quantitatively in the range up to 6 µg, Amido black showed a linear relation between intensity of staining and amount of protein up to 50 µg and Procion blue up to 200 µg protein. All analyses were carried out in duplicate.

(ii) Analytical ultracentrifugation.

Macromolecules can be characterized by their sedimentation rate in the ultracentrifuge. Accordingly, solutions of protein molecules were examined in a Beckman Model E analytical ultracentrifuge using schlieren optics (Beckman Instruments manual).

The analytical ultracentrifuge was calibrated for determining Fraction 1 protein as follows.

Solutions of bovine serum albumin (Sigma Fraction V) containing 5 and 10 mg of protein per ml of solution, were placed in a synthetic boundary cell and centrifuged at 15,220 r.p.m. in a Beckman Model E centrifuge. After diffusion of the initially sharp boundary between the solution and the solvent, photographs were taken of the schlieren profile of the protein concentration boundary, at bar angles of 70° and 80°.

From these photographs the areas under the schlieren peaks were determined. The concentration of protein in the solution producing the boundary is related to the area under the schlieren profile by the equation:

$$C = \frac{k.A.\tan\theta}{E^2} \frac{(d_c)^2}{(d_x)}$$

* see appendix

where C = concentration of protein in solution

k = a constant defined by the optical system of the ultracentrifuge

A = measured area under the schlieren peak

θ = the bar angle of the phase plate

E = the linear magnification of the photograph

d_o = the radial position of the sedimentation boundary at time t = 0

d_x = the radial position of the sedimentation boundary at time t = x

In the case of the synthetic boundary cell at low speeds, when no sedimentation is occuring, d_o is equal to d_x. The equation reduces to

$$C = \frac{A.k.\tan\theta}{E^2}$$

k can be determined from the gradient of the graph of $\frac{A.\tan\theta}{E^2}$ against C. For the instrument used in this research, k was equal to 12.6 mg ml^{-1} cm^{-2}, when units of C are mg ml^{-1} and the units of A are cm^{-2}.

The analytical ultracentrifuge, having been calibrated, was used to estimate Fraction 1 protein in leaf protein preparations where its concentration was at least 5 mg ml^{-1}.

Samples were centrifuged at 52,840 r.p.m. until the Fraction 1 protein had sedimented clear of the Fraction 2 proteins. Photographs of the schlieren profile were taken at bar angles of 70^o and 80^o, and the areas were

measured. The areas were corrected for dilution of the protein due to sedimentation, and by substitution into the equation relating area to concentration of protein, the Fraction 1 protein concentration was determined. Analyses were carried out in duplicate.

3. Determination of Fraction 2 proteins

Fraction 2 proteins, because of their heterogeneity, could not be determined by either of the methods used for measuring the concentration of Fraction 1 protein. They were therefore determined using either the Biuret method (Colowick and Kaplan, 1955) or as protein nitrogen (1.1 (a)). Analyses were carried out in duplicate.

4. Amino acid analyses.

Protein samples containing between 5 and 10 mg of protein were hydrolysed in 6N hydrochloric acid in sealed tubes at 110°C for 24 hours. The hydrolysate was filtered, evaporated to dryness under nitrogen and taken up in 5 ml of a pH 2.2 citrate buffer. A sample (0.2 ml) of this solution was analysed for amino acid composition or a Beckman* 120C amino acid analyser. The procedure and standardising of the apparatus are described in the Beckman amino acid analyser handbook*.

1.1 (b) Carbohydrate analyses

1. Total hexose

This was determined by the anthrone method (Dreywood, 1946). The anthrone reagent was added to 1 ml of sample in glass tubes in a water bath at 1-2°C. Air condensers were attached to the tubes, which were heated

* see appendix

to 100°C for 7 minutes. After cooling and allowing to stand for 20 minutes, the colour absorption at 625 nm was measured. Glucose (analar grade) dissolved in glass distilled water was used as a standard. Analyses were carried out in duplicate.

2. Pectin

Pectin was measured by the carbazole method of Dische (1927). The sample (1 ml) was pipetted into a glass tube and 6 ml of concentrated sulphuric acid added to the sample at 1-2°C. Air condensers were fitted to the tubes which were heated to 100°C for 20 minutes. The tubes were rapidly cooled by immersing them in ice, the carbazole reagent (0.2 ml) added, and after allowing to stand in a dark room for 3 hours, the colour absorption at 530 nm was measured. Glucuronic acid or galacturonic acid dissolved in glass distilled water were used as standards. Analyses were carried out in duplicate.

3. Sialic acid in salivary secretions

This was determined by the resorcinal method (Svennerholm, 1956). Samples (2 ml) were heated with resorcinol reagent (2 ml) in glass tubes in a boiling water bath for 15 minutes. The tubes were cooled and 5 ml of iso-amyl alcohol was added to the tubes which were shaken vigorously and allowed to stand in ice for 10 minutes. After centrifugation, the alcohol layer was transferred to cuvettes and the absorbancy at 580 nm measured. Analyses were carried out in duplicate.

4. Free sialic acid

Free sialic acid liberated on incubation of salivary secretions with neuraminidase, was determined by the method

of Warren (1959). To 0.2 ml of sample was added 0.1 ml of sodium periodate solution and the mixture allowed to stand for 20 minutes. Arsenite reagent (0.2 ml) was added and the mixture shaken until the yellow colour had disappeared. Thiobarbiturate solution (3 ml) was added to the mixture, which was then heated in a boiling water bath for 20 minutes. The tubes and contents were cooled, 4.3 ml of cyclohexanone added and thoroughly shaken. After centrifugation, the organic layer was transferred to cuvettes and the absorbancy measured at 550 nm. Sialic acid, dissolved in distilled water, was used as a standard. Analyses were carried out in duplicate.

5. Starch

Starch content of rumen liquor and rumen foam fractions was determined by the specific 'Agidex' enzyme method of MacRae and Armstrong (1968). This enzyme hydrolysed starch to glucose, which was then determined by the glucose oxidase method (Marks, 1959) or by the method of Somogyi (1945). Analyses were carried out in duplicate.

6. Hydrolysis of carbohydrate and chromatography of the sugars.

100 mg of freeze dried rumen fluid or fractions collected from rumen foams, was hydrolysed with 72% sulphuric acid for 4 hours at room temperature (Bailey, 1967). After neutralising this mixture with barium carbonate, the solution was concentrated and subjected to paper chromatography (Watman No. 1 paper) using ethyl acetate/water/pyridine, 2/2/1, v/v/v (Bailey and Pridham,

1962) as the developing solvent. Sugars were located by spraying the chromatogram with an aniline phosphate spray (Howard, 1957).

1.1 (c) Determination of calcium

1. Total soluble calcium was determined by atomic absorption spectrometry. Samples were wet ashed with a nitric acid/hydrochloric acid mixture, and the digest made up in 2M hydrochloric acid containing 0.65 g of caesium chloride per 100 ml. Dilutions were made so that the level of calcium in the final solutions was 5-20 p.p.m. Calcium was measured on these solutions by atomic absorption spectrometry using a Techtron*AA4 instrument. Glass distilled water was used to make up all solutions used in this method. Standards were prepared as described in the Techtron manual*.

2. Calcium bound to macromolecules in rumen fluids were measured as follows:

Rumen fluids were centrifuged at 16,000 g for 30 minutes to remove plant fragments and rumen microflora. 5 ml of this solution was pipetted into a Diaflo* 'centiflo' membrane and cone assembly. This membrane is reported to hold back all molecules of molecular weight over 50,000. The sample and cone assembly were centrifuged at 3,000 g for 30 minutes. The original sample and the solution that had passed through the membrane were analysed for calcium using the method described above. The difference in the amount of calcium in the original sample and the sample that passed through the Diaflo membrane was a measure

* see appendix

of the amount of bound calcium in solution.

1.1 (d) Determination of lipids

1. Total lipids

Total lipids in rumen liquor and foam fractions were measured as a dry weight percentage. The sample (200 mg) was extracted into 10 ml of chloroform/methanol, 2/1 v/v. The mixture was filtered and the solid re-extracted with another 10 ml of solvent. The extracts were combined and shaken with 4 ml of a 0.73% potassium chloride solution (Folch, 1957), and the chloroform layer collected, dried in a rotary evaporator and weighed. This preparation was called total lipid.

2. Thin layer chromatography

Thin layer chromatography of lipids was performed on plates coated with silica gel G, 250 μm thick. Silica gel G* was slurried with twice its volume of distilled water and spread on the plates using a Shandon Unoplan* thin layer spreader. The plates were dried at 110°C for 2 hours before use.

Samples (25 mm^3) of the lipid mixture in chloroform were applied to the plates by means of applicators similar to those described by Roughan (1967). For polar lipids, the developing solvent was chloroform/methanol/acetic acid/water, 85/15/10/3, v/v/v/v.

Neutral lipids were separated with petroleum ether (boiling point 57° - 59°C), diethyl ether, water mixtures.

Lipids were localised on the plates either by

* see appendix

spraying with a 0.2% w/v solution of 2'7' dichlorofluorescein in ethanol and examination of the plates under ultra-violet light or spraying the plates with concentrated sulphuric acid and heating of the plates to 130°C for 30 minutes.

PREPARATIVE METHODS

1.2 (a) The isolation and fractionation of the soluble leaf proteins.

Proteins are normally extracted from plant leaves by disrupting the tissue into buffer by grinding, blending, or shearing, followed by filtration and centrifugation to remove the insoluble plant material. While many plants yield a clear solution which is pale yellow, some plants such as red clover give an intensely dark brown solution. Protein precipitated from such extracts is similarly dark brown in colour, and is frequently difficult to redissolve.

This dark colour is believed to be the consequence of atmospheric oxidation, catalysed by the enzyme phenoloxidase, of the soluble polyphenolic constituents of the leaf cells. This leads to compounds with quinone-type structures, which form complexes with proteins, modifying their pigmentation, enzymic properties and solubilities.

In isolating the soluble plant proteins for study in this thesis, it was hoped to prevent this modification of

their properties, and preliminary experiments were carried out to test methods by which this might be achieved.

1. Preliminary investigations to obtain leaf proteins free of polyphenolic oxidation products.

Three methods were examined, the leaf source being red clover, Trifolium pratense L.

(i) Alcohol extraction of leaves will remove the phenolic substrate and precipitate the soluble protein. Fresh red clover leaves (20 g) were macerated into 80 ml of ice cold absolute ethanol in a Waring blender. The macerate was transferred to a basket centrifuge and washed with cold ethanol (0°C) until the ethanol wash was free of chlorophyll. The washed macerate was extracted overnight with 100 ml of phosphate buffer, ionic strength, I, 0.1 and pH 7.5 and dialysed against this buffer to remove ethanol. The extract was centrifuged at 16,000 g for 30 minutes and the supernate examined for soluble protein by acrylamide gel electrophoresis and by determination of protein nitrogen. 20 g of red clover leaves were also extracted by blending directly into 40 ml of phosphate buffer, centrifuged at 16,000 g for 30 minutes to sediment leaf fragments, and the supernate made up to 100 ml with more buffer. This solution provided a control extract.

The solution obtained by the ethanol extraction was colourless and had an optical density ratio 280 nm/260 nm of 1.6, whereas the control solution was dark brown in colour and had an optical density ratio of 1.2. The level

of soluble protein determined by the protein nitrogen method indicated that only 40% of the soluble protein of the control had been extracted by the ethanol extraction procedure. Acrylamide gel electrophoresis showed that none of the Fraction 1 protein had been extracted in this procedure.

(ii) Adsorption of phenolics on Polyclar AT.

Loomis and Bataille (1966) used insoluble polyvinyl pyrrolidone, Polyclar AT* (General Aniline Company), to adsorb plant phenolics and tannins during the extraction of plant enzymes, and so remove the substrate of the phenoloxidase system. These authors ground together equal weights of leaf tissue and Polyclar AT. Although this procedure is suitable for the extraction of small quantities of leaf, it was impracticable to apply this procedure to the large scale preparation of leaf proteins. The procedure was therefore modified as follows:

Polyclar AT was purified as described by Loomis and Bataille (1966), and equilibrated for 24 hours with phosphate buffer, I 0.1 pH 6.5, containing 0.1% $^{w}/_{v}$ ascorbate and 10^{-4}M mercaptoethanol. The fines were removed by repeated mixing of the buffer and polyclar, allowing it to settle for a few minutes and decanting off the supernate containing the fines. The Polyclar was slurried with more buffer and poured into a glass chromatography column (2.5 cm x 16 cm).

20 g of red clover leaves were extracted into phosphate buffer, I 0.1 pH 7.0, containing 0.1% $^{w}/_{v}$ sodium ascorbate.

* see appendix

The insoluble leaf fragments were sedimented at 16,000 g for 30 minutes and the supernate made up to 50 ml with phosphate buffer I, 0.1 pH 6.5. This solution (2 ml) was analysed for protein nitrogen and the remainder was applied to the Polyclar column. The effluent from the column was monitored at 280 nm and the effluent containing protein collected. Phenolics were absorbed as a narrow brown band at the top of the column. The protein solution was made up to 100 ml and 4 ml analysed for protein nitrogen. The solution was pale yellow and showed no sign of darkening when stored at 5°C for 5 days. The yield of protein was the same as that in the control extract.

(iii) Inhibition of the phenoloxidase enzyme has been reported when strong copper chelating agents are added to preparations of phenoloxidase (Kabowitz, 1937; Slack, 1966; Anderson, 1968). Sodium diethyldithiocarbamate, SDDC, has been reported to give 100% inhibition at a concentration of 10^{-4}M. SDDC is water soluble and easily incorporated into the extraction buffer and its effectiveness as a preventative of phenolic oxidation and subsequent interaction with soluble leaf protein was examined.

20 g samples of red clover leaves were extracted into 20 ml of phosphate buffer, I, 0.1 pH 7.5, containing SDDC at concentrations 0, 10^{-5}, 10^{-4} and 10^{-3}M. The macerates were transferred with another 20 ml of buffer into centrifuge tubes, and the leaf fragments sedimented at 16,000 g for 30 minutes. The supernates were made up to 50 ml and

stored at 5°C. Each extract (2 ml) was analysed for protein nitrogen.

The yield of protein nitrogen was 100% of the control extract for all extracts. The 10^{-5}M SDDC extracts had darkened considerably within 24 hours, the 10^{-4}M SDDC were pale brown after 24 hours and dark brown after 48 hours, whereas the 10^{-3}M SDDC extracts remained pale yellow for at least 7 days.

Of the three methods examined, the SDDC at 10^{-3}M concentration and the Polyclar AT extractions were effective in preventing interaction of the phenoloxidation system and the soluble red clover proteins during their extraction. Because SDDC was readily incorporated into the extraction buffer, it was more convenient to use than the Polyclar AT columns.

2. Fractionation of the soluble leaf proteins

Although leaf proteins contain a large number of different classes of protein, in this work a study was made of the two major classes, distinguishable by difference in size and sedimentation rate, viz. Fraction 1 and Fraction 2 proteins.

Preliminary experiments were therefore carried out to determine methods of isolating Fraction 1 protein in a reasonably pure form ($>$ 90% pure), and as a consequence Fraction 2 could be obtained from the remaining solution from which Fraction 1 had been removed.

For these experiments, a clear leaf protein solution

was obtained by precipitating total protein from a leaf extract with 60% ($^{w}/_{v}$) ammonium sulphate, clarifying the redissolved protein, and passing the solution through a column of G75 Sephadex* to remove low molecular weight contaminants.

The effluent was collected on an LKB fraction collector, and monitored for uv absorbing material at 254 nm using an LKB* Uvicord apparatus. A typical uv monitor of the elution profile from the G75 column is shown in Fig.1 (2.1). Biuret determinations of the fractions are shown in the same Figure.

The total protein was collected and fractionated as follows:

(i) Salt precipitation

The total protein solution was made 10% $^{w}/_{v}$ in ammonium sulphate, and the precipitate discarded. The supernate was made 24% $^{w}/_{v}$ in ammonium sulphate, allowed to stand for 1 hour at 5°C and the precipitate collected by centrifugation. This fraction contained 90% of the total Fraction 1 protein together with 10-25% of the Fraction 2 protein. The Fraction 2 proteins were isolated from the remaining supernate by increasing the ammonium sulphate concentration from 24-60% $^{w}/_{v}$, and collecting the precipitate.

The precipitates were dissolved in the minimum volume of phosphate buffer, I 0.1, pH 7.5 containing 10^{-4}M mercaptoethanol, and dialysed against this buffer to remove the ammonium sulphate.

* see appendix

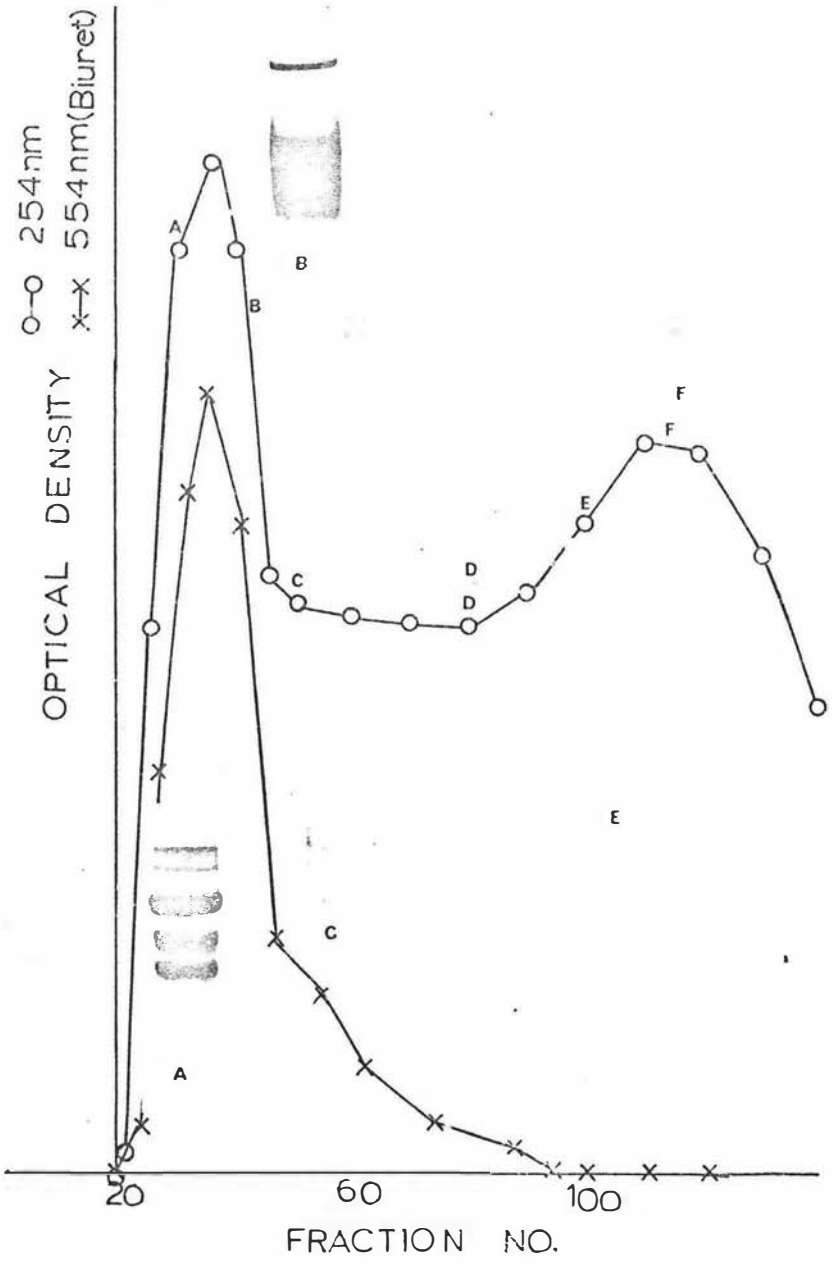


FIG. 1 (2. 1) ELUTION OF THE SOLUBLE LEAF PROTEINS OF RED CLOVER ON SEPHADEX G-75 DEXTRAN GEL.

O-O Optical density at 254 nm

X-X Optical density of Biuret determination of protein

Photographs are of the acrylamide gel electrophoretic analysis of the proteins eluted from the column.

(ii) Preparative ultracentrifugation was first used by Singer and Wildman (1952) to isolate Fraction 1 protein from tobacco leaves. These authors centrifuged their protein extracts at 40,000 g for 3 hours, during which time the Fraction 1 protein had sedimented as a pellet to the bottom of the centrifuge tube. The Fraction 2 proteins were distributed throughout the solution above the pellet.

The proteins that had been precipitated in the 10-24% ammonium sulphate fraction were redissolved in phosphate buffer, and centrifuged at 200,000 g for 90-120 minutes in either a Beckman* L-65 or a Spinco* L-50 preparative ultracentrifuge. Following ultracentrifugation, the contents of the centrifuge tubes were fractionated as follows.

(a) The pellet which had sedimented at the bottom of the tube. This was extracted into phosphate buffer.

(b) The viscous liquid above the pellet.

(c) The solution above the viscous liquid.

These fractions were examined by analytical ultracentrifugation and acrylamide gel electrophoresis. Fractions (a) and (b) contained more than 95% Fraction 1 protein whereas Fraction (c) contained mainly Fraction 2 proteins. As found by Cohen et al. (1956), the Fraction 1 protein pellet redissolved easily only if the phenoloxidase enzyme had been inhibited during the extraction and separation of the leaf proteins.

* see appendix

(iii) Molecular sieve chromatography has been the most common technique used to isolate pure Fraction 1 protein from a variety of plant extracts. Thus Pon (1967), Moon and Thompson (1968), isolated pure Fraction 1 protein from spinach leaves; Mendiola and Akazawa (1964) isolated Fraction 1 protein from rice leaves using Sephadex G 200 as the molecular sieve material. Wilson and Macalla (1967) isolated pure Fraction 1 protein from spinach, pea and bean leaves and chlorella by chromatography on Sepharose 4B*, an agarose matrix, followed by chromatography of the Fraction 1 protein on Sephadex*G 200.

The proteins that had been precipitated by ammonium sulphate at concentrations in the range 10 - 24% $^{w}/_{v}$ (see section 2(i) on page 47), were further fractionated on columns of Sephadex G 200, (100 cm by 5 cm). Fractions were collected on a fraction collector and ultra-violet absorbing material monitored at 254 nm using the LKB 'Uvicord' attachment*. Fractions were examined for Fraction 1 protein by acrylamide gel electrophoresis. Fraction 1 protein was eluted in the first peak from the column. Fraction 2 proteins were eluted over the region extending from the rear edge of the first peak through to the advancing edge of the second peak.

These experiments showed that preparative ultracentrifugation as a means of isolation Fraction 2 as well as Fraction 1 proteins had several advantages over the use of Sephadex G 200 chromatography. These were:

1. The centrifugation procedure took much less time than the molecular sieve chromatography (4 hours as

* see appendix

opposed to 12 hours).

2. Fraction 1 protein was concentrated and Fraction 2 proteins were at their original concentration when preparative ultracentrifugation was used, whereas both fractions were diluted, in particular Fraction 2 proteins, and had to be concentrated when Sephadex G 200 was used as the fractionation procedure.

3. Whereas preparative centrifugation cannot totally separate the two fractions in a single step, when starting with a fraction rich in Fraction 1 protein, the contamination of Fraction 2 was reduced to 5%, a satisfactory low level for this work.

3. The isolation and fractionation of the soluble leaf proteins of white and red clovers.

The extraction buffer was a disodium hydrogen phosphate, sodium dihydrogen phosphate mixed buffer, I = 0.1, pH 7.5 containing 10^{-3}M SDDC to inhibit the phenoloxidase, 0.1% sodium ascorbate to reduce any naturally occuring quinones, and mercaptoethanol at a concentration of 1×10^{-4}M to stabilize the Fraction 1 protein.

Method. White or red clover leaves (1 kg) were harvested from pure stands in the field. The leaves were infused with extraction buffer by immersing the leaves in 3 litres of extraction buffer at 4^{o}C in a large dessicator, which was then evacuated. Buffer infused into the leaves when the contents were brought to atmospheric pressure.

The leaves (100-150 g lots) were crushed into 50 ml of extraction buffer in a stainless steel disintegrator (Pirie, 1956), which had been precooled to 4°C. A further 50 ml of buffer was added to the crushed leaves and the slurry clarified through 3MM* filter paper in a basket centrifuge. The filtrates were centrifuged either at 75,000 g for 1 hour or at 45,000 g for 2 hours, and the protein precipitated from the resulting supernatant liquor by adding ammonium sulphate to 60% $^{w}/_{v}$. After standing for 1 hour at 0°C, the precipitated protein was collected by centrifuging at 16,000 g for 10 minutes.

The collected precipitates were suspended in the minimum volume of buffer required to transfer the protein to a dialysis bag, and dialysed against extraction buffer until the protein had redissolved. The protein solution was centrifuged at 105,000 g for 20 minutes to remove any remaining chloroplast fragments and applied for chromatography to a Sephadex G 75 column (60 x 10)cm which had been equilibrated with extraction buffer.

The fractions containing protein eluted from the Sephadex column were collected and ammonium sulphate added to 10% $^{w}/_{v}$. After allowing to stand at 5°C for 1 hour the protein solution was centrifuged at 16,000 g for 10 minutes and the supernatant made 24% $^{w}/_{v}$ in ammonium sulphate. The protein was collected by centrifugation. This fraction contained 90% of the total

* see appendix

Fraction 1 protein and about 10-25% of the Fraction 2 proteins.

The supernate was then made 60% $^{w}/_{v}$ in ammonium sulphate, and the remaining protein collected by centrifugation. This fraction contained 75-90% of the Fraction 2 proteins and less than 5% of the Fraction 1 protein. Both fractions were treated separately and dissolved in 50 ml of extraction buffer. The solutions were dialysed against 12 litres of 10^{-1}M sodium acetate buffer containing 10^{-4}M mercaptoethanol. The protein solution high in Fraction 1 protein was centrifuged at 200,000 g for 2 hours. The pellet and viscous liquid layer at the bottom of the centrifuge tube were separated from the remainder of the liquid. The pellet and viscous liquid, which contained approximately 95% pure Fraction 1 protein, were combined and dialysed against 2 x 12 litres of 0.1 M sodium acetate buffer, containing 10^{-4}M mercaptoethanol, to redissolve the protein pellet, and the Fraction 1 content of the solution determined by analytical ultracentrifugation. Two ml aliquots were pipetted into glass vials and stored at -70°C.

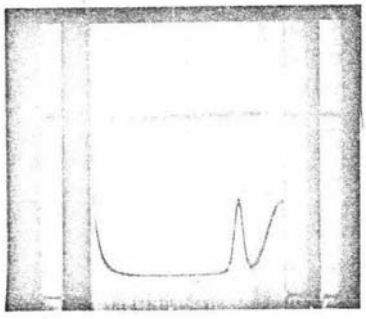
The Fraction 2 proteins (24-60% ammonium sulphate fraction plus the remaining liquor from the ultracentrifugation of the Fraction 1 protein preparation) were dialysed and stored in the same manner as for Fraction 1 protein. The protein content of this solution was determined by the biuret using Sigma (Sigma Chemicals)

fraction V bovine serum albumen as the protein standard. Fig. 1 (2. 2) shows a typical Fractionation of the soluble leaf proteins of white clover.

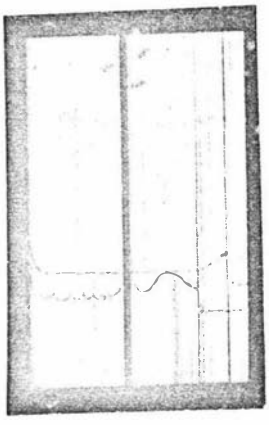
1.2 (b) Preparation of salivary secretions.

1. Salivary mucoprotein was isolated as described by Lyttleton (1964). Saliva was collected from the mouth of a non-lactating cow feeding on red clover hay. The collecting device (Reid, unpublished) consisted of a perforated copper tube which fitted under the tongue and round the lower jaw of the animal. On application of gentle suction from a water pump, saliva was collected into an ice-cooled buchner flask.

The saliva was diluted with one tenth of its volume of phosphate buffer, I 0.2, pH 6.0, and heated to 70°C for 10 minutes in a water bath. The saliva was centrifuged at 16,000 g for 30 minutes and the supernatant liquor collected. This was cooled to 0°C and an equal volume of 1% cetyl trimethyl ammonium bromide in water at 0°C added. The mixture was stirred vigourously for a few minutes when a bulky fibrous precipitate formed which contracted into a clot on standing for a few minutes. The clot was withdrawn on a glass rod, and washed at 0°C in 80% ethanol/20% 0.1 M sodium acetate and finally in 80% ethanol. The protein pellet was stored at -70°C in 80% ethanol. When required the protein was dissolved by dialysis against 1 M sodium chloride, followed by dialysis against 0.1 M sodium acetate. The solution was



a



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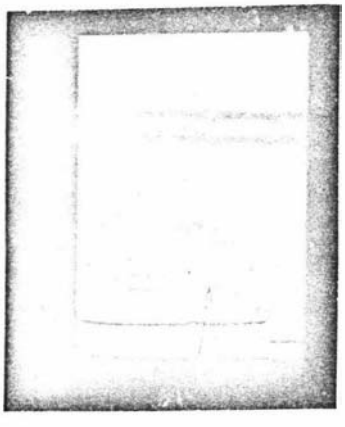

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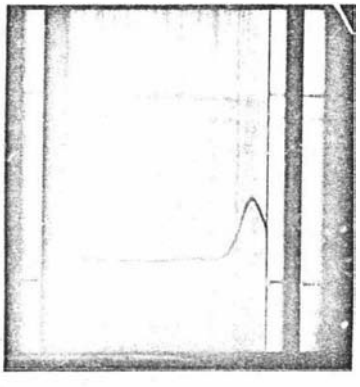

d

FIG. 1. (2. 2) THE FRACTIONATION OF SOLUBLE LEAF PROTEINS OF WHITE CLOVER

Photographs show the ultracentrifugation analyses of the total proteins and fractions.

(a) Total leaf proteins

(b) 10-24% ammoniumsulphate fraction

(c) Purified Fraction 1 protein (preparative centrifugation)

(d) Purified Fraction 2 proteins

clarified by centrifugation at 105,000 g for 20 minutes. The solution was analysed for sialic acid content and protein nitrogen (Analytical Methods). Analytical ultracentrifugation of this material showed a single hypersharp boundary, using schlieren optics. This material was termed salivary mucoprotein and was believed to originate from the submaxillary and sublingual glands (Phillipson and Mangan, 1959).

2. Oesophageal mucin was collected either as the free flowing secretion from an oesophageal fistula, with a water-filled balloon inserted through the fistula down the oesophagus, to act as a stimulus, or after emptying the rumen, the balloon was passed up the cardia into the oesophagus and the mucin collected by gentle suction (Reid, unpublished).

The mucin collected in these ways was a viscous material showing the phenomenon of spinbarkeit (Burnet, 1951), and was dispersed in one quarter of its original volume of phosphate buffer, and heated to 70°C for 10 minutes. Attempts to purify this material with the procedure used for mucoprotein failed because it was impossible to redisperse the precipitated mucin in a variety of buffers.

Examination of this material in the analytical ultracentrifuge revealed that it did not contain detectable amounts of the salivary mucoprotein isolated from the mouth secretion. A very high molecular weight material was observed to sediment rapidly, and also a low molecular polydisperse fraction was observed.

Analysis by acrylamide gel electrophoresis showed the mucin samples to contain several low molecular weight proteins and a large molecular weight component which did not stain for protein with Coumassie blue, but did stain for carbohydrate with the Schiff stain (Bodman, 1957) indicating the high molecular weight component to be carbohydrate in nature.

Sialic acid levels in these preparations did not differ greatly from one preparation to another, whereas the protein nitrogen determined on these samples varied by as much as 200% in different preparations.

1.2 (c) Protozoal proteins

Protozoa were isolated from non-lactating rumen fistulated cows fed on red clover hay, the rumen liquor being taken prior to feeding the animals.

To 2 litres of rumen liquor at 37°C, 0.3 g of glucose was added. The protozoa were allowed to feed on the glucose and settle to the bottom of the flask. As much as possible of the rumen liquor was withdrawn by suction, without disturbing the protozoa.

The protozoa and remaining rumen liquor were poured into a separating flask and mixed with 500 ml of protozoa wash solution (Clarke and Hungate, 1966). After allowing the protozoa to settle, they were separated into another separating flask together with a further 500 ml of wash solution. The procedure was repeated until the protozoa were free of plant fragments,

and then they were collected by centrifugation.

The contents of the holotrich protozoa were obtained by rapid freezing and thawing of a suspension of protozoa in phosphate buffer I 0.2, pH 7.5 containing 10^{-4}M mercaptoethanol, and grinding in a teflon/glass homogeniser. The suspension was clarified by centrifugation at 78,000 g for 30 minutes, and the protein was precipitated from the supernatant by making it 60% $^{w}/_{v}$ in ammonium sulphate. The precipitate was transferred, in the minimum volume of a 0.1 M sodium acetate solution containing 10^{-4}M mercaptoethanol to a heavy walled dialysis tube, and dialysed against 3 x 10 litres of 0.1 mol/litre sodium acetate solution containing 10^{-4}M mercaptoethanol. Protein nitrogen was determined on this solution, which was stored in 2 ml aliquots at -70°C.

1.2 (d) The isolation and fractionation of the polar lipids of red clover.

1. Extraction of total lipids of red clover (Trifolium pratense L.) was performed as described by Roughan (1968). Red clover leaves (2 kg) were collected from a pure stand in the field. Samples (200 g) were extracted in the cold with 10 volumes of methanol/chloroform, 7/3, v/v, in a Waring blendor and filtered. The residue was re-extracted with 5 volumes of chloroform and again filtered. The filtrates were combined and shaken with one fifth of their volume of a 0.73% aqueous sodium chloride solution. The mixture was left overnight for the phases to settle, and

the chloroform layer collected and evaporated to dryness under nitrogen. This total lipid residue was dissolved in chloroform/methanol 9/1 v/v.

2. Separation of the acidic lipids was achieved on a D E A E* cellulose resin as described by Rouser et al.(1963).

D E A E cellulose (200 g) was purified as described by Rouser (1963), by successive washing of the cellulose with 1 N hydrochloric acid, water and 1 N sodium hydroxide. It was then washed with methanol and air dried. The cellulose was dispersed into acetic acid in an homogeniser, and poured into a column (6.6 x 40 cm). After packing under a nitrogen pressure of 5 p.s.i. the column was washed with acetic acid, then methanol to remove all the acetic acid, and finally chloroform/methanol, 9/1, v/v.

The lipid mixture (equivalent to 250 g of fresh weight leaf) was applied to the top of the column, and by successive elution with three solvent mixtures were fractionated into three main classes.

1. Elution with chloroform/methanol, 9/1, v/v eluted the chlorophyll pigments, neutral lipids, monogalactosyl-diglycerides, and some phosphatydyl choline.

2. Elution with chloroform/methanol, 7/3, v/v eluted the digalactosyl diglycerides, phosphatydyl choline, and phosphatydyl ethanolamine together with some pigment material. The column was eluted with methanol which removed some non lipid pigments.

3. Elution with chloroform/methanol, 4/1 v/v, containing 0.05 M ammonium acetate and 20 ml of concen-

* see appendix

trated ammonium hydroxide per litre of solvent, eluted the acidic lipids phosphatydyl glycerol, phosphatydyl inisitol, and the sulpholipid.
The column was regenerated by washing the column with glacial acetic acid.

3. Separation of the lipids

Each of the fractions from the D E A E column was further resolved into individual classes of lipid by silicic acid chromatography, as described by Rouser et al. (1967).

Silicic acid, was transferred in chloroform to a glass chromatography column, 2.5 cm in diameter, and filled to a height of 20 cm. Each fraction from the D E A E column, after concentration and dissolution in chloroform, was subjected to silicic acid chromatography eluting the lipids with the following solvents in the order:

1 chloroform
2 chloroform/acetone, 8/1, v/v
3 chloroform/acetone, 1/1, v/v
4 acetone
5 methanol

The first fraction from the D E A E separation was fractionated into (a) pigments (chloroform); (b) neutral lipids (chloroform); (c) neutral lipid (chloroform/acetone, 8/1, v/v); (d) monogalactosyl diglyceride (chloroform/acetone, 1/1 v/v); and (e) phosphatydyl

choline (methanol).

The second fraction from the D E A E column was separated on silicic acid into (a) pigments (chloroform; chloroform/acetone, 8/1, v/v; chloroform/acetone, 1/1, v/v); (b) digalactosyl diglycerides (acetone); (c) phosphatydyl choline and phosphatydyl ethanolamine (methanol).

The third fraction from the D E A E column was separated into (a) pigments (chloroform; chloroform/acetone, 8/1 and 1/1); (b) sulpholipid (acetone); (c) phosphatydyl glycerol and phosphatydyl inisitol (methanol).

A flow diagram of the lipid separation is shown in Fig. 1 (2. 5).

1.2 (e) Isolation of the protein precipitating agents (tannins) from Lotus pedunculatus Cav.

The protein precipitants of Lotus pedunculatus Cav. were isolated from finely ground freeze-dried leaves using the procedure adopted by Feeny and Bostock (1968), for the quantitative measurement of tannins from oak leaves (Quercus robur L.).

50 g of freeze dried leaves were extracted with 500 ml of acetone/water 70/30 v/v and filtered. The plant residue was re-extracted with a further 500 ml of the acetone/water solvent and filtered. The combined filtrates were saturated with sodium chloride, and the acetone and aqueous layers separated.

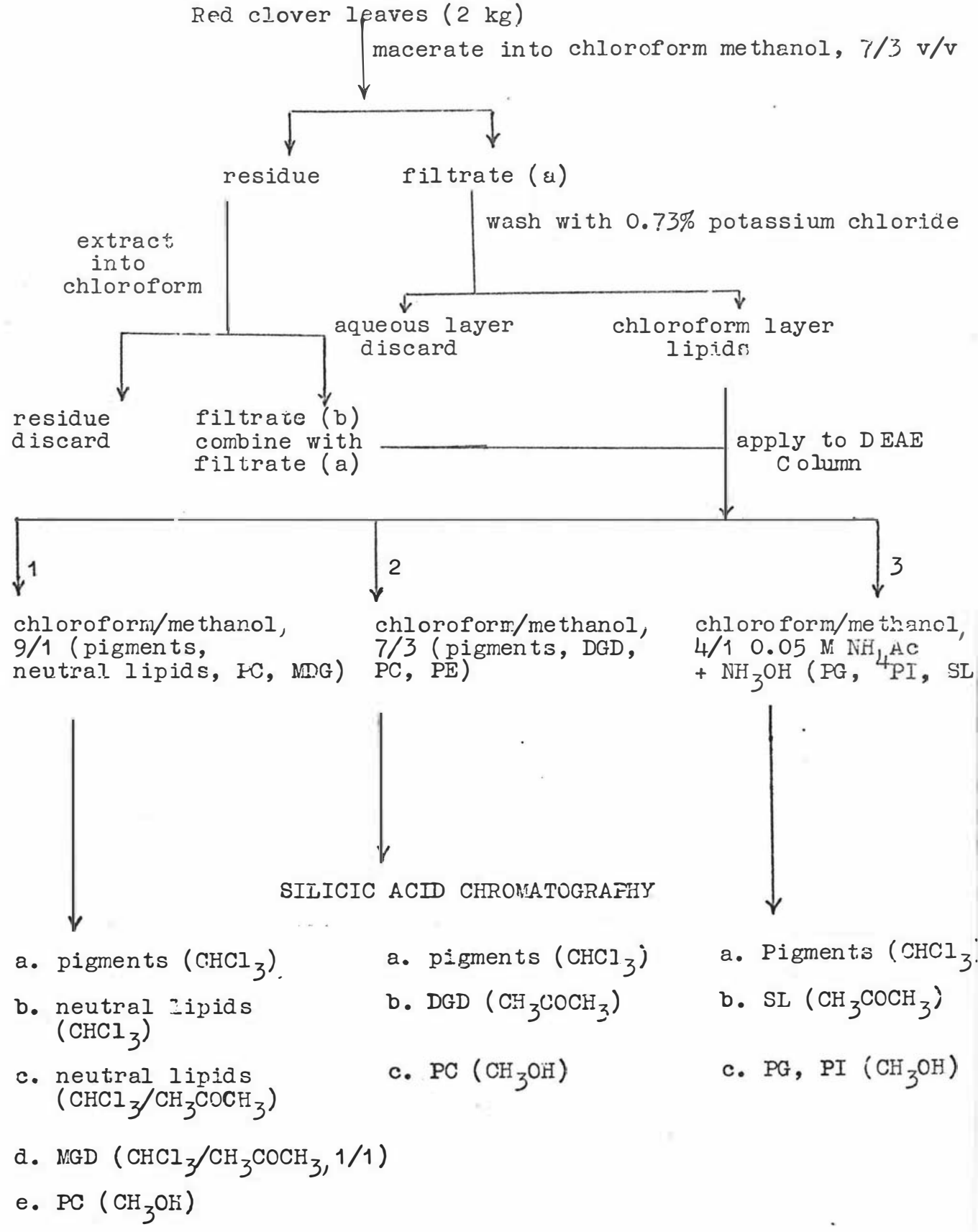

FIG. 1 (2. 5) FRACTIONATION OF THE POLAR LIPIDS OF RED CLOVER (TRIFOLIUM PRATENSE L.)

To the acetone layer, 2 volumes of ethanol and 15 volumes of anhydrous diethyl ether were added, and the precipitate of sodium chloride and tannins which formed was collected by centrifugation. The precipitate was dialysed against distilled water (10 litres) and the dialysed solution freeze dried.

The solid so obtained was a light brown fluffy solid which was completely soluble in 10% aqueous acetone.

Two dimensional paper chromatography using the elution solvent butanol/acetic acid/water, 60/15/25 v/v/v followed by elution with 2% acetic acid and spraying the chromatogram with ferric chloride/potassium ferricyanide reagent revealed only one component which had not removed from the origin though showing some streaking with acetic acid solvent.

This material gave an instantaneous red colouration when sprayed with a vanillin/hydrochloric acid reagent, indicating it to be a condensed tannin. Copious precipitates were produced when this material was added to solutions of soluble proteins.

1.3 Methods of Studying foams

(a) The measurement of protein foam parameters

Two systems were used to examine the nature of protein foams in the work described in this thesis. The temperature was controlled by immersing the respective apparatus in a water bath thermostatically controlled to ± 0.1%.

1. The modified Mangan apparatus. The original Mangan apparatus was designed to study properties which he believed were relevant to surface-viscous foams. The most useful measurement was termed 'foam strength', which he considered to give a measure of the ability of the foam to resist mechanical rupture. Mangan examined total red clover protein foams with this apparatus (Mangan, 1958; 1959).

Because the volumes of protein solution used in this apparatus were large (100 ml per measurement), the size of the apparatus was reduced so that the volume of solution used per determination was only 10 ml.

The apparatus consists basically of three parts: The foam tube, the gas supply and dispersion section and the falling weight. A diagram of the salient features of the modified apparatus is shown in Fig. 1 (3. 1).

The foam tube consisted of a high precision bore glass tube, 1.6 cm internal diameter and 31 cm long.

The gas supply and dispersion section. The flow of nitrogen gas from a cylinder of constant outlet pressure (10 psi) was regulated with a 'flow stat' (Platon instruments, Crawly, England) gas flow controller, the flow rate through the system being indicated visually on a 'gap meter' (Platon instruments). Gas was dispersed into

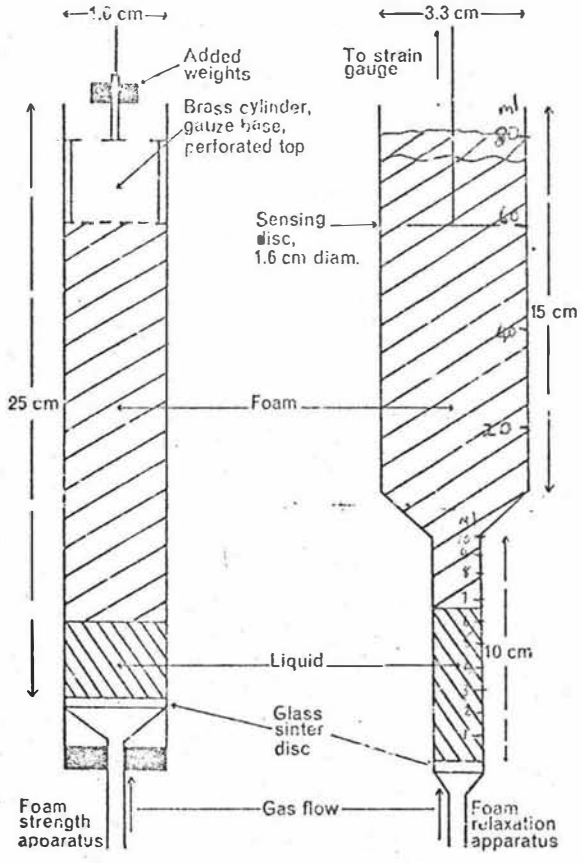

FIG. 1 (1. 3a)

LEFT The modified Mangan apparatus

RIGHT The Laby apparatus

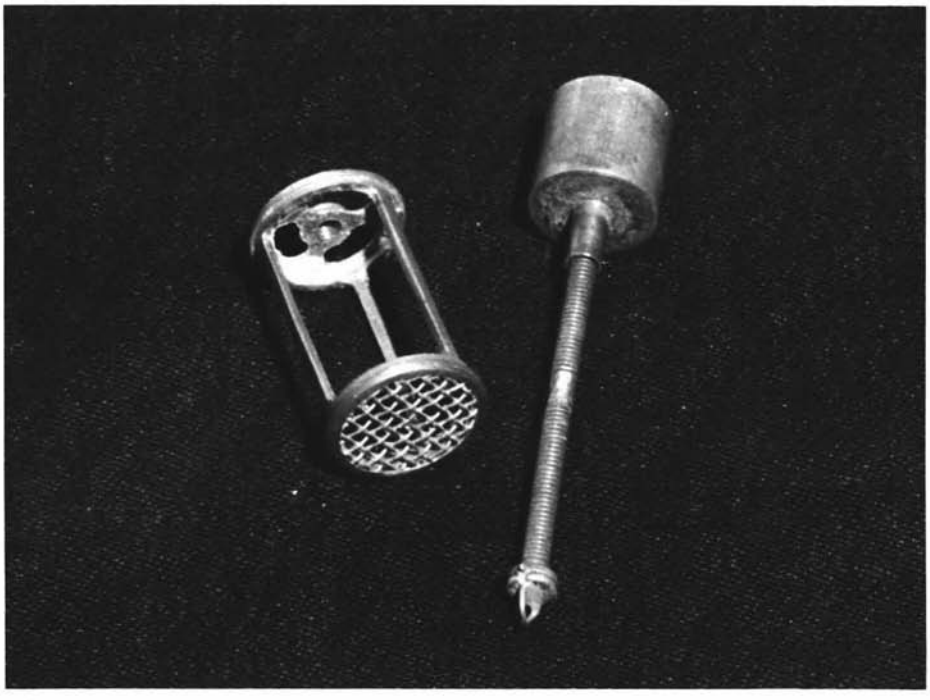

Fig. 1 (3. 1b) The falling weight assembly used in the Mangan apparatus.

Left — The basic weight (3.5 gms)

Right — Extra weight which can be added to the assembly. (14.5 gms)

the solution being studied using gas micro immersion-filters of different porosity sinters.

The falling weight. The weight used in this system was redesigned to achieve the minimum of friction between the falling weight and the walls of the tube.

A piece of brass rod 1.8 cm in diameter and 5 cm long, was turned on a lathe to decrease the diameter of the rod to give 2.5×10^{-3} inch clearance between the walls of the tube and the weight. The rod was cut away so that it touched the glass walls only at its top and bottom over a distance of 1 mm.

The rod was hollowed out leaving the top and bottom supported by three narrow strips of brass 2 mm wide by 1 mm thick. Holes were cut out of the top of the weight for the nitrogen released from the collapsed foam to escape. The base was fitted with a wire mesh (28 gauge wire, 64 holes per cm^2). Additional weights could be added to the top of the basic weight which weighed 3.5 g. A photograph is shown in Fig. 1 (3. 1b).

Measurement of foam strength. The base of the glass tube was fitted with a rubber stopper which carried a micro immersion filter to disperse the gas, and a tube connected to a reservoir of the solution from which the foam was to be generated.

5 ml of the solution was introduced into the apparatus, and nitrogen at a flow rate of 30 ml sec^{-1} was dispersed into the solution. The level of solution above the sinter was maintained by additions from the reservoir. The

column was filled with foam to a height 25 cm and the weight (total weight was 18 g) carefully lowered on to the top of the foam. It was then released and the time taken for the weight to fall through the total column of foam measured. Foam strength is defined as follows:

$$\text{Foam strength} = \frac{100 \times \text{time (sec) taken to fall through column of foam}}{\text{Length of foam column in cm}}$$

The fall times ranged from a few seconds to 1 hour.

2. The Laby apparatus. Laby (1969) described an apparatus suitable for measuring parameters which assist in characterizing persistent strong foams such as those derived from soluble leaf proteins. A diagram of the apparatus is shown in Fig. 1 (3.1). The apparatus consists of a foam tube in which the foam is generated, and the measuring equipment which detects pressure exerted by the foam.

The foam tube is made up of two sections (a) the drainage chamber and (b) the foam chamber. The drainage chamber consists of a barrel from a 10 ml graduated measuring cylinder which carries a porosity 3 glass filter disc at its base. This chamber is connected to a foam chamber of pyrex tubing 33 mm internal diameter and 105 mm long.

The measuring equipment consists of a stainless steel sensing disc 16 mm in diameter which is connected to the arm of a transducer (Shinkoh Communications Industry, unbonded strain gauge type UL-10-120) by a

stainless steel rod 1.6 mm in diameter. The input for the transducer was obtained from a stabilized DC power supply. The output from the transducer was recorded by a millivolt recorder (Hitachi Ltd, type QPD 54) using the circuit diagram given by Mansfield (1963) to balance the no load output from the strain guage.

Calibration of the transducer. The response of the transducer to different loads added to the arm of the transducer was determined using the 1 m volt range on a Hitachi chart recorder, which had been adjusted to give full scale deflection for 5 g and 10 g respectively. Weights 1-10 g were added to the transducer arm and the pen deflection of the recorder noted. This was repeated with the recorder giving full scale deflection for 5 g, and with weights in the range $\frac{1}{2}$-5 g added to the transducer arm.

The response was linear with the force applied to the arm of the transducer for both sensitivities measured.

Measurement of foam parameters was accomplished as follows:

The drainage chamber is filled to the 10 ml graduation mark with the solution to be foamed. The sensing disc and connecting rod is fitted to the arm of the transducer so that the disc is positioned at the 60 ml mark on the foam chamber and concentric with it. Nitrogen at a controlled flow rate of 30 ml per minute is passed via the sinter into the solution in the drainage chamber. The foam which is generated enters the foam chamber and

flows past the sensing disc. Its effect on the disc is recorded from the output of the transducer. Foam is generated to the 80 ml mark on the foam chamber, and the gas supply turned off. The volume of liquid remaining in the drainage chamber is recorded and the rate of drainage of liquid from the foam into the drainage chamber measured.

Two parameters are obtained from the recorder trace of the transducer output:

(a) The maximum compressive strength C_s. This is the maximum pressure exerted on the disc as the foam flows past the sensing disc. The units of compressive strength are g cm^{-2}.

(b) The stress relaxtion, S_r, is the rate at which the stress, applied to the sensing disc by the foam, decreases after the gas flow has been turned off. It is determined from the gradient of the recorder trace, and has the units g cm^{-2} sec^{-1}.

Two other parameters which were derived from the study of foams in the Laby method were the foam retention volume and the drainage constant.

When the foam is first formed it contains a volume of liquid v_o. The volume of liquid actually present in the foam at any time may be measured by subtracting the volume of liquid in the drainage chamber from the amount originally introduced (10 ml). With increasing time, the amount of liquid which drains from the foam approaches a volume v_∞, so that the liquid retained falls to the

limiting volume $v_o - v_\infty$, known as the foam retention volume.

The drainage of liquid from protein foams is best described by the equation of (Laby, 1969)

$$\frac{dv}{dt} = k_2 (v_\infty - v)^2$$

where k_2 is the second order rate constant, and v the volume drained from the foam at time t.

Integration of the equation and rearrangement leads either to the form

$$\frac{t}{v} = t.\frac{1}{v_\infty} + \frac{1}{v_\infty^2}\frac{1}{k_2}$$

whence v_∞ may be obtained from the slope of a plot of t/v against t, or to the form

$$\frac{1}{v_\infty - v} = k_2 t + \frac{1}{v_\infty}$$

whence k_2 may be obtained from the slope of the plot of $\frac{1}{v_\infty - v}$ against t.

3. Reproducibility of the foam measurements

Using a standard protein solution, 5 determinations of each parameter from both systems were measured, each time using a fresh aliquot from the same solution.

Foam strength data obtained from the modified Mangan apparatus had a maximum deviation from the mean of 15%.

Of the parameters measured in the Laby apparatus, compressive strength was the most reproducible coefficient with a maximum deviation from the mean of approximately 5%.

The deviation from the mean of the stress relaxation measurements was between 5-10% of the mean, foam retention volume ($v_o - v_\infty$) approximately 10-15% of the mean, and the least reproducible measurement was k_2 with a maximum deviation from the mean of 20%. In all the foaming studies in this thesis at least five determinations of each parameter on any solution were made in each experiment. Two standard deviations are represented on the graphs as a bar, Φ .

1.3 (b) Foam fractionation procedure

In experiments carried out to concentrate and determine the surfactant materials in foams derived in vitro from rumen liquor, the apparatus was similar to that used by Wace and Banfield (1966) which they termed the 'plateless' foam fractionator. The apparatus consisted of four stages, each section consisting of a wide (6 cm diameter) and a narrow tube (2 cm diameter) 12 cm long joined together.

Rumen liquor (1 litre) was taken, via a rumen fistula, from cows ¾ hour after feeding on red clover had commenced. The rumen liquor was squeezed through two layers of butter muslin to retain large plant fragments, and the liquor diluted with an equal volume of phosphate buffer, I 0.1, pH 6.0, and poured into a 3 litre flask. The flask and contents were placed in an ice-bath.

The fractionating column was connected to the flask, and foam was generated by bubbling carbon dioxide through the liquor via a porosity 3 sinter. The foam was subjected to a series of compressions and expansions as

it was forced through the narrow and wide sections of the apparatus. This resulted in a rapid drainage of the liquor that had been carried up in the foam. The gas flow was adjusted so that the liquid draining from the foam was able to drain back into the flask. The foam was collected at the top of the fractionating column in a 2 litre flask. The foam was collapsed with a few drops of ether and freeze dried. The freeze dried solid was stored at -70°C over calcium chloride. 200 ml of the original rumen liquor were also freeze dried and stored at -70°C.

CHAPTER 2

THE SOLUBLE PROTEINS OF BLOAT AND NON-BLOAT PREVOKING LEGUME SPECIES AND THE FOAMING PROPERTIES OF THE EXTRACTS DERIVED FROM THEM.

The soluble leaf proteins of legume pastures and correlation with bloat potential of the forage.

Bloat is associated with cattle foraging on pasture rich in legume species (Johns, 1954; Bartley, 1965). However, when cattle forage certain legume pastures such as Lotus corniculatus bloat does not occur.

Cooper et al. (1966) found that the extracts from leaves of legume forages considered not to induce bloat yielded smaller volumes of foam in vitro than extracts from bloat inducing forages. Kendall (1966) was able to produce large volumes of foam in vitro from similar non-bloating forages by addition to the leaf extracts of soluble polyvinyl pyrrolidone (PVP), an agent known to complex tannins and phenolics (Anderson, 1968). He was also able to decrease the volume of foam produced from bloating legume extracts by addition of a commercial tannin preparation to the extract. He postulated that the non-bloating pastures contained tannins which interacted with the protein/buffer system in such a way as to inhibit foam production. The nature of the interaction

was not explained, nor were tannins isolated from these plants which he examined.

A major group of non-bloating legumes is the tropical species. Bloat has been reported only on one of the main tropical legumes used as pasture for cattle in Australia (Hamilton and Ruth, 1968), but the absence of bloat could not be explained in terms of the tannin content of the leaves (Hutton and Coote, 1966).

McArthur et al. (1969) found that the level of Fraction 1 protein which he claimed to be the foaming agent responsible for bloat, was extremely low in the non-bloating temperate legumes he had examined. Fraction 1 is associated with the enzyme responsible for photosynthetic fixation of carbon dioxide in plants (Trown, 1965), and therefore McArthur's result seemed unusual because many of the non-bloating legumes have soft, green leaves which would be expected to be high in photosynthetic activity, and hence high in Fraction 1 protein.

In view of the results observed by Kendall (1966) and McArthur (1969), it seemed possible that the interaction between tannins and the soluble proteins was to produce an insoluble tannin/protein complex on grinding the leaves of the non-bloating legumes. This hypothesis was tested in this chapter.

2.1 Detection of protein precipitants in legumes

1 gm of leaf tissue was extracted in a conical, ground-glass homogeniser into 1 ml of either

(a) KH_2PO_4; Na_2HPO_4, ionic strength 0.1 pH 7.5 containing 0.1% ascorbic acid, 1 mM sodium diethyldithiocarbamate, and 0.1 mM mercaptoethanol; or (b) the same buffer containing in addition 2% soluble PVP/molecular weight 30,000).

After grinding until the leaf tissue was completely disrupted, the homogenates were centrifuged at 16,000 g for 30 minutes to sediment the insoluble material. The supernates were made 10% in sucrose and 25 mm^3 analysed by acrylamide gel electrophoresis (Section 1 (1 a.)) to reveal the soluble proteins in the extract. An increase in the level of protein in extract (b) compared with extract (a) indicated the presence of protein precipitants in the extract, presumably tannins, which complex preferentially with PVP rather than with leaf proteins.

2.2 The soluble leaf proteins of legume forages used in New Zealand.

The major legumes used in New Zealand are white clover (Trifolium repens L.), red clover (Trifolium pratense L.), subterranean clover (Trifolium subterraneum L.) and lotus major (Lotus pedunculatus Cav.). There is no record of lotus major used as a forage having produced bloat, and a closely similar plant, Lotus corniculatus L. is recognised as a non-bloating legume (Kendall, 1966). Bloat occurs occasionally in cattle grazing subterranean clover (McDonald, 1968), and it is very common in cattle

grazing both red and white clover.

The lotus major, white and red clover samples were taken from pure stands in the field, and the subterranean clover was grown in pots in a glasshouse during the summer months.

Extracts of these plants were examined for protein precipitants as described in Section 2 (1). Photographs of the acrylamide gel are shown in Fig. 2 (2). It can be seen that the addition of PVP to the extraction buffer had no measurable effect on the level of protein released into the extracts, of the forages associated with bloat. In the case of lotus major, the non-bloating forage, the addition of PVP increased the level of extractable soluble protein substantially. The leaves of lotus major appear to contain constituents which precipitate the soluble leaf proteins when the cells are broken, and PVP seems to bind these precipitating agents preferentially, enabling the protein to be released in a soluble form. This would suggest that these agents are tannin-like in nature (Loomis and Battaile, 1966). Further evidence to suggest that these materials were tannins was the demonstration that authentic tannins could be isolated from lotus major (analytical methods 1 (2. 6.)).

2.3 A survey of the legume forages which do and do not cause bloat.

Because of the results obtained above, the following experiments were undertaken firstly to find out if the

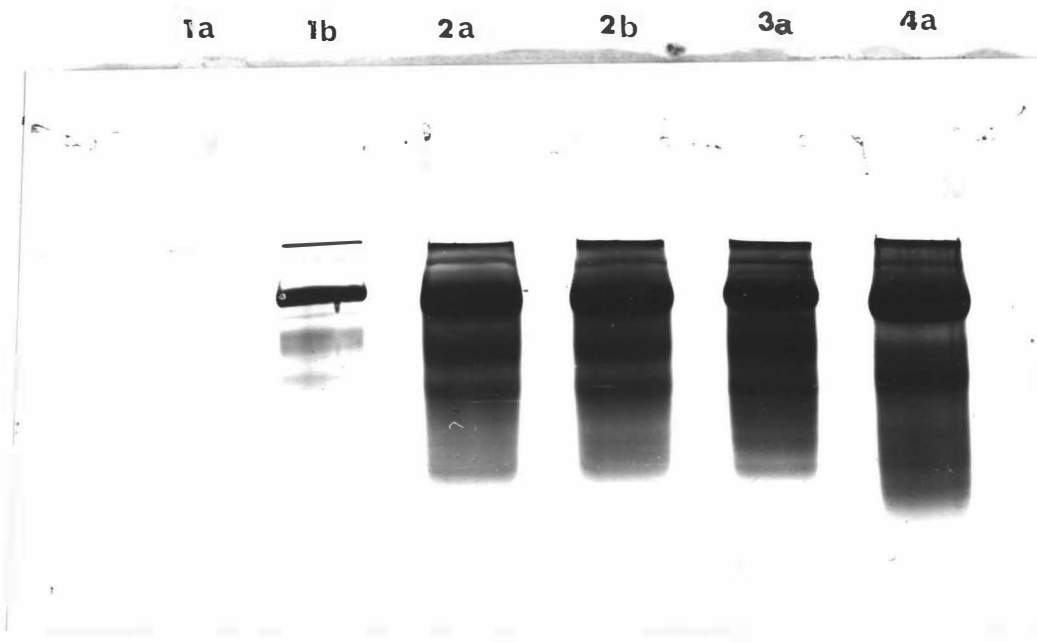

FIG. 2 (2) THE SOLUBLE PROTEINS EXTRACTABLE FROM THE LEAVES OF THE FOUR MAIN LEGUME PASTURES USED IN NEW ZEALAND.

Acrylamide gel electrophoresis.

1. Lotus pedunculatus Cav.
2. Subterranean clover
3. White clover
4. Red clover

a represents the leaves extracted into phosphate buffer

b " " " " " " " + PVP

absence of bloat on legume pastures could in general be correlated with the presence of tannins in these forages, and secondly to attempt to correlate the compressive strength (Methods 1 (3. 1b)) of foams derived from extracts of these legumes with their bloat potential. Tannins are defined by Swain (1965) as compounds with molecular weights 500-3000, containing 1-2 phenolic hydroxyl groups per 100 molecular weight, which can combine with proteins causing precipitation of the protein. No satisfactory chemical assay of tannins as such seems to have been devised (Feeny and Bostock, 1968) which led us in this thesis to detect their presence by their action as protein precipitants (2 (1)).

Plants 1-18 (Table 2 (3.1)) were grown in glasshouses during the summer months, the tropical legumes 12-18 being held at a minimum temperature of 60°C. The tropical legumes 19-21 were grown in the field at C.S.I.R.O., Division of Tropical Pastures, Brisbane, and extracts were made during a visit to that station by me.

Leaf samples of all the legumes in Table 1 (2. 3. 1) were examined for tannins using the technique described in part 2 (1) of this thesis. Results are shown in Table 2 (3. 1).

Compressive strength measurements (1. 3a) were made on leaf extracts prepared as follows: 5 gm of leaf were ground into 20 ml, 0.1 M sodium acetate buffer, pH 7.6, containing 1 mM sodium diethyldithiocarbamate until the leaf tissu

TABLE 2

(3. 1) THE PROPERTIES OF BLOATING AND NON-BLOATING LEGUME FORAGES

	Genera	% soluble protein	Presence of protein precipitants	(Mean of 9 determinations) Compressive strength (± 0.05)	Bloat Reported
	TEMPERATE LEGUMES				
(1)	Trifolium hybridum L.	-	(-)	-	(+)
(2)	Trifolium repens L.	2.06	(-)	2.43	(+)
(3)	Trifolium pratense L.	1.86	(-)	2.23	(+)
(4)	Medicago sativa L.	2.10	(-)	2.42	(+)
(5)	Onobrychis viciifolia (Scop.)	0.10	(+)	0.00	(-)
(6)	Ornithopus pinnatus (Mill.) Druce*	0.12	(+)	0.00	(-)
(7)	Ornithopus compressus**	0.83	(+) trace	1.02	(-)
(8)	Coronilla varia L.	0.04	(+)	0.00	(-)
(9)	Lotus corniculatus L.	0.13	(+)	0.33	(-)
(10)	Lotus pedunculatus Cav.	0.06	(+)	0.00	(-)
(11)	Lespedeza stipulacea L.	-	(+)	-	(-)
	TROPICAL LEGUMES				
(12)	Dolichos lab lab C.V. Rongai	0.63	(-)	2.02	(+)
(13)	Dolichos axillaris E. Mey (C.P.O. 17814)	-	(-)	-	(-)
(14)	Desmodium intortum (Mill.) Urb. (C.V. green leaf)	0.06	(+)	0.00	(-)
(15)	Desmodium uncinatum (Jacq.) DC C.V. silverleaf	0.04	(+)	0.00	(-)
(16)	Phaseolus atropurpureus DC C.V. Siratro	0.83	(-)	1.25	(-)
(17)	Lotononis bainesii Baker	0.33	(-)	0.56	(-)
(18)	Glycine javanica L. (C.V. Clarence)	-	(-)	-	(-)
(19)	Stylosanthes humilis Rich.	0.56	(-)	0.62	(-)
(20)	Leucaena leucocephala (Lam.)	0.34	(+) trace	0.56	(-)
(21)	Lotus purshianus de Wit	-	(+)	-	(-)

* Anthrolobium pinnatus (Allen N.Z. D.S.I.R. Bull. 83, 1940, p 131)

** Gladstones, J. S.; Barrett-Lennard, R. A.

was completely disrupted. The total extract was transferred with washings to a 50 ml volumetric flask and made up to the 50 ml mark with 0.1 mol/litre sodium acetate buffer. The compressive strength was determined in the Laby apparatus, on foam generated from 10 ml of the suspension, with pH adjusted to 5.8, held at 37°C. Three determinations of compressive strength were made on each of three different leaf extracts from each plant and the average result is shown in Table 2 (3. 1).

Soluble protein was also determined by nitrogen estimation on 2 ml aliquots of the above extracts after centrifugation at 16,000 g for 30 minutes. These results are also shown in Table 2 (3. 1).

It can be seen that in the case of all the legumes in which tannins were found, bloat has not been reported. With temperate legumes, only those containing tannins were not associated with bloat. In the case of tropical legumes, Table 2 (3.1) indicates that absence of bloat is not always to be correlated with the presence of tannins. In these plants, however, the level of extractable protein was markedly lower than with the temperate legumes on which bloat can occur. Probably as a consequence of the difference in protein concentration the compressive strength of all foams derived from extracts of non-bloating forages was low in comparison with foams derived from extracts of bloat producing legumes.

Although bloat is uncommon on tropical legumes, bloat has been reported on Dolichos lab lab (Hamilton and Ruth, 1968).

Here the level of soluble protein in the extract was lower than the level found in several other species which have not been associated with bloat. However the foam produced from extracts of this plant revealed a compressive strength which was comparable to that from temperate legumes which frequently give rise to bloat and considerably higner than those from other tropical species. Good correlation was obtained between compressive strength of foams derived from leaf extracts and the bloat potential of temperate and tropical legumes, whereas tannin content could only be correlated with the bloat potential of temperate legumes.

2.4 Examination of Trifclium species for tannins

The results of the preceding section suggest that if tannins could be bred into bloating legumes, then pastures containing such a legume may not cause bloat. The main legume forages in New Zealand are the clovers (Trifoliae). In order to find suitable material for cross-breeding with white clover or red clover, or to find material that might be suitable for mixed pastures with these clovers, a selection of Trifoliae was examined.

Plants were grown in the glasshouse in pots, the soil having been innoculated with the correct rhizobia. The plants were examined for tannins as described earlier (2. 1). The results of this investigation are shown in Table 2 (4. 1). Photographs showing the proteins of Trifolium arvense, Trifolium bocconei, Trifolium medium, are shown in Fig. 2 (4. 1). Tannins were only found in

Trifolium species	Tannins present
repens	-
pratense	-
hybridum	-
subterraneum	-
arvense	+
squammosum	-
squarrosum	-
dubium	-
achroleucum*	-
incarnatum	-
medium	-
striatum	-
scabrum*	-
stellatum*	-
bacconei*	-
cherleri*	-
hirtum*	-
lappaceum*	-
alexandrinum	-
escherinatum*	-
tricocephalum*	-
ligusticum*	-
diffusum*	-
purpureum*	-
pallidum*	-
phleoides*	-
nigrescens	-
glomeratum	-
fragiferum	-
semipilosum	-
burchillianum	-
vesiculosum	-
ambiguum	-

TABLE 2 THE PRESENCE OF CONDENSED TANNINS IN SELECTED TRIFOLIUM SPECIES

(4. 1)

* Trifolium species believed to be closely related genetically to Trifolium arvense.

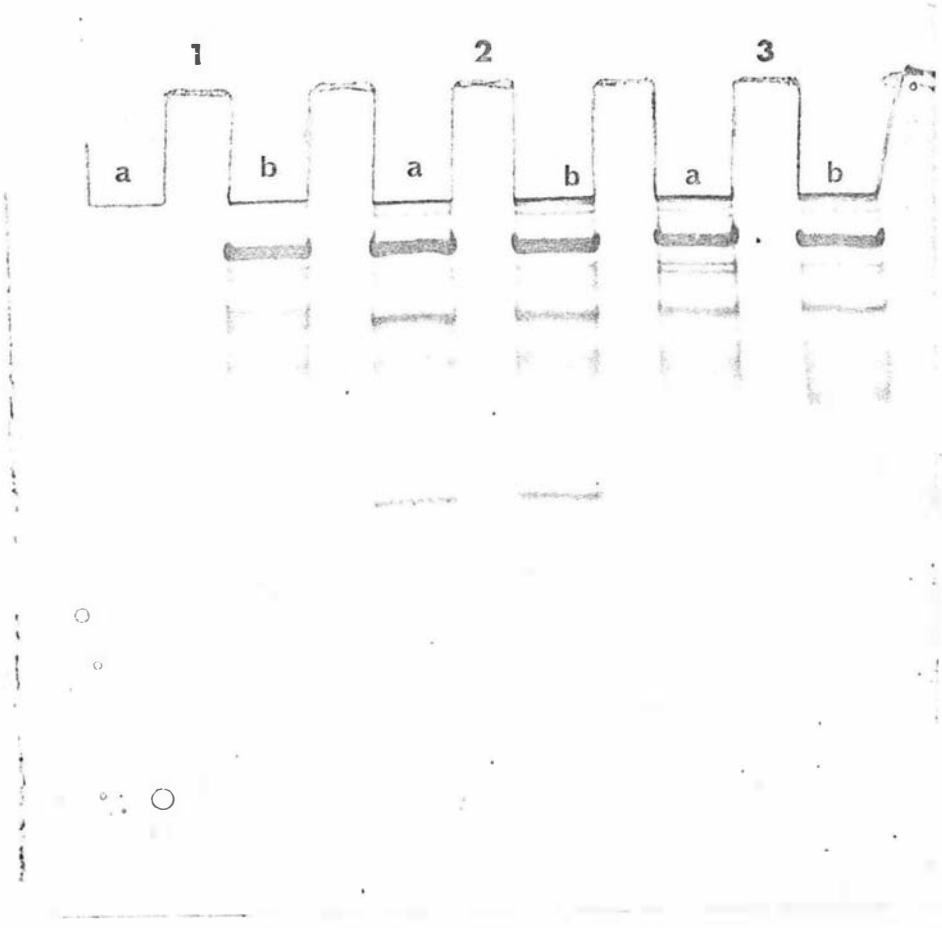

FIG. 2 (4. 1) ACRYLAMIDE ELECTROPHORETIC ANALYSES OF THE SOLUBLE PROTEINS EXTRACTED FROM THE LEAVES OF SOME OF THE TRIFOLIUM SPECIES

1. Trifolium arvense L.
2. Trifolium bocconei L.
3. Trifolium medium L.

a. Leaves extracted into phosphate buffer.

b. Leaves extracted into phosphate buffer containing 2% PVP.

Trifolium arvense. Although the plants marked with an asterisk * were believed to be closely related genetically to Trifolium arvense (Healy, 1970), no tannins were found in these species. Although Trifolium arvense is of no interest as a forage, and would be unsuitable for use as a mixed pasture, the fact that tannins have been found in the Trifolium species gives some hope of finding a suitable species or of inducing tannins into the bloating clovers.

2.5 The occurance of tannins in Lotus species.

Eight lotus species were grown in pots in glasshouses. They were examined for tannins using the procedure described in Section 2. 1 of this thesis. The results are shown in Table 2 (5. 1). The only species which did not contain measurable amounts of tannins was Lotus tenuis.

Lotus scoparius and Lotus angustissimus contained sufficient tannins to precipitate all of the Fraction 2 proteins and some of Fraction 1 protein. The remaining Lotus species contained enough tannin to precipitate all the soluble protein in these plants.

Summary

The examination of the proteins of legume forages indicates that

1. The non-bloating temperate legumes do not release soluble protein when their leaf tissue is disrupted, whereas the bloat provoking legumes release high levels of soluble protein. This difference is due to the presence of protein

Lotus species	Tannin content
corniculatus	4
pedunculatus	4
tenuis	0
scoparius	3
maroccanus	4
divaricatus	4
hispidus	4
angustisimus	3
purshianus	4

TABLE 2 THE TANNIN CONTENT OF LOTUS SPECIES

(5. 1)

0 No precipitation of soluble proteins.

1 Precipitation of some of the Fraction 2 proteins.

2 Precipitation of all the Fraction 2 proteins.

3 Precipitation of all the Fraction 2 proteins and some of the Fraction 1 protein.

4 Precipitation of all the soluble proteins.

precipitants, tannins, in the leaves of the former plants.

2. Tannins were not found to be present in the leaves of the non-bloating tropical legumes, with the exception of the Desmodium species. However, the compressive strength of foams derived from extracts of these legumes was low, correlating with their bloat potential.

3. Examination of some of the Trifolium species indicated that tannins were present in the plant Trifolium arvense and although this plant is useless as a forage, there is a possibility of obtaining a Trifolium species which might prove to be suitable material for breeding into the New Zealand pastures in an attempt to minimize the occurrence of bloat.

4. Tannins are common in the lotus species. If the problem of mixed regrowth that occurs in lotus major/white clover pastures could be overcome, a pasture containing clover and tannin-containing forage such as lotus, closely associated so that they are grazed together, might prove useful in controlling bloat.

5. These results add support to the hypothesis that soluble protein is an important factor in the aetiology of bloat.

CHAPTER 3

THE FOAMING PROPERTIES OF THE SOLBULE LEAF PROTEINS OF RED AND WHITE CLOVER, THE HOLOTRICH PROTOZOAL PROTEINS AND THE SALIVARY PROTEINS.

3.1 The foaming properties of the soluble leaf proteins

The research described in this chapter was undertaken to determine the conditicns required to produce stable protein foams. The types of apparatus used for measuring the foam properties are described in the methods, section (1. 3 a).

The results from the previous chapter (2) suggest that plant leaf proteins are the foaming agents involved in the bloat syndrome. The conditions required for stable foam production from these proteins are therefore studied in the following chapter. The proteins were dissolved in a sodium acetate buffer (0.1 mol/litre) which was adjusted to the desired pH with glacial acetic acid.

3.1 (a) Studies using the Mangan apparatus

(1) The effect of protein concentration on foam strength.

In order to determine the minimum concentration of protein required to produce strong foams, solutions of Fraction 1 and Fraction 2 proteins in 0.1 mol/litre acetate buffer, pH 5.8, of protein concentration 0.001 to 0.1% $^{w}/_{v}$ were foamed in the Mangan apparatus. The gas flow was 30 ml min^{-1}, and the temperature was maintained at $37^{o}C$. A porosity 2 micro-immersion filter was used to disperse the gas.

Results for Fraction 1 and Fraction 2 proteins are shown in Fig. 3 (1. 1). The minimum concentrations of Fraction 1 and Fraction 2 proteins required to produce a full column of foam were 0.005% and 0.008% $^{W}/_{V}$ respectively.

However, foams did not show any measurable foam strength until the concentration of Fraction 1 was 0.01% $^{W}/_{V}$ and Fraction 2 was 0.02%.

(2) The effect of bubble size on foam strength.

Solutions of Fraction 1 protein, at pH 5.8 and 37°C were foamed in the Mangan apparatus using different porosity sintered micro-immersion filters to disperse the gas. The porosity sinters were of the following pore size.

porosity 1	90-150 μm
porosity 2	40-50 μm
porosity 3	20-30 μm
porosity 4	5-10 μm

Foam strength was measured on these foams.

Results are shown in Fig. 3 (1. 2). The results indicate that bubble size is extremely important in determining the 'strength' of a foam. The large pore sinters produced loose large bubble foams of low 'strength' whereas the small porosity sinters produced solid minute bubble foams of high shear strength.

The number of interfacial films, the total surface area and therefore the amount of denatured protein per given volume of foam would be greater in the small bubble foam than in the large bubble foam and this possibly explains the differences found between the respective 'foam strengths'

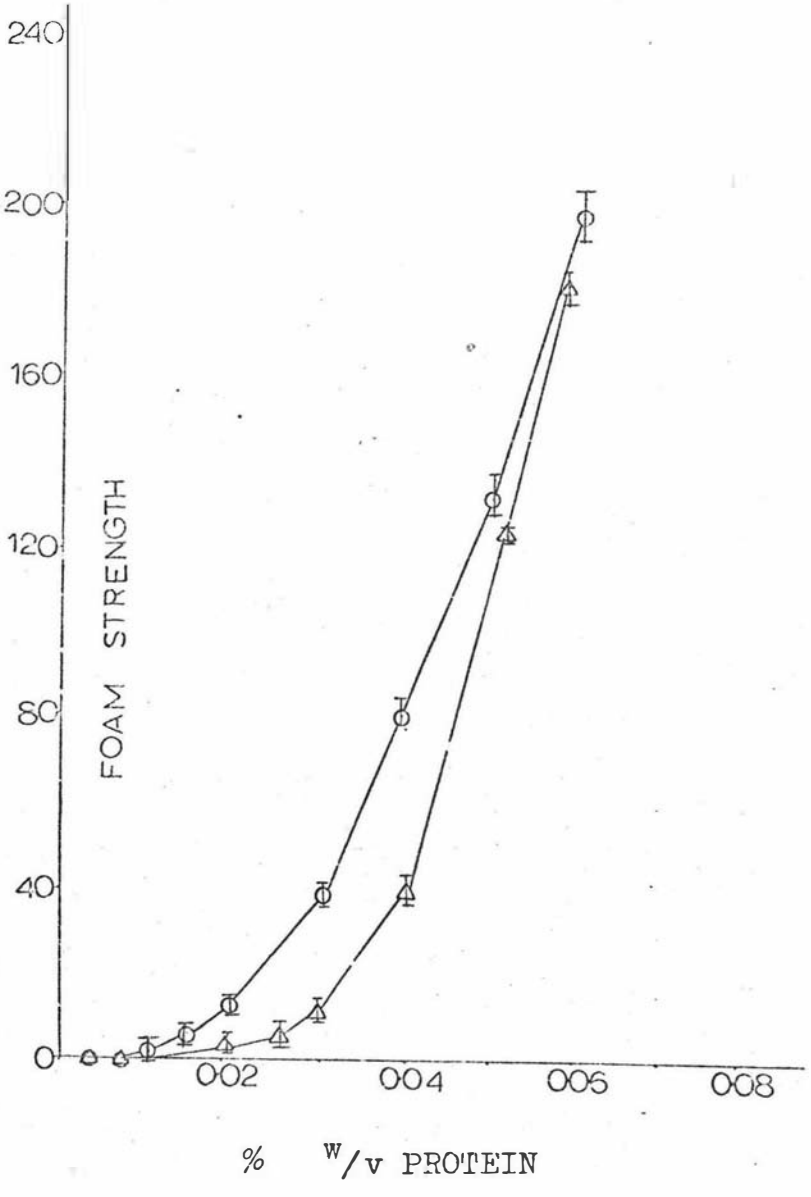

FIG. 3 THE EFFECT OF PROTEIN CONCENTRATION ON THE FOAM (1. 1) STRENGTH OF THE SOLUBLE PLANT PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre acetate(p.87); temperature 37°C; gas flow 30 ml min^{-1}; porosity sinter 2.

O——O Fraction 1 protein

Δ——Δ Fraction 2 protein

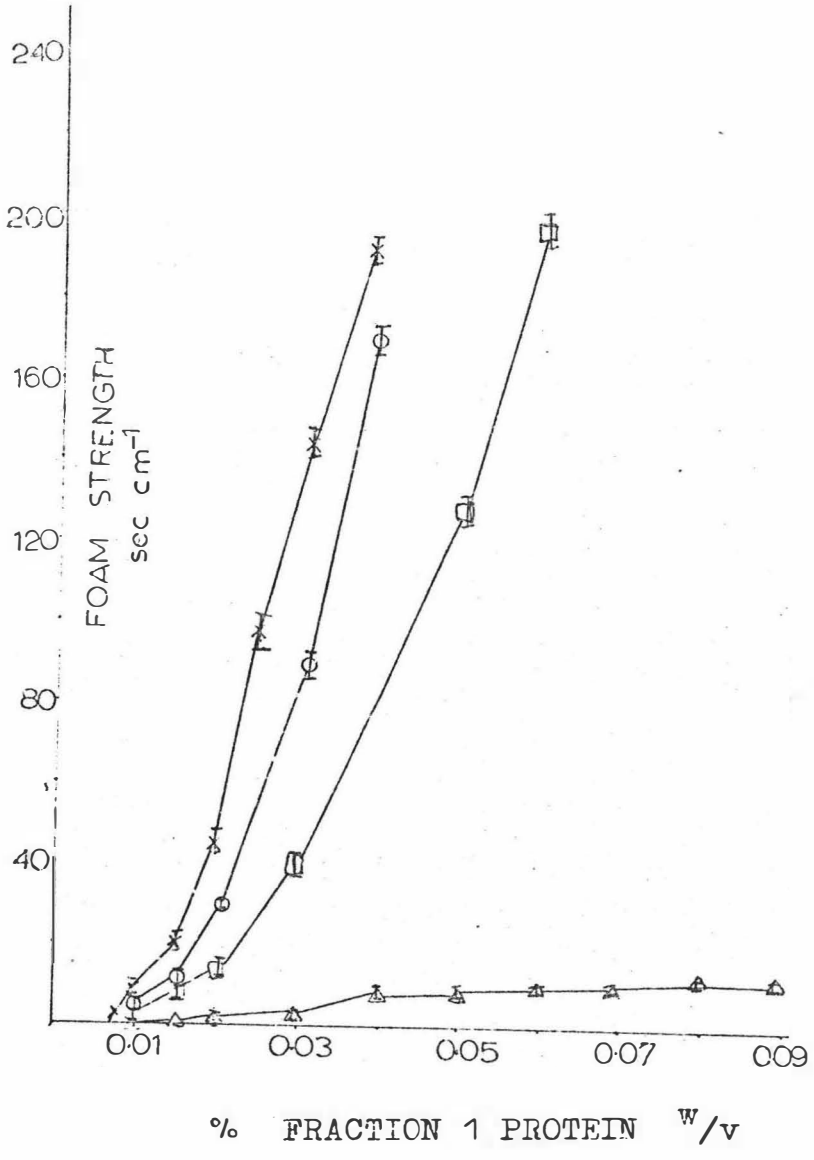

FIG. 3 THE EFFECT OF BUBBLE SIZE ON FOAM STRENGTH

(1. 2)

pH 5.8; buffer 0.1 mol/litre sodium acetate (p.87), temperature 37°C; gas flow 30 ml min^{-1}.

Δ——Δ porosity 1 sinter (90 to 150 μm)
□——□ porosity 2 sinter (40 to 50 μm)
○——○ porosity 3 sinter (20 to 30 μm)
×——× porosity 4 sinter (5 to 10 μm)

of these foams.

(3) The influence of temperature on 'foam strength'

An increase in temperature could be envisaged as decreasing the stability of interfacial films by decreasing the surface viscosity of the films, which in the case of foams would lead to an increase in the rate of drainage of the liquid from the foam. This would produce a rapid thinning of the film lamellae, thus hastening the rupture of the films and collapse of the foam.

Conversely in the case of protein films where the denatured protein in the surface is not in equilibrium with the protein in the bulk solution, an increase in temperature would increase the rate of diffusion of the protein molecules into the surface without causing an increase in diffusion of the molecules from the surface back to the bulk solution. An overall increase in the rate of denaturation of protein would result. However the rate of diffusion is proportional to the absolute temperature, and this effect would therefore be unimportant over the temperature range examined in this experiment.

Protein solutions containing 0.03% $^{w}/_{v}$ Fraction 2 proteins or 0.025% $^{w}/_{v}$ Fraction 1 protein in 0.1 mol/litre sodium acetate buffer pH 5.8, were foamed in the temperature range 5 to 55°C. A porosity 2 sinter was used to disperse the gas. Samples and apparatus were equilibrated to the desired temperature before the foam was generated.

Results are shown in Fig. 3 (1. 3).

The results indicate that increasing the temperature markedly reduces the foam strength of the plant protein

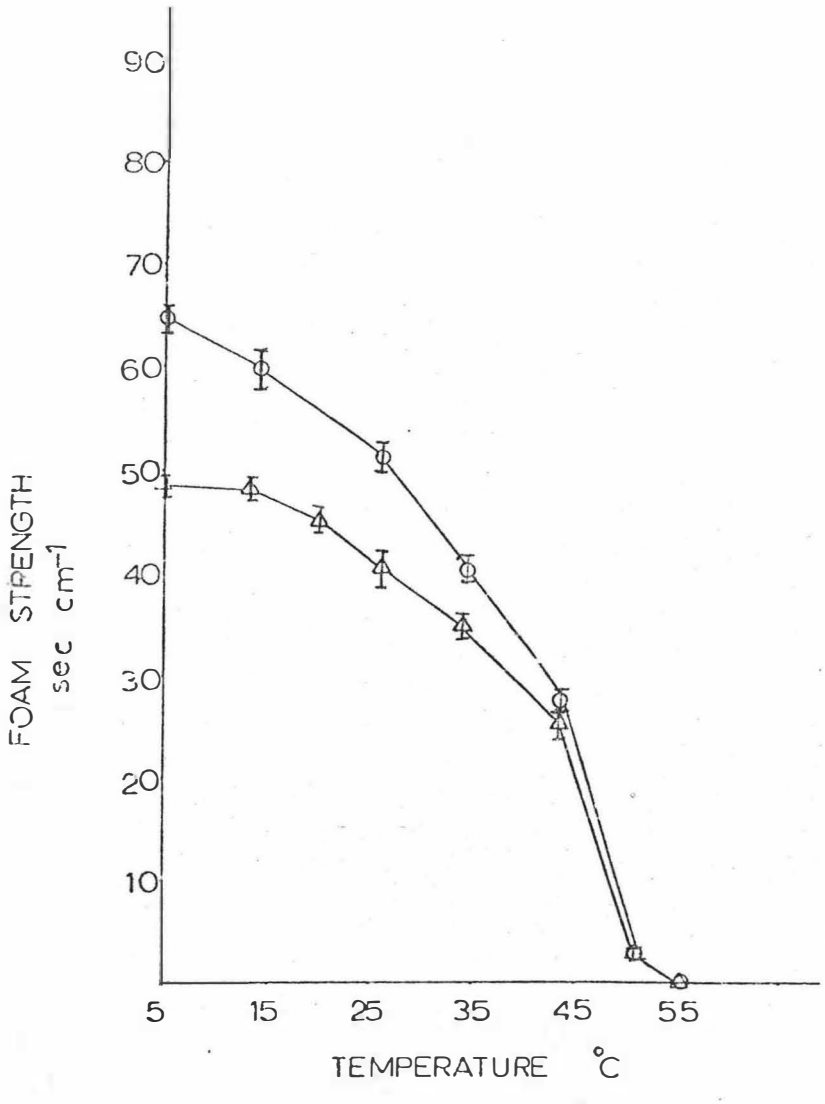

FIG. 3 THE INFLUENCE OF TEMPERATURE ON THE FOAM STRENGTH

(1. 3) OF PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre sodium acetate(p87)gas flow 30 ml min^{-1}; sinter porosity 2.

O——O (0.025% Fraction 1 protein)

Δ——Δ (0.03% Fraction 2 proteins)

foams. The decrease in foam strength as the temperature increases, is probably to be explained in terms of the dryness and brittleness of the foams at high temperatures, arising from the rapid drainage of liquid from the foam at these temperatures.

(4) The effect of pH on the foam strength of protein foams.

Studies on protein films (Cumper and Alexander, 1950) showed that the surface viscosity of protein films was maximum at pH's close to the isoelectric pH of the protein. At this pH the net charge on the protein molecule is zero, and the electrostatic repulsion between molecules is weakest, leading to maximum cohesion between the protein molecules that diffuse to the air liquid interface. The denatured protein, carrying zero net charge would be capable of intermolecular interaction by hydrogen bonding or Van der Waal's forces, giving rise to a rigid film of high surface viscosity.

Surface viscosity is an important factor involved in foam stability (Brown et al. 1953). If the maximum foam strength was related to surface viscosity it would be expected to occur at the isoelectric pH of the protein. Protein solutions containing 0.04% Fraction 1 or 2 in 0.1 M sodium acetate buffer were adjusted to pH's in the range 3.5 - 7.6. Foam strength was determined on the foam generated from these solutions. Results are shown in Fig. 3 (1. 4). The maximum 'foam strength' for Fraction 1 protein foams occur at pH 5.8. This pH is close to the

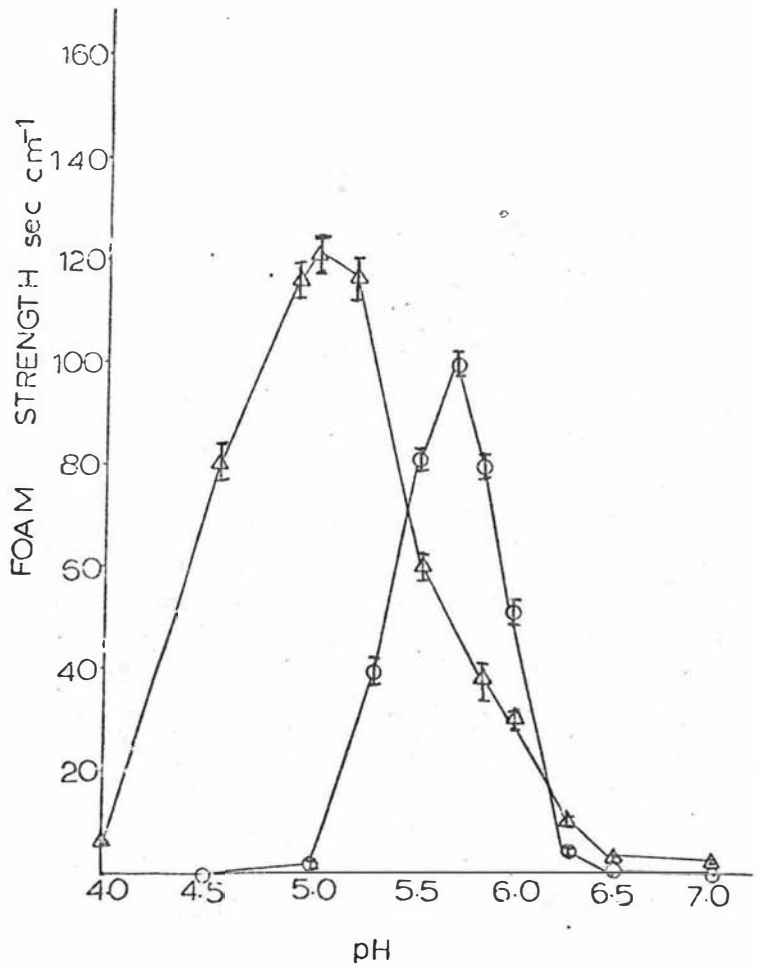

FIG. 3 (1. 4) THE EFFECT OF pH ON THE FOAM STRENGTH OF SOLUBLE LEAF PROTEINS OF WHITE CLOVER

Buffer 0.1 mol/litre sodium acetate(p.87), gas flow 30 ml min^{-1}; sinter porosity 2, temperature 37°C.

O——O 0.04% Fraction 1 protein

Δ——Δ 0.04% Fraction 2 protein

isoelectric pH of the protein molecule (Pon, 1967). The maximum 'foam strength' of Fraction 2 protein foams occurred at pH 5.1-5.2, although strong foams were produced over the pH range 4.0 to 6.3. This is not surprising, since Fraction 2 proteins are a heterogeneous mixture of proteins of different isoelectric pH's.

3.1 (b) Studies using the Laby apparatus

In 1969, Laby reported an apparatus which enabled him to characterise foams generated from a variety of surfactants. He measured four parameters of the foams. Two of these were derived from the foam drainage data by applying the second order relation $\frac{dv}{dt} = k_2(v_o - v)^2$ 1 (3. 1 b). The remaining two quantities are physical properties, the compressive strength and the stress relaxation, which are determined as described in part 1 (3. 1 b) of this thesis.

The variables, stress relaxation, the foam retention volume, and k_2 are reported to give some measure of persistence of foams (Laby, 1969). In this thesis k_2 was often difficult to interpret and therefore was not reported in the results which follow. However, an increase in persistence of foams was always accompanied by an increase in the foam retention volume, and a decrease in the stress relaxation.

The compressive strength gives a measure of the rigidity of the foams.

(1) The effect of protein concentration on the foam parameters.

Solutions of proteins containing 0.001% to 0.1% $^{w}/_{v}$ Fraction 1 or 2 protein were foamed in the Laby apparatus at 37°C and the parameters measured.

Results are shown in Fig. 3 (1. 5).

The minimum concentrations of Fraction 1 and Fraction 2 proteins required to produce foams having measurable stress relaxation were 0.02% and 0.03% $^{w}/_{v}$ respectively. The foam retention volume increased from 0.2 ml at 0.02% Fraction 1 to 3.6 ml at 0.1% protein. The foam retention volumes of Fraction 2 protein foams increased to 1.0 ml at 0.1% $^{w}/_{v}$ protein.

Although compressive strength of foams produced from solutions of Fraction 1 protein below 0.02% $^{w}/_{v}$ protein was high, the foams so produced either did not give a uniform column of foam or they collapsed instantaneously.

(2) <u>The relationship between compressive strength and bubble size</u>.

Foams were generated from solutions containing Fraction 1 protein (0.005 to 0.1%) in 0.1 mol/litre sodium acetate pH 5.8. The barrels of the liquid chambers of the Laby apparatus were fitted with sinters of porosity 1, 2 or 3. The gas flow was 30 ml min^{-1}. Results are shown in Fig. 3 (1. 6).

Compressive strength increased with decrease in the orifice size for any protein concentration, in a similar manner as did foam strength reported in 3 (1 a 2).

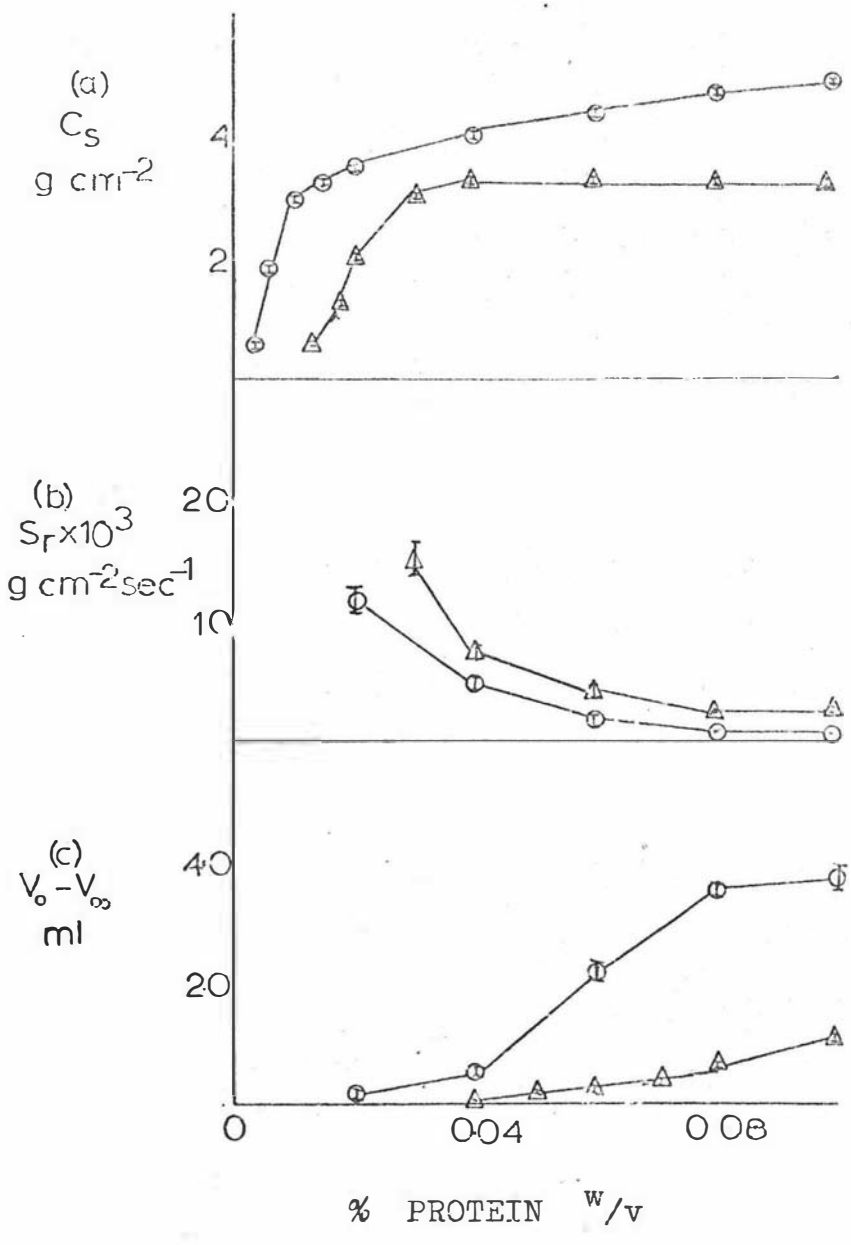

FIG. 3 (1. 5) THE EFFECT OF PROTEIN CONCENTRATION ON THE FOAM PARAMETERS OF SOLUBLE LEAF PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre sodium acetate (p.87); temperature 37°C; gas flow 30 ml min^{-1}; sinter porosity 3.

O——O Fraction 1 protein Δ——Δ Fraction 2 proteins

(a) compressive strength, C_s, g cm^{-2}

(b) stress relaxation, S_r, x 10^3 g cm^{-2} sec^{-1}

(c) foam retention volume, $V_o - V_\infty$, ml

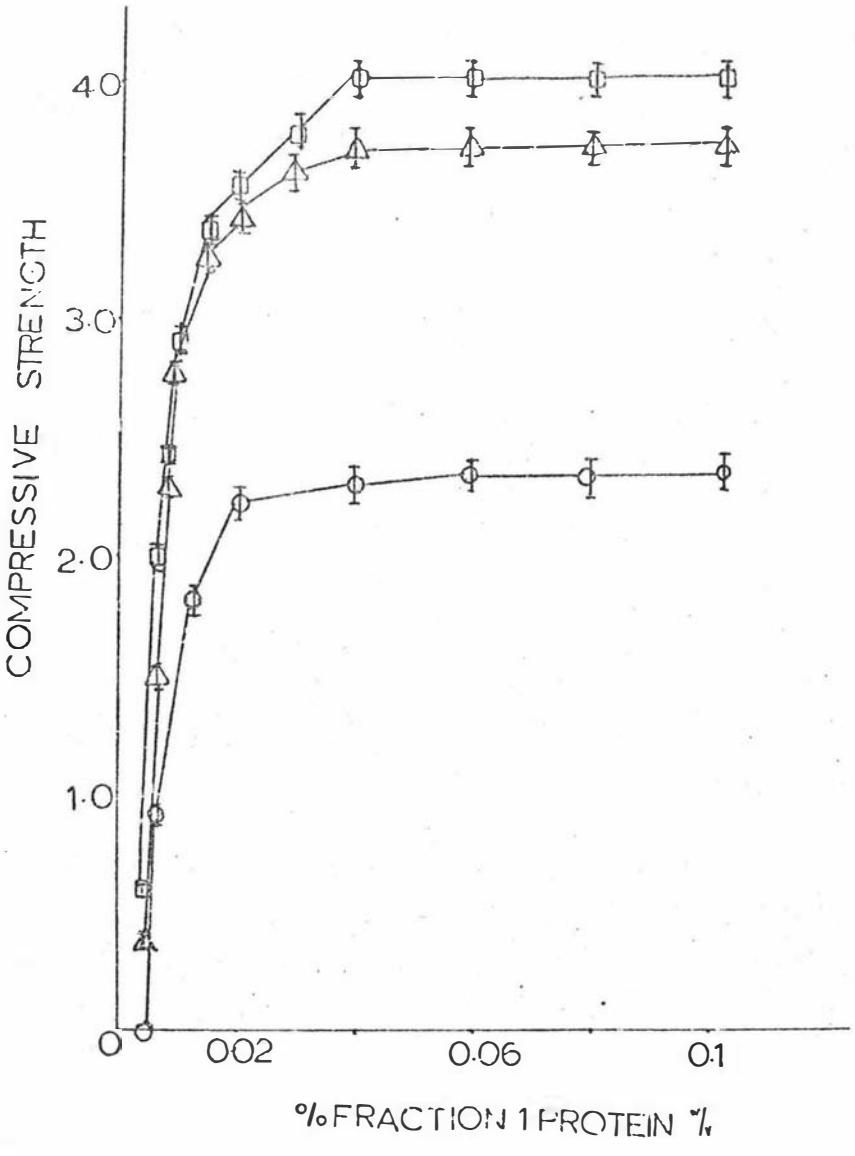

FIG. 3 (1. 6) THE EFFECT OF BUBBLE SIZE ON THE COMPRESSIVE STRENGTH OF FRACTION 1 PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre sodium acetate (p.87); temperature 37°C; gas flow 30 ml min^{-1}.

O——O porosity 1 sinter

Δ——Δ porosity 2 sinter

□——□ porosity 3 sinter

(3) Examination of compressive strength of foams at an early stage in their formation.

During the course of the previous experiments, it was observed that when solutions of low protein concentration were foamed, the foam chamber was filled with foam of a non-uniform structure. The upper part of the foam was composed of small bubbles, beneath which the foam was more open in structure and collapsed rapidly. On increasing the protein concentration, the volume of thick foam increased and the volume of open foam decreased. In order to determine whether the initial foam produced from solutions of low protein concentration was similar in properties to the initial foam produced from solutions of high concentration, a sensing disc was situated at the 15 ml volume mark on the foam chamber, and foam was generated to the 30 ml mark. Results for protein solutions in the range 0.003% - 0.10% $^{w}/_{v}$ Fraction 1 protein foamed at pH 5.8 and at 37°C for different porosity sinters are shown in Fig. 3 (1. 7).

Results indicate that foams are similar at concentrations above 0.008% $^{w}/_{v}$, but at lower protein concentrations there was a progressive increase in compressive strength with increase in protein concentration. The compressive strength was lower at this position of the sensing disc than when the disc was at the 60 ml position. This is presumably due to a difference in the liquid content of the foam at the two positions of the sensing disc. More liquid would have drained from the foam when

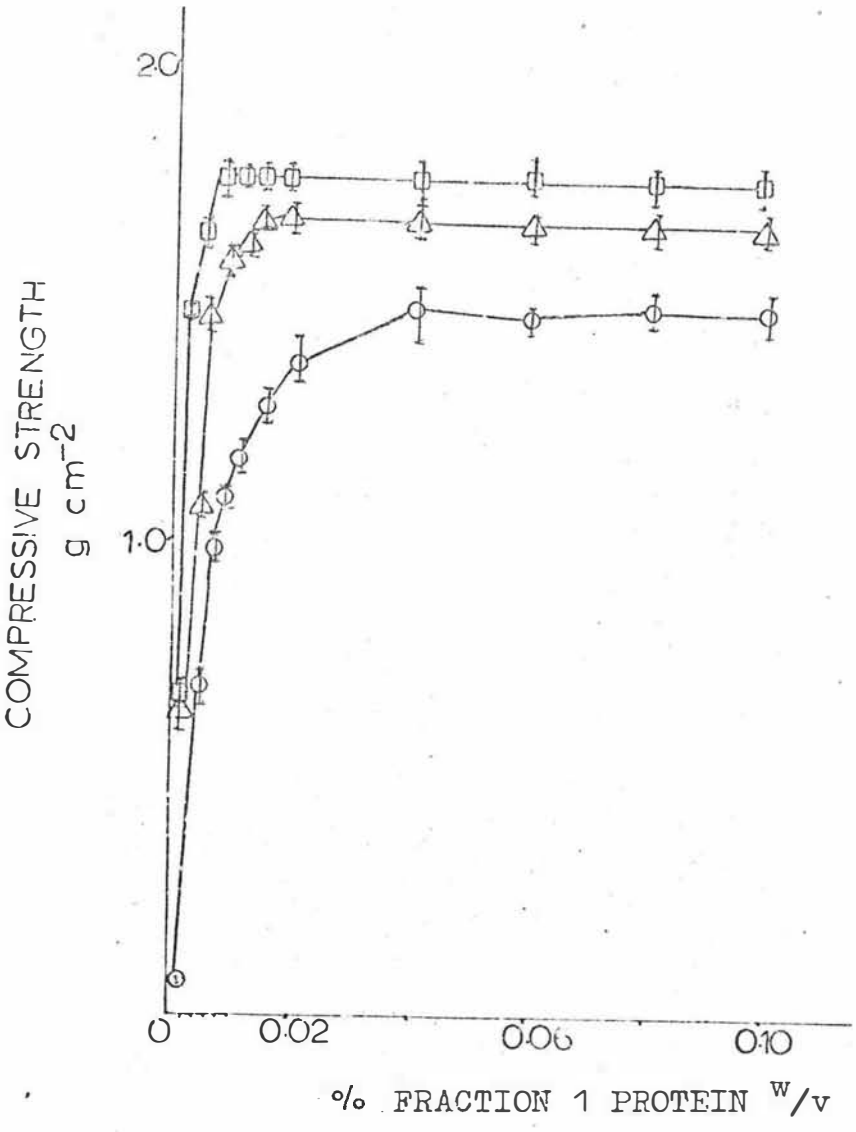

FIG. 3 (1. 7) THE COMPRESSIVE STRENGTH OF FOAMS AT AN EARLY STAGE IN THEIR FORMATION

pH 5.8; buffer 0.1 mol/litre sodium acetate (p.87), temperature 37°C; gas flow 30 ml min^{-1}; sensing disc at 15 ml mark; 30 ml foam generated.

O——O porosity 1 sinter
Δ——Δ porosity 2 sinter
□——□ porosity 3 sinter

the foam front was at the 60 ml mark than at the 15 ml mark and consequently the foam at the 60 ml mark is more rigid than the foam at the 15 ml mark, and would exhibit greater resistance to its flow past the sensing disc, giving a higher compressive strength.

The compressive strength reached a maximum at 0.008% Fraction 1 protein for the porosity 3 sinter, 0.012% for porosity 2 sinter and 0.04% $^{w}/v$ for the porosity 1 sintered filter.

(4) The effect of temperature on the compressive strength and stress relaxation of protein foams.

Foams were generated from protein solutions containing 0.02% $^{w}/v$ Fraction 1 protein or 0.03% Fraction 2 protein in 0.1 mol/litre acetate buffer, pH 5.8 in the temperature range 5° to 50°C.

Results are shown in Fig. 3 (1. 8) and Table 3 (1. 1).

Compressive strength increased with increase in temperature up to 10°C, remained constant from 10° to 25°C, then decreased at temperatures above 25°C.

Stress relaxation increased with increase in temperature. The most persistent foams were produced at low temperatures.

(5) The effect of pH on the foam properties of plant protein foams.

Foams were generated from solutions of 0.03% $^{w}/v$ Fraction 1 or 0.04% $^{w}/v$ Fraction 2 protein in 0.1 mol/litre acetate buffer over the pH range 3.5 to 7.5. The gas

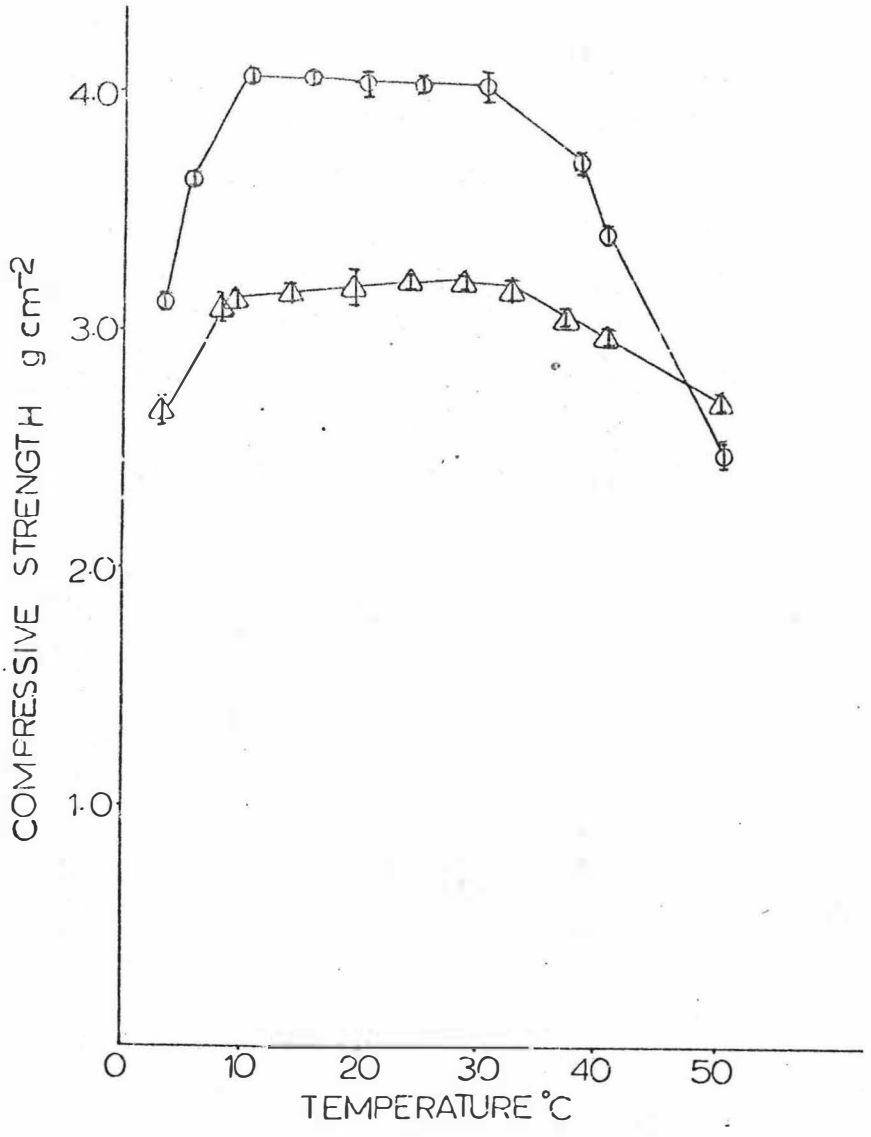

FIG. 3 (1. 8) THE EFFECT OF TEMPERATURE ON THE COMPRESSIVE STRENGTH OF PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre sodium acetate(p.87);gas flow 30 ml min^{-1}; porosity sinter 3.

O——O Fraction 1 protein

Δ——Δ Fraction 2 proteins

Temperature °C	Stress relaxation g cm^{-2} sec^{-1}	
	Fraction 1	Fraction 2
2	1.9×10^{-4}	3.3×10^{-3}
5	1.9×10^{-4}	3.3×10^{-3}
10.5	2.5×10^{-4}	6.0×10^{-3}
15	1.25×10^{-3}	6.1×10^{-3}
20	3.3×10^{-3}	6.1×10^{-3}
25	5.2×10^{-3}	6.1×10^{-3}
30	9.2×10^{-3}	9.0×10^{-3}
37	13×10^{-3}	24×10^{-3}
41	18×10^{-3}	
50		

TABLE 3 (1. 1) EFFECT OF TEMPERATURE ON STRESS RELAXATION OF LEAF PROTEIN FOAMS

pH 5.8; buffer 0.1 mol/litre sodium acetate(p.87); gas flow 30 ml min^{-1}; porosity sinter 3.

flow was 30 ml min^{-1}, and the foam was produced by dispersing the gas through a porosity 3 sinter into the protein solution in the Laby apparatus. The sensing disc was situated at the 60 ml mark and foam was generated to the 80 ml mark. Results are shown in Fig. 3 (1. 9).

Compressive strength was maximum at pH 5.8 and 5.1 for Fraction 1 and Fraction 2 proteins respectively. This is in agreement with the results reported for 'foam strength' measurements in 3 (1. a 4).

Strength relaxation showed greatest persistence for foams generated from solutions with pH's in the range 5.8 to 6.0 for Fraction 1 protein, and in the range 4.5 to 5.4 for Fraction 2 proteins, though the latter were stable over the pH range 4.5 to 6.3.

(6) Relationship between the compressive strength of Fraction 1 protein foams and the amount of denatured Fraction 1 protein in a fixed volume of foam as a function of pH.

Solutions of Fraction 1 protein in 0.1 mol/litre sodium acetate was foamed in the pH range 4.0 to 7.5 as described in 3 (1. b 5).

The amount of Fraction 1 protein denatured on foaming was measured as the difference in protein content of the solution, at the pH of the experiment, before foaming it and after foaming the solution to 80 ml in the Laby apparatus, and allowing the foam to collapse. The Fraction 1 protein was determined using the acrylamide gel electrophoretic technique in Chapter 1. Results are plotted in Fig. 3 (1. 10).

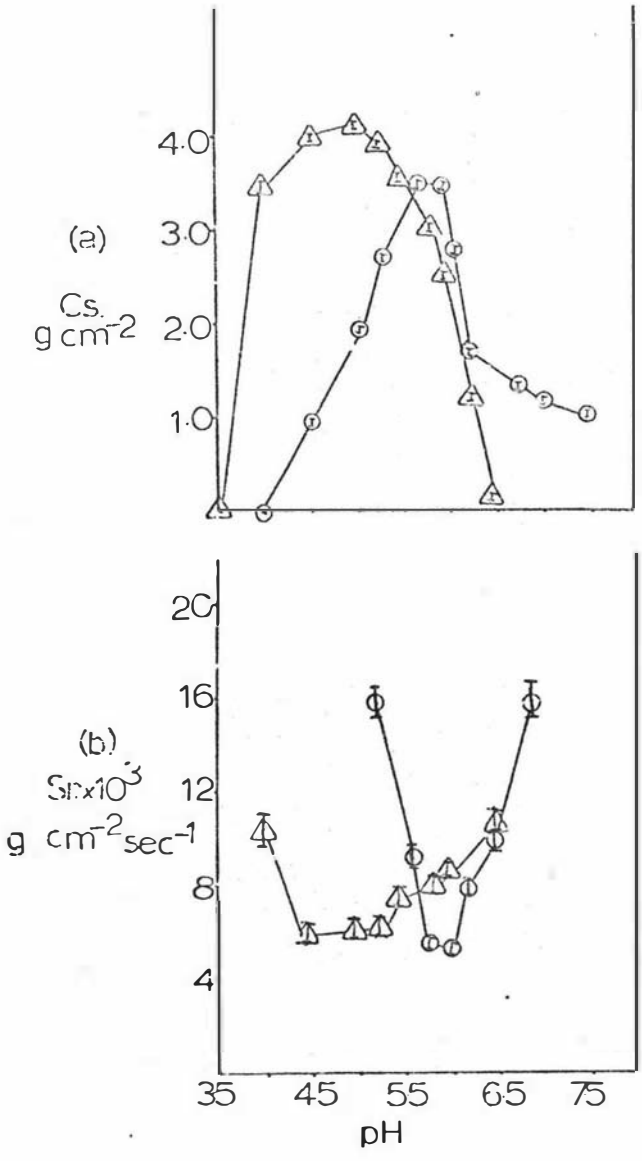

FIG. 3 (1. 9) THE EFFECT OF pH ON THE FOAM PROPERTIES OF THE SOLUBLE LEAF PROTEINS

buffer 0.1 mol/litre sodium acetate(p.87);temperature 37°C; gas flow 30 ml min^{-1}; sinter porosity 3.

Δ—— Δ Fraction 2 proteins O——O Fraction 1 protein

(a) compressive strength, C_s, g cm^{-2}

(b) stress relaxation, S_r, g cm^{-2} sec^{-1}

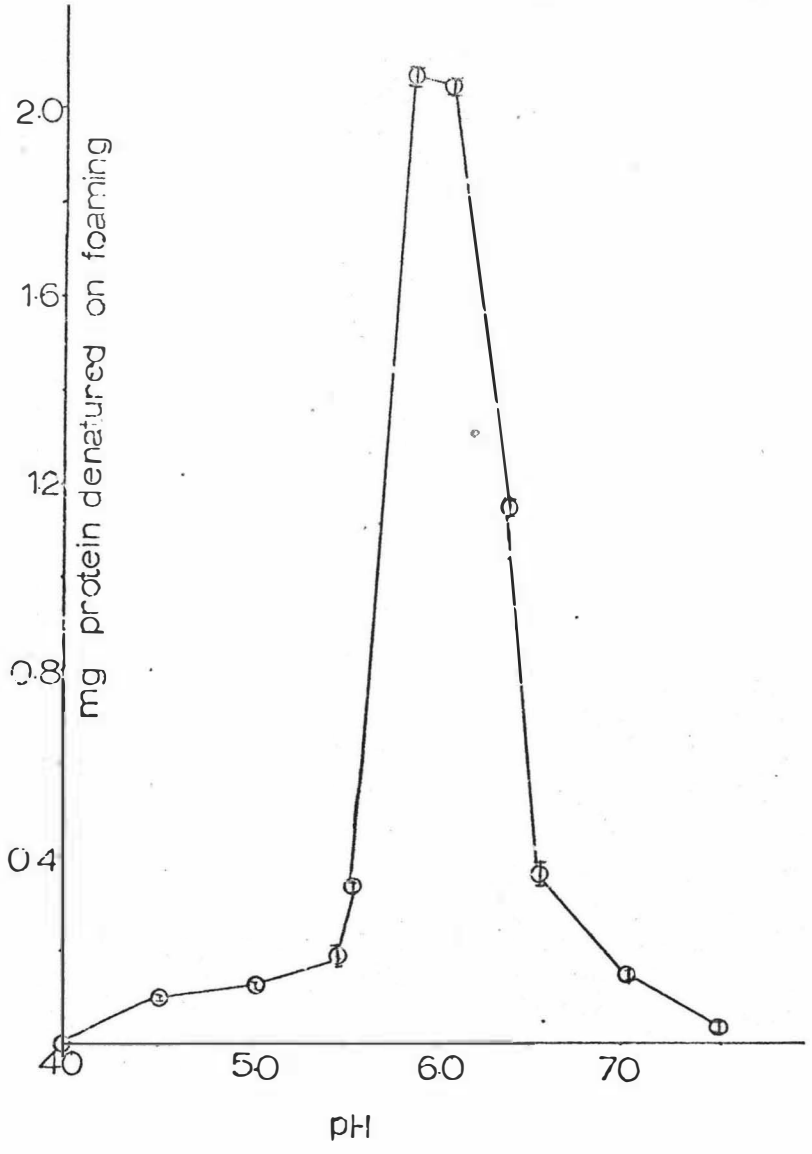

FIG. 3 (1. 10) THE RELATIONSHIP BETWEEN THE AMOUNT OF SURFACE DENATURED FRACTION 1 PROTEIN IN A FIXED VOLUME OF FOAM AT DIFFERENT pH's

buffer 0.1 mol/litre sodium acetate(p.87);gas flow 30 ml min^{-1}; temperature 37°C. sinter porosity 3.

It can be seen that the amount of Fraction 1 protein denatured on foaming is correlated with the compressive strength of the foam.

Fig. 3 (1. 10) shows a sharp maximum at pH 5.9 in the amount of denatured Fraction 1 in the foam. Below this pH, the amount of Fraction 1 protein denatured falls rapidly. This can be explained in terms of the low solubility of this protein at pH's lower than pH 5.8 3(1. b. 8). The protein has aggregated and is therefore not present in solution in a form suitable for foaming. Therefore the fall in the amount of denatured protein at pH values below 5.8 is not due to any decrease in the rate of denaturation of Fraction 1 protein but due to protein having aggregated out of solution as a precipitate prior to foaming.

(7) Fraction 2 proteins denatured in the range pH 3.5 to 7.5

Solutions of Fraction 2 proteins (0.1% $^{w}/_{v}$) in 0.1 mol/litre acetate buffer were foamed in the Laby apparatus at pH values in the range 3.5 to 7.5. The proteins removed from solution by surface denaturation in producing the foam were examined by acrylamide gel electrophoresis of the protein solution before and after foaming. The gels were stained for protein with Coumassie blue (Chapter 1) and scanned using a microdensitometer. Fig. 3 (1. 11) shows the microdensitometer tracings of these gels.

(8) The solubility of Fraction 1 and Fraction 2 proteins at various pH's.

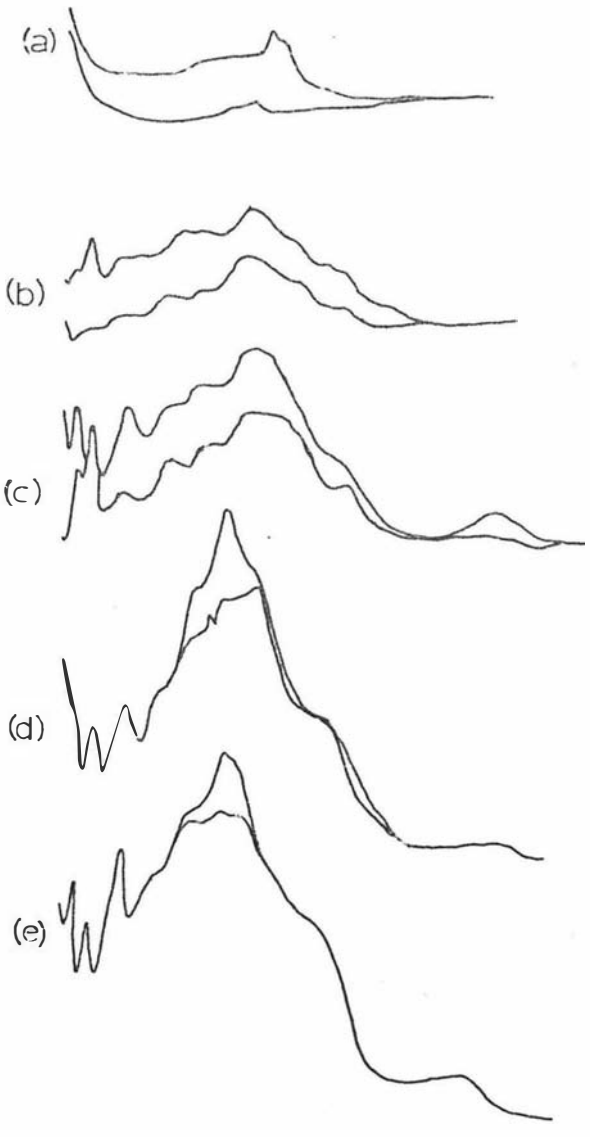

FIG. 3 (1. 11) MICRODENSITOMETER TRACINGS OF PROTEINS SEPARATED BY ACRYLAMIDE GEL ELECTROPHORESIS SHOWING FRACTION 2 PROTEINS DENATURED ON FOAMING AT VARIOUS pH'S

(a) pH 4.0; (b) pH 5.0; (c) pH 5.6; (d) pH 6.5; (e) pH 7.5.
Top line before foaming; Bottom line after foaming.

Solutions of plant proteins (0.1%) dissolved in 0.1 mol/litre sodium acetate were adjusted to pH's in the range 3.5 - 7.6. The solutions were allowed to stand at 0°C for 30 minutes and centrifuged at 16,000 g for 10 minutes to remove any aggregated protein. The optical density at 280 nm was measured on the supernate. Results are plotted in Fig. 3 (1. 12) as the % optical density at 280 nm of the protein at the particular pH, compared with the optical density of the solution at pH 7.5. Fraction 1 protein showed minimum solubility at pH's below 5.8. This is the pH at which maximum foam strength and persistence as well as maximum surface denaturation of this protein occurs.

Fraction 2 proteins showed a steady decrease in solubility over the range pH 7.5 - 3.5, although most of the protein was precipitated in the pH range 4.0 - 5.4.

Summary

1. Fraction 1 and Fraction 2 proteins produce foams of maximum 'foam strength', F_s, and compressive strength, C_s, and persistence at pH's close to the isoelectric pH of the proteins. In the case of Fraction 1 protein, the foam parameters indicate maximum strength and persistence at approximately 5.8, which is close to the isoelectric pH of the protein (Pon, 1967).

Fraction 2 proteins exhibited maximum F_s and C_s at pH 5.1 to 5.2, although persistent foams were generated over the

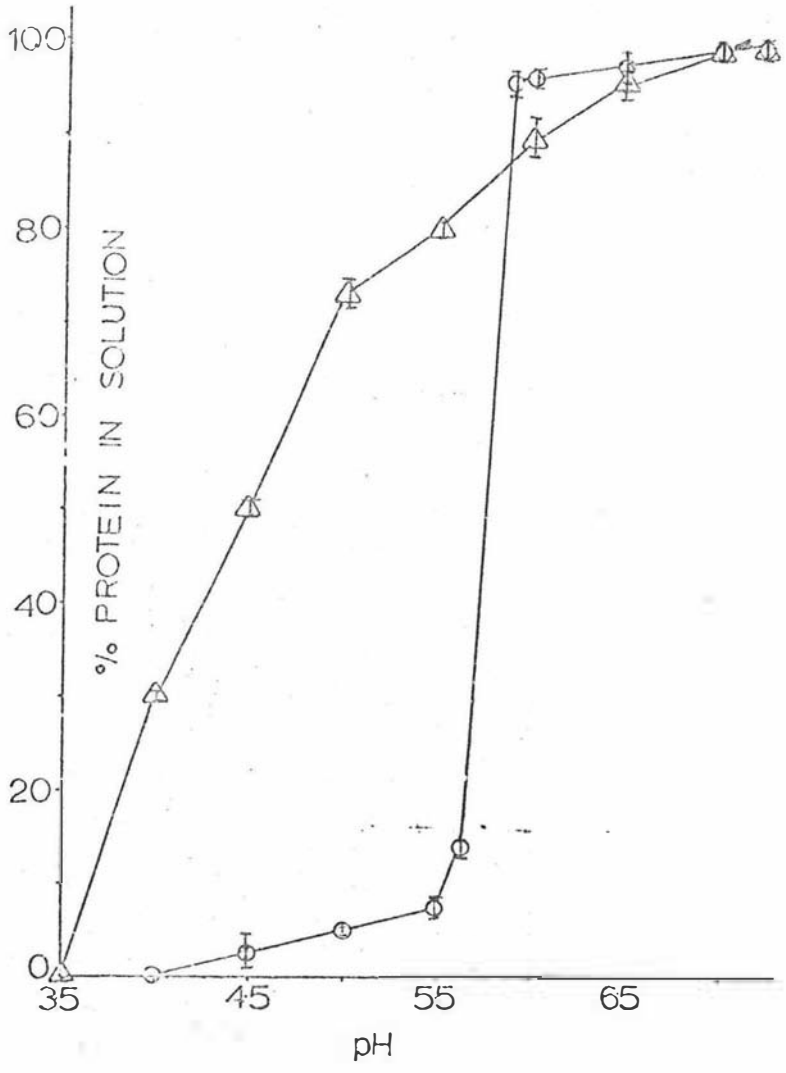

FIG. 3 (1. 12) THE EFFECT OF pH ON THE CONCENTRATION OF PROTEINS IN SOLUTION

Buffer 0.1 mol/litre sodium acetate (p.87).

O——O 0.1% Fraction 1 protein

Δ——Δ 0.1% Fraction 2 proteins

pH range 4.5 to 6.3. This wide pH range is presumably explained by the heterogeneous nature of these proteins giving rise to a range of isoelectric pH's.

The rate of denaturation and the amount of denatured protein in the foam, were also maximum at the isoelectric pH of Fraction 1 protein. The compressive strength of the foam was closely related to the amount of denatured Fraction 1 protein in the foam, and also indicates the rigidity of the foam or its resistence to flow past the sensing disc.

2. The limiting concentrations required to produce foams, of foam tc liquid expansion of 8/1 v/v and also showing measurable stress relaxation were 0.02% $^{w}/_{v}$ for Fraction 1 protein and 0.03% $^{w}/_{v}$ for Fraction 2 proteins. Increase in protein concentration above this limiting concentration produced an increase in the persistence of the foam, indicated by a decrease in the stress relaxation and an increase in the foam retention volume, and a slight increase in the compressive strength of the foam.

3. The most persistent foams were produced at low temperatures. Foam strength fell to zero at temperatures above 50°C.

4. Bubble size was shown to be an extremely important factor governing the foam strength and compressive strength of foams. The smaller the bubble size the greater the strength of the foams generated from a given protein concentration.

The growth of bubbles in the rumen could be an important factor governing the formation of rumen bloat foams. Any factor preventing coalescence of bubbles or limiting the size of the generated bubbles would favour the formation of a stable foam.

The Laby apparatus was more useful than the Mangan apparatus since:

(a) In the Laby apparatus four parameters could be measured which defined more precisely the nature of foams, whereas the Mangan apparatus defined only one parameter.

(b) The time dependent variable (stress relaxation), and the volume of liquid retained in the foam associated with the denatured protein gave a measure of the persistence of the foam.

It was decided that since the Mangan apparatus could not offer any additional information that was not obtainable with Laby apparatus, and that the Laby apparatus would be more useful in the study of the action of foam stabilising and antifoaming agents on protein foams, the Laby apparatus would be the only system used in further foam studies.

3.1 (c) <u>The action of the plant phenol/phenoloxidase system on plant proteins and its effect on the foams generated from them.</u>

When extracts of red clover are made, the solutions darken rapidly in air. The brown pigment is associated with soluble plant proteins and it is impossible to remove this pigment from the proteins. The pigment is

produced by the action of the polyphenoloxidase enzyme on the plant phenolic substrate to produce quinones. The quinones can react with hydroxyl, sulphydryl, or amino functional groups on the proteins to produce the pigmented phenolic-protein complexes.

Rideal and Schulman (1939) showed that the phenolics increased the rigidity of gliadin monolayers. In view of the findings of Brown et al. (1953) that surface viscosity is important in determining the stability of foams, the phenolics contained in leaf extracts could be important in stabilizing plant protein foams.

(1) Analyses on acrylamide gel electrophoresis of plant proteins which have and have not reacted with the phenoloxidase/phenolic system.

Red clover leaves were ground into the following buffers:

(a) KH_2PO_4/Na_2HPO_4, pH 7.0, ionic strength 0.1, containing 0.1 mol/litre mercaptoethanol.

(b) The same buffer containing in addition 10^{-4} mol/litre sodium diethyldithiocarbamate to inhibit the phenoloxidase enzyme.

The leaf extracts were centrifuged at 16,000g for 30 minutes to sediment the insoluble plant fragments.

Solution (a) turned dark brown very rapidly whereas solution (b) remained pale yellow in colour, indicating that phenoloxidase was active in solution (a) but inactive in solution (b).

Equal volumes of (a) and (b) were mixed and designated as solution (c). 25 mm^3 of solutions (a), (b) and (c) were subjected to acrylamide gel electrophoresis (Section 3 - 1. a) and the gels stained for protein with Coumassie blue. Photographs and scans of the gels are shown in Fig. 3 (1. 13).

It can be seen that the result of interaction between Fraction 1 protein and the phenoloxidase system was to increase the mobility of the protein in the gel. The mixed sample shown two distinct Fraction 1 protein bands. The band of higher mobility corresponds to the Fraction 1 protein, associated with the products of polyphenoloxidase action, and the slower moving band corresponding to the Fraction 1 protein of the system in which the phenoloxidase enzyme was inhibited by sodium diethyldithiocarbamate.

The difference in mobility can be explained by the Fraction 1 phenol complex having a greater net negative charge distribution on the molecule than the natural Fraction 1 protein at the pH (8.6) of the gel, due to the ionization of the hydroxyl groups on the phenolic molecule.

The action of the phenoloxidase system on the Fraction 2 proteins was to change the protein pattern on acrylamide gel from a series of well defined bands to a smearing of protein throughout the gel. This could arise from phenolics binding to different degrees to the proteins, producing from a single protein a series of

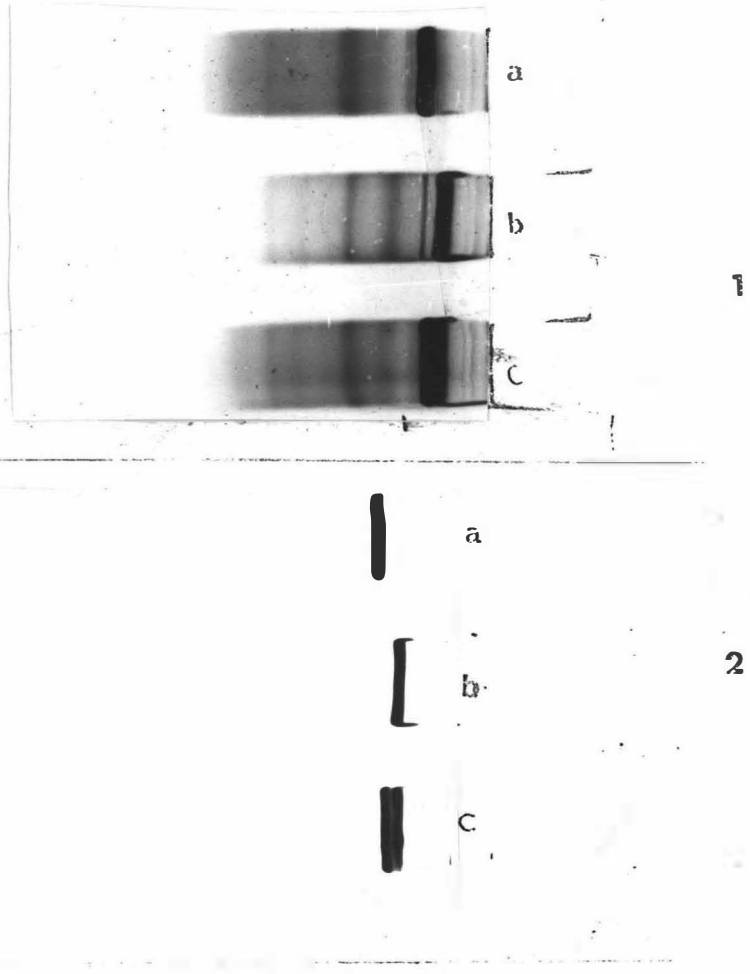

FIG. 3 THE ACTION OF PHENOL/PHENOLOXIDASE SYSTEM ON

(1. 13) THE SOLUBLE LEAF PROTEINS

(a) phenoloxidase active

(b) phenoloxidase inhibited with 10^{-4} mol/litre SDDC

(c) equal volumes of solutions (a) and (b)

2 is photograph 1 underexposed to show more clearly the two Fraction 1 bands in sample (c)

association products of different charge density and different molecular dimensions.

The phenol/phenoloxidase system has therefore modified the nature of the plant proteins, and could possibly affect their foam properties.

(2) The influence of the phenoloxidase interaction with plant proteins on the foam parameters of foams derived from them.

Plant proteins were prepared from red clover as described in Chapter 1 (2. a). Solutions of Fraction 1 or Fraction 2 proteins containing 1 mg ml^{-1} in 0.1 mol/litre acetate buffer, pH 6.5, were incubated with an amount of red clover phenolics to give an equivalent protein/phenolic ratio to that found in the plant. After addition of three units of phenoloxidase (mushroom source, Calbiochem), the samples were incubated at 30°C for 1 hour.

All measurements of foam parameters were made in the Laby apparatus at 37°C and pH 5.8. The gas flow was 30 ml min^{-1}. Foam was generated to the 80 ml mark and the sensing disc was situated at the 60 ml graduation.

The foams generated from the plant proteins treated with the phenol/phenoloxidase system differed from those from the natural proteins in the following respect:

(1) The limiting concentration of Fraction 1 protein and Fraction 2 proteins required to produce stable foams were 0.02% and 0.03%^{w}/v for the natural proteins and 0.03% and 0.02% respectively for the treated proteins.

(2) In the concentrations range 0.05% to 0.10%, stress relaxation and foam retention volume indicated that the treated Fraction 1 protein produced slightly less persistent foams than the natural protein, whereas the treated Fraction 2 proteins produced slightly more persistent foams than the natural Fraction 2 proteins. The compressive strength of the treated protein foams was reduced from 4.5 to 3.7 g cm^{-2} for 0.1% Fraction 1 protein foams whereas the compressive strength of the Fraction 2 protein foams had compressive strength 3.3 g cm^{-2} for 0.1% $^{w}/_{v}$ treated and natural protein foams.

3.2 The foaming properties of protozoal and salivary proteins

The other proteins that could have contributed to the high percentage of protein found in the rumen foam (Bartley, Bassette, 1961) are those from the animal's saliva and from the contents of the holotrich protozoa after lysis.

Mangan (1958) demonstrated that submaxillary mucoprotein produced very persistent foams of low foam strength.

Clarke (1964) observed a sudden decrease in the holotrich population when cattle were stall-fed lush bloat-producing red clover. The holotrich protozoa have a metabolic abnormality whereby in the presence of excess substrate, they are unable to stop synthesizing reserve polysaccharide (Sugden and Oxford, 1952). When

cattle are fed lush red clover, high levels of soluble sugars are released into the rumen. The holotrich protozoa feed on the soluble sugars to such an extent that they burst releasing their contents into the rumen. It has been suggested (Clarke, 1966) that these soluble contents, being high in proteins could assist in shifting the balance between foaming and anti-foaming agents in the rumen in favour of the bloating condition.

It would appear that the salivary proteins and the protozoal proteins could be important factors involved in the bloat syndrome and therefore their foaming properties were studied in this chapter.

(a) The effect of concentration on the foam properties of the salivary secretions and the protozoal proteins was studied with the materials prepared as described in Chapter 1 (2). Solutions of salivary mucoprotein, oesophageal mucin, and protozoal proteins were foamed at varying concentrations in the Laby apparatus at pH 5.8 and at 37°C. The oesophageal mucin did not generate stable foams under these conditions. Results for the compressive strength and stress relaxation of these foams are given in Fig. 3 (2. 1. a. b.). The salivary mucoprotein could produce persistent foams of low compressive strength. The minimum concentration of this material required to produce full columns of foam having measurable stress relaxation was approximately 0.015% $^{w}/_{v}$. At concentrations of mucoprotein above 0.02%, the compressive strength remained constant.

Protozoal proteins produced stable foams similar in

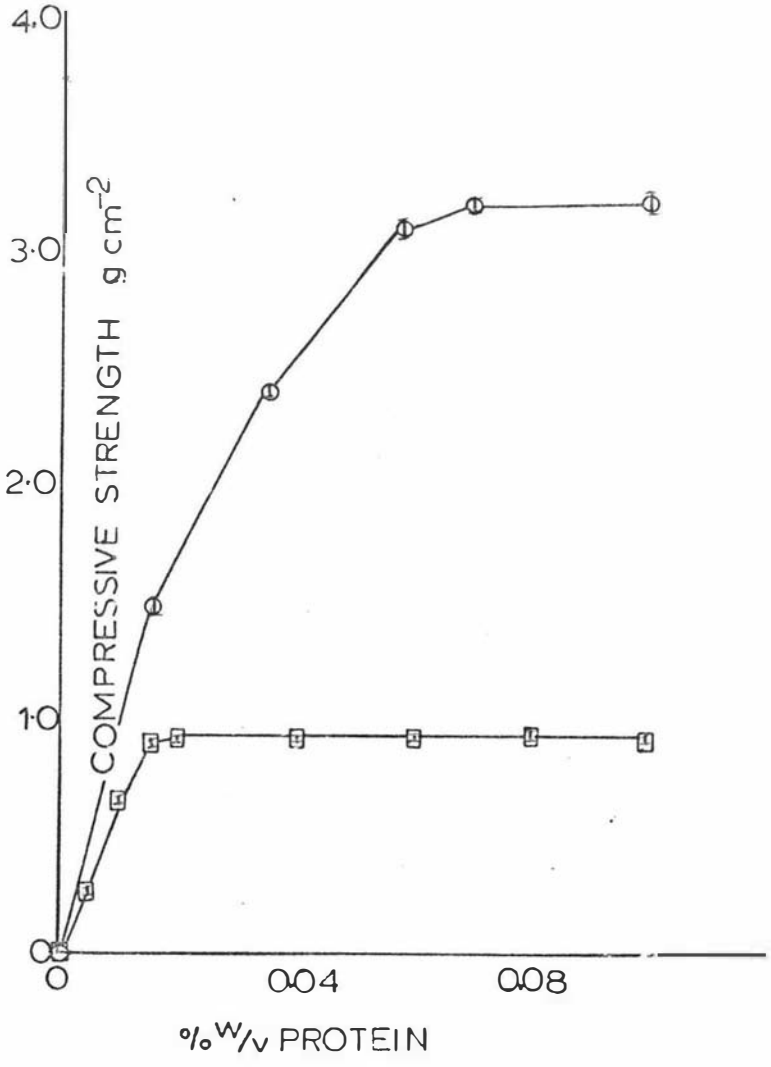

FIG. 3 (2.1a) THE EFFECT OF CONCENTRATION ON THE COMPRESSIVE STRENGTH OF HOLOTRICH PROTOZOAL PROTEIN AND SALIVARY MUCOPROTEIN FOAMS

O——O Holotrich protein foams

□——□ Salivary mucoprotein foams

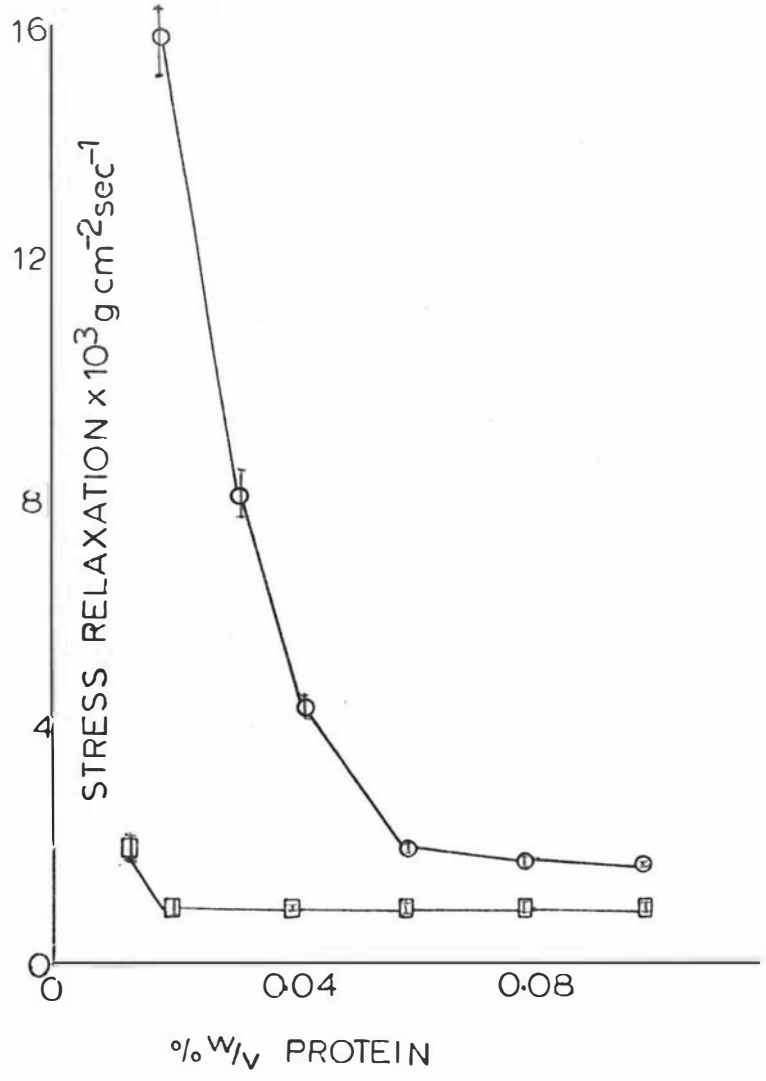

FIG. 3 (2. 1b)

THE EFFECT OF PROTEIN CONCENTRATION ON THE STRESS RELAXATION OF HOLOTRICH PROTOZOAL PROTEIN AND SALIVARY MUCOPROTEIN FOAMS

O——O Holotrich protozoal protein foams

□—□ Salivary mucoprotein foams

character to the plant protein foams. Compressive strength increased and stress relaxation decreased over the concentration range examined.

(b) The effect of pH on the foaming properties of salivary and protozoal proteins. Solutions of 0.04% bovine salivary mucoprotein and 0.065% protozoal proteins in 0.1 mol/litre sodium acetate buffers were foamed in the Laby apparatus over the pH range 4.0 - 7.6. Results for stress relaxation and compressive strength measurements are shown in Fig. 3 (2. 2.,a. b.)

The compressive strength and stress relaxation of the salivary mucoprotein foams were not affected by changes in pH over the entire pH range examined. The protozoal proteins however produced foams with parameters which were dependant on the pH of the solution. Compressive strength of these foams increased rapidly with decrease in pH below pH 6.5, levelling off at pH 5.5 - 5.9, then increasing and levelling off again at pH 5.0 - 4.0. Stress relaxation indicated a maximum persistence at pH's close to 5.9. Stress relaxation increased rapidly below pH 5.2.

Summary

Salivary mucoprotein foams are characterized by the following properties.

1. They produce foams of low compressive strength and low stress relaxation.

2. They are unaffected by changes in pH over the range pH 4.0 - 7.6.

Protozoal proteins behave more like the plant proteins producing foams that have high compressive strength and

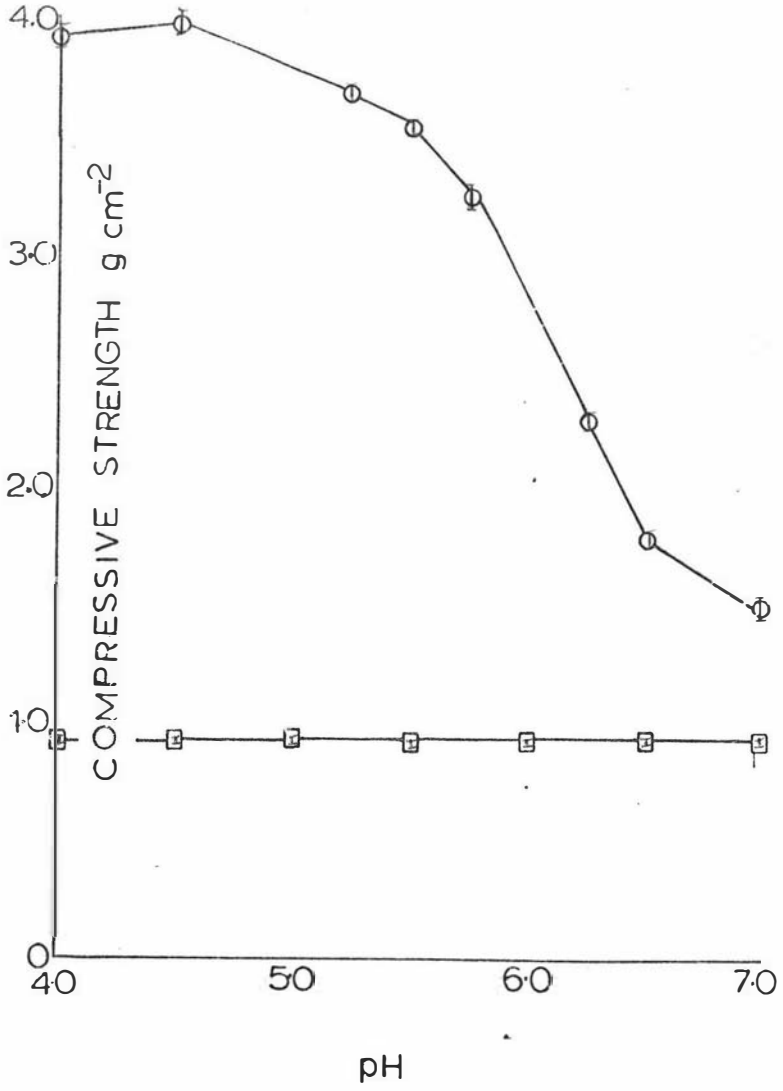

FIG. 3 (2. 2a) THE EFFECT OF pH ON THE COMPRESSIVE STRENGTH OF HOLOTRICH PROTOZOAL PROTEIN AND SALIVARY MUCOPROTEIN FOAMS

Δ- —Δ Holotrich protozoal protein foams

□ — □ Salivary mucoprotein foams

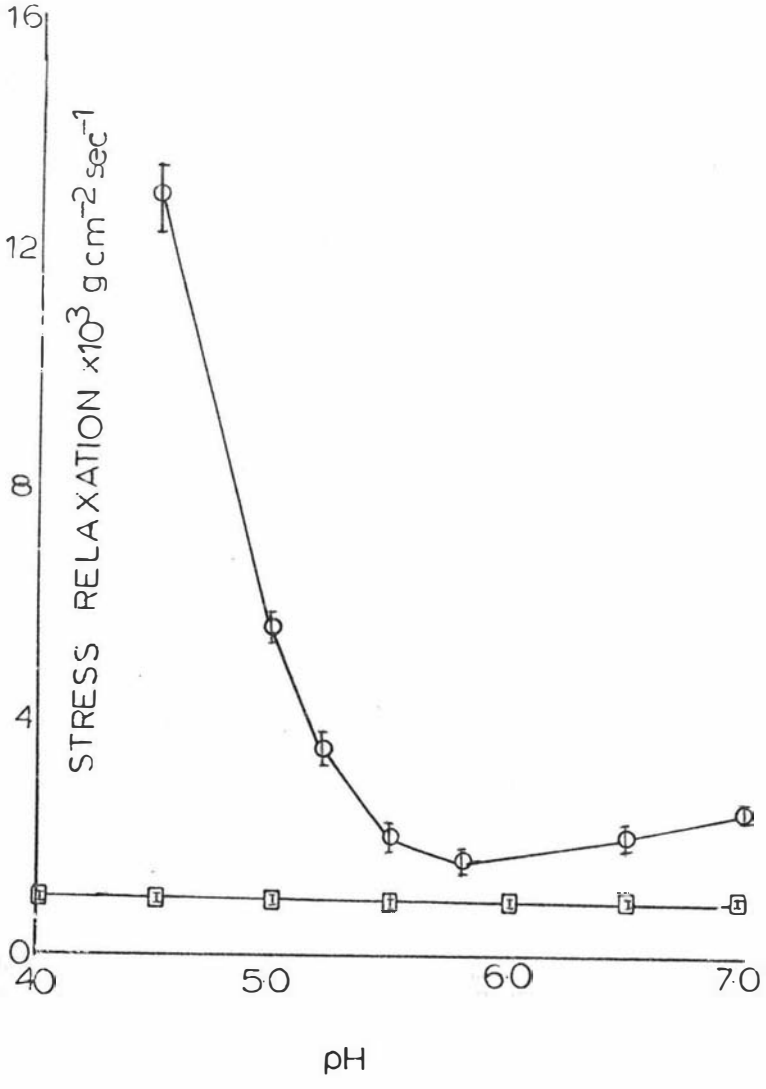

FIG. 3 (2. 2.b.) THE EFFECT OF pH ON THE STRESS RELAXATION OF HOLOTRICH PROTOZOAL PROTEIN AND SALIVARY MUCOPROTEIN FOAMS

O——O Holotrich protozoal protein foams

□——□ Salivary mucoprotein foams

low stress relaxation. Also the protozoal proteins show maximum persistence at pH's close to pH 5.9 and maximum compressive strength at pH's below 5.2.

CHAPTER 4

STUDIES ON RUMEN LIQUOR

Reports concerning the properties of rumen foams have been very sparse. Mangan (1958, 1959, 1962) showed that the foams generated *in vitro* from crude rumen liquor were of very low foam strength. The centrifuged rumen liquor produced foams of a higher foam strength with a maximum value at pH's close to 5.7.

Laby (1969) reported that the parameters of rumen foams measured in his apparatus showed the foams to be of low compressive strength but of high persistence.

In the previous Chapter of this thesis, the foaming properties of the soluble plant and animal proteins were studied, and in this Chapter the properties of the rumen foams generated *in vitro* will be examined.

Animals were graded for bloat severity according to Johns (1954). Ten dry fistulated cows, including four pairs of identical twins, were examined over a period of two years. The animals were stall-fed twice daily on a pure stand of red clover, at 9.00 am, and 1.30 pm for 2 hours, and starved in between feeds.

Prefeed samples of rumen liquor were taken $\frac{1}{2}$ hour before morning feeding. After feed samples were taken $\frac{1}{2}$ - $\frac{3}{4}$ hour after feeding had commenced. Samples (1 litre) were

taken via the rumen fistula and squeezed through two layers of cheese cloth, in order to remove large plant fragments and cooled in ice. This was termed rumen liquor (RL). Centrifuged rumen liquor (CRL) was obtained by centrifuging the rumen liquor at 30,000 g for 60 minutes.

4.1 The foaming properties of rumen liquor were determined at 37°C in the Laby apparatus. In these studies, the sensing disc assembly weighed 4.5 g instead of the usual 10 g, and the sensitivity range of the strain guage/recorder assembly was increased to give full scale deflection on the recorder for the weight of the sensing disc.

In the 1968/1969 season, differences were observed in the foam properties of *in vitro* generated foams derived from rumen liquor of bloating and non-bloating animals.

Compressive strength varied from 0.3 - 0.8 g cm^{-2}, the majority of the results being close to 0.7 g cm^{-2}, for the non bloat samples and 0.6 - 1.5 g cm^{-2}, 30 out of 42 results being 1.1 - 1.3 g cm^{-2} for the bloat samples. Stress relaxation was extremely low for the bloat samples having values in the range 0 - 0.3 x 10^{-3} g cm^{-2} sec^{-1}. The results for the non bloat samples ranged from 0.2 - 0.9 x 10^{-3} g cm^{-2} sec^{-1}, most values being close to 0.6 g cm^{-2} sec^{-1}.

In the 1969/1970 period the differences observed, in the 1968/1969 period, between the bloat and non-bloat

foams were not as clear. Most samples had compressive strength coefficients of 0.9 g cm^{-2}, stress relaxation of 0 - 0.4 x 10^{-3} g cm^{-2} sec^{-1}, and foam retention volumes of 2.0 - 3.0 mls. Differences were observed in the parameters of the foams derived from the animals of high susceptibility to bloat, 119, 120, on days when they did or did not bloat. The compressive strengths of these foams were in the range 0.6 - 1.1 g cm^{-2}, the stress relaxation was between 0 - 0.3 x 10^{-3} g cm^{-2} sec^{-1} for the bloat samples and 0.3 - 0.7 x 10^{-3} g cm^{-2} sec^{-1} for the non-bloat samples. The foam retention volumes varied from 1.5 - 2.2 ml for the non bloat samples and 1.9 - 3.0 ml for the bloat samples. These results indicate a difference in the persistence of the bloat and non-bloat foams.

The foams generated <u>in vitro</u> derived from the non-bloating rumen liquor of animals of low susceptibility to bloat did not show any significant differences in properties to the foams that were generated from bloating rumen liquor of the high susceptible animals.

4.2 <u>The effect of pH on the foam parameters of rumen foams.</u>

Rumen liquor samples were foamed in the pH range 3.5 - 6.8, in the Laby apparatus and the compressive strength and stress relaxation measured. Results are shown in Fig. 4 (2. a., b.).

Compressive strength was maximum at pH 4.0 in all centrifuged and uncentrifuged samples whether or not bloat had occurred. Stress relaxation

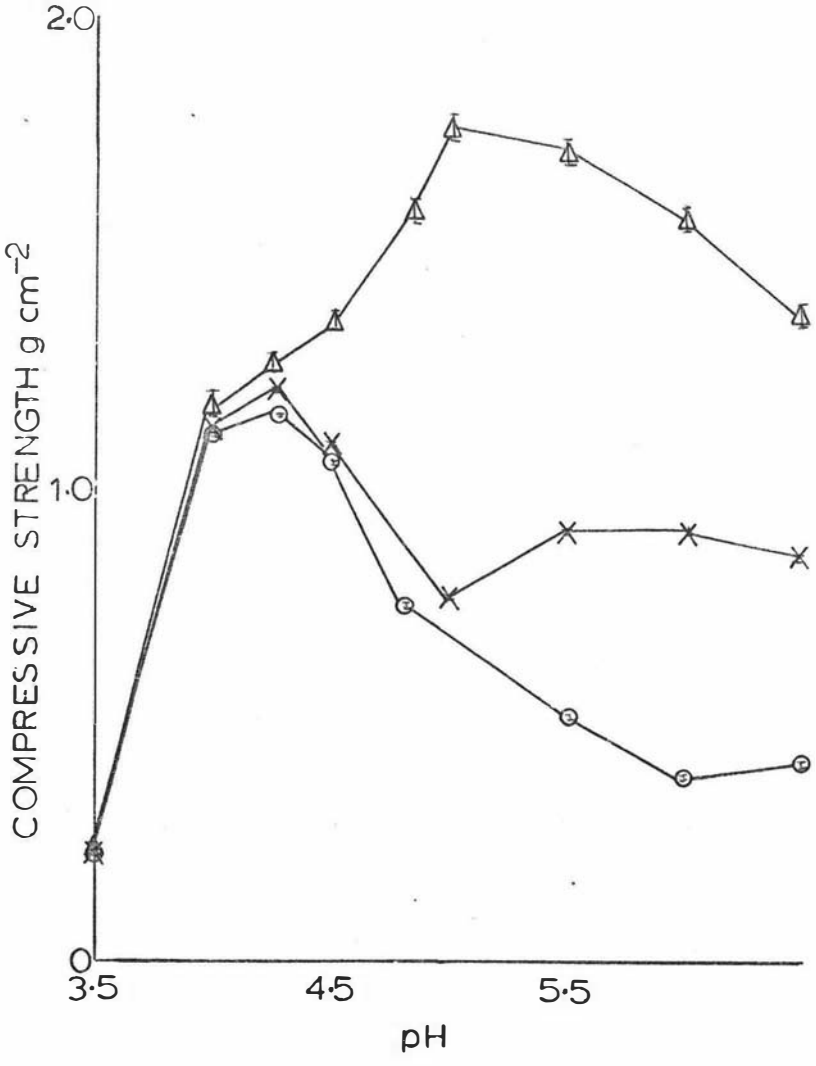

FIG. 4 THE EFFECT OF pH ON THE COMPRESSIVE STRENGTH OF FOAMS (2. a) DERIVED FROM RUMEN LIQUOR

temperature 37°C; nitrogren flow rate 30 ml min^{-1}; porosity 3 sinter.

X——X Uncentrifuged rumen liquor

Δ——Δ Centrifuged rumen liquor

O——O Prefeed

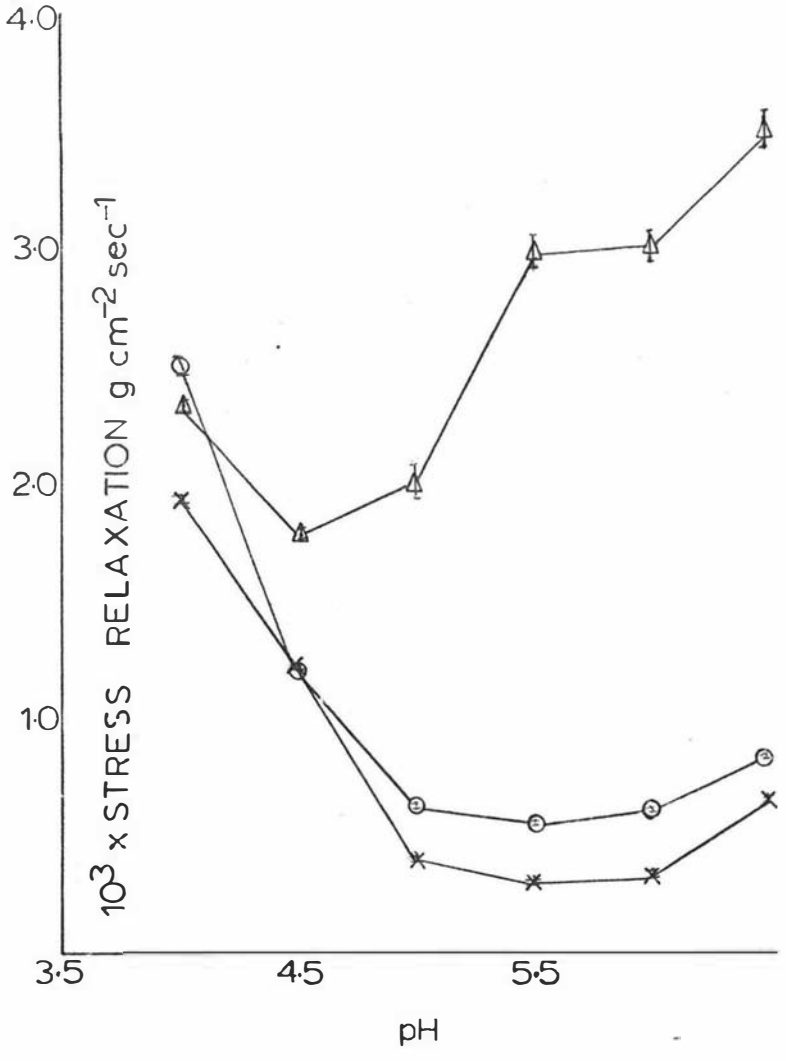

FIG. 4 (2. b) THE EFFECT OF pH ON THE STRESS RELAXATION OF IN VITRO RUMEN FOAMS

gas flow 30 ml min^{-1}; sinter porosity 3.

Δ——Δ centrifuged rumen liquor

O——O uncentrifuged rumen liquor no bloat

x——x uncentrifuged rumen liquor bloat sample

of the foam, when the compressive strength was at a maximum value, was very high indicating the foam was of low persistence.

In addition to the maximum at pH 4.0, a further maximum of compressive strength occurred at pH 5.6 - 5.8 in the case of samples taken after feeding.

Stress relaxation of the above foams was very low at pH's above 5.0 but increased rapidly below this pH. The foam retention volume decreased below pH 5.0.

Centrifuged rumen samples consistently showed a compressive strength maximum at pH 5.1 to 5.4. The compressive strength of the centrifuged samples was higher than that of the uncentrifuged samples. Centrifugation of the sample decreased the persistence of the foams that could be generated.

4.3 The effect of temperature on the foam parameters of rumen foams.

Rumen liquor from bloated cattle was divided into two portions, one of which was centrifuged at 30,000 g for 60 minutes to sediment the particulate material. The solutions were foamed at different temperatures in the range 5° - 45°C. Results are shown in Fig. 4 (3).

The compressive strength of the centrifuged rumen foams increased with increase in temperature.

The compressive strength of the uncentrifuged rumen foams was constant up to 15°C, decreased rapidly between 15 to 20°C and above 25°C remained constant.

The stress relaxation of the foams produced from the uncentrifuged rumen liquor increased suddenly at 15°C, remained constant up to 35°C and increased rapidly

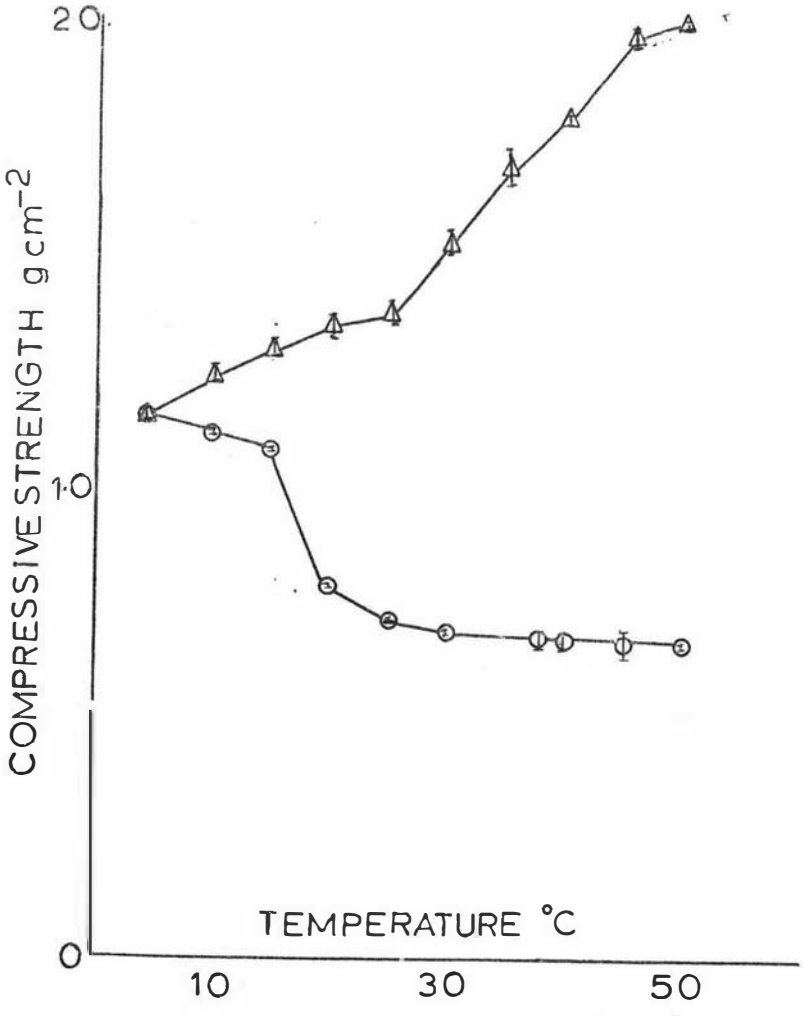

FIG. 4 (3) THE EFFECT OF TEMPERATURE ON THE COMPRESSIVE STRENGTH RUMEN FOAMS

pH 5.8; gas flow 30 ml min^{-1}; porosity sinter 3.

Δ——Δ Centrifuged rumen liquor

O——O Uncentrifuged rumen liquor

above 40°C.

The stress relaxation of the foams derived from the centrifuged rumen liquor was unaffected by temperature changes from 0°C to 40°C but increased with increase in temperature above 40°C.

4.4 Attempts to change the characteristics of rumen foams by addition of Fraction 1 protein to the rumen liquor.

The foaming properties of rumen liquor have been shown to be very different from those of the plant and protozoal proteins. The only similarity has been the observation that they have compressive strength maxima at approximately the same pH. This would suggest that while these proteins are contributing to some extent to the foam properties, other surfactants are present in the surface film. To determine how effective these other surfactants were in controlling the properties of the bloat foam, Fraction 1 protein was added to the rumen liquor, and the modified rumen liquor foamed. Compressive strength, stress relaxation and foam retention volume measurements were made on these foams.

The addition of Fraction 1 protein did not alter the stress relaxation nor the foam retention volume of the bloat foams. Compressive strength of the foams was slightly increased from 1.1 g cm^{-2} to 1.25 g cm^{-2} for addition of 0.02% Fraction 1 protein, and increased to 1.7 g cm^{-2} when the rumen liquor was made 0.2% in Fraction 1 protein. 0.02% and 0.2% Fraction 1 protein in 0.1 M sodium acetate, buffered to the pH of the rumen liquor with acetic acid, give rise to foams of compressive strength 3.6 and 4.5 g cm^{-2} respectively.

4.5 The surface active materials in rumen liquor.

(a) The concentration of Fraction 1 protein in the rumen was determined by acrylamide gel electrophoresis and staining with coumassie blue (Methods, Chapter 1).

Rumen samples were centrifuged at 30,000 g for 60 minutes and 100 mm^3 of the supernate examined for Fraction 1 protein. The level of Fraction 1 protein varied from 0.0008% to 0.025%. There did not appear to be any correlation between the level of Fraction 1 protein in the rumen liquor and bloat occurrence. The animals that were highly susceptible to bloat usually had lower Fraction 1 levels than the low susceptibility animals. Even the highest level of Fraction 1 protein found could not explain the high persistence of the bloat foams.

It is possible that the low level of Fraction 1 protein found in the bloating rumen liquor is due to the protein having already been surface denatured (foam was present in the rumen). In view of the findings in part 4.3 of this Chapter, that addition of Fraction 1 to the rumen liquor made very little difference to the compressive strength of the foam, this hypothesis is doubtful. However, to examine the relative rate of denaturation of Fraction 1 protein in rumen liquor as opposed to its rate of denaturation in acetate solution, the following experiment was carried out.

Rumen liquor from non-bloating and bloating animals were made 0.02% in Fraction 1 protein. The pH was

adjusted, by addition of acetic acid, to 5.8, at which maximum surface denaturation occurs, and the samples foamed in the Laby apparatus. Fraction 1 protein concentration was determined before and after foaming and the difference taken to be the amount of protein surface denatured. The amount denatured was consistently 24% of the amount denatured when 0.02% $^{w}/_{v}$ solutions of Fraction 1 protein in 0.1 mol/litre acetate buffer were foamed in the Laby apparatus under the same conditions. These results therefore indicate that the low level of Fraction 1 protein in rumen liquor cannot be totally explained in terms of its surface denaturation.

Another explanation is that the protein is being rapidly degraded or precipitated in the rumen. To test this, the rate of removal of soluble protein from the rumen liquor was examined as follows. 2 g of Fraction 1 protein was dissolved in 1 litre of artificial saliva (McDougall 1948) and warmed to 39°C. This was incubated at 39°C with 1 litre of prefeed rumen liquor in the artificial fermentors described by Clarke (1969). Samples were removed at ½ hourly intervals over a period of 3 hours, centrifuged at 74,000 g for 30 minutes, and analysed for Fraction 1 protein by acrylamide gel electrophoresis. Further experiments were carried out using minced red clover (500 g that had been macerated in a commercial meat mincer) in place of the Fraction 1 protein. Results showed that a rapid removal from solution of Fraction 1 protein had occurred, and that

the protein could not be detected in the rumen sample after a period of 90 minutes incubation. These results are sufficient to explain the low level of Fraction 1 protein found in the rumen.

(b) Calcium levels in rumen liquor were determined on 30 samples of rumen liquor by atomic adsorption spectrophotometry as described in Chapter 1 of this section.

The level of calcium in the soluble fraction ranged from 200 ppm to 400 ppm. There did not appear to be any correlation between the calcium levels and the occurrence of bloat.

The amount of calcium bound to the high molecular weight soluble fraction (MW greater than 50,000) was approximately 25% of the total soluble calcium in all samples analysed. The high percentage of calcium bound to the high molecular weight material might be a significant factor in the stability of the rumen foams (Mangan, 1959; Stifel et al. 1968b).

(c) Foam fractionation experiments (Chapter 1, Methods) were carried out on ten occasions on rumen liquor taken from animals ¾ hour after feeding on red clover had commenced. On 5 occasions bloat occurred and on the others no bloat was observed.

The foam and rumen liquor were analysed for protein nitrogen, total hexose, starch and lipids on a dry weight basis. Pectin could not be determined because the background colour of the rumen liquor interfered with the

colorimetric estimation.

The level of starch was the same in the rumen liquor as in the foam. The concentration of protein nitrogen in the foam was either the same or lower than in the rumen liquor. Total hexose was lower in the foam than in the rumen liquor. Lipids had concentrated in the foam approximately 2-2½ times. In general the lipid content of the first 50 cm^3 of foam from the bloating animal had increased from 7% in the rumen liquor to 17% in the foam but the later foam fractions contained 14% lipid. The non-bloat samples increased to 14% in all foam fractions. The lipids were examined by thin layer chromatography for polar and neutral lipids, using the solvents described in the methods section.

Equal weights of freeze-dried rumen liquor and foam fraction were extracted for total lipid and the extracted lipids dissolved in 1 ml of chloroform. The lipid extracts(25 mm^3) were applied to thin layer plates and separated as described in Chapter 1 of this thesis. Fig. 4 (5. c) shows typical thin layer chromatograms of the neutral lipids.

The major lipids present in rumen liquor and foams, were the neutral lipids and long chain fatty acids. The free fatty acids and material which had not moved from the origin in the neutral lipid separation appeared to have concentrated in the foams from both the bloat and non-bloat samples. The major band which ran close behind the diglycerides had concentrated in the bloat

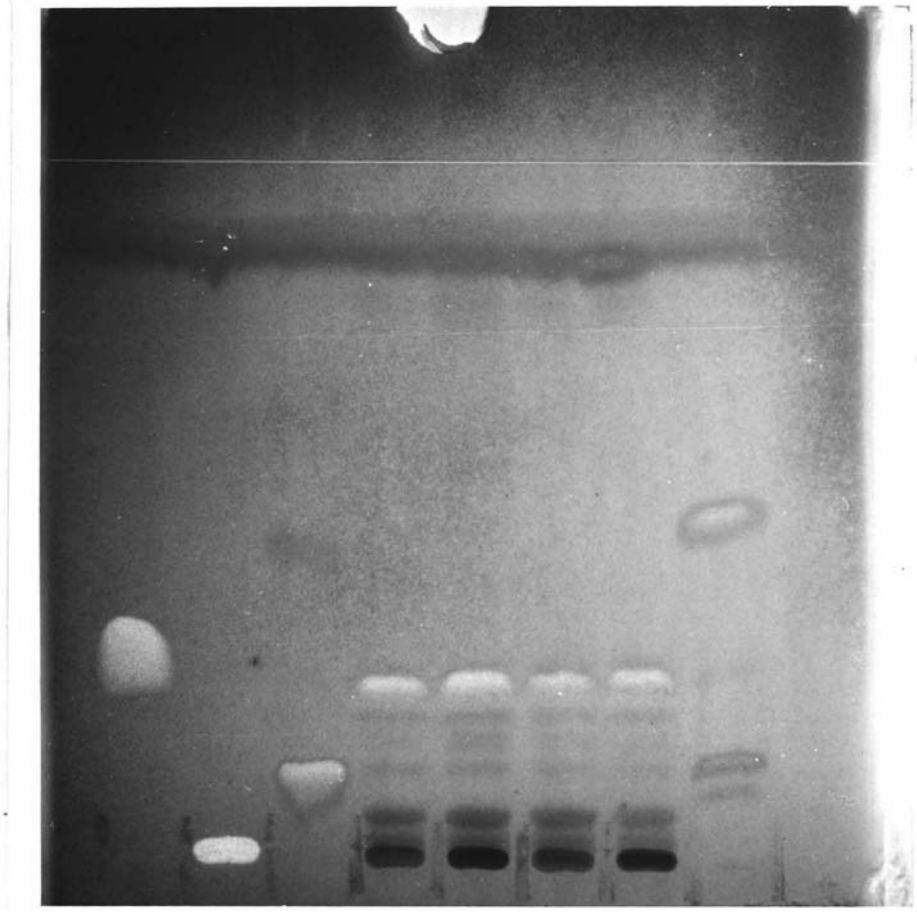

FIG. 4 (5. c.) THIN LAYER CHROMATOGRAPHY OF THE NEUTRAL LIPIDS THAT CONCENTRATE IN THE IN VITRO FOAMS DERIVED FROM RUMEN LIQUOR

(a) The neutral lipids

FA Fatty acids; DG Diglycerides; MG Monoglycerides, TG Triclycerides; RBL Rumen Bloating Liquor; RBF Rumen bloat foam; RNL Rumen liquor (No bloat); RNF Rumen foam (non bloat).

foam but appeared to have decreased in the non-bloat foam. This material was identified as sterol glycosides.

The polar lipids were less prominent. A weak band corresponding to phosphatydyl choline was observed in some samples. Other bands of lower mobility were observed, the major band corresponding in mobility to the sulpholipid though its identity was not confirmed. This material did not appear to concentrate in the foam although there was usually a higher concentration of it in the non-bloat samples.

(d) Examination of the soluble foaming agents in rumen liquor. The concentration of Fraction 1 protein in rumen liquor has already been discussed and shown to be insufficient to account for the persistence of the rumen foams. It was observed that other proteins were present in the rumen liquor of the bloating animals and that these were also denatured in foams. The following experiments were designed to isolate these proteins and to try to identify them.

Rumen liquor was taken from cattle ¾ hour after feeding and centrifuged to remove the particulate material. The clear supernate was adjusted to pH 5.8, with acetic acid, and foamed in the Laby apparatus to characterize its foaming properties. The remainder of the liquor was made 60% $^{w}/_{v}$ in ammonium sulphate and the precipitate collected by centrifugation. The supernate would not support foam and was discarded.

The precipitate was redissolved by dialysis (3 x 10 litres) against acetate buffer, pH 7.6 (0.1 mol/litre) containing 10^{-4} mol/litre mercaptoethanol. The solution was stored at -70°C. The solution, on diluting to its original concentration in the rumen liquor produced only very weak foams. When calcium was added to give the same level found in the rumen liquor, foams identical in properties to those produced from the original rumen liquor were produced.

Acrylamide gel electrophoresis of this precipitated material showed several protein components to be present. When the gel was stained for carbohydrates with the periodic acid/Schiff reagent (Bodin, 1963) a high molecular weight carbohydrate component which had moved approximately 1 mm into the gel was observed.

Analytical ultracentrifugation of this material, at 59,780 rpm, showed two components to be present (Fig. 4. 5. d. 1), a low molecular heterogeneous component which did not move from the meniscus, and a material showing a hypersharp schlieren pattern. The latter is very similar to the schlieren pattern of the salivary mucoprotein, and the material producing it was a major component of all the rumen samples examined. The low molecular weight material probably is the mixture of proteins observed on acrylamide gel.

The material which resembled mucoprotein in the ultracentrifuge was isolated from the rumen liquor as follows.

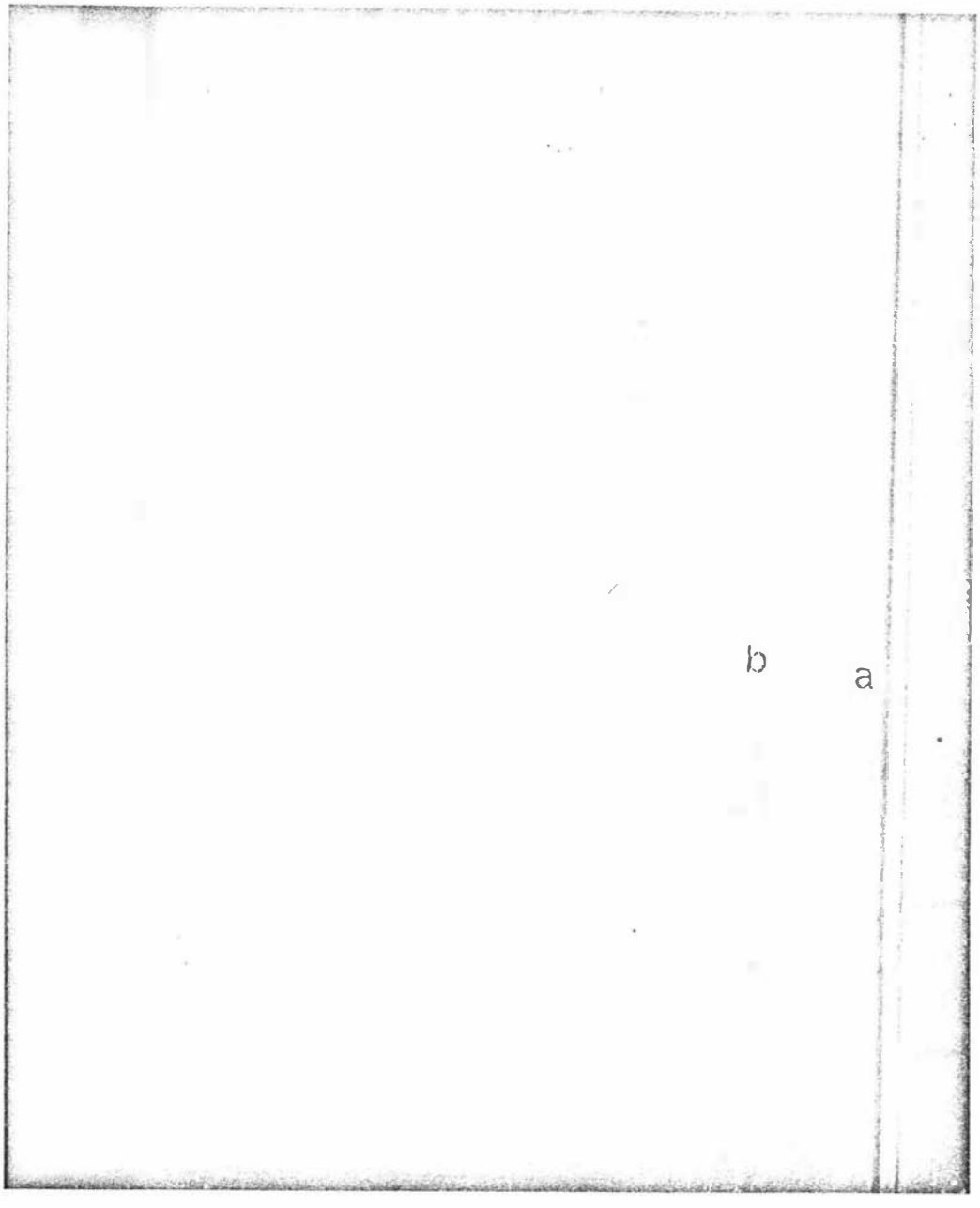

FIG. 4

(5. d. 1)

ANALYTICAL ULTRACENTRIFUGATION OF THE PROTEINS IN RUMEN LIQUOR

a. Heterogeneous proteins

b. The material resembling salivary mucoprotein

The concentrated rumen protein samples were centrifuged at 360,000 g for 4 hours, and the contents of the tubes divided into (1) the pellet that had sedimented out on the bottom of the tube, (2) the bottom 2 ml of viscous liquid, (3) the next 2 ml of liquid, (4) the next 3 ml of pale brown liquid and (5) the remainder of the liquid. Each of these fractions was analysed in the analytical ultracentrifuge and the material which resembled mucoprotein was located in fractions (2) and (3). The first fraction contained most of the heterogeneous proteins together with some Fraction 1 protein. Because of the resemblence of the major fraction to mucoprotein, all samples were analysed for sialic acid content. The maximum sialic acid occured in fractions (2) and (3) corresponding to the tubes in which the material was located.

An ultracentrifugation schlieren photograph of the purified material is shown in Fig. 4 (5. d. 2). The sedimentation coefficient of this material, determined in a Beckman model E analytical ultracentrifuge, was 6.6 S at a concentration of 4 mg ml^{-1}. Dilution of the sample slightly increased the sedimentation coefficient to a maximum of 7.4 S at a concentration of 1 mg ml^{-1}.

Cellulose acetate electrophoresis of this material showed a single component, which stained intensely for carbohydrate but only weakly for protein, indicating the molecule to be a glycoprotein.

Further characterization of the molecule was achieved by preparing the antibody to this material, by injecting

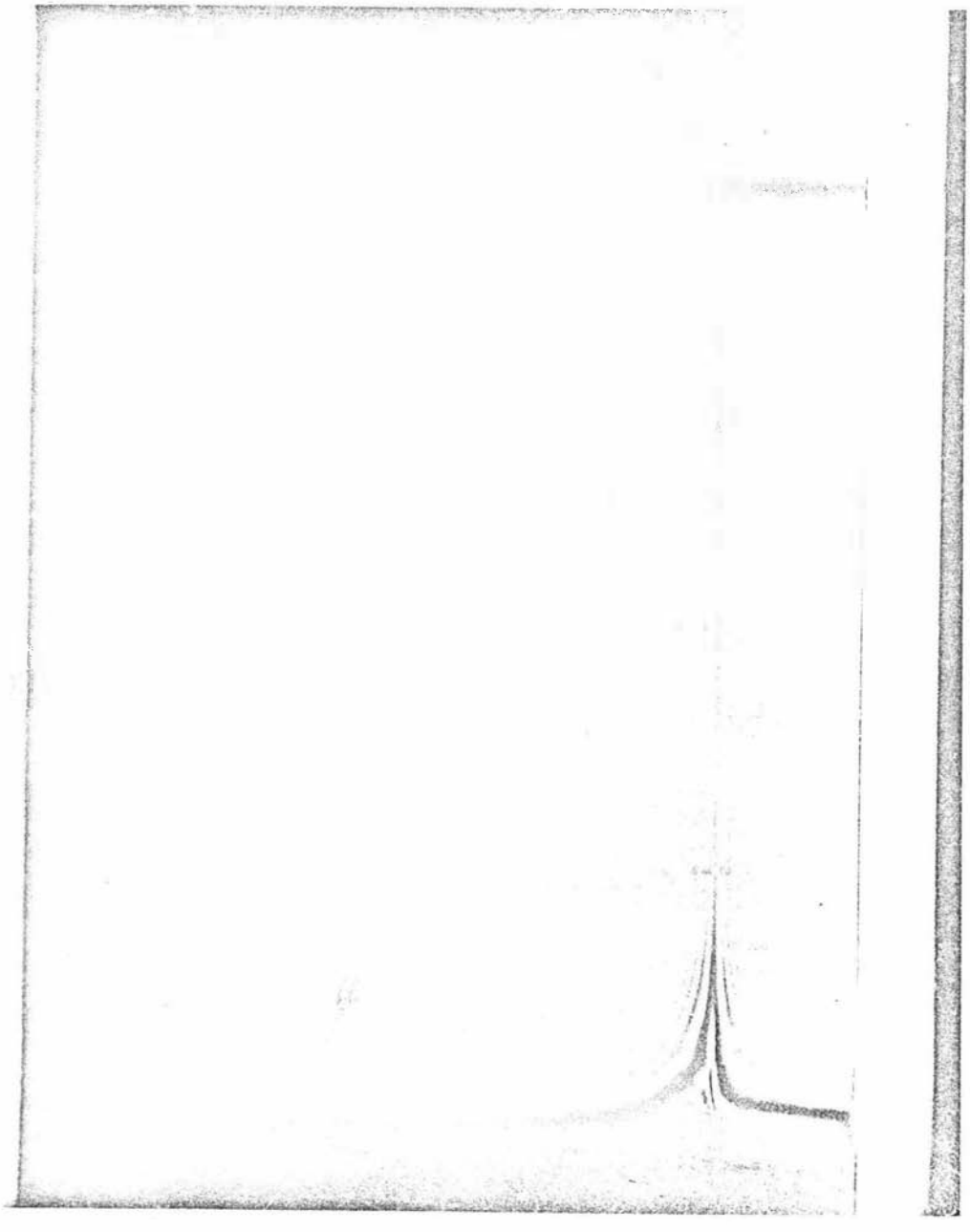

FIG. 4 (5. d. 2) ANALYTICAL ULTRACENTRIFUGATION OF THE MAJOR PROTEIN IN RUMEN LIQUOR OF BLOATING ANIMALS

it into rabbits. The antibody gave a precipitin reaction with the salivary mucoprotein and with the oesophageal mucin, though the degree of precipitation was not as great as with the mucoprotein. Removal of the sialic acid from the salivary mucoprotein by incubating it with neuraminidase, increased the sensitivity of the precipitin reaction ten fold. The antibody did not give precipitin reactions with Fraction 1 protein, Fraction 2 proteins nor protozoal proteins.

The material was not precipitated by 10% trichloracetic acid, but was precipitated by 60% ammonium sulphate, 80% ethanol, tannins from Lotus pedunculatus Cav., and by 1% Cetavlon indicating precipitation properties similar to those of the salivary secretions.

Summary

(1) The rumen foams differ from the plant and protozoal protein foams, though they are similar to the salivary mucoprotein foams in having low compressive strength associated with low stress relaxation and high foam retention volumes, the latter two parameters indicating foam persistence.

(2) Rumen foams show two maxima of compressive strength, one at pH 4.0 and the second at approximately 5.7 Maximum persistence is associated with the latter compressive strength maximum.

(3) Compressive strength of foams generated in vitro from rumen liquor decreased slowly on increasing the temperature from 5-15°C, then decreased rapidly between 15

and 20°C, and remained constant from 25-50°C. Foams from centrifuged rumen liquor did not show this phenomenon but increased steadily in compressive strength with increase in temperature over the range 5-50°C.

(4) The amount of Fraction 1 protein in the rumen liquor was too low to account for the high persistence of the rumen foams. Foam stabilizing materials other than plant proteins must be present to account for this stability. The low level of Fraction 1 protein in the rumen liquor could be explained by its rapid degradation and removal from solution.

Addition of 0.01 - 0.1% Fraction 1 protein to the rumen liquor only slightly increased the compressive strength, without affecting the stress relaxation or foam retention volume of the foams generated from these solutions. This suggests that a more surface active material than Fraction 1 protein is present in the rumen liquor. Further evidence for this is suggested by the fact that the amount of Fraction 1 protein denatured on foaming rumen liquor containing 0.02% Fraction 1 was only 24% of the Fraction 1 protein denatured on foaming a 0.02% solution of Fraction 1 protein in acetate buffer.

(5) Foam fractionation of rumen liquor showed that lipids, mainly neutral lipids and fatty acids, were concentrated 2-2½ times in the foam fractions.

(6) The soluble foaming agents in rumen liquor were isolated by salt precipitation, and fractionated by preparative

ultracentrifugation. One fraction contained a mixture of proteins, whose sources were not identified. A second fraction contained a component which resembled salivary mucoprotein in the ultracentrifuge and in its precipitation characteristics. This material had a sedimentation coefficient of 7.4 S, was homogeneous on cellulose acetate and shown to contain carbohydrate and protein. The antibody of this material gave precipitin reactions with the salivary secretions, particularly the salivary mucoprotein, but not with the soluble plant nor protozoal proteins. The sensitivity of the precipitin reaction was increased by prior removal of the sialic acid from the salivary mucoprotein by neuraminidase. It is suggested that this material is possibly a degradation product of the salivary mucoprotein.

CHAPTER 5

THE INTERRACTION BETWEEN THE SOLUBLE LEAF PROTEINS AND OTHER SURFACTANT INVOLVED IN THE BLOAT SYNDROME

In Chapters 3 and 4 of Section 2 of this thesis, the foaming properties of the plant and animal proteins and of rumen liquor were studied. Rumen foams were only similar to plant and protozoal foams in that they possessed compressive strength maxima at roughly the same pH. Whereas the plant and protozoal protein foams were persistent only when the compressive strength was high, the rumen foams were extremely stable although the compressive strength was low. Salivary mucoprotein foams were similar to the rumen foams in this respect, though they did not exhibit compressive strength maximum at any pH in the range which occurs in the rumen. A possible explanation of the character of rumen foams is therefore that a mixed system, with properties intermediate between the plant or protozoal proteins and the mucoprotein foams, is operating.

Another possibility is that the antifoaming lipids are producing mixed films with the plant or protozoal proteins of low compressive strength. This theory would require the presence of a foam stabilizing substance to be present to

account for the persistence of the rumen foams. Pectins (Conrad et al. 1961; Nichols et al. 1966) and bacterial slimes (Hungate et al. 1964) have been suggested to act as foam stabilizers on account of their high viscosity.

The results for calcium binding to the high molecular weight material in the rumen liquor shows that it could be another significant factor influencing the stability of the rumen foams.

In this Chapter the nature of the foams that can be derived from mixed systems between the soluble plant proteins and other surfactants, both foam stabilizing and antifoaming agents, are studied.

5.1 The influence of calcium on the compressive strength of protein foams.

The concentration of cations in the rumen liquor is usually in the range 0.1 - 0.2 M, and of this the level of calcium found in the soluble fraction of the rumen samples examined in this thesis (Chapter 4) was in the range 200 - 400 ppm. In this Section experiments are described in which solutions of proteins dissolved in 0.1 mol/litre acetate buffer, pH 6.3, containing varying amounts of calcium were foamed in the Laby apparatus.

Solutions containing 0.018% salivary mucoprotein, and Fraction 1 protein or 0.04% Fraction 2 protein were foamed at pH 6.3 and 37°C. Compressive strength was measured on these foams and the results for the leaf proteins are shown in Fig. 5 (1. a) for different concentrations of calcium.

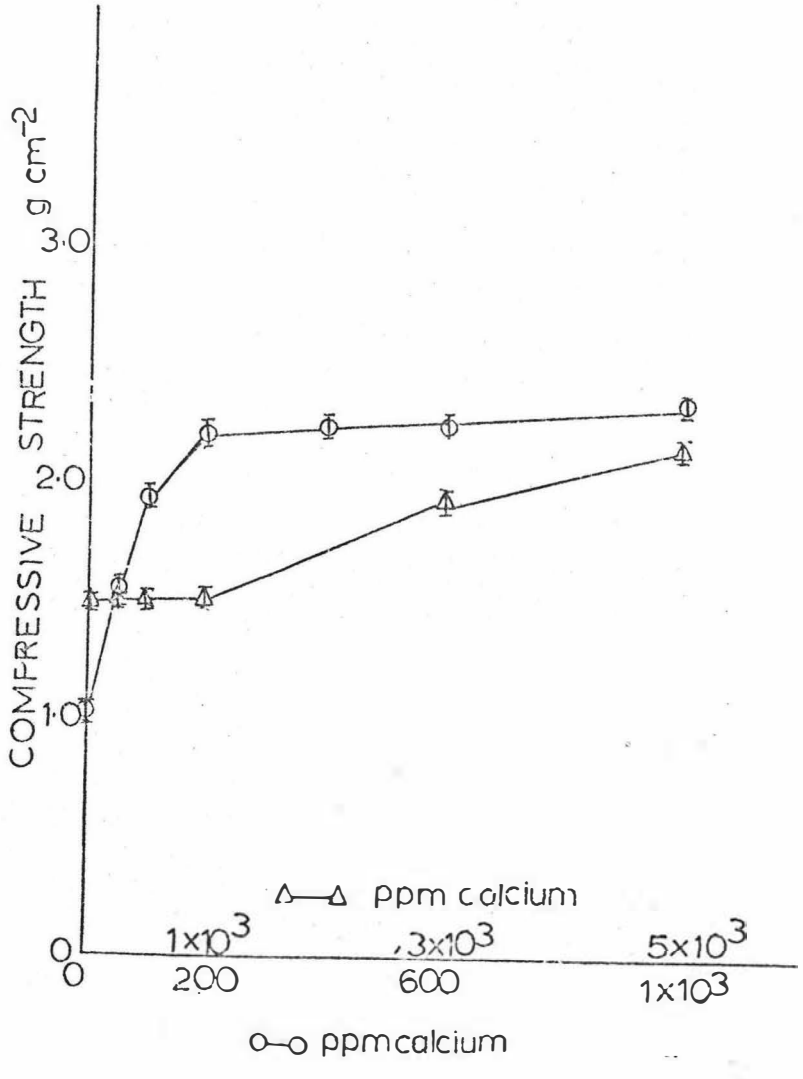

FIG. 5 (1. a) THE EFFECT OF CALCIUM ON THE COMPRESSIVE STRENGTH OF FRACTION 1 AND FRACTION 2 LEAF PROTEIN

buffer 0.1 mol/litre sodium acetate; pH 6.3(p.87); temperature 37°C; gas flow 30 ml min^{-1}; porosity sinter 3.

O——O Fraction 1 protein (0.018% $^{w}/_{v}$)

Δ——Δ Fraction 2 proteins (0.04%)

The compressive strength of Fraction 1 protein foams increased 1½ times as the calcium concentration increased from 0-200 ppm. Above this concentration no change in compressive strength was observed.

Fraction 2 protein foams showed no increase in compressive strength until a calcium concentration of 1000 ppm was reached. Above this concentration compressive strength increased slightly. Bovine salivary mucoprotein foams were unaffected by calcium in the range 0-5000 ppm.

The values of the isoelectric pH's for Fraction 1 protein, Fraction 2 proteins and salivary mucoprotein are 5.8, 5.1 and 2.5 respectively. It appears from the results that the further away the pH (6.3) of the experiment was from the isoelectric pH of the protein, then the higher was the concentration of calcium required to significantly modify the compressive strength of the foams derived from these protein solutions.

This is possibly due to the bound calcium decreasing the net negative charge on the protein molecules, in a manner similar to a lowering of the pH, resulting in a reduction of the electrostatic repulsion and hence a greater cohesion between them. The more distant the experimental pH of 6.3 is from the isoelectric pH of the protein, the more calcium would be required to reduce this repulsion to a value which would modify the foaming properties.

If this suggestion is correct, the effect of calcium should be less marked at the isoelectric pH of the protein, where the potential binding sites are occupied by hydrogen ions. To examine this effect, solutions of 0.028% $^{w}/_{v}$

Fraction 1 protein, one containing 200 ppm calcium, and the other without any calcium were foamed in the Laby apparatus at 37°C and in the pH range 5.4 to 6.8. Results are shown in Fig. 5 (1.b).

It was observed that the effect of calcium diminished as the pH of the protein solution approached the isoelectric pH of the protein. This suggests that calcium is neutralizing the charge on the protein molecule.

5.2 The effect of polygalacturonic acid on plant protein foams.

Fraction 1 (0.02%) solutions containing polygalacturonic acid (supplied by British Drug House Chemicals of citrus fruit origin) in the concentration range 0.01% - 0.5%, were foamed in the Laby apparatus at pH 5.8 and at 37°C. Compressive strength, stress relaxation and the foam retention volumes were measured. Results are shown in Fig. 5 (2.a., b., c.). Compressive strength decreased as the polygalacturonic acid concentration increased.

Measurements of stress relaxation and the foam retention volume indicated an increase in the persistence of the plant protein foams above a polygalacturonic acid concentration of 0.04% $^{w}/_{v}$.

In order to determine the effect of calcium on the Fraction 1/polygalacturonic acid system, solutions of Fraction 1 protein containing 200 ppm calcium and polygalacturonic acid ranging from 0.02% - 0.10% were foamed

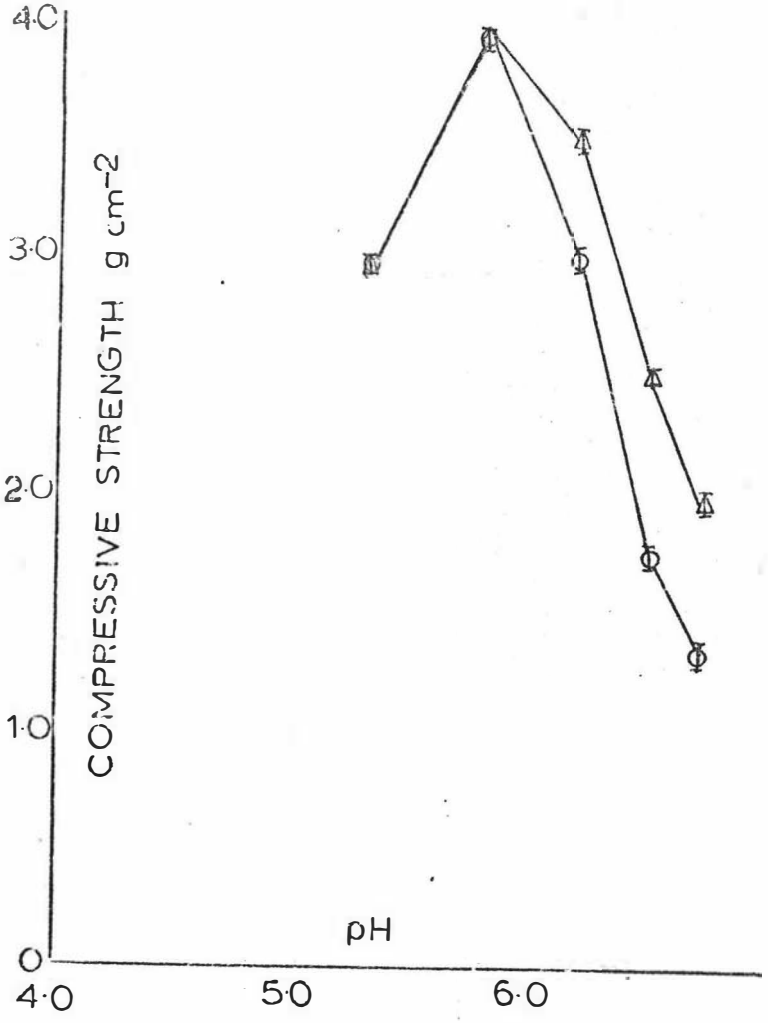

FIG. 5 (1. b) THE EFFECT OF pH ON THE INTERACTION BETWEEN CALCIUM AND FRACTION 1 PROTEIN AS SHOWN BY ITS EFFECT ON COMPRESSIVE STRENGTH OF THE FOAMS

buffer 0.1 mol/litre sodium acetate(p.87)temperature 37°C; gas flow 30 ml min^{-1}; porosity sinter 3.

O——O 0.028% Fraction 1 protein

Δ——Δ 0.028% Fraction 1 protein plus 200 ppm calcium

at pH 5.8 in the Laby apparatus. At polygalacturonic acid concentrations up to 0.06%, the calcium polygalacturonate precipitated and the foams were not significantly modified. At higher polygalacturonic acid concentrations the solutions gelled making it impossible to carry out the experiment. No significant data was therefore obtained regarding the possible role of calcium pectate gels as stabilizing agents.

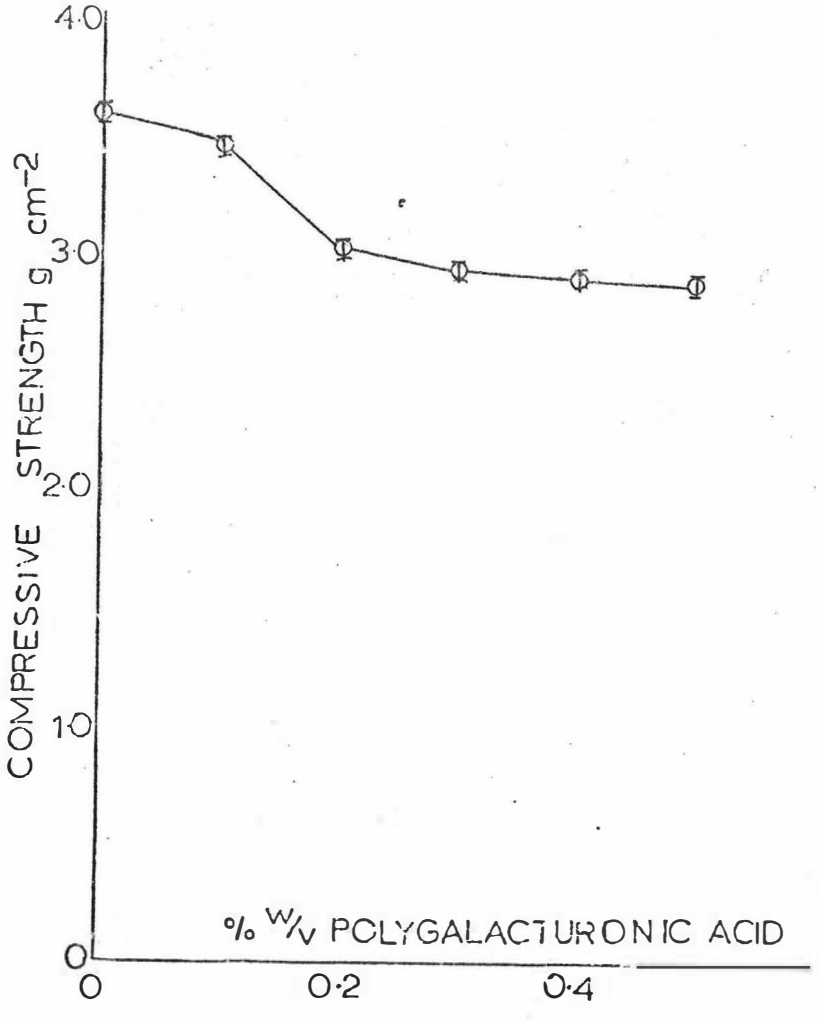

FIG. 5 (2. a) THE EFFECT OF POLYGALACTURONIC ACID ON THE COMPRESSIVE STRENGTH OF FRACTION PROTEIN FOAMS

buffer 0.1 mol/litre sodium acetate; pH 5.8(p.87); temperature 37°C; gas flow 30 ml min^{-1}; porosity 3 sinter. 0.02% $^{W}/_{V}$ Fraction 1 protein.

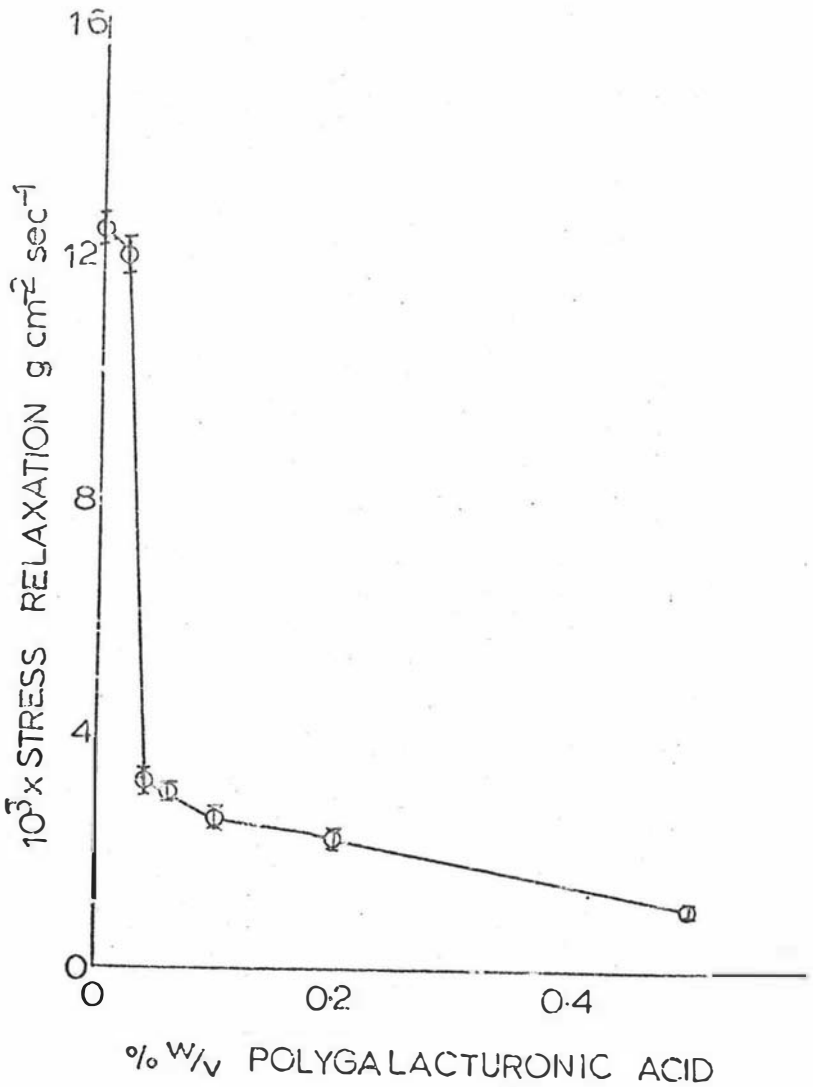

FIG. 5 (2. b.) THE EFFECT OF POLYGALACTURONIC ACID ON THE STRESS RELAXATION OF FRACTION 1 PROTEIN FOAMS

buffer 0.1 mol/litre sodium acetate (p.87); 0.02% W/V Fraction 1 protein; pH 5.8; temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.

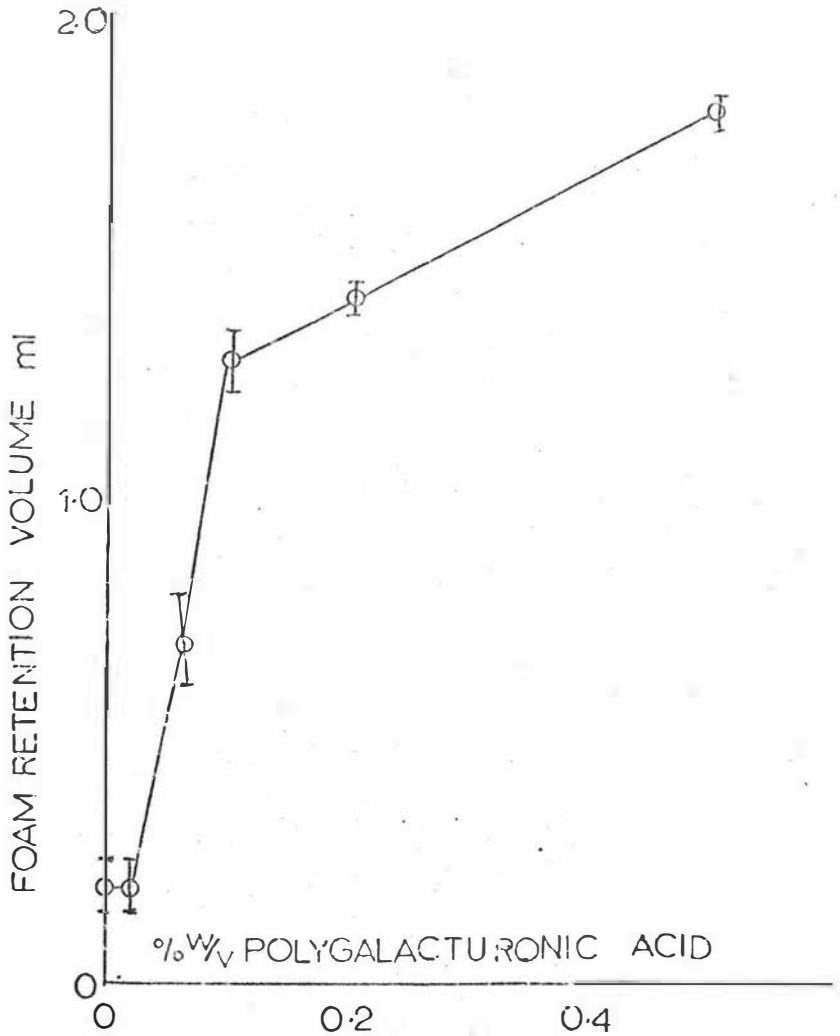

FIG. 5 (2. c.) THE EFFECT OF POLYGALACTURONIC ACID ON THE FOAM RETENTION VOLUME OF FRACTION 1 PROTEIN FOAMS

buffer 0.1 mol/litre sodium acetate (pH 5.8); 0.02% $^{W}/_{V}$ Fraction 1 protein; pH 5.8; temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.

5.3 The salivary secretions

The role of bovine salivary secretions in the bloat syndrome has been the subject of much controversy. Bartley (1967) favoured the idea that the salivary secretions act as foam destabilizers whereas Johns (1956) and Mangan (1958) believed they were capable of producing persistent foams. The results obtained in this thesis have shown that whereas the salivary mucoprotein can produce persistent foams, the oesophageal mucin did not support stable foams. In the following section the types of foams that can be derived from mixtures of soluble plant protein fractions and the salivary secretions have been studied.

Solutions containing 0.02% Fraction 1 protein and differing concentrations of salivary mucoprotein were foamed at pH 5.8 in the Laby apparatus at 37°C and the compressive strength, stress relaxation and foam retention volumes measured. The amount of Fraction 1 protein surface denatured on foaming these solutions was determined by acrylamide gel electrophoresis (Chapter 1).

The oesophageal mucin, which contains sialic acid, was added to a 0.028% solution of Fraction 1, to give solutions containing 0-15 µg of sialic acid per ml. These solutions were foamed at pH 5.8 in the Laby apparatus. Results obtained for the compressive strength

of the foams derived from these mixed systems are shown in Fig. 5 (3. a.).

The salivary mucoprotein decreased the compressive strength of Fraction 1 protein foams from 3.6 to 1.3 g cm^{-2} over the range 0-0.1% salivary mucoprotein. The compressive strength of foams derived from oesophageal mucin/Fraction 1 protein mixtures did not differ significantly from the pure Fraction 1 foams.

Stress relaxation of the foams derived from the mixed systems are shown in Fig. 5 (3. b.). The salivary mucoprotein/Fraction 1 mixed foams showed a minimum stress relaxation at 0.02% - 0.04% mucoprotein. The stress relaxation decreased to 1.5×10^{-3} g cm^{-2} sec^{-1} at 0.04% and increased to 3.5×10^{-3} g cm^{-2} sec^{-1} at 0.10% mucoprotein, indicating a maximum persistence of the mixed foam when the ratio of mucoprotein to Fraction 1 in the solution being foamed was 1/1 - 2/1.

The oesophageal mucin did not show any minimum in stress relaxation, but decreased from 6.0×10^{-3} to 0.9×10^{-3} g cm^{-2} sec^{-1} over the concentration range examined.

The measurements of foam retention volume are shown in Fig. 5 (3. c.). The foam retention volume of salivary mucoprotein Fraction 1 foams increased from 0-2.0 ml at 0.04% mucoprotein and decreased to 1.3 ml at 0.10% mucoprotein indicating a maximum persistence of foams derived from solutions containing 2/1 salivary mucoprotein/Fraction 1 protein.

The oesophageal mucin increased the foam retention

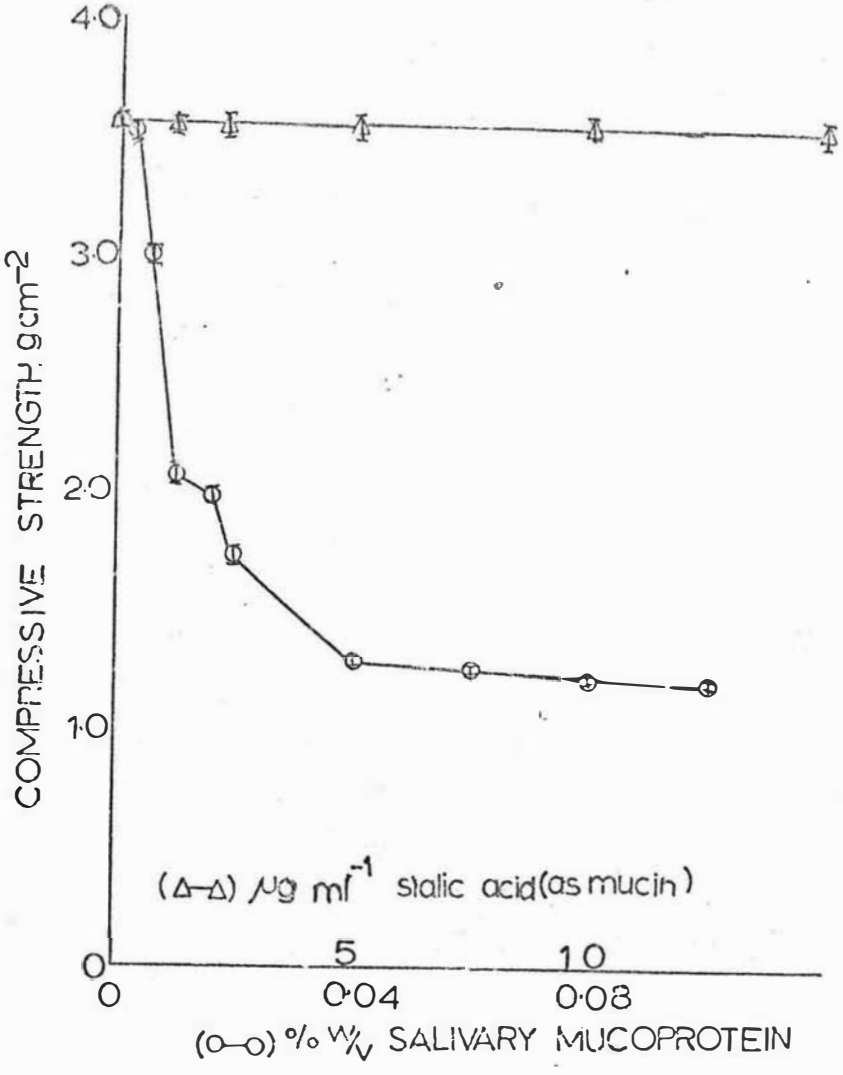

FIG. 5 (3. a) THE EFFECT OF SALIVARY SECRETIONS ON THE COMPRESSIVE STRENGTH OF FRACTION 1 PROTEIN FOAMS

acetate buffer 0.1 mol/litre (p.87); 0.02 % Fraction 1 protein; pH 5. temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.

o——o salivary mucoprotein

Δ——Δ oesophageal mucin

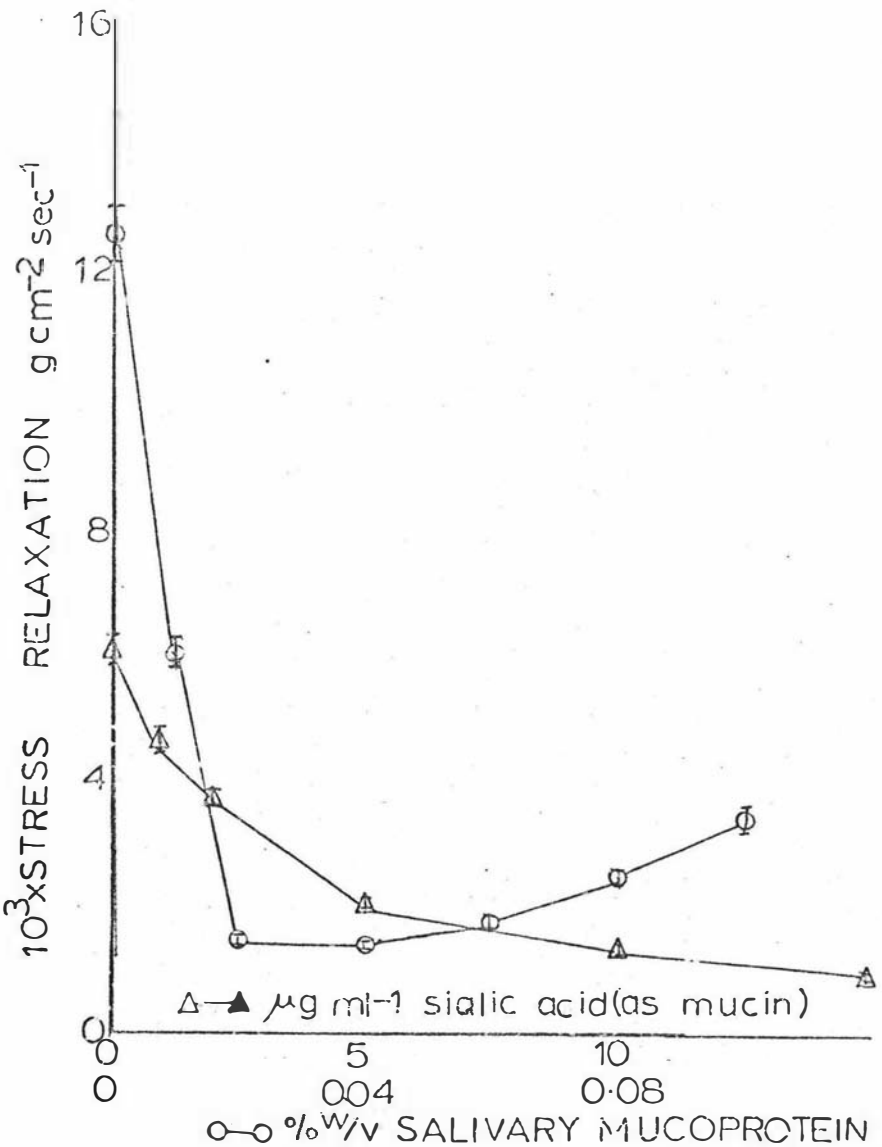

FIG. 5 (3. b) THE EFFECT OF BOVINE SALIVARY SECRETIONS ON THE STRESS RELAXATION OF FRACTION 1 PROTEIN

buffer 0.1 mol/litre sodium acetate; pH 5.8(p.87); temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.

Δ—— Δ oesophageal mucin; 0.028% Fraction 1 protein

O—— O salivary mucoprctein; 0.02% " " "

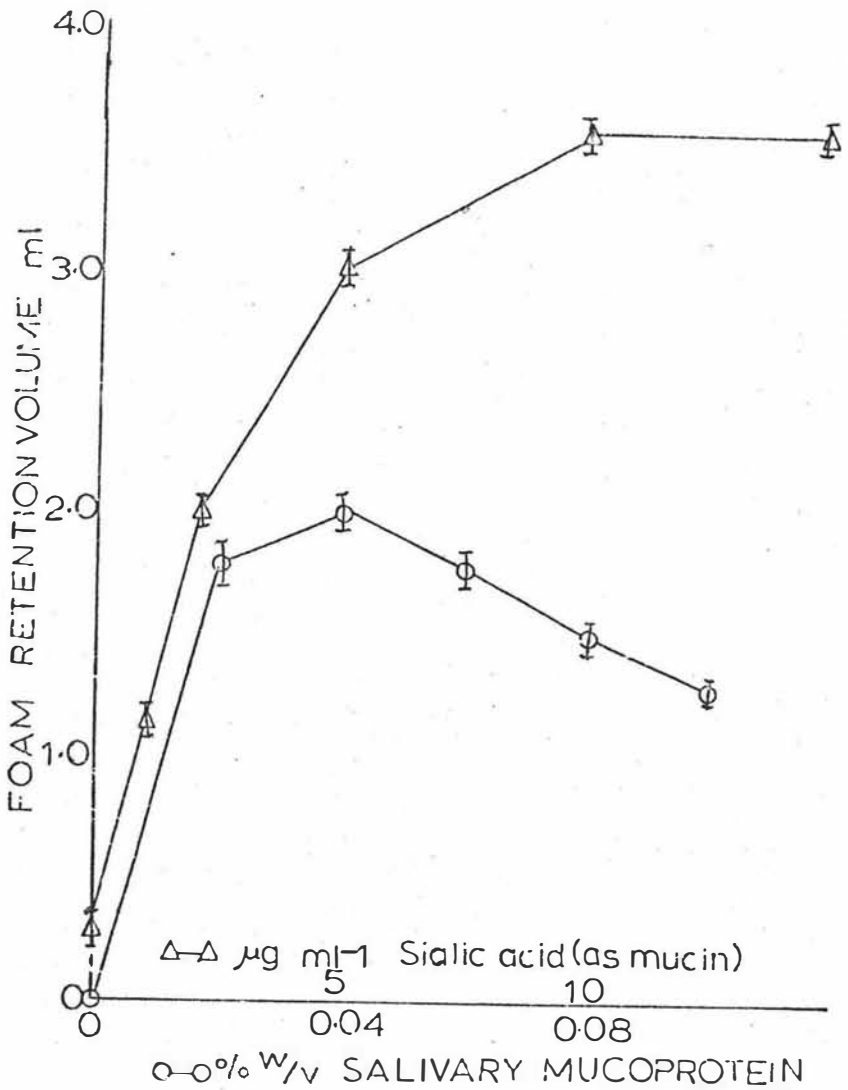

FIG. 5 (3. c) THE EFFECT OF BOVINE SALIVARY SECRETIONS ON THE FOAM RETENTION VOLUME OF FRACTION 1 PROTEIN FOAMS

buffer 0.1 mol/litre sodium acetate; pH 5.8(p.87); temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.

Δ——Δ oesophageal mucin; 0.028% Fraction 1 protein

o—— o salivary mucoprotein; 0.02% w/v " " "

volume dramatically from 0.3 ml for Fraction 1 protein alone to 3.2 ml for mixtures containing 10 µg of sialic acid per ml. Addition of salivary mucoprotein to Fraction 1 protein solutions decreased the amount of Fraction 1 protein being denatured in producing the foam (Table 5 (3. 1) to 56% at 0.04% mucoprotein.

The results indicate that the oesophageal mucin is a very efficient foam stabilizing agent, increasing the foam retention volume and decreasing the stress relaxation without modifying the compressive strength of the foams. The action of the salivary mucoprotein was not as readily characterized, for whilst the mixed foams were more persistent than the Fraction 1 protein foam, the compressive strength and the amount of Fraction 1 protein denatured in the foam decreased upon increasing the mucoprotein content of this solution.

If the rigidity of the foam, indicated by the compressive strength is the important factor in the bloat foam, mucoprotein must be considered to be an antifoaming agent, whereas if persistence is the controlling factor, the mucoprotein must be thought of as a foam stabilizing agent. From the results of Chapter 4 it would appear that the prime characteristic of rumen foams is their high persistence and relatively low compressive strength, and therefore on this basis, the salivary mucoprotein would be classed as a stabilizing agent.

% $^{w}/_{v}$ salivary mucoprotein	Fraction 1 protein denatured on foaming
0.00	94%
0.003%	94%
0.005%	91%
0.008%	89%
0.015%	76%
0.020%	69%
0.03%	62%
0.04%	56%

TABLE 5 (3. 1) THE EFFECT OF SALIVARY MUCOPROTEIN ON THE AMOUNT OF FRACTION 1 PROTEIN DENATURED ON FOAMING FRACTION 1 PROTEIN/SALIVARY MUCO-PROTEIN MIXTURE

buffer 0.1 mol/litre sodium acetate; pH 5.8(p.87);temperature 37°C; gas flow rate 30 ml min^{-1}; porosity sinter 3.
0.02% Fraction 1 protein.

In an attempt to explain the stabilizing properties of these secretions and of the polygalacturonic acid, the effect of other viscous materials on Fraction 1 protein was examined.

The materials used in this study were soluble polyvinyl pyrrolidone (BSAF K25) and linear chain dextran (Pharmacia Chemicals). Viscosity η_{rel}, was measured at 37°C relative to a 0.1 mol/litre solution of sodium acetate in microviscometers (Cannon instruments). Solutions of 0.02% $^{w}/_{v}$ Fraction 1 protein in acetate buffer pH 5.8, containing sufficient polyvinylpyrrolidone or dextran to produce solutions of viscosity equivalent to 0.01%, 0.02%, 0.06% and 0.10% salivary mucoprotein, were foamed in the Laby apparatus.

The effect of these viscous agents on protein foams was to decrease the compressive strength, and the stress relaxation, and to increase the foam retention volume. Thus polyvinyl pyrrolidone decreased the compressive strength of Fraction 1 protein foams from 3.6 to a limit of 2.0 g cm^{-2} and increased the retention volume 0.2 - 1.0 ml, and dextran decreased the compressive strength from 3.6 - 2.3 g cm^{-2} and the retention volume from 0.2 ml to 1.0 ml.

Compared with these results, it can be seen that polygalacturonic acid behaved very similarly. The mucin differed from these viscous materials in that it did not reduce the compressive strength of the foam, and increased the foam retention volume to a much greater

volume than was produced by any of these viscous materials.

The effect of viscosity could not explain the minimum observed in the stress relaxation nor the maximum in foam retention volume that occured with the Fraction 1/mucoprotein mixed foams.

5.4 The effect of tannins

A set of solutions containing 0.02% Fraction 1 protein or 0.04% Fraction 2 proteins, or 0.065% protozoal proteins or 0.06% mucoprotein or rumen liquor, to each of which Lotus tannin had been added to give protein/tannin (p/t) $^{w}/_{w}$ mixtures of 10/1-10/9, 1/1-1/4, were foamed in the Laby apparatus at 37°C, and the compressive strength measured. Results are shown in Fig. 5 (4. a.). Foam was not produced from Fraction 1, Fraction 2 protozoal proteins or rumen liquor at p/t mixtures above 5/1. 10/7, 5/3 and 5/2 respectively.

The compressive strength of the salivary mucoprotein/tannin mixtures increased from 1.0-3.2 g cm^{-2} at 1/1 and 1/2 mixtures and decreased to zero at p/t mixtures of 1/4. When the salivary mucoprotein/tannin mixtures were centrifuged prior to foaming, foams could not be produced from mixtures of p/t greater or equal to 1/1. If the mucoprotein was degraded with neuraminidase to remove sialic acid, prior to addition of the tannin, foams could not be generated from the degraded protein/tannin mixtures of greater than 5/3.

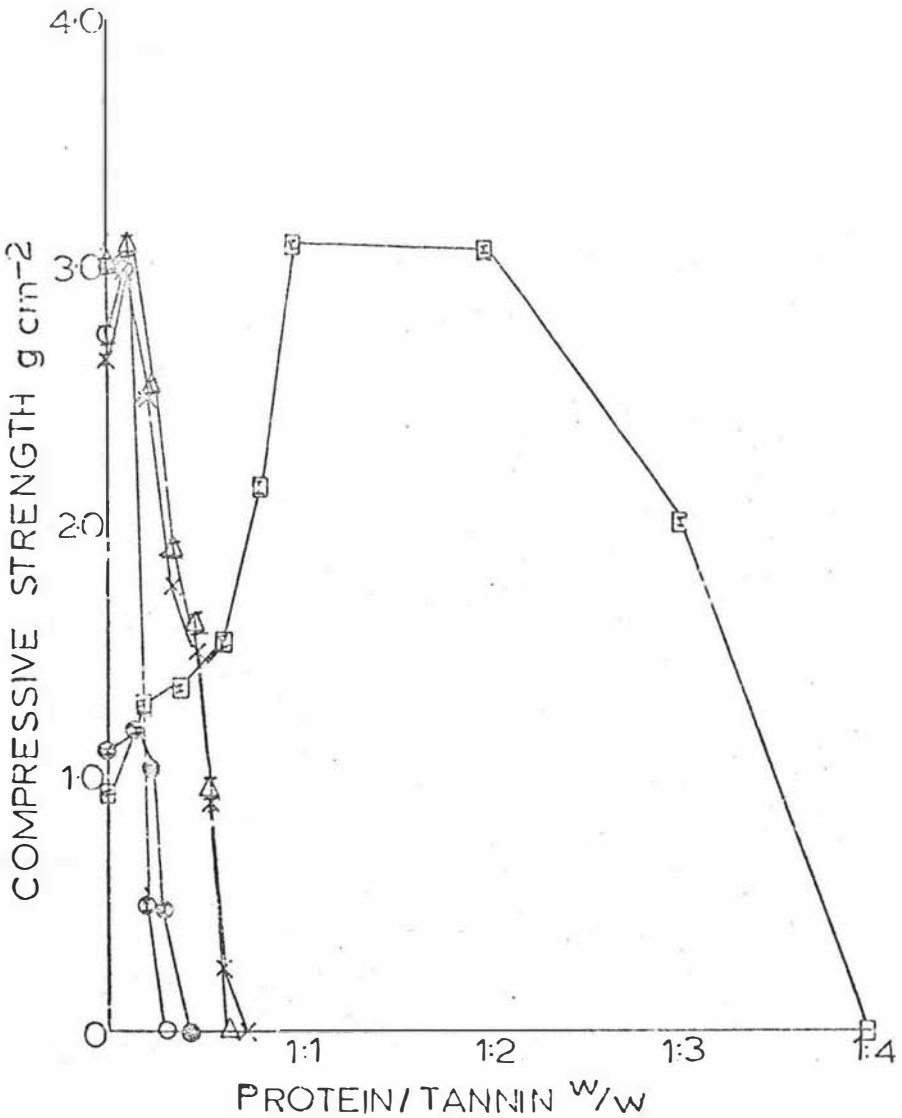

FIG. 5 THE EFFECT OF TANNINS ON THE COMPRESSIVE STRENGTH OF PROTEIN FOAMS

(4)

buffer 0.1 mol/litre sodium acetate; pH 5.8(p.87); temperature 37°C; gas flow rate 30 ml min^{-1}; porosity 3 sinter.

o---o	0.02% w/v	Fraction 1 protein
x—— x	0.04% w/v	Fraction 2 proteins
□—— □	0.06%	salivary mucoprotein
△—— △	0.065% w/v	protozoal proteins
●—— ●		rumen liquor from bloating animals

The amount of tannin required to produce insoluble complexes with Fraction 1 protein, Fraction 2 protein and salivary mucoprotein was determined as follows: 5 ml of 0.1% solutions of each of the proteins were mixed with 5 ml of tannin solution containing sufficient tannin to give the same p/t mixtures used in the above experiment. The tubes were sealed and incubated at 37°C for 2 hours and centrifuged. The protein remaining in solution was measured and the maximum p/t mixture in which no remaining protein was detected, was noted. As found with the foaming studies, the maximum p/t complex which was completely insoluble was 5/1, 10/7 and 1/1 for Fraction 1, Fraction 2 and mucoprotein respectively. The action of the tannin in inhibiting foam production was therefore that of precipitating the soluble protein.

5.5 The antifoaming properties of the polar lipids of red clover.

Mangan (1959) suggested that the plant lipids behaved as antifoaming agents in rumen liquor and that penicillin prevented bloat by destroying the bacteria which degrade the lipids present in the rumen. Bailey (1964) showed that the galactolipids were rapidly degraded in the rumen. Russell and Bailey (1966) also examined the sulpholipid of red clover and found it to be highly surface active, decreasing the surface tension of water by 36 dynes cm^{-1} at a concentration of 0.013%^{w}/v. Stifel (1968, Ph.D. thesis) observed a

preferential synthesis of phospholipid in the chloroplasts of low bloat alfalfa pastures.

The polar lipids of red clover leaves were fractionated as described in Chapter 1. Fig. 5 (5. a.) shows a thin layer chromatogram of the separated lipids. Mixtures of protein and individual lipids were prepared as follows. The lipids were dissolved in methanol, and aliquots (less than 0.5 ml) containing the required amount of lipid were placed in a 20 ml volumetric flask 10 ml of a 0.1 mol/litre solution of sodium acetate added and the mixture shaken to give a suspension of the lipid in the buffer. The solution of Fraction 1 protein was added and the contents made up to 20 ml with acetate buffer. The solutions were adjusted to pH 5.8 with acetic acid and foamed in the Laby apparatus. Results for compressive strength, stress relaxation and foam retention volume are given in Fig. 5 (5. b., c.d.). Of the lipids studied, phosphatydyl choline was the most effective antifoaming agent. The compressive strength decreased as the lipid concentration increased, falling from 4.5 g cm^{-2} to 0.35 g cm^{-2} for phosphatydyl choline and to 0.7 g cm^{-2} for the other lipids.

Stress relaxation increased from 0.18 g cm^{-2} sec^{-1} at zero lipid and showed complete instability at 50 µg per ml phosphatydyl choline, decreased to 3.5×10^{-3} g cm^{-2} sec^{-1} above 75 µg/ml digalactosyl diglyceride, showed complete instability above 150 µg/ml monogalactosyl diglyceride, whereas the sulpholipid had only increased the stress relaxation of the foam to 1×10^{-3}

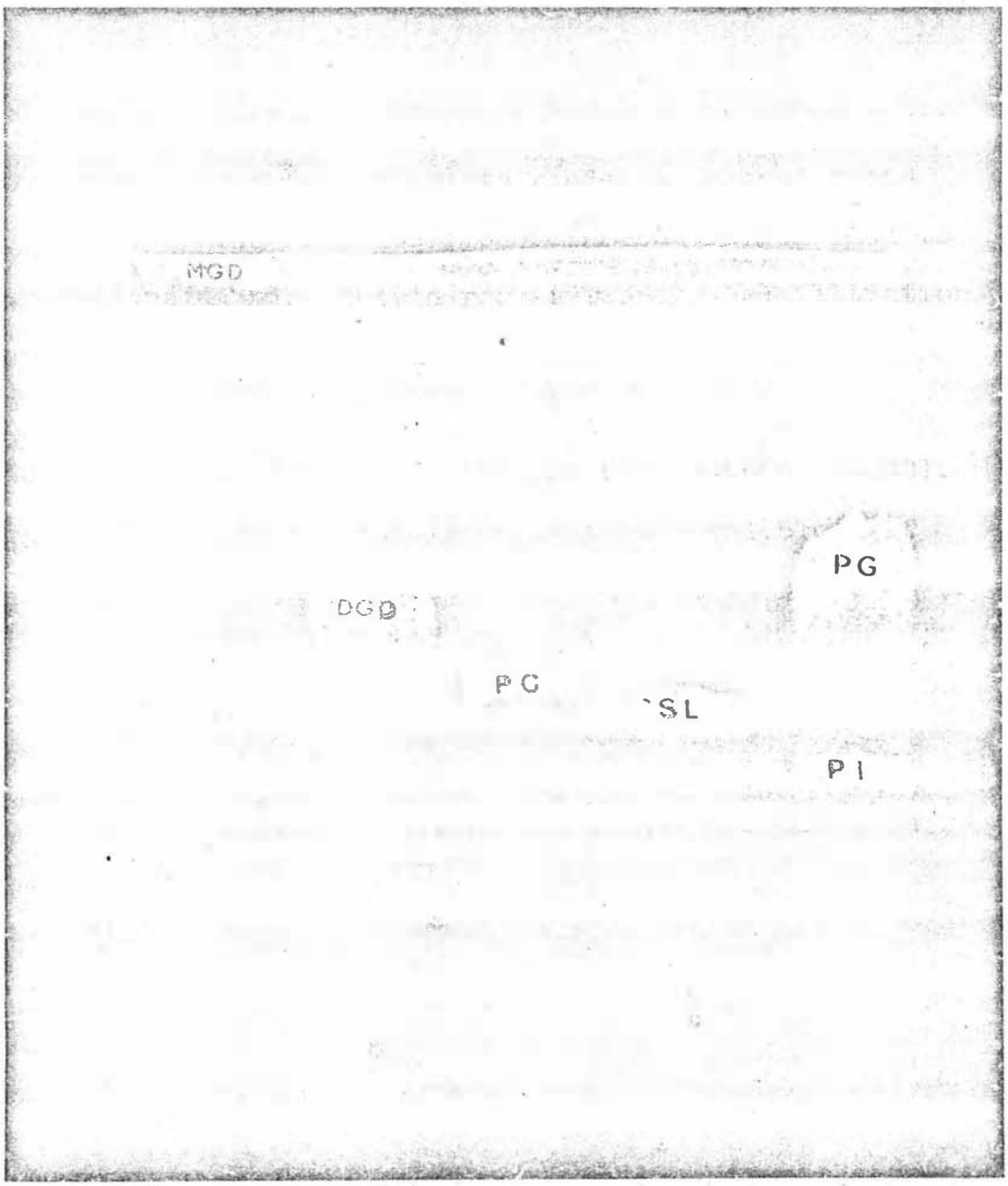

FIG. 5 (5. a) THE FRACTIONATION OF THE POLAR LIPIDS OF RED CLOVER (TRIFOLIUM PRATENSE L.)

MGD Monogalactosyl diglyceride
DGD Digalactosyl diglyceride
PC Phosphatydyl choline
PE Phosphatydyl ethanolamine
SL Sulpholipid
PG Phosphatydyl glycerol
PI Phosphatydyl inositol

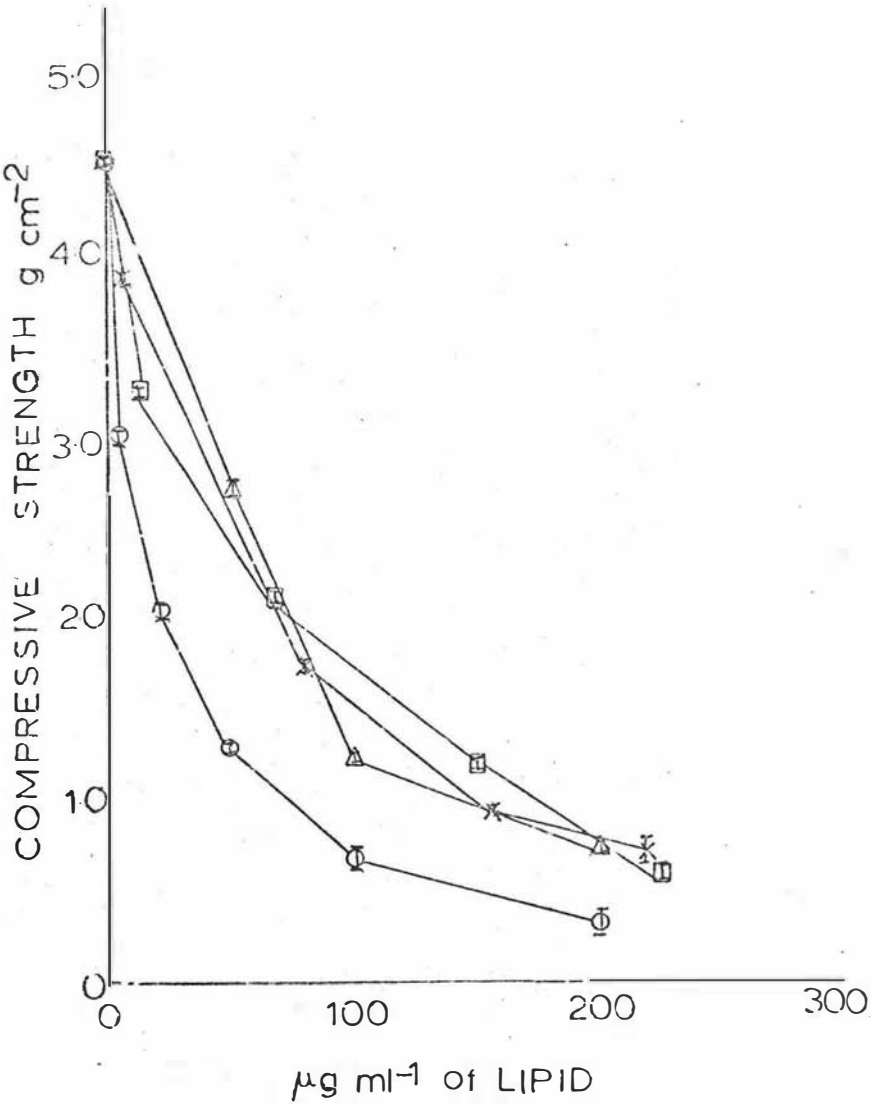

FIG. 5 THE EFFECT OF POLAR LIPIDS ON THE COMPRESSIVE

(5. b) STRENGTH OF FRACTION 1 PROTEIN FOAMS

buffer 0.1 mol/litre sodium acetate (p.8); 0.08% Fraction 1 protein; pH 5.8; temperature 37°C; gas flow rate 30 ml min^{-1}; porosity 3 sinter.

O—O Phosphatydyl choline

□—□ Monogalactosyl diglycerides

×—× Digalactosyl diglycerides

△—△ Sulpholipid

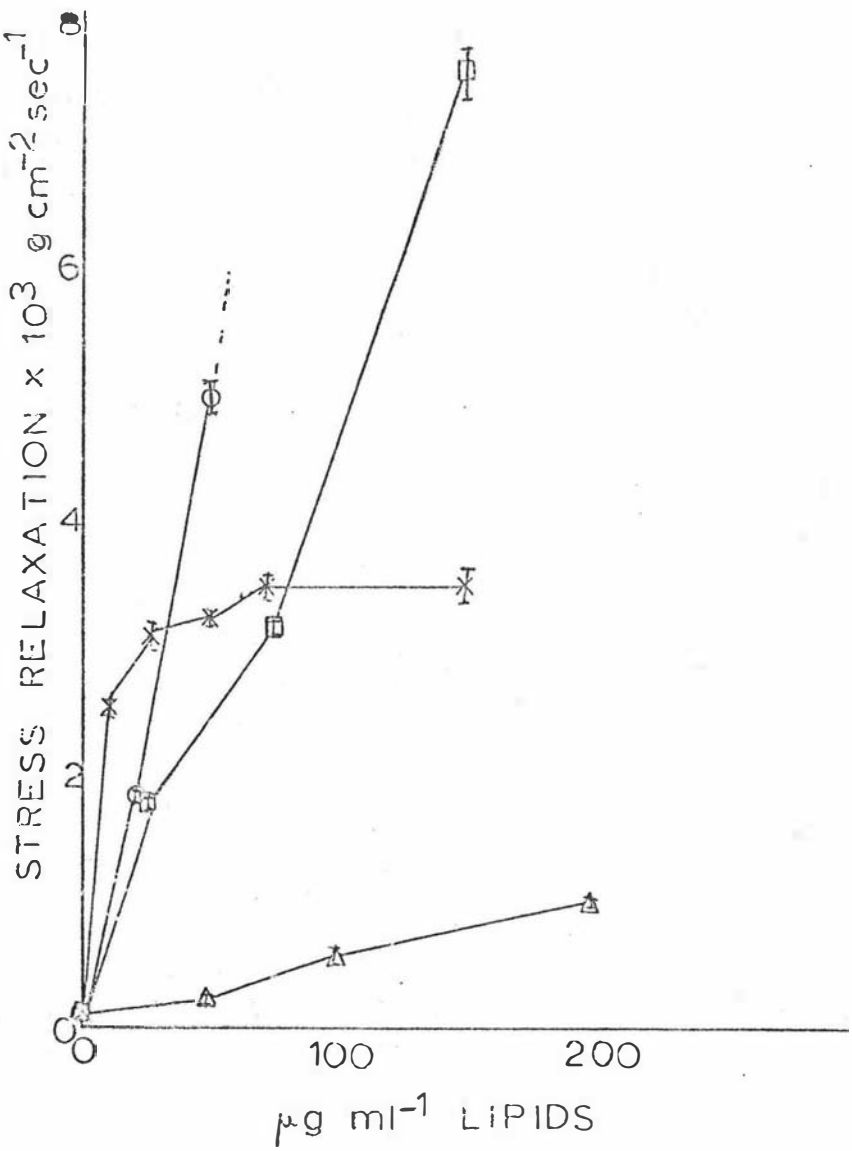

FIG. 5 (5. c) THE EFFECT OF LIPIDS ON THE STRESS RELAXATION OF FRACTION 1 PROTEIN FOAMS

0.1 mol/litre acetate buffer (p.87); 0.08% w/v Fraction 1 Protein; temperature 37°C; pH 5.8; gas flow rate 30 ml min^{-1}; porosity sinter 3.

○---○ phosphatydyl choline
□—□ Monogalactosyl diglycerides
x——x Digalactosyl diglycerides
△——△ Sulpholipid

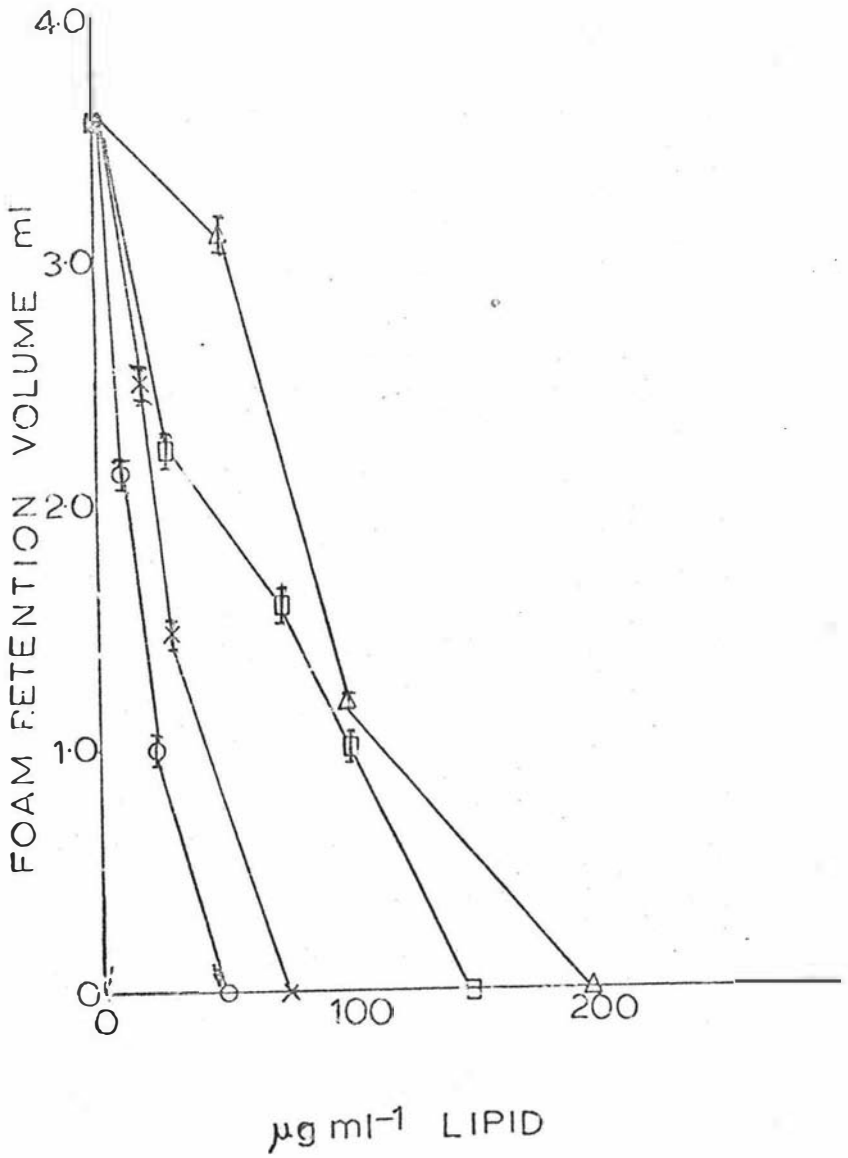

FIG. 5 THE EFFECT OF LIPIDS ON THE FOAM RETENTION VOLUME OF FRACTION 1 PROTEIN FOAMS

(5. d)

0.1 mol/litre acetate buffer (p.87); 0.08% $^{w}/_{v}$ Fraction 1 protein; temperature 37°C; pH 5.8; gas flow rate 30 ml min^{-1}; porosity 3 sinter.

o——o Phosphatydyl choline

□——□ Monogalactosyl diglycerides

x——x Digalactosyl diglycerides

Δ——Δ Sulpholipid

g cm^{-2} sec^{-1} at 200 µg/ml.

The foam retention volume decreased from 3.6 ml to zero at 50 µg ml^{-1} for phosphatydyl choline, to zero at 75 µg ml^{-1} for digalactosyl diglyceride, to zero at 150 µg ml^{-1} for monogalactosyl diglyceride, and to zero at 200 µg ml^{-1} for the sulpholipid. The effectiveness of the lipids as antifoaming agents were phosphatydyl choline > digalactosyl diglycerides > monogalactosyl diglyceride > sulpholipid. The sulpholipid itself produced a weak foam at a concentration of 200 µg ml^{-1} of compressive strength 0.35 g cm^{-2}, stress relaxation 3 x 10^{-3} g cm^{-2} sec^{-1}, and foam retention volume was zero.

In order to test the foam stabilizing properties of the salivary mucoprotein, mucoprotein was added to a solution of 0.08% Fraction 1 protein containing 150 µg ml^{-1} monogalactosyl diglyceride, which was sufficient to prevent formation of a Fraction 1 protein foam, to give a concentration of 0.04% $^{w}/_{v}$ mucoprotein. The solution was foamed in the Laby apparatus at 37°C and at pH 5.8. The foam so produced had a compressive strength of 1.5 g cm^{-2}, a stress relaxation of 0.5 x 10^{-3} g cm^{-2} sec^{-1}, and a foam retention volume of 3.2 ml. The lipid/Fraction 1 protein mixture without any mucoprotein present would not support a stable foam. This is therefore further evidence to suggest the role of mucoprotein as a foam stabilizing agent.

Summary

(1) Calcium (200 $\mu g\ ml^{-1}$) increased the compressive strength of Fraction 1 protein foams at pH's between 5.8 and 6.3, but did not affect Fraction 2 protein foams until the calcium concentration was greater than 1000 $\mu g\ ml^{-1}$. Salivary mucoprotein was unaffected by calcium.

(2) The sodium salt of polygalacturonic acid increased the persistence of Fraction 1 foams by increasing the viscosity of solution being foamed.

(3) Oesophageal mucin was shown to be a powerful foam stabilizing agent of Fraction 1 protein foams.

(4) Salivary mucoprotein increased the persistence of Fraction 1 protein foams, but considerably reduced the compressive strength of Fraction 1 foams. The reduction in compressive strength can be explained in terms of the mucoprotein modifying the surface composition of this film. The persistence of the mixed foams could not be explained in terms of the mucoprotein increasing the viscosity of the solution.

(5) Tannins could completely inhibit foam production from Fraction 1, Fraction 2, protozoal proteins, mucoprotein solutions and rumen liquor from bloating animals feeding on red clover, by producing insoluble complexes between the tannin and these foaming agents. The composition of these complexes, of minimum tannin to protein ratio, were 1/5, 7/10, 1/1 tannin to Fraction 1 protein, Fraction 2 protein and mucoprotein respectively.

(6) The concentration of polar lipid of red clover required to prevent formation of stable foams from a 0.08% solution of Fraction 1 protein at pH 5.8 were 50 μg ml^{-1}, 75 μg ml^{-1}, 150 μg ml^{-1}, and in excess of 200 μg ml^{-1}, for phosphatydyl choline, digalactosyl diglycerides, monogalactosyl diglycerides and sulpholipid respectively.

SECTION 3

DISCUSSION

DISCUSSION

This study was undertaken mainly to examine the properties of foams that can be generated from solutions of plant leaf protein fractions, and by comparison with the foams derived from the rumen liquor of bloating animals, to obtain information regarding the importance of the leaf protein fractions as the foaming agents responsible for the disorder in ruminants known as bloat.

Bloat in cattle is commonly associated with animals feeding on pastures containing a high percentage of legume species (Johns, 1954). It has been known for some time that the soluble leaf proteins of legumes could produce rigid viscous foams (Mangan, 1957). The most convincing evidence relating leaf protein foams to bloat was given by Mangan (1959) who showed that foams, derived from the soluble proteins of red clover and from the rumen liquor of bloating animals, possessed maximum rigidity at the same pH. This result formed the basis for the hypothesis that leaf proteins were responsible for the bloat foam.

Later work carried out by McArthur *et al*. (1966) suggested that of the soluble leaf protein fractions of lucerne, only Fraction 1 protein was capable of producing stable viscous foams. However Jones and Lyttleton (1969) showed that both Fraction 1 and Fraction 2 proteins of red and white clover were capable of producing these foams.

Apart from these preliminary studies on the leaf protein foams, no detailed study of the conditions necessary for the production of foams from the major leaf protein fractions has been made, though such an examination was made for the total leaf proteins of white clover (Laby, 1969).

The first part of the present study was therefore aimed at such an examination using Fraction 1 and Fraction 2 proteins of red and white clover leaves as the foaming materials. Before such a project could be carried out, pure leaf proteins had to be isolated from the clover leaves. The major contaminants of leaf protein preparations are compounds of quinone-type structure, produced by atmospheric oxidation, catalysed by the enzyme phenoloxidase, of the soluble polyphenolic constituents of the leaf. These quinones can produce covalent complexes with the leaf proteins, modifying their pigmentation, their electrophoretic mobility, and their foaming properties (Chapter 3 - 1. c). Red clover (Trifolium pratense) proteins become very highly contaminated with phenoloxidase products, unless the enzyme is inhibited.

The method used by Mangan (1959) and Buckingham (1970) for the isolation of leaf proteins from red clover would have led to serious contamination of the leaf proteins with polyphenoloxidase products.

Stifel (1967) isolated Fraction 1 protein from extracts of lucerne leaf chloroplasts, by first precipitating the total protein with trichloracetic acid, a known protein

denaturant, and redissolving the protein pellet in an alkaline borate buffer. This procedure was shown by Heyes (1968, personal communication) to render permanently insoluble most of the Fraction 1 protein. Stifel claimed to have fractionated his protein preparation by gel filtration on Sephadex G50. This gel has an exclusion limit of 50,000 and would not be expected to separate Fraction 1 protein (MW 500,000) from Fraction 2 proteins (MW 10,000-300,000). When gel filtration is used to separate the soluble leaf proteins, either Sephadex G200 (exclusion limit 800,000) or an agarose gel (exclusion limit 1,000,000) are suitable gels (Pon, 1967; McArthur, 1966). This would cast doubt on the significance of Stifel's results in relation to the binding properties of Fraction 1 protein for calcium in relation to bloat.

In this thesis considerable attention was given to isolating the soluble leaf protein fractions in as pure a form as possible and free of any phenoloxidase products, using methods which would minimize denaturation of the soluble proteins. The procedures used are described in Chapter 1 - 2 a of the experimental section.

Two systems were used for measuring the foam parameters of protein foams. One of these was a modified version of the Mangan apparatus (1958). In this apparatus the resistence to a weight falling through a column of foam was measured. This was termed foam strength and is a measure of the rigidity of the foam. The second system used to measure foam parameters was described by Laby (1969). This apparatus measured

in addition to rigidity (compressive strength), parameters (stress relaxation, foam retention volume, and drainage constant) which could be related to the persistence of foams. The Mangan apparatus was useful for studying viscous foams only, whereas the Laby apparatus was useful for studying all types of foams, especially those parameters which gave a measure of the persistence on non-rigid foams. The Laby apparatus was therefore used for characterizing all the foams studied in this thesis, whereas the Mangan apparatus was used only for studying the leaf protein foams.

The properties of leaf protein foams

The leaf protein foams were characterized primarily by their high rigidity. These foams were only persistent when their compressive strength was high. The proteins were irreversibly denatured in the foam, becoming insoluble as a result of their action on stabilizing the gas/water interface of the bubble films present in the foam. The insolubility of the denatured protein made it possible to determine the amount of denatured Fraction 1 protein required to produce a given volume of foam. Using this property it was shown that the rigidity (compressive strength) of Fraction 1 protein foams was closely related to the amount of denatured Fraction 1 protein in the foam.

The foams produced from solutions of Fraction 1 protein had maximum rigidity and persistence at pH 5.8. This pH is close to the isoelectric pH (Pon, 1967) of Fraction 1 protein. Fraction 2 protein foams exhibited maximum rigidity

at pH's close to 5.1, and produced persistent foams over the pH range 4.5 to 6.3. This wide pH range over which the foams are persistent is presumably explained by the heterogeneous nature of these proteins giving rise to a range of isoelectric pH's.

The maximum amount of Fraction 1 protein denatured on foaming also occurred at the isoelectric pH of the protein. This can be explained by recognising that the net electrostatic repulsion is a minimum and the cohesion between the protein molecules is a maximum at the isoelectric pH of the protein, when the net charge on the protein is zero.

Mangan (1959) and Laby (1969) showed that the total soluble leaf proteins of clovers produced foams of maximum rigidity at pH's between 5.4 and 6.0. It would therefore seem that the maximum rigidity that these workers reported was due to denatured Fraction 1 protein in the foam.

The minimum concentrations of Fraction 1 and Fraction 2 proteins at pH 5.8 and 37°C, required to produce foams having measurable stress relaxation were 0.02% and 0.03% w/v respectively.

The size of the bubbles making up the foam greatly affected the rigidity of the leaf protein foams. For any given protein concentration, rigidity increased as the bubble size of the foam decreased. This result can best be explained in terms of the amount of denatured protein in a given volume of foam of different bubble size.

For small-bubble foams, the total surface area of the bubbles constituting the foam is greater than in the

same volume of foam made up of larger bubbles. The greater surface area produces a greater amount of surface denatured protein. The compressive strength of the foam, which increases with the amount of surface denatured protein in the foam (Chapter 3 - 8), will therefore be greater the smaller the bubble size in the foam.

The properties of protozoal protein and the salivary foams.

The other main sources of protein that could be present in the rumen of bloating animals are the protozoal proteins and the proteins of the salivary secretions. The theories regarding the importance of these proteins was discussed in Section 1 (Chapter 1 - 3. a. 3 and 3. a. 5).

The holotrich protozoal proteins produced foams with properties very similar in nature to the leaf protein foams. The optimum pH for production of persistent viscous foams from protozoal proteins was close to pH 5.9. The holotrich protozoal proteins were surface denatured in stabilizing the gas/water interface of the bubbles comprising the foam.

The salivary secretions examined in this thesis were taken either from the oesophageal secretion (Section 2, Chapter 1 - 2. b. 2) or the salivary mucoprotein which was isolated from the mouth saliva of feeding animals (Chapter 1 - 2. b. 1. a). These secretions were very different in composition and in their foaming properties. The oesophageal secretion contained in addition to low molecular weight proteins a high molecular weight material which stained for carbohydrate but not for protein. This secretion did not produce stable foams.

The salivary mucoprotein (Lyttleton, 1964) produced persistent foams of low compressive strength, a result which agrees with the low foam strength reported by Mangan (1959). The foams generated from mucoprotein solutions were unaffected by changes in pH over the pH range 4.0 to 7.5. The salivary mucoprotein foams therefore, were very different in character to the plant or protozoal protein foams.

The relation of the protein foams to bloat.

The earlier implications of the role of leaf proteins was based on the evidence that rumen liquor and leaf proteins produced foams of maximum mechanical strength at the same pH. The idea that Fraction 1 protein was the cause of bloat resulted from the research of the Canadian workers (Section 1, McArthur et al.).

In this thesis it was shown that the optimum pH for the production of persistent viscous foams from protozoal proteins occured at pH 5.9. This pH is so close to the pH optimum for Fraction 1 protein foam production (pH 5.8) and for rumen liquor foams (pH 5.7) that it would be impossible on this basis to distinguish between plant proteins or protozoal proteins as the foaming agents causing the pH optimum in rumen liquor foams. It is therefore possible that Clarke's hypothesis (Introduction, 1. 3. a. 5) regarding the role of protozoa in the bloat syndrome is correct.

The foaming properties of rumen liquor taken from bloating animals were studied. The rumen foams were characterized by their low compressive strength but very

high persistence. In this respect they differed from the plant and protozoal protein foams, but bore a closer relationship to the mucoprotein foams. However, whereas the mucoprotein foams were unaffected by changes in the pH of the solution being foamed, the rumen liquor exhibited two maxima of rigidity at 5.7 and 4.0. Foam persistence was associated only with the pH 5.7 maximum. The only resemblence between the foams from protozoal proteins or the plant proteins and those from rumen liquor was the pH optima for persistent foam production. The important parameters associated with rumen foams were those measuring persistence rather than rigidity. The Mangan apparatus therefore was not as useful as the Laby apparatus in the study of these foams.

The differences between the rumen and the Fraction 1 protein foams prompted a closer examination of the role of Fraction 1 protein in the production of rumen foams. These studies revealed that:

(a) The level of Fraction 1 protein in the rumen liquor was too low to account for the persistence of the rumen foams. The maximum concentration in the rumen liquor was 0.02% $^{w}/_{v}$ which was the minimum required to produce Fraction 1 protein foams that had measurable persistence.

(b) When rumen liquor containing 0.02% $^{w}/_{v}$ Fraction 1 protein was foamed, only 24% of the Fraction 1 protein was surface denatured as opposed to 100% for a 0.02% Fraction 1 protein solution at pH 5.8.

(c) Addition of Fraction 1 protein to rumen liquor to give

a concentration of 0.2% $^{W}/_{V}$ only slightly increased the rigidity without affecting the persistence of the foams that could be generated from the rumen liquor.

These results suggested that while Fraction 1 protein is present as a component of the rumen foam, it is not the main surfactant responsible for the foam and that other materials are controlling the characteristics of the rumen foams.

The effect of temperature on the foam parameters of foams derived from rumen liquor and centrifuged rumen liquor differed greatly. The rigidity of the centrifuged rumen liquor foams increased with increase in temperature whereas the rigidity of the foams derived the uncentrifuged rumen liquor showed the presence of a possible phase change over the temperature range 15°C to 20°C. The compressive strength was constant up to 15°C, decreased rapidly over the range 15°C to 20°C, and remained constant from 25°C to 50°C.

A possible explanation of this phenomonen, which is apparently associated with the particulate material, is that above a critical temperature, antifoaming agents are spreading into the surface film from solid particles (the critical temperature for C_{18} fatty acids to spread from a solid into the surface is close to 18°C (Davies and Rideal, 1961). As a consequence some of the protein would be displaced from the surface film, thus lowering the compressive strength of the foam.

Another explanation is that a viscous material becomes soluble at this critical temperature. It has been shown in this thesis (Section 2, Chapter 5, 3) that the effect of viscous materials, such as polyvinylpyrrolidone, dextran and sodium pectate, was to decrease the compressive strength and increase the persistence of foams. The stress relaxation of the rumen foams increased slightly at this critical temperature, indicating that the foams became less stable. It appears therefore that the antifoaming agent hypothesis is the more likely explanation of the temperature effect.

It would therefore seem that the particulate material is modifying the properties of the rumen foams, and that further research should be carried out on this fraction. This phenomenon could possibly be best examined by a surface chemical study of the system, but such a study was outside the scope of this thesis.

Interactions between Fraction 1 protein and other surfactants.

In the above section it was suggested that while the plant or protozoal proteins or both could be responsible for the maximum in rigidity of the rumen foams at pH 5.7, these proteins were only minor components of the film, and the persistence of the foams was controlled by other factors. In Chapter 5 of the experimental section the type of foam that could be generated from mixtures of Fraction 1 protein and other surfactants was studied in an attempt to explain the characteristics of the rumen foams.

Although either Fraction 1 protein or the protozoal proteins could have been studied in this section, Fraction 1 protein was used because it is a well defined protein, was easily isolated in large quantities and also has been claimed as the plant protein responsible for the bloat foam (Introduction, 1. 3. a. 6).

One promising theory which has been put forward concerning the cause of bloat involves the idea of a balance between the foam stabilizing agents and the foam inhibitors or foam breakers. If the concentration of these substances in the rumen favours the production of a stable foam, then the balance is shifted in favour of the bloating condition (Stifel, 1967).

(a) Lipids

The presence of antifoaming agents in the rumen liquor has so far not been considered in this discussion. The plant lipids have been thought of as the main source of antifoaming agents (Introduction, Chapter 1, 3. b). In this study, the polar lipids of red clover leaves were examined for their effectiveness as antifoaming agents on Fraction 1 protein foams. The antifoaming potential of these lipids was in the order: phosphatydyl choline $\rangle$ digalactosyl dyglyceride $\rangle$ monogalactosyl diglyceride $\rangle$ sulpholipid.

The effect of the plant lipids was to reduce the rigidity and the persistence of Fraction 1 protein foams. To explain the properties of the rumen foams in terms of protein as the foaming agent and lipid as the antifoaming agent, the presence of extremely efficient foam stabilizing agents would be required to account for the high persistence

of the rumen foams.

Other materials were therefore examined for their potential as foam stabilizing agents of Fraction 1 protein foams.

(b) Pectins

Pectin has been suggested for some time as a possible stabilizing agent of the foam present in the reticulo-rumen of animals suffering from bloat (Introduction, Chapter 1, 3. a. 2.).

In the present study (Chapter 5, 2.) sodium pectate, though not producing a stable foam by itself, stabilized Fraction 1 protein foam when present in solution at a concentration of 0.04% or more.

Nichols and Deese (1966) suggested that pectin methyl esterase was an important factor in the bloat syndrome. This enzyme hydrolyses the methyl pectins to give pectic acid and methanol. They suggest that the pectic acid, in the presence of calcium or other ions increase the viscosity of rumen liquor by producing pectate gels, which trap rumen gases in a stable viscous foam.

In this study, attempts to examine the effect of calcium pectate on Fraction 1 protein foams failed, either because of precipitation of calcium pectate or, at higher concentrations of pectic acid, because of gelling of the solution before the foam could be generated.

(c) The salivary secretions

The salivary secretions have been implicated both as foam stabilizing agents and as antifoaming agents (a detailed

survey concerning the properties of the salivary secretions was given in the Introduction, Chapter 1, 3. a. 3.) Because of the possibility of the salivary secretions having foam stabilizing properties and because the properties of the salivary mucoprotein foams resembled to some extent the properties of the rumen foams (Section 2, Chapter 3, 2), the types of foam that could be generated from solutions of mixtures of Fraction 1 protein and the salivary secretions were studied (Section 2, Chapter 5, 3).

Two secretions were examined for their effect on Fraction 1 protein foams. The first of these was salivary mucoprotein which was purified from total mouth saliva as described by Lyttleton (1964), and the second secretion was collected from the oesophagus and termed the oesophageal mucin.

These secretions were shown to be different in composition and behaved differently in their interaction with Fraction 1 prctein.

The oesophageal mucin was an extremely effective stabilizing agent for Fraction 1 protein foams. The compressive strength of the foams were not affected by this mucin, but the foam retention volume increased from 0.2 ml for 0.028% Fraction 1 protein alone to 3.6 ml for the foams generated from a 0.028% $^{w}/_{v}$ Fraction 1 protein solution containing mucin of 15 $\mu g\ ml^{-1}$ sialic acid content. Stress relaxation also indicated an increase in persistence, the stress relaxation decreasing from 6.0×10^{-3} g cm^{-2} sec^{-1} for Fraction 1 alone to 0.9×10^{-3} g cm^{-2} sec^{-1} for Fraction

1 protein containing oesophageal mucin of 15 $\mu g\ ml^{-1}$ sialic acid content.

The high retention volume of the foams generated from the Fraction 1 protein/oesophageal mucin mixtures is probably due to the strong water-mucin interactions.

The salivary mucoprotein decreased the compressive strength of a 0.02% $^{W}/_{V}$ Fraction 1 protein foam from 3.6 g cm^{-2} to 1.1 g cm^{-2} at a mucoprotein concentration equal to or greater than 0.04% $^{W}/_{V}$. This reduction in compressive strength was of greater magnitude than could be explained by the effect of increased viscosity of the mucoprotein molecule. Thus when polyvinyl pyrrolidone and dextran were added to Fraction 1 protein solutions to give solutions of viscosity equal to the viscosity of a 0.04% mucoprotein solution, the compressive strength of the foams generated from these solutions were 2.6 g cm^{-2} and 2.8 g cm^{-2} respectively.

Examination of the Fraction 1 protein denatured on foaming these mucoprotein mixtures showed that the denatured protein in the foams generated from 0.02% $^{W}/_{V}$ Fraction 1/0.04% $^{W}/_{V}$ mucoprotein mixtures was only 56% of the Fraction 1 denatured in foaming a 0.02% Fraction 1 solution under the same conditions. Since compressive strength has been shown to be related to the level of denatured Fraction 1 protein in the foam, then the reduction of denatured Fraction 1 protein in the foams generated from these mixed solutions probably accounts for the observed reduction in the compressive strength of these foams.

The stress relaxation and foam retention volumes of the foams generated from the Fraction 1 protein/mucoprotein mixtures indicated a maximum persistence for foams generated from solutions containing mucoprotein/Fraction 1 protein of composition 2/1, $^{w}/_{w}$. Possibly Fraction 1 protein/mucoprotein complexes occur in the surface films of the foams generated from these mixtures, and the nature of the interaction could be explained by a surface chemical study of the system which was beyond the scope of the present thesis.

The results obtained in this thesis do not agree with the theory proposed by Bartley and Yavarda (1961) who claimed the salivary secretions to be foam destabilizers on the basis that addition of animal saliva or plant mucilages to a saponin foam increased the release of gas from the foam.

The results do agree with the hypothesis of Johns (1958), that the salivary mucoprotein helped to form a stable viscous foam, and with the observations of Clarke (1969, unpublished results) that addition of bovine saliva to rumen contents being studied in artificial fermentors (Clarke et al. 1969a) resulted in production of very persistent foams.

Salivary mucoprotein was added to a solution of Fraction 1 protein, containing sufficient lipid to prevent a stable foam being produced, and the mixture foamed. The foam resembled the rumen foams in its rigidity and its persistence. A mixed system, containing leaf protein, salivary mucoprotein, and lipid was able to support a foam which was very similar

to the foams produced from rumen liquor with respect to the parameters measured here. To test whether or not such a system could exist in the rumen of bloating animals, the surfactants present in rumen liquor were examined.

The surfactants in the rumen of bloating animals.

The use of the foam fractionation method to study the surfactants that concentrated in the in vitro foam generated from rumen liquor, showed that lipids had concentrated in the foam from 2 to 2½ times indicating that lipids were an important component of the rumen foam. The lipids were shown to consist mainly of long chain fatty acids and neutral lipids.

The soluble components responsible for foam production were precipitated from centrifuged rumen liquor with 60% $^{w}/_{v}$ ammonium sulphate, and were shown, by acrylamide gel electrophoresis, to contain several protein components, (including Fraction 1 protein) and also a glycoprotein. The glycoprotein resembled the salivary mucoprotein in its behaviour in the analytical ultracentrifuge, and in its precipitation properties. This material was purified and its antibody produced by injecting it into rabbits. The antibody when cross-reacted with the leaf proteins, protozoal proteins and the two salivary secretions that were studied in this thesis, gave positive precipitation reactions only with the salivary mucoprotein and to a lesser degree the oesophageal mucin.

Bartley and Bassette (1961) described their isolation of the foaming agent in rumen liquor and showed it to

contain 63% protein and 17% carbohydrate. Their procedure was to adjust the pH of the solution to pH 4.0, clarify the solution which still contained the foaming agent in solution, and subsequently precipitate the foaming agent with ethanol. The plant protein (Fraction 1 and most of Fraction 2 proteins; Section 2, Chapter 3, 1. b. 8.) and the protozoal protein are insoluble at pH 4.0 and therefore would not have been included in Bartley's foaming agent preparation. In fact, of the sources of protein (salivary, plant and protozoal), only the salivary mucoprotein, because of its low isoelectric pH, would still be in solution at pH 4.0 and consequently this would have been precipitated by addition of ethanol as described by Bartley et al. (1961). The material which was isolated in this study behaved as Bartley's preparation, and is probably similar material, which from its antigenicity and chemical properties would appear to be related to the salivary mucoprotein.

Incubation of salivary mucoprotein with neuraminidase, an enzyme which cleaves the sialic acid residues from the mucoprotein molecule, resulted in a degraded salivary mucoprotein molecule, which was more sensitive to the precipitin reaction of the antibody of the rumen foaming agent. This would suggest that the material isolated from the rumen liquor could be related to salivary mucoprotein which had undergone mucinolytic degradation.

Mishra et al. (1967, 1969) indicated that bloat was associated with large colonies of mucinolytic bacteria

in the saliva and rumen contents of the bloating animals. These workers believed that saliva was an antifoaming agent, which when degraded by mucinolytic bacteria destroyed the antifoaming properties of the saliva. However in view of the results described above, another explanation of Mishra's results could be that the mucinolytic bacteria degraded the saliva, and the sialic-free mucoprotein is the foaming agent or foam stabilizing agent present in the rumen.

The role of tannins in the bloat syndrome.

In this thesis, the soluble proteins (extractable in phosphate buffer - pH 7.5) from a group of bloating and non-bloating legume pastures were examined. All the temperate non-bloating species were extremely low in soluble protein, a result which confirmed the research of McArthur et al. (1968) who found that the non-bloating legumes contained very low concentrations of Fraction 1 protein. These results seemed unusual because many of the non-bloating legumes have soft, green leaves that would be expected to be high in photosynthetic activity, and hence high in Fraction 1 protein which is the protein associated with the photosynthetic activity.

In this study the material from non-bloating pastures was also extracted into phosphate buffer containing 2% $^{w}/_{v}$ polyvinylpyrrolidone, PVP, a substance which has been reported to form complexes with phenolics (Anderson, 1968). These extracts contained the normal levels of protein found in the extracts made from the bloating legumes. These

results suggested that the temperate non-bloating legumes contained constituents that are released on grinding the leaf tissue, and precipitate the soluble leaf proteins, and which preferentially combine with PVP rather than with proteins. These protein precipitants were isolated from Lotus pedunculatus Cav. and shown to be condensed tannins, which were shown to form insoluble complexes with the soluble leaf proteins.

Tannins have been defined by Swain (1965) as compounds of molecular weight 500 to 3,000, containing at least 1-2 phenolic hydroxyl groups per 100 molecular weight, which can combine with proteins causing precipitation of the proteins. No satisfactory chemical assay of tannins seems to have been devised (Feeney and Bostock, 1968) which led us in this thesis to detect their presence in legumes by their action as protein precipitants.

A great deal of misunderstanding has arisen over the term 'tannin'. Most reports of tannins in forages have measured tannin as total phenolic compounds or as the leucoanthocyanins, which are monomers of the condensed tannins. The leucoanthocyanins are common to most legume pastures and do not cause precipitation of the soluble leaf proteins. Thus Miltimore et al. (1970) claimed that 'tannins', measured as leucoanthocyanins, were present in the leaves of lucerne (Medicago sativa L.) to a concentration of 2% dry weight, and that the bloat potential of the forage increased as the 'tannin' content of the leaves increased.

In this thesis, lucerne was examined for the condensed tannins (by their ability to precipitate proteins) but these were not detected in any of the lucerne plants examined. We conclude that Miltimore et al. could not have been measuring true tannins, but probably the leucoanthocyanins which are very common in the legume pastures but do not cause precipitation of the soluble leaf proteins.

These results would appear to support the theory that leaf proteins are in fact the foaming agents responsible for the bloat foam. However, a closer examination of the precipitation properties of the condensed tannins of Lotus pedunculatus Cav. revealed that these tannins not only precipitated the soluble leaf proteins, but also the protozoal proteins, the salivary mucoprotein, and the foaming agents present in the rumen liquor taken from bloating animals. The tannins therefore appear to be unspecific for the precipitation of proteins. Therefore it would seem that if tannins could be incorporated into the bloat producing legume pastures, bloat could be eliminated by the action of the polymeric condensed tannins on the proteinaceous rumen foaming agents, causing their precipitation from solution and inhibition of foam production.

This could possibly be achieved in two ways:

(1) To breed tannins into the already existing bloating legume.

(2) To feed animals on mixed pastures of the bloating pasture and another species containing tannins.

In New Zealand, the major bloating species are the clovers and therefore a selection of species from the *Trifolium* genus were examined. Of the 30 *Trifolium* species examined in this thesis, tannins were found only in the species *Trifolium arvense L.* and although this particular species is useless as a foraging pasture, the finding of tannins in the *Trifolium* genus does show that the possibility exists of finding a species which might prove suitable for breeding into the New Zealand pastures in an attempt to minimize the occurence of bloat.

Tannins were shown to be common in the *Lotus* species examined in this thesis. If the problems of mixed regrowth that occurs in a white clover/*Lotus pedunculatus* pasture could be overcome, a pasture containing clover and a tannin-containing pasture such as *Lotus*, closely associated so that they are grazed together, might also prove useful in controlling bloat.

Concluding Remarks

Bloat in cattle is caused by the presence of a stable viscous foam in the rumen. The cause of the foam cannot be explained in terms of any single foaming substance, though leaf proteins have been implicated by others as the main foaming substance.

The results of this study indicated that although the leaf proteins are probably a necessary component of the bloat form, they cannot by themselves account for the persistence of the bloat foams produced in the rumen of

animals feeding on red clover pastures.

The basic requirements for the production of foams resembling the rumen foams are:

(1) Proteins that can produce foams of maximum persistence at pH 5.7. e.g. leaf or protozoal proteins.

(2) Foam stabilizing agents. The results of this thesis suggested the salivary secretions.

(3) The plant lipids.

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APPENDIX

Throughout this thesis, equipment and specialized chemicals have been marked with an asterisk*. Details of the manufacturers' addresses and chemical properties of these materials are given below:

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"Pluronic" is the trade name of a series of polymers composed of polyethylene glycol polymers that have been condensed on to both ends of a polypropylene glycol base unit. They can be represented by the chemical formula:

$$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_cH$$

where a, b, c are the average number of repeating units in the polymer.

These materials are extremely effective non-ionic detergents. They are manufactured by "Wyandotte" Chemicals, Wyanadotte, Michigan.

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"bis-acrylamide" - N,N'methylene bisacrylamide. Chemical formula $(CH: CHCO\ NH_2)_2\ CH_2$. It is the crosslinking monomer used in the preparation of polyacrylamide gels for electrophoresis. This material was manufactured by Eastman Organic Chemicals, Rochester, New York, U.S.A.

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Temed is the common name for N,N,N',N' tetramethylethylene diamine ($(CH_3)_2NCH_2CH_2N(CH_3)_2$). It is used as a catalyst in the polymerization of acrylamide by free radicals. The material was manufactured by Eastman Organic Chemicals, Rochester, New York, U.S.A.

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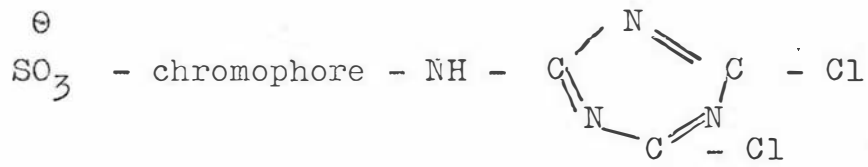
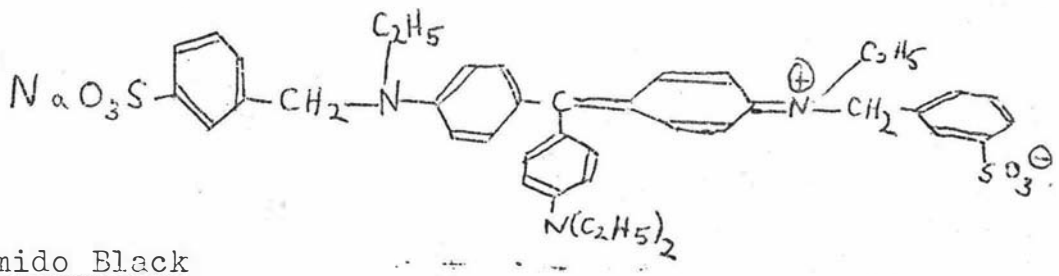
Coumassie Brilliant Blue, Procion Blue and Amido Black are dyes which have been found useful at detecting proteins following electrophoresis (Fazekas de St. Groth, S., Webster, R.G., Datyner, A. 1963, Biochem. Biophys. Acta 71, p.377). The chemical forumlae are:

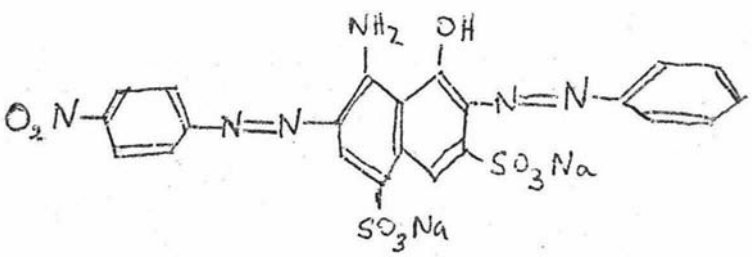
Procion Blue



Coumassie Brilliant Blue



Amido Black



All of these dyes were manufactured by Imperial Chemicals Industries, Great Britain.

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Microdensitometer, Model MK IIIC; an automatic scanning recording densitometer, double beam in operation, with density ranges 0 - 0.5, 0 - 1.0 and 0 - 2.0, and linear magnification adjustable between x1 and x20. Supplied by Joyce Loebl & Co., Ltd., Princesway, Team Valley, Gateshead 11 England.

page 35

Analytical Ultracentrifuge Model E; a mechanically driven ultracentrifuge, fitted with schlieren optical system, capable of rotational speeds up to 60,000 r.p.m., mean distance of sample cell from rotation axis 6.5 cm. Supplied by Beckman Instruments, Spinco Division, Palo Alto, California, U.S.A.

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Amino Acid Analyser Model 120C; an automated instrument using cation exchange chromatography on a strongly acid ($-SO_3$) resin (Dowex 50 x 8); separate columns used for basic amino acids and for neutral and acidic amino acids, the former eluted with citrate/HCl buffer, 0.35N, pH 5.25; the latter with similar 0.2N buffer at pH 3.25 followed by pH 4.25; the sensitivity of the instrument is 10^{-8}M for each amino acid; calibrated prior to use with mixture of 0.1 μ mol of 18 amino acids commonly resolved. Supplied by Beckman Instruments, Palo Alto, California, U.S.A.

page 40

Diaflo "Centiflo" membranes; conical ultrafilter membranes, to retain molecules of molecular weight greater than 50,000. Supplied by Amican Corporation, Lexington, Mass., U.S.A.

page 40

Atomic Absorption Spectrophotometer, Techtorn AA4; when fitted with the appropriate Ca hollow cathode source, this instrument gives 50 % absorption of the spectral line at 4227 Å at a sample concentration of 7 p.p.m. Ca^{++}, and has a limit (1% absorption) of 0.1 p.p.m. Supplied by Perkin Elmer Instruments Ltd., Beaconsfield, Bucks., England.

page 41

Silica gel G (for thin layer chromatography) was supplied by E. Merck, A.G. Darmstadt, Germany.

page 41

Thin Layer Spreader, Unoplan; will spread on uniform layer of material on glass plates for thin layer chromatography, of adjustable thickness from 0.1 to 1.5 mm. Supplied by Shandon Scientific Co., Willesden, London, N.W.10.

page 44

Polyclar A.T. is the commercial name for a water insoluble polyvinyl pyrrolidone. It is manufactured by the General Aniline Film Corporation, New York, U.S.A.

page 47

Column Effluent Monitor, Uvicord; measures the absorption of ultra-violet light (254 nm) by the liquid flowing from the column, and records the values on a strip chart recorder; flow cell used was 3 mm section. Supplied by L.K.B. Produkter , Stockholm 12, Sweden.

page 47,50

Sephadex and Sepharose are the trade names of crosslinked dextran and agarose gels respectively. The different types of Sephadex and Sepharose gels differ in the degree of cross-linking or agarose content, which results in different pore sizes in the gel matrix. The limiting molecular size which can diffuse into the gel matrix varies with the pore size in the gels, which act as molecular sieves and therefore separate molecules on the basis of their molecular size. In this thesis two Sephadex gels (G75 and G200) and one Sepharose gel (4B) were used. These gels are defined by the following limiting molecular weights which can diffuse into the gel.

		Limiting M.W.
Matrix:- Sephadex	(G - 75	75,000
	(G - 200	800,000
	Sepharose 4B	3,000,000

Sephadex and Sepharose gels are manufactured by Pharmacia Products Limited, Uppsala, Sweden.

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Preparative Ultracentrifuge L-50 and L-65; high speed centrifuges in which the rotor runs in an evacuated, refrigerated chamber; the rotor used was type 50, with a maximum sample radius of 7.1 cm and a top speed of 50,000 r.p.m. Supplied by Beckman Instruments, Spinco Division, Palo Alto, California, U.S.A.

page 52

3.MM filter paper was manufactured by Whatman (W. & R. Balston Limited, England).

page 59

DEAE cellulose, is a cellulose powder on which diethyl-aminoethyl (DEAE) groups have been condensed to produce an anion exchange material. The material used in this thesis was manufactured by Bio-rad Laboratories, Richmond, California. All other chemicals were of analytical grade quality.

page 67

Transducer UL-10-120; a strain gauge in which a varying thrust or load on a sensing arm differentially changes the tension on two pairs of resistence wires, thus changing the voltage output of a bridge circuit of which the wires are components; a change in load of 10 g gives an output of approx. 2 mV per 1 volt imput to the bridge; up to this

point the output is linear with load to ± 1%. Supplied by Shinkoh Communication Industry Co., Zushi, Japan.

page 68

Millvolt Recorder Model QPD54; a linear chart recorder with a D.C. imput range: 1, 2, 5, 10, 20, 50, 100, 200 and 500 mV in stepwise ranges, a sensitivity of better than 0.1% of full scale, and an accuracy of better than ± 0.5% of full scale deflection. The balancing speed of the recorder is less than one second for full scale movement of the pen. The chart speed is variable from 5 mm/min to 240 mm/min and the effective chart width is 250 mm. The zero adjustment is continuously adjustable over full scale. The recorder is supplied by Hitachi, Ltd. Tokyo, Japan.