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A STUDY OF BOVINE SALIVARY PROTEINS

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
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at the
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by

JAMES TREVOR McINTOSH

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ABSTRACT

This physiological genetics approach to the study of the bovine salivary proteins was designed to: (a) Test the hypothesis that the salivary proteins, other than mucoprotein, might be related to, or involved in determining an animal's susceptibility to bloat. (b) Gain some knowledge on the nature of the proteins in bovine saliva. This study paralleled the breeding programme selecting animals for high (HS) and low (LS) susceptibility to bloat. Two groups of adult cows, classified as HS or LS by field gradings (Group 1 and Group 2), and three groups of HS and LS progeny (1973, 1974 and 1975 animals), were studied.

Part I of this project involved studying saliva flow, total protein, and the eleven protein bands separated by polyacrylamide gel electrophoresis (PAGE) from saliva samples (collected from the mouth of cows), in relation to bloat grade or susceptibility.

Consistent results from these studies indicated that protein bands 4 and 6 were positively related to bloat susceptibility, and saliva flow and band 7 were negatively related to susceptibility. Band 7 was also negatively related to individual cow variance and positively related to saliva flow. It was demonstrated that a cow's saliva flow could be markedly stimulated by the oral administration of acetic acid and this stimulation also increased the proportion and concentration of band 7.

The time since a cow last had access to feed which did not induce bloat, made very little difference to the parameters measured in saliva. However, in a series of Pasture Type Trials, where saliva samples were collected from animals directly after coming off grass or 'bloat potent' clover pasture, the level of band 4 was increased (shown as an environmental correlation between bloat grade and band 4). Band 6 showed a positive phenotypic correlation with bloat grade and a positive environmental correlation with saliva flow, while band 7 showed a positive correlation with protein.

Changes in bloat grade and the salivary parameters with age were followed in the 1973, 1974 and 1975 animals.

Results from studies in Part Two, indicated that immunoglobulins were present in saliva and a semi-purified protein fraction, band 1-2-3 complex, when run on SDS-electrophoresis was composed of subunits corresponding in molecular weight to those of IgG and secretory-IgA. Comparisons between saliva collected from the mandibular gland, mouth and oesophagus indicated that band 1 was secreted by the mandibular gland.

Band 6 was shown to contain two proteins one of which cross-reacted with anti-albumin serum, had the same electrophoretic mobility and molecular weight as serum albumin.

Band 7, 8 and 9 were statistically related and this relationship was demonstrated chemically by incubating saliva or semi-purified proteins, band 7-8-9 complex, with neuraminidase. The molecular weight of this complex was 65 300 (2 800 SEM) and appeared to be composed of three sub-units with molecular weights of 26 000, 22 500 and 17 500.

Band 7 and 8 appeared to be secreted by the mandibular glands.

Other studies included, screening for enzymic activity, SDS-electrophoretic studies to determine the range of proteins present, screening for cationic proteins, and electrophoretic and immuno-electrophoretic comparisons of mouth saliva with secretions from the mandibular, nasal, and nasolabial glands using whole saliva antiserum.

It was concluded that protein bands 4, 6 and 7 and saliva flow were related to bloat susceptibility and that further work investigating the biological significance of these relationships was warranted.

PREFACE

The condition of bloat has been known for some time, ancient writings of nearly 2,000 years ago described the condition and a remedy for its cure (Tribe 1947). In New Zealand the incidence of bloat has increased with the widespread use of clovers in the pasture and it is now a major metabolic disorder in cattle. It would appear the fundamental cause of bloat is the formation of a stable foam in the rumen of cattle, thus preventing the normal eructation of gases (Clarke & Reid 1974).

Research work on bloat in cattle can be broadly classified into three areas of investigation: (1) The component in the pasture causing bloat (and more recently those which might prevent bloat). (2) Studies on bovine proteins which might form a stable rumen foam. (3) Means of preventing bloat in cattle.

However, a considerable body of evidence has accumulated over the last twenty years to indicate that susceptibility to bloat may be an inherited characteristic in cattle. Furthermore much of the evidence has indicated that genetic differences might reside in differences in the volume of saliva, or salivary proteins. Therefore this physiological genetics approach to the study of bovine salivary proteins was designed to investigate whether any of these proteins were important with respect to determining differences in bloat susceptibility amongst animals.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

> "The profound revolution begun by Darwin is not always appreciated by modern biologists, partly because the revolution has still not reached some of them, and partly because, for those it has affected, it is so much part of their thinking that they are unaware of it. Darwin's revolution was the assertion that the actual variation among living organisms is the proper subject of study, rather than any abstract ideal of a Platonic type. It was by his concentration on the actual variation between individuals that Darwin was able to solve the problem of how organic evolution could occur."
>
> Lewontin (1976)

1.1 INTRODUCTION

Bloat is a disorder of ruminants caused by the retention of gas in the stomach. Gas is produced continuously in the rumen by microbial fermentation, but when an animal is unable to eructate the excess gases, bloat develops (Clarke & Reid 1974). Bloat can be classified into two types, pasture bloat and feedlot bloat. The experimental work in this thesis was designed to study pasture bloat, and therefore the emphasis in the literature review is on this form of bloat. Studies in the literature review (1.3) on bloat refer to pasture bloat except where specifically indicated that the study was dealing with feedlot bloat. Feedlot bloat can develop in cattle when 50% or more of their diet consists of concentrates. Feedlot bloat usually develops slowly over a period of weeks and often becomes chronic, as opposed to the rapid onset of pasture bloat. Most traditional diets can give rise to feedlot bloat, but the most serious bloat usually occurs on the finishing ration (Clarke & Reid 1974).

Pasture bloat is a disorder of ruminants which occurs in all parts of the world where grazing legumes is an established practice. Lush grass, green cereals, turnips or chou moellier have also been reported to cause pasture bloat, but in the main it is considered to be caused by the ingestion of legumes (Johns 1954).

As early as the 1950's the formation of a stable foam in the rumen was believed to be the cause of pasture (or legume) bloat seen in New Zealand cattle (Johns 1954). Therefore a large proportion of the early bloat research in New Zealand was designed to find a bloat remedy which would be of practical benefit to the farmer in controlling this condition. More recently however, emphasis has been placed on the factors which might cause the stable foam found in the rumen of cows (Jones 1971). In the course of these studies on foams, Jones noted a difference between high susceptible (HS) and low susceptible (LS) animals in a lower molecular weight salivary protein separated by electrophoresis, which he designated band 4 (Clarke et al. 1974). This finding, combined with other evidence reviewed in this Chapter, led Reid et al. (1972) and Clarke & Reid (1974) to suggest that a study of the salivary proteins was warranted with respect to their involvement in bloat susceptibility.

One of the major problems in studying bloat in cattle in the past has been the large degree of animal variation present, thus masking or confounding the interpretation of results. This study using a physiological genetics approach to investigate bovine salivary proteins, differs from previous work in that instead of being hindered by the animal and environmental variation, it capitalises on the existence of this variation. Thus the ultimate aim of a physiological genetics approach is to enable selection for animals with low susceptibility to bloat (LS) to be based on some physiological or biochemical parameter which can be directly measured.

The selection of animals with desirable productivity traits on the basis of measurable physiological characteristics was first proposed in New Zealand by Cockrem (1962). This physiological genetics approach to animal selection could avoid high environmental variation in the phenotype of the trait itself. The requirements of such an approach are:

1) Animal variation in the trait with evidence that at least part of the variation is of genetic origin.

2) Sufficient information on physiological and biochemical determinants to suggest areas of study which are likely to be relevant to genetic control.

3) Availability of techniques so that sufficient animals can be measured to allow for the variability between them (Cockrem & McIntosh 1975).

Two reports in the literature suggested that there might be HS/LS differences in the lower molecular weight protein of saliva (Lyttleton 1960; Clarke et al. 1974). Furthermore, work on the salivary mucoprotein was already in progress at DSIR (Jones & Lyttleton, see literature review on bloat, Chapter 1.3). Therefore this study was designed to investigate the relationships of lower molecular weight salivary proteins with respect to: (i) bloat susceptibility, (ii) saliva flow, and (iii) individual protein band inter-relationships. Biochemical and immunochemical studies were initiated to provide fundamental information which could form the basis for future investigations into how these lower molecular weight proteins might be involved in determining an animal's susceptibility to bloat.

1.2
LITERATURE REVIEW ON SALIVA

The purpose of this review of literature on saliva is to illustrate the importance of saliva and the complexity of the salivary glands. For the sake of brevity the studies on the specific proteins produced or secreted by the salivary glands have been restricted to citing the references. Furthermore studies on human conditions such as dental caries (e.g. Molan 1969) which are not directly relevant to the problem of bloat, have not been reviewed.

Types of salivary glands

The structure of the salivary glands was reviewed by Leeson (1967) and will not be dealt with in detail in this thesis.

In the ruminant, saliva is produced from a series of different glands which can be broadly classified into three types:

a) Serous, those producing watery well buffered secretions with no mucin; these include the parotid and inferior molar glands.

b) Mucous, those producing mucin; these include the palatine, buccal and pharyngeal glands.

c) Mixed salivary glands have the properties of both serous and mucus glands; these include the mandibular, sublingual, and labial glands. (Kay 1960; Church 1975).

The parotid gland in the ruminant is located between the base of the ear and the caudal end of the mandible (Bartley 1976). It produces copious quantities of saliva and appears to have no counterpart in the non ruminant animal. In fact the parotid gland can be considered as much a secretory organ of the rumino-reticulum as the mouth (Kay 1966). It has been shown to secrete continuously in the ruminant as was first reported by Colin (1860), and may be stimulated by reflexes from the mouth, oesophagus and rumino-reticulum (Clark & Weiss 1952; Ash & Kay 1959; Kay 1960; Bartley 1976).

The inferior molar glands in cattle and sheep are well defined multilobular glands, each lobule drains by a separate duct into the lower nonpapillated area of the cheek. The secretion is continuous and augmented by the same stimuli that provoke parotid secretion (Bartley 1976).

The palatine glands are normal small glands in the caudal part of the hard palate and in the soft palate. The oral and laryngeal part of the pharynx contain another group of glands; they and additional glands in the lateral margins and root of the tongue are called the pharyngeal glands. Small buccal glands are found in the cheek. These mucous glands secrete continuously but slowly unless stimulated. They secrete mostly when stimulated by the mouth, oesophagus and rumino-reticulum.

The mandibular glands are in the submaxillary triangle medial to the mandible. These glands have a slow continuous flow that is strongly stimulated by feeding, but stimulating the oesophagus or rumino-reticulum gives little or no response (Meyer et al. 1965).

The sublingual glands are paired and lie immediately under the mucous membrane of the floor of the mouth under the tongue. They are drained by 5 to 15 or more small short ducts. Secretions from the sublingual glands flow continuously when not stimulated but respond moderately to stimulation of the oesophagus. Both the mandibular and sublingual are active when food is being masticated, but not during rumination.

Corners of the mouth contain numerous, small labial glands which appear similar to the mandibular and sublingual glands in performance and function (Kay 1960; Meyer et al. 1965; Bartley 1976).

Neurophysiology of salivation

The neurophysiology of salivation has been well reviewed by Emmelin (1967), and in this review it is not intended to cover the subject in detail.

Emmelin (1967) states that it seems safe to accept that the para-sympathetic contributes secretory and the sympathetic vasoconstrictor nerve fibres in the salivary glands. There is also evidence to suggest that some glands receive sympathetic secretory and motor fibres also, and there may be sympathetic vasodilators as well.

Spontaneous and continuous saliva secretion

The secretory cells of salivary glands are in a more or less pronounced state of activity, elaborating proteins and mucoproteins. Many of these cells do not discharge saliva except when activated by impulses from the central nervous system by way of secretory nerves.

Others, however, are continuously giving off saliva. Babkin (1950) proposed that the term 'spontaneous secretion' be reserved for flow from a gland occurring even in the absence of extraneous stimuli. This process usually goes on continuously and is not initiated by impulses carried to the gland along the nerve or by humoral regulators in the blood, although it may be increased or diminished by such agencies. (The term should not be confused with continuous, permanent, basal or resting secretion as is often done.)

There is little known about spontaneous secretion in man (Burgen & Emmelin 1961), but Schneyer et al. (1956) demonstrated that there was apparently no parotid secretion during sleep in humans, and only a very slow flow from the mandibular and sublingual glands.

Colin, the French physiologist (cited Emmelin 1967) was the first to report that in the ox the parotid, but not the mandibular gland, secretes continuously, and Eckhard (1867) made the same observation in sheep. Spontaneous parotid secretion seems to be characteristic of ruminants. Colin (1871) and Ellenberger & Hofmeister (1897), showed that the parotid gland of horses did not secrete between meals.

In cats and rats the sublingual glands secrete spontaneously (Emmelin 1955; Ohlin & Perec 1965). The rabbit lacks the major sublingual gland, but shows a continuous low secretion from the mandibular gland (Nordenfelt & Ohlin 1957). Smaje (1973) demonstrated that when all possible nerve influences had been excluded there was still a very slow spontaneous secretion from the rabbit mandibular glands. Smaje also showed that two active pumps were required for the regulation of this spontaneous secretion.

Parasympathetic system

The secretory activity of the salivary glands is regulated mainly by parasympathetic nerves; all salivary glands seem to be supplied with such fibres (which use acetylcholine as the transmitter at the ending of the nerves in the salivary glands). Whenever saliva is secreted reflexly to aid in digestion or the protection of the mucous membranes of the mouth and pharynx, impulses pass down the secretory parasympathetic nerves. Through these nerves the composition of the saliva poured into the mouth can be varied in the seemingly purposeful way originally described by Pavlov (Emmelin 1967).

It has been demonstrated in dogs (Ekstrom & Holmberg 1972) and cats (Ekstrom & Emmelin 1974) that the parasympathetic innervation of the parotid glands, responsible for secretion, was not restricted to the auriculotemporal nerve but other cholinergic fibres, in particular the internal maxillary artery nerve, were also responsible. The importance of the parasympathetic nerve supply to the normal development of the parotid glands in lambs and sheep has recently been demonstrated by Patterson et al. (1975). These authors studied the effect of the parasympathetic nerve supply on the development of the parotid gland in immature lambs and adult sheep by unilateral postganglionic denervation. They showed the adult denervated gland secreted at lower rates than the innervated and the atropine resistant secretory flow was reduced to as low as one fifth of that of the innervated glands. In two lambs an atropine resistant flow did not develop. These authors concluded that the integrity of the parasympathetic innervation is essential for the development of the parotid gland of the sheep and for its maintenance in the adult animal.

The diet on which animals are fed has also been shown to play a role in the development and function of the salivary glands. When rats were maintained on a liquid diet from 4 to 21 days the weight of the parotid glands, but not the mandibular or sublingual glands were reduced in weight. However response to sialogogue agents showed the maximal secretory capacity to be the same except for a smaller response to adrenaline (Ekstrom 1973). When rats were fed on dry cellulose rich diets there was an increased acetylcholine transferase activity in the parotid gland (Ekstrom 1974). This work demonstrated that dietary factors were important in regulating the weight and neuronal activity of the parotid glands in rats.

Parasympathetic stimulation and vasodilation

The vasodilator response due to parasympathetic stimulation has been a matter of dispute as to whether it is due to vasodilator nerve fibres, or to some chemical activator. Heidenhain's discovery, 1872 (Emmelin 1967) showed that atropine was able to abolish secretion and yet not affect the vasodilatory capacity. Ungar & Parrot (1936) suggested the vasodilation seen during parasympathetic stimulation might be due to the depressor agent (kallikrein) in saliva. On the other hand Schachter and co-workers have questioned the role of kallikrein, and have strongly

advocated the view that parasympathetic vasodilator fibres for salivary glands exist (Bhoola et al. 1965). However, Emmelin (1967) concluded that it seemed reasonable to assume that when secretion is elicited from the central nervous system by way of secretory parasympathetic fibres, vasodilatory fibres are activated as well. During the high activity in the gland cells, vasodilator agents in addition may appear which contribute to the very marked increase in the blood flow.

Prostaglandins

Recent work on prostaglandin (PG) PG $F_{2\alpha}$ (Taira & Satoh, 1973) has demonstrated that this compound causes salivation and vasodilation through excitation of the parasympathetic postganglionic neurons. However although PG E_2 appeared to exert its sialogenous effect through parasympathetic excitant action; its vasodilator action was in the larger part due to a direct effect on the vasculature (Taira & Satoh 1975).

Sympathetic secretory and motor nerves

There is a marked difference in the effects of sympathetic stimulators between the different species. In the dog sympathetic stimulation causes only a minor response from the mandibular gland and even less for the parotid. In the cat the parotid response is small, but there is often a lively response from the mandibular gland with respect to saliva flow. In some cats though the effect is very small and in extreme cases completely lacking (Langley 1878; Emmelin 1955; Emmelin 1967). In the rabbit the parotid flow is more rapid than the mandibular gland (Nordenfelt & Ohlin 1957), while in rats both these glands give a marked response but the sublingual gives scarcely any (Emmelin et al. 1965; Emmelin 1967). In man, stimulation of the sympathetic trunk in the neck has been shown to produce a flow from the mandibular but not from the parotid (Emmelin 1967). Adrenaline injected through the salivary duct evokes salivary flow from the mandibular but not from the parotid gland (Emmelin & Stromblad 1954). Even in glands that respond to sympathetic stimulation it is always more difficult to evoke increased saliva flow with sympathetic than with parasympathetic stimulation. Sympathetic stimulation usually causes an intense vasoconstriction which does not favour secretion.

Part of the evidence for the presence of sympathetic motor fibres in the salivary glands is that the flow obtained on sympathetic stimulation is in many cases of brief duration, quickly diminishing, and even ceasing despite continued stimulation, (Emmelin 1967). Bradford (1888) discovered that the salivary response to sympathetic stimulation could be increased by previous excitation of parasympathetic fibres. Langley (1889) called this phenomenon 'augmented salivary secretion' and suggested as an explanation that the parotid stimulation leaves the gland cells in a state of heightened excitability which persists for a short time. Babkin (1950) thought the effect was not due to the sympathetic fibres exerting their action on the secretory cells but in many instances the effect was probably due to a motor effect of the sympathetic on a gland already filled with saliva by parasympathetic stimulation.

In the parotid gland of the sheep the effect of sympathetic stimulation on the contractile mechanism has been demonstrated. This gland contained myoepithalial cells (Silver 1954), and stimulation experiments suggest that these cells can be exited by sympathetic stimulation and also by injection of oxytocin (Kay 1954). The gland cells are spontaneously active and continuously secrete at a constant rate even when not influenced by extraneous stimuli (Coats et al. 1956). Sympathetic stimulation temporarily increased the salivary flow but was followed by a compensatory decrease.

Physiological evidence thus indicates that the sympathetic impulses (and catecholamines) contract the myoepithelial cells. These cells decrease in size after injection of adrenaline. Travill & Hill (1963) and Garrett (1965) concluded that the myoepithelial cells, parotid and mandibular gland of cats have dual innervation and suggests the parasympathetic fibres might cause relaxation (Stromblad 1955). In sheep the only effect of sympathetic stimulation of the parotid glands (apart from the vascular effect), seems to be on a contractile mechanism which expels saliva (Emmelin 1967).

In the mandibular gland of the cat the sympathetic stimulation appears to be mediated through α-adrenoceptors (Dale 1906; Emmelin 1955b; Emmelin & Muren 1951). However sympathetic stimulation attributed to β-adrenoceptors has also been detected in the mandibular glands of the cat, when studied against a background of slow secretion evoked by parasympathetic stimulation (Emmelin & Gjorstrup 1975). These authors

TABLE 1.2.1 Inorganic Constituents of Cattle Saliva (m mol/l)

	HCO_3^-	HPO_4^-	Cl^-	Na^+	K^+	Ca^{++}
(a)						
Parotid	107.5	20.6	15.4	136.6	13.5	6.7
Submaxillary (mandibular)	15.7	0.5	30.9	13.6	13.9	14.2
Sublingual, cheek and buccal	98.4	20.2	48.7	110.0	17.1	8.6
(b)						
Parotid	127	23	7.4	157	7.0	
Mixed	126	26	7.1	161	6.2	
(c)						
Parotid	90-140	10-70	10-20	160-180	4-10	2 or less

(a) Phillipson & Mangan (1959)

(b) Bailey & Balch (1961a)

(c) Blair-West et al. (1967)

also demonstrated a marked secretory effect on the parotid gland under these conditions.

The rat mandibular gland appears to be richly supplied with β-adrenoceptors and stimulation with isoprenaline will cause an abundant secretion (Schneyer 1962; Emmelin & Stromblad 1963; Ohlin 1964; Emmelin et al. 1965). Receptors of the β type have also been demonstrated in the rabbit parotid gland (Nordenfelt & Ohlin 1957; Ohlin 1964), and in the rat, catecholamine induced parotid saliva flow was shown to be mediated by β-adrenoceptors (Woodruff & Carpenter 1975). It has been shown that in the male mouse mandibular gland, the release of epidermal growth factor (Byyny et al. 1974) and the release of renin (Menzie et al. 1974), appears to be mediated via the α-adrenoceptors. Serotonin has been shown to enhance enzyme secretion from the rat and rabbit parotid glands as well as from the guinea pig mandibular gland (Carlsoo et al. 1975). The results from these studies indicated that there might be a close relationship between the postulated receptors for serotonin and those for catecholamines.

In summary it can be concluded that the complex process of salivary secretion is a result of the parasympathetic and sympathetic nervous system working synergistically to control the secretion. The proportion of secretions from the different glands, and possibly the release of specific substances from any particular gland would be expected to vary according to the type of stimulus evoking salivation.

Salivation in cattle

Salivation in ruminants has been briefly reviewed by several authors including Hungate (1966), Kay (1966), Church (1975) and Bartley (1976).

In the ruminant animal the saliva is an important fluid as the rumen has no indigenous secretions (McDougall 1948). Saliva aids mastication, deglutition and provides a suitable media for the microbial fermentation in the rumen; buffering against increasing acidity which occurs during the production of volatile fatty acids (VFA) by the microorganisms. Its buffering capacity is greater against acids than alkalis (Turner & Hodgetts 1955). The inorganic ion composition of cattle saliva is shown in Table 1.2.1.

Kay (1960), has shown that the osmotic pressure of mixed saliva as measured by freezing point is isotonic to plasma and he concluded that the salivary glands as a whole are required to do no osmotic work. Kay feels this is one explanation of why the salivary glands of ruminants can produce such copious quantities of saliva.

Numerous workers have attempted to measure total salivary flow in ruminants, however the data collected by different laboratories is difficult to compare owing to the different methods of collection used. These include cannulation of the parotid glands (but this ignores the contribution from the other glands), cardial collections from rumen fistulated animals and oesophageal fistulated animals. Most work has been carried out over short periods of time and then extrapolated for a 24 h collection period. Various estimates for the total salivary flow over a 24 h period have been made, examples of these are listed below:

Bailey (1959) quotes 74 to 110 l, exclusive of rumination in dairy cows; Bailey (1961) 98 to 190 l; Mendel & Boda (1961) 100 l; Meyer et al. (1964) 117 to 183 Kg; Yarns et al. (1965) 25 to 51 l in steers; Putnam et al. (1966) 33 to 54 l in steers.

From reviewing the literature Church (1976) concluded that mature cattle probably secrete somewhere around 150 l of saliva per day.

The type of feed consumed by a cow affects the amount of saliva produced. Figures quoted by Bailey (1959) illustrate the effects of different feeds (when the bolus was collected from the cardia), and these are shown below:

Feed	Salivary Production g/g food	Salivary Production ml/min	Eating Rate g food/min
Pelleted ration	0.68	243	357
Fresh grass	0.94	266	283
Silage	1.13	280	248
Dried grass	3.25	270	83
Hay	3.63	254	70

Balch (1958) showed a tenfold differences in saliva production between feeding hay and beets, 50 lbs (22.7 Kg) and 5 lbs (2.27 Kg) of saliva respectively being produced during the consumption of 10 lbs (4.5 Kg) of the above feeds. Meyer et al. (1964) demonstrated a large

difference of 1.53 compared with 3.3 Kg in the saliva added per Kg dry matter, when two sets of identical twin cows were fed lush alfalfa as compared to mature alfalfa.

Bartley (1976) concludes from his review of the literature that feeds that do not induce large quantities of saliva to be secreted are those with either low dry matter content (such as lush pasture) or those eaten rapidly such as ground or pelleted hay or grain concentrates.

Both the parotid saliva (Bailey & Balch 1961a) and the mixed salivary secretions (Bailey & Balch 1961b) reach their lowest ebb in secretions immediately after a meal when the rumino-reticulum is newly filled with food and fermentation is rapid. These authors found little or no depression of secretion when food was given through the rumen fistula. However, studies by Wilson (1963) on sheep indicated the distention of the rumen was an important factor in the control of salivary secretion. Phillipson & Reid (1958) demonstrated that stimulation of parotid salivation occurred at intraruminal pressures from 5 to 22 mm Hg and that pressures of 15 - 40 mm were required for inhibition. There was a large animal difference in the response to these intraruminal pressures.

Many reflex arcs have been described which seem to account largely for the salivary secretion that is stimulated by feeding and rumination and for the especial effectiveness of fibrous diets and these reviewed by Kay (1966) are listed below:

Parotid secretion is readily stimulated by brushing between the tongue and molar teeth, mainly an ipsilateral effect, and by brushing the lower oesophagus and cardiac region, or distending the thoracic oesophagus with gas, fluids,or balloons. It is also stimulated by stretch of the cardiac and reticulo-omasal orifices and of the rumino-reticular fold and by tactile stimulation of these and nearby structures in the rumino-reticulum. The inferior molar glands and the small mucous glands of the mouth respond equally well but stimulation of the oesophagus and rumino-reticulum causes little or no secretion by the mandibular or sublingual glands.

Kay (1966) concluded that it seemed likely that the salivary secretion during and after feeding is determined by the balance between excitatory and inhibitory stimuli.

Aldosterone influence on saliva in ruminants

Using unilateral parotid fistulae in bilaterally adrenalectomised sheep, Goding & Denton (1956) demonstrated the importance of aldosterone in regulating the electrolyte levels in saliva. The control of electrolyte levels in saliva by aldosterone has been reviewed by Blair-West et al. (1967).

Bailey & Balch (1961b) studied the salivary ionic composition in cattle and found the $Na^+ + K^+$ constituted 98% of the cations of mixed saliva. During resting an increase in Na^+ concentration was accompanied by a decrease in K^+ and vice versa. The total $Na^+ + K^+$ concentration tended to be about 166 m mol/l, but decreased slightly when the sodium was less than 120 m mol/l. When the Na^+ concentration increased to greater than 164 m mol/l the K^+ concentration did not decrease below the mean plasma K^+ concentration. These authors suggested that the salivary glands are apparently incapable of transferring water to the saliva without K^+.

Relative to other animals the salivary secretory process in ruminants virtually represents a second circulation. Approximately twice the sodium content of the extracellular fluid passes down the oesophagus and is reabsorbed from the gut each day (Blair-West et al.1964).

Blair-West et al. (1964) showed that Na^+ replete sheep which had adrenal transplants had a Na^+ to K^+ ratio consistently in the range of 25 - 45 and aldosterone secretion rate of less than 2 µg/h, while sodium deficiency was associated with a commensurate decrease in Na^+ to K^+ ratio and an increase in aldosterone secretion rate. The increase in aldosterone was not associated with any consistent augmentation of cortisol or cortisosterone. The conclusion drawn from this work that hypersecretion of aldosterone in Na^+ deficiency was not determined by an increase of ACTH secretion, has been verified by comprehensive experiments on sheep and other species (Blair-West et al. 1967). From studies on adrenalectomised sheep, Blair-West et al.(1965) suggested that the parotid gland was more sensitive to aldosterone when the animal was Na^+ deficient. The changes in Na^+ to K^+ ratio which may occur with dietary change and grazing have been reviewed by Dobson (1965). Changes from lush to dry diet were shown to be associated with a decrease in the Na^+ to K^+ ratio of the saliva. A substantial reduction of the Na^+ to K^+ ratio of saliva and rumen fluid was also noted when the diet of sheep and cattle was changed from grass low in sodium and high in potassium content.

This effect was attributed to aldosterone activity.

Changes in salivary sodium and potassium composition occur under natural field conditions, and the extent of this interchange of sodium and potassium is a striking factor of the electrolyte organization of ruminant animals. The hypothesis has been advanced that the extent of endocrine control of a digestive secretory mechanism is an important adaption with considerable survival value in the evolution of the capacity of these species of mammals to adapt to stringent ecological conditions involving pasture change and episodic feeding (Denton et al. 1957a,Blair-West et al. 1967).

Blood and rumen constituents important in control of saliva secretion

Annison et al. (1957) showed a good correlation between the concentrat of total volatile fatty acids (VFA) in portal blood and rumen contents. Under normal feeding condition there was a much lower concentration of butyrate relative to the levels of acetate and propionate in the portal system than in the rumen content. The concentration of ketone bodies in the portal vein usually exceeded that in the peripheral blood. Dosage with butyrate resulted initially in high ketone body levels in portal blood, followed by a steady rise in concentration in the peripheral blood.

McManus (1959) using anesthetized and conscious sheep fitted with oesphageal fistulae, collected saliva diverted from the rumen and the result was an increase in VFA and concomitant decrease in pH in the rumen. Bailey & Balch (1961b) investigated the effect of lowering the rumen pH from 6.8 to 5.4 with acetic acid, on the secretion rate of mixed saliva but could not detect a change in the flow rate. Likewise, Yarns et al. (1965) using seven steers and two sheep equipped with oesophageal and ruminal fistulae, were unable to establish a significant effect on salivary secretion when VFA, water and CO_2 or saliva was infused into the rumen. These authors concluded that if receptors for a salivary reflex were present in the rumen, they were relatively insensitive to the materials infused in these trials.

However, Obara et al. (1972a) using a sheep fitted with a unilateral parotid fistula and a rumen fistula kept the rumen pH at 5 or 6 by the administration of acetic, propionic, butyric, or hydrochloric

acids. At pH 5, acetic acid did not affect the salivary flow rate but propionic acid decreased the flow rate and butyric acid gave an even greater decrease in flow rate. When the rumen pH was maintained at 6 the result in parotid saliva flow was an increase with acetic acid, no change with propionic, and a decrease with butyric acid. Hydrochloric acid did not affect the flow rate, although a decrease was observed after the recovery of the ruminal pH. The significance of these results are questionable considering only one animal was used.

Obara et al. (1972b) measured the saliva flow from unilateral parotid glands of six sheep while the total VFA concentration of the carotid arterial blood was elevated to around 2 m mol/l for 2 hours by infusion of acetic or butyric acids. Acetic acid infusion showed a tendency to increase the saliva flow, but butyric acid did not affect the flow. Acetic acid infusion resulted in a decrease of CO_2 level and an increase of total VFA level. A marked increase in blood ketone bodies, glucose, and VFA level and a decrease of CO_2 content was observed with butyric acid infusion.

In a third experiment on two sheep, the ruminal pH was maintained at 5 by administration of acetic, propionic, butyric, or hydrochloric acids into the empty rumen. The results of saliva flow and blood values were similar to those quoted above (Obara et al. 1972c).

These workers postulated that the stimulative effect of acetate on the parotid gland or on its secretory centre was due to the direct action through the humoral pathway, but the inhibitory effect of butyrate introduced into the rumen was due to nervous action through nerve endings in the rumen wall.

Taste

Another factor which could influence the quantity and composition of saliva secreted by cattle is their ability to taste certain compounds.

A description of the taste buds and their neurophysiology has been outlined by Bowman et al. (1972), thus explaining the arcs involved in the reflex secretion of saliva in response to a taste stimulus. Work is at present in progress to determine the biochemical and physiological properties of the taste buds (e.g. Lum and Henkin 1976). As proteins which can modify the taste stimulus have now been characterised (Cagan 1973) the importance of taste with respect to salivation in cattle cannot be ignored.

It is also possible that cows might have genetic differences in their ability to taste certain compounds. Humans and other primates have been shown to differ in their ability to taste certain substances. The classical example is phenylthiocarbamide. In humans the ability to taste this compound resides in a single gene difference with two alleles, T and t. The allele for tasting, T, is dominant over t, so that heterozygotes Tt are tasters and the only non-tasters are tt (Strickberger 1968).

Proteins and protein-like molecules found in mammalian saliva

The following section gives a selection of proteins and protein-like substances found in mammalian saliva. The number of substances which have been detected in saliva from different species of mammals is extensive, and therefore the list of references cited in this section is far from complete. However it is hoped that these examples will illustrate the large diversity of functional proteins which can be detected in mammalian saliva.

Protein and protein-like molecules found in mammalian saliva

Enzymes in Saliva

Enzyme	Source	Reference
α-Amylase	Human	Squires (1953) Keller et al. (1971) Ward et al. (1971) Birkhed & Soder (1973) Merritt et al. (1973) Rosenmund & Kaczmarek (1976)
	Cattle & Buffalo (nasolabial glands)	Majeed et al. (1970) Toutain et al. (1973) Church (1976)
Arginine esterase	Mouse	Greene et al. (1969)
Deoxyribonuclease	Human	Zollner et al. (1975)
Hyaluronidase	Human	Hopps & Prout (1972)
Kallikrein	Cat Guinea Pig Human Mice Rat	Barton et al. (1975) Abano et al. (1976) Fujimoto et al. (1973) Bhoola et al. (1973) Gauturk & Kritz (1974)
Lipase	Calf (bovine)	Dukes (1947) Grosskopf (1965) Edwards-Webb & Thompson (1977)
Lysozyme	Human	Masson et al. (1965)
Pre-gastric esterase	Calf (bovine)	Ramsey et al. (1960), Ramsey (1962)
Renin	Mouse	Cohen et al. (1972) Michelakis et al. (1974)
Salivain	Rat	Reikkinen et al. (1966) Reikkinen & Ekfors (1966) Levitskii & Vovchuk (1974)

Hormones in Saliva

Epidermal growth factor	Mouse	Cohen (1962), Cohen & Taylor (1974) Byyny et al. (1974) Cohen & Carpenter (1975)
Gastrin-like molecule	Bovine	Takeuchi et al. (1973)
Glucagon	Human Mouse Rabbit Rat	Lawrence (1977)

Nerve growth factor	Mouse	Bueker (1948) Levi-Montalcini & Cohen (1960) Greene et al. (1969) Boyd et al. (1974) Frazier & Hogue-Aggeletti (1974)
Parotin	Bovine, Equine, Human & Porcine	Ito (1960)
Sialotonin	Cat	Moriwaki et al. (1968) Barton et al. (1976)

Immunoglobulins in Saliva

Anti-C_3 antibodies	Human (neonatal)	Price et al. (1975)
Immunoglobulins	Human	Tomasi & Zigelbaun (1963) Tomasi et al. (1965) South et al. (1966) Mandel & Khurana(1969) Strober et al. (1970) Bellavia et al. (1972)
Immunoglobulins	Bovine or Ovine	Sullivan et al. (1969) Pahud & Mach (1970) Mach & Pahud (1971) Watson & Lascelles (1971) Butler et al. (1972) Duncan (1972) Watson & Lascelles (1973) Smith et al. (1975) Chipps & Lascelles (1976)
J chain	Human & Rabbit	Halpern & Koshland (1970)
Secretory component	Bovine	Mach (1970) Mach & Pahud (1971)
	Human	Tomasi & Bienenstock (1968)
	Ovine & Caprine	Pahud & Mach (1970)

Unclassified proteins in Saliva

Acidic proteins	Human	Friedman & Karn (1977)
Albumin	Ovine	Watson & Lascelles (1973)
	Human	Masson et al. (1965) Selmer et al. (1968) Mandel & Khurana (1969) Strober et al. (1970)
Albumin-like molecule	Human	McKeen & Beeley (1974)

Basic glycoproteins	Human	Friedman et al. (1971) Azen (1973)
α_{2u} globulin	Rat	Roy & Byrd (1976)
Lactoferrin	Human	Masson et al. (1965) Hurliman & Zuber (1968) Schulze & Heremans (1968)
β_2-microglobulin	Human	Talal et al. (1975)
Mucoprotein	Bovine	Lyttleton (1960 & 1964) Bettelheim et al. (1962) Jones (1971) Jones & Lyttleton (1972)
	Bovine, Ovine and Porcine	Gottschalk et al. (1972)
	Human	Baig et al. (1973)
Peroxidase	Human	Azen (1977)
Phosphoprotein	Human	Bennick (1977)
Proline rich proteins	Human	Azen & Oppenheim (1973)
Proteinase inhibitor	Dog	Fritz & Hochstrasser (1976)
Transferrin	Monkeys & Rabbits	Thorbecke et al. (1973)
Vitamin B_{12} binding protein	Human	Grasbeck et al. (1972)

Sexual dimorphism of proteins in salivary secretions

The concentration of certain proteins or protein-like substances in the saliva of mammals have been shown to differ between males and females; this is known as sexual dimorphism.

The tubular cells of the mandibular glands of rodents exhibit sexual dimorphism (Sreebny & Meyer 1964). The quantity of Epidermal growth factor present in the mandibular glands closely parallels the development of the tubular cells (Cohen 1965)., The levels of Epidermal Growth Factor has been shown to be seventy fold higher in male as compared to female mandibular glands of mice (Frati 1976). Nerve Growth Factor is also present in higher concentrations in mandibular saliva from male mice compared with females (Levi-Montalcini & Cohen 1960).

An androgen-dependent rat urinary protein α_{2u} Globulin has recently been shown to be concentrated by the parotid and mandibular salivary glands of male rats but not by these glands in the female rat (Roy & Byrd 1976). The possible role of this protein in the regulation of spermatogenesis has recently been indicated (Roy et al. 1976).

No sexual dimorphism has been reported in the salivary proteins of cattle, but the possibility that a sexual dimorphism might be present, cannot be ignored when studying these salivary proteins.

1.3
LITERATURE REVIEW ON BLOAT IN CATTLE

The second part of the literature review is concerned with bloat in cattle, the main emphasis being on the animal factors which might be involved.

Bloat is a disorder of ruminants caused by the retention of gas in the stomach. Gas is produced continuously in the rumen by microbial fermentation, but when an animal is unable to eructate the excess gases, bloat develops. The resulting inflated stomach may have an intra-ruminal pressure as high as 70 mm Hg, and this can mechanically interfere with respiration. Absorption of CO_2 from the rumen can further compound the condition and the animal can die of bloat within an hour of commencing to feed (Clarke & Reid 1974).

There are two forms of bloat; feedlot bloat which develops in animals in a feedlot situation where their diets consist of over 50% concentrates (Chapter 1.1), and pasture bloat which predominantly develops when animals are grazed on lush pasture, and in particular pasture containing legumes. This latter form of bloat is investigated in this project, and therefore references to bloat in this review refer to pasture bloat unless otherwise stated.

The stable foam theory and factors important to the foam formation

A number of authors have suggested that acute pasture bloat is caused by the development of a stable foam in the rumen contents, these include Quinn (1943), Clark (1948), Johns (1954), Hungate et al. (1955), Boda et al. (1956), Reid (1960), Walker (1960), Clarke & Reid (1974).

Johns (1954) was able to demonstrate a difference in the foam stability of clover juice and grass juice when foamed in a graduated cylinder. The rye grass juice foam disappeared in 1 h while the clover foam remained stable for 24 h. Rumen fluid was removed from two groups of sheep pastured for seven days, one group on red clover the other on cocksfoot. Rumen content was foamed in a graduated cyliner, the rumen foam from cocksfoot fed sheep disappeared in 90 - 120 s while the foam from the clover fed sheep was stable for 12 h and had decreased very little in 24 h. Mangan & Johns (1957) attempted unsuccessfully to show a difference in foaming properties of bloat producing and non bloat producing clover. They showed that freezing at -20°C was the only method

of preserving the foaming properties of the clover, while drying apparently denatured the foaming agents.

Fergusson & Terry (1955) have assumed that foam [illegible] the important factor in pasture bloat. These authors were able to produce bloat by administering lucerne juice to sheep, lucerne juice heated to 60°C and passed through an anion or cation exchange resin, or even after precipitating the proteins with 80% alcohol and using the supernatent. However these experiments were of limited value owing to the lack of controls; only one or two animals were used for each of these experiments. These authors also tried combinations of lucerne juice, sodium bicarbonate, egg albumin, sugar+saponin and grass juice. Sufficient acetic acid was added with the bicarbonate to release carbon dioxide. These results were inconclusive because of the small number of animals used, but they did attempt to investigate a large number of possibly relevant factors, some of which have still not been conclusively tested to date.

Because bloat can occur within 15 to 30 min after commencing grazing, Fergusson & Terry (1955) suggested that it would be more likely that material already in the rumen would supplement the stable foam than the material being eaten at the time of bloating. However, subsequently Reid et al. (1962) and Bryant (1964) have shown that chewing of the ingested clover resulted in a rapid release of soluble protein, the amount of which was dependent on the age and condition of the plant material. On the other hand the proportion of chloroplast material released, measured as galactolipid, remained fairly constant, the absolute amount depending on the amount contained in the herbage. The importance of the rate of release of plant proteins in vivo in determining the bloat potency of a pasture has been recently reiterated by Howarth et al. (1977). These workers are at present developing a more accurate index of the rate release in vivo to evaluate this hypothesis.

Mangan (1958; 1959) tested the foam strength of the cytoplasmic proteins from red clover and showed their maximum foam strength was at pH values between 5.4 - 6.0. The foaming properties were markedly dependent on the salt concentration. Mangan showed the rumen liquor from cows fed red clover had a pH optimum for foam strength between 5.4 - 5.7 which was similar to that found for the cytoplasmic proteins from the red clover. Saponin from red clover and lucerne, had a maximum foam strength at pH values between 4.5 - 5.0, and calcium was found necessary for the formation of a rigid foam. Mangan also showed that the mucoprotein

aspirated from under the tongue of cattle was a powerful foaming agent and may have had some significance in bloat. The mucoprotein, however would not have been pure but have included other salivary proteins. Mangan concluded that the cytoplasmic proteins from red clover were of major importance in causing bloat in cattle. This conclusion was based on in vitro studies which showed similar pH optima for rumen content and cytoplasmic protein foams. These findings of Mangan provided the preliminary evidence upon which much of the subsequent work on bloat foams and the involvement of the pasture proteins was based.

Head (1959) collected rumen foam from bloating cows and concluded that the foam could not be protein because the precipitate formed in 70% ethanol was not denatured. He concluded it was carbohydrate in nature and by hydrolysis demonstrated the presence of sugars associated with pectin and hemicellulose. This author did not test for the presence of protein because the precipitate could be redissolved. However, Bartley & Bassette (1961), collected foam during the period of maximum bloat in a cow, and using the same procedure as Head (1959) demonstrated that their foam contained 63.3% protein, ether extract 1.5%, crude fibre 0%, carbohydrate 17% and ash 18.2%. They also qualitatively determined the amino acids present. This work gave good preliminary evidence that the foam was at least partly composed of protein.

Van Horn & Bartley (1961) investigated the effect of adding 80 ml of either bovine saliva or linseed meal mucin to 200 g of frothing rumen contents. Both the saliva and the plant mucin increased the rate of release of trapped gas from the foam. The addition of saliva to non frothing rumen contents did not cause an increase in gas evolution, indicating the evolved gas must have come from the foam rather than being produced from the saliva. This indicated that whole saliva and the plant mucin had anti-foaming properties; Meyer et al. (1964) concluded that reduced salivation which may occur when an animal is pastured on lush pasture could be an important factor in bloat aetiology.

Miltimore et al. (1964) examined the incidence of bloat in cattle in relation to the levels of a number of different pasture variables. These authors used a multiple regression approach for this study, with bloat

percent dry matter, nitrogen, phosphorus, sulphur, calcium, magnesium contents of alfalfa as the independent variables. They found 29% of the

variation in bloat incidence was accounted for by nitrogen content of alfalfa. They suggested that this was further evidence that protein is the bloat producing agent in alfalfa. They did not take into account individual animal susceptibility but looked at average daily bloat of the fourteen Jersey cows.

McArthur et al. (1964) isolated an 18 S protein from the leaves of alfalfa which they suggested was responsible for the formation of foam in the rumen of bloating cattle. This 18 S fraction 1 protein appears to be the major protein in the chloroplasts of green leaves (Lyttleton & Ts'o, 1958) and has been identified with the enzyme ribulose diphosphate carboxylase* (Trown 1965). Stifel et al. (1968) purified 18 S protein from bloat producing and non bloat alfalfa pasture. Sedimentation patterns on ultracentrifugation suggested the protein was pure but as pointed out by Jones & Lyttleton (1969) their method of purification would not necessarily result in a pure fraction 1 18 S protein. These authors (Stifel et al. 1968) showed that the 'bloat producing' fraction 1 protein had more of the amino acids, aspartate, serine and glutamine and less proline than the non bloat alfalfa fraction 1. They studied the calcium and magnesium binding to fraction 1 from these two sources. They showed that the 'bloat producing' fraction 1 could bind more of these cations and suggested there was a difference in the number of binding sites on the fraction 1 protein between bloat producing alfalfa and the non bloat alfalfa. Lecithin was shown to inhibit the binding of cations in non bloat fraction 1. These authors suggested that metal ions may 'salt out' fraction 1, thereby stabilizing the rumen foam. They stated that lipids compete for the same sites and hence are antifoaming.

McArthur & Miltimore (1969a) demonstrated that severe bloat occurred only when the pH of the rumen contents of four fistulated cows was in the range of 5.2 - 6.0. The bloat producing legumes, alfalfa (Medicago sativa)and ladino clover (Trifolium repens) produced lower pH than orchard grass (Dactylis glomarata). When rumen pH was depressed to the range obtained with the legumes by adding grain to the orchard grass ration there was no bloat. These authors concluded that four important conditions were necessary to produce bloat:

1) Vigorous gas production to produce the foam gas phase and rumen pressure
2) Adequate 18S protein to produce and maintain the foam

* 3-phospho-D-glycerate carboxy-lyase EC 4.1.1.39

3) Sufficient acid to lower the rumen pH below 6.0

4) Cations to bind the protein molecule in the surface film

However, these Canadian workers have masked the animal variation by pooling the bloat data from all cows and have thus ignored the animal components of bloat. The same authors (McArthur & Miltimore 1969b) studied the soluble proteins in five bloating and six non bloating forages and claimed that there was more total protein in the bloating forages and furthermore the proportion of 18S protein to total protein was higher in the bloating forages. However, Jones & Lyttleton (1970) suggested that these protein differences may be due to differences in the condensed tannins contained by these plants, as tannins have been shown to precipitate plant proteins (Kendall 1966).

Jones & Lyttleton (1969) demonstrated that both fraction I and II proteins could produce strong foams over the pH range which exists in the rumen when bloat occurs. Foam production at pH 5.8 was shown to cause surface denaturation of both fraction I and II proteins in extracts from both red and white clover. This in vitro study would indicate that both fraction I and some fraction II protein are capable of causing bloat if the foaming properties of the plant proteins is the important factor.

Miltimore et al. (1970) claimed that there was a threshold of 1.8% fraction I for alfalfa below which bloat did not occur, and that fraction I protein was most closely associated with bloat as the average bloat incidence increased from 2 - 35% over the bloating range of fraction I protein.

Jones (1971) and Jones & Lyttleton (1972) showed that the salivary mucoprotein produced a persistent foam of low compressive strength which was unaffected by pH changes over the range of 4.0 - 7.0. These authors also demonstrated that proteins from the holotrich protozoa produced a very rigid foam, some of the proteins becoming insoluble as a result of surface denaturation at pH 5.8. The maximum persistence of this foam occurred within the pH range of 5.5 - 6.5.

The three major sources of soluble proteins in the rumen are, plant proteins, salivary proteins, and proteins from the rumen ciliates, (Jones & Lyttleton 1972; 1973). These authors were unable to show differences in persistence and compressive strength of foams produced from rumen liquor from cows fed red clover which subsequently produced bloat and the rumen liquor of cows fed red clover which did not produce

bloat. These workers isolated a major protein component in the rumen liquor involved with the formation of foam, which in the presence of $CaCl_2$ had almost identical foaming properties to that of whole centrifuged rumen liquor. On electrophoresis this glycoprotein molecule entered 1 mm into a 7.5% polyacrylamide gel, and antisera produced against this purified protein cross reacted with salivary mucoprotein.

The evidence cited above indicates that salivary mucoprotein might be involved in the foam formation in pasture bloat. However these authors (Jones & Lyttleton 1973) were unable to demonstrate consistent differences in foaming properties of rumen liquor between cows that were about to bloat and those that did not bloat. Furthermore HS/LS differences were confounded with bloating and non bloating animals. No adequately designed experiment has been carried out to investigate HS/LS differences in the foaming properties of rumen liquor. The above results could suggest that the primary difference between HS and LS animals does not reside in the foaming potential of the rumen liquor. Therefore further work is required to establish whether there is a difference in the foaming properties of the rumen liquor between HS and LS animals.

Importance of Microorganisms in bloat

This subject has been discussed by Hungate (1966) and well reviewed by Clarke & Reid (1974). These authors conclude that any difference in the microbial population between bloating and non bloating animals or between HS and LS animals is not reflected in the end products of metabolism. Gases, volatile acids and nitrogenous compounds do not appear to be different in such animals. Hungate et al. (1955) attempted to demonstrate a difference in gas production between bloating and non bloating animals, but owing to inadequate experimental design including confounding results of not differentiating between phenotypically LS cows and cows not bloating for other reasons, no valid conclusions could be drawn.

Jacobson & Lindahl (1955) suggested that slime production by bacteria might be associated with bloat. These authors calculated the percentage of encapsulated microorganisms present in the rumen over an eight week period and obtained a correlation coefficient of 0.94 between the percent encapsulated organisms and the occurrence of feedlot bloat.

Bailey & Oxford (1958; 1958b) demonstrated the production of dextran slime by S.bovis in liquid culture only when growing on sucrose in the presence of CO_2. Bailey (1958) demonstrated that leaves and petioles of fresh red clover grown in his laboratory contained 2.5 - 3.5% (dry wt) of sucrose. However, Clarke (1959) demonstrated Lactobacillus bifidus, an active dextran splitting organism, to be present in the rumen in significant numbers. The possible importance of S.bovis with respect to feedlot bloat has been further investigated by Bartley et al. (1975), who suggest the build up of chains of Streptococci may play a role in trapping the gas. Horacek et al. (1977) have recently demonstrated the presence of agglutinating immunoglobulins to encapsulated S.bovis in bovine serum and saliva. These authors suggest that the animal component important in feedlot bloat might be the salivary immunoglobulins. Canadian workers at Lethbridge have suggested that the levels of alkaline phosphatase in cell free rumen fluid of cattle may indicate the onset of conditions associated with feedlot bloat (Bailey 1975).

Mendel & Boda (1961) proposed that saliva might act as an anti-foaming agent and that HS animals secrete less saliva than LS animals. They proposed that the HS animals have a more active population of mucinolytic organisms which effectively destroy the antifoaming properties of saliva.

Bartley & Yadava (1961) using animal mucin concluded that mucins had antifoaming properties. Fina et al. (1961) stated that the effectiveness of mucins as antifoaming agents may be lost if desialated by mucinolytic microrganisms. These authors were able to isolate five different organisms from the rumen which were capable of breaking down salivary mucin and utilizing free sialic acid. These authors using three pairs of rumen-fistulated animals were able to demonstrate more bloat in the member of each pair, of which the rumen had been inoculated with mucinolytic organisms. Mishra et al. (1967) in the same laboratory isolated several aerobic bacteria from the rumen capable of utilizing bovine salivary mucin as the sole source of organic nutrient. They showed oral saliva drippings to be a potential source of these aerobic mucinolytic bacteria. They also showed that members of twin pairs receiving hay before pasturing had fewer aerobic (facultative) mucinolytic bacteria in the rumen content than did the twins receiving pasture alone. They also concluded (from two pairs of twins) that the rumen contents of the bloat susceptible twins were more mucinolytic than from non susceptible twins.

Mishra et al. (1967; 1968) suggested the role of the mucinolytic bacteria might be to desialate the salivary 'mucin' and thus destroy its antifoaming properties. However Jones & Lyttleton (1973) after studying the main protein isolated from the rumen of cows, proposed that the role of the mucinolytic bacteria (with respect to bloat), might be to produce the foam stabilizing molecule by desialating the mucoprotein molecule.

Clarke & Hungate (1971) studied four sets of non-lactating monozygotic twins fitted with rumen cannulae. One set of animals were HS, two were of intermediate susceptibility and one set were LS. They attempted to demonstrate differences in the microbial activities in the rumino-reticulum of these animals differing in bloat susceptibility, but results proved negative. These results however cannot be considered conclusive owing to the inadequate experimental design.

Protozoa

Oxford (1958) first studied the ciliates of the rumen of the New Zealand domestic cow. Oxford found Epidinium ecaudatum the predominant oligotrich in the rumen of two cows fed exclusively on red clover. Clarke (1964) undertook an extensive study of the rumen ciliate fauna in the rumen of four cows fed grass hay and fresh red clover. This author (Clarke 1965a) demonstrated a marked increase, (2-4 fold) in the number of protozoa during the two hours of feeding in cows fed once per day and a marked fall in the numbers of protozoa in the 2-5 hours after the commencement of feeding. The marked fall in dasytrich numbers in the period 2-5 h after feeding commenced was too grate to be solely the result of passage down the alimentary tract. Rumen holotrich protozoa have a metabolic abnormality whereby, in the presence of excess substrate, they are unable to stop synthesizing reserve polysaccharide.

The bursting of these protozoa will be dependent on the availability of substrates. Isotricha can use soluble sugars and starch granules of small size (Oxford 1955) but Dasytricha can only use soluble sugars (Mould & Thomas 1958).

Clarke noted that the cell contents of burst isotrichs can have considerable foam-stabilizing properties, hence the bursting of Isotricha in the period immediately following the commencement of feeding could have some significance in the production of bloat in cattle. He showed

that while the isotrich protozoa increased in number on feeding hay, they decreased when fed clover. Clarke (1965b) also showed a positive relationship in two pairs of twin cows, between their subsequent bloat score and the weight of protozoa in rumen liquor taken before feeding. Clarke suggested the presence of ciliates full of ingested food and storage polysaccharides could allow maximum gas production by bacteria.

Jones & Lyttleton (1972) confirmed the observation of Clarke (1965a) that the contents of burst holotrich protozoa have considerable foam stabilizing properties. They demonstrated that the components from holotrich protozoa produced very rigid foams, some of the proteins becoming insoluble as a result of surface denaturation at pH 5.8. The maximum persistance of the protozoa foam occurred within the pH range 5.5 - 6.5.

However, more recently the importance of protozoa as a primary cause of bloat was questioned by Clarke et al. (1969) who showed that anti-protozoal agents such as dimetridazole reduced the incidence of bloat, but did not prevent bloat entirely. The role of protozoa is presently being investigated by Australian workers (Leng 1973).

Pectin

Conrad et al. (1958) and Head (1959) first proposed pectins as being capable of causing the stable foam in pasture bloat. Wright (1960) demonstrated that rumen protozoa could degrade pectic substances by showing that these protozoa possessed pectin esterase and polygalacturonidase activity. Wright (1961) stated that red clover (Trifolium pratense L) under New Zealand conditions may contain up to 6.6% pectin. Rumen bacteria and protozoa contain pectin methyl esterase (PME) and poly-galacturonase. The galacturonic acid produced by these enzymes is utilized by the organism with the formation of gas and a mixture of acids, predominantly acetic acid. Wright concluded that the gas produced by fermentation of pectin by rumen liquor was due to the action of bacteria rather than protozoa, and that hemicellulose as well as pectin from the plant fibre was responsible for gas production.

Mangan (1959) demonstrated that the protein foams were markedly dependent upon adequate Na^{++} or Ca^{++} ion On this evidence Wright (1961) postulated that a high degree of esterification of pectin would mean the ready availability of ions, thus increasing protein foam strength or alternatively a low degree of esterification would allow Na^{++} and Ca^{++} to

bind to the free carboxyls in pectin and thus decreasing the protein foam strength.

Pressey et al. (1963) found no correlation between pectin levels and bloat frequency, but Gufta & Nichols (1962) suggested PME might be involved in bloat and Nichols and Deese (1966) showed a direct correlation between PME activity and the moisture level of freshly harvested alfalfa. On bloating days the alfalfa feed was significantly lower in dry weight and higher in PME activity than on non bloating days. When corrected to average 6.8 Kg dry matter intake/day, average amount of PME on bloating days was estimated to be twice that of non bloating days.

The enzyme PME, converts the plant pectin to pectic acid and methanol. The pectic acid in the presence of calcium ions can form a pectate gel (Penn et al. 1966). These authors demonstrated the presence of a heat labile inhibitor of this pectate gel, in the strained rumen contents from a cow feeding on alfalfa hay. Alkyl aryl sulphonate was also shown to inhibit the gel formation and it could also prevent bloat in cattle.

Jones (1971) showed that sodium pectate stabilized foams produced by leaf proteins, but at higher concentration 0.06% (wt/vol) in the presence of calcium, the calcium polygalacturonate precipitated. At still higher polygalacturonic acid concentrations the solutions gelled making it impossible to carry out the experiments. The importance of pectin and PME activity has still not been satisfactorily resolved.

Saponins

Saponins are a group of glycosides widely distributed in plants of agricultural importance. They are composed of a carbohydrate and non-carbohydrate portion or aglycone portion which is often referred to as a sapogenin. The sapogenin nucleus may be either of steroid or triterpenoid structure, the latter being most common in forage legues (Cheeke 1971).

Lindahl et al. (1957) examined the effects of alfalfa saponin on the motility of smooth muscle. Intraruminal and intravenous administration of saponin reduced rumen motility, and inhibited eructation. Coulson and Davies (1960) suggested that saponin differences could possibly explain the transient nature of bloat potency in herbage, exerting its effect either by its foaming properties or by its ability to inhibit smooth muscle motility. The physiology of eructation in relation to bloat has been discussed by Dougherty et al. (1958), Dougherty (1961) and Clarke & Reid

(1974); it would appear that in the case of 'free gas' bloat eructation is important. However in pasture bloat present evidence (Chapter 1.3, the stable foam theory) would suggest that it is the stable foam in the rumen which prevents the normal eructation of gases. With respect to this stable foam Mangan (1958; 1959) demonstrated that the saponin foams *in vitro* had a maximum foam strength at a pH below that for the rumen liquor *in vitro*, or that found during bloat in *in vivo* studies, (McArthur & Miltimore 1969a).

At present it is thought that saponins do not play a major role in bloat in cattle (Clarke & Reid 1974).

Lipids

The possible importance of plant lipids in bloat in cattle was reviewed by Clarke & Reid (1974).

Mangan (1959) demonstrated that the plant lipids may have some degree of antifoaming properties. Wallace (1959) found 11-24% lipid in rumen foam from bloating animals feeding on fresh alfalfa. Wallace detected yellow bubbles in the froth which had a high content of particulate material in their surface layer.

Laby & Weenink (1966) used foam separation to examine components in the rumen liquor. Foam fractions of rumen liquor drawn prior to feeding bloating and non bloating red clover showed differences in the concentration of non-acid lipid, fatty acid, and carbohydrate components, but these were not reflected in the rumen liquor samples themselves. However yellow bubbles containing more than 30% lipid were present in the leading fraction of foam separated from rumen liquor collected prior to feeding. These authors detected more yellow bubbles when bloat resulted from the following feed than when no bloat occurred. These yellow bubbles were also observed in foam obtained from bloated cattle. This evidence would indicate that an animal is already 'primed' to bloat prior to consuming the feed which causes the bloat.

Stifel et al. (1968) proposed a competitive situation between the fraction I proteins and the polar lipids for metal ions. Fraction I protein can be denatured or salted out in the presence of Ca^{++} or Mg^{++} which results in a stable protein foam, but in the presence of polar lipids there is competition for the metal ions. The inhibitory effect of lecithin upon metal to protein binding diminished as bloat increased.

Palmitic acid inhibited Ca^{++} to fraction I protein binding less than did lecithin but consistently more than linolenic acid.

Jones (1971), examined various plant lipids for their antifoaming properties and found their effectiveness to be in the following decreasing order phosphatydyl choline, digalactosyl diglycerides, monogalactosyl diglycerides, sulpholipid, when tested gainst fraction I protein. However, a stable foam was produced when mucoprotein was added to the fraction I protein/monogalactosyl diglyceride solution which would not produce a stable foam.

Jones & Lyttleton (1973) showed a change in compressive strength of foams derived from uncentrifuged rumen liquor. This change was accompanied by a decrease in persistence which these authors attributed to antifoaming agents spreading into the surface films. These antifoaming agents were thought to be lipids.

Tannins

Cooper et al. (1966) showed that the bloat potential of forage was positively correlated with the volume of foam that could be generated from extracts of leaf. Kendell (1966) tested foams produced from red clover, white clover and alfalfa compared with non bloating legumes, birdsfoot trefoil (Lotus corniculatus), Lespedesa (Lespedeza stipulacea maxim) and Crownvetch (Cornilla varia). Non bloating legumes contained about 10% dry weight of tannin whereas legumes associated with bloat contained less than 2%. The foams produced from bloating legumes were inhibited by commercial tannins, but this inhibition was overcome by polyvinylpyrrolidine (PVP), a reagent which binds tannins. PVP enhanced the foam production from legumes which did not cause bloat.

Jones et al. (1970) showed that leaves of white, red, and subterranean clover ground into buffer, liberated soluble proteins but leaves of lotus major (Lotus pendunculatus Cav.) did not unless PVP was present. However Miltimore et al. (1970) showed a positive correlation of tannin with fraction I protein, but these authors were not differentiating between condensed and hydrolysable tannins.

Jones & Lyttleton (1971) suggested the absence of bloat in cattle when grazing temperate and tropical legumes may be related to the presence of protein precipitants in the plant. Their studies, however, did not involve any feeding trials to test the bloating potential of the legumes.

McLeod (1974) in a review article stated the biological properties of tannins is mainly a result of their ability to complex with protein. Tannins may affect intake of feed by astringent reaction in the mouth by binding to salivary proteins and receptor sites of oral mucous membrane. The condensed tannins do not appear to be digested by animals. However, Jones (W.T. Jones pers. comm.) stated that the plant protein bound by tannins are released by the time the tannins reach the duodenum.

Work is in progress at the DSIR, Palmerston North, New Zealand to breed plants of high tannin content and to study the digestibility of the same, and to determine their effectiveness in controlling pasture bloat.

Importance of saliva in bloat

Saliva could be of importance in bloat aetiology for a number of reasons:

1) Quantity of saliva secreted
2) Buffering capacity of saliva
3) Amount of carbon dioxide evolved from bicarbonate
4) The amount of mucoprotein present
5) The amount of mucin secreted
6) The amount and/or type of other proteins or functional molecules present (including inorganic ions)

Over thirty years ago Cole & Mead (1943) proposed bloat was due to diets containing insufficient roughage to stimulate eructation. Weiss (1953), discovered that mechanical stimulation of the rumen wall could cause salivation as a reflex response. The hypothesis was put forward that frothing was the main cause of bloat in sheep on green lucerne. Formation of the froth was dependent on the consistency of the ruminal ingesta, which in turn was influenced by reflex salivary secretion. The reflex salivary secretion was thought to be stimulated by the presence of coarse materials in the fore-stomach.

Johns (1954) concluded that while the agent producing the foam may well be both saponin and plant protein and their hydrolysis products, the contribution of saliva with its variation in surface activity and volume may well be all or part of the genetically controlled animal factor.

Results from experiments performed by Fergusson & Terry (1955) were inadequate to support or disprove Johns hypothesis on the importance of

saliva in bloat. These authors showed that one sheep given 750 ml of cows saliva plus 2.5 l of lucerne juice bloated, as did the two control sheep which only received the lucerne juice. Their results from two groups of six sheep given either lucerne juice or lucerne juice plus artificial saliva were inconclusive, as both groups of animals bloated. Johns et al. (1958), reviewed the current state of knowledge on bloat, and reiterated the probable importance of saliva with respect to the animal factors involved in bloat.

The hypothesis that components in saliva were the important animal factors in bloat started a series of investigations on factors influencing the amount of saliva produced, and the components in saliva.

Phillipson & Reid (1958) demonstrated an increased salivation in four out of five calves in response to an increased intra-ruminal pressure of 5-20 mm Hg. The response of the mandibular gland was small in comparison to the parotid gland except in one animal where a seven-fold increase was seen in the mandibular secretion, but only a small increase was seen for the parotid in this animal. These authors suggested that the increased parotid response, may in the case of bloat, be sufficient to precipitate a dangerous state, due to the increased liberation of CO_2 from the bicarbonate in the parotid secretion. However, this could be equally interpreted the other way, where the increased parotid saliva flow in response to increased intra-ruminal pressure could be effective by preventing a drop in pH in the rumen. Sheep were less responsive to an increased intra-ruminal pressure; only one out of five animals increased its parotid saliva flow when the pressure was raised to 20 mm Hg.

Phillipson & Mangan (1959) demonstrated an increase in protein nitrogen content of the mandibular saliva in cows when the intra-ruminal pressure was increased to 5-15 mm Hg. They described this as an increase in mucoprotein but the methods used would not have been sufficient to differentiate between the mucoprotein and other proteins found in secretions from this gland.

Mangan (1959) showed that the 'mucoprotein' of saliva collected by means of a perforated bit which fitted under the tongue of the cow, was a powerful foaming agent and he suggested it may play some significance in bloat. The 'mucoprotein' would not have been pure but would have included other salivary proteins as mentioned earlier.

Using the Tiselius electrophoresis apparatus, Lyttleton (1960) investigated the bovine salivary proteins collected by means of a perforated copper bit fitted under the cows tongue. This was the first reported attempt to see whether recognised differences between animals in susceptibility to bloat were parallel by similar differences in the protein composition of the saliva. Although only three protein components could be identified by this technique, Lyttleton showed that the one pair of twins which were reported to be noticeably less prone to bloat, had a lower content of mucoprotein and a higher content of a faster moving protein, termed fraction A. Only six animals were used in this study. This work was followed by a paper by the same author, (Lyttleton 1964) describing an improved method of purifying the mucoprotein component of saliva. He demonstrated the low isoelectric point (about 1.5) of this protein and the very high molecular weight of 1.1×10^7. However, he did not investigate further the fraction A protein which was increased in the low bloating twins. In fact no further work was undertaken on these smaller molecular weight proteins until Clarke et al. (1974) showed a possible difference between HS and LS cows in a protein designated band 4.

Mendel & Boda (1961) proposed the theory that saliva might act as an antifoaming agent and that animals susceptible to bloat (HS) secrete less saliva than animals of low susceptibility (LS). These workers found that the HS cows had a higher content of dry matter in the ruminal digesta than did LS animals. Yadava (1960) demonstrated that a reduced saliva flow produced by subcutaneously injecting Atropine sulphate (50 mg/cow/day) increased the foam in the rumen of identical twin cows grazing mature alfalfa pasture. It was also demonstrated _in vitro_ (Van Horn & Bartley 1961) that saliva added to foaming rumen contents could liberate the trapped gas. Bartley & Yadava (1961) reported a reduction in bloat for up to 4 h when animal mucins were introduced via the rumen fistula 30 min before pasturing. They suggested these mucins appeared to serve as a foam inhibiting and foam breaking agent and postulated that bloat results when feeds containing foaming constituents fail to induce sufficient salivary secretions. The quantity of saliva produced per feed has been shown to depend on the animals diet (Chapter 1.2, salivation in cattle). Meyer et al. (1964) showed a large difference in the saliva produced per feed for cows feeding on freshly cut alfalfa (8.0 Kg), compared with the quantity produced when fed hay (15.8 Kg). However these authors were unable to

demonstrate differences between bloaters and non bloaters for resting saliva flow in the three pairs of twins used in these trials.

Jones & Lyttleton (1972) using salivary mucoproteins purified by the method of Lyttleton (1964) demonstrated, using the Laby apparatus (Laby 1969), that this mucoprotein produced a very persistent foam of low compressive strength that was unaffected by pH over the range 4.0 - 7.0. In contrast to this they showed that the salivary mucin although producing a viscous solution, did not produce persistent foams. Furthermore these workers (Jones & Lyttleton 1973) demonstrated the presence of a high molecular weight glycoprotein from the rumen liquor, which on electrophoresis was the major protein entering the 7.5% polyacrylamide gel. The chemical properties of this protein were similar to those of the salivary mucoprotein. Furthermore antisera prepared against this protein cross reacted with purified mucoprotein, and with an increased sensitivity to desialated mucoprotein. In view of these findings Jones & Lyttleton (1973) suggest the mucinolytic bacteria might produce the foam stabilizing molecule in the rumen by desialating the mucoprotein. This is in contrast to Mishra et al. (1967, 1968) who proposed that mucinolytic bacteria destroyed the antifoaming agent in saliva. However no comparisons have been made of the foaming properties of complete and desialated mucoprotein. The only evidence available (Jones & Lyttleton 1973) would suggest the mucoprotein has similar foaming properties before and after the removal of sialic acid.

The mucin molecule, which is quite distinct from the mucoprotein may be worth studying with respect to antifoaming properties as this appears to be produced in large quantities and can be isolated from saliva collected from the base of the oesophagus in rumen fistulated cows (Jones 1971, Jones & Lyttleton 1972). The mucin appears to be produced by the palatine glands and can be purified by preparative ultracentrifugation (W.T. Jones and M.P. Gurnsey pers. comm.).

Clarke et al. (1974) demonstrated that rumen liquor from a given cow tended to degrade homologous saliva faster than saliva from other cows. They also qualitatively demonstrated more of one protein band, (designated band 4), in HS animals as compared to LS animals. This protein was analytically separated by electrophoresis on a 7.5% polyacrylamide followed by staining with Coomassie Brilliant Blue R250. The method of collection and concentration of the saliva is open to criticism as well as the very small number of animals used, but this work coupled with earlier findings,

(Lyttleton 1960) and the report by Reid et al. (1972) prompted a physiological genetics approach to be undertaken in this laboratory (Reid et al. 1975a; Cockrem 1975). Preliminary results showing salivary protein band inter-relationships have been reported (McIntosh 1975), and results in line with the findings in this laboratory have been published by Reid et al. (1975b).

Inheritance of susceptibility to bloat

Numerous researchers and farmers alike have observed differences between animals in their susceptibility to bloat. Some selected examples from the literature are as follows. Knapp et al. (1943) observed differences between Hereford progeny groups in the frequency of bloat; this was feedlot bloat, the diet being a grain mixture and alfalfa hay. They noted there was less bloat in the cross breeds than the pure bred animals. Brundage & Petersen (1952) while studying four sets of identical twins on a rotational grazing trial noticed one set of twins bloated while the other animals did not.

Johns (1954) noted the similarity in bloating behaviour within-twin sets as compared to between-twin sets. Johns concluded the saliva may be all or part of the genetically controlled animal factor.

Hancock (1953; 1954) observed wide differences in bloat susceptibility between sets of twins and great similarity within twin sets.

Cole & Boda (1960) stated "the evidence is clear that ingesta of bloating animals is more frothy than that of non bloaters on identical rations. This means there must be differences in the ruminal ingesta." They conclude that the evidence favoured variation in saliva flow as an explanation.

Mendel & Boda (1961) showed salivary secretion and composition differed significantly between high susceptible (HS) and low susceptible animals (LS). The LS animals secreted 5% more saliva during rest than HS,while during feeding the LS secreted 28% more saliva than the HS cows. There was a difference in salivary bicarbonate concentration, the HS animals being greater than the LS, but this was only shown in one trial. They observed that the LS animals ingesta was characteristically watery whereas the HS was thick and viscous with a high dry matter content in spite of similar water intakes and feed consumption.

Mishra et al. (1967) reported that the rumen contents from bloat susceptible twins were more mucinolytic than were the contents from non susceptible twins when inoculated into a saliva substrate.

Reid et al. (1972) stressed the apparent differences between animals in their susceptibility to bloat and suggested it may be possible to breed for low susceptible animals. This was further stressed by Clarke & Reid (1974). These authors emphasized the importance of either finding the cause of low susceptibility, or a marker that correlates with susceptibility which would thus shorten the breeding programme for LS animals. Work investigating possible genetic markers was started by Clarke et al. (1974). These authors observed a marked difference in the proportion of one protein component of saliva separated by electrophoresis which they designated band 4; the proportion of band 4 in HS animals was much higher than in LS animals. Results from these studies prompted a physiological genetics approach to investigate bloat susceptibility in cattle. Cockrem (1975) outlined the physiological genetics approach to breeding HS and LS animals, and gave a heritibility of 0.6 for the trait calculated from two different sources of data. The preliminary work on breeding HS and LS animals, which is in progress at Ruakura, and the initial data on the salivary proteins in relation to bloat was reported by Reid et al. (1975a). Relationships between the individual protein bands in saliva, saliva flow, and bloat susceptibility was discussed in more detail by McIntosh (1975). Subsequent work on the salivary proteins and their relationship to bloat susceptibility is discussed in the experimental section of this thesis and in two published papers (Cockrem & McIntosh 1976; McIntosh & Cockrem 1977).

1.4

REASONS FOR A PHYSIOLOGICAL GENETICS STUDY OF THE LOWER MOLECULAR WEIGHT SALIVARY PROTEINS IN RELATION TO BLOAT SUSCEPTIBILITY IN CATTLE

Part one of the literature review (Chapter 1.2) illustrates the diversity of functions of the salivary glands. With respect to ruminant animals, only a very small amount of work has been undertaken to investigate the role of salivary proteins in their digestive system.

Bloat in cattle is a digestive disorder of ruminants which occurs most frequently when animals are grazed on pasture, containing legumes such as clover. The day to day variation of bloat in cattle is one of the major problems associated with this metabolic disorder, both from the point of view of the farmer, and the researcher alike. Therefore a repeatable bloat grading system is of fundamental importance to the study of bloat and the variation in the trait. Because of the large degree of variation made up of animal and environmental components, the only approach likely to lead to a complete understanding of the factors responsible for the animal variation is one based on the statistical analyses of results from an adequate number of animals. No previous research has attempted to systematically study the animal variation amongst animals with respect to bloat.

The current hypothesis on bloat suggests that it is caused by the formation of a stable foam in the rumen which prevents the normal eructation of excess gases produced by microbial fermentation, and hence the build up of gases causes distention of the rumen wall which is the characteristic appearance seen in bloated animals (Clarke & Reid 1974). It has been suggested that the fraction I and possibly some fraction II cytoplasmic proteins from plants might be involved in the formation of this stable foam, while the mucoprotein from saliva might be the major animal protein involved. Lipids might also be involved in the formation of a stable foam.

Studies on the physical properties of foams produced from the rumen liquor <u>in vitro</u> have not revealed differences between animals about to bloat and those that did not bloat. Furthermore no satisfactory differences have been demonstrated in the foam properties between cows of high (HS) and those of low (LS) susceptibility to bloat (Jones 1971; Jones & Lyttleton 1973). Therefore the work of Jones & Lyttleton (1973) places some doubt on whether the ability to form a stable foam <u>in vitro</u>

from the rumen contents of a cow bears any relationship to why some animals bloat while others do not. Further studies looking at *in vitro* foaming properties of rumen liquor or saliva and plant protein constituents may be of little value, without first studying in more detail the HS/LS animal differences. Whether or not differences can be detected in the physical properties of foams generated *in vitro* from rumen liquor can only be resolved by experimentation based on a correctly designed experimental plan. This would involve testing HS and LS animals on bloating and non-bloating (control) pasture in a cross-over design where animals tested on the bloating pasture were subsequently tested on the control pasture and vice versa.

The evidence from the literature (summarized above), would indicate that the genetic component or components involved in determining bloat susceptibility differences amongst animals might not be revealed by physical studies on protein foams. Three reasons for indicating that further complexities might be involved are as follows:

a) Not all cows bloat when pastured on 'bloat potent' feed
b) All cows produce mucoprotein in their saliva
c) No difference has been shown in the foam properties of rumen liquor from cows that were about to bloat and those that did not bloat

Therefore, although the formation of a stable foam might be the end point in determining whether a cow will or will not bloat, the important factors likely to differ amongst animals of different bloat susceptibility might be those preceeding the foam formation. To understand the reason how animals differ in bloat susceptibility, it is therefore necessary to search for these factors which differ between HS and LS animals. The components which differ between HS and LS animals are not necessarily involved in the formation of a stable foam in the rumen.

Much of the evidence in the literature indicated that saliva might be important with respect to components which differ between animals of HS and LS bloat groups, and this was discussed in the literature review on bloat (Chapter 1.3). A study of the fundamental difference in the salivary proteins between HS and LS animals is important for three reasons:

(i) It should give an insight into the factors which might differ between HS and LS animals and thus provide a starting point for future detailed research on these factors.

(ii) It might allow selection of LS animals on the basis of this (or these) differences, and thus form the basis of a practical breeding programme to reduce the incidence of bloat in cattle.

(iii) A study of the salivary proteins might provide some general information on the function of these proteins in cattle, and in particular their role in ruminant digestion.

The work described in this thesis concentrated on the lower molecular weight proteins in bovine saliva. Three reasons (apart from the time available), for restricting this investigation to the lower molecular weight proteins, were as follows:

a) No evidence was available to suggest the mucoprotein molecule in saliva differed in quantity or its properties between HS and LS animals.

b) The mucoprotein was already being investigated by other workers engaged in bloat research*.

c) Evidence was available (Chapter 1.3) to suggest that differences might exist in the lower molecular weight salivary proteins between HS and LS cattle.

The fundamental requirements necessary for a physiological genetics approach to animal selection were outlined in Chapter 1.1. There were four major requirements peculiar to this physiological genetics investigation into the involvement of the salivary proteins in relation to bloat susceptibility:

(i) An accurate and repeatable bloat scoring procedure

(ii) A means of collecting representative samples of saliva from an appropriate number of animals

(iii) A means of measuring the total protein in saliva

* Dr W.T. Jones and the late Dr J. Lyttleton, Applied Biochemistry Division DSIR, Palmerston North, New Zealand

(iv) A suitable electrophoretic procedure for studying individual protein bands

The experimental work covered in this thesis is divided into two parts. The work described in Part One tested the hypothesis that the lower molecular weight proteins in bovine saliva might be related to bloat susceptibility in cattle. Furthermore it examined the inter-relationships between these proteins and some factors (animal or environmental) which might influence the quantity and/or composition of the saliva secreted.

Part Two of this thesis reports the results of studying the lower molecular weight proteins using biochemical and immunochemical procedures. The specific questions asked about these proteins are given in the Introduction to Part Two. The purpose of this research was to obtain fundamental information regarding the properties of these proteins which would provide a starting point for future work.

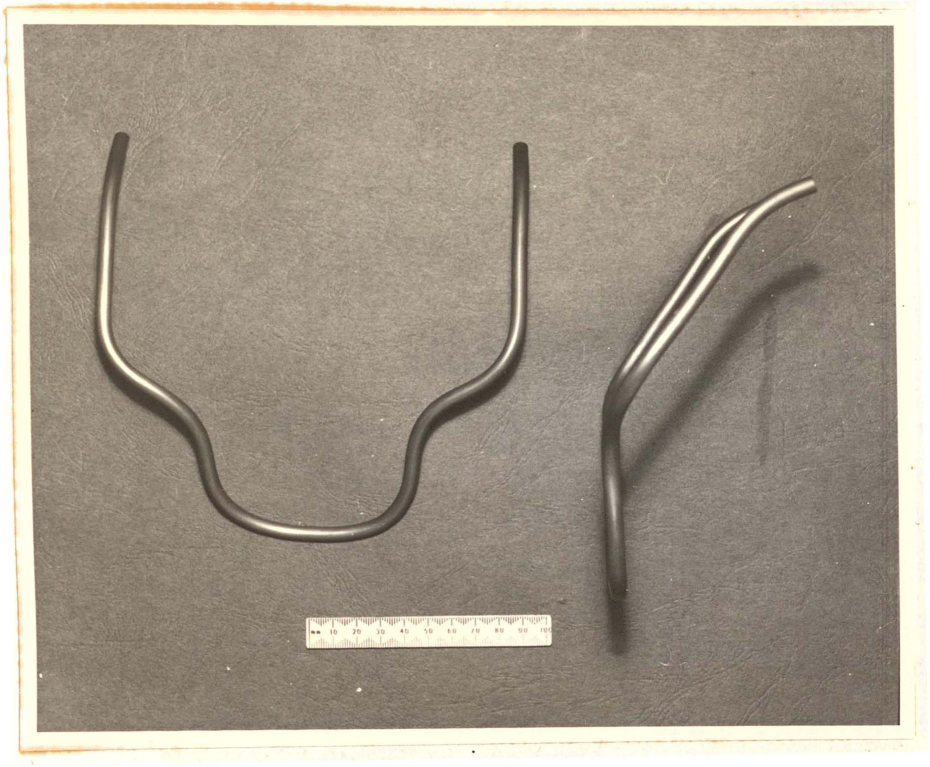


PLATE 2.1.1 Photograph showing the basic design of the copper bit used for the saliva collection.

CHAPTER 2

EXPERIMENTAL METHODS

The experimental methods described in this Chapter were the routine procedures used throughout this research project. Where methods were adapted directly from published work, only the outline of the technique is reported and the appropriate reference cited.

2.1
COLLECTION METHODS

2.1.a
Collection of saliva from the mouth by means of a standard bit

Saliva samples collected by the mouth bit did not give a representative sample of whole saliva, nor could the total saliva flow be measured by this technique. Instead the saliva collected by the mouth bit appeared to be secreted predominantly by the mandibular glands and other glands, such as the sublingual, which have ducts opening in the region of the positioned bit (this is discussed in Chapter 8). This sampling technique was repeatable both for the proportion of protein and the saliva flow and because of the simplicity of the collection procedure. It allowed an adequate number of animals to be studied without the necessity for surgical insult, which apart from the small number of animals that could be used, might alter the saliva composition and flow rate.

Saliva was collected by means of a plastic covered perforated copper bit (Plate 2.1.1) which fitted under the tongue and posterior to the opening of the ducts of the mandibular glands, (Plate 2.1.2). This procedure was based on the technique described by Mangan (1959). The saliva was collected by suction into a 250 ml Ehrlinmeyer flask which was kept on ice and connected to an Edwards 1/6th horsepower vacuum pump through a water trap (Plate 2.1.3). The collected saliva was centrifuged for 10 min at 1 500 g-min and the volume, colouration, amount of deposit, and viscosity (as determined by the rate of flow from a 3 ml pasteur pipette) were recorded. The saliva was then dispensed in vials and frozen until required for analyses.

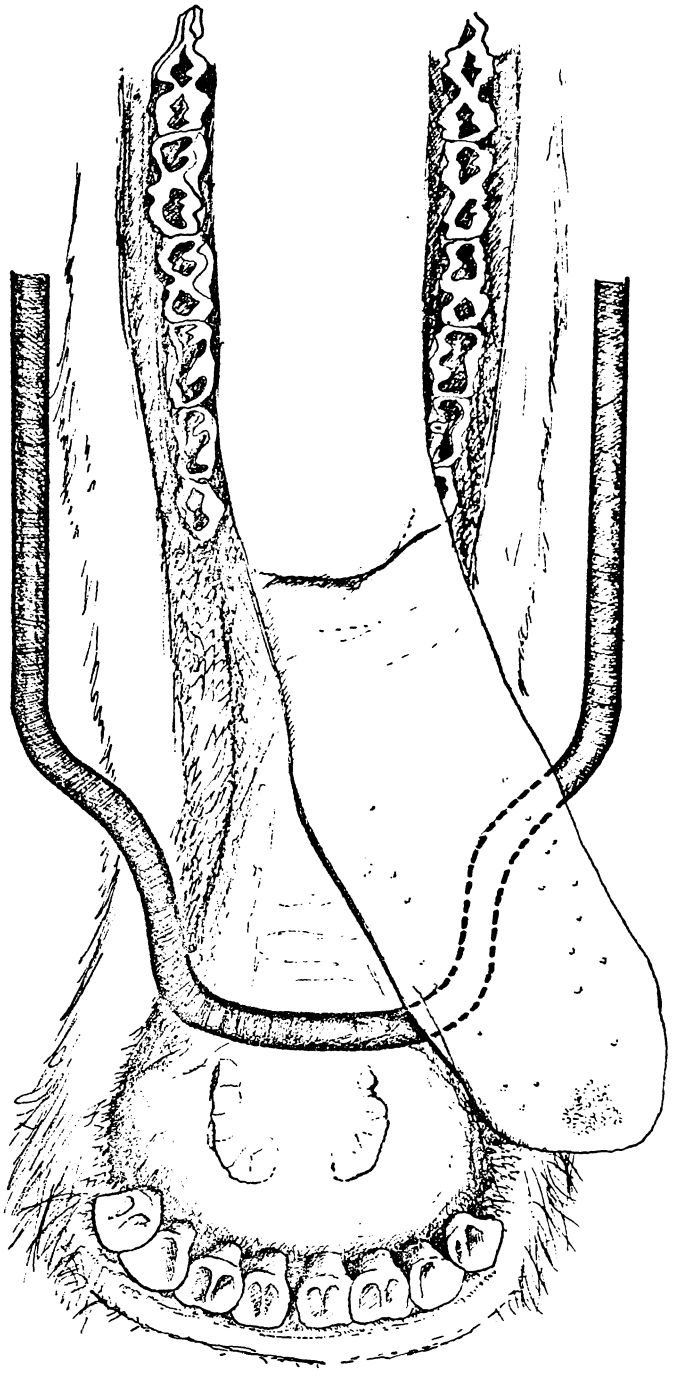


PLATE 2.1.2 Sketch of the lower jaw showing the mouth bit in position (Sketch by R.D. McLaren)

PLATE 2.1.3 Photographs showing the collection of saliva from a cow using the mouth bit.

2.1.b

Collection of nasal secretions

Secretions from the nose were obtained by swabbing each nostril with a pre-moistened cotton wool swab. The swabs were gently wiped over the walls of the nostril and then placed into 0.5 ml of physiological saline which was kept on ice. On return to the laboratory, the cotton wool swabs were rung out into the physiological saline and the sample was then frozen until required for analyses.

2.1.c

Collection of saliva from the mandibular gland

After swabbing dry the general area in the front of the mouth, saliva was collected from the mandibular gland of cows by means of a small rubber suction cup measuring 37 mm in external diameter, which fitted over either the left or right Whartons duct. Gentle suction* was applied using the Edwards vacuum pump, the pressure being regulated by means of a three way tap. Collections were carried out for short timed periods, not exceeding a five minute interval. The saliva was collected into 100 ml Ehrlinmeyer flasks which were kept on ice throughout the collection. After collection the saliva obtained was treated the same as the saliva obtained by means of the bit.

2.1.d

Collection of secretions from the nasolabial glands

Nasolabial glands are small glands situated in the dermis of the muzzle skin of ruminant species such as cattle and buffalo. The watery secretions from these glands keep the muzzle moist (Church 1975).

Prior to collection the muzzle was cleaned and wiped dry with paper towels. After about one minute the glands had completely moistened the muzzle and these secretions were collected by means of two cotton wool swabs**. After swabbing the area the swabs were placed into 0.5 ml of physiological saline and kept on ice until returning to the laboratory. The swabs were then rung dry and the diluted secretions frozen until required for analyses.

* The pressure used was just sufficient to hold the suction cup to ones hand.

** No licking of the muzzle occurred during the period of collection.

2.1.e

Collection of blood samples

Blood was collected from the jugular vein of all the 1973 and 1974 progeny groups*, EDTA was used as the anticoagulant and after mixing the blood sample by inversion, it was placed on ice until returning to the laboratory.

* (immediately after saliva sampling in September 1976)

2.1.f

Collection of saliva from the oesophagus

Oesophageal saliva samples were collected from animals at the DSIR* which were fitted with oesophageal fistulae. The collections were performed over a 5 min interval, with and without the mouth bit sampling being performed at the same time. All samples were kept on ice during and immediately after collection. On returning to the laboratory these samples were treated the same as the mouth saliva samples.

2.2

BIOCHEMICAL METHODS

2.2.a

The polyacrylamide gel electrophoresis technique (PAGE) used for studying the bovine salivary proteins

The electrophoretic separation of the protein bands in saliva was one of the most important analytical procedures used in this research work. The tube technique described here was based on the flat gel polyacrylamide electrophoresis method of Jones (1971) and Jones & Lyttleton (1971). Preliminary trials comparing the tube polyacrylamide gel electrophoresis technique (PAGE), with the flat gel method, showed the resolution and repeatability of the tube technique to be superior under the conditions used in this laboratory. A major problem encountered with the flat gel method at the start of this project was scanning the gels as the Densicord Densitometer used in the preliminary work would not scan these flat gels without first slicing the acrylamide gel into individual sample strips and this introduced an additional error into the quantitation of the bands.

* Dr C.S.W. Reid, Applied Biochemistry Division,
The Department of Scientific and Industrial Research,
Palmerston North, New Zealand.

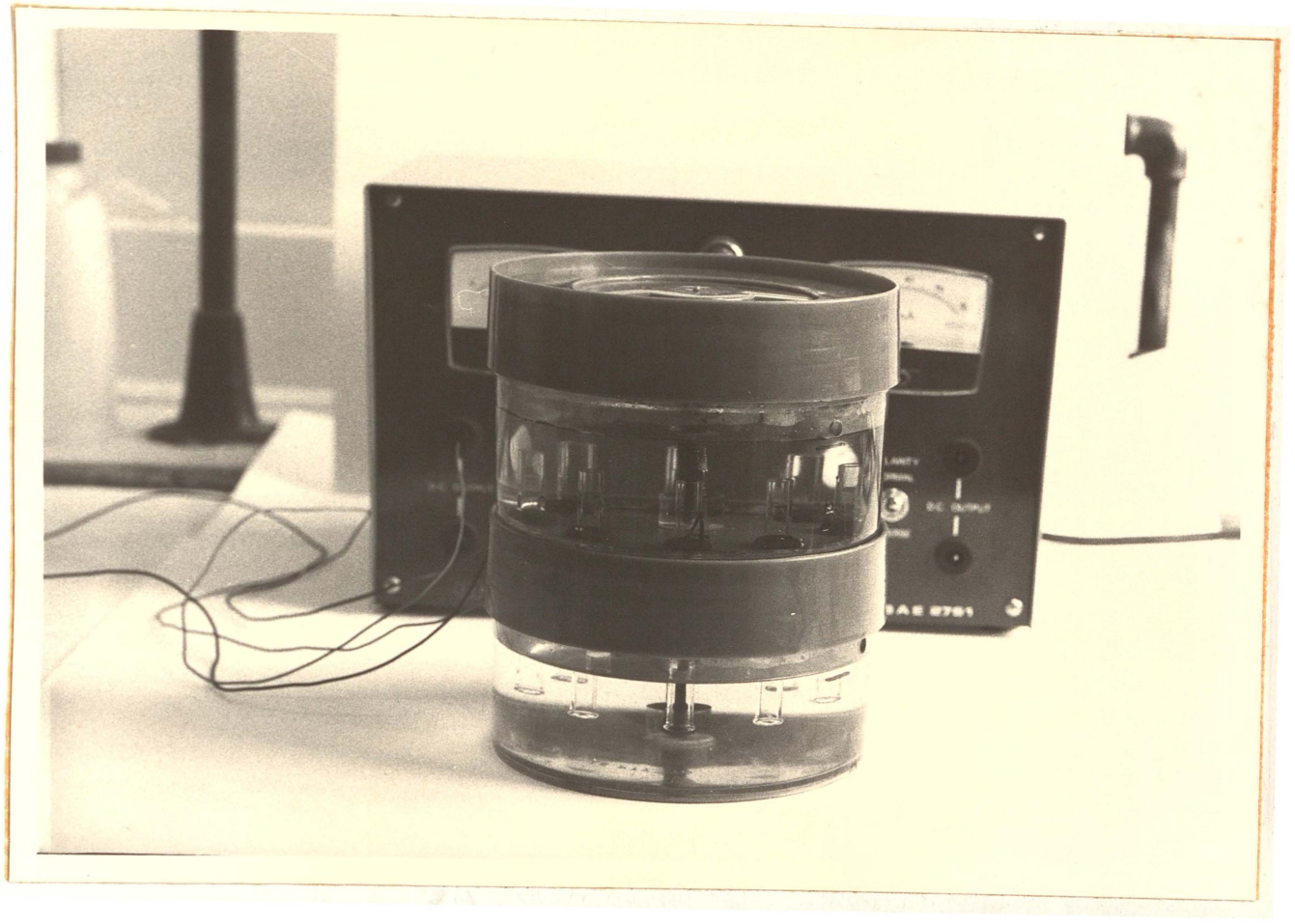


PLATE 2.2.1 The electrophoresis apparatus used for all quantitative PAGE in this project

The tube PAGE technique was used throughout this work and with the inclusion of a control saliva within each electrophoretic run (and in later work a standard saliva) the repeatability of the system could be consistently monitored.

Preparation of saliva samples

Thawed, well mixed mouth saliva (100 µl) was placed into a test tube containing 15 mg of glucose. Marker dye (25 µl of 0.1% bromophenol blue in 50% ethanol) was added to the tube. The contents were mixed on a vortex test tube mixer and placed on ice until required.

Polyacrylamide gel electrophoresis apparatus

The electrophoresis apparatus was constructed in this laboratory from two commercially available screw-capped plastic containers, which measured 15 cm in diameter and were 9 cm in height. In both the anode and cathode chambers a piece of perspex tubing 10 mm in height with an internal diameter of 8 mm was glued in the centre of the bottom of each chamber. Into this tube, a carbon rod electrode (obtained from a standard dry cell battery) 66 mm in height was inserted. Two holes were drilled in the sides of each chamber at a height of 56 mm, these allowed evolved gases to escape and formed the exit for the electrode flex.

The screw-capped top on the anode chamber had a circular portion 120 mm in diameter cut out, this formed the seal and support between the top and bottom chambers when the apparatus was in operation.

In the cathode chamber eight equidistant holes were drilled in the bottom of the chamber, forming a circle with a radius of 46 mm. Each hole was fitted with a rubber grommet, giving a final internal diameter of 9 mm.

A photograph of the electrophoresis apparatus is shown in Plate 2.2.1.

Gel tubes

The gel tubes were 10 cm long with an internal diameter of 6 mm. The bottom end of the tube was lipped to facilitate removal of the polymerised gel. This lipped end was sealed with Parafilm before the tubes were placed in the polymerizing rack. All tubes were siliconized in a 1% solution of Siliclad*.

* Clay Adams, Division of Becton Dickinson, New Jersey, USA.

Stock solutions for polyacrylamide gel electrophoresis

Stock buffer: 1 mol/l tris, 1 mol/l glycine in distilled water

Running buffer for gels: 14 ml stock buffer diluted to 100 ml with distilled water

Electrolyte buffer: 100 ml stock buffer diluted to 2 l with distilled water

Stock TEMED: 10% N, N, N', N'-tetramethylethylene diamine in absolute ethanol.

Ammonium persulphate: 10% aqueous solution (prepared daily).

Stock acrylamide solution: Acrylamide 15.0 g, and N, N'-methylenebisacrylamide 0.3 g, were made to 100 ml in distilled water

Polyacrylamide gels:

Running buffer	15 ml
Stock acrylamide	15 ml
TEMED	0.3 ml
Ammonium persulphate	0.3 ml

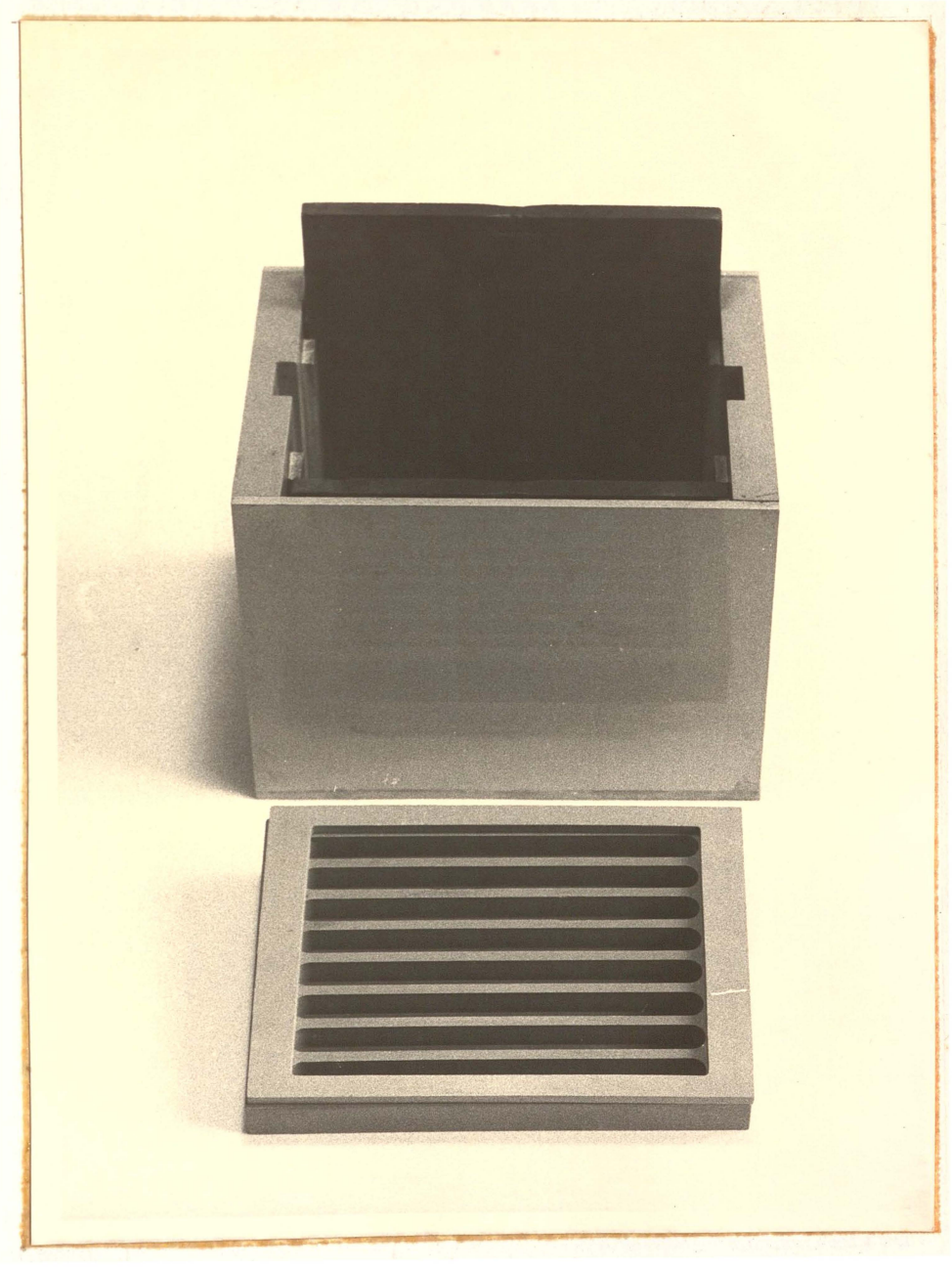
Reagent for staining polyacrylamide gels with Coomassie Brilliant Blue R 250

Protein Fixative: 12.5% aqueous solution of trichloroacetic acid was used to fix all proteins.

Coomassie staining solution: The dye used was Coomassie Brilliant Blue R 250 (this is a triphenylmethane dye CI number 42660, it is also known as Acid Blue 83).
250 mg of the coomassie dye was dissolved in 25 ml of analar methanol, and the dissolved dye was transferred to a 100 ml volumetric flask. Seven ml of glacial acetic acid was then added to the container in which the dye was originally dissolved, and the residual dye dissolved in this acid and this was also transferred to the 100 ml flask*. The volume of dye solution was then made to 100 ml, and the staining solution was ready for use. This staining solution was always prepared daily.

* (A white crystalline substance which did not dissolve in either the methanol or acetic acid was noticed with two brands of coomassie, (Sigma and Colab).)

PLATE 2.2.2 The electrolytic destainer used for destaining all the quantitative PAGE in this project; showing one of the carbon electrodes and the gel holder.



Gel polymerization

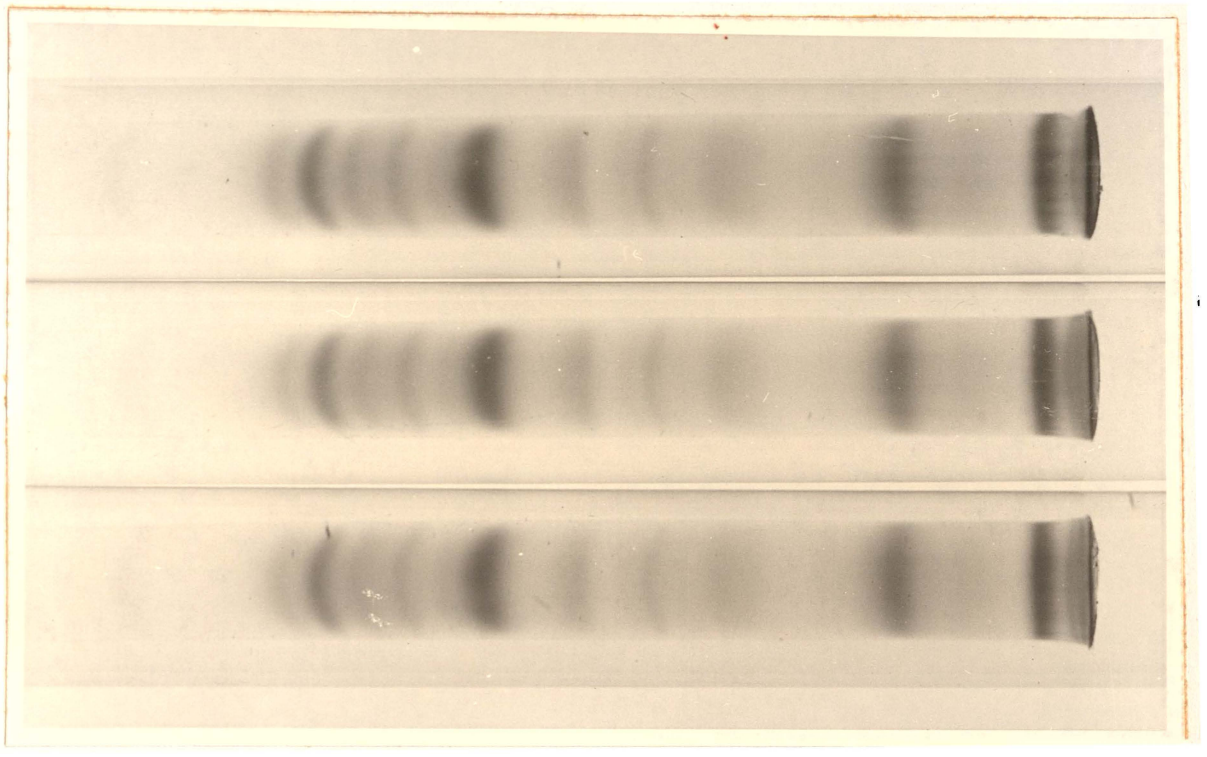
The acrylamide solution was prepared by mixing the running buffer with the stock acrylamide solution, both being brought to room temperature prior to mixing and de-gassing. The TEMED and ammonium persulphate solutions were then added and mixed with acrylamide/buffer solution. This mixed acrylamide solution was then added, by means of a 10 ml plastic syringe, to the vertically held polyacrylamide gel tubes. The acrylamide solution was added to a height of 90 mm and 25 µl of water was then carefully layered on the acrylamide surface, forming a sharp flat interface. The polymerization took 30 min at room temperature.

Running the electrophoresis

Eight tubes were placed in the electrophoresis chamber such that 7 cm projected down into the anode reservoir, which was filled with 650 ml of electrolyte buffer. A few drops of buffer were placed on the end of the acrylamide gel before lowering into the buffer so as to prevent air bubbles from being trapped. The upper cathodal chamber was then filled with 600 ml of buffer and the electrophoresis apparatus was then connected to the power source. Prior to the application of samples, the gels were run for a ten minute period at 13 mA constant current. 25 µl of sample was then applied to each tube by means of a Hamilton syringe. One control was included within each electrophoretic run. The gels were run for 15 min at 13 mA and then for a further 60 min at 26 mA. During electrophoresis the voltage increased from 180 to almost 300 volts.

Fixing and staining the gels

At the completion of the run, the gels were carefully removed from the tubes by gently inserting a fine gauge needle and rimming around the tube while pressure was exerted on the syringe forcing buffer into lubricate the needle. When both ends of the gel had been rimmed with the syringe needle the gel was tipped out of the tube into the protein fixative. After 20 min, the gels were transferred to freshly prepared 0.25% Coomassie and stained for 60 min. On completion of the staining the gels were washed with two changes of destaining solution before being placed in the electrolytic destaining apparatus (Plate 2.2.2). Electrolytic destaining was carried out for a period of 16 hr at 80 mA; the gels were then transferred



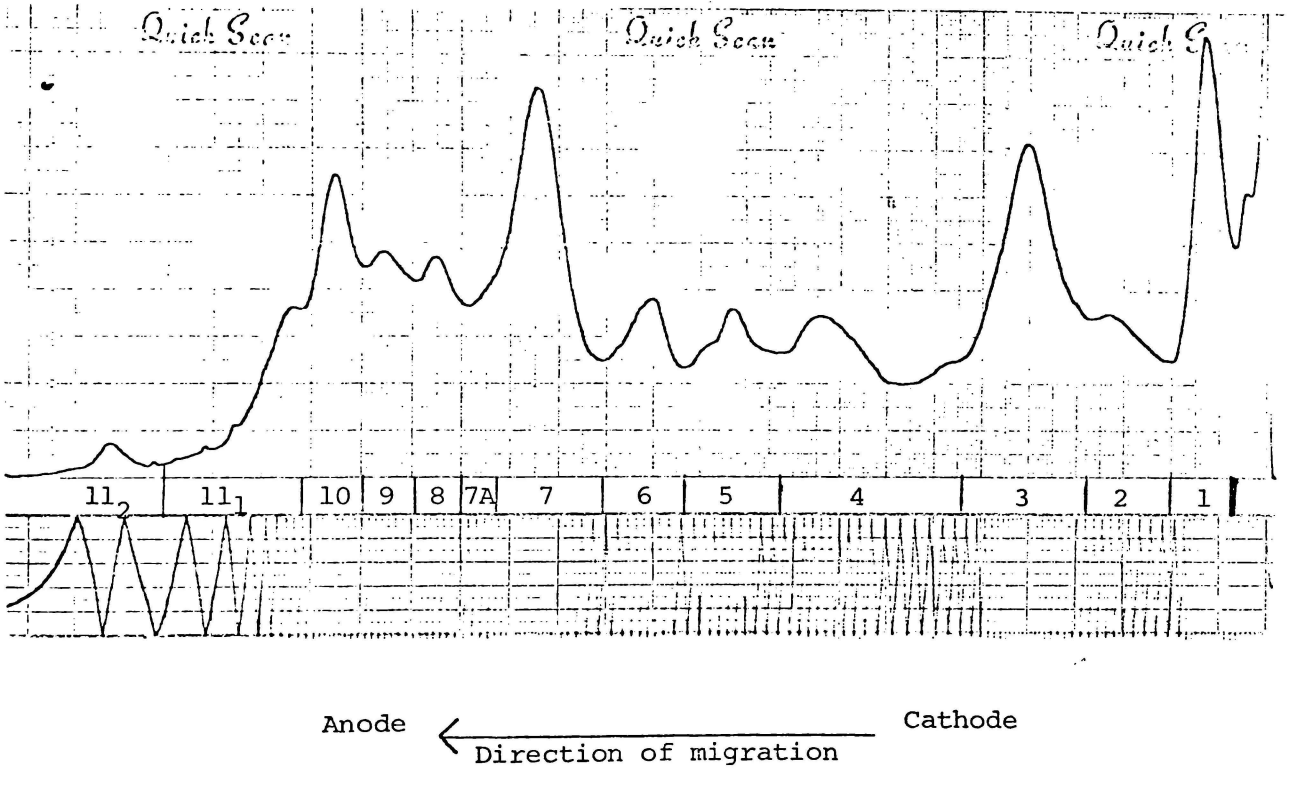


PLATE 2.2.3 A photograph and reduced (2-fold) electrophoretic scan of standard saliva showing the numbering system used for PAGE

into a fresh destaining solution and placed on a rotary tube mixer where they were slowly rotated for a further 4 h. The gels were then scanned in the densitometer* at 595 nm and the band proportions computed automatically.

2.2.b.i

The numbering system used for the salivary protein bands separated by polyacrylamide gel electrophoresis (PAGE)

The numbering system adopted for the anionic protein bands separated by PAGE from mouth saliva was based on the system of Jones (pers. comm; Clarke et al. 1974). The numbering of bands started at the sample end of the gel (cathode end) and numbered from 1 to 11; the fastest migrating band having the highest band number. This numbering system is illustrated in Plate 2.2.3.

Any one protein band separated by electrophoresis might contain more than one protein, as indicated by Clarke et al. (1974) for band 4. It is also possible that two or more bands might differ only by the number of sialic acid or carbohydrate molecules present on the protein molecule (McIntosh 1975). However this numbering system described above, for the different electrophoretic bands separated by PAGE, formed the bases upon which to start the investigation into the possible differences in the salivary proteins between animals, and between HS and LS sire groups, and the effect of environmental factors (within animals) such as different feeds. Furthermore the statistical relationships of these bands with total protein, saliva flow, and the inter-relationships between bands could be investigated. Finally, this numbering system formed an identification system upon which the chemical relationships between bands, and the identification of individual proteins within the electrophoretic bands could be made.

2.2.b.ii

The reason for expressing the salivary protein bands as percentages

This project was designed to investigate the lower molecular weight proteins in bovine saliva. However, because it was not possible to determine the exact quantity of mucoprotein in saliva, or the quantity of these lower molecular weight proteins without mucoportein, an additional

* Helena Quick Scan densitometer coupled to a Helena Quick Quant computer. (Helena Laboratories Ltd., Texas, USA)

complication was introduced. This was overcome by measuring the total protein in saliva (mucoprotein + lower molecular weight proteins) and expressing the individual protein bands separated by polyacrylamide gel electrophoresis, as a percentage of the total protein which entered the 7.5% gel rather than as a concentration. Provided the limitations of the percentage technique are fully appreciated then the use of percentages can be justified. Results reported in Chapters 3.3, 3.5 and 4.5 compare measuring the individual protein bands as percentages with that of expressing the bands as corrected integrations. This latter technique can be considered almost as close to an absolute quantitation of the concentration of individual proteins in a heterogenous mixture as can be achieved by conventional electrophoresis. No electrophoretic procedure relying on dye binding can give absolute concentrations of individual proteins in a heterogeneous mixture because of the large differences in dye binding affinities amongst proteins.

A discussion of this quantitative study of proteins compared with the clinical biochemistry approach to the study of serum proteins, or the biochemical genetics approach to the study of isozymes is given in Appendix A.

2.2.c

Production of standard saliva

A mixture of all the proteins seen in the mouth and oesophageal saliva collections was made; this was termed the Standard Saliva. The purpose of this Standard Saliva was to enable a saliva of known composition (containing all the salivary proteins) to be included with each batch of electrophoresis as a check on the technique. Equally well the Standard Saliva was necessary for the immunochemical procedures, and furthermore the use of this sample containing all the proteins in crossed electroimmunodiffusion studies enabled further information to be gained on the number of immunochemically distinct proteins present in saliva.

The Standard Saliva was produced from three different sources of salivary proteins. The first was mouth saliva (from cow 9120) which had been concentrated five-fold, this was added to the second saliva which was a five-fold concentrated sample collected from the oesophagus (cow 1125). Both these saliva samples had low levels of band 6, therefore the proportion of band 6 was increased by the addition of a semipurified band 6 preparation which also contained a small proportion of bands 1, 3 and 8.

After thoroughly mixing the three samples of salivary proteins the Standard Saliva was dispensed in small test tubes and frozen until required. The total protein value of this Standard Saliva was 1 480 µg/ml.

2.2.d

Constant absorbance standard (CAS)

The Quick Scan Densitometer has a continuously variable gain setting and it was thus impossible to scan gels at the same gain setting unless the gain selector was not moved between gels. To overcome this problem, a film with a constant absorbance value was scanned with each gel.

A strip of X-ray film measuring 90 x 25 mm, which had been cleared by washing in concentrated hydrochloric acid overnight, followed by washing in water, was used as a standard absorbance standard for the densitometer. A 65 mm length was marked off and this formed the constant area which was scanned with each gel.

Gels scanned at variable gain settings were thus equated with one another by the simple expression,

$$\frac{\text{Sample gel}}{\text{CAS}} \times \frac{1000}{1}$$

the corrected integration values obtained were termed Gain-corrected integrations. (This is further discussed in Chapter 4.5)

2.2.e

Periodic acid schiff stain procedure

The Periodic Acid Schiff (PAS) staining procedure was used as the main method for detecting glycoproteins or carbohydrate containing molecules in the polyacrylamide gel electrophoresis and acrylamide/agarose gel electrophoresis techniques. The method used was adapted from that of Zacharious et al. (1969).

Reagents

Schiff reagent: this was prepared according to the method of McGuckin and Mckenzie (1958)

Trichloroacetic acid: A 12.5% aqueous solution was used

Periodic acid: 1% periodic acid in 3% acetic acid

Potassium metabisulphite: 0.5% aqueous solution of potassium metabisulphite (prepared fresh).

Method

After running the electrophoresis, the gels were removed from their tubes and placed in trichloroacetic acid for 30 min to fix the proteins. This was followed by washing several times in distilled water. The gels were then placed into the periodic acid solution for 50 min. This was followed by several washings in distilled water in which they were left to stand overnight. The gels were then placed in the Schiff reagent for 50 min, and then transferred to the freshly prepared potassium metabisulphite solution for 30 min. The metabisulphite step was repeated several times, after which the gels could be stored in this solution for up to five days. Gels were scanned at 525 nm on the Helena Quick Scan Densitometer.

2.2.f

The Alcian Blue staining procedure for glycoproteins

The Alcian Blue staining procedure used in these studies was based on the method described by Wardi and Michos (1972). This is an alternative technique to PAS for the staining of glycoproteins and is based on the improved Alcian Blue staining procedure of Mowry (1956). Alcian Blue has an affinity for acid mucosubstances and this affinity is dependent upon the pH of the dye solution. At a pH of 2.5 (3% acetic acid) weakly acid sulphated mucosubstances (heparain, chondroitin sulphates), hyaluronic acid and epithial mucins containing sialic acid (sialomucins) show strong affinities.

Reagents

Trichloroacetic acid:)
Periodic acid:) As for the PAS technique
Potassium metabisulphite:)
Alcian Blue: 0.5% Alcian Blue made up in 3% acetic acid. This stain can be kept at room temperature.

Method

On the completion of the electrophoretic run, gels were removed from their tubes and placed in trichloracetic acid for 30 min. They were then rinsed lightly with distilled water and placed in the periodic acid solution for 50 min, after which the excess periodate was washed off using several

changes of distilled water. The gels were then placed in the potassium metabisulphite solution for 30 min, and this was followed by washing in distilled water and then staining for 4 h in the Alcian Blue stain. Gels were destained electrolytically in 7% acetic acid.

2.2.g

The staining of proteins with 'Stain All'

The procedure used for staining the salivary proteins with 'Stain All' was that of Green et al. (1973). Interaction of this dye with different polymers results in complexes which cause changes in the absorption spectrum of the dye. At least five different complex states of polymer dye interaction exist according to Bean et al. (1965), each with a specific spectral band maxima. Thus this technique can be used to distinguish the presence of various macromolecules by their differences in staining. According to Dahlberg et al. (1969) RNA stains bluish purple, DNA stains blue, protein stains red, and acid mucopolysaccharades various colours. Green et al. reported differential staining with phosphoprotein which stained blue and non phosphorylated proteins which stained red or remained unstained.

Reagents

Stock Stain All: 1-ethyl-2-{3-(ethylnaphtho(1,2d)thiazoln-2-ylidene) -2-methyl-propenyl}-naphthol(1,2d)thiozolium bromide (Available from Eastman Kodak Co. USA) 0.1% Stain All solution in formamide. This stock solution was kept in the dark.

Working Stain All: (prepared immediately prior to use) 10 ml of stock Stain All was added to 10 ml of formamide and to this 50 ml of isopropanol was added plus 1 ml of 3.0 mol/l tris/HCl buffer (pH 8.8). The final volume was made up to 200 ml with distilled water.

Method

The polyacrylamide or acrylamide/agarose gels after electrophoresis were placed into the working staining solution and allowed to stain overnight in the dark. The following morning the staining solution was replaced with distilled water and allowed to destain (in the dark) until a good contrast

between the bands and the background was obtained. This could take up to 72 h. The gels were scanned at 625 and 525 nm.

2.2.h

Sodium Dodecyl Sulphate polacrylamide gel electrophoresis (SDS-electrophoresis)

SDS-electrophoresis is a technique which can be used to study the subunit structure of proteins. This procedure is also a useful technique when studying membrane bound proteins or where protein-protein interaction might occur. The vigorous conditions used in this procedure include treating the sample with the detergent (SDS), a sulphydryl reagent (β-mercaptoethanol), and heat. Thus non covalent bonds are disrupted as well as the disulphide bonding in the proteins. The SDS-electrophoresis technique was used in this work to gain information on the subunit composition of the proteins in bovine saliva.

Reagents

Stock Acrylamide solution:

Acrylamide	40.0 g
N,N'Methylene bis acrylamide	1.5

This was made up to 100 ml with distilled water

Stock buffer (pH 7.4):

Tris	48.4 g
Sodium acetate	27.2
Disodium EDTA	7.4

The pH was adjusted to 7.4 with acetic acid and the final volume made to 1 l.

Stock 10% sodium dodecyl sulphate (SDS):

SDS (BDH specially purified)	100.0 g

This was made up to 1 l with distilled water

Ammonium persulphate, 1.5% working solution:

Ammonium persulphate	0.75 g

This was made up to 50 ml with distilled water

Working TEMED solution: N,N,N',N'-tetramethylethylene diamine (TEMED) 0.25 ml

This was made up to 50 ml with distilled water

Working electrophoresis buffer:

Stock buffer	100 ml
Stock SDS solution	100 ml

This was made up to 1 l with distilled water

Preparation of gel tubes

The same 10 cm glass tubes as used for the analytical PAGE were used. These tubes were acid-washed, rinsed thoroughly in distilled water and dried. They were then siliconized in a 1% solution of Siliclad and dried in a hot air oven. After drying the tubes were placed in a 1% SDS solution for 1 h, rinsed with distilled water and dried in a hot air oven.

Preparation of 30 ml of SDS acrylamide for polymerization

Acrylamide stock solution	4.2 ml
Stock buffer	3.0
SDS stock solution	3.0
Distilled water	15.3

These solutions were mixed well, deaerated and the following two reagents added,

Ammonium persulphate working solution	3.0
Working TEMED solution	1.5

The 'acrylamide solution' was then gently mixed and the electrophoresis gel tubes were filled to the desired 9 cm length. The solution in each tube was then layered with 50 µl of a freshly prepared solution containing 0.1% SDS, 0.15% ammonium persulphate, and 0.05% TEMED solution.

Sample preparation

Reagents

Pyronin G solution:	This was used as the tracker dye, 10% pyronin G in a 5% sucrose solution
Stock SDS:	As for the gel preparation
Sucrose-tris-EDTA:	A solution containing Sucrose 1 mol/l, tris 50 m mol/l, and EDTA 5 m mol/l was prepared, and the pH was adjusted to 8.0 with hydrochloric acid, prior to diluting to the final volume
β-mercaptoethanol:	This was used without diluting

To prepare 500 µl of sample:

Stock SDS	50 µl
Sucrose-tris-EDTA	100

β-mercaptoethanol	50
Saliva or working solution of the control proteins	300

The prepared sample was then boiled for 15 min after which 5 µl of Pyroninin G solution was added.

Standard solutions

β-Galactosidase (MW 130 000), bovine serum albumin (MW 67 000), bovine Ig G (MW 50 000 and 25 000), and cytochrome c (MW 13 000), were used as the known molecular weight standard proteins. These were prepared as for the saliva samples starting from 1 mg/ml stock solutions. Equal quantities of the four stock solutions were mixed together to form the working solution of control proteins.

Running the electrophoresis

The electrophoresis apparatus (as used for the standard PAGE system) was set up as for the PAGE system. The gels were pre-run at 50 V constant voltage for 30 min prior to adding the samples. The samples were then added (25 µl of saliva, 40 µl of control proteins) and the electrophoresis was commenced, the run being carried out at 50 V constant voltage for approximately 3 h.

Staining and destaining the gels

On completion of the electrophoresis run the gels were removed from their tubes and placed in 12% trichloroacetic acid for a period of two to three hours. The tubes were continuously mixed during the fixing period by means of a rotary test tube mixer, and the trichloroacetic acid solution was changed every 30 min. The fixed gels were then stained in Coomassie Brilliant Blue R 250 (as used for the standard PAGE system Chapter 2.2.b) for a period of six hours. Destaining was achieved by elution in the methanol/acetic acid/water solution as used for the standard PAGE system.

2.2.i

Low pH polyacrylamide gel electrophoresis

The method of low pH electrophoresis used in this work to screen for the presence of cationic proteins in mouth saliva was that of Reisfeld et al. (1962). All reagents used were as described by these authors. The gels were polymerized in the standard siliconized tubes as

described for the routine PAGE (2.2.a). They were allowed to polymerize for at least 90 min before use, and were then run in the same electrophoresis apparatus as described for the standard PAGE (2.2.a). The sample was prepared as for the routine PAGE and 25 µl was added per gel.; 0.1% methyl green was used as the marker dye. The electrophoretic run was commenced at 13 mA for 10 min and then the current was increased to 52 mA for the remainder of the run.

Staining procedures

i) Coomassie Brilliant Blue R 250. The same procedure was used for the routine anionic PAGE.

ii) A modified Amido Black procedure was used according to the method of Sung and Smithies (1969).

iii) Fast Blue B (3, 3' dimethoxybenzidine)/hydrogen peroxide procedure was used as described by Azen & Oppenheim (1973).

2.2.j

Lowry protein estimations

This protein determination method used was that of Lowry et al. (1951), and reagents used were as described by these authors.

Principle

The final colour is a result of:

a) Biuret reaction of protein with copper ion in alkali

b) Reduction of the phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan present in the treated protein.

Protein standards.

These were prepared from stock quality control serum diluted to give values from 100 - 1 500 µg/ml. This serum was cross checked with Standard solutions prepared from crystalline bovine albumin, the value of which had been established by the Kjeldahl nitrogen technique.

Quality control serum

Dilute solutions of Moni-trol 1 * were run with each batch of salivary proteins. The diluted solution was prepared and dispensed into small vials, and frozen, a fresh vial was used with each batch of proteins.

* Dade, Miami, Florida, USA

Method

A 100 µl sample of saliva was pipetted into a test tube by means of a constant volume pipette, and 3 ml of alkaline copper solution was then added and the solutions were mixed on a vortex test tube mixer and allowed to stand. After 10 min, 300 µl of diluted Folin reagent was added, and the contents of the tubes were immediately mixed. After standing for 30 min the absorbance of the solution was read at 500 nm. All saliva samples were run in duplicate and protein standards run with each batch of samples. The protein values were determined from the standard curve (the regression of A on protein µg/ml for the standards run with each batch. Quality control sera was run with each batch of proteins as a check on the between run repeatibility.

2.2.k

Thin layer gel chromatography

The thin layer gel chromatography (TLG) was used as the method to determine the molecular weights of the partially purified salivary proteins.

Apparatus

The thin layer gel apparatus was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The apparatus and its operation are described in 'Thin-Layer gel filtration with the Pharmacia TLG-apparatus' (booklet supplied with the apparatus).

Reagents

Phosphate buffer: The working buffer used in these studies was a 0.02 mol/l sodium phosphate buffer pH 8.6

Sephadex: Both Sephadex G100 superfine and Sephadex G150 superfine were used. The slurry used for pouring the plates was prepared according to the method described in the Pharmacia booklet.

Blue Dextran: Blue dextran was used as the indicator to determine the end point of the run

Reference Protein: Thyroglobulin, 5 mg/ml dissolved in the working buffer was used as the reference protein

Standard proteins for molecular weight studies:

Cytochrome c MW 13 000
Soybean trypsin inhibitor MW 21 500
Bovine serum albumin MW 67 000
β-galactosidase MW 130 000
Bovine Ig G MW 150 000

Method

The slurry was spread to a height of 0.8 mm on the 20 x 20 cm glass plate which was used for all the TLG runs. Buffer was added to both reservoirs of the TLG-apparatus, 60 ml to the top and 25 ml to the bottom reservoir. The TLG plate was positioned in the apparatus and the 20 x 4 cm wicks (pre soaked) were placed on the surface of the Sephadex. The apparatus was raised to a 10^{o} angle and allowed to equilibrate overnight. The samples were applied the following morning.

Sample spots containing 10 - 20 µl of sample (with protein concentrations ranging from 1 - 5 µg/ml) were applied 15 mm apart on the thin layer gel. After the samples were applied, the gel was inclined at an angle of 10^{o} and run until the blue dextran had reached the end of the gel. The position of the proteins after the chromatographic run was determined by the replica technique, that is the proteins were blotted from the gel layer to a paper which was then treated as a normal chromatogram. Coomassie Brilliant Blue R 250 was used to stain the dried replica print (details are described in the operators booklet).

Molecular weights were determined by measuring the distance migrated from the origin to the mid point of the spot on the chromatogram for thyroglobulin and the standard proteins and the test proteins. The regression of the inverse migration distance (relative to thyroglobulin), on the log MW for the standard proteins was calculated for each run, and thus the molecular weights of the unknown test samples could be determined.

2.2.1

Screening for α-amylase activity in saliva

The Calbiochem Stat-Pack was used for the determination of α-amylase; (Calbiochem, San Diego, California, USA). The substrate for α-amylase used in this method is a special grade of amylopectin labelled with a blue dye. The amylase hydrolyses amylopectin azure to form soluble coloured

reaction products. The intensity of the released blue colour is proportional to the level of the enzyme in the sample.

The amylase assays were carried out as described by the manufacturers. Undiluted saliva (100 µl) was used for both the test and blank tubes. After 10 min incubation the enzymic reaction was stopped; the test and blank tubes were read at 595 nm. The concentration of amylase in the sample was calculated by reference to the amylase standard and checked by the Moni-Trol 1 quality control serum run with each batch of assays (Moni-Trol 1, produced by Dade, Division American Hospital Supply Corporation, Miami, Florida, USA). Because this assay was used to screen for the presence of amylase in cows saliva, the amylase values were left as Somogyi units and not converted to S.I. Units.

2.2.m

Screening for Kallikrein activity (or other enzymes capable of hydrolysing BAEE)

The method used for screening for possible Kallikrein activity in bovine saliva was based on the procedure of Trautschold and Werle (1965). This method is not specific but will include all enzymes which are capable of hydrolysing the substrate benzoyl-L-arginine ethyl ester (BAEE). The activity is determined by measuring the increase in absorbance at 253 nm.

Reagents

Trypsin EC No 3.4.4.4. from Hog pancreas, was used as the positive control as no Kallikrein was available. The trypsin was obtained from Sigma Chemicals, product number T-0134.

Working trypsin solution: 1 mg/ml in the buffer

Triethanolamine buffer 0.067 mol/l ; 1.24 g triethanolamine HCl was dissolved in about 70 ml of distilled water, the pH was then adjusted to 8.0 with 0.2N NaOH, and then diluted to 100 ml with distilled water.

Substrate; benzoyl L-arginine ethyl ester (BAEE) 3 m mol/l in buffer.

Method

Two ml of buffer plus 0.5 ml of substrate were mixed in a 1 cm light path quartz spectrophotometer cuvette. The temperature of the buffered substrate was brought to 25°C and 0.5 ml of test saliva was then added and mixed with the substrate by inversion. The reaction rate at 25°C was measured over a 5 min period at 253 nm on the Gilford 2100 spectrophotometer. Working trypsin solution was used as the positive control, and both

test and control were read against a blank (0.5 ml of buffer instead of saliva).

2.2.n

Screening for esterase activity in salivary proteins separated by polyacrylamide gel electrophoresis

Reagents

Phosphate buffer: 0.1 mol/l phosphate buffer pH 7.4

Substrate coupler solution: prepared fresh prior to use. 20 mg of either α- or β-naphthyl acetate were dissolved on 4 ml of N,N-dimethyl formamide; this was then added to 36 ml of 0.05 mol/l phosphate buffer (pH 7.4) containing 20 mg of Diazo Blue B (C.I. No.37235), synonyms Fast Blue B, O-Dianisdine,(tetrazotized).

Stop bath: 2% acetic acid solution

Electrophoresis: the standard PAGE procedure was used

Method (Adapted from the method of Rosenberg et al. (1975)

The salivas were applied to the polyacrylamide gels as for the standard system. After completion of the electrophoretic run the gels were removed from their tubes and incubated for 15 min at 37°C in the 0.1 mol/l phosphate buffer. The gels were then placed in the substrate coupler solution and incubated for 1 h at 37°C. At the end of the incubation the reaction was stopped by replacing the substrate coupler solution with the 2% acetic acid solution. Gels were then scanned at 480 nm for α-naphthyl acetate and 590 nm for β-naphthyl acetate. A negative control was used in this work, this consisted of 4 ml of N,N-dimethyl formamide added to the Diazo Blue solution (i.e. without substrate), thus non-specific staining could be detected.

2.2.o

Acrylamide agarose gels used for the study of mucoproteins

The acrylamide/agarose gel procedure of McDevitt and Muir (1971) was the only electrophoretic procedure which was capable of allowing the large molecular weight mucoprotein to enter the gel matrix. The reagents and the preparation of the acrylamide/agarose gels were as described by these authors. The gels were polymerized and the electrophoresis run in the flat gel polyacrylamide electrophoresis apparatus described by Reid & Bieleski (1968).

2.2.p

Mucoprotein purification

The mucoprotein purification procedure used in this work was that of Lyttleton (1964). Saliva collected from the mouth by means of the copper bit was used for this purification.

Reagents

The reagents used for this procedure were as described by Lyttleton (1964).

Method

After collecting the saliva it was centrifuged for 60 min at 16 000 g-min. The supernatant was removed and diluted with one tenth of its volume with phosphate buffer I = 1.0, pH 6.0. This was then heated to 70°C for 30 min and the denatured protein removed by centrifugation at 16 000g-min for 30 min. The supernatant was removed and an equal volume of ice cold 1% cetyl trimethyl ammonium bromide (aqueous solution) was added and the mixture was stirred vigorously for a few minutes. A bulky fibrous precipitate (clot) formed which was wound onto the glass stirring rod and withdrawn. The clot was then washed twice at 0°C in a solution containing 80% ethanol and 20% 0.1 mol/l sodium acetate (v/v), and once with 80% ethanol, each washing lasting one hour. The clot was then dialysed against 0.1 mol/l sodium acetate, followed by ultrafiltration. The mucoprotein was then frozen until required for electrophoretic studies.

2.2.q

Rapid staining of the salivary proteins with Coomassie Brilliant Blue G 250

Where the position of the protein bands separated by PAGE were required to be detected quickly for qualitative work, Coomassie Brilliant Blue G 250 was used in perchloric acid (Chapter 7). The method used was that of Reisner et al. (1975).

2.3
IMMUNOCHEMICAL METHODS

2.3.a
Immunoelectrophoresis

The immunoelectrophoresis procedure used throughout this work was based on the procedure of Arquembourg et al. (1970).

Reagents

Stock barbitone buffer:

Diethylbarbituric acid	5.6 g
Sodium diethyl barbiturate	20.0
Sodium chloride	4.0

This was made to a volume of 1 l with distilled water

Working barbitone buffer:
Stock buffer was diluted 1 in 4 with distilled water

Agarose (as described in Chapter 2.2.o)

Agarose Gel:
The agarose gel was prepared by making a solution of 1% agarose in the working barbitone buffer, this was dissolved by heating in a boiling water bath for 30 min; 4 ml were required for each microscope slide (76 x 25 mm).

Method

The prepared agarose solution was poured over the individual microscope slides, 4 ml per slide. This was allowed to cool and the immunoelectrophoresis pattern was cut with a 'Shandon Immunoelectrophoresis Pattern Cutter'. The gel from the antigen wells was removed and 2 µl of antigen was placed in each well. Up to six immunoelectrophoresis slides could be accommodated in the Shandon Model U77 electrophoresis apparatus, these were placed face down on the Whatman 3MM chromatography paper wicks which dipped into the working electrophoresis buffer. The immunoelectrophoresis were run at constant voltage (150V) for 2 h. The distance of migration was indicated by the migration of a spot of bromophenol blue which was applied on the slide adjacent to the sample wells prior to commencing the electrophoresis.

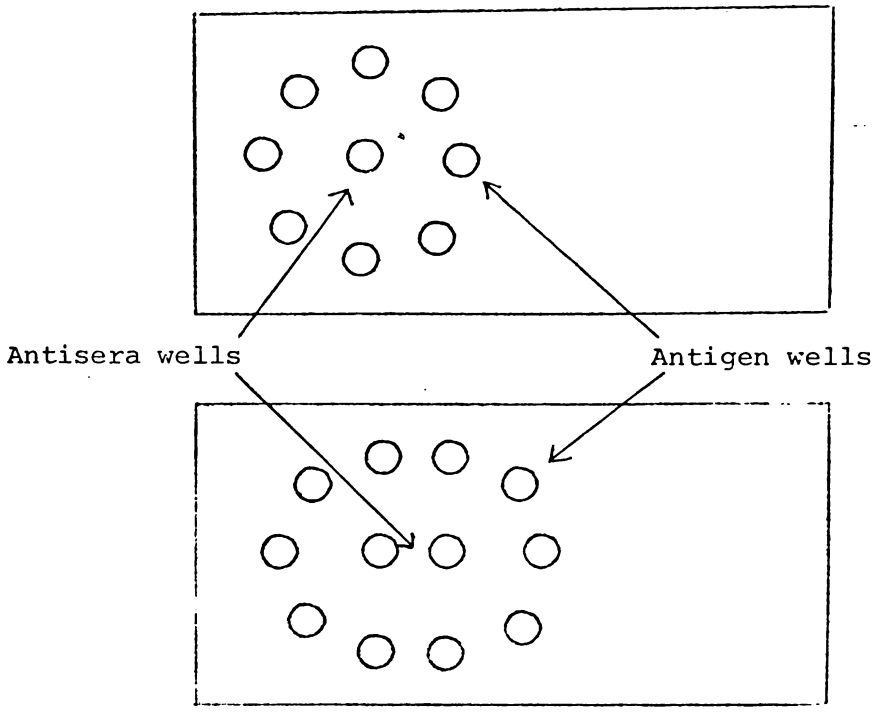


PLATE 2.3.1 The templates (single and double antiserum well designs) used for double immunodiffusion

On completion of the electrophoretic run the centre strip of agarose was removed from the antigen trough and this was lined with a layer of agarose prior to adding 100 µl of antisera. The slides were then placed in a moist chamber at room temperature, and the precipitin arcs were allowed to develop for 24 h before reading the results. The slides were then wrapped in Whatman filter paper (9 cm diameter) and immersed in physiological saline at 4°C. The slides were washed in several changes of physiological saline over a 48 h period. The slides were then dried in a hot air oven and the filter paper removed prior to staining in 0.2% Coomassie Brillian Blue R 250 (which was dissolved in methanol/acetic acid/distilled water, 45/10/45) for 5 min. The slides were destained in the methanol/acetic acid/distilled water solvent.

All photographs of the immunoelectrophoresis slides were taken after staining the precipitin arcs.

2.3.b

Double immunodiffusion

The double immunodiffusion technique was used in this work to check for cross reactivity between different antigens.

Reagents and Materials

The agarose and the buffer used were the same as for the immuno-electrophoresis. Glass slides measuring 76 x 38 mm were used as the support plates.

Method

The agarose solution which had been prepared as for the immuno-electrophoresis was dispensed 6 ml per slide. When set the well pattern was cut with a number 1 cork borer (external diameter 5 mm), using preformed templates of the dimensions shown in Plate 2.3.1. The antibody well was 8 mm from each of the antigen wells, while each of the antigen wells were 5 mm apart. To each antigen well, 10 µl of sample was added, and likewise 10 µl of antisera was added to the central antibody well. The slides were then placed in a moist chamber and left at room temperature until the precipitin arcs had formed. Routinely 48 h was allowed for complete diffusion. The diffusion plates were then washed and stained according to the procedure used for the immunoelectrophoresis.

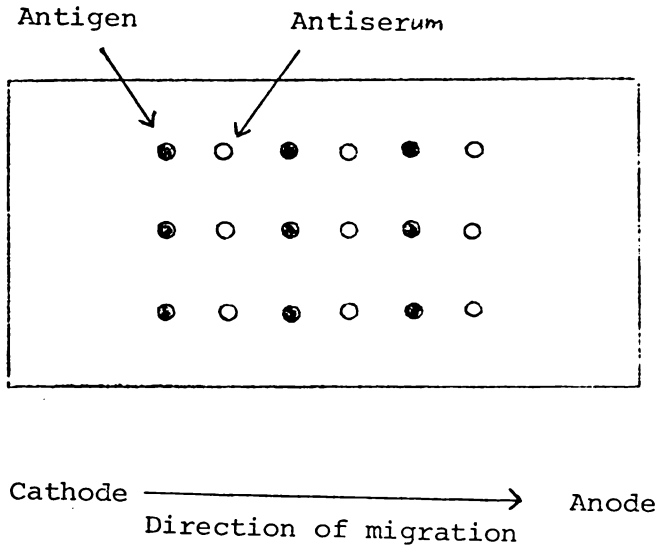


PLATE 2.3.2 The template used for crossed-over electrophoresis

2.3.c

Crossed-over immunoelectrophoresis

Crossed-over immunoelectrophoresis is a simple, rapid, highly sensitive technique, which in this work was used as a screening procedure to detect the presence of specific antigens. Although more sensitive than the double diffusion technique, this technique cannot give the same information with respect to multiple precipitin arcs present in a sample. Crossed-over electrophoresis is limited to a positive or negative result only. The principle of the technique was described by Culliford (1964); the antigen is placed in the cathodal end well and its corresponding antibody in the well opposite which is nearer the anode. On applying an electric current the antigen and antibody migrate towards opposite electrodes and a precipitin arc is formed at the junction

Reagents and Materials

These were the same as used for the double diffusion technique, and the electrophoretic system was the same as that used for the immunoelectrophoresis. After pouring the slides, the pattern(Plate 2.3.2) was cut using a 2 mm diameter gel cutter which was used in conjunction with the preformed template. Each system consisted of 2 mm wells, which were 5 mm apart. Nine pairs of wells were formed per slide.

Method

The antigen to be tested was applied to the cathodal well and the antibody against which it was to be tested was placed in the opposite anodal well, 4 µl of each were used per well. After the samples and antisera had been applied to the plates, these were placed in the electrophoresis tank at 4°C and run for 60 min at constant voltage (150V). After completion of the electrophoretic run the slides were washed and stained as described for the immunoelectrophoresis.

2.3.d

Quantitative electroimmunodiffusion

This method is based upon the electrophoretic migration of antigens in an antibody-containing gel, thus individual precipitin rockets are formed for each different antigen/antibody reaction present. The area enclosed by the precipitin rocket is proportional to the antigen/antibody ratio. This technique which was first described by Laurell (1966), can be used as

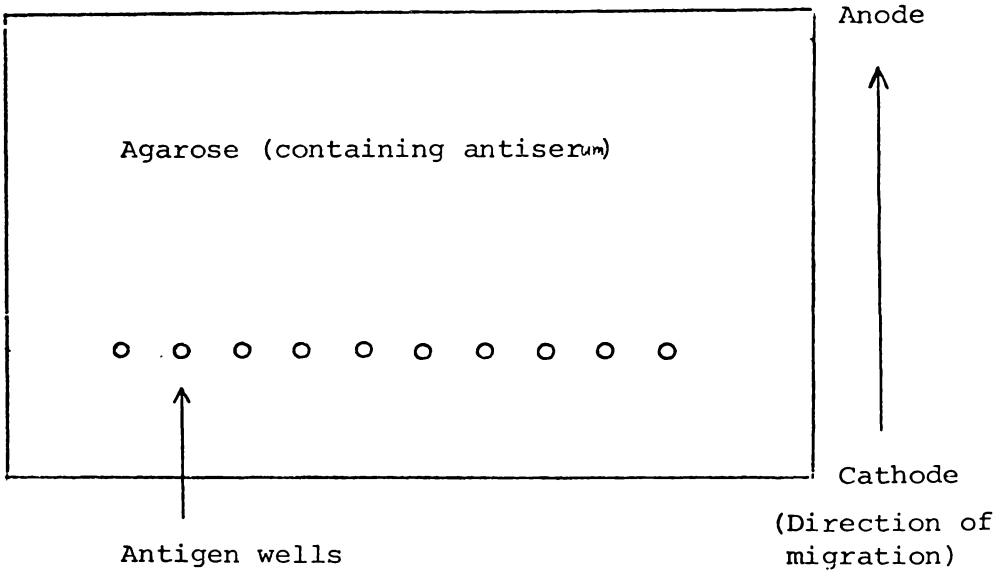


PLATE 2.3.3 The template used for quantitative electro-immunodiffusion

an accurate measure of the concentration of a protein in a solution. By measuring the heights of the precipitin rockets for both the standards and the test samples, the protein concentration of the test samples can be determined.

Reagents and Materials

Agarose and buffer; as for the immunoelectrophoresis

Antiserum; bovine serum albumin antiserum, prepared in the rabbit was obtained from Miles Laboratories

Glass plates; plates measuring 100 x 60 mm were used

Standard proteins; a solution of stock bovine serum albumin was prepared and the protein concentration determined by the Lowry Protein Procedure. Dilutions of the stock standard were made ranging from 2.5 - 30 µg/ml, (depending on the antiserum concentration used in the particular electroimmunodiffusion gel).

Method

The electroimmunodiffusion method used in this work was adapted from that of Laurell (1966,1972). The agarose solution was prepared as described for the immunoelectrophoresis, and then cooled to 50°C. When cool the appropriate amount of antiserum was added to the agarose. For albumin determinations in saliva, the final antiserum concentration in the agarose was between 0.05 and 0.1%, depending upon the concentration of the albumin in the saliva, and on the antibody titre of the particular batch of antiserum being used. After the agarose and antiserum had been mixed, 8 ml was delivered to each glass plate (prewarmed to 50 - 60°C). When set the pattern shown in Plate 2.3.3. was cut, each antigen well being 2 mm in diameter, 7 mm from the next well, and 20 mm for the bottom edge (Cathode end) of the plate.

The samples were then added to the wells (3 µl per well), three wells were used for running the standard proteins and the other seven wells were used for the test samples. The electroimmunodiffusion plates were then run at constant voltage (150V) overnight at 4°C. At the end of the electrophoretic run the plates were wrapped in moistened filter paper and

dried for 4 - 6 h in a hot air oven at between 50 - 60°C. Staining and destaining with Coomassie was the same as that described for immuno-electrophoresis.

After staining, the height of the precipitin rockets were measured. Measurements were made from the centre of the antigen well to the tip of the rocket. A standard curve was plotted from the three standard proteins and the values for rocket heights for the test proteins were read off this graph. For accurate repeatable results, rocket heights between 10 - 30 mm in height were necessary. Test samples with rockets too high to read were repeated using a higher concentration of antiserum and higher concentrations of standard proteins. Likewise test samples with rocket heights too low were repeated using lower concentrations of antiserum and standard proteins.

2.3.e

Crossed (two-dimensional) electroimmunodiffusion

The crossed electroimmunodiffusion technique combines the separating power of polyacrylamide gel electrophoresis with the specificity of the electroimmunodiffusion technique. The method consists of a high resolution electrophoretic separation in the first dimension, followed by electro-immunodiffusion in the second dimension. The individual precipitin rockets formed can be equated with the protein bands seen on the electrophoretic run used in the first dimension. The crossed electroimmunodiffusion technique was used in this study for two purposes:

i) To indicate how many serum proteins cross react with the proteins in the mouth saliva

ii) To indicate the number of individual precipitin rockets seen in standard saliva when run against whole saliva antiserum. The purpose of looking at the cross reactivity between the salivary proteins using whole saliva antiserum was to gain some information as to the number of different proteins within each protein band separated by PAGE.

Reagents and Materials

The first dimension electrophoretic run was the standard PAGE technique which was described in 2.2.b.

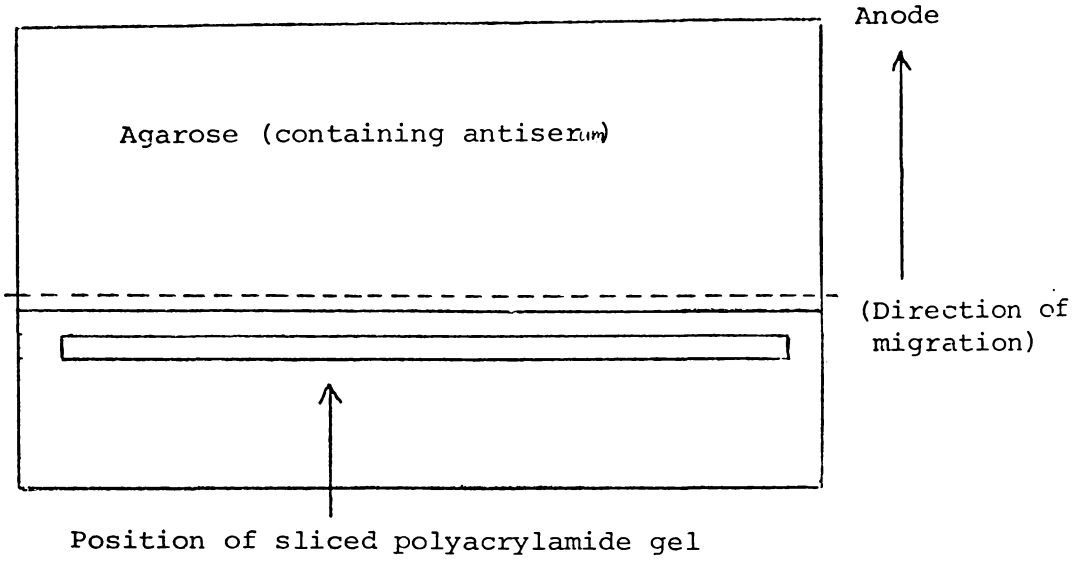


PLATE 2.3.4 The template used for crossed electroimmuno-diffusion

The second dimension was based on the electroimmunodiffusion technique described in 2.3.d.

Slicing the polyacrylamide gel. A glass gel slicer was made in this laboratory which consisted of a glass tube 92 mm long, with an internal diameter of 6.5 mm. A wire grid was cemented to the end of this tube which longitudinally split the polyacrylamide gel into four equal segments when the gel was forced through the tube by means of a close fitting plastic plunger.

Method

The saliva being studied was run on PAGE and while running, an agarose plate containing the appropriate amount of antisera for the specific run was poured. This differed from the agarose/antisera plate poured for electroimmunodiffusion in that a plastic or metal bar was placed across the glass plate 36 mm from the top edge (and parallel to it). and only 5 ml of the agarose/antisera solution was required. When cool the bar was removed from the plate and a 3 mm wide strip was trimmed from this edge of the agarose gel (Plate 2.3.4).

On completion of the first dimension electrophoresis, the polyacrylamide gel was sliced longitudinally. One segment of the gel was placed face downwards on the electroimmunodiffusion plate 3-5 mm from the cut agarose/antisera gel surface, (and parallel to it). Three ml of 1% agarose solution at 50^{C}C was then poured over the bottom portion of the glass plate and when set the crossed electroimmunodiffusion plate was run as for electroimmunodiffusion.

At the completion of the second dimension run, the plates were removed from the electrophoresis tank and the strip of polyacrylamide gel was carefully peeled off. The plate was then washed in physiological saline for 2-3 days, followed by drying and staining as for immunoelectrophoresis. Another segment of the gel was stained to locate the position of the bands seen in the first dimension PAGE run using the method described in Chapter 7.3. A method similar to the one used in these studies, except that a gel slab rather than the tube technique was used, has recently been published by Lindahl & Liljas (1975).

2.3.f

Production of whole saliva antisera

Reagents and Materials

Sagatal; Pentobarbitone sodium 60 mg/ml
May & Baker Ltd., Dagenham, England

Freunds Complete Adjuvant; Supplied by Commonwealth Serum Laboratories, Melbourne, Australia

Rabbits; Six ten week old New Zealand White rabbits were obtained from Massey University, Palmerston North, New Zealand

Preparation of the salivary proteins

Saliva collected from the cardia region of the oesophagus was centrifuged at 16 00 g-min for 30 min to remove most of the mucin. The supernatant was removed and divided into two samples (a) 20-40% ammonium sulphate, (b) 40% ammonium sulphate.

Preparation of the 20-40% ammonium sulphate sample

Ammonium sulphate, 20% (wt/vol) was added to the first sample and mixed until dissolved. This sample was kept at 4°C overnight followed by centrifugation at 10 000 g-min for 20 min. The supernatant was then removed and the ammonium sulphate concentration made up to 40% (wt/vol) mixed until dissolved and allowed to stand at 4°C overnight. This was then centrifuged at 10 000 g-min for 20 min and the supernatant was discarded. The proteins in the deposit were washed several times in 0.1 mol/l tris/glycine buffer pH 9.0 by ultrafiltration (10 000 molecular weight cut off filter) and finally washed in 0.001 mol/l ammonium bicarbonate and concentrated down to 3 mg/ml.

Preparation of the 40% ammonium sulphate sample

This second sample was prepared as for the first sample except that the 20% ammonium sulphate step was omitted.

Both samples were then dispensed in vials, labelled, and frozen until required for inoculation.

Preparation of the inoculum

To prepare the inoculum 250 µl of protein solution (containing 750 µg of protein) was added to 250 µl of Freund's complete adjuvant and these were emulsified. The emulsion was then used for inoculating the rabbits. Both samples (a) and (b) were prepared in the same way.

Inoculation of the rabbits

Six rabbits were used for the preparation of the antisera, three rabbits per sample. Each rabbit was sedated with an intramuscular injection 0.5 ml of Sagatal and an area of fur approximately 7 x 10 cm clipped on the back of each animal. The emulsified inoculum was then injected subcutaneously by a series of injections, each animal receiving 500 µl of inoculum. In each animal three inoculations were carried out at 14 day intervals. After a further seven days all animals were bled from the ear vein, and the serum collected from each animal was checked for the presence of antibodies against the salivary proteins by immunoelectrophoresis. Fourteen days later all animals were given a further inoculation. Eleven days after the fourth inoculation, four of the animals were sedated with 2 ml of sodium barbitone administered intravenously, and the blood was then collected from these animals by means of cardiac puncture. The final two animals were then bled from the ear vein 13 days after their fourth inoculation. These two animals were kept as a potential source of whole saliva antiserum for future work. The samples collected from the second bleeding were also tested for their antibody content by immunoelectrophoresis.

There was no obvious difference in the antibodies present from the animals inoculated with sample (a) compared with sample (b). Antibody titres were not performed on this sera because of the large number of antibodies present in the whole saliva antiserum. Antiserum collected from the second bleeding was used for the qualitative studies described in Chapters 7 and 8.

2.3.g

Source of antisera not prepared in this laboratory

Antiserum prepared against band 4 protein from bovine saliva

This monospecific antiserum was prepared and supplied by Dr W.T. Jones, Applied Biochemistry Division, The Department of Scientific and Industrial Research, Palmerston North, New Zealand.

Antiserum prepared against bovine serum proteins

Bovine serum protein antiserum was prepared in the rabbit. It was supplied by Miles Laboratories Ltd., P.O. Box 37, Stoke Poges, Slough,SL2 4LY, England, Production number 64-021.

Antiserum prepared against bovine immunoglobulin G

Bovine Ig G antiserum was prepared in the rabbit against both the heavy and light chains of Ig G. This antiserum was supplied by Miles Laboratories Ltd., Product number 64-113.

Antiserum prepared against bovine immunoglobulin A

This monospecific antiserum was prepared against the heavy chains of bovine Ig A. It was prepared and supplied by Dr D. Mackenzie, Department of Dairy Husbandry, Massey University, Palmerston North, New Zealand.

Antiserum prepared against human immunoglobulin A

Two sources of monospecific human Ig A antiserum were used, each being specific for the heavy chain.

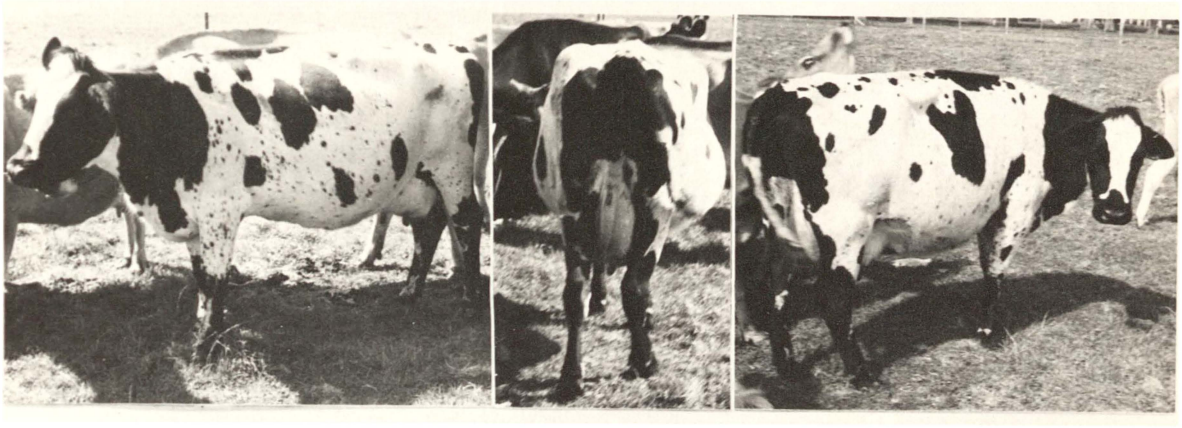
i) Miles Laboratories Ltd., Product number 65-065

ii) Behringwerke A.G. Marburg-Lahn, Germany. Product number R 7117.

Antiserum prepared against bovine serum albumin

This monospecific bovine serum albumin antiserum was prepared in the rabbit. It was supplied by Miles Laboratories Ltd., Product number 64-111.

VISUAL ASSESSMENT OF BLOAT



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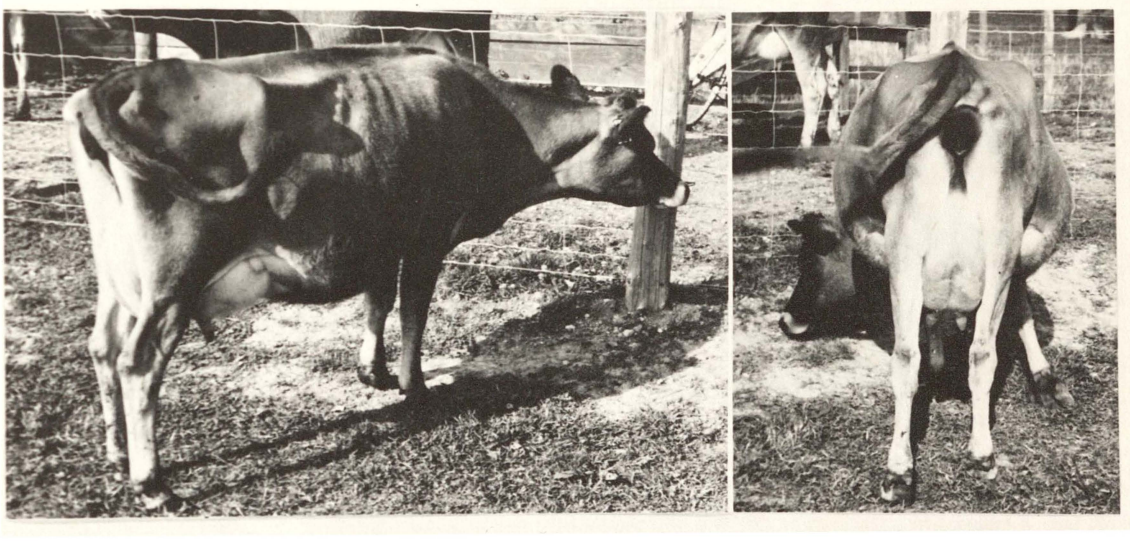

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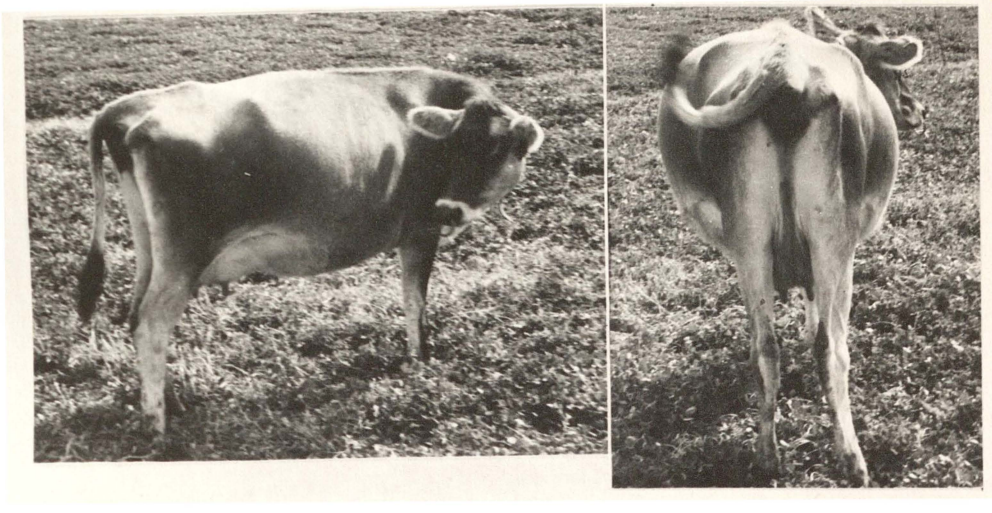
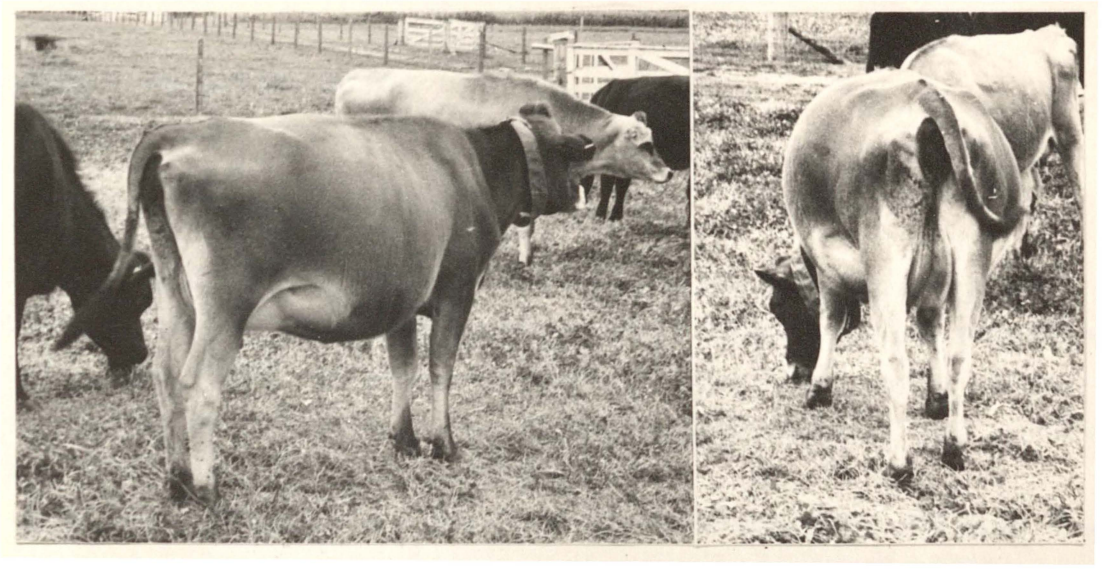


bloat score 2

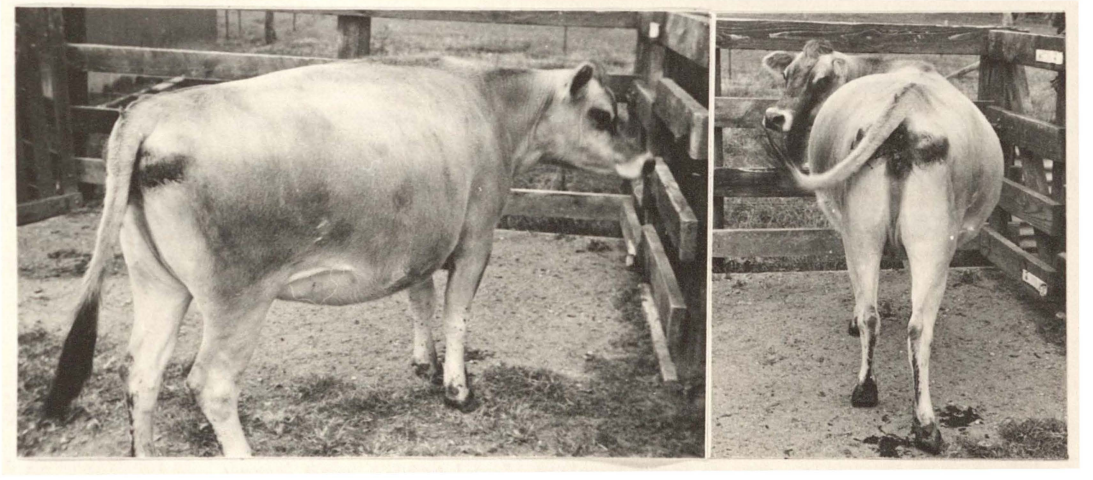
PLATE 2.4.1

(By kind permission of G.H. Derrick)

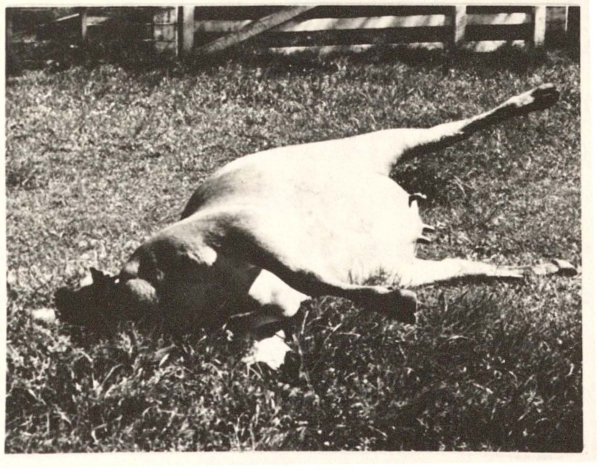
VISUAL ASSESSMENT OF BLOAT



bloat score 3



bloat score 4



bloat score 5

PLATE 2.4.2

2.4
BLOAT GRADING AND ANIMAL GROUPS

2.4.a
Field Grading of Bloating Cattle

Bloat in cattle was measured by visual gradings of the degree of distention of the cows on a 0 to 4 scale. This method of bloat scoring was described by Sim (1977) and Derrick (1976), and was based on the methods of Johns (1954) and Wright (1971). The method of bloat grading used in this work is illustrated in Plates 2.4.1 and 2.4.2.

Cows were break fed on a pure red clover stand with sufficient feed for about one hour's grazing, as judged by causing temporary satiety. The animals were pastured immediately after milking, at about 8 am and 4 pm. Gradings were made 30 min and one hour after feeding started. It took two observers about 15-20 min to grade the whole herd, walking through it and recording the results by radio. No bloat treatment was used, except when a cow reached grade 4 (it was then drenched with paraffin because it was at risk). After grading the cows wre moved to a grass-dominant pasture. Dry cows were held off pasture during milking and were graded with the lactating herd.

2.4.b
The origin of the individual groups of animals

All the cows from the identical twin herd at No.4 Dairy at Ruakura were graded for bloat according to the scheme outlined in 2.4.a. (Cockrem and McIntosh 1976; Derrick 1976; Sim 1976). These animals had previously been used for bloat trials including drenching trials of prophylactic agents.

For mating in 1972 the No.4 Dairy herd was divided into two on the basis of bloat grade. The half with the higher grades (called HS) were mated to an HS Jersey bull and the lower half (called LS) were mated to an LS Jersey bull. The bulls were selected by the NZ Dairy Board on a progeny test basis. From this herd five pairs of extreme HS cows and five pairs of extreme LS cows were selected for the preliminary saliva sampling in 1973, these cows constituted the Group 1 animals. After a series of saliva samplings two extreme pairs of HS and two extreme pairs of LS animals were sent to the DSIR in Palmerston North for detailed physiological work.

The first calf crop from this bloat breeding programme were born in 1973. Saliva samples were collected from these first progeny at 10 and 90 days of age. After grading for bloat at six months of age, 6 HS and 6 LS heifers were selected for regular saliva sampling, selection being on their bloat grade within their sire groups. These animals were designated the 1973 progeny group. The dams of this heifer group were used to make up a second cow group for saliva sampling in 1974. These adult animals were designated Group 2 animals but the Group contained some of the Group 1 animals.

In 1973 the HS and LS animals from the main herd were again mated with either an HS or LS Dairy Board bull, however Friesian bulls were used instead of Jerseys. The Friesian cross progeny born in 1974 were designated the 1974 progeny, and 6 HS and 6 LS females were selected to form the 1974 progeny group.

In 1974 the main herd of cows were mated, according to their bloat group, to either HS or LS Jersey bulls supplied by the Dairy Board, while the 1973 progeny females were mated with either an HS or LS 1973 progeny bull. The animals born in 1975 were therefore either F_1 or F_2. There was only 5 HS and 3 LS females in the 1975 (F_1) progeny owing to a sex imbalance for this year.

Summary of the individual groups of animals

Group 1 cows. The five pairs of extreme HS and the five pairs of extreme LS animals were selected from the identical twin herd at No. 4 Dairy.

Group 2 cows. These were the dams of the 1973 progeny saliva group, plus the Saliva Group 1 animals excluding the eight sent to the DSIR. There were 22 animals in this Saliva Group.

1973 Progeny Group. After bloat grading the 1973 progeny at six months of age, the 6 extreme HS and the 6 extreme LS female Jersey animals were selected.

1974 Progeny Group. The 6 extreme HS and the 6 extreme LS Friesian cross female animals were selected.

1975 Progeny Group. As there was a sex imbalance for this year there were only 5 HS and 3 LS female Jersey progeny available to form the 1975 (F_1) progeny group. There were also 2 HS and 5 LS F_2 animals born in 1975.

Mated 1972: Main Number 4 Dairy Herd mated to HS or LS Jersey Bulls (Dairy Board)

HS♀ x HS♂ → HS progeny (born 1973)

LS♀ x LS♂ → LS progeny (born 1973)

The Six highest HS females and Six lowest LS females made up the 1973 progeny group.

Mated 1973: Main Number 4 Dairy Herd mated to HS or LS Friesian Bulls (Dairy Board)

HS♀ x HS♂ → HS progeny (born 1974)

LS♀ x LS♂ → LS progeny (born 1974)

The Six highest HS females and Six lowest LS females made up the 1974 progeny group.

Mated 1974: Number 4 Dairy Herd mated to HS or LS Jersey Bulls (Dairy Board)

HS♀ x HS♂ → HS progeny (born 1975)

LS♀ x LS♂ → LS progeny (born 1975)

Only five HS and three LS female animals were available to make up the 1975 (F_1) progeny group.

Mated 1974: 1973 HS and LS females mated to 1973 HS and LS bulls respectively.

HS♀ x HS♂ → HS F_2 progeny (born 1975)

LS♀ x LS♂ → LS F_2 progeny (born 1975)

Two HS and four LS females made up the 1975 F_2 progeny group.

FIGURE 2.4.1 The origin of the 1973, 1974 and 1975 animals used for bloat grading and saliva studies

Figure 2.4.1 illustrates diagramatically the origin of the animals studied in this project.

The breeding programme for selection of animals for either HS or LS bloating characteristics is at present only at the first stage. During the first stage of the breeding programme the emphasis has been placed on building up the numbers of HS and LS animals in the two base herds. It is from these base herds that the future selection for HS and LS animals will be made.

2.5
STATISTICAL METHODS

Statistical analyses were made by Dr F.R.M. Cockrem using the Forest Research Institute statistical package (Cockrem & Shanks 1972). The analyses of data from the saliva samples included:

i) Analysis of variance to study HS and LS group differences and sampling date differences and interactions. Treatment differences were also studied by the analysis of variance. For example in the Pasture Interaction trials HS, LS group differences were studied as well as differences between periods, feeds and repeats and their interactions. The form of the analyses of variance and the tests used when there were repeated measurements on the same cow is given in McIntosh & Cockrem (1977).

ii) Simple regressions to study overall relationships and the consistency of relationships within HS, LS and sampling date groupings. This was done to test for relationships with bloat grade and between band proportions. Simple regressions and correlations were also used to study treatment effects on bloat' grade and band proportions in saliva.

iii) Stepwise multiple regression were used to study the complex inter-relationships indicated by the simple regression approach. However, the limitations of this technique and the use of protein band proportions, together with the small numbers of animals and a lack of knowledge of cause and effect, mean that it is used for defining hypotheses rather than testing them. In this respect Brownlee (1965) states, "In most circumstances therefore any indications produced by multiple regression analysis of observational data are merely a good hint to try for confirmation by a proper experiment."

The work in Part 1 of this thesis, where statistical analyses were an important part of the work, was designed to provide the fundamental information upon which to:

a) Design future animal trials with respect to studying bloat susceptibility, and factors which influence whether or not a particular cow will bloat.

b) Indicate which protein bands might be important with respect to bloat, and therefore warrant a detailed biochemical investigation.

c) Form future bloat hypotheses, which are possible to test experimentally.

Thus although the results from the statistical analyses of this work will not provide the full answer to explain the biological differences between animals of different bloat susceptibility, they can suggest the direction for future experimentation which might ultimately explain these differences.

PART ONE

EXPERIMENTAL WORK

The main objective of the first part of the experimental work in this thesis was to test the hypothesis that bovine salivary proteins, other than mucoprotein, might be related to, or involved in determining bloat susceptibility in cattle.

Saliva samples collected by the mouth bit were used throughout this investigation. Five groups of animals were studied; these consisted of two groups of mature cows (Group 1 and Group 2) classified into HS and LS groups on the basis of field gradings, and three groups of progeny from the bloat breeding programme (1973, 1974 and 1975 animals) also classified into HS and LS groups.

Part One of this thesis reports the results of the parameters studied in saliva which were; saliva flow, total protein, and the eleven protein bands separated by polyacrylamide gel electrophoresis. These parameters were examined in relation to the cows bloat grade at the time of sampling, or the bloat susceptibility of the animal (average bloat grade) and individual cow variance in bloat grade. Furthermore the inter-relationships between these parameters were examined as were factors which could influence the level of the salivary constituents.

A section reporting the accuracy and source of errors involved in electrophoresis is also presented in Part One of the experimental work.

CHAPTER 3

AN INVESTIGATION INTO THE STATISTICAL RELATIONSHIPS OF THE SALIVARY PROTEINS WITH BLOAT SUSCEPTIBILITY IN CATTLE

INTRODUCTION

In Chapter 1 the evidence was reviewed which suggested that the animal differences in bloat susceptibility might be inherited characteristics, and furthermore these animal differences might reside in the saliva. However all investigations into the relationships of salivary proteins and saliva flow with bloat susceptibility, ultimately depend on being able to grade cows for the degree of bloat (and hence determine their susceptibility), by a repeatable field technique. The repeatability of the grading system and the variation in the degree of bloat in cows grazing red clover is reported in 3.1.

Clarke et al. (1974) suggested that the band 4 protein might be present in a higher proportion in the saliva of HS cows compared with LS cows, but these authors had been unable to detect band 4 protein in the saliva collected from the mouth of cows. The results investigating whether or not band 4 protein was detectable in the mouth saliva are reported in 3.2. Initial studies on the Group 1 cows investigating the individual protein band inter-relationships and their relationship to saliva flow, total protein, bloat susceptibility and cow variance in bloat, are reported in 3.3, along with studies on the effect of stimulating salivation with acetic acid.

The protein band inter-relationships and their relationships with the parameters described above, were further investigated using the Group 2 cows, and these results are reported in 3.4 along with a study to determine the effect of saliva sampling directly off bloat-potent clover pasture. Because the results for the individual protein bands were reported as percentages of the total integrations (total protein entering the polyacrylamide gel), the relationship between individual band integrations and percentages were examined and these are reported in 3.5.

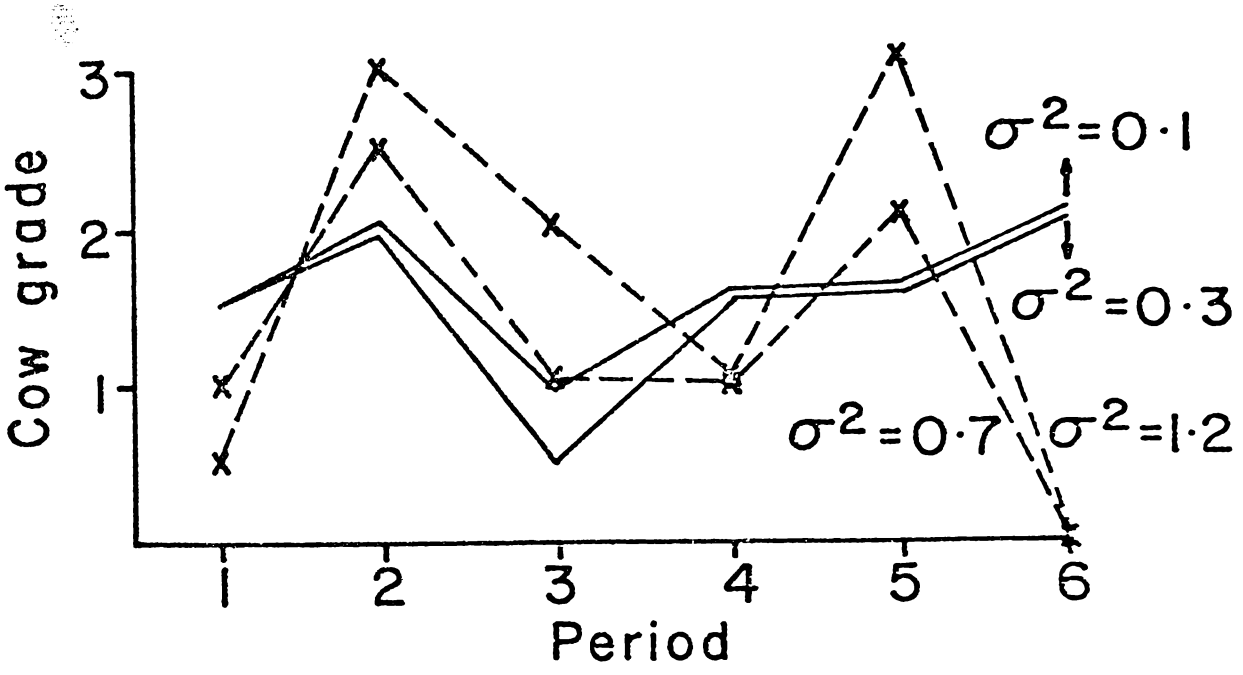


FIGURE 3.1.1 Individual cow grades for six periods in Autumn 1973 plotted for high and low variance twin pairs

High variance - - - ; low variance ——, σ^2 variance

TABLE 3.1.1 Means and "cow variances" by selection groups

Susceptibility	N	Mean grade	Mean "cow variance"
High	11	1.66	0.64
Medium	5	1.40	0.60
Low	7	0.93	0.71
SE	(5)	0.18	0.09

3.1

AN ANALYSIS OF VARIATION IN THE DEGREE OF BLOAT IN COWS GRAZING RED CLOVER

One of the greatest problems encountered by researchers studying bloat in cattle has been the large variation amongst gradings, both within and between cows. In contrast to methods used by previous workers studying bloat, the physiological genetics approach makes use of the variation. Provided there is sufficient animal variation, partly of genetic origin, then defining the source of variation becomes a key part of the experimental plan.

These trials on the analysis of variation in the degree of bloat in cows grazing red clover were designed to establish variation in the description of bloat in the field using the Ruakura Number 4 Dairy identical twin herd. That is, to determine what proportion of the variance of bloat was due to the year of bloat grading, the season (whether Spring or Autumn), the time (whether morning or afternoon), and the cows. The repeatability between individual operators in determining individual cow grades was also examined. The cows used in these trials were the same animals from which the Group 1 and Group 2 animals (Chapter 2.4.b) were selected.

Detailed results have already been published on these trials (Cockrem & McIntosh 1976) and a copy of this paper is included at the back of this thesis.

Summary of Results

The analyses of the results from these trials demonstrated that approximately 35% of the variance of bloat grades measured on twelve days over two years was the result of the pasture. There was as much or more variation in a single day or between consecutive days as there was between spring and autumn or between the two consecutive years.

Variation among cows was affected by some animals being more variable from day to day than others. This is illustrated in Figure 3.1.1 where the individual cow grades for six periods in autumn 1973 are plotted for high and low variance twin pairs. This cow variability was not related directly to bloat susceptibility, as HS cows had a similar average cow variability to LS cows (Table 3.1.1). There was some indication to suggest that this variability was associated with live weight, the larger twin pairs being more variable.

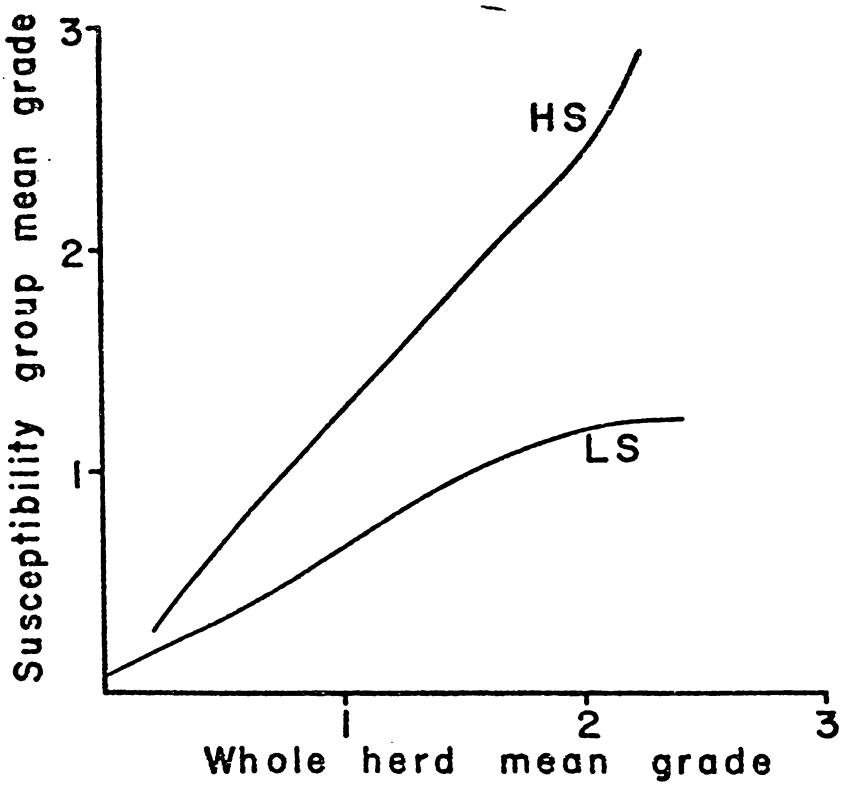


FIGURE 3.1.2 The relationship of HS and LS group means (y) to the whole herd means (x).

Another factor affecting variation among cows was the level of 'bloat potency' of the pasture. The full animal variation of susceptibility showed only when the pasture bloat potency was such that the herd mean grade was above 2. This is illustrated in Figure 3.1.2 where the susceptibility sub-group (HS/LS) mean bloat grade is plotted against the whole herd mean grade.

Differences between observers was not an important source of error. Part of the very small observer effect was due to effects of the actual time of grading. There was a difference of about 15 min between grading the first and the last cow, but as cows were graded twice at approximately 30 min intervals, the use of the larger of the two gradings appeared to successfully overcome this problem.

Lactation, age, calving date, and butterfat production had only minor relationships, if any, in affecting or being affected by bloat under the conditions of these trials.

3.2

A PRELIMINARY STUDY OF BAND 4 PROTEIN IN MOUTH SALIVA

Clarke et al. (1974) demonstrated that the band 4 protein found in saliva collected from the cardia region of the oesophagus, was higher in an HS animal compared with the LS animals tested. A description of the numbering system for the protein bands separated by electrophoresis (PAGE) was given in Chapter 2.2.b. Jones (pers comm) had been unable to demonstrate the presence of band 4 protein in mouth saliva collected by means of the perforated copper bit, (Mangan 1959).

The mouth bit procedure for collecting saliva samples from cows which was described in Chapter 2.1.a, was suited to collecting from an adequate number of animals daily. However no method was available for collecting saliva from the oesophagus without surgical interference of the animals. Therefore this experiment was designed to establish whether or not the band 4 protein was present in the mouth saliva.

Materials and Methods

Band 4 Antiserum (supplied by Dr W.T. Jones*)

This antiserum was produced in rabbits against band 4 protein, purified by Dr W.T. Jones.

Immunoelectrophoresis

Details of the immunoelectrophoresis technique were given in Chapter 2.3.

Cardia saliva samples

Samples were collected by Dr C.S.W. Reid* from the cardia region of the oesophagus. Two cows were sampled (264 and 320) both being fitted with a rumen fistula.

Mouth saliva samples

Mouth saliva samples were collected from all the Group 1 animals (Chapter 2.4.b) by means of the perforated copper bit (Chapter 2.1.a).

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Results

Saliva samples collected from the cardia region of the oesophagus from cows 320 and 264 were examined by immunoelectrophoresis using the band 4 antiserum. Four immunoelectrophoretic runs were performed on each animal and in each case cow 320 gave a positive result, one precipitin arc being present, however cow 264 was consistently negative.

All the twenty samples of mouth saliva collected from the Group 1 animals were positive when examined by immunoelectrophoresis using the band 4 antisera. The results were semi-quantitatively graded on a 0 - 4 scale, (0 = negative, 4 = strong positive). This semi-quantitative assessment of the concentration of band 4 did not show a significant difference at the 5% level between HS and LS cows ($P = 0.1$) and there was insufficient antiserum available to perform quantitative electroimmunodiffusion studies on these saliva samples.

When bovine serum proteins were tested against the band 4 antiserum by immunoelectrophoresis and double diffusion plates no precipitin arcs were detected.

Discussion

These results demonstrated that band 4 protein could be detected in the mouth saliva samples of the twenty Group 1 animals tested. There was also some indication that the levels of band 4 protein might be greater in HS as compared to LS animals.

Clarke et al. (1974) suggested that the structure of the band 4 protein may be different in HS and LS animals, but in both the HS and LS animals band 4 protein cross-reacted with the same antiserum. The common precipitin arcs seen on immunoelectrophoresis and double diffusion plates generally indicates some degree of chemical homology between the proteins being studied, and therefore these results do not support the above hypothesis of Clarke et al. (1974)

3.3

PRELIMINARY RESULTS FOR SALIVA FLOW, TOTAL SALIVARY PROTEIN, AND INDIVIDUAL PROTEIN BANDS FROM SALIVA SAMPLES COLLECTED FROM GROUP 1 COWS OF HIGH AND LOW SUSCEPTIBILITY TO BLOAT

The evidence reviewed in Chapter 1.3 suggested that differences in saliva constituents might be associated with differences in susceptibility to bloat. Details of the project investigating the possibility of breeding cattle with low susceptibility to bloat was discussed by Cockrem & McIntosh (1976) and the overall experimental plan was discussed by Cockrem (1975). An early requirement of the plan was to establish an initial technique of saliva sampling and analyses of the samples so that the major sources of variation could be identified. This knowledge could then be used for the determination of the research areas most likely to be effective.

An initial study of the variation in the degree of bloat in cattle, was summarised in Chapter 3.1, and in Chapter 3.2 it was established that band 4 protein was detectable in the saliva collected from the mouth. This band 4 protein, which Clarke et al. (1974) suggested might differ between HS and LS cattle, had previously only been detected in saliva collected from the oesophagus (Jones pers.comm.).

This section (Chapter 3.3) reports the results of the initial saliva samplings where the salivary proteins were separated and quantitated by analytical electrophoresis, and their relationships with bloat susceptibility investigated.

Methods

The five pairs of twins with the highest bloat grades (HS group) over the season of 1972-73 and five with the lowest grades (LS group) (Cockrem & McIntosh 1976) were sampled for saliva. They were of mixed age and breed. Samples were taken in May, June, and September 1973. One LS cow died before the September sampling.

The cows were grazed on a mixed grass-dominant pasture, which did not cause bloat over the period. All cows were pregnant for the first two samplings but only one cow in each group had not calved by September.

The cows were yarded about 7 a.m. off pasture and sampling started about 9 a.m. As it took about two hours to sample six or seven cows, sampling was done on three successive days in random groups.

TABLE 3.3.1 Analyses of variance and mean squares of band percentages, total protein, for the three sampling times (May, June and September), without acetic acid stimulation (arcsin transformed data)

Source	d.f.	E. (Mean square)+	Band 1	Band 2	Band 3	Band 4	Band 5
Bloat Group	1	$\sigma^2 + 3\sigma^2_C + 2\sigma^2_{TS} + 9.1\sigma^2_{TB} + 6\sigma^2_S + f(b)$	0.0066	0.0084	0.0116	0.3040*	0.0251
Sets within bloat	7	$\sigma^2 + 3\sigma^2_C + 2\sigma^2_{TS} + 6\sigma^2_S$	0.1173**	0.0180	0.0138	0.0335	0.0288**
Cows within sets	9	$\sigma^2 + 3\sigma^2_C$	0.0149	0.0080	0.0043	0.0165	0.0032
Time	2	$\sigma^2 + 2\sigma^2_{TS} + 18\sigma^2_T$	0.0464	0.0978**	0.0208	0.0337+	0.0005
Bloat x time	2	$\sigma^2 + 2\sigma^2_{TS} + 9.1\sigma^2_{TB}$	0.0466	0.0220	0.0005	0.0070	0.0062
Sets x time	14	$\sigma^2 + 2\sigma^2_{TS}$	0.0223	0.0150	0.0149	0.0119	0.0039
Residual	18	σ^2	0.0129	0.0099	0.0092	0.0208	0.0106
Variation between sets (%)			56**	7	5.6	22.5	53**

Band 6	Band 7	Band 8	Band 9	Band 10	Total integrations	Protein
0.0742	0.0143	0.0008	0.0336+	0.0677	36 145	6 073
0.0622**	0.1149**	0.0411**	0.0078	0.0307	59 881	685 859*
0.0093	0.0185	0.0066	0.0134	0.0200	20 625	77 067
0.0443*	0.0500+	0.9107	0.0235+	0.0147	11 776	149 993
0.0070	0.0040	0.0032	0.0033	0.0099	8 104	136 311
0.0115	0.0141	0.0057	0.0073	0.0132	18 530*	305 164
0.0086	0.0167	0.0187	0.0117	0.1050	8 502	179 973
45**	51**	46.8**	0	35.7	21.7	28*

+ Variance symbols, σ^2 error, σ^2_{TS} set x time, σ^2_{TB} time x bloat, σ^2_T time, σ^2_C cows, σ^2_S sets, f(b) fixed bloat effect.
P + <0.1, * <0.05, ** <0.01

Saliva was collected using the method described in Chapter 2.1.a by using a perforated bit fitted under the tongue and over the openings of the mandibular gland ducts. Saliva was collected in a flask in ice by use of a vacuum pump. In June the time taken for each collection was noted. Collections took 5 to 10 minutes. September collections were standardised to 5 minutes. Saliva flow rates are expressed as ml/min.

In June an additional collection was made to test the effect of increasing saliva flow on the protein constituents. The flow was stimulated by placing 5 ml of acetic acid (diluted to pH 2.0) on the tongue (Kay 1966) immediately after the normal collection.

Sample preparation and electrophoresis

The preparation of the saliva samples and the analytical separation and quantitation of the salivary proteins by electrophoresis (PAGE) was described in Chapter 2.2.a.

Total protein in the saliva was measured using the technique of Lowry et al. (1951) described in Chapter 2.1.h. Bloat grade and individual cow variances were those for the 1972-73 season, (Cockrem & McIntosh 1976; Chapter 3.1).

Analyses of variance

Five HS and four LS twin sets had complete electrophoretic results for all three sampling times. An analysis similar to those for split plot designs was used (Table 3.3.1). The relevant tests, with bloat susceptibility group as a fixed effect, are obtainable from the expected mean squares. The effects of stimulation were analysed by the same form with stimulation replacing time, and the degrees of freedom being adjusted accordingly. An arcsine transformation was used so that variances of the bands should be comparable.

Regression analyses of all cows alive (n = 19) were used to check whether differences between HS and LS groups were reflected in relationships within the groups.

Results

The standard samples represented repeated runs of the same saliva and as such their variation covers all that due to the gel and scanner variation and that due to identification of the boundaries of the peak areas.

TABLE 3.3.2 Means for the salivary protein band percentages, total protein, total integration, saliva flow, and bloat grade for the Group 1 animals. (1) Time: Means of the above parameters for all the Group 1 animals showing the different times of the year saliva was collected (May, June and September). (2) Susceptibility: Means of the above parameters for the HS and the LS groups for the three sampling times. (3) Acetic acid stimulated: Means of the above parameters for the acetic acid stimulated saliva samples collected in June.

	(1) TIME			(2) SUSCEPTIBILITY		(3) ACETIC ACID	
	May	June	September	HS	LS	STIMULATED	P#<
Band 1	13.4	15.2	16.6	14.1	15.5	10.8	0.05
Band 2	5.2	8.7	7.2	7.2	6.6	7.6	n.s.
Band 3	13.8	14.1	15.9	15.2	14.1	11.7	0.1
Band 4	3.4	3.6	5.0	5.2	2.7	4.4	n.s.
Band 5	5.4	5.9	5.7	5.2	6.1	6.3	n.s.
Band 6	4.4	3.2	3.0	4.1	2.7	3.2	n.s.
Band 7	19.7	18.5	15.9	17.7	18.5	28.7	0.001
Band 8	18.2	16.6	17.4	17.4	17.7	16.2	n.s.
Band 9	9.3	7.6	7.1	7.4	8.7	3.4	0.05
Band 10	4.1	3.6	3.0	3.0	4.1	0.3	0.001
Total Integrations	409	464	445	469	410	309	0.1
Protein µg/ml	1192	1232	1382	1281	1255	701	0.001
Bloat Grade				1.8	0.8		
Saliva Flow +		0.9	2.00	1.7	2.4	5.2	0.001

\+ ml/min June and September only; Difference P<0.1

\# From variance analysis of June results. Stimulation effect tested against sets x stimulation effect

Standard deviations (n = 9) for some key bands were: Band 1, 0.82, Band 4, 1.39, and Band 7, 1.47. The units are percent of total area under the curves.

The mean squares for the analyses of bloat susceptibility group and sampling time are shown in Table 3.3.1 (HS and LS). The time x bloat mean square tested against the sets x time mean square was negligible for all the variables so the bloat group mean square was tested against the sets within bloat mean square. Time was tested against sets x time, and the sets mean square was tested against the cows within sets mean square for which test times x sets variance is assumed negligible.

Mean values transformed back to percentages are shown in Table 3.3.2. There was a higher proportion of Band 4 for HS cows at each time (no interaction) and in September compared with other times (Tables 3.3.1 and 3.3.2). Bands 2 and 6, and possibly 7 and 9 varied with time, Band 6 having a higher proportion in May, and Band 2 a lower one.

The band percentages are not independent as they all depend on the total integrations. Thus an increase in one band must correspond with a decrease in at least one other band. Thus Band 9 may be in greater proportion for LS cows but the test is suspect because of the very low set variation. An alternative test against cows within sets gives $P > 20\%$

Table 3.3.1 also shows the proportion of variation resulting from the twin sets. The saliva flow result based on June and September only, was 85% of the variance due to sets, which suggests that this variable together with Bands 1, 5, 6, 7, and 8, and possibly protein concentration, have some genetic or congenital determination.

Overall the agreement between times shown by the small time differences and lack of interactions and the detection of a high between set variation in saliva flow, would suggest that the techniques are reasonably reliable.

Effects of stimulation

These are shown in the right hand column of Table 3.3.2 together with the result of the test of the stimulation effect. There were no HS/LS susceptibility group interactions with the stimulation effect.

However, some twin pairs were not markedly stimulated by the treatment, as there was a significant set x stimulation interaction for Bands 1 and 7.

TABLE 3.3.3 Standardised partial regression coefficients from the multiple regression analysis for bloat susceptibility and cow variance. Calculations were made for the overall data and within sub-classes of sampling time and HS and LS groups

x Variable	Band 4	Band 6	Band 7	Saliva flow	R^2	Res d.f.
Y variable log bloat grade						
Total regression	0.30*			-0.30*	0.24	54
Within groups		-0.36*		-0.66**	0.30	49
Y variable log cow variance						
Total regression		0.24+	-0.35**		0.24	54
Within groups		0.32*	-0.36*		0.32	49

+ P <0.1 * P <0.05 ** P <0.01

The main effect, apart from the increased saliva flow, was for Band 7 which increased from 18.5% to 28.7%. This was associated with a decrease in Bands 1, 9 and 10. The last two bands were present in samll amounts so their proportions could have been affected by the large increase in Band 7. Saliva flow was increased in both HS and LS animals when stimulated by acetic acid; the flow rate for the HS animals increased from 0.9 to 5.5 ml/min, while for the LS animals the pre and post stimulation flow rates were 0.8 and 4.8 ml/min respectively.

Protein concentration and the total integrations which reflect it, both showed a decrease in the acetic acid stimulated samples. As saliva flow increased by a factor of five, but protein decreased by only 0.6, it seems likely that the stimulation was not confined to the parotid gland which produces very little protein.

As stimulation could affect relationships of the band proportions or salivary flow with bloat grades and individual cow variances these were examined by simple regression analyses.

For the simple regressions on bloat grade, Band 5 and total integrations had negative relationships for all subclasses (HS stimulated, LS stimulated, HS normal, LS normal). Band 6 gave inconsistent results.

For simple regressions on cow variance, Band 2 had a negative relationships consistent for stimulated subclasses while Bands 4 and 6 showed positive relationships consistent for the normal subclasses only. Band 7 had a consistent borderline effect ($P<10\%$).

For the scanner used the total integrations were proportional to the amount of protein which entered the gel. The regressions of total integrations on total protein in the saliva were consistent for the stimulation effect. The overall correlation was 0.8 (36 d.f. $P<0.05$). However, HS cows had adjusted mean value 415 (SEM 20) and LS cows 359 (SEM 21) integrations at a mean total protein value of 968 µg/ml. This suggests that a lesser proportion of protein enters the gel for LS cows and that this was independent of the stimulation effect.

Relationships calculated for non-stimulated samples

Results from stepwise (adding variables) multiple regression analyses are presented in Table 3.3.3. Calculations were made for the overall data and within the subclasses of sampling time and HS and LS groups. Standardised partial regression coefficients are presented so as to indicate their relative importance.

TABLE 3.3.4 Product-moment correlations of band proportions which showed significant relationships with saliva flow rates

	d.f.	Band 4	Band 6	Band 7		
September HS	8	-0.53	-0.86	0.30	p=5%	r=0.63
September LS	7	-0.48	-0.61	0.42		
Total	55	-0.28	-0.57	0.33	p=5%	r=0.23
Within	50	-0.20	-0.56	0.34		

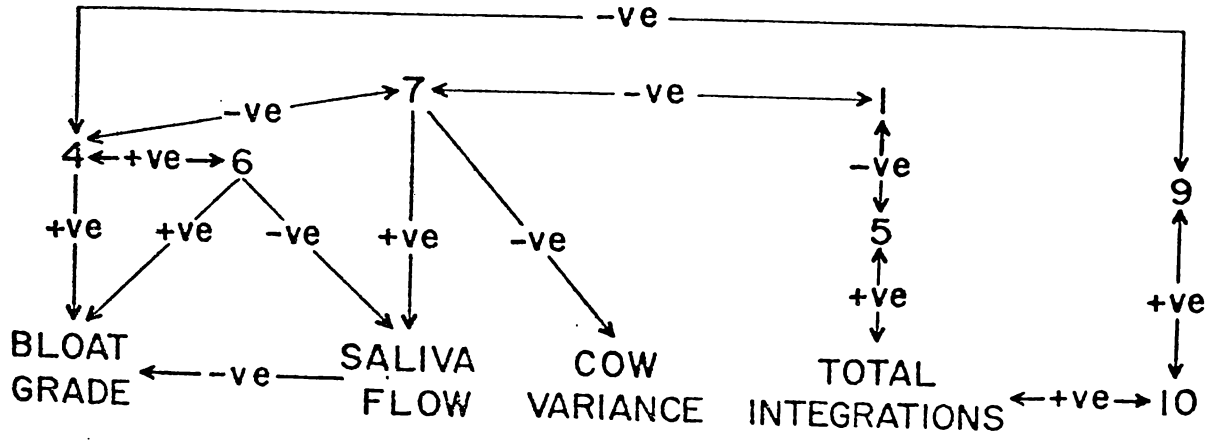


FIGURE 3.3.1 Band inter-relationships (↔), and hypothesis of effects on bloat (→)

Bands 4 and 6 and salivary flow rate showed relationships with bloat grade, however those for Band 4 were not consistent within groups as they were confined to the LS subclasses.

Band 7 showed consistent relationships with cow variance but not with bloat grade.

Those product moment correlations of saliva flow rates which showed a significant relationship with a band proportion are shown in Table 3.3.4 . Those variables related to bloat susceptibility (partial coefficients) are also related to saliva flow rates.

With these levels of inter-relationships between independent variables and no knowledge of cause and effect (except bloat grade as an effect), interpretation at this stage must be confined to the statement that the proportions of Bands 4, 6 and 7 together with salivary flow rate are related to a cow's susceptibility to bloat.

Inter-relationships of band proportions

Initial simple regressions indicated that the proportions of some bands were correlated between animals. Therefore, a number of multiple regressions were computed with the proportions of Bands 1, 4, 6 and 10 as dependent variables and the various relevant bands as well as total integrations as independent variables (Figure 3.3.1).

Figure 3.3.1 illustrates the relationships which probably exist between the proportions of the bands. Causal relationships reflect the order of the simple relationships with bloat grade. Thus Band 7 showed no direct relationship with grade but shows a partial relationship when the Band 4 - Band 7 relationship is allowed for. Band 4, however, shows both direct and partial relationships with grade. The diagram thus represents a general hypothesis of inter-relationships to be investigated.

Discussion

The trial was made to develop techniques for the separation and quantifying of salivary proteins from samples from a number of animals under field conditions. The lack of interaction of time with bloat susceptibility groups or twin sets would suggest that repeatable results were obtained. That is, the differences were consistent over the three sampling times. The detection of significant set variances for some of the bands and of relationships with bloat would confirm that the approach

used should be capable of detecting those band proportions which are of importance.

Saliva flow rates had not been expected to be measured meaningfully by the bit sampling method used. However the high proportion of variance between twin sets would indicate that a real variable was being measured. As the two twins of a set were not measured on the same day then the various external effects on saliva flow described by Denton (1957) may not be of immediate importance. Other factors affecting flow rate are the relative amounts of saliva from the various glands. Parotid saliva has a low protein content while that of the mandibular gland is relatively high (Kay 1966; Phillipson & Mangan 1959). The protein contents of the present samples and their repeatability would suggest that they are mainly and consistently from the mandibular and possibly sublingual glands. In addition, the calculated low absolute flows (about 2 - 15 l/24 h) compared with the reported volumes of total saliva (110 l/24 h) (Bailey 1961) would suggest that the parotid flow was not providing a major contribution to total flow (Kay 1966).

Results for the band proportions were consistent over the three samplings except for Band 2 and Band 9. These two bands also had no significant part of their variation between twin pairs which suggests that they are more subject to environmental effects. The remainder of the bands, except possibly 3, 4 and 10 appear to have some genetic control or marked effects of a common maternal environment.

There was also considerable evidence for a role of saliva in bloat. Band 4 was in greater proportions for HS animals and this was significant, although it was present in small amounts and had a high variance relative to its mean value. Some inaccuracies, apart from poor resolution, arose from a random drift on the scanner affecting some low readings and a non-linearity for high readings. That significant effects were obtained over this background noise suggests that this band is worth detailed investigation. Immunoelectrophoresis has shown that Band 4, which corresponds to the Band 4 found by Clarke et al. (1974) to be in low proportions in LS cows but present in higher proportions than found here in some HS cows. Clarke's samples were obtained by collection from the cardia region.

Stimulation with acetic acid led to a lower protein concentration which could have been a direct effect or could be a partial dilution from fast flowing parotid saliva. The main effect was to increase the

proportions of Band 7. In unstimulated cows this band was positively correlated with saliva flow but cause and effect is not clear at this stage. For example, Band 7 could be stimulated via the taste buds and in turn stimulate flow or vice versa. HS cows did not show any relationships of band proportions and bloat grade or salivary flow on stimulation. Therefore, the conditions of sampling could be important, as there may be plant constituents which have stimulatory effects similar to that of acetic acid.

The regression analyses showed some correlations which indicated that real effects could be detected, but their relative importance should be treated with some caution. Consistent direct effects on bloat grade were shown by Bands 4, 6 and 7 and also salivary flow, and these variables were themselves all interrelated. Although mean bloat grade and cow variance were not related they both appeared to be related to Bands 4 and 6. However, Band 7 was important for cow variance only. This band was also markedly affected by the acetic acid stimulation.

Summary

Data from five HS and five LS identical twins, saliva sampled three times are presented. Ten zones (bands) were obtained by electrophoretic separation on polyacrylamide gels; the relative proportions for the individual protein bands were obtained by scanning with a densitometer. Bands 2 and 6 showed differences between sampling times. Band 1, 5, 6, 7 and 8, together with protein concentration and saliva flow rate, had significant between-set variances indicative of congenital effects. Bands 4, 6 and 7 and saliva flow rate showed relationships with bloat susceptibility; band 4 was higher for the HS group. Data from a fourth saliva collection where salivation was stimulated with acetic acid (orally administered); saliva flow was markedly increased, protein concentration was decreased and the proportion of band 7 was increased.

3.4

STUDIES ON THE GROUP 2 ANIMALS

The origin of the Group 2 animals was described in Chapter 2.4.b. These animals were used in this preliminary work to check on the relationships of saliva flow, total protein, and protein bands with respect to bloat susceptibility differences reported in Chapter 3.3. However because the extreme HS and LS animals had been culled from the herd for detailed physiological studies at the DSIR, these Group 2 animals did not maximise the HS/LS differences seen for the previous animals.

Apart from the three standard saliva collections on all of the Group 2 animals, a fourth saliva sample was collected from a sub-group of cows which had been pastured on 'bloat potent' clover. The purpose of collecting these samples was to look for possible effects of bloat potent feed on the saliva flow and protein composition of the saliva.

Materials and Methods

The Group 2 animals (described in Chapter 2.4.b) were sampled by a similar system to that used for collecting saliva from the group one cows, with samples being collected in November 1973, May 1974, July 1974. These cows were confined to the yard after milking in November and removed from feed (silage) at 7 a.m. in May and July. Both the May and July polyacrylamide gels were scanned with both the Quickscan and Densicord densitometers. A comparison of these two densitometers is given in Appendix B.

Collecting saliva samples from animals directly after grazing on clover, compared with the standard procedure used for collecting saliva samples

One week after the standard trial in November, cows were sampled after approximately 90 min on a bloat-producing mixed clover pasture. The object of this trial was to preliminary examine the possibility that the standard sampling system was failing to detect differences which were only associated with feeding or specific feeds. The original technique, taking the cows off pasture at least two hours prior to saliva sampling, had been introduced to avoid contamination of saliva samples with feed, however in this trial only one sample was contaminated, and in this case a

TABLE 3.4.1 Mean squares from the analyses of variance for saliva flow and band percentages for the HS and LS Group 2 animals sampled off pasture or held in the yard

Source	d.f.	Saliva flow	Band 1	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8
HS/LS	1	32.20+	101.2	6.6	22.1	22.1+	30.6#	36.5	17.8
Treatment	1	0.58	38.5	49.9	86.5*	4.7	14.5#	37.1	46.9
Interaction	1	0.14	14.3	6.4	1.4	6.2	1.9	1.4	19.3
Cows within HS/LS	14	9.39	38.9	20.0	14.5	6.3	7.8	49.8	28.9
Residual	14	0.56	29.9	16.7	11.0	3.2	3.7	25.1	23.3

P + <0.1 # =0.07 * <0.05

TABLE 3.4.2 Means relevant to Table 3.4.1 showing the significant differences

	Saliva flow	Band 5	Band 6		Band 4	Band 6
HS	2.3	4.8	5.4	Held yard	4.3	3.7
LS	4.3	6.5	3.4	Off pasture	7.5	5.1
SEM	1.08	0.89	1.00		1.3	0.7

Based on Cows within and n=8
SEM based on residual and n=8
Note: these are the residuals used for the tests in the analyses of variance

second saliva sample was collected from this animal. Where possible, cows showing bloat were selected for sampling, but none were still bloating at the moment of sampling.

Experimental design

Eight HS and eight LS cows were sampled off the clover pasture and results compared with the same animals sampled a week previously on the standard system. (Note the confounding effect of time and pasture effects, this was justified by the lack of time effects in the bands of interest.)

The analysis of variance was of a split plot design (Brownlee 1965) as the animals were measured twice.

Thus:

Item		df	Test
HS versus LS sire groups	(A)	1	A/B
Standard versus Off Pasture	(C)	1	C/E
A x C interaction	(D)	1	D/E
Cows within HS/LS	(B)	14	
Residual	(E)	14	

The twin pairs were not allowed for in the analysis so the error term B might be slightly low, but this would only affect probabilities by 1 or 2%.

Results and Discussion

3.4.a

The comparison between saliva samples collected from animals immediately after grazing on 'bloat potent' clover with samples collected by the standard system

Analysis of bands of knowninterest, or with possible differences, are shown in Table 3.4.1, with the relevant means in Table 3.4.2. The treatment effects on the saliva flow and salivary proteins, of grazing animals on 'bloat potent'clover were similar for HS and LS animals, this was indicated by no interactions in the analysis of variance. There were possible susceptibility group differences for saliva flow, band 5 and band 6,

TABLE 3.4.3 Means for the protein band percentages, total protein, and saliva flow for both the Group 1 and Group 2 animals listed according to the month of sampling

	GROUP ONE			GROUP TWO				
	May	June	September	November	November* clover	May	July	SEM#
Saliva flow			2.0	3.6	3.2	4.2	3.7	0.50
Band 1	13.4	15.2	16.6	12.9	16.3	17.1	22.4	2.1++
Band 2	5.2	8.7	7.2	10.9	8.3	7.6	11.2	0.84++
Band 3	13.8	14.1	15.9	15.5	13.3	14.6	13.0	0.93
Band 4	3.4	3.6	5.0	4.1	7.5	3.9	5.4	0.72
Band 5	5.4	5.9	5.7	5.9	6.1	4.8	4.7	0.58
Band 6	4.4	3.2	3.0	3.9	5.1	3.0	3.5	0.41
Band 7	19.7	18.5	15.9	18.9	16.5	18.4	17.3	1.59
Band 8	18.2	16.6	17.4	17.3	16.0	19.7	12.9	1.03++
Band 9	9.3	7.6	7.1	6.8	7.3	7.3	7.4	0.48
Band 10	4.1	3.6	3.0	3.6	3.7	3.2	2.1	0.48
Protein	1192	1252	1382	1757	1474	1232	1264	114.1

* Clover group did not contain all Group 2 cows

\# SEM for Group 2 (not clover group) from within HS/LS and 3 samplings mean square

++ Significant difference ($P < .01$) between Group 2 sampling times shown by analysis of variance

Saliva flow ml/... Total prote... µg/ml

and there was a possible treatment effect for band 6 and a very likely effect for band 4. The treatment effects were that grazing bloating pasture increased the proportion of these two bands.

There was no correlation between band 4 values for treated and untreated animals, which suggested that the animals varied in their response to the treatment.

3.4.b

The comparison between saliva samples collected in November 1973, May 1974 and July 1974

All results for the saliva flow, total protein, and individual protein band percentages for both Group 1 and Group 2 animals are summarised in Table 3.4.3. In the Group 2 animals band 1 was up and band 8 down in July, (this contrasts with the positive correlation previously found between animals). Band 2 was low in May in these Group 2 animals, which was similar to that found for the group one animals.

For Group 2 animals HS versus LS differences were shown for band 1, 21.0 versus 14.0 respectively, and band 5,4.1 versus 6.2 respectively, these differences were consistent over the three standard samplings. Band 6 was higher in HS but this was mainly from the standard November sampling result. The band 1 differences were greatest in the May and July collections. These differences were not detected for the Group 1 cows.

3.4.c

Protein band inter-relationships, and their relationship with bloat

The Group 2 animals did not maximise the possible HS/LS differences because the extreme HS and LS cows had been selected out and sent to the DSIR for physiological studies. Therefore the relationships between bloat and salivary proteins were examined by multiple regression.

Results from the Group 1 cows, indicated that there were some strong relationships between band proportions. With small numbers such relationships can vary by chance and within the context of multiple regression techniques this could result in different bands showing as contributing to the variance of bloat grade and other dependent variables. These results from the Group 1 animals indicated that certain groups of bands were inter-related, presumably from certain chemical or biological

TABLE 3.4.4 Protein band inter-relationships for the Group 2 animals, and the Group 1 and Group 2 animals combined

(1) Correlations between band percentages for Group 2 cows within HS/LS and sampling times

Band	Band 3	Band 5	Band 7	Band 8	Band 6	Band 9
3	-	0.34	0.30	-	n.s.	n.s.
5	-	-	0.40	-	n.s.	n.s.
1	-0.50	-0.65	-0.70	+0.23	n.s.	-0.3
8	-0.33	-0.59	-0.15	-	n.s.	n.s.
4	n.s.	n.s.	n.s.	n.s.	0.36	-0.39
10	n.s.	n.s.	n.s.	n.s.	n.s.	0.45

61 d.f. P=0.05 for r = 0.25 n.s. P less than 10%

(2) Correlations between band percentages for Group 1 and Group 2 HS cows within September and November

3	-	0.58	0.20	-	n.s.	-0.50
5	-	-	0.63	-	n.s.	n.s.
1	-0.49	-0.81	-0.70	0.67	n.s.	n.s.
8	-0.63	-0.77	-0.56	-	n.s.	0.48
4	n.s.	n.s.	n.s.	n.s.	0.41	n.s.
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

20 d.f. P=0.05 r=0.42

(3) Correlations between band percentages for Group 1 and Groups 2 LS cows within September and November

3	-	0.21	-0.23	-	n.s.	n.s.
5	-	-	0.33	-	n.s.	n.s.
1	-0.29	-0.69	-0.74	0.66	n.s.	n.s.
8	-0.11	-0.74	-0.60	-	n.s.	-0.47
4	n.s.	n.s.	-0.68	n.s.	0.25	-0.61
10	n.s.	n.s.	n.s.	n.s.	n.s.	0.56

++

16 d.f. P=0.05 r=0.47

reasons. These possible relationships, shown for Group 1, were checked in the Group 2 animals and Groups 1 and 2 combined. These results are presented in Table 3.4.4 as simple correlations, and confirm the groupings found for the Group 1 animals.

Bands 3, 5, and 7 form a group with positive correlations centered on band 5 (i.e. band 3 and 7 were not closely related). However the correlations between bands 3, 5 and 7 were uncertain for the LS animals for Groups 1 and 2, and this might be due to a difference relationship with the band 3 protein. The band 3 protein in these LS animals did not show a negative correlation with bands 1 and 8.

Bands 1 and 8 as a group were negatively associated with bands 3, 5, and 7 as a group. This could be partially artificial as some combinations of proportions must be negatively related if the total (100%) is to remain constant. The limitations of the percent method are more fully discussed in Chapter 3.5, where the results of the saliva Group 2 animals are compared using protein band integrations and protein band percentages.

Results in Table 3.4.4 also suggest that bands 4 and 6 have a common factor and possibly bands 9 and 10. These bands are too small a proportion of the total for negative relationships to be generated by the use of percentages.

3.4.d
Multiple regression analyses with bloat grade (y) as the dependent variable

The bloat grade was in effect the average of a number of grades for 1972 and 1973 which was a measure of an animals bloat susceptibility and was therefore no longer a discrete variable with any special properties that such a variable might confer on a multiple regression. The results are given in Table 3.4.5 where only the standardised partial regression coefficients* are shown. (Group 1 results are repeated in this Table for comparative purposes.)

There was a consistent picture of a negative relationship with band 6 and a positive relationship with band 5. The remaining relationships were inconsistent although band 8 and/or band 1 might have some significance during the spring season.

* Standardised partial regression coefficients; these are regression coefficients divided by the standard deviations of the variable concerned and are therefore on a comparable scale.

TABLE 3.4.5 Multiple regression analysis with bloat grade (y) as the dependent variable and protein band percentages and saliva flow as the independent variables; showing only the significant standardised partial regression coefficients (partial F <2.8, and t <2.0)

Sample	Band 1	Band 8	Band 5	Band 7	Band 4	Band 6	Band 2	Saliva Flow	R^2	Res.d.f.
HS										
November	-	-	-	-	-	-	-	-	n.s.	11
May	-	-	-	-	-0.65	-	-	-	0.42	10
July	-	-0.51	-	-	-	-	-	-	0.26	10
LS										
November	-	+1.50	2.3	-	-	-	-0.75	-	0.95	5
May	-	-	0.54	-	-	-	0.68	-	0.72	6
July	-	-	-	-	-	-	0.54	-	0.30	7
Both Groups September/November 1973										
HS										
Total(overall)	-		-	-	-	-0.72	-	-0.72	0.39	19
Within Groups	-	0.96	0.90	-	-	-	-0.37	-	0.51	17
LS										
Total	0.77	-	0.87	-	-	-	-	-	0.40	15
Within	-	-	0.81	-0.64	-	-0.37	-	-	0.53	13
Group off clover pasture November 1973										
HS	-	-	-	-	-	-	-	-	n.s.	7
LS	-	-	-0.48	-	-	-1.33	-	-0.57	0.88	4
Group 1 cows (From Table 3.3.3)										
Total	-	-	-	-	0.3	-	-	-0.30	0.24	49
Within	-	-	-	-	-	-0.36	-	-0.66	0.30	44

Pooling the results of both groups of animals for the Spring of 1973 gave results which were generally in line with the groups considered separately at other times of the year as well. Sampling off the clover pasture indicated a negative relationship with band 5 but of low order relative to the band 6 effect.

The main additions from the Group 2 results were the implication that the band 5 group and bands 1 and 8 might be important in relation to bloat susceptibility in cattle.

The results from this sort of analysis are tentative, and provided they are used as a guide to further experimentation then they are of value, but they must not be regarded as confirmation of the involvement of the salivary proteins in bloat. Bands 5 and 6 seemed to give relationships which were opposite to those which might be expected from the group differences. The negative band 6 result might be accepted as real, but the band 5 and bands 1 and 8 may be subject to disturbances from band inter-relationships.

3.4.e

Multiple regression analyses with individual cow variance (y) as the dependent variable

Multiple regressions, similar to those for bloat grade, are shown in Table 3.4.6 for individual cow variances as the dependent variable. Cow variance is a measure of the extent of day to day differences in grade in individual cows. A high value means that a cow has shown high bloat on a few days but could still have a medium average grade. A low value implies any mean grade but a much steadier level.

The Group 1 animals gave a positive standardised partial regression coefficient for band 6 and a negative results for band 7 (results repeated in Table 3.4.6). These results were confirmed by the Group 2 cows. Those classes giving non-significant results in Group 2 had R^2 of the order of 25%, mainly from negative correlations with band 7.

The difference in sign for band 6 and grade, and band 6 and variance may arise from band 6 being higher under bloating conditions. Bloat grade and variances are uncorrelated, and there was no animal correlation for band 6 values between control and treatment (animals fed bloat potent pasture). The positive correlations with variance might reflect the highest values achieved by the particular band, whereas the

TABLE 3.4.6 Multiple regression analyses with individual cow variance (y) as the dependent variable and protein band percentages as the independent variables; showing only the significant standardised partial regression coefficients

Sample	Band 1	Band 3	Band 7	Band 4	Band 6	Band 2/9	R^2	Residual d.f.
HS								
November	-	-	-	-	0.61	0.45(9)	0.52	9
May	-	-	-	-	-	-	n.s.	11
July	-0.60	-	-	-	-	0.89(2)	0.56	9
LS								
November	-	-	-	-	-	-	n.s	8
May	-	-	-	-	-	-	n.s.	8
July	-	-	-0.54	-1.10	+0.71	-	0.80	5
Both Groups September/November 1973								
HS								
Total (Overall)	-	0.45	-0.41	-	-	-	0.45	19
Within	-	0.47	-0.38	-	-	-	0.49	18
LS								
Total	-	-	-	-0.45	+0.68	-	0.48	15
Within	-	-	-	-	+0.55	-	0.31	16
Group 1 Cows (from Table 3.3.3)								
Total	-	-	-0.35	-	+0.24	-	0.24	54
Within	-	-	-0.36	-	+0.32	-	0.32	49

negative ones might reflect some between cow effect. In this context the positive correlations reflect the animal x environment interaction.

Band 7 was very variable and these results might be interpreted as those cows which can achieve a high proportion or concentration of band 7 are prevented from bloating severely, compared with cows which are unable to produce such high levels. In this context one might expect a negative rather than a positive relationship between band 5 and bloat grade, in view of its positive relationship with band 7.

The conclusions drawn from the Group 1 and 2 animals can be summed up by saying that the salivary proteins relate to bloat in cows but how this relationship works can not be derived from this data.

3.4.f

Saliva flow and its relationship to salivary proteins

In Table 3.4.7 correlations of protein band proportions with salivary flow are shown for the same groupings as the multiple regressions. In general bands 6 and 4 were negatively related and bands 5 and 7 positively related. Bands 1 and 8 might also be negatively related but this could arise from band inter-relationships. Partial regression shows that band 7 is the key protein and the negative relationship with band 6 being the next most consistent. The acetic acid stimulation of saliva flow carried out on the Group 1 cows showed that band 7 was markedly increased as was saliva flow, this result being in line with the results from the unstimulated animals where band 7 was positively correlated with saliva flow.

The negative relationship of band 6 with saliva flow, coupled with the negative relationship of saliva flow and bloat grade, make the negative bloat grade to band 6 relationship difficult to explain. Pasturing on clover did not change this relationship. If bloat grade and band 6 were related then a positive relationship would have been expected, as illustrated below;

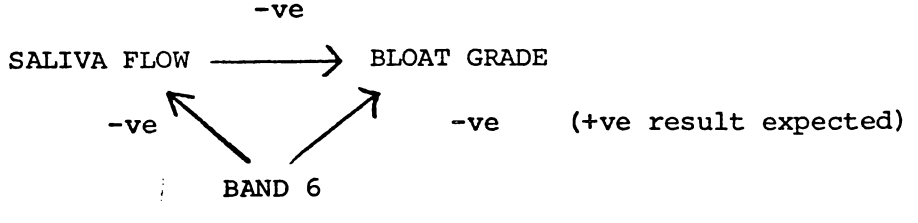


TABLE 3.4.7 Saliva flow and its relationship to salivary proteins; correlations of protein band percentages with saliva flow, for HS and LS groups, different months of sampling, and pasturing on clover for the Group 2 cows compared with correlations for the Group 1 cows (as reported in Table 3.3.4)

Sample	d.f.	Band 4	Band 6	Band 5	Band 7	Band 1	Band 8	Significant partials bands
HS								
November	10	-0.23	-0.64	0.83	0.56	-0.67	-0.68	5 and 9
May	10	-0.16	n.s.	0.57	0.90	-0.70	n.s.	7 and 10
July	10	n.s.	-0.44	0.40	0.81	-0.77	n.s.	7
LS								
November	7	-0.56	n.s.	0.22	0.53	-0.38	n.s.	4
May	7	n.s.	+0.30	n.s.	n.s.	n.s.	-0.54	8
July	7	n.s.	n.s.	0.56	-0.37	-0.20	-0.37	2 r=0.77
Total (Overall)	61	-0.23	-0.29	0.45	0.64	-0.51	-0.13	5 and 7
Within	56	-0.19	-0.18	0.40	0.63	-0.54	-0.23	7
Off Clover Pasture, November								
HS	6	n.s.	-0.71	0.84	0.58	-0.59	n.s.	
LS	6	n.s.	-0.49	0.40	0.37	-0.53	n.s.	
Saliva Group 1 as reported								
Total	55	-0.28	-0.57	-	0.33	-	-	
Within	50	-0.20	-0.56	-	0.34	-	-	

P = 0.05, 10 d.f. r = 0.58, 7 d.f. r = 0.67, 61 d.f. r = 0.25, 56 d.f. r = 0.27

Note 1. Band 2 also showed r = 0.59 for November HS
2. n.s. those correlations obviously not different from zero
3. Partial bands are those showing significance from multiple regression (See Table 3.4.5)

However these relationships are not absolute and they imply that other factors are involved. For example, the demonstration of more than one protein in band 6 (Chapter 7.1.f), would introduce an added complexity which might partly explain the above relationships.

Summary

The results from the Group 2 animals showed that band 4 was increased when animals were sampled directly off clover as compared with the routine saliva collection where animals were standing in the yard after milking. There also appeared to be HS/LS susceptibility group differences for bands 5 and 6 and saliva flow.

In the comparison of all the sampling times for these animals, band 1 was higher in HS compared with the LS animals, whereas band 5 was lower. In the saliva samples collected in November 1973 band 6 was higher in the HS than the LS animals.

Simple correlations between protein bands confirmed the results seen for the Group 1 animals. Bands 3, 5 and 7 were positively correlated while bands 1 and 8 showed a low positive correlation ($P<0.1$). Bands 4 and 6 have a common factor as do bands 9 and 10.

The results from the multiple regression of bloat susceptibility (dependent variable) on protein bands, showed band 6 to be negative related, and band 5 positively related to bloat susceptibility. These results also indicated that bands 5 and possibly bands 1 and 8 might have some importance to bloat susceptibility.

The results from the multiple regression for individual cow variance (dependent variable) on protein bands, were in line with the results from the Group 1 animals. Band 6 had a positive standardised partial regression coefficient while band 7 was negative.

Bands 4 and 6 were shown to be negatively related to saliva flow, while bands 5 and 7 were positively related. Band 7 appears to be the key protein in the relationship with saliva flow.

3.5

A STUDY OF THE PERCENTAGE METHOD COMPARED WITH BAND INTEGRATIONS AS A MEANS OF MEASURING THE SALIVARY PROTEIN BANDS SEPARATED BY ELECTROPHORESIS

One of the limitations in this study was the use of percentages rather than concentrations for the individual protein bands. All protein bands were expressed as a percentage of the total protein entering the polyacrylamide gel because it was not possible to measure the concentration of proteins in the bands as there was no method quantitating the mucoprotein present in each saliva sample. Results of Lyttleton (1960) suggested that perhaps 50% of the protein in mouth saliva might be mucoprotein, but the proportion (and concentration) of this protein might vary between samples and between animals.

In the original work, using the Densicord Densitometer all gels were scanned on a fixed gain setting. Thus all integration values within runs would be proportional to the amount of protein entering the gel, varying between bands depending on their dye binding affinities, and the range over which the dye binding was linear. Between-run variation would include any differences in staining intensities which could arise from differences in batches of stain, and differences due to the length of time used for staining and destaining. In this work all of these variables were standardized as much as practicable and checked between runs by the inclusion of a control saliva sample within each run.

Results and Discussion

The individual electrophoretic bands for the salivary proteins from the Group 1 and Group 2 cows were expressed as percentages of the total protein entering the polyacrylamide gel, and these results, given in Chapters 3.3 and 3.4, showed that bands 1 and 8 as a group were negatively associated with bands 3, 5, and 7. This could be partially artificial as some combinations of proportions must be negatively related if the total (100%) is to remain constant. In this section this aspect was checked by comparing the band proportions and the absolute integrations for the individual bands when scanned at a constant gain. However when integrations are used the resulting correlations have a common factor in the total protein, and therefore results would be dependent on changes in the total protein in the gel, and on the strength of the relationship of band integration with this.

TABLE 3.5.1 A comparison of the percentage method with the integration method, and the relationship of individual band integrations with total integrations

(a) Correlations between percentages and actual integrations at constant gain for protein bands 1, 5, 8 and 7, in the saliva of the Group 2 HS cows sampled in November. (n = 12 10 d.f.)

	Percentage Units				Integration Units		
Band	8	5	7	Band	8	5	7
1	0.78	-0.79	-0.69		0.39	-0.57	-0.15
8	-	-0.86	-0.49		-	+0.51	+0.61
5	-	-	+0.55		-	-	+0.94

(b) The correlations of Total integrations and individual protein band integrations with the total protein in saliva.

Total integrations	Band 1	Band 5	Band 7	Band 8	Band 6
+ 0.75	+0.07	+0.77	+0.73	+0.61	+0.25

The results in Table 3.5.1, assume that samples analysed in differen. electrophoretic runs are comparable. Further discussion on this method is given later (Chapter 4.5). Effects of differential dye binding could affect the linearity of the percentage results but not the integration units if relationships between bands are linear.

In Table 3.5.1 the same correlations based on percentage units are compared with those obtained with the original integration unit data. It can be seen that where there is a high correlation between a band's integrations and total protein then the correlation of that band with other bands are moved in a positive direction. Bands 5, 7, and 8 are highly correlated with total protein. Band 1 is uncorrelated with total protein, and Table 3.5.1 illustrates the correlations for band 1 are reduced independently of sign, suggesting that the band relationships with band 8 may be dependent on the variability and levels of protein in a particular sample series. If two bands had a constant absolute amount of protein then their proportions would vary only according to the total integrations and they would show a perfect correlation with each other. This situation would almost certainly be detected by observation but nevertheless its occurrence as a partial effect means that these relationships obtained from percentages are never more than a guide to experimentation.

Summary

When measured as integrations bands 5, 7 and 8 were shown to be highly correlated with total protein, and the correlation of these bands with other protein bands were moved in a positive direction compared to the correlations seen for the percentage method.

Band 1 however, showed no correlation with total protein and its correlation with other bands was reduced independent of sign.

CHAPTER 4

FURTHER INVESTIGATIONS INTO THE STATISTICAL RELATIONSHIPS OF THE SALIVARY PROTEINS WITH BLOAT SUSCEPTIBILITY IN CATTLE USING THE HS AND LS PROGENY FROM THE BLOAT BREEDING PROGRAMME

INTRODUCTION

In Chapter 3 the statistical relationships of the salivary proteins with bloat susceptibility were investigated in the mature cows; these animals formed the founder population from which the bloat breeding programme started. These cows had been classified as either HS or LS on the basis of their field gradings. In this Chapter the animals studied were the progeny resulting from the bloat susceptibility breeding programme, where two lines of animals (HS and LS) were being selected. The majority of the work in this Chapter deals with the animals born in 1973 (1973 progeny), but studies on the 1974 and 1975 progeny are also included.

The work reported in this Chapter on the progeny groups commenced with a more detailed examination of the bloat scoring procedure, and compared the mean bloat grade method with the other means of assessing bloat susceptibility from the field grading system (4.1). The effects of collecting saliva from animals directly after pasturing on bloat potent pasture were further examined by a series of Pasture Type Trials where a comparison was made of the effects on the saliva (quantity and composition), of collecting samples from animals directly off clover compared with grass (4.2) . The effect of different time intervals post-feeding on the saliva was also examined by two repeat 'Time of Sampling Trials'. These trials investigated this time effect on saliva flow and the protein components in saliva, (4.3). The effect of age (or year born) on the saliva composition and bloat grades was examined for the 1973, 1974 and 1975 progeny, as well as the effect of sampling all age groups at a constant season where ages differed; these results are reported in 4.4. Finally, the errors involved in assessing polyacrylamide gel electrophoresis on a quantitative basis were examined. A further acetic acid saliva stimulation trial on the 1973 progeny, similar to the one reported for the Group 1 animals was evaluated, using both the percent (or proportional) method for reporting the individual protein bands, and the 'corrected integration' method.

4.1

FURTHER STUDIES ON THE BLOAT GRADING SYSTEM, USING THE 1973, 1974 AND 1975 PROGENY

The purpose of the grading system was to detect animal differences within a period of bloat grading, and the group of animals being graded. The grade in the paddock was the criterion of difference or relationship for all variables. It was also the criterion of selection. Thus a complete understanding of the relationship of the bloat grades, with the probability of an animal dying of bloat was essential. Selection for LS animals, the ultimate objective of the breeding programme, relies on an accurate method of field grading. The three points considered here are:

a) Inter-relationship of various ways of expressing grades
b) Repeatability of bloat grades
c) Age and HS/LS group differences

Methods

The standard grading system was described in Chapter 2.4.a and examined in Chapter 3.1. The same system was used for the progeny groups described here. Animals were graded at 6 months of age (March or April) and one year of age (September or October) and at six monthly intervals thereafter. In March 1976 gradings were made in two successive weeks as a repeatability check. Gradings were usually continued until three days of 'reasonable' bloat had been obtained, and days (time) with no or very little bloat are ommitted from the mean values. Thus grading is a combination of compromises of management and season which contribute to the bloat 'potency' of the pasture.

In the second week of grading in Autumn 1976 and subsequently, the 1973 and 1974 animals were graded together. In September 1975, gradings were on the same strip of pasture but separated by an electric fence, however the 1973 and 1974 and HS and LS animals were run separately at night for mating. The dry cows were joined with the lactating cows several days before grading started.

TABLE 4.1.1 Average bloat grade measurements for the 1973 and 1974 progeny classified by the number of periods in which a peak of grade 4 was reached

Bloat grade measurement		Number of Grade 4 periods 0	1	2	3	4	R^2_{xy}
Average grade	1973	0.8	1.03	1.36	1.60	1.71	0.52
	1974	0.8	1.26	1.68	-	1.66	
Average peak grade	1973	1.70	2.12	2.51	2.89	2.90	0.62
	1974	1.88	2.50	2.94	-	3.08	
Average + 2 SD	1973	1.86	2.21	2.63	2.66	3.23	0.51
	1974	1.61	2.32	2.71	-	3.13	
Number of cows	1973	3	6	7	2	1	9*
	1974	6	5	4	0	1	7*

* possible periods

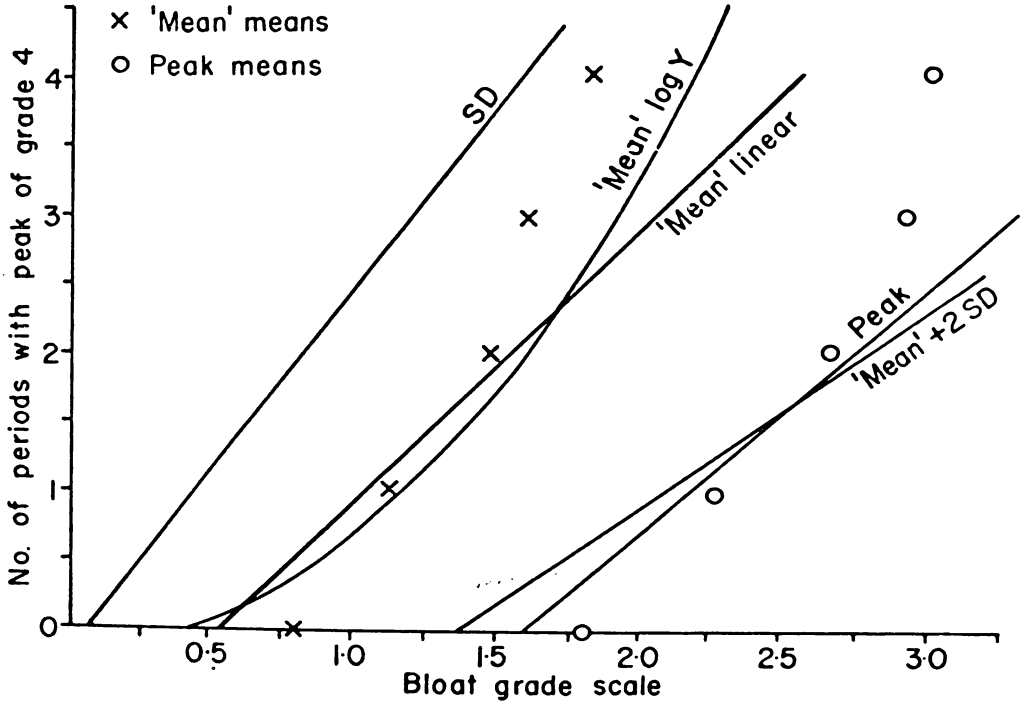


FIGURE 4.1.1 The relationships between the different bloat estimation methods for the 1973 and 1974 animals (plotted from the data shown in Table 4.1.1)

Results and Discussion

4.1.a Inter-relationships of various ways of expressing grades

The grade at one feeding was the highest of the two grades taken at half hourly intervals. The mean of these grades over the period of three or more grading days was the period mean. The bloat grade used for relating to saliva was either the relevant period mean, or the average of all the period means available at the time of analysis.

Other possible systems are:

a) The maximum grade achieved in a period with its corresponding overall average of maximum grades.

b) The number of times a cow reached grade 4 (at risk and drenched) or the number of periods in which 4 was the peak grade. This latter method avoids correlated effects within a period. The number of periods with a peak grade of 4 relative to the total periods is some measure of the probability that a cow might die of bloat if not drenched.

c) The overall average of period plus two standard deviations (where period means are the observations). This gives the maximum grade likely to be reached in 97.5% of the periods.

Table 4.1.1 shows the various measurements for 1973 and 1974 cows classified by the number of periods in which a peak of grade 4 was reached.

The 1973 cows had two extra periods in which to attain grade 4 but for one of these no animals reached this grade, thus the 'probabilities of death' were about similar for the number of grade 4 periods. It can be seen from this Table that mean values for the 1973 and 1974 animals were also much the same.

Figure 4.1.1 shows the relationships for all the cows of each measurement predicting 'the probability of death', the R^2 values (percent variance y accounted for) are given in Table 4.1.1. The logistic fit (for average grade) gave R^2 0.55 which was only a slight improvement on the linear fit. The relationship between average and peak grades gave an R^2 of 0.77, such that peak grade equalled average grade plus 1.1. The peak and mean plus two standard deviations regression lines were coincident.

Thus in general any of these measurements should be equally effective in predicting possible death from bloat, and selecting on mean grade should

TABLE 4.1.2 Analysis of variance of the 1973 progeny bloat grades

Source	d.f.	Mean square	Variance component	% variance
Cows	18	1.4011	0.1311	26
Periods	8	2.9507	0.1436	29
Cows x Periods	144	0.2216	0.2216	45

be phenotypically equivalent to selecting on peak grade. When data becomes available it will be possible to check the relationship of parents' mean grade with the offsprings' probability of death to see whether or not separate genetic mechanisms exist which were not revealed by this sort of analysis.

On the basis of the relationship between average and peak grades, it was concluded that a mean grade of less than 0.5 - 0.75 or a mean peak less than 1.5 - 2.0 was required for reasonable confidence that a cow was unlikely to die from bloat, (this does not exclude the occasional unexpected death).

4.1.b Repeatability of bloat grades

Table 4.1.2 shows the analysis of variance of the 1973 female bloat grades. There was much the same variance between cows as between periods, the latter depended on the bloat potency of the pasture while the former was the variance which this study was attempting to explain. The 45% variance in the interaction term could be regarded as error at present in that it resulted from cows not having the same ranking and/or the same order of difference at each period. This could be partly from smaller cow differences when the bloat 'potency' of the pasture was lower, as well as appetite and other such differences which might have occurred over the three and a half years.

If it was taken as error at this stage then we could estimate:
95% of values for one cow at one period would be mean $\pm$ 1.0 grade
95% of values for one cow, mean of 9 periods would be mean $\pm$ 0.34 grade
95% of means of 6 cows (i.e.'saliva group') for two periods (one year) would be mean $\pm$ 0.3 grade.

In March 1976 gradings of the 1974 animals were made in two successive weeks as a repeatability check. The correlation (r) between these two weeks was 0.74.

Table 4.1.3 shows the correlations between grades for various period combinations. The March 1977 correlations for the 1973 cows fell off only when separated by about two to three years. The March 1974 grades for these animals had a low correlation with most other times, and in particular the correlation with the September 1974 grading was particularily low (r = 0.08). This low correlation of the March 1974 gradings with the other gradings arose from HS/LS differences in bloat grade which were present at 6 months

TABLE 4.1.3 Correlations between bloat grades for various period combinations

(a) For the 1973 progeny

	Sept 1974	March 1975	Sept 1975	March 1976	Sept 1976	March 1977
March 1974	0.08	0.25	-	0.42	0.19	0.34
September 1974	-	0.62	0.64	0.36	0.30	0.23
September 1975	-	-	-	-	0.28	-
March 1976	-	-	-	-	0.53	0.71
September 1976	-	-	-	-	-	0.63

(b) For the 1974 progeny (n=16)

	March 1976	March 1977
March 1975	0.71	0.35
September 1976	0.77	0.42

TABLE 4.1.4 Mean bloat grades for the 1973, 1974 and 1975 HS and LS progeny at each bloat grading period from March 1974 until March 1977

		March 1974	Sept 1974	March 1975	Sept 1975	March[@] 1976	Sept[@] 1976	March[@#] 1977	N	Deaths from bloat
1973	HS	1.20	1.94	0.74	0.81	1.32	1.03	1.46	9	2
	LS	0.67	2.09	0.70	1.00	1.52	1.09	1.72	10	1
1974	HS	-	-	2.13	1.47[+]	1.34	1.61	0.91	8	3
	LS	-	-	1.59	1.46[+]	0.91	0.97	0.65	8	Nil
1975	HS*	-	-	-	-	0.47	0.63	0.23	5	Nil
	LS*	-	-	-	-	0.76	0.97	0.28	3	Nil
BULLS 1975	HS*	-	-	-	-	0.44	1.47	1.80	6	Nil
	LS*	-	-	-	-	0.26	1.00	1.15	5	Nil

@ 1973 and 1974 grazed and graded together
+ HS & LS run separately overnight
N = number of animals
# 1975 joined 1973 and 1974 for grading
* F_1 (by Dairy Board bulls) only

of age but not subsequently repeated. The correlations of bloat grade at one year of age (September 1974) with subsequent grades decreased with time for the 1973 animals. Thus the one year of age bloat grade for these animals was of little value in predicting their future bloating behaviour; both HS and LS animals had high mean bloat grades at one year of age, but at six months of age these animals had shown HS/LS differences. Furthermore there was some indication from the correlations shown in Table 4.1.3 (higher March 1976 onwards), to suggest that the bloating behaviour of the 1973 animals had changed in some way around March/ September 1976.

Owing to confounding of sire differences with overnight feed differences the one year old bloat grades for the 1974 animals could not be used to test for correlations with grades at other ages. An unexplained result for these 1974 progeny shown in Table 4.1.3 was that September 1976 gradings gave a lower correlation with the March 1977 grading ($r = 0.42$) than the March 1976 grading ($r = 0.77$) although the time intervals were approximately the same. Whether the differences in correlations pre and post two years of age was indicative of a change similar to that suggested for the 1973 animals after two years of age could only be proven with more data.

The results from the 1973 and 1974 progeny would indicate that some aspects of the bloating behaviour of animals appear to change during the first two to three years of an animals life. From the point of view of breeding LS cows, this was an important observation which will be examined in more detail in subsequent progeny.

4.1.c Age and HS/LS group differences

Results presented in Table 4.1.4 illustrate the lack of difference in mean bloat grades between the 1973 HS and LS progeny groups (apart from the March 1974 six months of age grade). The low bloat grade for the LS animals in March 1976 would account for the lack of correlation of this grade with grades at subsequent ages. The 1974 progeny showed consistent bloat grade differences between the HS and LS animals, but for the 1975 progeny the mean bloat grades were low and there were no differences between the HS and LS animals. The low bloat grades recorded for these 1975 animals might indicate that these animals have not been sufficiently challenged with bloat potent pasture and therefore the results of these gradings might be random effects.

TABLE 4.1.5 Mean bloat grades at April 1977 for cows and bulls born in 1976

	F_2		G_2		F_1	
	HS	LS	HS	LS	HS	LS
Bulls	2.30	0.89	1.27	0.29	0.43	0.18
n	3	2	2	5	3	11
Cows	2.12	0.54	1.48	1.46	1.30	1.25
n	4	2	4	2	7	11
Bloat deaths	1	0	0	1	1	0

F_2 = 1974 x 1974 progeny

G_2 = 1973 progeny x Dairy Board bull

F_1 = Main herd x Dairy Board bull

n = number of animals

All three age groups of animals (1973, 1974 and 1975 progeny), were all pastured together for the March 1977 bloat gradings, nevertheless there were large differences in mean bloat grades for the three age groups. This means that the pasture bloat potency, as assessed by the mean bloat grade of a group of animals, would differ if assessed by the 1973 progeny as opposed to the 1974 progeny. These results would indicate that one group of animals is more sensitive than another to presumably the same dose rate, but this data is inadequate to draw firm conclusions. The possibility of grazing interactions between the age groups seems unlikely because there was surplus pasture in each break, and furthermore the F_2 cows graded with these animals in March 1977 (HS and LS) averaged 1.2. Other factors which would influence these results are parturition and lactation, and hence appetite effects, if these are important. However it would be expected that these effects would be largely confined to the September bloat gradings.

These results would indicate that the differences in bloat grade for the three different age groups (1973, 1974 and 1975 progeny) are apparently genuine but with a large element of chance. In addition to these results, the six months of age bloat gradings for the 1976 F_2, G_1 and F_1 progeny showed large differences in their mean grades (Table 4.1.5). The mean grades for the F_2 animals would suggest that the average period peak grades for these animals would be greater than 3, indicating that selecting animals on the basis of their mean grades would appear to be effective in selecting for HS and LS animals. However owing to the very small number of animals these results have to be interpreted with caution.

SUMMARY

The correlation between the two methods of assessing bloat grade, (1) mean grade, and (2) peak grade, had an R^2 of 0.77. Furthermore the peak grade (method 2) and the mean plus two standard deviations, regression lines were coincident. Thus all methods should be equally effective in assessing an animals bloat susceptibility.

Bloat grades at one year of age were of little value in predicting future bloat grades. There was some indication from the correlations to suggest that bloating behaviour of both groups of animals might have changed at about two to three years of age.

Pasture potency, as assessed by the mean bloat grade of a group of animals would differ if assessed by the 1973 animals as compared with the 1974 animals.

The 1974 (Friesian crosses) showed HS/LS progeny group differences for mean bloat grades, but there were no HS/LS differences for either the 1973 or 1975 (Jersey) animals. However results from the 1976 F_2 animals showed large mean bloat grade differences between their HS/LS progeny groups.

4.2

CLOVER/GRASS PASTURE TYPE TRIALS TO INVESTIGATE THE EFFECT ON THE SALIVARY PROTEINS AND SALIVA FLOW, OF FEEDING COWS ON DIFFERENT PASTURES

In Chapter 3.4 it was shown that when saliva samples were collected from the Group 2 cows, immediately after grazing on a bloat-potent mixed clover pasture, there was a treatment effect consisting of an increase in the proportion of both protein bands 4 and 6. Therefore a series of repeat trials using the 1973 progeny were designed to examine in more detail the treatment effects described in Chapter 3.4.

Materials and Methods

Saliva from cows was sampled and analysed as described in Chapter 2. The parameters being measured were, saliva volume, total protein (Lowry technique described in Chapter 2.1.h) and individual protein band proportions (PAGE described in Chapter 2.1.a). The gels were scanned at variable gains in these trials as opposed to the studies in Chapter 3 where a constant gain densitometer was used.

Experimental design

Three trials (repeats) were made in November 1974, April 1975 and March/April 1976 using the twelve animals which formed the 1973 progeny group. However cow 320 died, and was replaced by cow 318 in the last trial.

Each trial was divided into two periods. In the first period three LS and three HS cows were pastured on grass and the remainder were pastured on a bloat-producing clover or clover/grass pasture. In the second period the treatments were reversed. The animals had three days on the respective treatments before being sampled. Six animals (three from each treatment and sire at random) were sampled on day 1 and the rest were sampled on day 2. Thus the day effects were partially confounded with animal effects. The groups were reversed for the second period, so that in any one trial all animals were measured on both treatments.

After one hours grazing the animals were graded for the degree of bloat at half hour intervals using the scale of Cockrem and McIntosh (1976) this was described in Chapter 2.4.a. The maximum grade obtained for a given cow was used as its bloat record for the day.

TABLE 4.2.1 Means for the repeats of the Clover/Grass Pasture Type Trials showing bloat grades, saliva flow (ml/min) and protein bands; (a) expressed as a percentage of total protein entering the gel; (b) as a percentage of the total minus band 1

Repeat		Saliva flow	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8	Band 9	Band 10	Band 11
(a)	1	2.68	30.8	7.2	8.9	9.5	3.8	3.3	7.4	16.6	7.6	2.6	0.8
	2	2.50	40.9	4.9	7.8	6.9	2.7	2.6	3.2	14.8	9.3	3.2	2.6
	3	3.34	22.7	4.8	11.4	11.9	4.8	5.2	7.0	16.1	6.8	2.8	4.2
	Mean	Bloat	Grade	Band 1 omitted from the percentage calculations									
(b)	1	0.56	-	10.5	13.0	13.8	3.8	4.7	10.7	23.8	11.1	2.6	1.1
	2	0.42	-	8.1	12.9	11.9	4.4	4.3	5.1	26.2	15.6	5.2	4.0
	3	0.77	-	6.2	14.8	15.3	6.2	6.7	8.9	20.8	8.9	3.6	5.4
SEM based on 24 animals				0.77	1.04	1.15	0.51	0.55	1.38	2.07	1.29	0.64	0.68

Statistical analyses

The analysis of variance used to analyse this data was of a split plot design (Brownlee 1965). Because the animals were re-randomized for each repeat, the basic block was one cow, the whole plot was the repeat, and the sub-plot the period. The pasture (clover/grass) effects were then sub-divided out of the sub-plot error.

Thus the analysis consisted of:

Blocks	Sires (S)
	Cows within sires (CWS)
Whole plot	Repeats (R)
	Repeat x sire (R x S)
	Repeat x cows within sires (R x CWS) (whole plot error)
Sub-plot	Periods (P)
	Periods x sires (P x S)
	Periods x cows within sires (P x CWS) = A = error term
	Subdividing remaining interaction
	Feeds (F)
	Feeds x sires (F x S)
	Periods x repeats (P x R)
	Periods x repeats x sires (P x R x S)
	Residual (B)
	Error term = A + B

The sires were tested against cows within sires (with repeat observations summed. Repeats and repeats x sires were tested against repeats x cows within sires. All sub-plot terms were tested against the A + B error term. (A = P x CWS, was not tested.)

A Table showing the mean squares from the analysis of variance is given in Appendix C.

Regression analyses were also made to examine any differences revealed by the analyses of variance. That is, if a difference occurred between clover and grass it was assumed to be an effect of different 'bloat potency' and average bloat for repeat/treatment means can be related to the appropriate saliva characteristic. These and other relationships which also involve the means of all animals can be considered to be largely environmental in origin. However the converse animal effects will be phenotypic and contain

TABLE 4.2.2 Significant means from the analysis of variance for: saliva flow between HS and LS animals, and clover/ grass treatments for bands 3 and 4 and bloat grade

	HS	LS	
Saliva flow ml/min	1.59	4.08	
	Clover	**Grass**	**Total**
Band 4 %			
Repeat 1	10.8	8.3	9.6
2	6.9	7.0	7.0
3	13.1	10.7	11.2
Band 3 %			
Repeat 1	7.6	10.3	8.9
2	7.3	8.4	7.8
3	10.7	12.1	11.4
Bloat grade			
Repeat 1	0.8	0.3	0.56
2	0.7	0.1	0.42
3	0.8	0.7	0.77

See Appendix C for the Mean Squares from the Analysis of Variance

some day effects which will be environmental. Thus the analyses of variance together with some regression analyses enables a test to be made of the occurrence of the induction effects postulated by McIntosh (1975).

Results

Means for the 11 salivary protein bands expressed as percentages of the total protein entering the polyacrylamide gel are given in Table 4.2.1. Because of the large degree of variation in band 1 between repeats ($P<0.001$), the other bands are also given as percentages of the total with band 1 omitted.

Results from the analysis of variance

Results from the analysis of variance demonstrated the only significant HS/LS sire group difference was for saliva flow. The LS animals had a flow rate of 4.08 ml/min, while the HS animals had a flow rate of 1.59 ml/min ($P<0.01$) (Table 4.2.2). The proportion of proteins differed between repeats for bands 1 to 7 inclusive and band 11. The large difference between repeats for band 1 could have affected the proportion of the other bands which differed between repeats. However after allowing for band 1 differences between repeat trials, band 2 appeared higher for repeat 1, while band 7 was lower for repeat 2, and band 8 and 9 appeared higher for this repeat (Table 4.2.1).

Total protein, band 2 and band 6, ($P<0.05$) and bloat grade ($P<0.01$) differed between periods, while saliva flow and band 5 were not significant at the 5% level ($P<0.1$)

There was a higher proportion of band 4 protein in the saliva of animals pastured on clover compared with those pastured on grass, regardless whether HS or LS ($P=0.06$), whereas band 3 protein was higher in the saliva of animals pastured on grass ($P=0.06$), Table 4.2.2).

The bloat potency of the pasture was varied in these trials by the clover and grass treatments. The greater proportion of band 4 protein on the clover treatment indicated that the bloat potency of the pasture could be one factor affecting this band. This was examined further by looking at the relationships between the repeat and period measurements as well as those between animals.

TABLE 4.2.3 Correlations between repeat/treatment sub-classes (environmental) and within repeat/treatment/sire sub-classes for; (A) protein band percentages (band 1 omitted) with total protein, saliva flow, and bloat grade, (B) protein band percentages (Band 1 omitted) with each other (Band inter-relationships)

(A) Between band proportions (No Band 1) and other variables

Variable	Band 4	Band 6	Band 7	Band 8	Band 9
Protein					
Environmental	0.67	0.45	0.86*	-0.74	-0.83*
Phenotypic	-0.19	0.04	0.39**	-0.07	-0.22
Saliva Flow					
Environmental	0.45	0.92**	0.38	-0.72	-0.74
Phenotypic	0.44**	0.18	-0.38**	-0.26*	-0.10
Bloat Grade					
Environmental	0.81*	0.55	0.34	-0.71	-0.69
Phenotypic	-0.02	0.26*	-0.04	-0.02	-0.0003

(B) Between various band proportions

Bands		6	7	8	9
4	Environmental	0.62	0.44	-0.54	-0.80
	Phenotypic	0.27*	-0.42**	-0.34**	-0.18
6	Environmental	-	0.36	-0.85*	-0.81*
	Phenotypic	-	-0.08	-0.32**	-0.19
7	Environmental	-	-	-0.60	-0.78
	Phenotypic	-	-	-0.14	-0.38**
8	Environmental	-	-	-	0.86*
	Phenotypic	-	-	-	0.05

P * <0.05, ** <0.01

The correlations shown in Table 4.2.3 are those between Repeat and Treatment sub-classes, that is environmental ($n = 6$, $r = 0.81$ at the 5% level of significance), and those between animals within Repeat, Treatment, and Sires, that is phenotypic without the possible genetic weighting of the sires, (59 DF $r = 0.25$ at the 5% level of significance).
Band 1 was omitted for the calculations of proportions.

The correlations shown in Table 4.2.3 are those between Repeat and Treatment subclasses, that is environmental (n=6, r=0.81 at the 5% level of significance) and those between animals within Repeat, Treatment and Sires, that is phenotypic, without the possible genetic weighting of the sires, (59DF, r=0.25 at the 5% level of significance). Band 1 was omitted for the calculations of proportions.

Relationships of protein bands with bloat grade, saliva flow and total protein

Band 4 protein showed a positive environmental correlation of 0.81 with bloat grade ($P<0.05$), but the band 6 correlation of 0.55 was not significant. When the proportion of band 4 protein (without band 1) was plotted against the mean bloat grades, on both grass and clover for the three repeat trials, it was shown that the variation in pasture potency within grass contributes to the correlation as much as the clover/grass differences (Figure 4.2.1).

Band 6 protein did however show a significant phenotypic correlation of 0.26 with bloat grade ($P<0.05$), these results are shown in Table 4.2.3A.

Saliva flow had a significant environmental correlation of 0.92 with band 6 ($P<0.01$), while bands 4,7 and 8 also had significant phenotypic correlations of 0.44 ($P<0.01$), -0.38 ($P<0.01$), and -0.26 ($P<0.05$) respectively with saliva flow. The proportion of band 7 protein was shown to increase with an increase in total protein both between animals and between sampling times, but the environmental relationship was not associated with pasture potency as there was no relationship with bloat grade, (Table 4.2.3A). Band 3 protein showed no relationship with the bloat potency of the pasture.

Relationships between the various band proportions

The relationships between the various bands are shown in Table 4.2.3B. Bands 4 and 6 were shown to be phenotypically related as were bands 4,7,8 and 9. In addition to these phenotypic relationships, bands 8 and 9 have environmental relationships with band 6 and with each other.

Discussion

The results from these studies indicated that some environmental component associated with the bloat potency of the pasture caused an increased proportion of band 4 protein to be secreted in the saliva of cows.

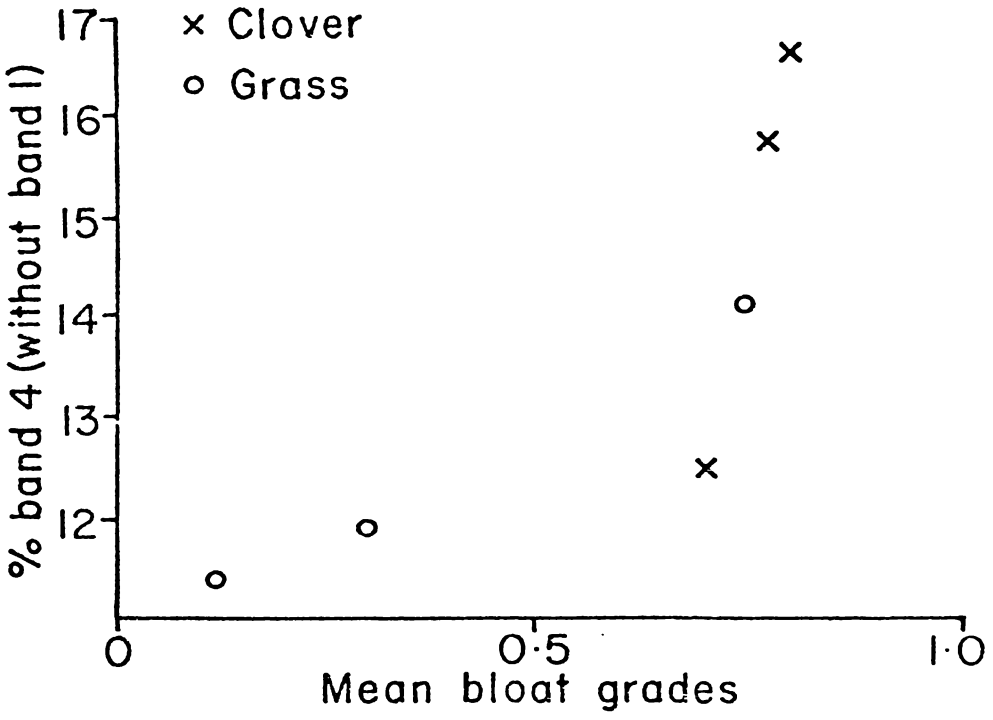


FIGURE 4.2.1 The relationship of percentage Band 4 (without Band 1) with mean bloat grades, for animals pastured on clover compared with animals pastured on grass.

This increased proportion of band 4 protein was related to the bloat grade of the animal ($r=0.81$, $P<0.05$), and there was a feed effect for band 4 protein; the proportion of band 4 being higher on clover than on grass, regardless of HS or LS sire groups. There was also a bloat effect of grass on band 4, (i.e. the higher the mean bloat grade on grass, the higher the band 4% in the saliva). These results suggested that an environmental factor in the pasture might be causing an induction of protein synthesis and secretion of this specific protein by the salivary glands.

The band 6 protein was phenotypically related to bloat grade, but the environmental correlation was not significant. However in these trials there was an additional environmental component which stimulated saliva flow and the proportion of band 6, but this environmental stimulus did not appear to be related to the bloat potency of the pasture.

The relationships between the individual protein bands were in line with those seen for the Group 1 and Group 2 animals.

Summary

The analysis of variance results of the three repeat clover/grass trials on the 1973 animals showed that LS animals had a higher mean saliva flow than the HS (4.08, 1.59 ml/min respectively). The band 4 levels on clover were higher than on grass (10.25% and 8.67% respectively) but did not differ between HS and LS groups. Band 7 proportions increased with an increase in total protein but this increase was not associated with pasture potency. Phenotypically saliva flow was related positively to band 4 and negatively to band 7, but bands 4 and 7 were also negatively related. There was a high environmental correlation of band 6 and salivary flow, and no phenotypic relationship. This would suggest that an environmental factor could be affecting both these variables.

The results indicated that the different pasture potency within grass contributes to the correlation of bloat with band 4 as much as the clover/grass differences.

The phenotypic relationships between bands already demonstrated for the Groups 1 and 2 animals also exist for these animals, and in addition bands 8 and 9 have environmental correlations with band 6 and with each other.

TABLE 4.3.1 Timetable for the Time of Sampling Trial showing the plan of sampling and the number of hours since the animals last had access to feed

Time of Day	09:00-10:00	10:00-11:00	14:00	16:00	17:00-18:00
Group A	Sampled (15)*	Feeding	Sampled (3)	Yards	Feeding
Group B	Feeding	Sampled (0)	Yards	Sampled (6)	Feeding

* Figures in brackets represent the time in hours since the animals last had access to feed

4.3

TIME OF SAMPLING TRIALS USING THE 1973 PROGENY TO INVESTIGATE THE EFFECT OF TIME AFTER FEEDING ON THE SALIVARY PROTEINS AND SALIVA FLOW

The Time of Sampling Trials were designed to investigate the effect on the salivary proteins and saliva flow, of collecting saliva samples by the standard mouth bit procedure at different times after the animals last had access to feed. The routine standard sampling procedure which had been used for all the initial work on the Group 1 and 2 animals had been standardized with respect to time after the last feed so as to eliminate one further variable. However the questions to be asked here were firstly, was the standard sampling time the optimum to show maximum differences between high and low animals? Secondly, were there differences in saliva flow, protein content or composition, changing with time of sampling after feeding, but not necessarily relating to bloat susceptibility?

Methods

Two repeat trials were made in November 1974 and April 1976, using the same cows (the 1973 progeny), as used for the clover/grass trials described in Chapter 4.2. Samples of saliva were collected by the standard mouth bit as described in Chapter 2.1.a. Samples were taken from cows immediately off pasture and three, six and fifteen hours after coming off pasture. As it was not possible to sample all cows at all times on one day the animals were randomised into two groups and sampled according to the sampling plan shown in Table 4.3.1.

At all other times and overnight the animals were held in the yards. The sampling was repeated on the second day with groups A and B reversed. Six cows (3 HS and 3 LS) were sampled at all four times over the two days of each trial.

In this design the day, group and time effects are partially confounded, (i.e. for one group only the comparison of day 1 with day 2 will be the same as the comparison of times +3 and +15 with times 0 and +6). Also the effects due to the time from feeding are confounded with any diurnal trend. However as the primary purpose of this trial was to determine the validity of the sampling procedures these confoundings were not considered important as they only affect the estimation of some interactions which were not of immediate relevance.

TABLE 4.3.2 Means for time effects on band 6 protein

Number of hours since animals last had access to feed	Band 6 percentage
0	4.2
3	3.4
6	2.8
15	3.8

TABLE 4.3.3 Band 8 means for the four sampling times and two repeat trials

Number of hours since animals last had access to feed	Band 8 percentage Repeat 1	Repeat 2
0	15.7	13.2
3	13.9	9.8
6	13.2	16.3
15	12.9	17.2

TABLE 4.3.4 Significant means showing HS/LS difference for the Time of Sampling Trials compared with the means for the Clover/Grass Trials

	Time of Sampling Trial	Clover/Grass Trial
Saliva flow (ml/min)		
HS	1.96	1.59
LS	4.29	4.08
Total protein (mg/l)		
HS	815	778
LS	1104	932
Band 2 (%)		
HS	5.8	5.1
LS	7.0	6.1
Band 8 (%)		
HS	12.7	14.8
LS	15.3	16.9

Protein bands in these tables are expressed as a percentage of the total integrations

Analyses of Samples

The saliva volume was measured, and the total protein content was measured by the Lowry method described in Chapter 2.1.h. PAGE were performed on all samples according to the method described in Chapter 2.2.a.

Statistical analyses

The analysis of variance was of a split plot design (Brownlee,1965) similar to that used for the clover/grass Pasture Type Trial (Chapter 4.2), except that due allowance was made in the model for the fact that cows were not re-randomized to the treatments for successive repeats. Day effects were tested if there were no significant time effects.

A Table showing the mean squares from the analysis of variance is given in Appendix D.

Regression relationships were calculated between animals and were also considered for the possible environmental effects represented by the day/repeat differences when no time effects occurred. Any relationships were considered relative to those obtained from the grass/clover trials.

As band 1 was very variable in both sets of trials, relationships were calculated on band percentages based on totals omitting the values for band 1.

Results

Time of sampling after feeding effects

Results from the analysis of variance showed that band 6 (Table 4.3.2) was the only salivary protein which differed significantly over the four sampling times ($P<0.05$). However band 8 showed a repeat x time interaction indicating that the proportion of this protein varied with the time of sampling but was not consistent in the two repeat trials (Table 4.3.3.).

HS/LS sire group differences

Significant HS/LS (sire group) differences were demonstrated for saliva flow, total protein, and the proportion of bands 2 and 8, and these results are shown in Table 4.3.4, where they are compared with the means from the clover/grass trials for these parameters. The HS animals had a saliva flow of 1.96 ml/min and a total protein of 815 mg/l, while the

TABLE 4.3.5 Band 6 means for the HS and LS animals for the two repeat trials

	Band 6 protein	
	Repeat 1	Repeat 2
HS	2.9	3.5
LS	2.6	5.1

TABLE 4.3.6 Means for band 1 protein showing both day and day x sire (HS/LS) differences

	Repeat 1	Repeat 2
HS animals		
Day 1	22.4	22.2
Day 2	39.4	32.3
LS animals		
Day 1	23.9	21.9
Day 2	24.4	24.7

TABLE 4.3.7 Means for protein bands 4 and 7 showing day and day x sire effects

	Band 4 percentage	
Day 1	9.7	
Day 2	7.5	
	Band 7 percentage	
	HS	LS
Day 1	9.9	8.6
Day 2	7.4	10.8

TABLE 4.3.8 Means for protein bands 4,6,7 and 11 showing significant differences between repeat trials

	Repeat 1	Repeat 2
Band 4	6.7	10.4
Band 6	2.8	4.3
Band 7	11.5	6.8
Band 11	0.6	4.3

Protein bands in these tables expressed as a percentage of the total integrations

LS animals had a flow rate of 4.29 ml/min and a total protein of 1 104 mg/l. Band 2 in the HS and LS animals were 5.8 and 7.0 percent respectively, while the percentage band 8 in the HS and LS animals were 12.7 and 15.3 respectively. Band 6 protein showed a significant repeat x sire interaction which could be attributed to the increase in band 6 protein in the LS animals only, in the second trial, and these results are shown in Table 4.3.5.

Differences between days of sampling and/or between repeat trials

Other significant effects were those between days or between the two trials (Repeat 1 and Repeat 2). Saliva flow was consistent for days and repeats but protein was higher on Day 1 (1 060 mg/l) compared with Day 2 (859 mg/l). Band 1 showed both day and day x sire differences. The means in Table 4.3.6 show that HS cows in particular had a marked increase in the proportion of band 1 for Day 2, and this was consistent for both repeats. Bands 2 and 4 showed significant day effects ($P<0.001$), however when these results were re-analysed without band 1, only the band 4 differences were significant, Day 1 having 9.7% band 4 and Day 2, 7.5% band 4, (Table 4.3.7).

A day x sire interaction was shown for band 7, independently of band 1, where the HS animals were higher on Day 1 and the LS higher on Day 2 (Table 4.3.7).

Significant repeat effects were shown for protein bands 4,6,7 and 11. Bands 4, 6 and 11 were higher in the second trial (Repeat 2) while band 7 was highest in the first trial (Repeat 1), these results are shown in Table 4.3.8. The results from the Repeat 1 (November 1974) Time of Sampling Trial, except for band 7, were similar to those seen for the clover/grass trial, Repeat 2 (April 1975). Also the results for Repeat 2 Time of Sampling Trial (April 1976) were similar to the Repeat 3 clover/grass trial (April 1976).

Significant relationships between protein bands, saliva flow and total protein

These relationships were calculated within groups of sires, days and repeats and are thus the average estimates for a group of HS or LS offspring. They are thus comparable to the phenotypic regressions of the clover/grass trials. The 5% level of significance for these correlations was $r = 0.22$.

Band 4 was shown to be positively related to saliva flow ($r = 0.23$), while band 1 had a negative relationship ($r = -0.23$). Band 7 and band 8 were negatively related to band 1, ($r = -0.39$ and $r = -0.43$ respectively). Because of the relationship of band 1 protein with the other bands, and for comparison with the clover/grass trials, calculations were made with band 1 omitted from the integration totals. Bands 3, 5 and 7 were positively correlated with each other. Band 7 had a correlation of 0.27 with band 3, while the correlation between bands 7 and 5 was 0.52. Band 4 was positively correlated with band 6, ($r = 0.30$), and negatively correlated with band 7, ($r = 0.52$).

Total protein was positively correlated with protein bands 3, 4, 5 and 7, and negatively correlated with bands 8, 9, 10 and 11.

Discussion

The time interval from 0 - 15 hours between a cow coming off pasture and collecting the saliva sample did not show much effect on the salivary protein bands. The band 6 protein was the only one showing a significant difference between the sampling times in both trials, however band 8 protein changed significantly with time, but this change was not consistent between the two repeat trials. Therefore the relationship between the collection of saliva and the time after an animal was fed would appear to be of only minor importance to the interpretation of salivary protein results with respect to their involvement in bloat susceptibility in cattle.

The total salivary protein measured in these trials include all the lower molecular weight proteins which enter the polyacrylamide gels, plus the mucoprotein, and any other proteins present in the mouth saliva. In both the Time of Sampling Trials, and the Clover/Grass Trials the total protein was higher in the LS animals. The higher protein values, coupled with the higher saliva flow rate for the LS animals compared with the HS, would suggest that more protein secreted by glands sampled by the mouth bit would enter the rumen of LS animals. The mouth bit samples were predominantly from the mandibular gland (Phillipson & Mangan, 1959; Chapter 8) and as the mucoprotein is known to be secreted by this gland (Gottschalk et al. 1972), the higher protein results for the LS animals suggests that the level of mucoprotein entering the rumen would be higher for LS compared with HS animals.

Consistent Day effects were observed in the two trials for total protein, Day 1 being higher than Day 2, despite the 18 month time interval between the repeat trials. The lower protein values on the second day of each trial would suggest that some real but unidentified effect was influencing the levels of the salivary proteins. The proportion of band 1 was higher on Day 2 but these results did not establish whether the actual concentration of band 1 was increased or whether this increase was due to a proportional effect due to the lower total protein concentration on Day 2.

The significant repeat effects for protein bands 4, 6, 7 and 11 could perhaps be explained by the 18 month age difference between the two trials. Age effects on protein bands are discussed more fully in Chapter 4.4.

The band inter-relationships shown in these trials were in line with the results from the Clover/Grass Trials which used the same animals They were also in line with the results obtained for the Group 1 and Group 2 animals, indicating that the band inter-relationships are consistent meaningful results.

Summary

Differences between HS and LS sire groups for saliva flow, band 8, and possibly band 2, were in agreement with the findings of the Clover/Grass Trials. Total protein was lower in the HS compared with the LS progeny groups. For the time effect the only significant difference was for band 6. More important effects detected, were between days or between the two repeat trials. The differences seen between repeat trials could be an age difference of one and two and a half years of age. Relationships between protein bands were similar to those seen for animals in Groups 1 and 2, but total protein was positively related to bands 3, 4, 5 and 7 and negatively to bands 8, 9, 10 and 11.

There was an indication from these results that the LS animals produced more mucoprotein than the HS animals, but no specific quantitative studies on mucoprotein were carried out.

4.4

CHANGES WITH AGE, IN BLOAT GRADE AND SALIVA COMPOSITION FOR ANIMALS BORN 1973,1974 and 1975

This study was designed to follow changes in saliva flow, protein concentration, salivary protein band proportions, and mean bloat grade for the three progeny groups over a three year period. Saliva samples studied in this work were collected at nine months, one year, 18 months, two years and three years of age. Although saliva samples were collected at ten days of age from some of these animals, these results have not been included in this study. Band patterns in the saliva collected at ten days of age were different from later samples. This was presumably due to the presence of pre-ruminant enzymes in the saliva such as the salivary lipase reported by Grosskopf (1965) which disappear from the saliva when the animal is about three months of age. However this work was concerned primarily with the proteins present in the saliva of adult animals which could have some importance in determining bloat susceptibility differences amongst animals.

All the electrophoretic results of the individual protein bands were expressed as percentages and therefore a large fluctuation in one band (e.g. band 1) could affect other bands because of the proportionate effects. It has been assumed in this work, that the band 4 and band 6 variations were associated with pasture bloat potency, even at low levels, while the fluctuation in band 7 levels were due to some environmental effect of unknown nature. These assumptions affect the approach to the analyses in that no attempt was made to analyse seasonal (age) fluctuations directly.

Comparisons are difficult in any situation where seasonal and annual effects exist and one crop of animals are born each year. Animals can be compared at one time at different ages, or at one age at different times. To do both requires that data be available from all times and ages over sufficient time for the effects to be independent. At this stage when seasonal effects (bands 4 and 6) are known to occur, then age effects cannot be taken beyond the stage of informed discussion.

Materials and Methods

The animals used in the first part of this work were the male and female 1974 and 1975 progeny. These animals were saliva sampled at nine months and one year of age. HS/LS sire groups were tested for differences

in saliva flow, total protein and the individual salivary protein band differences.

The 1973, 1974 and 1975 female progeny were used for the second part of this work. Saliva samples were collected at nine months, one year, 18 months, two and three years of age (constant age). All animals were also sampled in September 1976 at a constant season (age varies). Saliva flow, total protein, and individual protein bands, were tested for year born and sire group (HS/LS) differences. (Note in both cases year born is confounded with the season.)

All results were analysed by the analysis of variance. Where unequal sub-classes were used in the analysis of variance, the means shown in the Tables (results section) were derived by fitting least squares. The differences shown for significance at the 5% level were based on the residual mean squares, and the number of animals was based on the number in the 1973 progeny group. These differences shown at the 5% level for the year born were approximately 20% higher than would be required for significance at the 5% level for HS and LS sire group differences.

Results

4.4.a
The analysis of variance for the 1974 and 1975 male and female progeny at nine months and one year of age

The table of means from the least squares estimates and analysis of variance for the 1974 and 1975 male and female progeny is given in Appendix E. The main purpose of this analysis was to test for sex effects on saliva flow, total protein, and individual protein bands in the saliva collected at nine months and one year of age. The one year of age saliva sample was assumed to represent saliva from sexually mature animals.

The only significant sex difference demonstrated was that for saliva flow at one year of age, where the females saliva flow rate was 1.66 ml/min and the males had a flow rate of 2.63 ml/min. However the importance of this result is questionable as the saliva flow rate of 2.56 ml/min for the females at nine months of age was as high as that of the males at one year of age. There was a year born x sex interaction for band 6 where the 1974 males had a lower band 6 level than the females,

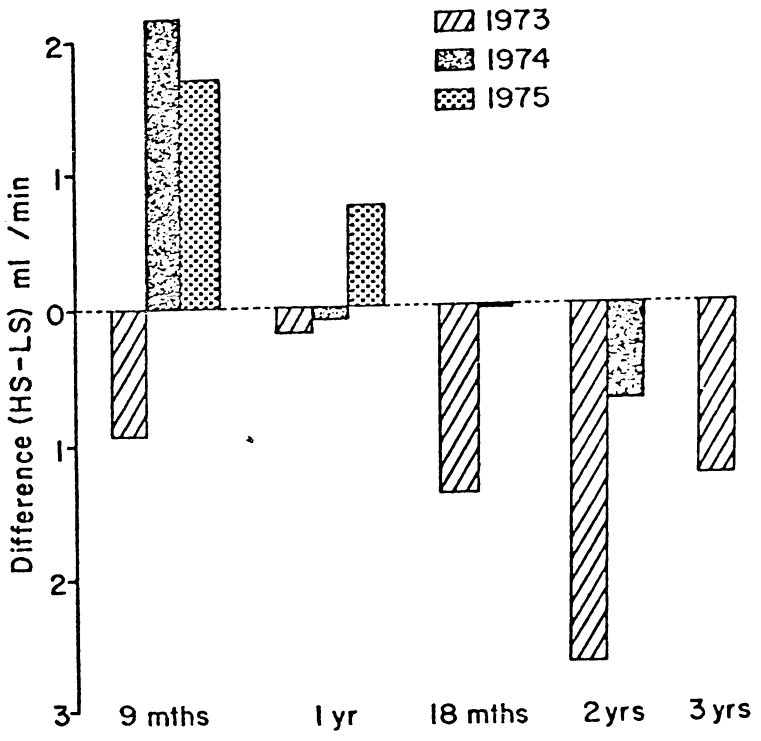


FIGURE 4.4.1 Mean saliva flow differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for saliva flow

		9 months	1 year	18 months	2 years	3 years
1973	HS	1.50	2.56	1.90	1.78	3.82
	LS	2.43	2.75	3.28	4.40	5.05
1974	HS	4.55	2.25	3.23	3.85	-
	LS	2.42	2.35	3.33	4.55	-
1975	HS	2.60	1.46	-	-	-
	LS	0.90	0.73	-	-	-
5% Difference*		1.77	1.26	1.64	1.83	3.29

* Differences required for significance at the 5% level

3.1 and 4.7% respectively, while the reverse was true for the 1975 progeny (males 5.0%, females 3.9%).

Other effects of interest were those on total protein and band 7. At nine months of age the 1974 progeny showed a higher protein value of 1 260 µg/ml compared with 751 µg/ml for the 1975 progeny ($P<0.01$). This high protein value was associated with a high band 7 value of 12.5% for the 1974 progeny compared with 5.3% for the 1975 progeny ($P<0.001$). However this finding was not repeated at one year of age which indicates some environmental factor was effecting the 1974 animal at nine months of age. In both the 1974 and 1975 progeny there was an HS/LS sire group differences for band 7 of 7.2 and 10.5% respectively at nine months of age ($P<0.05$). However at one year of age the HS/LS difference of 8.1 and 10.5% respectively, was not significant.

4.4.b

The analysis of variance for the 1973, 1974 and 1975 females for saliva samples collected at a constant age (season varies), and constant season (age varies)

Saliva flow

Figure 4.4.1 illustrates the consistent difference between HS and LS animals for saliva flow in the 1973 progeny. However as can be seen from Figure 4.4.1, the 1974 LS animals did not show a greater flow rate than the HS animals until two years of age. Significant HS/LS differences (at the 5% level) were not obtained for all the groups of animals owing to the high error variation (probably mainly cow variation) for saliva flow, and this was illustrated by the differences required for significance at the 5% level. At two years of age however, there was an HS/LS difference of 2.82 and 4.47 respectively, which was significant at the 10% level.. From the means of the saliva flow rates at the different ages, there was some indication to suggest that saliva flow increased with increasing age.

Salivary protein

There was no significant differences shown for total protein owing to the high variation in protein content, but it was possible that there might have been a decrease in protein concentration with increasing age, (Figure 4.4.2). The results in Figure 4.4.2 suggested that the 1973 LS

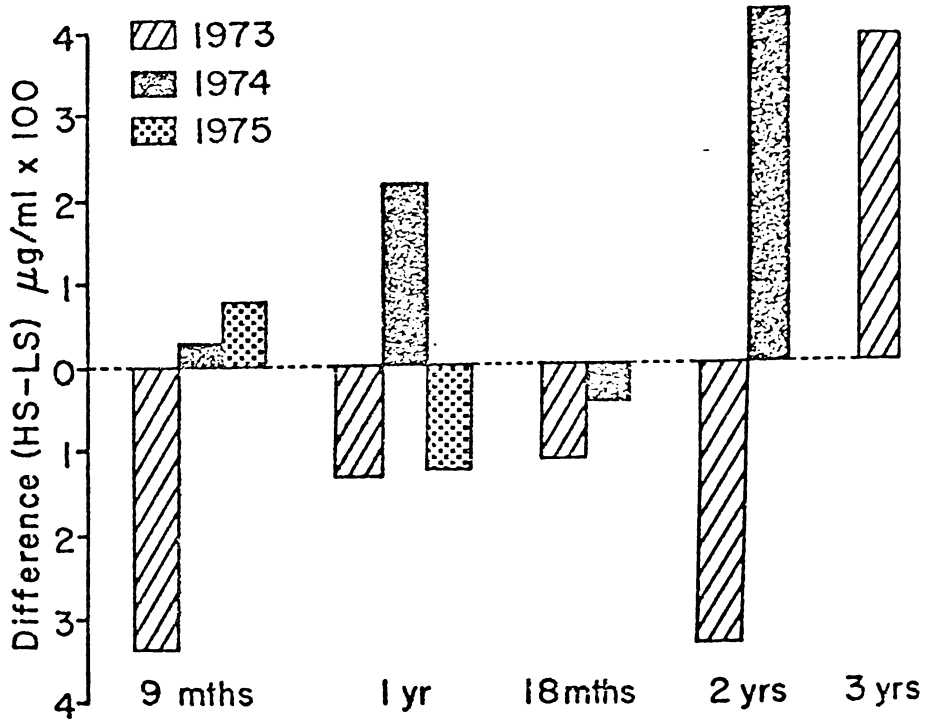


FIGURE 4.4.2 Mean total protein differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for salivary protein

		9 months	1 year	18 months	2 years	3 years
1973	HS	955	861	873	568	1046
	LS	1288	985	983	896	668
1974	HS	1078	1177	1004	1142	-
	LS	1049	966	1046	730	-
1975	HS	833	963	-	-	-
	LS	760	1084	-	-	-
5% Difference*		357	494	418	503	574

* Difference required for significance at the 5% level

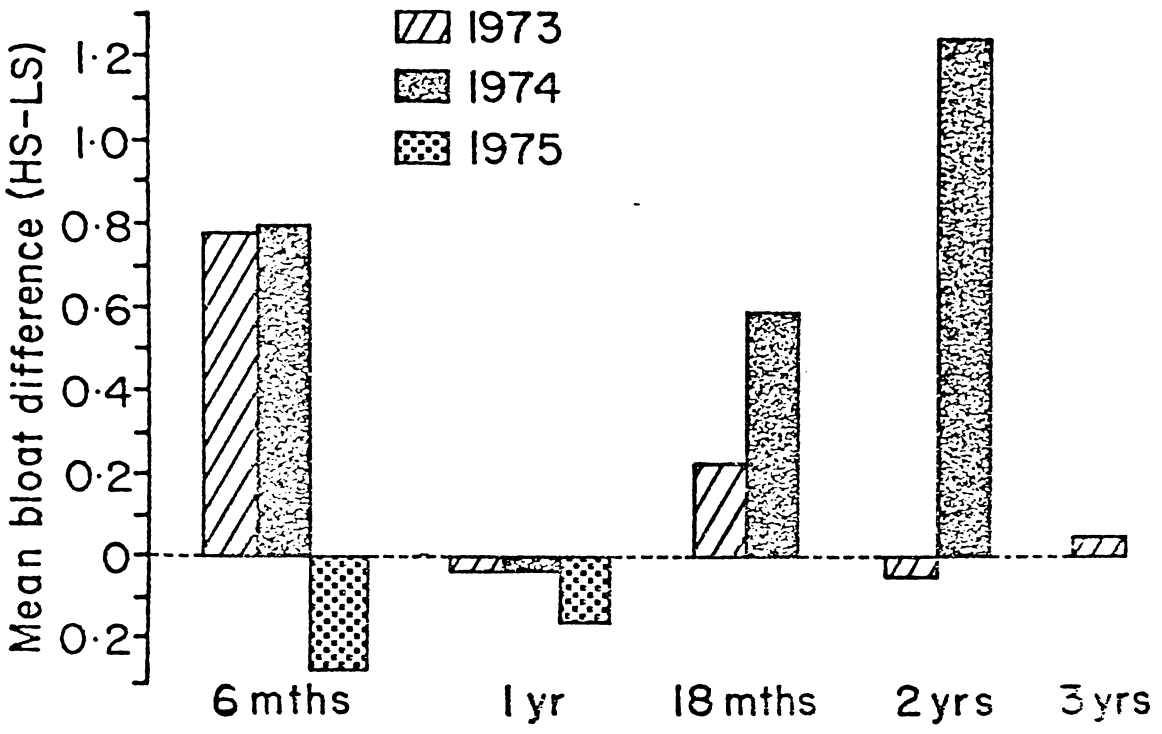


FIGURE 4.4.3 Mean bloat grade differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for bloat grade

		6 months	1 year	18 months	2 years	3 years
1973	HS	1.20	1.93	0.90	0.80	0.96
	LS	0.43	1.95	0.68	0.85	0.92
1974	HS	2.32	1.58	1.28	1.93	-
	LS	1.53	1.60	0.70	0.68	-
1975	HS	0.50	0.84	-	-	-
	LS	0.77	1.00	-	-	-
5% Difference*		0.36	0.63	0.41	0.42	-

* Difference required for significance at the 5% level

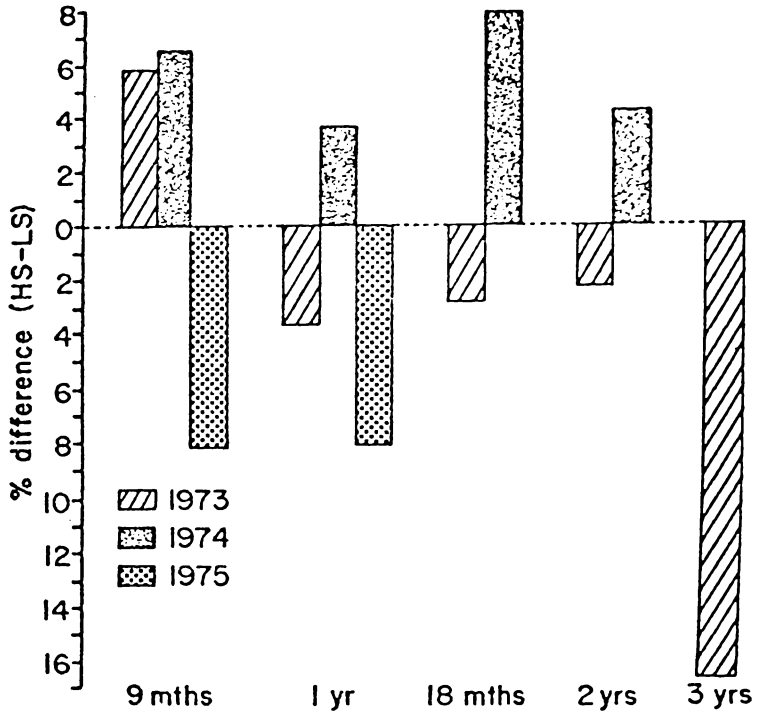


FIGURE 4.4.4 Mean Band 1 percentage differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for Band 1

		9 months	1 year	18 months	2 years	3 years
1973	HS	32.3	27.7	40.6	27.9	39.8
	LS	26.6	31.3	43.4	30.1	56.2
1974	HS	30.6	26.8	36.8	37.6	-
	LS	24.2	23.2	28.9	33.4	-
1975	HS	28.8	26.2	-	-	-
	LS	36.9	34.2	-	-	-
5% Difference*		9.6	9.6	9.3	10.4	12.2

* Difference required for significance at the 5% level

animals up to two years of age, had a consistently higher protein concentration in their saliva compared with the HS animals, but this was not demonstrated for the 1974 animals.

Bloat grade

The analysis of variance results for the mean bloat grades demonstrated significant year born effects at six months, one year, and 18 months of age, and a significant year born x sire group (HS/LS) interaction at six months of age, however the interpretation of these results was difficult as age and season were confounded in the analysis for bloat (See Appendix F. for details). At six months of age there were mean bloat grade differences for HS and LS animals for both the 1973 and 1974 progeny (Figure 4.4.3), but at one year of age there were no HS/LS differences present despite a high mean bloat grade. However unlike the 1973 progeny, the 1974 animals subsequently showed consistent HS/LS bloat differences.

Band 1 protein

There were large differences in the proportion of band 1 protein present between animals and between samplings, but the 1974 animals showed a higher mean value for the HS compared with the LS animals at the four different age groups, (Figure 4.4.4), but these differences were not significant at the 5% level. This HS/LS difference was not demonstrated for either the 1973 or the 1975 progeny. The results from the September 1976 sampling when analysed by the analysis of variance showed there could be an age/year born effect for band 1 ($P<0.05$), the older the animals the greater the proportion of band 1 present; 1973, 1974 and 1975 progeny had 48.0%, 35.5%, and 30.2% band 1 respectively.

Band 4 protein

The analysis of variance demonstrated a significant year born x sire group (HS/LS) interaction ($P<0.01$) at one year of age for the band 4 protein. There was a high band 4 proportion of 15.5% for the 1973 HS animals compared with 8.6 and 8.7% for the 1974 and 1975 animals respectively. The means for this interaction are given in Table 4.4.1.

At the constant sampling time, when all animals were sampled in September 1976 there was a significant HS/LS difference in band 4 protein for the three age groups, (9.47 and 6.41, HS and LS means respectively).

TABLE 4.4.1 Protein bands and bloat grade sub-class means for significant interactions from the analysis of variance, plus some non-significant results for clarification

Born	Band 4 HS	Band 4 LS	Band 6 HS	Band 6 LS	Band 7 HS	Band 7 LS	Bloat HS	Bloat LS
Sampling at constant time, variable age, September 1976								
	ns		ns		*			
1973	10.1	6.8	4.1	2.8	4.3	1.6	0.96	0.92
1974	9.6	6.8	5.8	3.5	2.3	9.9	1.93	0.70
1975	8.7	5.6	3.2	3.9	10.7	11.5	0.84	1.00

Born	One Year age HS	One Year age LS	18 months age HS	18 months age LS	Two years age HS	Two years age LS
Sampling at constant age, different times (see Fig.4.4.3 for relative bloat)						
Band 4	**		ns		ns	
1973	15.5	7.7	6.5	6.9	10.7	9.4
1974	8.6	9.6	6.3	5.1	9.6	6.8
1975	8.7	5.6	-	-	-	-
Band 6	*		*		ns	
1973	6.4	2.7	2.5	2.8	4.9	5.0
1974	3.0	4.3	5.1	3.3	5.8	3.5
1975	3.2	3.9	-	-	-	-
Band 7			**		*	
1973	No		4.2	2.2	7.0	5.9
1974	interaction all additive		4.9	10.5	2.3	9.9

ns not significant

P * <0.05, ** <0.01

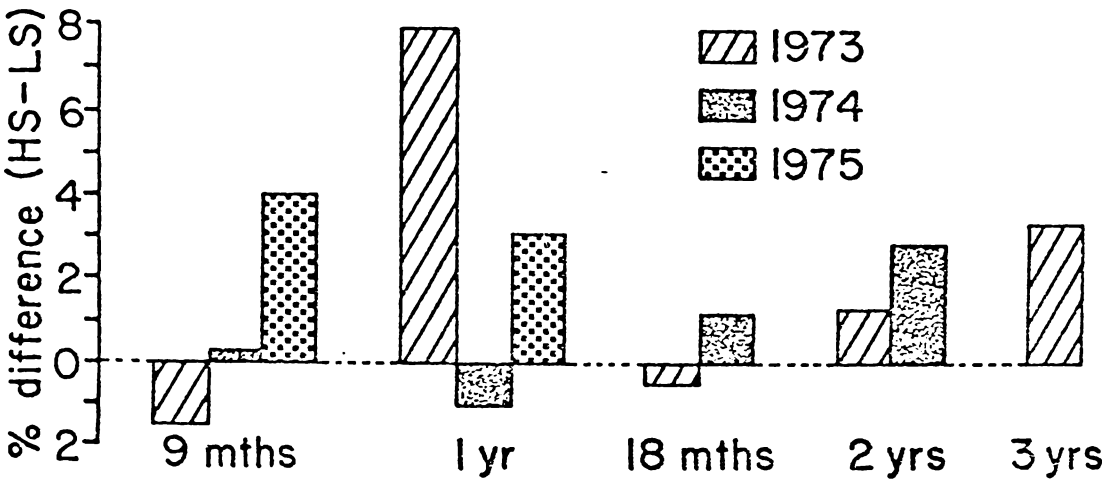


FIGURE 4.4.5 Mean Band 4 percentage differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for Band 4

		9 months	1 year	18 months	2 years	3 years
1973	HS	7.9	15.6	6.5	10.7	10.1
	LS	9.3	7.7	6.9	9.4	6.8
1974	HS	9.3	8.6	6.3	9.6	-
	LS	9.0	9.6	5.1	6.8	-
1975	HS	10.3	8.7	-	-	-
	LS	6.3	5.6	-	-	-
5% Difference*		3.7	2.9	1.6	3.0	3.44

* Difference required for significance at the 5% level

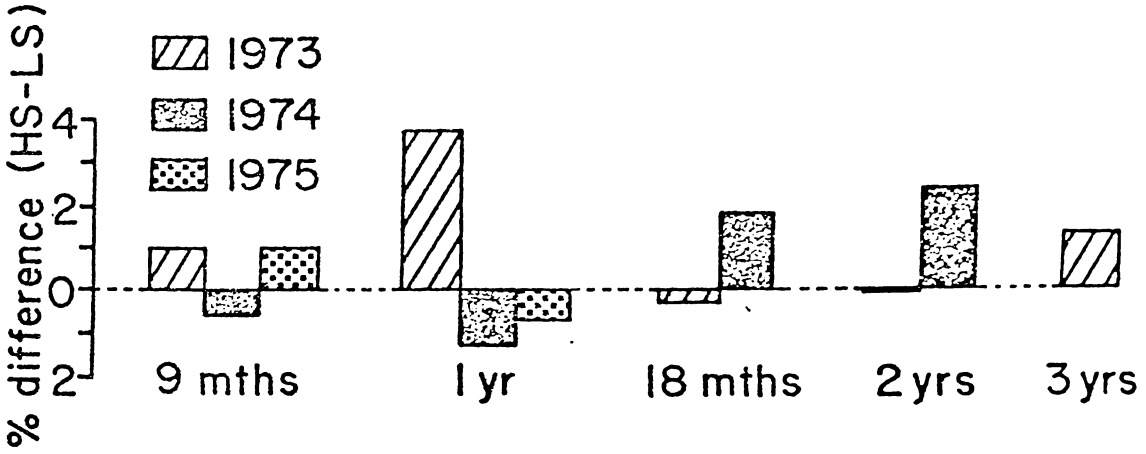


FIGURE 4.4.6 Mean Band 6 percentage differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months two years and three years of age.
The original from which this data was derived is presented below.

Mean values for Band 6

		9 months	1 year	18 months	2 years	3 years
1973	HS	3.4	6.4	2.5	4.9	4.1
	LS	2.4	2.7	2.8	5.0	2.8
1974	HS	2.8	3.0	5.1	5.8	-
	LS	3.4	4.3	3.3	3.5	-
1975	HS	5.4	3.2	- =	-	-
	LS	4.4	3.9	-	-	-
5% Difference*		1.5	2.3	2.1	1.8	1.9

* Difference required for significance at the 5% level

The 1973 HS animals had a higher band 4 proportion than the LS animals at one year of age, but at two and three years of age, the differences were not significant at the 5% level (Figure 4.4.5). There were no significant differences in mean bloat grades between the 1973 HS and LS animals at these different ages (Figure 4.4.3). However the 1974 animals showed band 4 levels more in line with their HS/LS bloat differences.

Band 6 protein

At nine months of age there was a significant year born effect for band 6 (2.9, 3.1 and 5.0% respectively, for the 1973, 1974 and 1975 progeny), and a year born x sire group (HS/LS) interaction at one year of age. This interaction resulted from the high band 6 proportion of 6.4% for the 1973 HS animals, compared with 2.7% for the LS animals, while for both the 1974 and 1975 progeny the mean band 6 values for the HS animals were lower than for the LS animals (Table 4.4.1). There was also a significant year born x sire group interaction at 18 months of age and this was the result of the low band 6 proportion of 2.5% for the 1973 HS animals compared with 5.1% for the 1974 HS animals (Table 4.4.1).

The mean HS - LS differences for band 6 protein tended to be in line with the mean HS - LS bloat differences at 18 months, two years, and three years of age (Figure 4.4.6).

Band 7 protein

At nine months of age there was a significant year born effect for band 7, the 1975 animals had 5.5% band 7 compared with 11.3 and 11.1% for the 1973 and 1974 animals respectively. At 18 months and two years of age there was a significant year born x sire group interaction, the means for these interactions are given in Table 4.4.1. These interactions resulted from the 1973 HS animals having higher band 7 values than the LS animals at both ages, whereas the converse was true for the 1974 HS animals. The average bloat grade at these two age groups were similar for the HS and LS 1973 animals, but there were large HS/LS bloat differences for the 1974 animals (Figure 4.4.3).

The HS - LS animal differences for band 7 protein shown in Figure 4.4.7 illustrate the consistently higher band 7 value for the 1974 LS compared with the HS animals. These 1974 progeny were shown to have consistent HS/LS differences in average bloat grade, except at one year of age (Figure 4.4.3).

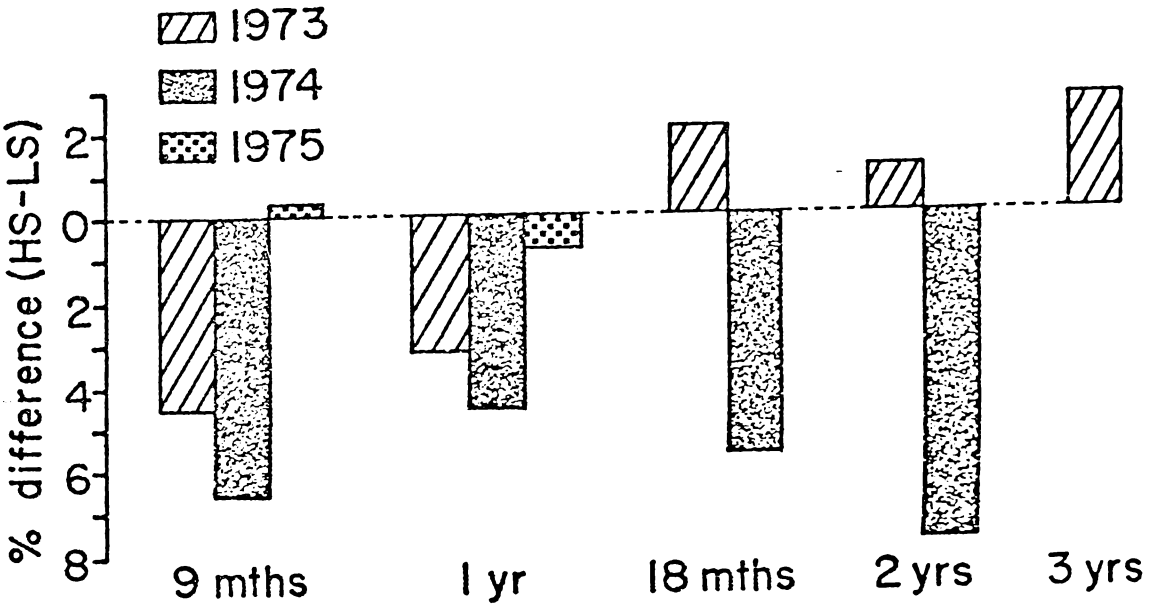


FIGURE 4.4.7 Mean Band 7 percentage differences (HS/LS) between the HS and LS sire groups for the 1973, 1974 and 1975 progeny at nine months, one year, 18 months, two years and three years of age.
The original from which this data was derived is presented below.

Mean values for Band 7

		9 months	1 year	18 months	2 years	3 years
1973	HS	9.0	6.8	4.2	7.0	4.3
	LS	13.5	10.0	2.2	5.9	1.6
1974	HS	7.9	6.3	4.90	2.3	-
	LS	14.4	10.8	10.50	9.9	-
1975	HS	5.1	10.7	-	-	-
	LS	4.8	11.5	-	-	-
5% Difference		4.3	6.2	2.6	4.2	4.6

* Difference required for significance at the 5% level

Relationships between variables

The relationships between bloat, saliva flow, total protein, and protein bands for the 1973, 1974 and 1975 progeny were tested for by a simple regression and multiple regression approach, however as the results did not differ from those previously reported for these variables, they are not reported here.

Discussion

The greater saliva flow rate shown for the male animals at one year of age for the 1974 and 1975 progeny compared with the females, was of doubtful biological significance, because the saliva flow rate at nine months of age for the females was virtually as high as the males at one year of age (2.53 and 2.63 ml/min respectively). The high band 7 and high total protein seen at nine months of age for the 1974 animals suggested that some environmental component was influencing the levels of the proteins in saliva.

There tended to be a decrease in total protein and an increase in saliva flow with increasing age, but this could have been a sampling artifact resulting from collecting more parotid saliva in the older animals, because the same size bit was used to collect saliva from all the animals from nine months of age and older. Whether the increase in mouth size with increasing age, influenced the proportion of parotid saliva collected, could be tested by measuring the amount of sodium and potassium in the saliva samples collected at the different age groups, because there is a marked difference in the concentration of these parameters between the secretions from the parotid and mandibular glands in cows (Chapter 1.2).

Whether or not one year of age is of special significance in determining an animals future bloat susceptibility is not known, but at one year of age none of the three progeny groups showed mean bloat grade differences between HS and LS animals, despite the high bloat potency of the pasture. Subsequently, only the 1974 progeny showed HS/LS bloat differences, but both the 1973 and 1974 progeny tended to have higher mean band 4 levels in the HS sire group compared with the LS sire group. Although the saliva was not collected at the time of bloat grading, the results showed that bands 4 and 6 in saliva tended to be highest around the age when the bloat grade was at its maximum. This is in line with the results from the Clover/Grass Pasture type trials (Chapter 4.2).

In general these results would support the hypothesis that bloat susceptibility is under multifactorial control and that the level of band 4 is merely one factor which is important in determining the bloat susceptibility of an animal.

Another salivary protein thought to be of importance to bloat susceptibility in cattle was band 7. This protein had previously been demonstrated to be related to saliva flow and individual cow variance, and these results demonstrated that the 1974 LS animals consistently had a higher proportion of this protein compared with the 1974 HS animals. These 1974 animals were the only progeny group to show consistent HS/LS bloat grade differences and therefore the higher levels of band 7 in the LS animals might suggest that band 7 is an important anti-bloat factor.

Summary

At nine months and one year of age the levels of band 7 in the HS animals were lower than the LS for both the 1974 and 1975 progeny, but results were only significant at nine months of age. There was a considerable environmental effect at nine months of age, both the percent band 7 and total protein being markedly increased in the saliva of the 1974 animals.

Studies on the three age groups, animals born in 1973, 1974 and 1975 (at constant age and constant time) showed the following results.

Greater saliva flow for the LS than the HS progeny groups was consistently demonstrated for the 1973 progeny, but only at two years of age for the 1974 progeny. These 1974 progeny had the highest saliva flow of the three groups. There was some indication to suggest that saliva flow increased with age while total protein decreased. The 1974 animals were the only ones showing consistent sire group differences for bloat susceptibility.

Protein bands: There was an age/year born effect, the older animals having a higher proportion of band 1. HS/LS sire group differences for band 4 were shown at all ages for the 1973 animals except at 18 months of age when the band 4 values were low for all three age groups. At 18 months, and two years of age the 1974 sire groups showed band 4 differences. When all three age groups were sampled at a constant time, the HS progeny had a significantly higher mean band 4 value than the LS progeny, despite only the 1974 animals having consistent HS/LS bloat

differences. Band 6 values showed a similar pattern to band 4 except that the band 6 results were more in line with the bloat differences.

The 1974 progeny, the only animals with consistent bloat differences between HS,and LS sire groups, also showed band 7 differences. The HS progeny having a lower proportion of band 7, the values of which were in line with the low values seen for both HS and LS sire groups for the 1973 progeny.

4.5

A STUDY OF ERRORS INVOLVED IN ASSESSING POLYACRYLAMIDE GEL ELECTROPHORESIS ON A QUANTITATIVE BASIS

In running a series of gels to compare salivary components between animals or groups of animals there will be a number of sources of variation. If treatments or other effects are to be compared then animal variation itself is a source of 'error'. Thus 'error' is variation relative to the hypothesis being examined, or variation that is not of immediate experimental interest in itself but which is liable to conceal that which is of interest. In general 'error' will conceal real results whereas bias in methods may produce false ones.

Possible sources of variation in the standard saliva group samplings were;

At the animal level:

1) Between animals or groups (sires) of animals
2) Between samplings of the same animal
   a) Under apparently the same conditions
   b) Different ages or seasons
   c) Different imposed treatments
   d) Different times of day, (e.g. since last feed)

At the sampling level:

1) Effect of different bit shape and size
2) Different times of day
3) Animal handling
4) Handling of samples up to electrophoretic or other analytical procedures
   a) Freezing and thawing of saliva samples (including temperature effects in general if not kept at a constant $4^{o}C$)
   b) Centrifugation
   c) Time of storage

At the electrophoretic level:

1) Any factors affecting protein entering the gel
2) Gel position during electrophoresis and destaining

3) Dye binding affinities and the range of linearity for the individual proteins
4) Dye fading, or less stable protein-dye complex for some proteins
5) Homogeneity of gel, or gels
6) In general variations (a) between gels within runs
(b) between runs
7) Any random variation of the scanner, including effects associated with varying gain and zeroing
8) Determination of the band boundaries on the scan

Animal and sampling errors are dealt with elsewhere in this thesis. This section is mainly concerned with the errors and problems of interpretation at the electrophoretic level. A preliminary discussion of the relationships between band integrations and bands expressed as a percent was discussed in Chapter 3.5.

This section describes the results of investigating the effects of the following:-

(a) Salivary proteins in PAGE measured by total integrations:
(1) Relationship with total protein in saliva
(2) Variation between repeats of the standard saliva, and position variations between and within electrophoretic runs
(3) Variation between electrophoretic runs for standard saliva in one position in the electrophoretic destainer

(b) Variation measured in various ways for bands 1, 4, 7 and 8:
(1) When repeats of standard saliva were used
(2) When repeat saliva samples collected from the same animals were used

(c) Band inter-relationships using different measurements systems, (corrected integrations compared with percentages)

(d) The acetic acid stimulation trial, using the 1973 progeny. Individual protein bands expressed as integrations and as a percent of the total.

Materials and Methods

Animals used in this work were the 1973 progeny, these animals were sampled in June 1976, during the course of an acetic acid/clover stimulation experiment. This experiment was designed to reinvestigate the acetic acid stimulation effects demonstrated in the Group 1 animals (Chapter 3.3) and secondly, to test for a red clover smell or sight effect (psychic response).

Experimental design

The twelve animals were all routine sampled (pretreatment). Six animals were then given 5 ml of acetic acid (pH 2) on the tongue, followed immediately by the saliva collection. The second group of six animals were shown red clover (some being crushed) at the commencement of the saliva collection. The animals were divided into three HS and three LS per group, and the sampling was done in a random order on two successive days (six per day).

Three repeat PAGE runs (seven gels per run) of standard saliva were carried out. Standard saliva (described in Chapter 2.2.c) should have a constant effect of dye affinities as proteins in each sample should be present in the same proportion. Therefore the total integrations should vary only with total protein in the gel and overall dye intensity and destaining effects.

Measurement systems in these experiments were:

A) Normal PAGE run using band integrations as a percent of the total integrations

A2) Percent method as above, but calculated from B below

B) Integrations for each band corrected to a standard gain with the constant absorbance standard; these were termed, gain corrected integrations (Chapter 2.2.d)

C) Gain corrected integrations (B) of individual bands, as a percent of the respective bands in standard saliva

D) Gain corrected integrations (B), corrected to standard total integrations (4 000) by use of the standard saliva for each run

Results and Discussion

4.5.a

Salivary proteins in PAGE measured by total integrations

Relationship with total protein in saliva

At a constant gain the total integrations will be proportional to the protein in the gel and the band proportions. However because of different dye binding affinities for the different protein bands, an increase in a protein with a high dye binding affinity will increase the total integrations more than the other proteins. A secondary effect would be the decrease in the proportion of all other bands over and above the normal proportion effect discussed in Chapter 3.4.

If there were no dye binding affinity effects and if exactly the same proportion of the total protein entered the gel, then (apart from unknown random errors) there should be a correlation of 1 between total protein and total integrations at a constant gain. The regression slope would be a function of the proportion entering the gel. This gives one test of the general system for which a positive result indicates that differential dye binding affinities are not an undue distrubing factor. As 'protein entering the gel' also contributes to the variation about the regression line this will also affect the degree to which the correlation falls short of 1. Thus the sum of two unknowns are being tested.

Results from the acetic acid/clover experiment showed the regression of protein concentration on total integration (gain corrected integrations,B) had a correlation coefficient (r) of 0.68 or 46% of the variation was accounted for. When these were corrected for run differences as well as gain (measurement D) r = 0.69, or 48% of the variation was accounted for. Thus protein concentration in the gel as measured by total integrations was dependent on total protein concentration (with considerable variation remaining) but clearly integrations were a function of protein. The similar R^2 values for both measurements above, indicated that between run variation was not important in this relationship.

A subdivision of data from all control saliva samples (including the 'clover' samples) gave an r of 0.72 while the saliva samples from the acetic acid treated animals gave an r of 0.85 for the regression of protein concentration on total integrations. Thus there were more integrations per unit of protein in the saliva from the acetic acid stimulated animals,

indicating that a larger proportion of the total protein entered the polyacrylamide gel (see 4.5.d).

The total integrations and the total protein were experimentally completely independent estimates. The amount of mucoprotein present in saliva was included in the measurement of total protein and its variation, and that of the total protein measurement must account for some of the unexplained variation in the relationship between total protein and total integrations. Thus it seemed unlikely that dye binding factors were causing much 'errors' although obviously they will cause scale effects.

Variation between repeats of the standard saliva, and position variations between and within electrophoretic runs

Analysis of variance of three repeated runs with standard saliva in all seven gels gave 24% of the variation between runs and 76% within runs and a 5% coefficient of variation (average within run standard deviation used). The mean and standard deviation were 3 900 and 197. This within run error arose mainly from the two end positions in the destainer. A further run was made in which the electrophoretic positions did not correspond with destainer positions, but the lower values were still obtained for the end positions of the destainer.

Variation between electrophoretic runs for standard saliva in one position in the electrophoretic destainer

The above results suggest running a standard always at the end position of the destainer was probably a good policy. This position was correlated with the remaining positions in the run for correction purposes but was biased within the run for comparative purposes with the other gels in the run. This end position effect could be overcome by several ways; (i) avoid using the end positions in the destainer, (ii) modification of the destainer, (iii) or by use of duplicate samples, but the large within and between animal variation would indicate the use of duplicate samples is not warranted at this stage. (The band proportions remained constant despite the wide range in total integration values.)

TABLE 4.5.1 Coefficients of variation and proportions of variation between runs for corrected integrations (D) and proportions of total (A2) for bands 1, 4, 7 and 8

	Band 1	Band 4	Band 7	Band 8
Band Integrations (D)				
Coefficients of Variation %				
Within	6.6	9.7	5.4	7.8
Total	6.4	9.8	7.1	9.6
% Variation between	0.0	3.8	51.4	41.8
Band Proportions (A2)				
Coefficients of Variation %				
Within	4.5	6.5	3.7	4.8
Total	4.8	7.6	4.1	5.8
% Variation between	13.8	35.8	26.8	39.7

4.5.b

Variation measured in various ways for bands 1, 4, 7 and 8

When repeats of standard saliva were used

Three repeat PAGE runs (seven gels per run) of standard saliva were carried out. The coefficients of variation and the proportion of variation between runs for corrected integrations (D) and proportions of the total (A2) were calculated, the results are shown in Table 4.5.1.

The results in Table 4.5.1 showed that by dividing by the total (proportion method A2), decreased the coefficient of variation in every case, presumably because it removed the variation between gels as well as within gels while the method D, or method C (described above) adjusted for between runs only.

For integrations (method D), both bands 1 and 4 had almost all the variation within a run, (i.e. the correction for total integrations between runs had been successful, for these bands). However bands 7 and 8 still varied as much between runs as within, suggesting that for the standard saliva these bands were varying for factors besides those affecting total integrations between runs.

For the proportions (method A2) variance between runs was more uniform for all bands, being increased for bands 1 and 4, and decreased for bands 7 and 8. This proportioning of variance was perhaps to be expected as the proportion method should have ironed out the variation reflected in the totals for both between and within runs in the same way.

The high coefficient of variation for both proportions and integrations for band 4 after most other sources of variation were accounted for was disturbing, (note the same standard saliva was used for each gel). Presumably this high coefficient of variation arose from the difficulty of delineation of this band.

When repeat saliva samples were collected from the same animals

Only the proportion method repeatability (method A above) could be checked as all these samples were run twice (runs). Assuming no clover effect for these animals, the 'clover stimulation' saliva samples were regarded as repeats.

Analysis of variance for bands 1, 4, 7 and 8 showed no variation for band 1, and none from repeat or repeat x runs for the other bands.

TABLE 4.5.2 Correlations between band 1 and band 7 in standard saliva expressed as; (a) percentages, (b) integrations, and the correlations of the protein band integrations with total integrations

	Band 7		Total
	Integrations	Percentage	Integrations
Band 1			
Integrations	+ 0.47		+ 0.67
Percentage		- 0.22	
Total integrations	+ 0.82		

Standard saliva, based on 21 repeat samples
At the 5% level of significance, r = 0.43

TABLE 4.5.3 Correlations between band 1 and band 7 in saliva collected from the 1973 progeny expressed as; (a) percentages, (b) integrations, and the correlations of the protein band integrations with total integrations, and total protein

	Band 7		Total	Total
	Integration	Percentage	Integrations	Protein
Band 1				
Integrations	- 0.41		+ 0.65	+ 0.95
Percentage		- 0.77		+ 0.56
Band 7				
Integrations			+ 0.27	+ 0.23
Percentages				+ 0.34

Correlations for the 1973 progeny based on 12 animals
At the 5% level of significance (10 d.f.) r = 0.57

However the other bands showed 10 - 20% of the variation associated with runs. This showed as a small decrease in bands 7 and 8. Of the variation remaining, that for band 7 was mainly between animals, 72% of the total variation, but for band 4 it was factors other than animals, i.e. errors (unknown, delineation etc) or inconsistent run differences between animals. This confirmed the standard saliva results in suggesting that band 4 was a problem area and that inconsistent relationships and differences found may be associated with techniques as well as the environmental effects detected. (Chapter 4.2 showed the effects of bloat potent clover pasture on band 4.) Using the present technique a large number of animals were required to observe significant effects on this band.

4.5.c

Band inter-relationships using different measurement systems (Bands 1 and 7)

A preliminary discussion of these inter-relationships was given in Chapter 3.4 for the Group 2 animals. The points discussed here are (i) the generation of negative relationships in the percent method because of the common divisor of total integrations, and (ii) the positive relationships for corrected integrations (method D) because of the common effect of the amount of protein entering the gel.
These relationships have been checked by the following data:

(i) Results from the standard saliva run, where all the correlations would have been generated by the methods (and chemical relationships of proteins), and none due to the between animal effects. Results from the standard saliva are shown in Table 4.5.2. Variation in integrations in both band 1 and band 7 were related to total integrations, hence the correlation of band 1 with band 7 was probably generated. The negative sign but low correlation for the percentage, suggested that the high significant negative correlation usually observed had an animal factor in it.

(ii) The pretreatment saliva sample and the 'clover stimulation' saliva sample, results where between animal covariance might also exist. In contrast to the repeated standard saliva the band 1/band 7 negative correlation occurred for both integration and percentage units (Table 4.5.3). This suggested that previous indications of a negative between animal relationship were correct. Correlations with total integrations were less than or similar to those for the standard saliva, suggesting that such correlations came from the methods used. Correlations with total protein

reversed the band 1 and band 7 results obtained for the Group 2 animals (Chapter 3.4). The reason for this was not clear but could be due to the comparatively high percentage of band 1 in the saliva of these 1973 animals thus contributing more to the total protein. Secondly, the numbers were small in this study, and thirdly there was a dichotomy of band 7 values in this sample which could affect the result.

Discussion

The main result in addition to those already discussed for the Group 2 animals, was that of repeated runs with all gels being of the same standard saliva. This confirmed that the percentage method gave lower errors than the other methods, as shown by the coefficent of variation. Thus the advantage of the different interpretation of the actual integrations must be weighted against its higher coefficient of variation.

Correction for between run differences by using a standard improved the integration results slightly but left the major sources of error, that between gels within runs, and that which was probably from the delineation of bands. This latter error in the standard meant that correcting for individual bands (method C above) could introduce more errors than it adjusts. Such error would not be random but would bias all gels in a run and could be dangerous where samples were not randomised for running and position in the destainer.

For integrations of individual bands the different distribution of between and within run variance would suggests that method C could give differen levels of error between the different bands.

Position effects in the destainer could be minimized by having the standard in an end position and the remaining gels randomised to position. However, ultimately a modification to the destainer will be made to eliminate the end position effects, but at present the additional destaining in these end gels could be overcome by a reduced destaining time for the end positions. (Flat gels are also likely to have similar position effects as well as other problems associated with scanning the gels on the densitometer.) The percentage method had the advantage over the other methods, in that it adjusted 'automatically' for the biases which appeared to affect all bands similarily.

TABLE 4.5.4 Results of the acetic acid stimulation trial on the 1973 progeny analysed by the analysis of variance, and comparing the corrected integration method (D) with the percentage method (A2)

	Bands				Total Integrations	
	1	4	7	8		
Means as integrations						
Control	392	72	52	103	1063	
Acetic acid	351	67	238	144	1410	
$F_{1,10}$ (Anova)	n.s.	n.s.	12.1	n.s.	1.46	
			P<0.01		n.s.	
					Protein µg/ml	Saliva flow ml/min
Means as percentages						
Control	36.6	6.9	5.1	9.7	884	3.2
Acetic acid	23.6	5.2	17.0	9.6	815	8.1
$F_{1,10}$ (Anova)	2.1	n.s.	9.3	n.s.	n.s.	very large
	n.s.		P=0.02			P<0.01

This study of errors in the electrophoresis technique would indicate that band 4 measurements had a high variance other than between animals, indicating a delineation problem or some other error in the technique of measuring this protein.

4.5.d

Acetic acid stimulation trial, using the 1973 progeny

This experiment was designed to reinvestigate the acetic acid stimulation effects demonstrated in the Group 1 animals, and secondly to test for a psychic response to the sight and /or smell of red clover. There were no effects of red clover and hence the results were analysed by the analysis of variance for pre and post acetic acid stimulation of saliva flow and band constituents.

Results and Discussion

Results of the analysis of variance are given in Table 4.5.4 where the two measuring systems (described above) method D, and Method A2, are compared.

There was a clear cut increase in saliva flow for all animals. The HS animals increased five fold while the LS increased two fold.

The only significant increase was for band 7 and this was shown by both methods of measurement. Band 4 and band 8 were unchanged by either method of measurement. Band 1 did not show a significant drop in integrations or proportions, but the effect on proportions of increasing band 7 shows in a greater decrease for other bands than was indicated by the change in integrations. The Group 1 animals showed a significant drop in band 1 in the saliva samples collected post acetic acid stimulation (Chapter 3.2). However the small decrease in band 1 as measured by either integrations or proportions in the post acetic acid stimulation saliva samples from these 1973 animals was not significant, but it did not disprove the findings for the Group 1 animals.

While the total integration increase in the post acetic acid stimulated saliva samples could be due to chance, this result together with the regression results shown in Figure 4.5.1, would suggest that the band 7 increase was associated with more protein entering the polyacrylamide gel and not an increase in protein concentration. However there was a big increase in total protein secreted post acetic acid stimulation because of the large increase in saliva flow.

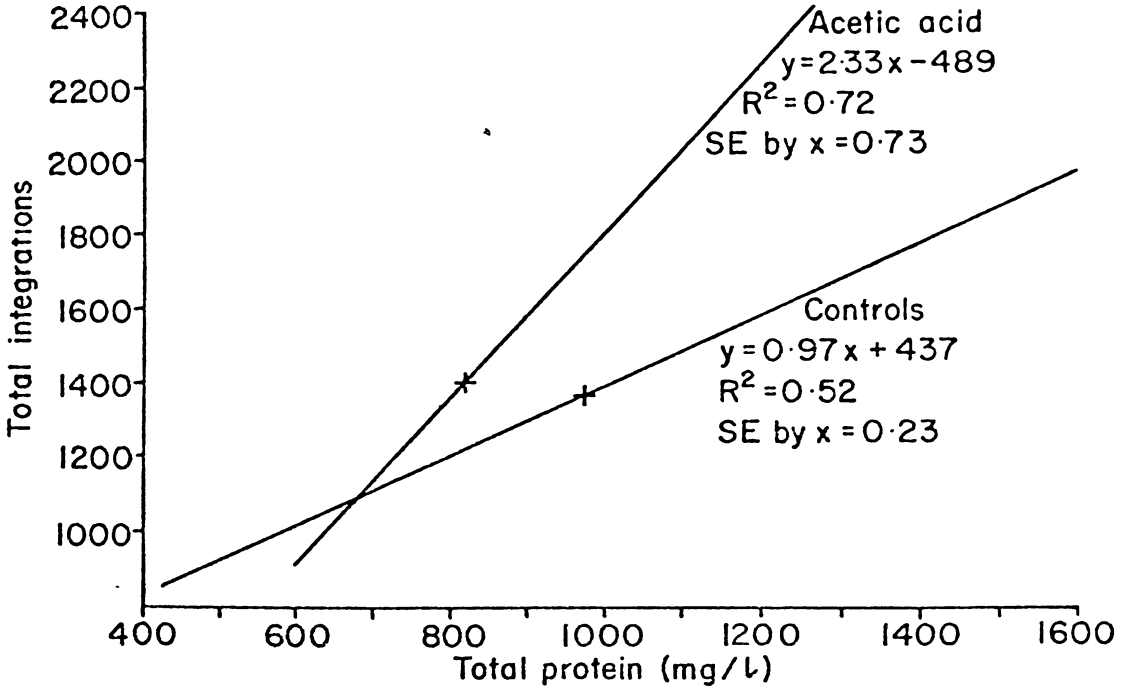


FIGURE 4.5.1 The regression of total integrations on total salivary protein (mg/l) for the control and 'acetic acid stimulated' saliva samples.

This acetic acid stimulation experiment using the two methods of measuring the protein bands, integrations (method D), and proportions (method A2), gave fundamentally the same results, which would indicate the use of percentages to measure changes in protein bands in saliva was unlikely to be misleading provided the method and its limitations were understood.

Summary

When salivary proteins in PAGE were measured by total integrations there was a clear indication that integrations were a function of total protein, furthermore the between run variation was not important. In the acetic acid stimulated saliva samples the regression of total protein on total integrations, indicated that a larger proportion of the total protein entered the polyacrylamide gel.

Three repeat runs using standard saliva showed that 76% of the variation was within runs. This was demonstrated to be due to the end positions in the electrolytic destainer.

The proportion method (A2) as compared with the corrected integration method (D) was shown to decrease the coefficient of variation in all bands. There appears to be a considerable error in the measurement of band 4. Part of the negative correlation seen between bands 1 and 7 by the proportion method was generated by the use of percentages. However the corrected integration method (D) also had a negative but low correlation (between animals) suggesting there was an animal factor in this relationship.

The acetic acid saliva stimulation trial performed on the 1973 progeny showed the same conclusions whether assessed by the band proportion method (A2) or the corrected integration method (D). The main result being that band 7 and saliva flow were increased and more of the total protein entered the polyacrylamide gel.

PART TWO

EXPERIMENTAL WORK

INTRODUCTION

In Part One of this thesis it was demonstrated that there were statistical relationships between some of the salivary proteins, separated and quantitated by PAGE, and bloat in cattle. The three proteins which appeared important in these relationships were protein bands 4, 6 and 7. Protein bands 4 and 6 appeared positively related to bloat susceptibility, and band 7 appeared negatively related to individual cow variance (in bloat grade), and to bloat susceptibility. The quantity of saliva collected by the mouth bit (which sampled saliva from under the tongue), appeared to have a negative relationships with bloat susceptibility in cattle.

Part Two of this thesis describes the investigation of the lower molecular weight proteins using biochemical and immunochemical screening procedures to gain some insight into the nature of the salivary proteins. This section of the investigation set out to answer the following questions:

a) Was it possible to develop procedures to purify these proteins?

b) What biochemical information could be gained from;
   i) general studies of whole saliva,
   ii) from semi-purified proteins?

c) Could the mucoprotein molecule in saliva be studied by electrophoresis;
   i) for quantitation,
   ii) for investigating relationships with the other proteins?

d) Were there any cationic proteins present in the saliva collected by the mouth bit?

e) Could any of these proteins be provisionally identified by either biochemical or immunochemical procedures, if so, could immunochemical procedures be used to quantitate the protein(s)?

f) Is it possible to produce antibodies against the salivary proteins, if so, could the number of protein bands seen by crossed electroimmunodiffusion be equated with those seen on polyacrylamide gel electrophoresis?

g) What relationships could be shown between salivary proteins and proteins from other secretions, and/or the serum proteins, by using bovine salivary protein antisera?

h) What was the relationship between the saliva collected by the mouth bit and that collected via the oesophagus?

Both the quantitation and characterization of the individual proteins were of some importance to understanding the relationship of these proteins to bloat susceptibility in cattle. One of the limitations of the work described in Part One of this thesis was the degree of accuracy and specificity of the electrophoretic method for quantitating the individual salivary proteins. Work described in this following section might facilitate a more accurate means of quantitating individual salivary proteins, and provide a starting point for the purification and characterization of these proteins which might prove to have some biological importance to bloat.

CHAPTER 5

PURIFICATION OF THE SALIVARY PROTEINS

In part one of this thesis it was established that various protein bands separated by analytical PAGE were statistically related to bloat susceptibility, and/or variance of bloat. These relationships were consistently reported in different groups of animals. To find the biological significance of these relationships, establishing the identity of these protein bands is of major importance. The fundamental first step towards the conclusive identification of a particular protein is to be able to separate this protein in a completely pure form.

5.1
AN INVESTIGATION INTO THE TECHNIQUES WHICH MIGHT BE APPLICABLE TO PREPARATIVE SEPARATION OF THE SALIVARY PROTEINS

Strategy for protein purification

Three important molecular properties can be made us of when purifying a protein from a heterogeneous mixture, these properties are; the pI value of the protein; the electrical charge on the protein molecule; and the molecular weight of the protein. The techniques used for purifying proteins, based on these properties vary in their severity with respect to protein denaturation. Therefore because proteins are less stable when purified, then ideally the severity of the technique should decrease with the increasing purity of the protein. The following techniques are described in order of decreasing severity with respect to possible protein denaturation. Proteins can be separated according to their pI values by isoelectric focusing, and upon their electrical charge by ion exchange chromatography, isotachophoresis, or preparative electrophoresis. Finally gel filtration (e.g. Sephadex column chromatography) can be used to separate the proteins on the basis of their molecular weights.

Reasons for purifying band 7 protein

The majority of work on the purification of the salivary proteins has concentrated on purifying band 7 protein. This was for two main reasons: (i) Band 7 was in a higher proportion than most other proteins in the mouth saliva used for the preliminary purification work.

(ii) Band 7 had been shown to be negatively related to individual cow variance (in bloat grade), and furthermore the 1974 progeny which showed HS/LS differences for mean bloat grade also had HS/LS differences for band 7, the LS animals having a higher proportion of band 7 protein compared with the HS. Therefore it was of some importance to purify this protein so that its physiological role and its possible biological relationship with bloat could be investigated.

Viscosity of saliva

The purification of salivary proteins is an especially difficult task owing to the presence of the large carbohydrate-rich mucin molecule (Bonilla 1972; W.T. Jones pers.comm.). The mucin molecule in cows saliva appears to be produced by the palatine glands (W.T.Jones & M. Guernsey, pers.comm.). There is also a high molecular weight muco-protein present in bovine saliva which is produced by the mandibular glands (Lyttleton 1960; Lyttleton 1964; Gottschalk et al. 1972; Jones & Lyttleton 1972). Saliva collected from the mouth and oesophagus of cows has a high viscosity and this has been attributed to the presence of the mucin and mucoprotein molecules (Lyttleton 1964; Jones & Lyttleton 1972). It would appear as if these glycoproteins of high molecular weight might interact with themselves and with other proteins in saliva (W.T. Jones, pers. comm.). As a result of the high viscosity and possible interaction between molecules, conventional biochemical procedures for separating proteins are of limited value unless most of these high molecular weight mucin molecules are removed.

DEVELOPMENTAL WORK ON PROTEIN PURIFICATION

As no isoelectric focusing apparatus was available for purifying the proteins, no attempts were made (at this stage) to separate the proteins on the basis of their pI values.

Both ion exchange chromatography and Sephadex gel filtration were unsuccessful in separating the salivary proteins which had been concentrated by ultrafiltration. Likewise these procedures gave poor results when the salivary proteins were concentrated by precipitating with ammonium sulphate and redissolved in the appropriate buffer.

Centrifuging saliva at 10 000 g-min for 60 min was shown to remove most of the mucin, and therefore this procedure was adopted as the first step in the protein purification; this saliva was termed

centrifuged saliva. Attempts were made to separate by ultrafiltration, the proteins of low molecular weight from the large molecular weight mucoprotein and remaining mucin molecules. Filters with a cut off point of up to 300 000 daltons were all unsuccessful becuase the mucin and mucoprotein molecules adhered to the surface of the filtration membrane and prevented the majority of the lower molecular weight proteins from passing through

Protein precipitation

Ammonium sulphate successfully precipitated most of the proteins from saliva when used at a concentration of 45% (w/v). However, fractionation of the salivary proteins using different concentrations of ammonium sulphate gave inconsistent results between the different batches of saliva. It was thought that the lack of repeatability between batches of saliva might have been due to the variation in the amount of mucin and mucoprotein in the saliva.

Precipitation of the proteins with acetone proved too severe and resulted in most of the proteins being denatured. Ethanol was tried as a protein precipitant and was shown to work satisfactorily; it proved a successful method of fractionating the proteins in saliva. An 80:20 ethanol:saliva (v/v) ratio was shown to selectively purify the bands 7, 8 and 9 proteins. Ethanol precipitation of the salivary proteins was therefore adopted as the next step in the purification of the band 7 protein.

Ion exchange chromatography

Ion exchange chromatography using DEAE Sephadex had previously given poor results when attempts were made to use this procedure to separate the salivary proteins. However, the proteins recovered from the ethanol precipitation of centrifuged saliva, when fractionated on ion exchange chromatography, gave a semi-pure preparation of band 7 protein which contained some band 8 and 9 proteins. Ion exchange chromatography was therefore incorporated as the next stage in the purification procedure.

Preparative electrophoresis

Prior to commencing work on the purification of the salivary proteins, it was thought that preparative electrophoresis and/or isotachophoresis might have some advantages over ion exchange

chromatography when separating proteins on the basis of their electrical charges. An LKB Uniphor preparative electrophoresis apparatus was purchased for this work, however because of the complexity of the apparatus, and being the first such apparatus in New Zealand, several small technical problems were encountered which resulted in delays in developing suitable purification procedures.

A series of different polyacrylamide gel techniques were tried in the Uniphor, using saliva which had been concentrated by ultrafiltration, or proteins precipitated by ammonium sulphate. From these trial runs it was concluded that although small yields of semi-purified proteins could be recovered, the mucin or mucoprotein present appeared to hinder the separation. The ability to separate proteins by preparative polyacrylamide electrophoresis can be illustrated by the results of an early test run where a mixture of albumin and transferrin were being separated on a 10 cm column; the main albumin peak and the main transferrin peak were separated in time by 14 hours.

Recently a 6% polyacrylamide electrophoresis technique incorporating a 2.5% stacking gel has been tested, and this procedure appeared to enhance the resolving power of the polyacrylamide gel electrophoresis when separating the proteins with lower molecular weights from preparations containing mucins and mucoproteins. However, this technique is at present at an early stage of development and will require further testing before being used as an alternative procedure for separating proteins according to the electrical charge on the protein.

A powder gel preparative electrophoresis technique, base on the method of Rosenbaum & Baker (1971), was also used for protein purification. The two theoretical advantages of this procedure were: (i) It made use of the Kohlrausch principle (Routs 1971), thus allowing a relatively large sample to be concentrated into a compact zone at the start of the electrophoretic run. (ii) The nature of the separating media should allow more protein to be separated in any electrophoretic run. This procedure, like the previous two procedures gave the best results when the proteins to be separated had been partially purified by centrifuging the saliva and selectively precipitating the band 7 protein with ethanol. This method was either used in conjunction with, or instead of ion exchange chromatography.

A flow diagram for the partial purification of the band 7 protein is given in Figure 5.1.1. The next subsection (5.2) gives the details on the methods resulting from these developmental investigations.

Figure 5.1.1 Protein Purification Flow Diagram

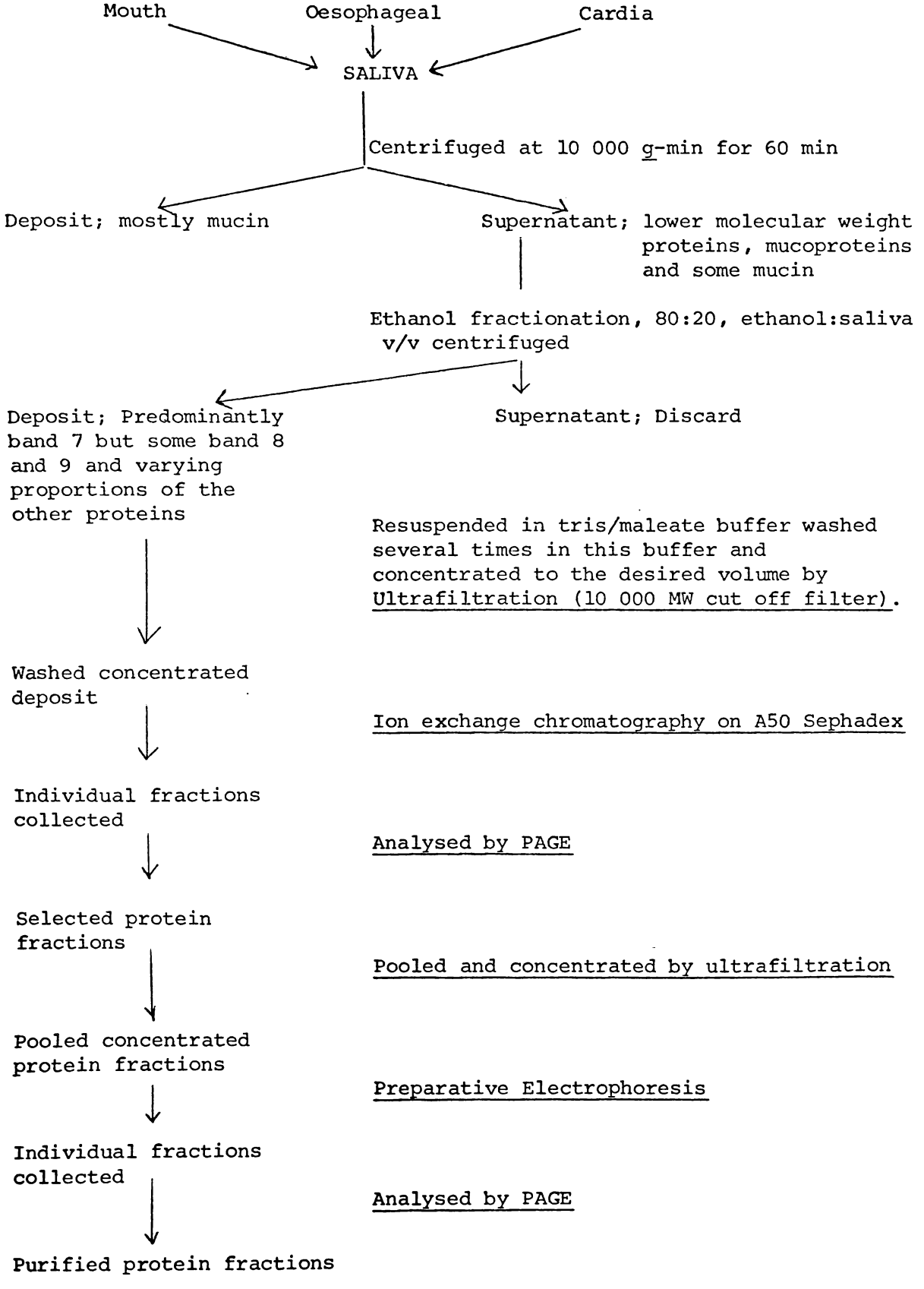


Table 5.2.1 Fractionation of the Salivary Proteins by Ethanol Precipitation

Ethanol concentration (v/v)	Protein bands increased in proportion	Total protein concentration (ug/ml)
10%	4 & 6	30
20%	6 & 11	240
50%	No protein bands	200
70%	5, 8, 10 & 11	140
80%	7, 8, 9 & 10	480

5.2

TECHNIQUES USED FOR PURIFYING THE SALIVARY PROTEINS

This section describes the techniques used to purify the band 7-8-9 complex, as outlined in the flow diagram ullustrated in Figure 5.1.1. No specific description is given for the ultrafiltration as this was carried out according to the manufacturers recommendations using 50 ml capacity ultrafiltration apparatus in conjunction with 10 000 molecular weight cut off filters,

5.2.a

Removal of Mucin by centrifugation

Saliva was collected from one of three sources, mouth collection using the standard bit, oesophageal collection, or cardia collection. The saliva was placed into 250 ml centrifuge flasks and centrifuged at 4°C for 60 min at 10 000 g-min using the MSE 18 High Speed centrifuge. After centrifugation the saliva was decanted into a large Ehrlenmeyer flask, ready for the next stage of purification.

5.2.b

Ethanol precipitation of the salivary proteins

Saliva and absolute ethanol were brought to 4°C and mixed. The saliva/ethanol mixture was stirred several times and then allowed to stand at 4°C overnight. The mixture was then centrifuged at 10 000 g-min for 10 min and the deposit obtained was resuspended in the appropriate buffer (depending upon the next stage of purification). The proteins in the deposit were then washed several times using the same buffer, and concentrated to the desired volume by ultrafiltration using a 10 000 molecular weight cut off filter.

An 80:20 (v/v) ethanol:saliva mixture was shown to produce the best recovery of band 7 protein. Table 5.2.1 illustrates the type of protein fractionation achieved at different ethanol:saliva ratios.

5.2.c

Ion exchange chromatography using DEAE-Sephadex

Reagents

Tris/maleate (tris/maleic acid) buffer; 0.5 mol/l, adjusted to pH 8.0 with sodium hydroxide.

DEAE-Sephadex A-50; this is an anionic media produced by Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of the A-50 Spehadex slurry

A-50 Sephadex was washed in 0.5 mol/l tris/maleate buffer. This buffer was then removed and 0.01 mol/l tris/maleate buffer, pH 8.0 added. The A-50 Sephadex was then soaked overnight, followed by washing five times in this buffer. This washed slurry was used to pack the chromatography column.

Chromatography column; internal diameter 26 mm, height 400 mm.
Ionic gradient; an LKB gradient former was used to form a linear gradient.
Ionic gradient solutions; Flask A 0.01 mol/l tris/maleate buffer, flask B sodium chloride 2.0 mol/l in 0.01 mol/l tris/maleate buffer.

Sample and sample application

Saliva which had been centrifuged to remove mucin was then treated with ethanol (80/20; ethanol/saliva) and the proteins which were precipitated were washed by ultrafiltration using the 0.01 mol/l tris/maleate buffer. Up to 100 ml of these semi-purified salivary proteins in 0.01 mol/l tris/maleate buffer were added to the packed washed chromatography column by means of a small reservoir which was connected to the column inlet tube and controlled by a four way valve.

Running the chromatography column

After the sample was added, working buffer was run through the column so as to elute any unbound proteins. The column outlet was monitored through a Uvicord 11 and the fractions collected by means of an LKB Minirac fraction collector* When the transmittence had settled down to the base line the ionic gradient was commenced.

5.2.d

Preparative electrophoresis using a 6% polyacrylamide gel

The 6% polyacrylamide gel preparative electrophoresis used in the Uniphor was adapted from the analytical PAGE used routinely for the study of salivary proteins.

Reagents

Stock buffer; tris/glycine 1 mol/l, pH 9.0
Electrode buffer; 50 ml stock buffer diluted to 1 l with distilled water

* The only fraction collector available throughout these investigations was basically unsuitable for ion exchange chromatography as fractions could only be determined on the basis of time rather than volume.

Running buffer; 14 ml of stock buffer diluted to 100 ml with distilled water.

TEMED (N,N,N',N'-tetramethylethylenediamine) (Eastman Kodak Co., Rochester, New York 14650, USA) Product No. 8178. 10 ml of TEMED made up to 100 ml with ethanol.

Acrylamide working solution; Acrylamide (Eastman Kodak Product No.5521) 12.0 g, Bis (N,N'-methylenebisacrylamide) (Eastman Kodak Product No. 8383) 0.2 g, made up to 100 ml with distilled water.

Ammonium persulphate 10% solution; ammonium persulphate 1.0 g, made up to 10 ml with distilled water.

Preparation of acrylamide for polymerization

Acrylamide working solution	25 ml
Running buffer	25 ml
TEMED	1 ml
Ammonium persulphate	1 ml

Air was evacuated from the acrylamide solution and running buffer and these solutions were mixed on a magnetic stirrer. TEMED and ammonium persulphate were then added and gently mixed.

The unpolyermized gel solution was then poured into the Uniphor preparative electrophoresis column which had been fitted with its gelling stopper. A small quantity of distilled water was then layered onto the unpolyermized gel solution so as to form a sharp flat interface when polymerization took place.

After polymerization was complete (at least 2 h at room temperature) the gelling stopper was removed and the column was attached to the electrophoresis apparatus, which in turn was set up according to the manufacturers instructions. An electrophoretic pre-run was carried out to enable the air to be evacuated from the elution stopper and allow the system to stablize before applying the sample.

Sample application and running the electrophoresis

After the electrophoretic pre-run, about 3 ml of concentrated salivary proteins were added to the top of the polyacrylamide gel by means of a long glass tube. The current was then turned on to give 300 V constant voltage, (approximately 9 mA), and this voltage was held for 30 min in which time the proteins entered the polyacrylamide gel.

The voltage was then increased to 600 (approximately 16 to 20 mA), and this was maintained throughout the fractionation of the salivary proteins.

The eluant from the electrophoresis column was monitored and collected as described for ion exchange chromatography.

5.2.e

Powder gel

The powder gel preparative electrophoresis used in this study was based on the method of Rosenbaum & Baker (1971).

Reagents and materials

Tris/glycine buffer, pH 8.3; tris 3.0 g, glycine 14.4 g, made up to 1 l with distilled water.

Tris/HCl buffer, pH 8.2; tris 3.0 g, titrated to a pH 8.2 with 1N HCl and made up to 1 l with distilled water.

Biogel P60; produced by Bio-Rad Laboratories, Richmond, California, USA.

Biogel P60 slurry; The Biogel P60 was soaked in tris/glycine buffer. The Biogel was allowed to settle to the bottom of the flask and the supernatant removed by suction. This was repeated several times over a period of 24 h.

Procedure

The Uniphor electrophoresis column was connected to the elution stopper and mounted on the Uniphor apparatus. It was then filled with tris/glycine buffer and the powder gel packing tube attached to the column and filled with the gel slurry. The outlet of the elution stopper was opened and the gel packed under atmospheric pressure. Tris/glycine buffer (200 ml) was then allowed to flow through the column. This was followed by 20 ml of tris/HCl buffer, after which the sample (dissolved in 25 ml tris/HCl) was applied, and allowed to run into the gel. After the sample had entered the gel the flow through the column was stopped and tris/glycine buffer was placed above the column of powder gel and in the upper buffer reservoir. The power was then turned on to 500 V constant voltage (approximately 16 mA) and this voltage was maintained throughout the separation.

The monitoring of the eluant and collection of fractions were performed, as described for the ion exchange chromatography.

DISCUSSION

The results from the work on the development of procedures for purifying the salivary proteins can only be considered qualitative, as no purification tables have been constructed to indicate the degree of purification achieved at each step. Although the PAGE was used to monitor the presence of band 7 protein throughout the various steps in the purification, the actual concentration of protein recovered at each step in the purification was not determined.

The procedure developed for purifying the salivary proteins resulted in purifying a preparation of band 7 which contained some band 8 and 9 proteins. The proportion of bands 8 and 9 present in the semi-purified band 7 preparation was about the same for each purification, regardless of the saliva used, and therefore this preparation was termed the band 7-8-9 complex; studies on this band 7-8-9 complex are discussed in subsequent chapters.

For future work, the recovery of a good yield of band 7 protein will be facilitated by using saliva collected from cows which have first been stimulated with acetic acid (Chapters 3.3 & 4.5). Additions to the present purification procedure for band 7 protein will include incorporating a preparative isoelectric focusing method following the ethanol precipitation of the salivary proteins, and a gel filtration chromatography method after the proteins have been separated by preparative electrophoresis. This final purification procedure should result in the preparation of chemically pure band 7 protein suitable for biochemical characterisation and the preparation of monospecific antiserum.

SUMMARY

A partial purification procedure was worked out for some of the salivary proteins. This involved the following steps. centrifugation to remove some of the mucin, ethanol precipitation to enhance the specific band proportions, centrifugation to precipitate the proteins, ultrafiltration to remove the ethanol and concentrate the protein fraction. Ion exchange chromatography using A-50 Sephadex, ultrafiltration of the collected fractions to concentrate these, and finally preparative electrophoresis. PAGE was used to detect the protein bands at each stage of purification. The main purification was designed to specifically purify the band 7, 8 and 9 proteins.

CHAPTER 6

BIOCHEMICAL STUDIES OF BOVINE SALIVARY PROTEINS

INTRODUCTION

At least three of the salivary proteins present in the saliva collected by the mouth bit appeared statistically related to bloat susceptibility in cattle. However, nothing was known as to the biochemical nature of these protein bands, nor was it known whether more than one protein was present within an electrophoretic band, except in the case of band 4 (Clarke et al. 1974).

The biochemical screening described in this Chapter was designed to give more information on the unpurified proteins in saliva from the point of view of electrophoretic migration, the amount of carbohydrate present, whether sulphydryl groups were present, the effect of neuraminidase on the proteins, and screening for the presence of some enzymes. Studies using SDS-electrophoresis are also reported, these were undertaken to determine the range of molecular weights and the number of protein subunits present in the saliva samples. Electrophoretic studies on the mucoprotein present in the saliva collected by the mouth bit are also reported, as are the results from the screening procedures for cationic proteins in saliva.

The work described in this Chapter also includes studies on the semi-prufied proteins using the same procedures as for the unpurified proteins.

6.1
ELECTROPHORETIC BAND MIGRATION AND RELATIVE DYE BINDING AFFINITIES OF PROTEINS

The ultimate aim of the biochemical approach to the study of the salivary proteins is to identify all the protein bands present in bovine saliva to enable their biochemical and physiological roles to be determined. The purpose of this study was to look at the relative electrophoretic migrations of proteins known to be present in bovine saliva and compare these with the electrophoretic migrations of the protein bands seen in mouth saliva.

The immunoglobulins are the most extensively studied class of protein found in the saliva of ruminants; examples of these studies are cited in Chapter 1.2. Albumin has also been reported to be present in saliva (Chapter 1.2) and in the developmental stages of this work albumin was used as a control protein to enable an accurate check to be made on the relative electrophoretic migrations between runs. The first part of this chapter discusses the electrophoretic migration of immunoglobulin G and albumin in relation to the protein bands detected in mouth saliva, (6.1.a).

The relative dye binding affinities of different proteins vary considerably. However for a standard electrophoretic separating and staining procedure, the dye binding affinity of any particular protein should be relatively consistent between runs, (for further information on electrophoresis see Chapter 4.5 and Appendix A). However an important point from the point of view of quantitating proteins by electrophoresis, is whether there is a linear relationship between the amount of dye bound and the concentration of the protein; i.e., is Beers Law obeyed over the concentration range being studied?

A resume of the results comparing the relative dye binding affinities of three serum proteins, immunoglobulin G, albumin, and transferrin is given in Chapter 6.1.b. The individual protein bands in the standard saliva were studied to determine whether there was a linear relationship between the amount of dye bound and the amount of protein present. However the scope of this work on the salivary proteins was limited because the concentration of the individual protein bands were not known; the results are summarised in Chapter 6.1.c.

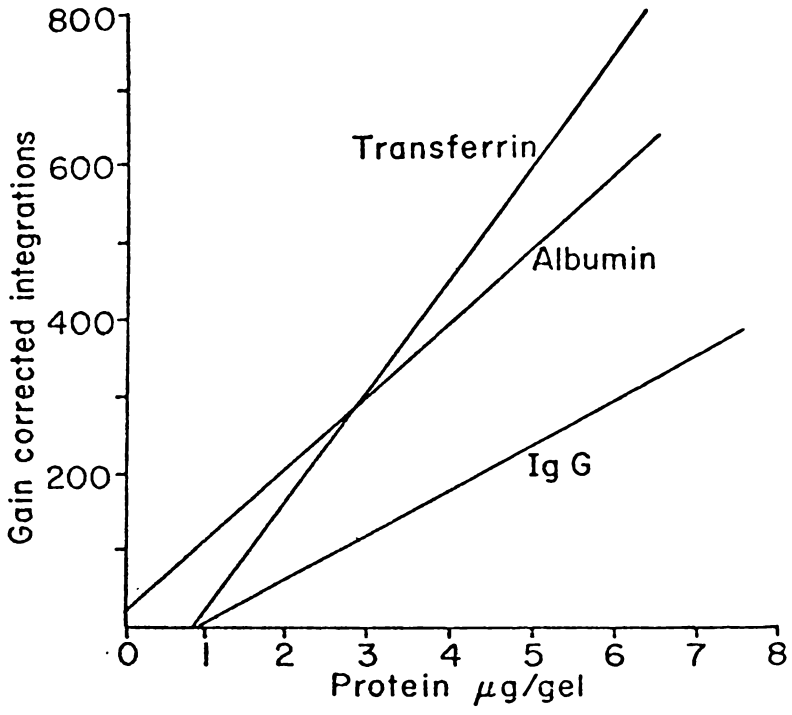


FIGURE 6.1.1 Relative dye binding affinities for the three serum protein, albumin, transferrin and IgG.

Materials and Methods

Bovine serum albumin; obtained from Sigma Chemical Company, Missouri, USA; product number A 4378.

The purified immunoglobulins, Ig G, Ig G_1, and Ig G_2 were obtained from Miles Laboratories Ltd., Slough, England; their product numbers were 64-140, 64-341 and 64-342 respectively.
Note - no purified Ig A was available commercially

Results

6.1.a

The relative electrophoretic migrations of albumin

The mobility of bovine serum albumin was compared to the relative electrophoretic mobility's of the individual salivary protein bands when run on PAGE. The monomer of the albumin had an identical electrophoretic migration to band 6 protein, while the dimer of albumin migrated within the band 4 region. When bovine serum albumin was added to saliva an increase in the band 6 region was detected.

Electrophretic migration studies on the immunoglobulins

Bovine Ig G was shown to migrate within the band 1 region on PAGE while the purified Ig G_1 spanned the bands 1 and 2 regions and purified Ig G_2 migrated in the pre-band 1 and the band 1 regions of the gel.

No purified Ig A was available commercially, and the only Ig M available for testing appeared impure, being composed of a number of bands most of which were confined to the pre-band 1 and bands 1, 2 and 3 regions.

6.1.b

The relative dye binding affinities of albumin, transferrin, and immunoglobulin G

Three serum proteins, transferrin, albumin, and immunoglobulin G were examined for their relative dye binding affinities. The protein values for these samples were determined by the Lowry method. The results are illustrated in Figure 6.1.1. Both the slope and intercept of the lines differed (regressions not calculated), albumin having twice the dye binding affinity of the immunoglobulin G sample, while transferrin had the highest dye binding affinity of all three proteins.

6.1.c

The linearity range for the dye binding/protein concentration relationships for the salivary proteins

The standard saliva had a protein concentration of 1 480 ug/ml, and all the proteins seen in the mouth saliva samples were present in this preparation (described in Chapter 2.2.c). The standard saliva at varying concentrations (20, 40, 60, 80, 100, 150 and 200% of the normal sample) was studied by PAGE, the gels being stained with Coomassie Brilliant Blue R250. Integration for the individual protein bands were plotted against the total protein concentration, and in all cases the results suggested a linear relationship over the range of concentrations tested. Because the individual protein band concentrations were not known it was not possible to work out relative dye binding affinity's for the individual bands.

Discussion

The presence of albumin in saliva has been reported by numerous workers (Chapter 1.2), and these results showed that bovine serum albumin and band 6 protein had the same relative electrophoretic migration. It was therefore postulated that band 6 protein might be partially or wholly composed of albumin. Further work to test this hypothesis was undertaken and this is described in Chapters 6.7.a, 7.1 and 7.2

The predominant immunoglobulin found in bovine saliva is secretory - Ig A; this molecule is thought to have a molecular weight of 420 000 (Pahud & Mach 1970). No purified Ig A or Secretory - Ig A was available to test the relative electrophoretic migration of this protein but the Ig G molecules were shown to migrate in the pre-band 1 to band 2 region on PAGE. It was concluded from this initial work on the immunoglobulins, that the band region at the cationic end of the gel (at least up to band 2) could contain some of the immunoglobulins found in saliva. Electrophoretic studies on human saliva indicate that the immunoglobulin molecules are located in the protein bands at the cathode end of agar gels when run at an alkaline pH (Hurlimann & Zuber 1968) and in the three slowest migrating bands seen on polyacrylamide gel electrophoresis (Bellavia et al. 1972).

Further work on the immunoglobulins is described in Chapters 6.3, 6.6, 6.7 and 7.3.

The three serum proteins tested showed a large difference in their relative dye binding affinities. An increase of one unit of albumin gave the same increase in integrations as two units of immunoglobulin G. Therefore it is quite probable that differences of this magnitude exist for the relative dye binding affinities of the salivary proteins. The relative dye binding affinities of the individual salivary proteins could only be tested by obtaining purified samples of the proteins. Once purified samples of these proteins are available, immunochemical techniques having a higher degree of accuracy than the electrophoretic procedure will be utilized to quantitate the individual proteins in saliva.

6.2

ELECTROPHORETIC SCREENING FOR GLYCOPROTEINS IN MOUTH SALIVA

As part of the overall plan to characterise the proteins found in mouth saliva a series of studies were undertaken to determine which of the salivary protein bands entering the 7.5% polyacrylamide gel on electrophoresis, were strong glycoproteins. Two different staining techniques were used to screen for carbohydrate containing molecules (glycoproteins), these were the periodic acid-Schiff method and the alcian blue method. The periodic acid-Schiff reaction involves breaking the carbon bonds of 1:2 glycol groups (CHOH-CHOH) or glycol-amino groups, and converting them to aldehydes. The strongly acidic Schiff reagent then reacts with these aldehyde groups forming a purplish-red complex. The alcian blue staining reaction is based on the formation of salt linkages with the acidic groups of acid mucopoly-saccharides (Lynch et al. 1964).

The limitations of this approach to studying the glycoproteins in saliva are numerous, and two major limitations are listed below. Firstly, there might be molecules present which can enter the poly-acrylamide gel and stain positively with either periodic acid-Schiff or alcian blue, but not stain with the protein stain coomassie. Therefore a band which gives a positive reaction with one of the 'carbohydrate' stains used for detecting glycoproteins will not necessarily correspond to the protein band which stained with coomassie. Secondly, a negative reaction with either periodic acid-Schiff or alcian blue might merely indicate that the staining reaction was not sufficiently sensitive to detect the carbohydrate moiety present on the glycoprotein molecule.

Materials and Methods

Periodic acid-Schiff method for staining carbohydrate containing molecules; described in Chapter 2.2.e.

Alcian blue staining method; described in Chapter 2.2.f.

Bovine transferrin; Miles Laboratories, Slough, England, product number 82-054.

Bovine serum albumin; as described for 6.1.a.

The bovine transferrin was used as a positive control and the albumin as a negative control in these studies.

TABLE 6.2.1 Selected results showin the range of Periodic Acid Schiff to Coomassie Brilliant Blue (PAS/Coomassie) Ratios for five different saliva samples

BANDS	1	2	3	4	5
2077 (cow sample)					
PAS	36.7	29.9	22.2	7.7	3.6
Coomassie	25.9	14.4	40.0	2.1	17.5
PAS/Coomassie	1.4	2.1	0.6	3.7	0.2
9120 (cow sample)					
PAS	31.1	27.4	24.1	12.8	4.6
Coomassie	41.8	11.6	28.5	4.3	13.7
PAS/Coomassie	0.7	2.4	0.8	3.0	0.3
Standard saliva					
PAS	29.2	17.2	29.7	17.3	6.6
Coomassie	21.8	11.3	34.9	19.6	12.4
PAS/Coomassie	1.3	1.5	0.9	0.9	0.5
603 (calf sample)					
PAS	40.6	26.7	17.1	12.0	3.6
Coomassie	32.4	20.9	19.9	4.3	13.7
PAS/Coomassie	1.3	1.3	0.9	0.8	0.3
19 (calf sample)					
PAS	43.0	23.3	25.0	8.3	0.4
Coomassie	44.4	11.2	28.1	4.5	11.7
PAS/Coomassie	1.0	2.1	0.9	1.8	0.1

Calculation of Results: Bands 1-5 were all PAS positive whereas 6-11 showed no positive staining reaction. Hence total integrations for PAS staining were counted and all bands from 1-5 worked out as a percentage. Likewise for the Coomassie results bands 1-5 integrations were counted and the individual bands expressed as a percentage.

$$\text{Thus the PAS/Coomassie Ratio} = \frac{\%\text{ of integrations (bands 1-5) for PAS}}{\%\text{ of integrations (bands 1-5) for Coomassie}}$$

Results

Staining with Periodic Acid Schiff (PAS)

A good linear relationship of transferrin integrations plotted against protein concentration was found over the 50-500 µg/gel range. 250 µg of transferrin gave approximately the same number of integrations as 25 µg of mixed proteins as found in the mouth saliva.

In almost every experiment carried out to determine the PAS positive proteins, bands 1-3 gave strong positive results while bands 4 and 5 gave weak positive results, but all other bands were negative. Results from five different salivas are shown in Table 6.2.1. It was noticed that most gels stained with PAS gave a very intense band at the extreme end of the gel. The position of this band would indicate the presence of a large molecular weight glycoprotein or mucopolysaccharide such as the mucoprotein or mucin molecule, which was too large to completely enter the 7.5% polyacrylamide gel.

Band 5 and band 7-8-9 complex purified by preparative polyacrylamide gel electrophoresis (Chapter 5.2.d) and band 7-8-9 complex purified by the complete purification procedure were all negative when stained with PAS.

Staining with Alcian Blue

The Alcian Blue staining procedure is more specific for sialic acid than is the PAS procedure. Transferrin was used as the positive control for this qualitative study of the salivary proteins, however gels up to and including 250 µg of transferrin were negative when stained with Alcian blue.

Qualitatively the bands giving a positive reaction with PAS and Alcian blue were virtually the same, except that band 5 failed to give a positive result with Alcian blue. Qualitative results indicated that band 2 had a more intense Alcian blue staining reaction than the other bands, while the band 3 region gave a very low staining intensity.

Differences in the staining reactions between PAS and Alcian blue would indicate differences in the proportion of sialic acid to carbohydrate in a semi-quantitative manner. For instance, a greater proportion of staining for band 3 with PAS may suggest there is less sialic acid on this protein compared with bands 2 and 4.

The interpretation of staining reactions of whole saliva is confounded by molecules which may enter the cathode end of the gel and not stain with Coomassie but stain with PAS. Evidence for this comes from finding high molecular weight molecules in SDS-electrophoresis which only stain with PAS and not with Coomassie. It is likely that breakdown components from the mucin or mucoprotein molecules could behave in this way.

Discussion

The PAS/Coomassie staining ratio for bands 1, 3 and 5 were quite consistent for most animals, this is illustrated by the samples shown in Table 6.2.1. For band 2, three animals had a very similar PAS/Coomassie ratio, however the standard saliva and the fourth animal had considerably lower results. Saliva making up the standard saliva came from three different sources, these were; cow 9120 (concentrated saliva), cardia saliva from cow 1125 (concentrated) which had been centrifuged to remove as much mucin as possible, and thirdly a semi-purified band 6 preparation which would have been almost completely free of mucin or mucoprotein. From the studies on SDS-electrophoresis stained with PAS, and the differences found between the salivas from three of the animals and the standard saliva preparation which had most of the mucin removed, it would appear as if some of the PAS activity may be due to the presence of the mucin molecule. The band 2 protein region also had the highest Alcian blue/Coomassie ratio of all the bands when salivas from these three cows were studied. This finding is in line with the hypothesis that some of the PAS activity is due to a molecule which does not stain well with Coomassie such as the mucin molecule, because the Alcian blue is more specific for sialic acid than is PAS. Jones (pers. comm.) has shown that this mucin molecule in bovine saliva is very rich in sialic acid.

The band 4 protein region for the two animals 2077 and 9120 had a high PAS/Coomassie ratio, but a low absolute amount of band 4 protein as determined by Coomassie. The standard saliva which had a high concentration of band 4 protein, had a low PAS/Coomassie ratio, indicating that it is not the main band 4 protein which gives a strong PAS reaction but something apart from this protein. Like band 2, it is thought that this may be due to the mucin molecule.

The sensitivity of PAS technique may be inadequate to detect glycoproteins such as transferrin at the low concentrations (2-10 µg/gel) at which most of the individual salivary proteins would be present, however when available higher concentrations of purified salivary proteins will be tested.

6.3

THE EFFECT OF FREEZE DRYING, EDTA, β-MERCAPTOETHANOL AND ETHANOL ON PROTEINS IN BOVINE SALIVA

A series of experiments were carried out to investigate the effects of freeze drying, EDTA*, β-mercaptoethanol, and ethanol on saliva routinely collected from the mouth of cow 9120. Freeze drying is a convenient procedure for both concentrating saliva and storing salivary proteins and it was therefore necessary to investigate whether this treatment effected the salivary proteins. The effects of treating the saliva with EDTA was investigated as this chelating agent was found to assist in dissolving the freeze dried proteins. Thus it was important to establish whether any changes in these proteins occurred when EDTA was used. β-mercaptoethanol, a sulphydryl reagent was investigated for two main reasons, these were: (i) β-mercaptoethanol assisted with reconstitution of the freeze dried salivas when incorporated in the buffers; (ii) It was known that some proteins in bovine saliva contained disulphide bonding and thus it was important to establish which proteins changed with the β-mercaptoethanol treatment, (see Chapter 1.2 immunoglobulins). Ethanol treatment of saliva was shown to reduce the viscosity of fresh mouth saliva as judged by the time taken for a sample to run from a 0.2 ml pipette. The viscosity of the saliva was one of the major problems encountered in the purification of the salivary proteins thus any treatment which would reduce the viscosity could have importance in increasing the resolution of the individual protein bands in preparative and analytical PAGE.

Methods

In the first experiment, illustrated in Table 6.3.1 five different treatments were used, these were as follows:

a) Untreated mouth saliva from cow 9120, 930 µg/ml protein
b) Two mls of the above saliva mixed with 0.5 ml of ethanol
c) Freeze dried saliva diluted in buffer containing no additives, 1535 µg/ml protein
d) Freeze dried saliva diluted in tris/glycine buffer containing 1% EDTA and 1% β-mercaptoethanol
e) Freeze dried saliva diluted in tris/glycine buffer containing 3% EDTA and 3% β-mercaptoethanol.
(Tris/glycine buffer; 0.05 mol/l, pH 8.9)

* Ethylenediamine-tetra-acetic acid

TABLE 6.3.1 Treatment of saliva with β-mercaptoethanol, EDTA, Freeze Drying and Ethanol

Treatment	A	B	C	D	E
Band					
1	13.6	12.6	11.6	1.2	2.9
2	3.0	4.6	3.0	19.3	14.1
3	18.1	18.9	18.1	20.6	15.6
4	8.5	7.0	6.6	7.0	4.9
5	7.7	5.9	7.3	5.9	7.3
6	3.0	2.7	3.3	4.5	4.5
7	20.0	19.8	21.3	19.6	23.4
7A	5.2	6.0	5.0	2.6	2.2
8	7.8	8.0	8.6	8.1	12.9
9	6.7	8.0	7.8	6.1	6.5
10	3.5	3.2	4.7	2.8	3.2
11	3.0	3.3	2.7	2.2	2.5

Treatments:
(a) Untreated control saliva
(b) 2 ml of saliva mixed with 0.5 ml of ethanol
(c) Freeze dried saliva diluted in buffer, no additives
(d) Freeze dried saliva diluted with buffer containing β-mercaptoethanol and EDTA
(e) Freeze dried saliva with buffer containing EDTA and three times the concentration of β-mercaptoethanol

Results

Results from the above experiment are shown in Table 6.3.1. There was a marked decrease in band 1 in the saliva which has been treated with β-mercaptoethanol + EDTA. Freeze drying or ethanol treatment did not cause any change in the band proportions. Band 2 increased in the saliva samples treated with β-mercaptoethanol + EDTA but the increase was less pronounced in the sample with a higher β -mercaptoethanol + EDTA concentration. In the latter sample there appeared to be a decrease in band 4, while bands 7 and 8 have both increased slightly. Further experiments carried out showed the β -mercaptoethanol + EDTA effect was due to the sulphydryl reagent and not the chelating agent. More severe treatment* with β-mercaptoethanol caused all bands from 1 to 4 to decrease while there was a two-fold increase in the percent band 8 in these saliva samples. Untreated saliva incubated for 150 min at 45°C showed no changes except for a slight increase in band 3.

Discussion

Ethanol, EDTA, or freeze drying did not appear to have any effect on the salivary proteins, but β-mercaptoethanol treatment resulted in a marked reduction in band 1 and an increase in band 2. These results would indicate that band 1 probably contains, or is a protein which has a considerable amount of disulphide bonding.

* The more severe treatment of the salivary proteins consisted of 10 µl of concentrated β-mercaptoethanol being added, and the saliva was incubated at 40°C for 150 minutes.

6.4

THE EFFECT OF NEURAMINIDASE ON SALIVARY PROTEINS

This experiment was initially designed to test the hypothesis that the PAS positive band 1 salivary protein was a 'subunit' of the sialic acid containing mucoprotein molecule. Jones & Lyttleton (1973) had shown that on electrophoresis a desialated fraction of mucoprotein could enter the 7.5% polyacrylamide gel.

Other proteins present in the saliva were PAS positive, (Chapter 6.2) and if these contained sialic acid then the desialated form of the protein might be formed by treatment with a neuraminidase. It was postulated that the sialated and desialated forms of the same protein may be present in the mouth saliva (McIntosh 1975), thus one protein could represent two or more protein bands. Furthermore by merely varying the proportion of protein present in each form, without necessarily changing the total protein concentration, two protein bands could statistically appear negatively correlated.

To investigate these hypotheses a series of experiments were carried out using neuraminidase prepared commercially from Cl. perfringens.

Materials and Methods

Neuraminidase

Commercially purified neuraminidase (N-acetyl-neuraminic glycohydrolase EC 3.2.1.18) purified from Cl. perfringens; obtained from Sigma Chemical Company, Product Number N 2876.

Neuraminidase solution

A solution containing 2 mg/ml was prepared in 0.02 M sodium phosphate buffer, pH 7.0.

The final neuraminidase concentration in each test was 1 mg/ml.

Samples used in the neuraminidase experiments

Experiment 1: Three samples were chosen for this first experiment;

(i) Saliva from animal 318 which was rich in band 1 (protein concentration 1 mg/ml).

(ii) Commercially prepared bovine mandibular mucin (protein concentration 2 mg/ml).

(iii) A saliva sample which had been treated according to the mucoprotein purification procedure of Lyttleton (1964) but without removing the fibrous clot.

This sample had a high concentration of bands 1 and 7 (protein concentration 1.45 mg/ml).

Each sample was split three ways; (a) no incubation; (b) incubation for 15 min with neuraminidase at 37°C; (c) incubation for 45 min with neuraminidase at 37°C. A control saliva was used with this experiment, this was incubated for 45 min at 37°C without neuraminidase.

Experiment 2: Purified band 7-8-9 complex purified by preparative electrophoresis (described in Chapter 5.2.d) was used. This sample contained 58% band 7, 27% band 8 (+ 7A) and 14% band 9 (+ some band 10). This sample was treated in four ways; (a) control, no incubation; (b) incubation with neuraminidase for 15 min; (c) incubation with neuraminidase for 45 min; (d) incubation with neuraminidase for 24 h. All test samples were incubated at 37°C.

Two further controls were included in this experiment to test for neuraminidase activity and possible proteolytic action due to impurities in the enzyme preparation. Transferrin and albumin were used as the control proteins, at a concentration of 0.5 mg/ml in pH 7.0 phosphate buffer (0.02 mol/l). These control proteins were incubated for the same length of time as for the test samples.

Electrophoresis of test and control samples

All test and control samples in Experiment 1 and 2 were analysed by the routine PAGE procedure, and stained with Coomassie. However in Experiment 2 periodic acid-Schiff and Alcian blue were also used.

Results

Experiment 1

(i) Saliva from animal 318 showed a decrease in band 7 and an increase in band 8, this was most pronounced in the 45 min incubation.

(ii) The submaxillary mucin contained a considerable amount of band 7 but on incubation with neuraminidase this was progressively converted to band 8.

(iii) The change in band 7 percentage on incubation with neuraminidase was quite marked in the saliva sample which had been partially purified for mucoprotein. With no incubation there was 21.1% band 7 present but at 15 min incubation this had decreased to 17.2% and after 45 min incubation there was only 5.7% band 7 remaining. However there was only

TABLE 6.4.1 Effect of neuraminidase on the protein band 7-8-9 complex

	7	(7A)8	9/10
No incubation	58%	27%	14%
15 min incubation	22%	67%	12%
45 min incubation	6%	78%	16%
24 hr incubation	6%	65%	29%

11.6% band 8 present before incubation, and this rose to 20.3% and 23.7% after 15 and 45 min incubation respectively. There was no change in the other salivary protein bands after incubation.

(iv) The control saliva which was incubated for up to 45 min without neuraminidase showed no change in the percentages of the various protein bands. *

Experiment 2

The effect of neuraminidase on purified band 7-8-9 complex

In the first experiment band 7 was the only protein decreased when the various protein preparations were incubated with neuraminidase. Therefore this second experiment was carried out with the band 7-8-9 complex, purified by preparative electrophoresis, to answer the following questions:

1) Does band 7 contain any carbohydrate or sialic acid?
2) Is 'purified' band 7 converted to band 8?
3) Does band 8 contain any detectable carbohydrate or sialic acid?
4) What is the effect of neuraminidase on the control proteins albumin and transferrin?

Table 6.4.1 shows the change in percentages of the three protein bands when stained with the protein stain Coomassie.

Band 7 decreased from 58% prior to incubation to 6% after 45 min incubation but there was no further decrease after 24 h. Band 8 increased from 27% prior to incubation to 78% after 45 min, however after 24 h there was a slight decrease in the percent band 8. Band 9 was present as 14% of the protein in the pre-incubation sample and did not change much up to 45 min incubation, but after 24 h had increased to 29%.

The control protein albumin after incubation for 24 h showed an increase in the percent monomer, but no additional bands, or any evidence of proteolytic enzyme activity was apparent.

* In an additional experiment carried out with calf saliva incubated at 37°C with no neuraminidase added, a spontaneous reduction in band 7 was observed. Other proteins also decreased on prolonged incubation. Not all calf salivas tested showed this 'enzymic activity' but in the course of studying salivas from calves up to 90 days of age this spontaneous effect was observed when repeat PAGE were performed on some calf samples.

Glycoprotein staining

PAS and Alcian blue staining were negative for all the gels run in this experiment using the band 7-8-9 complex.

After 30 min incubation the two fastest moving bands of the transferrin, which were present in the PAS stained control gel (without neuraminidase), had disappeared.

Discussion

These experiments do not support the first hypothesis that band 1 protein might be a 'subunit' of the mucoprotein molecule, but they do not exclude the possible presence of some form of partially degraded (desialated) mucoprotein molecule in this band region.

The finding that band 7 was decreased by the action of the neuraminidase enzyme gives support to the second hypothesis which was tested; that two protein bands, in this instance bands 7 and 8 (and possibly band 9) may be chemically related and possibly differ only by the amount of sialic acid present. However if sialic acid only was removed from band 7, then a reduced rather than an increased electrophoretic migration would be expected. Also the PAS and Alcian blue staining for these bands in whole mouth saliva, or in semi-purified forms of these proteins were negative, thus casting some doubt on the interpretation of these results. It is possible that these negative results with the glycoprotein stains could be due to the low protein concentrations available, and thus beyond the sensitivity of the techniques. This will be tested in the future, now that techniques for purifying larger quantities of band 7-8-9 complex are available (Chapter 5).

Albumin was not changed on incubation for 24 h with neuraminidase, and this indicated that it was unlikely that the activity of the neuraminidase was due to the presence of a proteolytic enzyme as an impurity in this commercial preparation. However it is of some importance to note that some saliva samples collected from young calves showed a spontaneous reduction in band 7, and on prolonged incubation other bands were also affected. This would suggest that an enzyme is present in some calf saliva samples, but is either absent or inhibited in saliva collected from adult animals.

6.5

SCREENING FOR ENZYMIC ACTIVITY IN SALIVA

6.5.a

Screening for amylase activity in saliva

The saliva of most mammals contains amylase but this does not appear to be the case in the ruminant animal, (Ramsay et al. 1960; Ramsay et al. 1962). However Toutain et al (1973) and Majeed et al (1970) studied the nasolabial gland in the cow and found its chemical composition to be similar to saliva but noted a high level of amylase present. Majeed et al. (1970) observed that cows and buffalo frequently thrust their muzzles into the feed and during rumination often run their tongues into the nostril and over the muzzle. It is thought that the amylase produced by the nasolabial gland may be the source of amylase sometimes found in the ruminant saliva, (Church 1975).

The screening for amylase undertaken in this work was aimed at establishing whether amylase was present in bovine saliva.

Materials and Methods

Amylase was determined by the Calbiochem procedure (Chapter 2.2.1) which uses a coloured dye coupled to the substrate. The amylase activity is quantitated by the amount of dye released by the test enzyme, compared with the standard enzyme under the same conditions. Saliva samples from under the tongue were collected from four 10 day old animals, born 1976, and six 90 day old animals born in the same year. Twelve 10 months old animals were tested, these were the 1973 progeny.

Results

The results are shown in Table 6.5.1. The mean amylase activity for the 10 animals tested (born 1976) was 3.9 Somogyi units/100 mls. The 10 day old animals had a slightly higher mean value of 6.2 Somogyi units/100 mls, than the 90 day old animals whose mean value was 2.3 Somogyi units/100 mls. The twelve 1973 progeny tested at 10 months of age had a mean amylase activity of 1.2 Somogyi units/100 mls.

Table 6.5.1 Salivary Amylase Results from Animals at Three Different Ages

Animals	Age at testing	Number of animals	mean ± SEM
1973 progeny	10 months	12	1.2 ± 0.4
1976 progeny	90 days	6	2.6 ± 0.9
1976 progeny	10 days	4	6.3 ± 1.8

Discussion

From the limited data collected on amylase, it would appear as if this enzyme is present at a low concentration in bovine saliva. Furthermore there is some indication to suggest that the highest concentration is found in the calves,but further work would be required to substantiate such a claim. Church (1975) has suggested that the amylase sometimes found in bovine saliva may be produced by the nasolabial gland. No attempt was made in this study to determine from which glands the enzyme was secreted.

6.5.b

Gamma - Glutamyl Transpeptidase (GGT)

The presence of secretory immunoglobulins in saliva was reviewed in Chapter 2.2. Recently it has been suggested by Binkley & Wiseman (1975) that the secretory component of the secretory immunoglobulins may be one and the same protein as gamma-glutamyl transpeptidase. However results to the contrary have recently been presented (Labib et al. 1976).

This very brief screening of the bovine saliva was undertaken to establish whether any GGT acitivity was present in the routine collections of mouth saliva, and secondly, if present, was there any indication as to which bands were responsible for this enzymic acitivity.

Materials and Methods

The method employed was based on that of Szasz (1969).

These analyses were performed by Mr B. Coe, Experimental Pathology Section, Ruakura Animal Research Station.

Three animals were chosen for the initial screening, these were from three different age groups, calf 641 (90 days of age), cow 307 (1973 progeny group), and cow 9120. This latter animal had been used as a control animal in much of the analytical and preparative electrophoresis work. Purified band 1-2-3 complex and band 7-8-9 complex were also tested for GGT acitivity.

Results

Results from this initial screening for GGT activity are given in Table 6.5.2.

Table 6.5.2 Gamma-Glutamyl Transpeptidase Screening of Saliva

Animal or proteins tested	GGT activity (mU/ml)
Calf 641	10
Cow 307	12
Cow 9120	18
Band 1-2-3 complex	5
Band 7-8-9 complex	0

The maximum activity was found in cow 9120 saliva and the minimum activity was found in the saliva from the 90 day old calf. There was no activity in the purified band 7-8-9 complex but the band 1-2-3 complex did have a small amount of activity present.

TABLE 6.5.3 Relative activity in saliva and semi-purified proteins of enzymes capable of hydrolysing BAEE

Ranking Order	Animal number	Comments	Tan
1	9120	five-fold concentrated saliva	0.42
2	641	saliva from a 90 day old calf	0.40
3	Band 7-8-9 complex	purified protein	0.20
4	Band 7-8-9 complex	purified protein	0.18
5	Standard saliva	five-fold concentrated saliva	0.08
6	640	saliva from a 90 day old calf	0.07
7	1125	five-fold concentrated saliva	0.04
8	639	saliva from a 90 day old calf	0.03
9	Band 1-2-3 complex	purified protein	0.00

Relative enzymic activity is indicated by the slope of the line, expressed as the Natural Tangents, (Tan).

Discussion

The presence of GGT activity in the band 1-2-3 complex does not necessarily support the hypothesis that the secretory component and GGT enzyme are one and the same protein. However the presence of specific enzymic activity in the saliva, and in particular within the band 1-2-3 complex, should provide useful information towards the final characterization of the individual protein bands.

6.5.c

Screening for enzymes which can hydrolyse benzoyl L-arginine ethyl ester (BAEE)

The presence of kallikreins in the salivary glands of numerous animals have been reported by several different groups of workers, (Chapter 1.2). The discovery that kallikrein had hydrolytic action on benzoyl L-arginine ethyl ester has enabled kallikrein to be determined by a non-biological method, (Trautschold & Werle 1965).

Materials and Methods

The method used to determine the relative enzymic activity was described in Chapter 2.2.m. This method is based on measuring the increase in absorbance at 253 nm, when the enzyme is incubated with benzoyl L-arginine ethyl ester as the substrate (BAEE).

Samples analyzed for activity were as follows:

Cow 9120 saliva (five times concentrated), cow 1125 saliva (five times concentrated), standard saliva (five times concentrated), three samples of saliva from 90 day old calves, 641, 640 and 639. Purified proteins, band 1-2-3 complex, and band 7-8-9 complex were also examined.

Results

Results are tabulated in Table 6.5.3., these are ranked according to the enzymic activity present. The enzymic activity was high in both samples of the purified band 7-8-9 complex, the only two samples having a higher enzymic activity were the 90 day calf sample and the five times concentrated saliva from cow 9120. The band 1-2-3 complex did not show any activity.

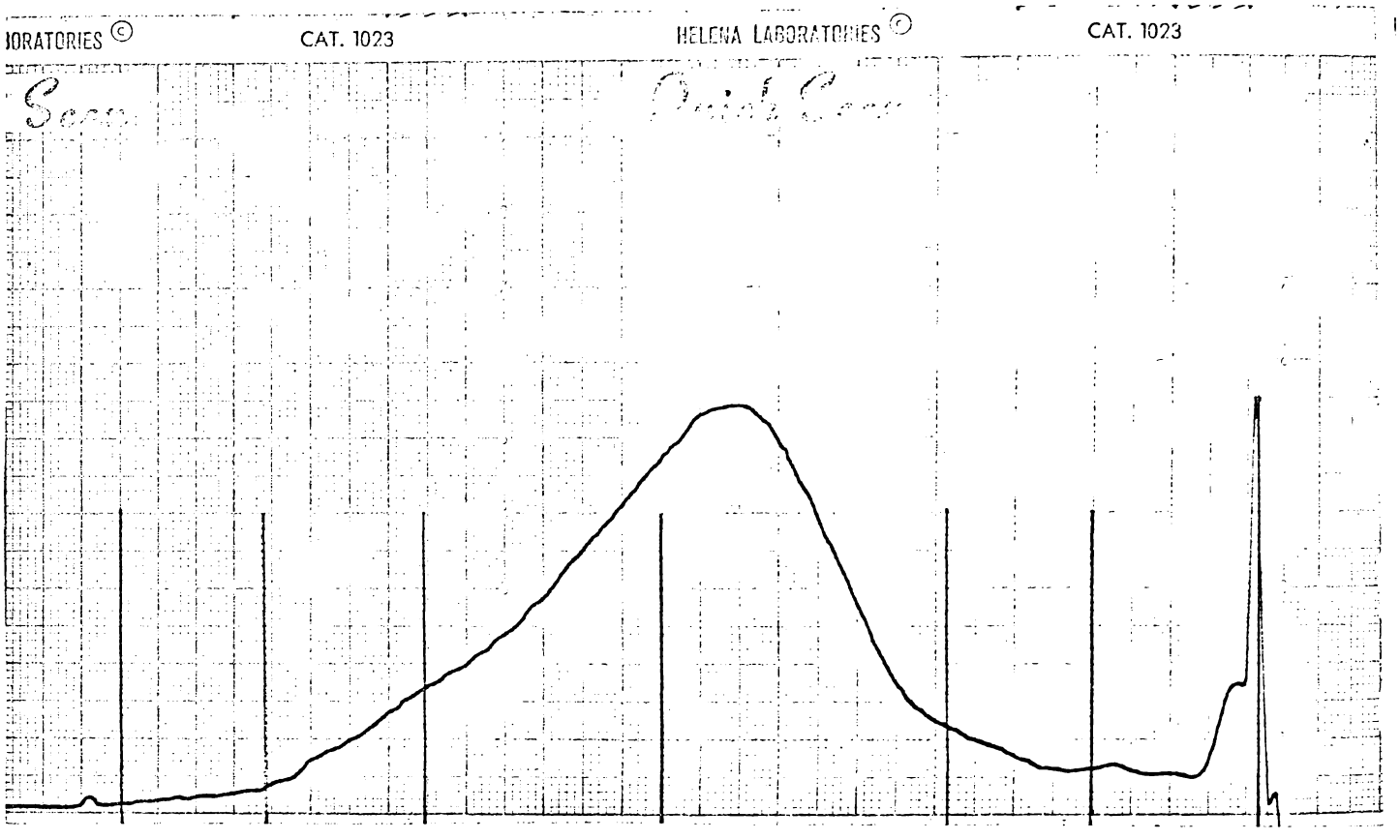


FIGURE 6.5.1 Zymogram of the α-naphthyl acetate hydrolysing esterase in saliva, showing the protein electrophoretic bands superimposed

Discussion

These results show that an enzyme (or enzymes) capable of hydrolysing BAEE was present in the saliva of all animals tested, furthermore this activity was present in the purified band 7-8-9 complex. However although kallikrein activity can be measured by its ability to hydrolyse BAEE (and these results might indicate that kallikrein was present), it should be noted that other enzymes including trypsin can hydrolyse this substrate.

6.5.d

Screening for esterase activity in saliva, using α-and β-naphthyl acetate as substrates

To gain further information on the properties of the proteins present in saliva, zymograms were performed testing for the presence of esterases capable of hydrolysing α-and β-naphthyl acetate.

Materials and Methods

Details of the zymogram technique was given in Chapter 2.2.n. The samples chosen to test for the esterase activity were; standard saliva (five times concentrated), serum (tenfold dilution), and saliva from two calves (90 days of age), 640 and 639.

Results

Although there was a difference in the electrophoretic migration of the bands in serum having either α- or β-naphthyl acetate esterase activity, the results from the saliva samples were very much the same for both substrates. The majority of this esterase activity was in the band 3 region. This is illustrated in Figure 6.5.1, which shows a zymogram of the standard saliva using α-naphthyl acetate as the substrate. The position of the protein bands, as determined by staining with Coomassie, are superimposed on the zymogram.

Discussion

Esterase activity, capable of hydrolysing α- and β-naphthyl acetate was present in the saliva samples tested. The majority of this activity was in the band 3 region. This was only a screening procedure to test for either the presence or absence of esterases, but by using a zymogram technique some information was given to indicate which bands were positive for these enzymes.

TABLE 6.6.1 Molecular weight determinations of semi-purified proteins using thin layer gel chromatography

(a) Semi-purified protein band 1-2-3 complex

6 independent molecular weight determinations

Molecular weight >150 000

(b) Semi-purified protein band 7-8-9 complex

7 molecular weight determinations

Molecular weight = 65 276 ± 2 775 (SEM)

(5% confidence limits = 72 065 - 58 487)

(c) Calculation of results; the reciprocal of the relative migration (thyroglobulin used as marker protein) was plotted against log Molecular weight.

Standards used for each run;

Protein	Molecular Weight
Cytochrome C	13 000
Soybean Trypsin Inhibitor	21 500
Bovine Serum Albumin	67 000
β-Galactosidase	130 000
Bovine IgG	150 000

regression calculated $y_{1/R} = a + b \log x \, MW$

e.g. $y = 7.40008 - 0.5235 x$

$R^2 = 0.9766$

6.6

MOLECULAR WEIGHT STUDIES BY THIN LAYER GEL CHROMATOGRAPHY (TLG)

The use of TLG to determine the molecular weights of proteins has become a feasible proposition with the commercially available TLG apparatus. This procedure utilizes the molecular sieving chromatographic properties of Sephadex beads on a thin layer gel, very similar to that used for thin layer chromatography. The major advantages from this procedure are threefold:

1) The speed of separation and molecular weight determinations.
2) The simplicity of the entire procedure.
3) This technique allows several unknown proteins to be compared with the standard proteins in any single run.

A full description of the technique is given in Chapter 2.2.k.

Results

A series of developmental runs were carried out to perfect the TLG procedure, but only the accurately standardized results are reported here. Molecular weights were determined for semi-purified proteins of the band 1-2-3 complex. For the six independent determinations a MW greater than 150 000 was obtained. This purification consisted of 54.4% band 1, 16.9% band 2 and 15.5% band 3, as determined by the standard PAGE system.

The band 7-8-9 complex molecular weight was determined from seven independent TLG runs using proteins from four different purifications all of which had very high levels of band 7 and smaller proportions of bands 8 and 9. The mean molecular weight for the band 7-8-9 complex was 65 300. These results are shown in Table 6.6.1.

Discussion

The semi-purified proteins seem to behave as some form of protein complex, this may be real, in the case of bands 1-2-3 as subsequent results would indicate that these bands are probably largely composed of the secretory immunoglobulin complex (Chapter 6.7.a and 7.1). However the band 7-8-9 complex has not been chemically identified and could be a physical artifact, for instance, the proteins may be bound together non specifically by the mucin molecule.

Evidence for this comes from the finding of a PAS positive band, which did not stain with Coomassie in one of the band 7-8-9 complex preparations. However chemical evidence from studies with neuraminidase, (see Chapter 6.4) would suggest the band 7-8-9 complex to be composed of bands differing only by the presence or absence of sialic acid and/or some other molecule hydrolysed by neuraminidase. Also the very repeatable molecular weight values obtained for the four different purifications used would suggest this is not just a random physical association.

TABLE 6.7.1 Molecular weight determinations of the protein subunits present in the semi-purified protein preparations as determined by SDS-electrophoresis

(a) Purified protein band 1-2-3 complex

SDS-electrophoretic band	Approximate molecular weight
E	56 500
F	51 500
I	23 000
H	26 500
C	76 000
C (shoulder of band C)	82 000
G (very low proportion)	39 000

(b) Purified protein band 7-8-9 complex

H	26 000
I	22 500
J and K	17 500
Two very small peaks	38 500
	60 000

(c) Calculation of results: the relative migration (R) was plotted against the log MW by a fitted regression.

$$y_R = a + b \log x \, MW$$

$$y_R = 3.244 - 0.2549x \qquad R^2 = 0.9959$$

Molecular weight standards used:

Protein	Molecular weight
Cytochrome C	13 000
IgG light chain	25 000
IgG heavy chain	50 000
Bovine serum albumin	67 000
β-Galactosidase	130 000

The individual protein peaks separated by SDS-electrophoresis were numbered as illustrated for the standard saliva shown in Figure 6.7.1, and these are listed in order of decreasing proportion as judged by the height of the protein peak.

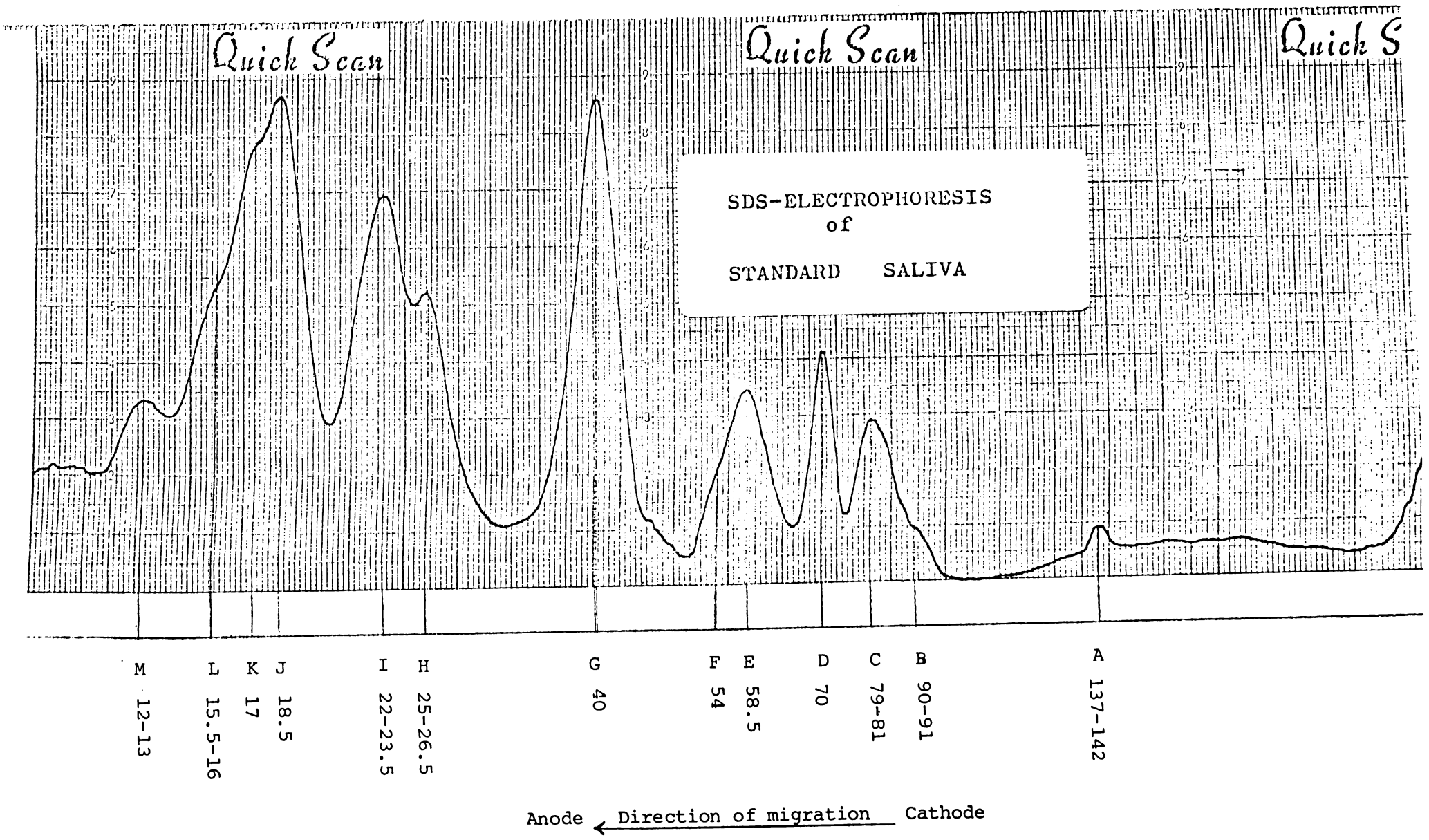


FIGURE 6.7.1 Densitometer scan of standard saliva run on SDS-electrophoresis; showing the numbering system used and the approximate molecular weights (x 10^3)

6.7

SODIUM DODECYL SULPHATE (SDS) ELECTROPHORESIS

SDS-electrophoresis is widely used in biochemistry and one of its widest applications has been the determination of protein subunit molecular weights. Considerable insight into the tertiary structure of the molecule can be achieved when subunit molecular weights are compared to the whole protein molecular weight. Secondly, proteins which are bound to membranes or to other 'protein' molecules, either by disulphide bonding, or ionic bonding can be separated and studied by this procedure.

6.7.a

SDS-electrophoretic studies on standard saliva, semi-purified salivary proteins, and saliva from cow 9120

Materials and Methods

The SDS-electrophoretic procedure used in this study is described in detail in Chapter 2.2.h. The protein molecular weight standards used in this study were cytochrome C, bovine serum albumin, immunoglobulin G (Ig G heavy and light chains) and β-galactosidase. The methodology adopted for calculating the molecular weights is given in Table 6.7.1.

Results

The standard saliva was run on SDS-electrophoresis and at least 13 different proteins (subunits) could be distinguished. The results from the standard saliva are illustrated in Figure 6.7.1. This figure shows the numbering system adopted which is from A to M commencing with the highest molecular weight subunit. The molecular weights for each of these protein subunits are also given.

Purified band 1-2-3 complex was run on SDS-electrophoresis and five major bands could be distinguished. These were bands E,F,I,H & C. The results are given in Table 6.7.1.

Purified band 7-8-9 complex was also run on SDS-electrophoresis and the major bands seen in this run were H, I, and J,K. However the molecular weights for the H and I for the band 7-8-9 complex were slightly different than the molecular weights for the bands 1-2-3 complex, which would suggest that these subunits from the two different protein purifications are different.

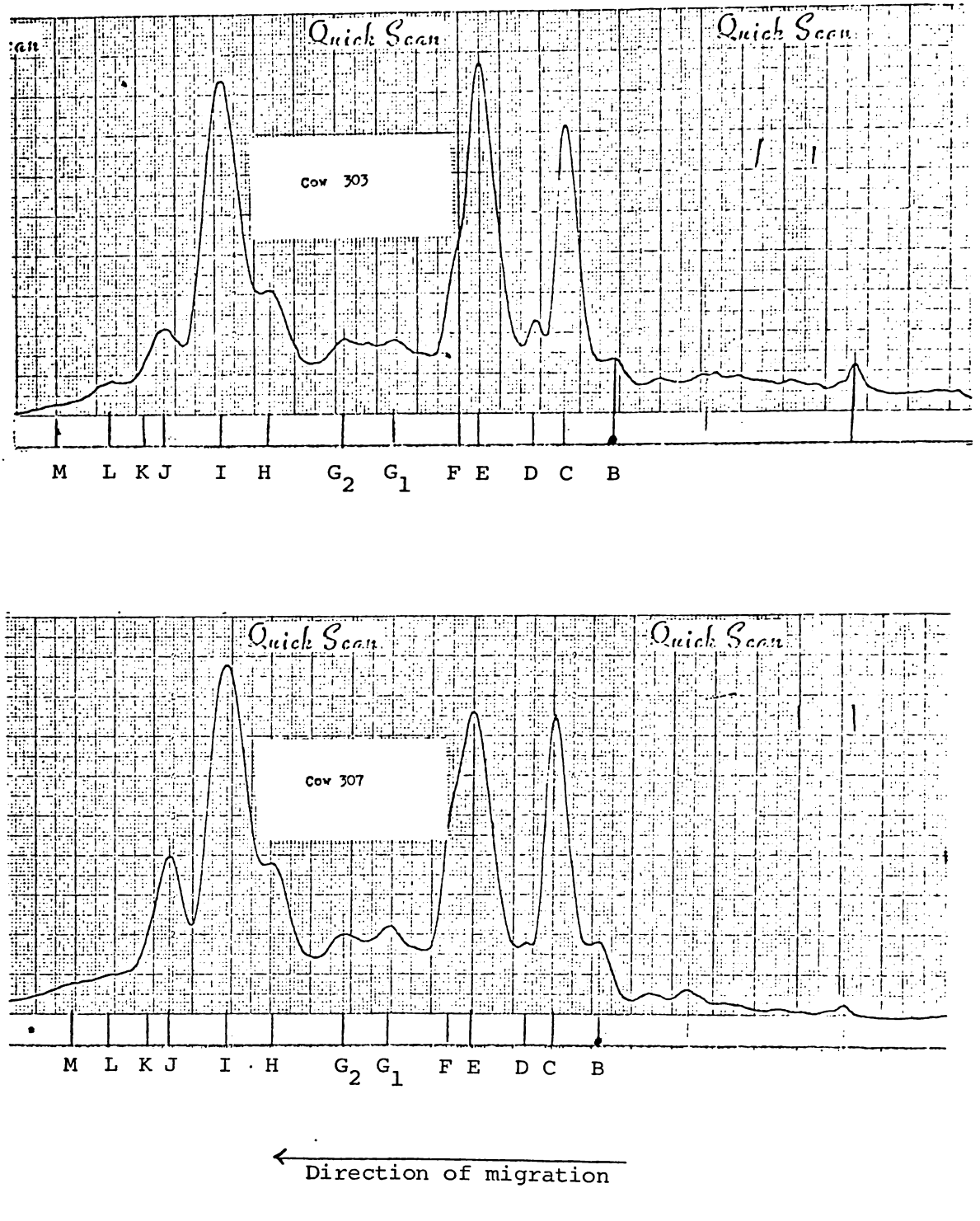


FIGURE 6.7.2 Reduced SDS-electrophoretic scans for cows 303 and 307, showing bands numbered according to the system used for standard saliva

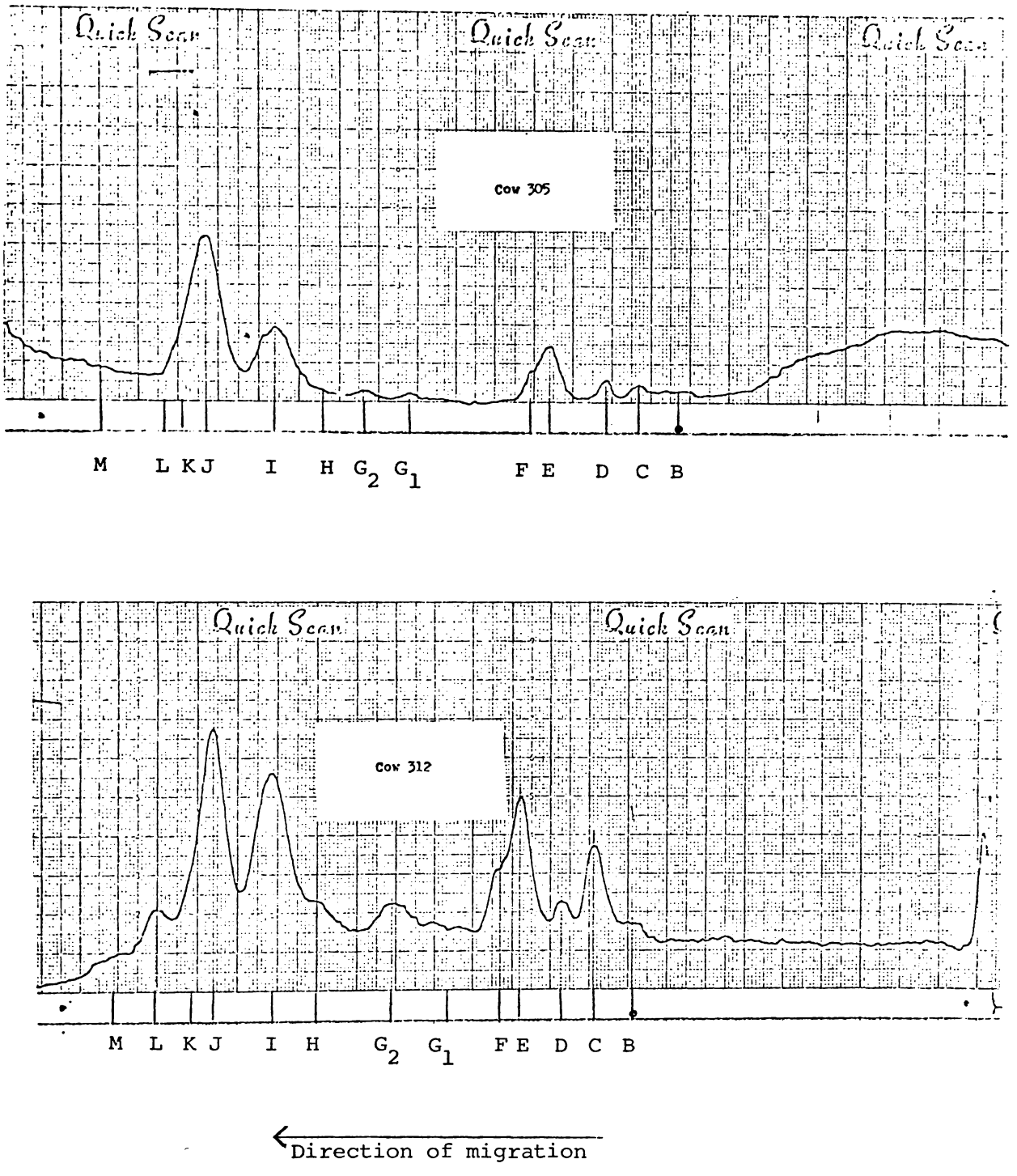


FIGURE 6.7.2 Reduced SDS-electrophoretic scans for Cows 305 and 312

TABLE 6.7.2 Molecular weight determinations by SDS-electrophoresis of the protein subunits found in saliva collected by the mouth bit from Cow 9120 listed in order of decreasing concentration as judged by the peak heights

SDS-electrophoretic band	Approximate molecular weight
I	(23 500 (21 000
H	25 500
J & K	17 500
G	39 000
G_2	33 500
E	55 500
F	51 500
L	14 300
C	79 000
B	88 000
M	12 500
D	68 000
A	138 000

Calculations: fitted regression as for purified band 1-2-3 complex and band 7-8-9 complex

TABLE 6.7.3 PAGE and total protein result from the September/ October 1976 saliva collection

Animal	Total protein (µg/ml)	Electrophoretic bands expressed as percentages											
		1	2	3	4	5	6	7	7A	8	9	10	11
303	1115	74.8	4.2	6.9	3.5	2.2	2.1	0.6	0.7	2.2	1.2	0.5	1.2
307	2340	40.9	5.1	10.9	8.2	5.0	3.1	9.6	1.5	9.6	2.8	1.0	2.3
305	413	25.3	8.0	22.3	12.6	2.3	3.5	0.4	0.7	14.5	6.7	2.8	0.9
312	520	32.3	7.2	9.8	9.0	4.5	3.5	2.2	1.2	17.3	8.3	3.5	1.4

A semi-purified band 6 preparation which on PAGE had a small proportion of bands 1,2,3 and some band 8, when run on SDS-electrophoresis had a major protein band with a molecular weight of 67 000. (This preparation had been used to supplement the band 6 proportion when preparing the standard saliva, Chapter 2.1.c.)

A similar pattern of protein subunits as for the standard saliva, was detected for cow 9120 mouth saliva (which had been used as a control saliva in the routine PAGE studies), except that the relative proportions of each subunit varied. These results are given in Table 6.7.2.

6.7.b

SDS-electrophoresis studies on the three year old 1973 progeny

Saliva samples collected in September/October 1976 from the twelve 1973 progeny were run on SDS-electrophoresis and the results were compared with the routine PAGE results. The purpose of this experiment was to verify the results seen for the purified band complexes. That is, where there was a high band 1 level and low band 7, 8, and 9 , was there a corresponding high band E, F, I, H and C, and where there was a moderately high band 7, 8 and 9 (or 8 and 9) was there an increase in bands H and I and in particular were the bands J and K increased.

Results

The four animals showing the greatest difference between band 1 and band 7 and/or 8 in their saliva as determined by PAGE were chosen to illustrate the differences in bands seen on the SDS-electrophoresis. The PAGE results are given in Table 6.7.3 and the SDS-electrophoresis results are illustrated in Figure 6.7.2.

Cow 303 had 74.8% band 1 on PAGE. The SDS-electrophoresis showed a high quantity of bands E and F and almost an equal quantity of band I. There was a reasonably high concentration of bands C and H, however there was on a low level of bands J and K present.

Cow 307, had 40.9% band 1 while both bands 7 and 8 were 9.6%. The SDS-electrophoresis showed a high level of bands E and F, but the band I level was considerably increased compared with cow 303. Bands C and H were present in moderately high concentrations, while bands J and K were increased in proportion compared to cow 303.

Cow 305 had 25.3% band 1, 0.7% band 7 and 14.5% band 8. The SDS-electrophoresis showed low levels of E and F. Band I was higher than either E or F while bands J and K were in the highest proportion, (in contrast to the results for cow 303). Both bands C and H were present in very low proportions.

Cow 312 had 32.3% band 1, 2.2% band 7 and 17.3% band 8. The SDS-electrophoresis showed an increased proportion of band I compared with E and F. Bands J and K were present in high proportions while there was a moderate proportion of bands C and H present.

6.7.c

Periodic Acid Schiff staining of saliva and semi-purified salivary proteins run on SDS-electrophoresis

Saliva (25 ul) from cow 9120 was run on SDS-electrophoresis and stained with PAS as described in Chapter 2.2.e. About eight PAS positive bands were detected all of which were in the high molecular weight region of the gel, four bands being greater than 150 000 whilst the other four had molecular weights of approximately 50 000, 80 000, 90 000 and 120 000.

When purified band 1-2-3 complex (25 ul) was run on the SDS-electrophoresis and stained with PAS no positive bands were detected. However when the band 7-8-9 complex (25ul) was run under the same conditions a PAS positive peak was detected which just entered the gel and faint residual dye (no definite bands) remained from the molecular weight range of 120 000 and greater. There was also a very small peak at about the 80 000 MW range.

Discussion

The SDS-electrophoresis of standard saliva demonstrated the presence of at least thirteen protein subunits all of which were present in the mouth saliva from cow 9120. However certain bands present in standard saliva in moderate concentrations but in low amounts in 9120 saliva could be provisionally identified when the two gels were compared,(e.g. band 6 was provisionally classified as band D in the SDS-electrophoresis gels. This was subsequently verified when semi-purified band 6 was analysed by SDS-electrophoresis).

The purified band 1-2-3 complex gave five major peaks on SDS-electrophoresis. Evidence from electrophoretic migration, treatment with sulphydryl reagents, and immunochemical cross reactivity, indicated that the band 1 and 2 region as determined by routine PAGE, was largely composed of the immunoglobulins found in ruminant saliva (Chapters 6.1, 6.3 and 7.1). The subunit molecular weights could be accounted for as follows; band E, 56 500 MW, Ig A heavy chain, band F 51 500 MW, Ig G heavy chain, band I 2 00 MW Ig light chain, band H 26 00 MW secretory-Ig A J chain, and band C 76 000 MW Ig A secretory component. The TLG chromatography had shown the molecular weight of this complex to be in excess of 150 000. The presence of these same subunits in the saliva of the three year old 1973 progeny, was demonstrated, and in particular cow 303 which had almost 75% band 1. The major bands present in the saliva on SDS-electrophoresis for this animal were the five bands seen for the purified band 1-2-3 complex.

Purified band 7-8-9 complex run on SDS-electrophoresis showed four major bands, H, I, J and K, however it would appear that the bands H and I from the band 7-8-9 complex were different as the molecular weights were not exactly the same and furthermore immunochemical evidence discussed in Chapter 7.1, showed no cross reactivity in the band 7, 8 and 9 region with antisera prepared against bovine Ig A and Ig G. Evidence from cow 9120 which had a high band 7 and cow 307 with moderate amounts of bands 7 and 8 suggested that the band 7 and 8 region in PAGE was largely accounted for by protein subunits which on SDS-electrophoresis migrated in the H, I, J and K regions. Cows 305 and 312 which had low band 7 values but high band 8 levels showed that the band H and I region to be proportionally increased compared to the almost equal amounts of band 1 and E + F seen in cow 303. Bands J and K were considerably increased in these two animals compared to cow 303. Thus results from routine saliva collections run on SDS-electrophoresis verify the findings from the purified bands.

The results from the PAS staining, indicated that high molecular weight carbohydrate containing molecules were present and detectable when 25 µl of saliva was used. These PAS positive molecules in most cases appear to be different molecules from those detected by the

Coomassie stain because of their different electrophoretic mobilities on SDS-electrophoresis. It is quite likely that the PAS positive molecules are part of the mucin or mucoprotein molecules.

The purified band 1-2-3 complex gave negative results when stained with PAS but this was attributed to the relative in-sensitivity of the PAS technique. However the band 7-8-9 complex did show some positive PAS staining in one purified sample, but this was thought to be due to the presence of a residual amount of mucin in this preparation.

6.8

ELECTROPHORETIC STUDIES ON THE MUCOPROTEIN FOUND IN BOVINE SALIVA

Mucoproteins appear to represent about 50% of the total protein in bovine saliva (Lyttleton 1960). This mucoprotein molecule has a molecular weight of up to 1.1×10^7 and has a very high viscosity in solution (Lyttleton 1964). It is though that these very large glycoprotein molecules may play a significant role in the formation of the stable foam found in the rumen of cattle suffering from pasture bloat. (Jones 1971; Jones & Lyttleton 1973; Clarke & Reid 1974). The bovine mucoprotein molecule purified from the saliva by the procedure of Lyttleton (1964) has a very high sialic acid content of approximately 30% w/w (W.T. Jones pers. comm.). This high sialic acid content might allow interaction with other proteins such as plant proteins (W.T. Jones pers. comm.) or other salivary proteins. Physical biochemical studies on the mucoprotein molecule with respect to bloat in cattle have been undertaken by Jones (1971) and Jones & Lyttleton (1972).

The high molecular weight, high viscosity and high sialic acid content of the mucoprotein molecule, coupled with the presence of the mucin molecule in bovine saliva add considerable difficulty to the study of the lower molecular weight proteins. This experimental work on the mucoprotein was designed to look for obvious inter-relationships between the mucoprotein and lower molecular weight salivary proteins, and to determine whether it would be possible to quantitate the amount of mucoprotein present in the individual salivas by an electrophoretic procedure.

The primary requirements for this study were:

a) The development of a suitable electrophoretic technique which would allow the mucoprotein to enter the matrix.

b) The availability of suitable staining procedures to detect the mucoprotein in the polyacrylamide gel.

Search for electrophoretic methods

The first procedure used was based upon the 6% preparative polyacrylamide gel electrophoresis and effectively only half the final strength acrylamide (3%) was used. Photopolymerization was first tried using riboflavin as the catalyst but this did not work.

Chemical polymerization was achieved with the next gel using ammonium persulphate. However, the gel was very 'tacky' and difficult to handle.

Because the 3% gel appeared very difficult to handle the next gel tried was a gradient gel. This method was based on the procedure of Robard et al. (1971). The gradient former used was the LKB gradient former designed and used for ionic and pH gradients in ion exchange chromatography. This gradient former appeared to work well even with the small quantities of acrylamide solutions added to each reservoir. This was shown by incorporating some bromophenol blue dye in the second reservoir, which showed up as an even gradient down the gel. Polyermization took place in the presence of CO_2 and gradients from 3.4 - 14.5% were formed. Unfortunately the 3.4% part of the gel would not polymerize completely. Further investigations showed that the TEMED was in low concentration compared with the standard PAGE used in this work and the Bis was in a much lower concentration than used by Edgell et al. (1969). These authors undertook an extensive investigation into the optimum Bis concentration for large pore polyacrylamide gels and found 15% Bis the optimum.*

The large pore gels, described by these authors, was then tried. This system used a 3% acrylamide gel and a high degree of crosslinking with Bis (3.0 g acrylamide 0.5294 g Bis per 100 ml). This was a discontinuous technique using a tris glycine electrode buffer and a tris/HCl gel buffer. Ammonium persulphate was used to polymerize the gels.

On running this system no evidence of the mucoprotein entering the gel was obtained. There appeared to be a high molecular weight protein component on the outer edge of the gel, this was probably the mucoprotein component. This system was tried with both the flat polyacrylamide apparatus and the tube technique. A series of experiments were run using reduced Bis concentration to see what effect this had on the high molecular weight protein component which did not enter the gel properly. No improvement was seen when the percent Bis was decreased even as low as 4.5% of the acrylamide + Bis.

* Bis (N,N'-methylenebisacrylamide) expressed as a % of acrylamide + Bis in the gel.

This 3% system was then tried using a sample gel, i.e. the flat gel was poured routinely, and then the sample was incorporated into a 3% gel which was polymerized on top of the main gel. When saliva was run on this gel a very intensely stained sample gel and only one diffuse zone which had entered the main running gel was seen. Most of the mucoprotein must have been blocking the pores of the acrylamide.

It was concluded from these studies that acrylamide concentrations around 3% were not low enough to give adequate pore sizes for the mucoprotein to enter. Acrylamide concentrations much lower than 3% are difficult, if not impossible to polymerize, hence a technique giving a larger pore size was required.

McDevitt & Muir (1971) used a large pore acrylamide/agarose gel to study glycosaminoglycans. This technique was tried in this electrophoretic study of the salivary mucoproteins. Details of this procedure are described in Chapter 2.2.o.

The acrylamide/agarose gel was placed vertically in the flat gel electrophoresis chamber. The gel was prevented from sliding downwards by inserting a 3MM paper wick into the bottom of the flat gel mould which dipped into the cathode buffer reservoir. Both reservoirs were filled with buffer and the sample applied to the top of the gel

Results

Staining the salivary proteins, separated by analytical acrylamide/agarose electrophoresis, with Coomassie and Stain All

The protein stain Coomassie and general stain 'Stain All' (Chapter 2.2.g) were used to stain the proteins in saliva when separated analytically by acrylamide/agarose electrophoresis. Saliva collected from cow 9120 was concentrated to one tenth its volume. 300 µl of this saliva plus 100 µl of glycerol and 50 µl of bromophenol blue were mixed and applied to the top of the flat gel. The sample was allwed to enter the gel slowly, the initial current being 15 mA for 20 min. The current was then increased to 35 mA and the run continued for a total of 130 min.

Staining

The gel was sliced in half, one half being stained in Coomassie brilliant blue R while the second half was stained in 'Stain all'. At least five different bands were seen in the gel stained with 'Stain all'. The zone which appeared to be the very high molecular weight protein stained up a yellow colour with a reddish cathodal margin. The other four regions stained up greenish, blue-green, red blue and purple respectively.

Less bands were clearly visible in the Coomassie stained gel but the high molecular weight protein region was clearly visible as there appeared to be a distortion of the gel where this band had entered.

Considerable difficulty was experienced in obtaining good repeatable gels which did not split. Total running times used for most gels was 130-140 min.

Periodic acid-Schiff staining of salivary glycoproteins on acrylamide/agarose gels

The glycoprotein stain periodic acid-Schiff was used to stain the glycoproteins in saliva when separated analytically on acrylamide/agarose gel electrophoresis. Saliva collected from cow 9120 was concentrated to one quarter its volume, and 400 µl of this sample mixed with 100 µl of glycerol and 50 µl of bromophenol blue was added to the gel.

The gel was electrophoresed for approximately 130 min and then removed from the apparatus and sliced longitudinally, one half being stained with Coomassie and the other half being stained with PAS (Chapter 2.2.e).

Six or seven bands were seen in the Coomassie stained gel, the very high molecular weight component which was thought to be the mucoprotein was the slowest moving and its relative migration varied between runs.

The PAS stained gel showed at least five bands, the most intensive stained zone being the high molecular weight component.

Acrylamide/agarose gel electrophoresis of purified mucoprotein

The method of purification of the mucoprotein was described in Chapter 2.2.p.

Purified mucoprotein (400 µl) was applied to the acrylamide/agarose gel and the electrophoretic run commenced at 15 mA for 20 min,

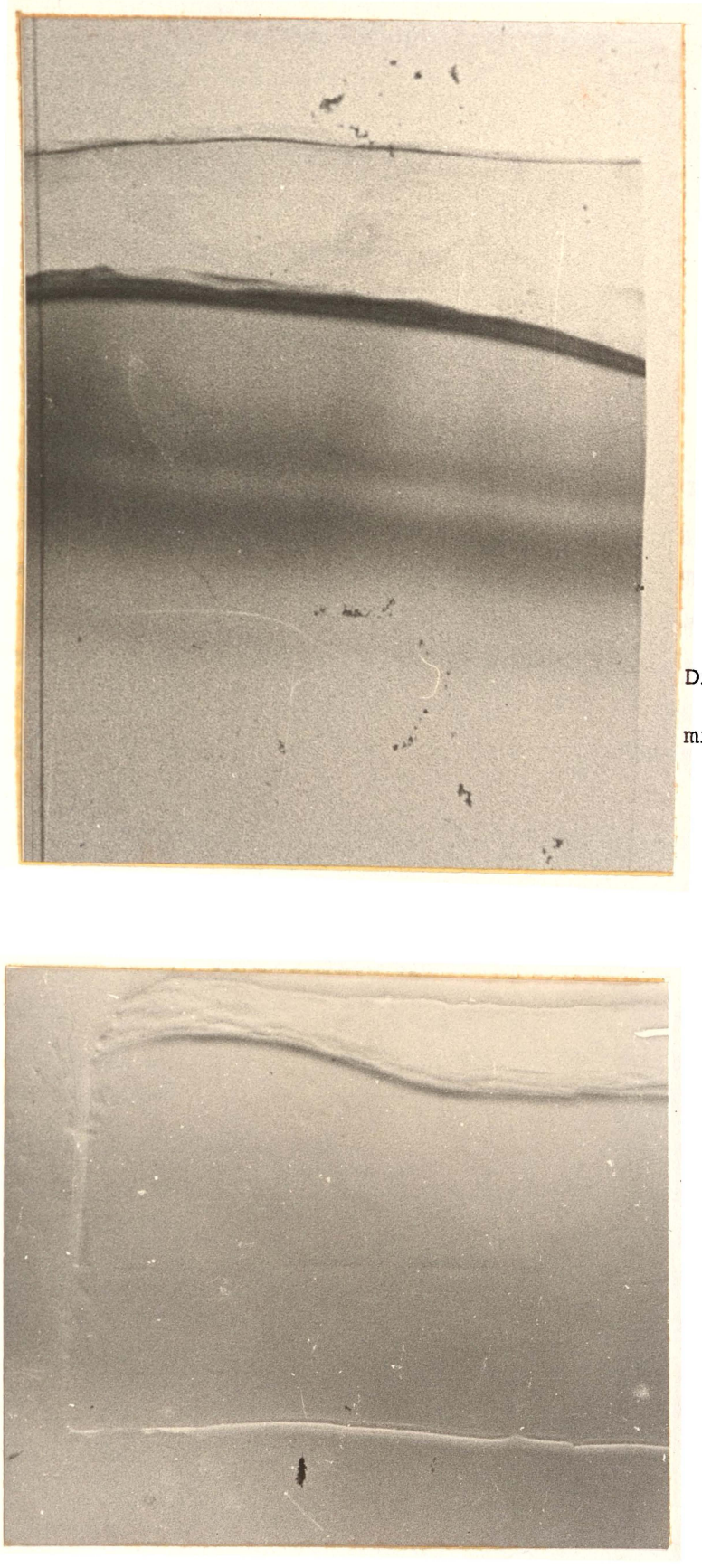


PLATE 6.8.1 Acrylamide/agarose electrophoresis of saliva (A) and purified mucoprotein (B) stained with PAS.

then increased to 35 mA for 110 min. On completion of the run the gel was removed from the electrophoresis apparatus and sliced longitudinally, one half being stained with Coomassie the other being stained with PAS.

The purified mucoprotein entered as one major band in the same position as the high molecular weight component in the mouth saliva collected from cow 9120. This band stained with Coomassie but the best staining reaction was obtained with PAS (Plate 6.8.1).

Discussion

It was possible to show that on electrophoresis at least a major portion of mucoprotein in saliva could enter the acrylamide/agarose gel and that it could be stained with Coomassie, PAS and 'Stain all'. However, the procedure suffered from many inadequacies which would require more time to resolve. The gels had a tendency to split and were very dependent on the particular batch of agarose used. One batch worked quite well most times, while a later batch would not produce a gel that did not split.

Most of the comparisons achieved with these gels were comparing one staining procedure with another using only one sample at a time. This was because no simple method was successful in allowing multiple samples to be compared on the one flat gel.

A single band, accompanied by a considerable distortion of the gel matrix was observed when purified mucoprotein was electrophoresed on the acrylamide/agarose gel. This single band corresponded to the high molecular weight component found in the mouth saliva samples It stained intensely with both the carbohydrate/sialic acid stain PAS and the general stain, 'Stain all'. However the staining reaction of the mucoprotein band was less intense with the protein stain Coomassie. Although the mucoprotein stained satisfactorily with PAS, the variation in the electrophoretic migration and the shape of the band on acrylamide/agarose gels would have made quantitation by electrophoresis an inaccurate procedure.

6.9

SCREENING FOR CATIONIC PROTEINS IN MOUTH SALIVA

Only a brief study to screen for the presence of cationic proteins was undertaken in this study of the bovine salivary proteins. The only cationic 'protein' which had been suggested might be present in bovine saliva was the polypeptide ubiquitin (Shannon et al. 1975). However cationic proteins are found in human saliva especially in parotid secretions, these references are cited in Chapter 1.2.

Materials and Methods

The two electrophoretic procedures used to screen for the cationic proteins were; (a) reverse electrode procedure, (i.e. the standard PAGE system run towards the cathode); (b) low pH electrophoresis according to the method of Reisfeld et al.(1962). This procedure is a discontinuous PAGE technique using β-alanine/acetic acid as the electrolyte buffer, (pH 4.5), and acetic acid/potassium hydroxide as the running buffer (2.2.i).

Four different staining procedures were investigated;

a) The standard Coomassie procedure described in Chapter 2.1.b
b) Modified amido black procedure according to the method of Sung & Smithies (1969).
c) 3,3'dimethoxybenzidine/hydrogen peroxide procedure described by Azen & Oppenheim (1973).
   This reagent appears to precipitate in the gel and has been used for detecting proline rich proteins in human saliva.
d) 'Stain all', as described in Chapter 2.2.g. However this procedure was unsuccessful in detecting proteins at this low pH.

Animals from which the saliva samples were collected

Saliva samples were collected from the following animals to screen for cationic proteins: Cows, 2100, 2077, and 9120 and calf 312 at ten months of age and the twelve 1973 progeny at one year of age. These were all screened by the reverse electrode procedure and the low pH electrophoresis method. Saliva samples, collected at one year of age from the 28 male and female 1975 progeny were all screened for cationic proteins using the low pH electrophoresis method.

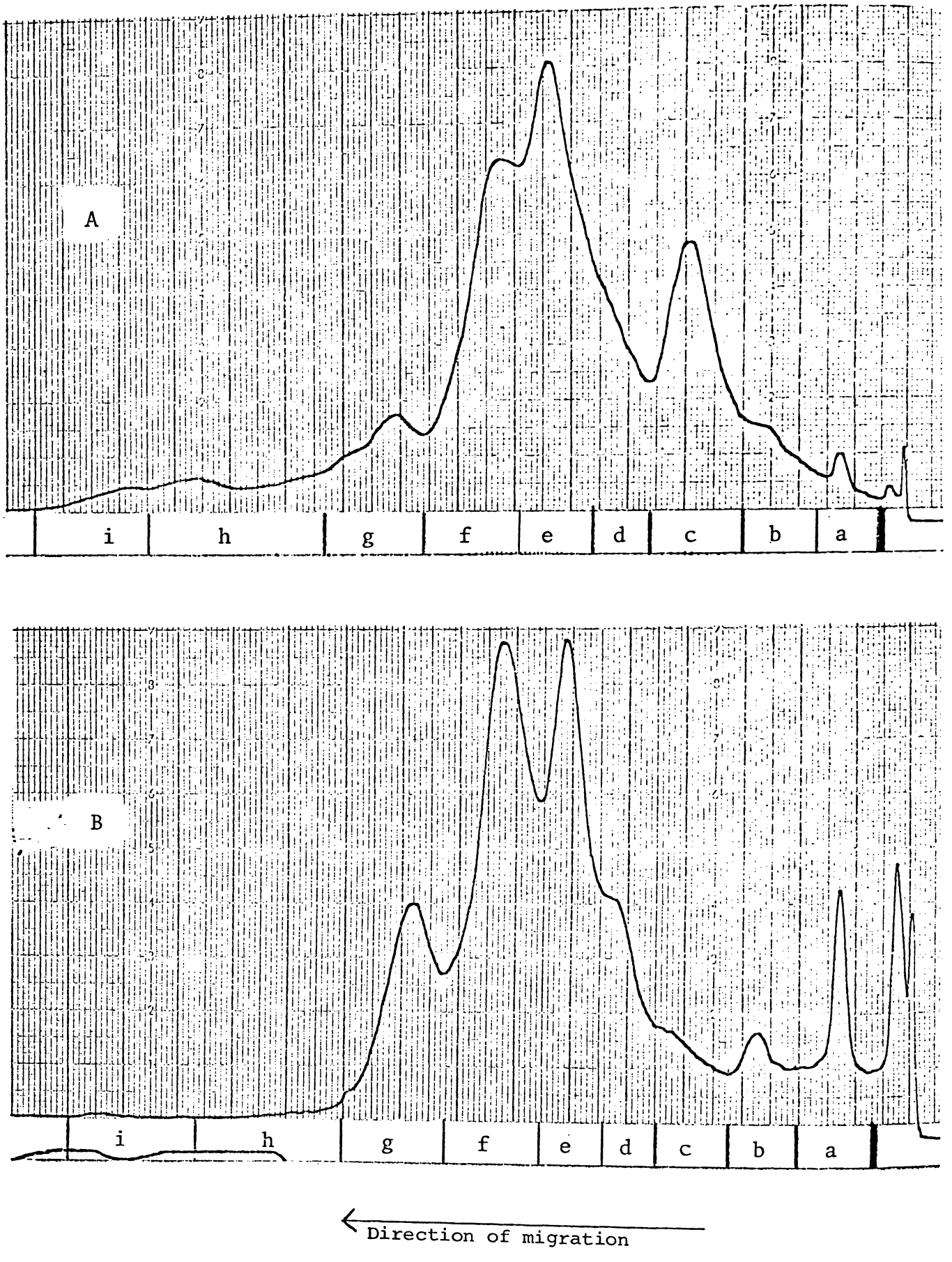


FIGURE 6.9.1 Low pH electrophoresis; two electrophoretic scans (A) standard saliva and (B) cow 520 showing the numbering system used.

Control samples used in this study were, the standard saliva, band 1-2-3 complex, band 7-8-9 complex, and serum albumin.

Results

a) Reverse Electrode Procedure

Studies of the salivary proteins using the reverse electrode procedure did not detect any cationic proteins.

b) Low pH Electrophoresis

A numbering system was devised for the protein bands separated by the low pH electrophoresis. This system consisted of alphabetically numbering the bands from a to i in order of increasing migration rate. The nunbering system is illustrated in Figure 6.9.1 where the standard saliva control sample and a saliva sample from the 1975 progeny heifer number 520 are shown.

It was concluded from this study that there were no cationic proteins detectable (by the methods used) in the mouth saliva of the animals tested. However all the saliva samples showed a diffuse band near the migration boundary which did not stain evenly with Coomassie but had a tail of stained material behind it; this substance also stained with amido black. Gels run without the marker dye still showed this band, whereas it was not present in the albumin control which did not contain saliva.

Discussion

Although this was not an exhaustive search for cationic proteins in saliva, it served as a method to screen for any cationic proteins present which were not detectable by the routine anionic electrophoresis. No cationic proteins were detected by this study, but the nature of the fast moving 'unidentified band' seen with both Coomassie and amido black staining procedures, will require further investigation.

All saliva samples used in this investigation were collected by the standard mouth bit; this bit appears to predominantly collect saliva from the mandibular and sublingual glands and therefore cationic proteins secreted by other salivary glands could have been missed by this study.

SUMMARY

Bovine serum albumin was shown to have the same electrophoretic mobility as band 6 protein in saliva as determined by PAGE. Although no bovine secretory Ig A was available to test its electrophoretic mobility, Ig G, Ig G_1 and Ig G_2 and Ig M spanned the pre-band 1, band 1 and band 2 regions of the polyacrylamide gel.

Bands 1-5 were positive when stained with PAS and bands 1-4 were positive when stained with Alcian blue. However, owing to the sensitivity of the technique it was thought that these positive results might be due to the presence of mucin in the polyacrylamide gel.

Freeze drying, EDTA and ethanol did not effect the band composition of saliva seen on PAGE, but β-mercaptoethanol caused a reduction in band 1 and an increase in band 2. This suggested that the band 1 protein contained disulphide bonding.

Incubation with neuraminidase resulted in a reduction in the proportion of band 7 in whole saliva. When purified band 7-8-9 complex was incubated with neuraminidase there was an initial increase in band 8 and a decrease in the proportion of band 7, but on further incubation band 8 was decreased and band 9 was increased.

α-Amylase was present in low concentrations in the saliva of cattle, the youngest animals having the highest enzymic activity.

There was a low gamma-glutamyl transpeptidase activity in the saliva of the cattle tested. The purified band 1-2-3 complex had a low enzymic activity, but the band 7-8-9 complex had no activity.

A low level of enzymic activity capable of hydrolysing BAEE was present in cattle saliva. Although not specific for kallikrein, this could suggest that kallikrein might be present in bovine saliva. Furthermore enzymic activity was present in the band 7-8-9 complex but not in the band 1-2-3 complex.

Enzymes capable of hydrolysing α- and β-naphthylacetate were shown to be present in the bovine saliva. The majority of this enzymic activity was located in the band 3 protein region.

The thin layer gel chromatography was used to determine the molecular weights of the two purified band complexes. The band 1-2-3 complex had a molecular weight greater than 150 000 and the band 7-8-9 complex had a molecular weight of 65 300.

Molecular weight studies of the salivary proteins by SDS-electrophoresis demonstrated that at least thirteen different molecular weight 'subunits' could be distinguished in the standard saliva. Five major subunits were present in the band 1-2-3 complex and these corresponded in molecular weight to the subunits of Ig G and secretory -Ig A. Either three or four subunits were shown to be present when purified band 7-8-9 complex was run on SDS-electrophoresis. A study was also made to compare the SDS-electrophoretic patterns from the 1973 progeny saliva samples collected in September 1976.

An acrylamide/agarose flat gel electrophoresis technique was successfully adapted to examine the mucoprotein component in bovine saliva. The mucoprotein component in saliva was compared to mucoprotein purified by the method of Lyttleton (1964). The mucoprotein molecule stained intensely with PAS and 'Stain all' but gave rather weaker staining with the protein stain Coomassie.

No cationic proteins were identified in the mouth saliva by the low pH electrophoresis procedure of Reisfeld (1962). The very fast moving diffuse band detected in the saliva sample (but not in the albumin control) has not yet been identified as a protein.

CHAPTER 7

IMMUNOCHEMICAL STUDIES ON BOVINE SALIVARY PROTEINS

INTRODUCTION

Immunochemical procedures offer a means of investigating a mixture of proteins, both for identification and quantitation. Their advantage over most biochemical procedures lie in the specificity of the antigen/antibody reaction. Thus proteins in a heterogeneous mixture can be identified by the use cf specific antisera in any one of the immunochemical techniques described in Chapter 2.3. Furthermore it is possible to quantitate the proteins present, e.g. using the quantitative electroimmunodiffusion assay (Chapter 2.3.d).

The major limitation common to all of the immunochemical procedures is the availability of monospecific antisera. With respect to cattle only a very limited range of antisera is available commercially and hence for screening and identification purposes their usefulness is strictly limited.

The work in this chapter was designed to investigate the following points:

a) Which protein bands cross reacted with bovine Ig G antisera?
b) Which protein bands cross reacted with bovine Ig A antisera?
c) How many protein precipitin arcs could be detected when saliva was run on two-dimensional electroimmunodiffusion, using bovine serum protein antisera?
d) Did band 6 cross react with bovine serum albumin antisera?
e) Was there more than one protein in the band 6 region?
f) Which bands had common cross reactivity between serum and saliva when tested by immunoelectrophoresis using 'whole saliva' antisera?
g) What information could be gained from a study of saliva by crossed electroimmunodiffusion, using 'whole saliva' antisera?
h) Could the electroimmunodiffusion assay be used to quantitate the salivary proteins, and if so, could this technique be used to give further information on the relationship of the salivary proteins to bloat susceptibility?

TABLE 7.1.1 Immunochemical studies using double diffusion and immunoelectrophoresis to test the cross reactivity between samples of saliva and serum and purified immunoglobulins with antiserum prepared against bovine IgA

Test sample	Result
Human saliva	negative
Bovine fraction II gamma globulin	positive
Saliva from cow 9120	"
Saliva from cow 2100	"
Saliva from cow 1121	"
Saliva from calf 04	"
Saliva from calf 09	"
Saliva from calf 20	"
Bovine IgM (semi-purified preparation)	"
Bovine IgG_1	"
Bovine IgG_2	"
Bovine serum	"

Materials and Methods

The work covered in this chapter utilizes several immunochemical procedures, the details of which are given in Chapter 2; immunoelectrophoresis (2.3.a); double diffusion (2.3.b); crossed over electrophoresis (2.3.c); and crossed (two-dimensional) electroimmunodiffusion (2.3.e).

Each subsection of this chapter specifies which antiserum was used, the details on the source of the individual antisera were described in Chapter 2.3.f and g.

The animals tested with each antiserum are listed in each subsection where appropriate.

7.1
IMMUNOCHEMICAL SCREENING OF SALIVARY PROTEINS USING DIFFERENT ANTISERA

7.1.a
Antiserum produced against bovine serum proteins

Antiserum produced against all the serum proteins was used to test for cross reactivity with the saliva proteins.

Immunoelectrophoresis

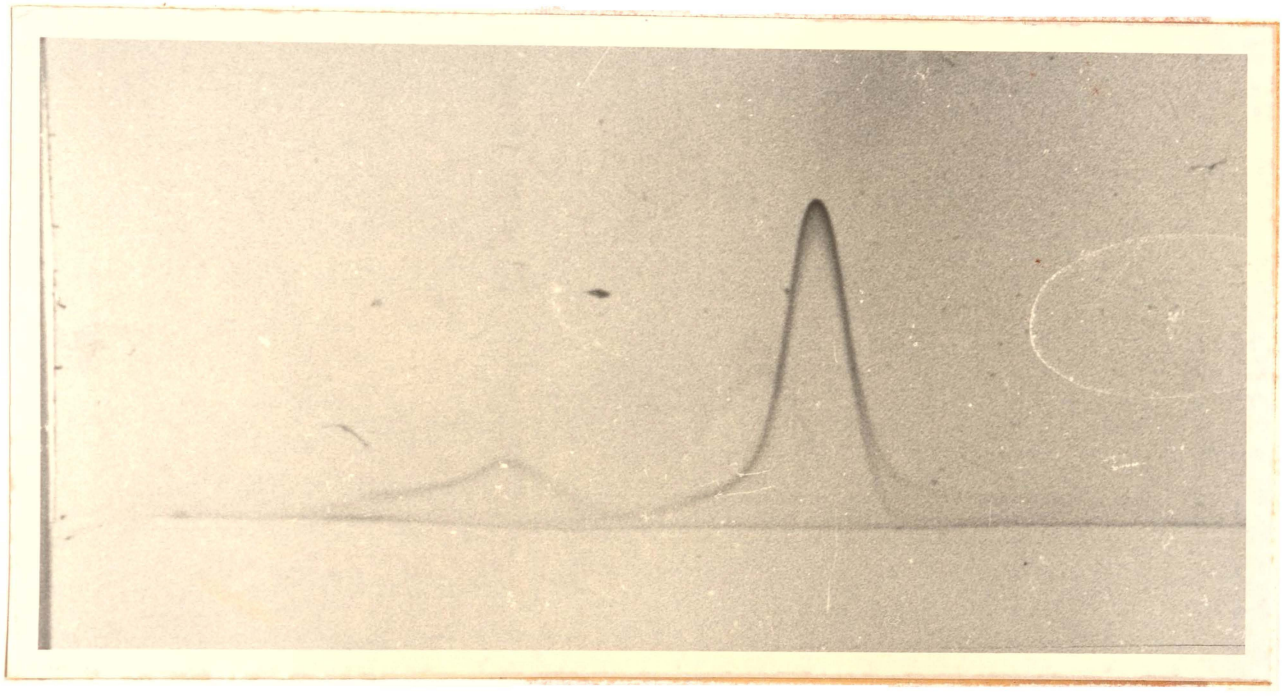
Two precipitin arcs were formed when saliva was run on immunoelectrophoresis. The first, fastest moving precipitin arc corresponded to that of bovine serum albumin, whilst the second precipitin arc was seen near the sample application well.

Crossed electroimmunodiffusion

Concentrated saliva run on crossed electroimmunodiffusion showed three precipitin rockets, the fastest corresponding to bovine serum albumin (band 6 position), the second rocket was within the band 4 region, while a third precipitin rocket was seen at the origin. The precipitin rocket at the origin was extremely faint. The results are illustrated in Plate 7.1.1.

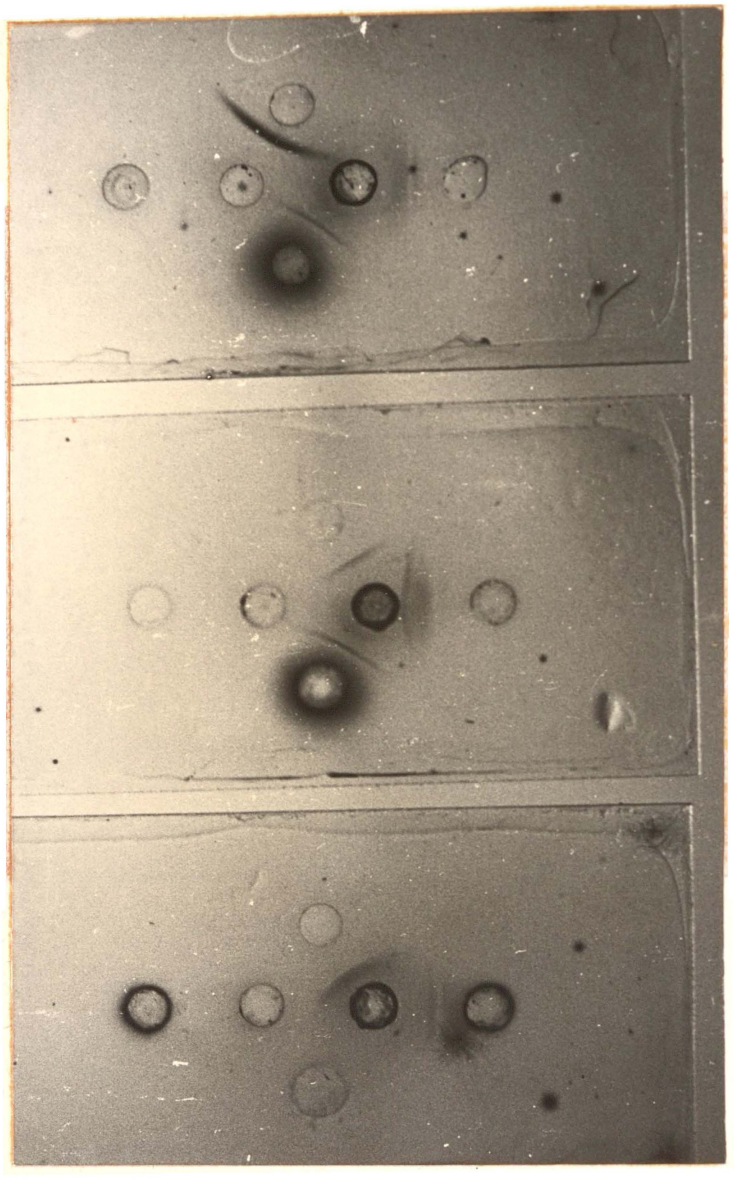
7.1.b
Antiserum prepared against bovine Ig A (Massey University)

A series of studies were made using bovine Ig A antiserum, and the results from these are summarised in Table 7.1.1. This antiserum did not cross react with human saliva which would have contained appreciable quantities of human Ig A. There was cross reactivity with all the bovine



3 2 1

PLATE 7.1.1 Crossed electroimmunodiffusion of saliva using bovine serum protein antiserum. Three precipitin rockets are present, the slowest being poorly resolved in the photograph.



A

B

C

Antigen wells

1 Human saliva

2 Cow 9120 saliva

3 Bovine serum

4 Cow 9120 saliva

1 Bovine fraction II globulins

2 Calf 4 saliva

3 Bovine serum

4 Calf 4 saliva

1 Bovine IgG_1

2 Bovine IgM

3 Bovine IgG_2

4 Bovine IgM

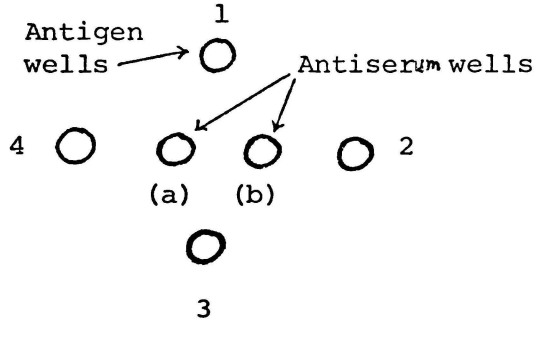


PLATE 7.1.2 Double diffusion studies on bovine and human anti-IgA; antiserum well (a) contains anti-human IgA and well (b) contains anti-bovine IgA

saliva tested, either by double diffusion or immunoelectrophoresis. There was cross reactivity with the commercially prepared bovine Ig M, but this had been shown by electrophoresis to be an impure fraction and could have contained some bovine Ig A. There was cross reactivity with commercially prepared bovine Ig G_1 but not Ig G_2, (Plate 7.1.2). Crossed electroimmunodiffusion studies on bovine saliva, using this antiserum, showed one single broad based peak, with its centre corresponding to the band 1 peak on the first dimension electrophoresis.

7.1.c

Antiserum prepared against human Ig A

Antiserum prepared in rabbits against human Ig A was shown to react positively against the positive control human saliva when tested by three different procedures; crossed over immunoelectrophoresis, immunoelectrophoresis, and double diffusion. However, this antiserum was negative when tested against bovine serum, bovine saliva, bovine fraction II immunoglobulins, bovine Ig G, and bovine Ig M, when tested by the same techniques as used for the human saliva (Plate 7.1.2).

7.1.d

Antiserum produced against bovine immunoglobulin G (Anti-Ig G)

This Ig G antiserum was produced by Miles laboratories (Chapter 2.3.g) against both heavy and light Ig G chains. Therefore because the immunoglobulin light chains are common between the different classes of immunoglobulins, this antiserum would cross react with other classes of immunoglobulins apart from Ig G.

The bovine serum gave two or three strong precipitin arcs on immunoelectrophoresis which spanned into the anodal and cathodal parts of the gel. The bovine Ig G, Ig G_1, Ig G_2 and Ig M were all positive both on double diffusion plates and by immunoelectrophoresis. The Ig G_1 precipitin arc on immunoelectrophoresis was predominantly anodal while the Ig G_2 precipitin arc was predominantly cathodal. There were two precipitin arcs seen when the Ig M was run on immunoelectrophoresis, one of which was quite straight and close to the sample well, similar to that seen for Ig M in human serum, (Arquembourg et al. 1970), while the second precipitin arc was similar to that seen for Ig G_1, but with a

TABLE 7.1.2 Immunochemical studies using double diffusion and immunoelectrophoresis to test the cross reactivity between samples of saliva and serum and purified immunoglobulins with antiserum prepared against bovine IgG

	Double diffusion	Immunoelectrophoresis
	Number of precipitin arcs	Number of precipitin arcs
Bovine serum	2 - 3	2 - 3
Bovine IgG	1	2 or 3
Bovine IgG_1	2	1
Bovine IgG_2	1	1
Bovine IgM (semi-purified)	2	2
Bovine fraction II (gamma globulin)	1	not tested
Saliva from cow 9120	not tested	1
Saliva from cow 1125	" "	1
Saliva from cow 302	" "	1
Saliva from cow 306	" "	1

PLATE 7.1.3 Immunoelectrophoresis using bovine anti-IgG.

Numbering from left to right

1a Purified IgG
1b Saliva from cow 9120

2a Purified IgG_1
2b Saliva from cow 302

3a Purified IgG_2
3b Saliva from cow 306

4a Semi-purified IgM
4b Bovine serum

5a IgG, IgG_1, and IgG_2
5b Saliva from cow 1125

6a IgG and IgM
6b Bovine serum

1 2 3 4 5 6

more hooked tail. All the saliva samples tested from cows and calves gave precipitin arcas similar to Ig G_1. Results are given in Table 7.1.2 and immunoelectrophoresis results are illustrated in Plate 7.1.3.

7.1.e

Antiserum prepared against bovine serum albumin

In Chapter 6.1 it was shown that bovine serum albumin had an identical electrophoretic migration to that of band 6 protein. Therefore commercially prepared bovine serum albumin antiserum was used to test for immunochemical cross reactivity with the salivary proteins. The two procedures used to screen for cross reactivity were, crossed over immuno-electrophoresis (Chapter 2.3.c), and immunoelectrophoresis (Chapter 2.3.a).

Saliva from two cows (2077 and 2100) were run on immunoelectrophoresis using albumin as the positive control; both samples were positive. The position of migration was identical for the test saliva precipitin arc and the arc seen for the serum albumin positive control. This was further evidence that the band 6 region seen in the PAGE was partially or wholly composed of a protein with electrophoretic and immunochemical properties of bovine serum albumin.

As only one precipitin arc was detected in saliva when run on immunoelectrophoresis, crossed over electrophoresis was used to screen saliva from other animals for proteins which cross reacted with the bovine serum albumin antiserum. Saliva samples collected at 10 and 12 months of age from the twelve 1973 progeny, were screened for cross reactivity, and all results were positive except for the sample collected from heifer 302 at 12 months of age.

Nasal secretions were collected from all the 1973 progeny at 10 months of age, and from five mature cows (9127, 2099, 2100, 8130 and 1128); all samples were positive when tested on crossed over immuno-electrophoresis using bovine serum albumin antiserum.

7.1.f

Further immunochemical studies on the band 6 region

This experiment was designed to establish whether or not the band 6 protein seen on the PAGE was a homogeneous protein with specific cross reactivity with bovine serum albumin antiserum, or whether more than one protein was present in the band 6 electrophoretic region.

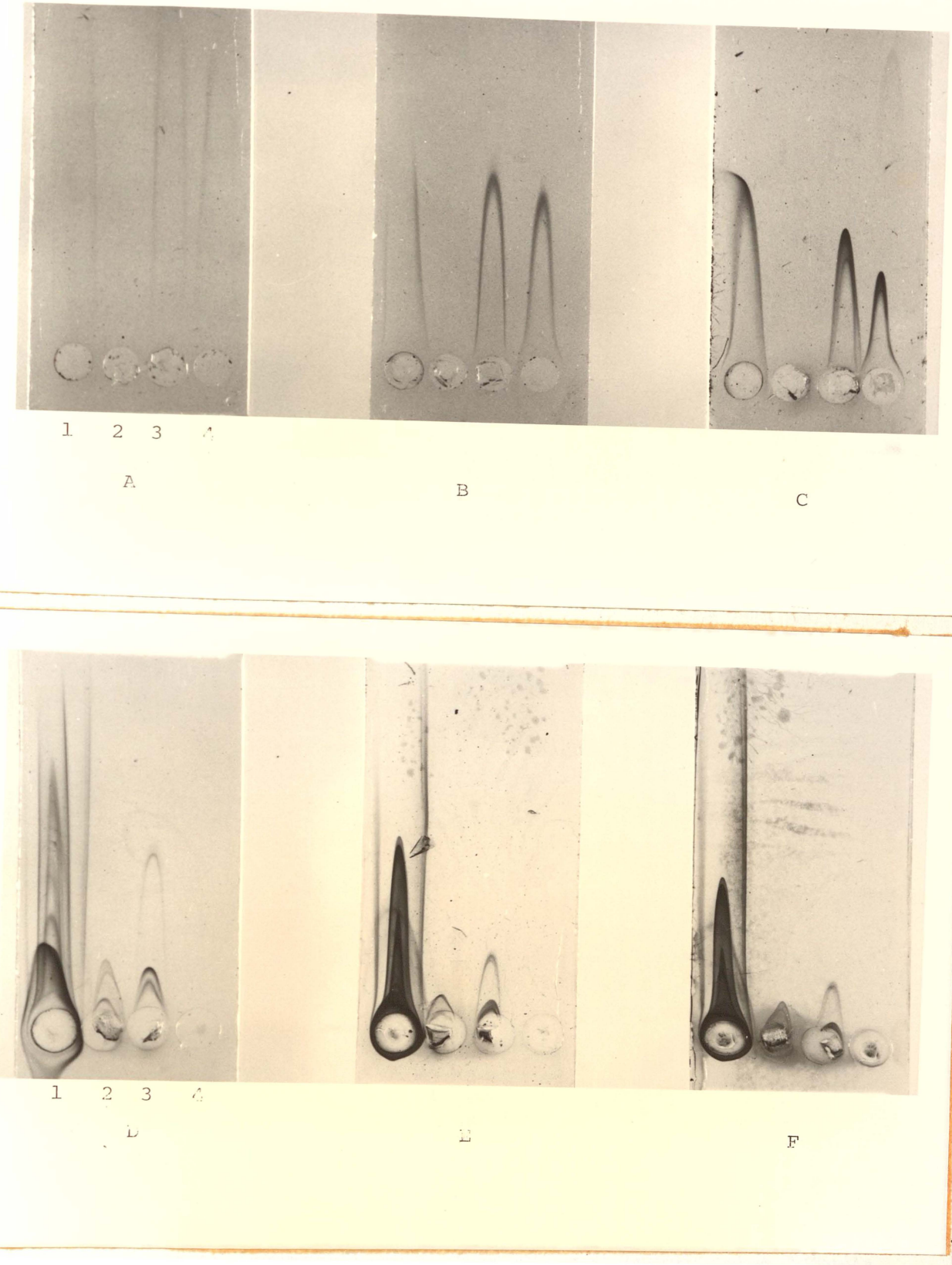


PLATE 7.1.4 Electroimmunodiffusion studies on standard saliva (1), band 4 (2), band 6 (3) and albumin (4), at three different anti-albumin concentrations 0.05% (Plate A), 0.1% (B), 0.2% (C), and three different salivary protein antiserum concentrations, 2% (D), 5% (E) and 7% (F).

Band 6 region was localised on the polyacrylamide gel by means of the rapid stain Coomassie G, (Chapter 2.2.q). The quantitative electroimmunodiffusion procedure was then used to run the dissected bands in the second dimension (Chapter 2.3.d); thus the results were seen as the presence or absence of precipitin rockets. The antigens tested in this experiment were, standard saliva, dissected band 4, dissected band 6, and purified bovine serum albumin. Bovine serum albumin antiserum and bovine whole saliva antiserum were used in this experiment.

Three different concentrations of bovine serum albumin antiserum were used for the electroimmunodiffusion studies; these were, 0.05, 0.1, and 0.2%. The results are illustrated in Plate 7.1.4. The standard saliva showed a good precipitin rocket. Band 4; no precipitin rocket was detected. Band 6; there appeared to be two precipitin rockets present. Albumin; there appeared to be two precipitin rockets present.

Three different concentrations of whole saliva antiserum were used for the electroimmunodiffusion studies; these were 2, 5 and 7%. The results are illustrated in Plate 7.1.4. Standard saliva; several precipitin rockets were present. Band 4; one or two small precipitin rockets were present. Band 6; at least two small precipitin rockets were seen. Bovine serum albumin; no precipitin rockets were seen.

The antiserum prepared against the bovine salivary proteins was devoid of anti-bovine serum albumin activity as determined by the immunochemical procedures employed in these studies. Thus the result showing no precipitin rocket when bovine albumin was the antigen and the salivary protein antiserum the antibody, was to be expected. However the band 6 region showed two precipitin rockets with this antiserum, indicating that these must be different proteins from bovine serum albumin. Band 4 region had been used in this study because when purified bovine serum albumin was run on PAGE, the dimer migrated in this band 4 region. Using the antiserum prepared against the salivary proteins there was one or two precipitin rockets produced by the band 4 protein on electroimmunodiffusion, but when the bovine serum albumin antiserum was used, there were no precipitin rockets present; indicating that the band 4 protein, corresponding in migrational distance to the dimer of bovine serum albumin, did not show immunochemical cross-reactivity and therefore was most likely a different protein. The height and shape

of the rockets produced from the band 4 and band 6 regions dissected from the polyacrylamide gel were not directly comparable to those for the standard saliva or bovine serum albumin. These former proteins were subjected to different conditions at the start of the electroimmunodiffusion as they were still imbedded in the acrylamide matrix, whereas the standard saliva and albumin were in aqueous solutions.

Discussion

When bovine saliva was run on crossed electroimmunodiffusion, using antiserum prepared in the rabbit against the bovine serum proteins, three precipitin rockets were detected. On immunoelectrophoresis only the slowest and fastest migrating proteins were detected. The slowest migrating precipitin arc seen on immunoelectrophoresis was similar to that seen for bovine Ig G_1, while the fastest arc corresponded to that seen for bovine serum albumin. The second precipitin rocket seen on crossed electroimmunodiffusion was not detected by immunoelectrophoresis. Its electrophoretic migration was similar to that seen for the dimer of albumin, but it would appear to be a different protein as the tails of the precipitin rockets crossed each other (Plate 7.1.1).

The first precipitin rocket was very faint on the crossed electroimmunodiffusion but on immunoelectrophoresis was a good precipitin arc. This could be accounted for by the fact that most of the immunoglobulin in bovine saliva is secretory-Ig A, (Pahud & Mach, 1970) while the predominant immunoglobulin in serum is Ig G. Thus on immunoelectrophoresis there will be a good precipitin arc due to the cross reaction of the antibodies against light chains of the immunoglobulin molecules with the Ig A in the saliva. However on crossed electroimmunodiffusion the rocket will appear faint because the height is a measure of the concentration of the total intact molecule present whereas the intensity of the rocket is determined by the number of different antibodies present to interact with the intact molecule. If equal numbers of antibodies were present for the heavy and light chains then the intensity would be expected to be increased compared to when almost only light chain antibodies are present.

The bovine Ig A antisera used in this work was deemed specific for the α-chain (D. Mackenzie pers.comm.), it did not cross react with the

purified Ig G_2 and therefore it probably did not contain any antibodies against the light chains. However it did cross react with Ig G_1 the reason for this is not clear from this data, but could be due to two reasons, (i) that the Ig G_1 was not pure but contained some Ig A or, (ii) that the antisera contained some antibodies against the γ_1-chain.

All the cows and calves tested with this antisera were positive, but human saliva was negative. Human Ig A antisera, which was positive for human saliva was negative for the cows and calves tested, thus suggesting that there was no cross reaction between the human and bovine Ig A molecule. However other workers have demonstrated that ruminant Ig A is cross-precipitated by an antiserum against human colostral secretory Ig A (Pahud & Mach 1970).

The Ig G antisera used was prepared (commercially) against the heavy and light chains of this bovine immunoglobulin. Therefore positive results seen for the saliva samples did not specifically indicate the presence of Ig G in the saliva, but more generally indicated the presence of any immunoglobulin molecule, (all have common light chains). The position of the precipitin arcs in the saliva was very similar to that seen for the Ig G_1, but unfortunately because no purified bovine Ig A or bovine secretory-Ig A was available, the relative position of the Ig A and Ig G_1 precipitin arcs could not be established.

Attempts were made to quantitate the immunoglobulins in bovine saliva using the Laurell Rockets technique, but the commercially available antisera proved unsuitable owing to its lack of specificity.

Virtually all saliva samples tested gave positive precipitin arcs with bovine serum albumin antiserum, as did the nasal samples from the same animals. The electrophoretic migration of the precipitin arcs formed for band 6 and bovine serum albumin on immunoelectrophoresis were coincidental. Whether or not the band 6 protein region was composed entirely of albumin was resolved by using two antisera, the first being the bovine albumin antiserum, and the second being the bovine salivary protein antiserum which was devoid of anti-albumin activity. The results from this experiment demonstrated that at least one other protein apart from albumin was present in the band 6 region.

FIGURE 7.2.1 The numbering system used to characterize the protein precipitin arcs seen when semi-purified salivary proteins or saliva were run on immunoelectrophoresis using bovine salivary protein antiserum

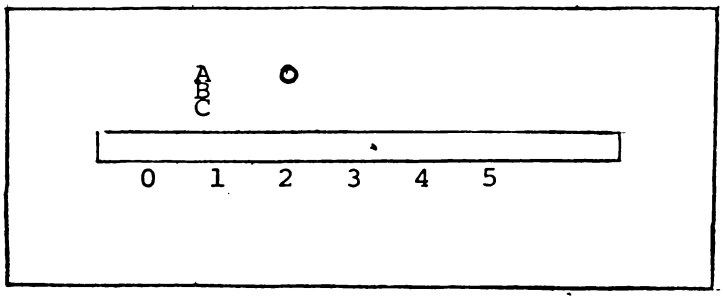


TABLE 7.2.1 Preciptin arcs present when the semi-purified proteins, band 1-2-3 complex, and band 7-8-9 complex were run on immunoelectrophoresis using bovine salivary protein antiserum

		Position of precipitin arcs			
		2	3	4	5
Band 1-2-3 complex	A	2A			
	B	2B*,2B			
	C				
Band 7-8-9 complex	A	2A**			
	B			2-4B	
	C			4C	5C

* faint
** very faint

7.2

IMMUNOELECTROPHORETIC STUDIES OF SEMI-PURIFIED PROTEINS AND SERUM AND SALIVA SAMPLES COLLECTED FROM THE 1973 PROGENY, USING ANTISERUM PREPARED AGAINST BOVINE SALIVARY PROTEINS

7.2.a

A numbering system for immunoelectrophoresis, and a study of the semi-purified salivary proteins

Salivary proteins separated by immunoelectrophoresis gave a series of precipitin arcs when polyvalent antiserum prepared against all the salivary proteins was used. To enable a comparison to be made of these protein precipitin arcs seen in the saliva samples collected from different animals, and the secretions from different glands, a numbering system was devised. This principle behind this simple numbering system was inspired by the method dev ised by Grabar for human serum proteins (Arquembourg et al. 1970).

Using the new numbering system for identifying the precipitin arcs on immunoelectrophoresis, purified band 1-2-3 and band 7-8-9 complexes were characterized.

Precipitin arcs seen in immunoelectrophoresis were numbered according to the system shown in Figure 7.2.1. The position of the arc along the length of the microscope slide was numbered from 0 - 5; 1 being on the cathode side and 3 being on the anode side of the sample well. The distance from the sample well to the antiserum trough was measured by the letters A - C. A being at the sample well, B being almost half way between the well and the trough, whilst C was nearest the antisera trough. Thus any precipitin arc could be described, e.g 2B, is a precipitin arc which lies half way between the sample well and the antiserum trough and approximately below the sample well.

Immunoelectrophoresis used in this work was described in Chapter 2.3.a. The antiserum used was prepared in the rabbit against the bovine salivary proteins (Chapter 2.3.f), and the purified proteins run on immunoelectrophoresis were the band 1-2-3 complex and the band 7-8-9 complex.

TABLE 7.2.2 Immunoelectrophoretic studies on the saliva and serum from the 1973 progeny using bovine salivary protein antiserum

Cow		Saliva 2	Saliva 3	Saliva 4	Serum 2	Serum 3	Serum 4
300	A	2A					
	B	2B*			2B		
	C			4C		1-3C	
302	A	2A					
	B	2B		4B	2B		
	C					1-3C	
303	A	2A					
	B	2B			2B		
	C			4C		1-3C	
305	A	2A					
	B	2B**		4B	2B		
	C			4C		1-3C	
306	A	2A					
	B	2B		3-4B	2B		
	C			4C		2-3C**	
						1-3C	
307	A	2A					
	B	2B			2B**		
	C			4C*		1-3C	
312	A	2A					
	B	2B			2B		
	C			4C		1-3C	
314	A	2A					
	B	2B		4B	2B	2-3C**	
	C			4C		1-3C	
316	A	2A					
	B	2B			2B**		
	C			4C*		1-3C	
320	A	2A					
	B				2B		
	C					1-3C	
332	A	2A					
	B	2B			2B	2-3C**	
	C			4C		1-3C	
333	A	2A					
	B	2B		4B	2B	2-3C**	
	C			4C		1-3C	

* faint ** very faint

The immunoelectrophoresis results of the band 1-2-3 complex and band 7-8-9 complex run against bovine salivary protein antiserum are given in Table 7.2.1. The band 1-2-3 complex gave three precipitin arcs, 2A and two labelled 2B. The second 2B precipitin arc was very faint but was quite distinct from the amjor 2B arc but their centres coincided. The band 7-8-9 complex showed a very faint 2A arc, and a flat diffuse 2-4B arc, however the two major precipitin arcs were 4C and 5C.

7.2.b

Immunoelectrophoretic studies on the saliva and serum collected from the 1973 progeny as three year olds

Immunoelectrophoresis were carried out on the saliva and serum samples collected in September 1976 from the 1973 progeny, using the antiserum prepared in the rabbit against the salivary proteins of the cow (Chapter 2.3.f). The numbering system used to characterize the precipitin arcs was described in Chapter 7.2.a.

The purpose of this work was to identify which bands were present in the saliva from this collection and compare the precipitin arcs with those seen for the serum collected at the same time.

The results are tabulated in Table 7.2.2, for saliva and serum samples for the twelve 1973 animals. All the cows had both 2A and 2B precipitin arcs present in the saliva samples. All animals except 302, and 320 had band 4C present in the saliva while cows 302, 305, 314 and 333 had 4B precipitin arcs. Cow 306 had a slightly slower migrating arc 3-4B. None of the cows had the 2A precipitin arc seen in the saliva, in their serum immunoelectrophoresis, but they all showed the 2B arc. All animals possessed the 1-3C precipitin arc in their serum, and four of the animals had a very faint additional arc, designated 2-3C.

Discussion

The only band in common between the saliva and the serum when this antiserum was used was the 2B precipitin arc. However, it was established that this antiserum was almost devoid of antibodies against bovine albumin; this was attributed to the very low amount of albumin present in the saliva used for the preparation of the antiserum.

The serum would have been expected to cross react with the bovine salivary protein antisera, if sufficient albumin had been present in the original saliva. The 2B precipitin arc corresponded in position to that seen when saliva was run on immunoelectrophoresis, using bovine Ig G antiserum (Chapter 7.1.d). The purified Ig G_1 had a similar but slightly faster precipitin arc to that seen in the saliva samples examined in this experiment.

When purified band 1-2-3 complex was run on immunoelectrophoresis, the two major precipitin arcs seen were 2A and 2B with a third faint precipitin arc also in the 2B region.

From immunochemical studies, and SDS-electrophoresis the band 1-2-3 complex appears to be composed largely of the immunoglobulin molecules and their subunits; it therefore appears almost definite that the 2B precipitin arc is due to an immunoglobulin. In most cases this is probably the Ig A molecule, as the Ig A immunoglobulin has been shown to be the predominant immunoglobulin in bovine saliva (Mach & Pahud 1971) whereas in serum the predominant immunoglobulin is Ig G. It would appear likely that the 1-3C precipitin arc seen in the serum sample collected from these animals is due to the Ig G molecule in the serum. There was also an additional arc, 2-3C present in some of the serum samples, but this was a very faint precipitin arc.

The band 7-8-9 complex gave two major precipitin arcs, these were 4C and 5C, there was an additional diffuse arc 2-4B as well as a very faint 2A preciptin arc. The salivas from these animals showed band 8 to be present in the highest proportion when studied by electrophoresis, and it would appear as if the 4C precipitin arc seen in the saliva was due to this protein band. Some animals showed a 4B or a 3-4B precipitin arc which may be similar to that seen for the band 7-8-9 complex. Serum samples from these animals did not show any bands corresponding to those seen for the purified band 7-8-9 complex.

7.3

CROSSED ELECTROIMMUNODIFFUSION STUDIES ON STANDARD SALIVA

This experiment was designed to investigate the complexity of the protein bands being studied by PAGE, as some of the band regions were thought to be composed of more than one protein. Crossed electro-immunodiffusion was used to determine the rockets present and these were related to the protein bands seen on PAGE by two methods; (1) by using corrected measurements from the gel scan; (2) by dissecting out individual bands from the polyacrylamide gel and running these by the quantitative electroimmunodiffusion (Laurell Rocket) procedure.

Materials and Methods

Standard saliva (Chapter 2.2.c) was used in this study because all the salivary proteins detected by the PAGE technique were present. The routine 7.5% PAGE technique was used for the first dimension run. The second dimension run used an electroimmunodiffusion technique, the antiserum used for this second dimension was prepared against bovine salivary proteins (Chapter 2.3.f).

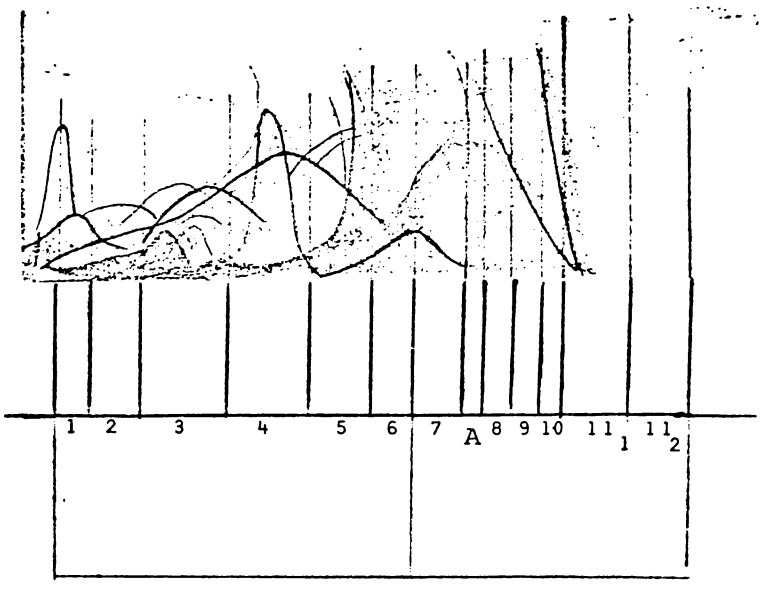
After running the standard saliva on the PAGE, the gel was sliced longitudinally into four segments. One segment was used for the crossed electroimmunodiffusion, the second segment was stained with rapid stain, Coomassie G, to locate the protein bands, and this was then dissected into the various bands as located by the above stain. The dissected bands were then placed on an electroimmunodiffusion slide and run as described in Chapter 2.3.d. Details of the immuno-chemical procedures were given in Chapter 2.3.

A second identical polyacrylamide gel was stained with Coomassie (R250) and scanned according to the routine procedure. The length of the electrophoretic migrations were measured for individual protein bands and were corrected to the band distance seen on the crossed electroimmunodiffusion by the formula $B = \frac{d}{c} \times S$

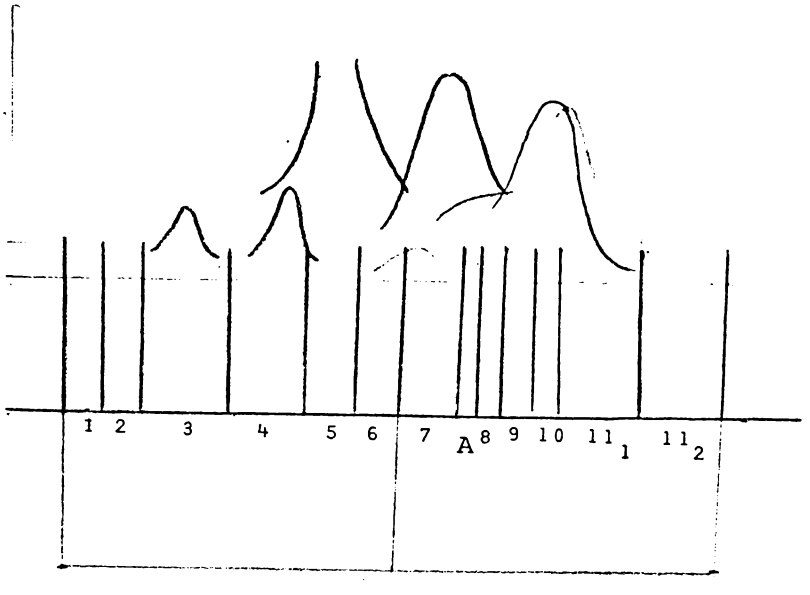
where, B = band location on the crossed electroimmunodiffusion

d = distance migrated for individual bands measured on the densitometer scan

c = constant $\left(\frac{\text{densitometer scan length}}{\text{gel length used for scanning}} \right)$

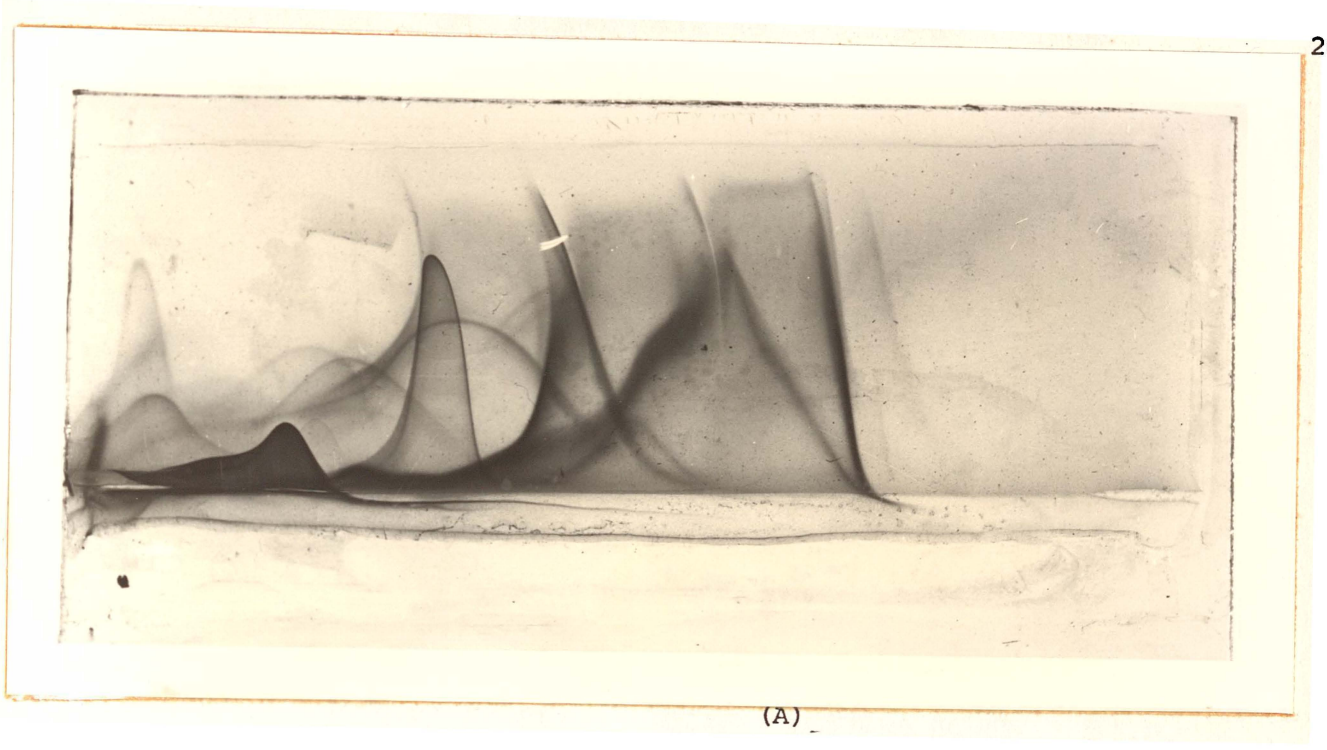


(A)

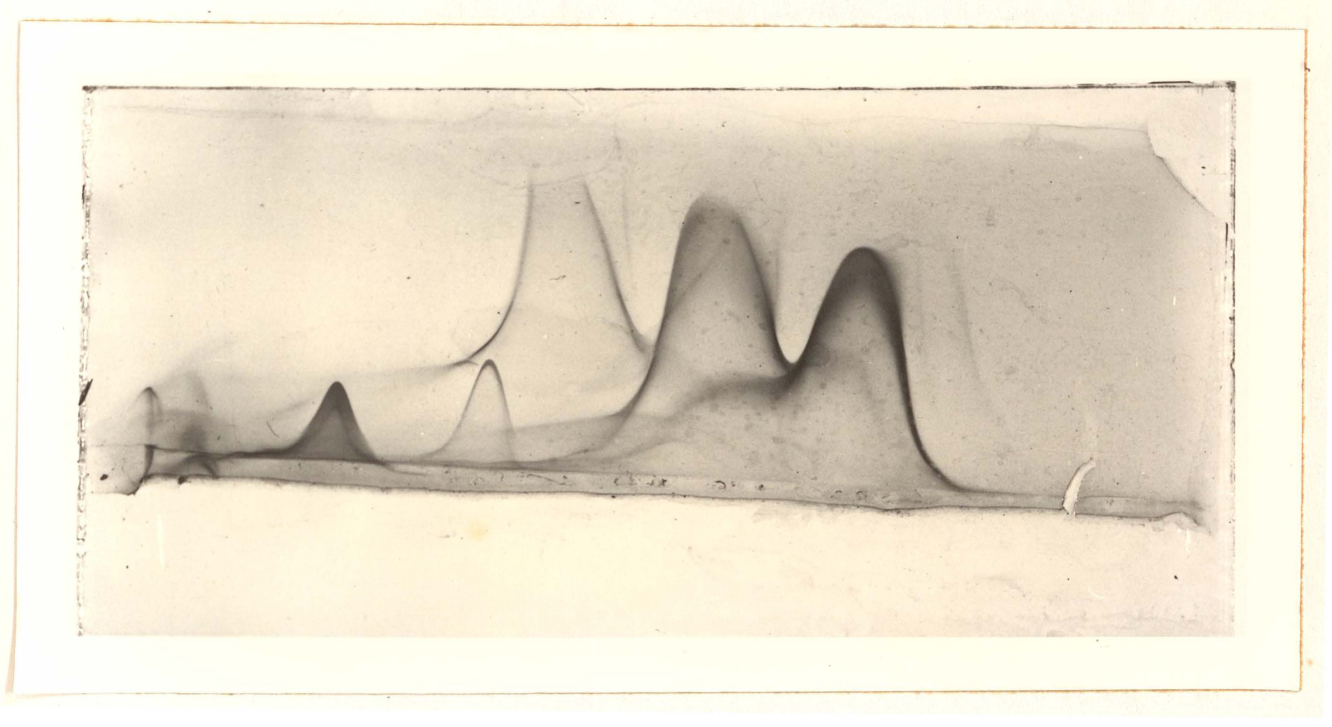


(B)

PLATE 7.3.1 Crossed electroimmunodiffusion of standard saliva using salivary protein antiserum;
A = saliva 5-fold concentrated;
B = unconcentrated saliva;
showing electrophoretic bands superimposed



(A)



(B)

PLATE 7.3.2 Crossed electroimmunodiffusion studies of standard saliva using 5% salivary protein antiserum;
A = saliva 5-fold concentrated;
B = unconcentrated saliva

$$S = \text{Shrinkage or expansion ratio}$$

$$= \frac{\text{gel length used for crossed electroimmunodiffusion}}{\text{gel length used for scanning}}$$

Results

The above procedure was repeated several times using different concentrations of antiserum, but the 5% concentration gave the most detailed results with standard saliva. The standard saliva was run as unconcentrated and as five times concentrated.

Results of the crossed electroimmunodiffusion are shown in Plate 7.3.1 and 7.3.2.

Using five times concentrated standard saliva with 5% salivary protein antiserum the following precipitin rockets were present and classified with respect to the PAGE protein band regions.

Pre-band 1, a faint 20 mm high rocket was present, the centre of which was on the pre-band/band 1 boundary.

Band 1, an 8 mm rocket was present, the centre of which corresponded to the electrophoretic mobility of band 1.

Band 2, a faint 9 mm high rocket was present.

Band 3, five precipitin rockets were present within this region. Their heights were 6, 7, and 8 mm and two further peaks 12 mm in height.

Band 4, a 22 mm high narrow based rocket was present as well as a 16 mm very broad based rocket, this latter rocket spanned from band 1 to band 6.

Band 6, a 5 mm high rocket was present, the centre of which was on the band 6/band 7 boundary.

Bands 5 to 11, this portion of the gel showed at least three additional rockets to the one seen in the band 6 position, but they were all too high to measure or detect their centres. Therefore an unconcentrated standard saliva was run using the 5% salivary protein antisera. The major precipitin rockets detected in the unconcentrated saliva, not defined in previous runs were as follows:

A large rocket centered over the band 5 region was present, the tip of which was not clearly defined. The second largest rocket was 24 mm high, the centre of which coincided with that of band 7, however the rocket completely spanned the band 8 region. An additional smaller rocket, centered over band 8 was also present. The centre of the fastest migrating 21 mm high rocket corresponded to the band 10 region but also spanned band 11.

Discussion

At least 15 protein precipitin rockets were detected by crossed electroimmunodiffusion studies using whole saliva antisera. Single individual proteins were clearly identified at the boundary of pre-band 1/band 1, and in the band 1, and band 2 regions, however five rockets were seen in the band 3 zone. Previous results indicated the immuno= globulins were located within these three bands.

Band 4 was shown to be important with respect to bloat susceptibility, and this protein showed a very clearly defined precipitin rocket. Therefore it should be possible to quantitate this protein when further specific antiserum is available. The broad based rocket which peaked in band 4 might be due to the mucin molecule which by PAS staining on PAGE appeared to span up to band 5.

The salivary protein antiserum being devoid of anti-albumin activity did not show a rocket coinciding with the centre of band 6, but there was a small rocket on the boundary of band 6 and band 7 which might be the second protein which is present in this region (Chapter 7.1.f).

The only explanation offered for the very high rocket seen for band 5 is that this protein might be a poor antigen, and therefore this antiserum would have a low level of band 5 antibodies present. The large rocket coinciding with band 7, also spanned band 8, but also a less clearly defined rocket was present spanning bands 7A and 8. Although not proving some degree of homology between bands 7, 8 and 9, the results from the two-dimensional studies do not negate the possibility. The fastest migrating rocket coincided with the centre of band 10 but spanned into the band 11 region.

The results from the crossed electroimmunodiffusion studies of the standard saliva, provided some idea of the number of individual proteins present in the individual protein bands separated by PAGE. Furthermore it was concluded from this study, that it should be possible to use a quantitative electroimmunodiffusion assay to quantitate some of the salivary proteins (e.g., bands 4 and 6) when monospecific antiserum is available.

7.4

ALBUMIN IN SALIVA AND SERUM

Introduction

Results given in Part I of this thesis indicated that band 6 was important with respect to bloat in cattle. In Part II, Chapter 6, results were given which showed band 6 had a molecular weight and electrophoretic mobility corresponding to bovine serum albumin. Immunoelectrophoresis results given in Chapter 7 demonstrated a cross reactivity between band 6 and bovine serum albumin antiserum, however, electroimmunodiffusion studies using 'whole saliva' antiserum revealed that a second protein was also present in the band 6 position.

This study, using bovine serum albumin antiserum to quantitate the albumin in saliva, was designed to investigate the relationship of salivary albumin with the band 6 protein, with total salivary protein, and the relationship with bloat susceptibility.

The second part of this work on albumin involved a preliminary investigation of serum albumin. Albumin in saliva of cattle and sheep is believed to be derived from the plasma (Watson & Lascelles 1973) and thus it was of interest to establish whether the levels of albumin in saliva were related to the concentration of serum albumin.

Materials and Methods

Albumin in saliva was determined by the quantitative electroimmunodiffusion assay (Chapter 2.3.d), based on the method of Laurell 1966. The concentration of serum albumin was derived by determining the total serum protein concentration, and the proportion of albumin was calculated from the electrophoretic scan. (Serum albumin assays and serum protein electrophoresis were performed in the Animal Health Laboratories, Ruakura.)

The saliva samples used in this study were from the 1973 progeny collected for the Pasture Type (clover/grass) Trials (Chapter 4.2), and the 1973 and 1974 animals sampled in September 1976. Serum samples were also collected from the 1973 and 1974 animals during September 1976.

TABLE 7.4.1 Regressions of band 6 (y) on salivary albumin (x) for the 1973 animals using the data from the Clover/Grass Pasture Type Trial, and the regression of band 6 on salivary albumin for the 1973 and 1974 animals using the data from the September 1976 collection

	Number of animals	Regression Coefficient	SE	Intercept	SE	R^2
1973 Clover/Grass						
Total (overall)	72	.178	0.037	2.052	0.22	26%
Between cows	12	.218	0.089	1.664	0.25	38%
Within cows - between repeats	60	.176	0.040	2.056	0.26	25%
Within Sires & treatments between cows & repeats		.194	0.039	1.898	0.22	27%
September sample (all between animals)						
Total	24	.290	0.080	1.002	0.38	37%

Animals within sires and years were almost the same

TABLE 7.4.2 Regressions of salivary albumin on total protein in saliva for: (1) Six observations per cow from the Clover/Grass Pasture Type Trial (1973 animals). (2) Means for Grass, Clover and September collections for the 1973 animals

	df	Regression Coefficient	SE	Intercept	SE	R^2
1) For six observtions per cow: (Clover/Grass Trial)						
Total	71	0.0062	0.0011	3.98	0.59	32
Between animals	11	0.0051	0.0026	3.98	0.75	28
Within	60	0.0063	0.0011	4.64	0.64	32
2) Means Grass/Clover/September Three observations per cow						
Total	35	0.0025	0.0014	7.35	0.62	8
Between cows	11	-0.0037	0.0010	12.92	0.90	30
Within	23	0.0077	0.0011	2.76	0.44	58

Results

7.4.a
The relationship of band 6 with salivary albumin

The relationship of band 6 and salivary albumin are summarized in Table 7.4.1, where the regressions of band 6 % (y) on salivary albumin are given for the data from the Pasture Type (clover/grass) Trials, and the September 1976 saliva collections from the 1973 and 1974 animals. All the relationships shown in Table 7.4.1 were much the same when the standard errors were considered. They were all significant but left a lot of variance of band 6 unexplained. When the albumin value was zero (see intercepts Table 7.4.1) there was about 1.5% band 6 protein present, and this was significantly different from zero.

Because band 6 protein was expressed as a percentage of the total protein entering the polyacrylamide gel and not a concentration, it was an unrelated measuring system to the concentration of albumin (µg/ml). Therefore the corrected integration method was used to test the correlation between band 6 and albumin in the saliva samples collected in September from the 1973 and 1974 animals as this was a more accurate measure of the concentration of protein in the band 6 region. However, the results for the two methods of measurement (percentage and corrected integrations) gave similar results for the correlation between band 6 and albumin in saliva. The correlations for the 1973 and 1974 HS animals sampled in September were; band 6 percentage, $r = 0.61$ ($R^2 = 37\%$); band 6 as corrected integrations, $r = 0.72$ ($R^2 = 52\%$).

Although the total protein entering polyacrylamide gel was not known, because it was not possible to quantitate the amount of mucin and mucoprotein which did not enter the gel matrix, it was possible to measure the total protein in saliva; therefore the relationship of salivary albumin with total protein could be looked at and allowed for if present.

7.4.b
The relationships of albumin and total protein in saliva

Results from the Clover/Grass Trials showed that the correlation coefficient (r) of albumin and protein was 0.57, but for the September 1976 saliva collection the correlation coefficient was only 0.26. This was looked at further by dividing the Clover/Grass results into its animal and

TABLE 7.4.3 Means and SEM for salivary protein, and bands 1, 4, 6 and 7 and salivary albumin for the Clover/Grass Pasture Type Trials (HS and LS 1973 animals); plus the same parameters for the HS and LS, 1973 and 1974 animals for the saliva samples collected in September 1976 as well as results for serum albumin, haematocrit and γ-globulins

Variable	Clover		Grass		September 1973 Cows		September 1974 Cows	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
HIGH SUSCEPTIBLE SIRES								
Band 4	10.4	0.91	9.3	0.98	10.1	2.03	9.6	1.67
Protein	834	122	721	108	1046	294	1142	382
Band 6	3.6	0.57	3.5	0.48	4.1	1.47	5.8	0.44
Salivary Albumin	8.9	1.60	6.3	0.52	9.3	0.94	14.9	2.40
Band 1	35.5	3.60	32.1	3.33	39.8	7.55	37.6	4.02
Band 7	5.2	0.94	5.3	0.86	4.3	2.03	2.3	0.70
Serum Albumin					30.8	2.60	27.2	0.60
Haematocrit (%)					27.0	1.79	25.3	0.84
γ-globulin					12.0	1.44	16.1	1.08
LOW SUSCEPTIBLE SIRES								
Band 4	10.1	1.42	8.0	0.92	6.8	0.94	6.8	1.31
Protein	970	185	894	93	668	73	730	59
Band 6	4.0	0.58	3.7	0.42	2.8	0.39	3.5	0.32
Salivary Albumin	10.1	1.72	11.9	1.37	8.6	1.67	9.0	1.82
Band 1	27.0	2.70	31.3	2.69	56.2	5.50	33.4	4.59
Band 7	6.9	1.56	6.0	1.37	1.6	0.35	9.9	2.92
Serum Albumin					29.9	1.61	28.2	1.22
Haematocrit (%)					24.8	0.83	29.7	1.09
γ-globulin					12.1	1.45	13.4	1.35

within animal component, and also for the 1973 cows looking at between and within animals where within animals was represented by the means of clover, the means of grass, and the September data (i.e. three observations per cow). The results of these regressions are shown in Table 7.4.2.

The within cow relationships were consistent in both calculations. About 50% of the variation in albumin concentration was associated with variation in total protein. Between cows the relationship was indefinite as the addition of the September data made it negative, while the relationship was hardly real and positive for the clover/grass data. Therefore the between animal relationship for albumin and protein could be taken as zero until further evidence is available.

Adjustment of the band 6/albumin relationships for protein variation on Clover/Grass data increased the between animal R^2 to 54% and that within animals to 29%. Thus conclusions relative to band 6 were largely applicable to albumin. As crossed electroimmunodiffusion results showed a protein other than albumin in the band 6 region and as the quantitative variation of this was unknown the R^2 of 54% suggested reasonable sampling and estimation techniques for initial investigations of both albumin and band 6.

7.4.c

The relationship of salivary albumin with bloat and other variables for the data from the Pasture Type Trials

The occurrence of environmental relationships with pasture bloat has already been discussed for bands 4 and 6, and these relationships contrasted with phenotypic relationships between animals bloat susceptibility or average bloat (Chapter 4.2). The data used for these analyses were from the Clover/Grass Trials and all variables except salivary albumin were analysed in Chapter 4.2.

Table 7.4.3 shows the relevant means and their standard errors for HS/LS and Clover/Grass. Results from the 1973 and 1974 animals sampled in September 1976 are also shown in this table for comparison.

The means from the salivary albumins from the Clover/Grass Trials indicated that if a clover effect on salivary albumin existed, it was for the HS cows. However the concentration of albumin was higher in LS compared with the HS animals (ANOVA $F = 10.03$, $P < 0.01$), and the band 6 results tended to parallel this result. No significant relationships

TABLE 7.4.4 Summary of results for the analysis of variance for the saliva samples collected in September 1976 from the 1973 and 1974 animals, showing the probabilities of obtaining differences by the chances of sampling

Variable	Year Born	Sire	Interaction
Band 4	ns	0.06	ns
Protein	ns	ns	ns
Band 6	ns	<0.05	ns
Salivary Albumin	ns	0.05-0.10	ns
Band 1	<0.05	ns	ns
Band 7	ns	ns	<0.05
Serum Albumin	ns	ns	ns
Haematocrit (%)	ns	ns	<0.05
γ-globulin	0.05-0.1	ns	ns

ns (not significant) P <0.10

were obtained for salivary albumin with bloat potency, even when protein concentrations were allowed for, nor were there between animal relationships with average bloat.

7.4.d

Blood and saliva samples from the 1973 and 1974 progeny collected in September 1976

Parameters measured on these samples were; on saliva, the flow rate, total protein concentration, and protein bands as percentages; and on blood, the albumin and γ-globulin concentration, and haematocrit.

(i) Analysis of variance

Table 7.4.3 shows the mean and SEM for the 1973 and 1974 progeny (September 1976 collection), classified as sire groups and year born. Table 7.4.4 shows a summary of the significant results from the analysis of variance.

The sire effect for the salivary albumin might be a real effect for the 1974 animals as these animals differ in bloat susceptibility. The results for band 6 were similar to the salivary albumin results, the HS sires having a higher band 6 value than the LS ($P < 0.05$), however the LS tended to have a high albumin concentration relative to band 6. There was a borderline sire effect on band 4 ($P = 0.06$), and a year born effect on band 1, and a possible year born effect ($P = 0.05 - 0.10$) on the serum γ-globulin.

The haematocrit results showed a significant interaction, ($P < 0.05$), this would have been mainly due to the LS being greater than the HS for the 1974 animals, which is the reverse of the salivary albumin results. There was also a significant interaction ($P < 0.05$) for band 7, and this was in line with the blood haematocrit results, the 1974 LS animals having the highest value.

(ii) Relationships; simple regressions and multiple regressions

Simple regressions were calculated with average bloat grade (y) as the dependent variable and the saliva flow, total protein and the protein bands, and the blood parameters, albumin, γ-globulin, and haematocrit as the independent variables. Salivary protein, saliva flow, and salivary albumin were all tested as the dependent variable (y) against the blood parameters as the independent variables.

TABLE 7.4.5 Simple regressions for average bloat grade (y) showing the significant correlations on bands 4, 6 and 7, and plasma albumin and haematocrit (and the non significant correlation with salivary albumin), and other significant relationships between the variables for the 1973 and 1974 progeny sampled in September 1976.

		Total		Within Year & Sire	
	Sign	R^2%	Probability	R^2%	Probability
1. y = Average Bloat Grade (as susceptibility measure)					
Band 4	+	18	<0.05	9	ns
Band 6	+	23	<0.01	9	ns
Band 7	-	13	0.10	2	ns
Salivary Albumin	+	8	ns	0	ns
Plasma Albumin	+	4	ns	14	0.05-0.10
Haematocrit	-	22	<0.01	12	ns
2. y = Protein, saliva flow, salivary albumin, NOT related to to plasma variables					
3. Possible relationships (All others ns)					
Band 4 Plasma Albumin	+	16	00.08	21	<0.05
Band 5 γ-globulin	-	9	ns	19	<0.05
Band 6 Salivary Albumin	+	38	<0.01	25	<0.05
Band 10 Plasma Albumin	+	9	ns	18	<0.05

ns = not significant

R^2 (i.e., percent variation of y attributable to x)

All other possible relationships were tested for, and the significant results are given in Table 7.4.5.

There was no relationship between salivary albumin and plasma albumin but band 4 protein sbowed a significant within group positive relationship with plasma albumin and a possible 'total' correlation. Band 10 also showed a similar relationship with plasma albumin. Band 5 protein showed a negative within group relationship with γ-globulin and as shown previously there was a high positive correlation between band 6 and salivary albumin for both the 'total' and within group classification.

The results for the regression of bloat susceptibility on bands 4, 6 and 7 were in line with previous results for these bands. The relationships of bloat susceptibility with plasma albumin and haematocrit were of sufficient interest to warrant further investigation, and these relationships were investigated by a simple regression of average bloat grade (y) on the serum albumin/haematocrit ratio (x), and by a multiple regression approach. The physiological explanation underlying the possible relationship between average bloat grade and the serum albumin/haematocrit ratio is described below:

Plasma albumin results were expressed in terms of concentration (i.e., g/l). However the total plasma albumin present in the blood will depend on the total blood volume and the haematocrit value. If we assume the total blood volume is constant, then the amount of total plasma albumin present will depend on the haematocrit value;

Thus total plasma albumin $A = CV$ (where C = plasma albumin concentration, g/l and V = plasma volume)

therefore $C = A/V$

Haematocrit (percentage) $H = \frac{P}{P+V} \times 100$ (where P = packed cell volume)

therefore
total plasma albumin $A = C/H$

If A and H are assumed independent and/or variation in haematocrit is largely from V, then the relationship between C and H can be used as a correction for plasma volume. Haematocrit variation can be interpreted as blood volume variation but will not be an absolute correction as it will be affected by differences in red cell production and destruction.
The simple regressions of bloat susceptibility on the plasma albumin/haematocrit ratio is shown in Figure 7.4.1. The R^2 for the 1974 animals was 74% while that for the 1973 animals was 31%. The corresponding R^2

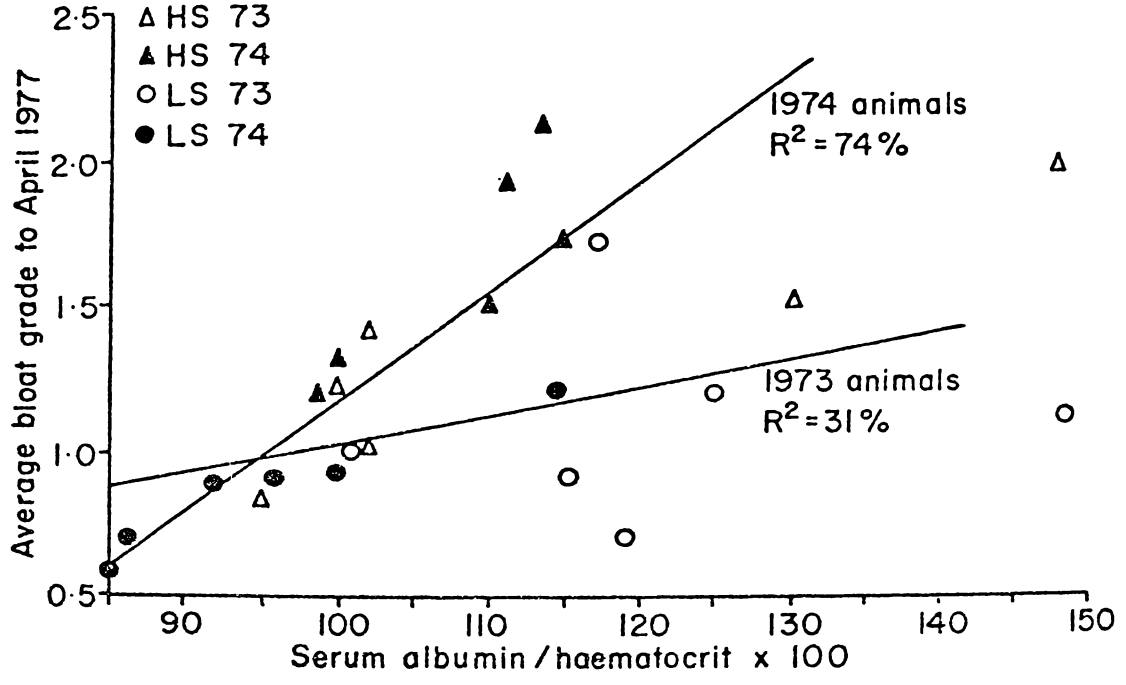


FIGURE 7.4.1 The regressions of bloat susceptibility (average bloat grade to April 1977) on the plasma albumin/haematocrit ratio for the 1973 and 1974 animals showing the R^2 values.

from the multiple regression were; 1974 animals 79%, 1973 animals 14% and the within group regression 25%.

Discussion

The relationship between band 6 percentage and salivary albumin was consistent both within and between animals for the data from the Clover/Grass Pasture Type Trials (1973 animals) and the 1973 and 1974 animals when saliva samples were collected in September. This consistent relationship would indicate that the relationship between these parameters was chemical rather than physiological. The low R^2 for this relationship could have arisen from; (a) Errors in determination of one or both parameters. (b) Variation between and within animals in proportions of the other component(s) of band 6 (as it had been demonstrated that there were at least two proteins in the band 6 region). (c) The different units being used for the measurements, (i.e., band 6 was measured as a percentage of the protein entering the gel, while albumin was an unrelated concentration).

However when this latter point was further investigated using the corrected integrations as a measure of band 6, the correlations between band 6 and salivary albumin were similar to those obtained with the percent method.

When the relationship of salivary albumin to the total protein in saliva was examined by a correlation and regression approach it was shown that within cows there was a consistent relationship with about 50% of the variation in albumin concentration being associated with variation in total protein; however between cows the relationship could be taken as zero. Thus a cow which produces more protein does not necessarily produce more albumin, but when an individual animal has more protein on a particular occasion she will tend to produce more albumin. This means that the proportion of albumin differs between cows but will be less variable within cows than the absolute concentration.

In the data from the Clover/Grass Trials the salivary albumin results were significantly higher for the LS animals compared with the HS. There was no significant relationship between salivary albumin and bloat potency even when protein concentration was allowed for; nor was there between animal relationships with average bloat grade which is in contrast with the results shown in Chapter 4.2 for band 6, despite the positive significant regressions of band 6 on salivary albumin (Table 7.4.1) and the regression of average bloat grade (as susceptibility measure) on band 6 having an R^2 of 23% ($P < 0.05$, Table 4.2.3).

Explanations for this could be:

(a) Real relationships were being concealed by errors.

(b) The other band 6 component might be important with respect to bloat.

(c) The bloat potency of the pasture might have been insufficient to show significant relationships.

The 1974 animals, in contrast to the 1973 animals, showed HS and LS bloat grade differences.

Blood and saliva samples were collected form both groups of animals in September 1976 and the 1974 HS animals showed a considerably higher salivary albumin level than the LS animals. Both the 1973 and 1974 HS animals had a higher band 6 value than the LS animals but the concentration of albumin in saliva relative to band 6 tended to be higher in the LS, which implied that the 'second'protein in the band 6 region might be lower in the LS animals.

When analysed by a simple regression and correlation approach average bloat grade, as a susceptibility measure, was not related to salivary albumin nor was the salivary albumin related to the blood parameters, albumin, γ-globulin or haematocrit. However the within group regression of average bloat grade on plasma albumin and a significant total regression ($P < 0.01$) of average bloat grade on haematocrit were investigated further.

The high R^2 value of 74% for the regression of average bloat grade on the albumin/haematocrit ratio was interpreted as an indication that either the albumin levels or blood volume might be important in determining bloat susceptibility in cattle. When analysed by a multiple regression approach the R^2 value for the 1974 animals was increased, while the lower R^2 value for the 1973 animals was further decreased. The low R^2 value for the multiple regression (14%) compared with the simple regression (31%) was the result of a low correlation between haematocrit and albumin for these 1973 animals. Also some of the LS low bloat grade animals had high plasma and salivary albumin concentrations.

Speculation on the importance of these relationships on the basis of the limited amount of data could be misleading. However, as it had previously been demonstrated that saliva flow was negatively related to bloat susceptibility and band 7 protein positively related to saliva flow and negatively related to bloat susceptibility, a knowledge of factors

which could influence saliva flow would be worth a more detailed investigation. McFarlane (1975) has reviewed the present state of knowledge in sheep on changes in blood volume after feeding resulting from the loss of fluid from the plasma to the saliva, and the effect of other factors on blood volume, including the importance of albumin. As very little is known on the fluctuation in plasma albumin levels in ruminants a long term study on the albumin and haematocrit in relation to animals of different bloat susceptibility might provide useful data to explain the possible relationship between blood parameters and bloat susceptibility.

SUMMARY

Three protein components were detected by crossed electroimmunodiffusion when bovine saliva was tested against bovine serum protein antiserum, but only the two peaks corresponding to bands 1, 2 and 6 were detected by immunoelectrophoresis.

Bovine salivary proteins gave a precipitin rocket corresponding to band 1 when tested against bovine IgA antiserum. This antiserum which was specific for the α-chain of the IgA molecule,was positive with all the cows and calves tested by either double diffusion or immunoelectrophoresis. However, it did not cross react with human saliva, and conversely human IgA antisera did not cross react with cows saliva but was positive against human saliva.

Bovine IgG antiserum containing antibodies against both the heavy and light chains cross reacted with cows saliva from all animals tested.

Band 6 appeared to be composed of at least two different proteins, one which cross reacts with bovine albumin antiserum, and the other which cross reacted with bovine salivary protein antiserum, which was devoid of anti-albumin activity.

A numbering system was devised for the identification of the precipitin arcs seen on immunoelectrophoresis. Saliva from the 1973 progeny tested by immunoelectrophoresis using the salivary protein antisera, showed 2A and 2B precipitin arcs and these appeared to be due to the immunoglobulin molecules. 4B and 4C precipitin arcs were also present and corresponded to the arcs seen for the band 7-8-9 complex. The serum from these animals gave two precipitin arcs, 2B and 1-3C, these were attributed to IgA and IgG respectively.

At least 15 precipitin rockets were detected in standard saliva by crossed electroimmunodiffusion using salivary protein antiserum. Results from these studies gave some indication to the number of separate proteins present in individual bands as determined by PAGE.

Band 6 and salivary albumin were significantly correlated and the relationship appeared chemical rather than physiological. When an individual cow produced more salivary protein it also produced more albumin.

Although the 1973 animals did not show a relationship between bloat grade and salivary albumin, the 1974 animals which showed HS and LS bloat grade differences also showed higher salivary albumin levels in the HS animals.

There was no correlation between salivary and plasma albumin, but there was a significant relationship between average bloat grade and the plasma albumin/haematocrit ratio.

CHAPTER 8

A COMPARISON OF SECRETIONS FROM DIFFERENT SITES AND GLANDS

INTRODUCTION

Although whole saliva samples would have been preferable for this investigation into salivary proteins with respect to bloat susceptibility, it is not possible to obtain whole saliva samples without subjecting the animals to surgical insult. Whole saliva can be collected from the oesophagus of cows which have been fitted with a suitable fistula, however this is not practicable when large numbers of animals are to be sampled (Cockrem 1975). Therefore, the mouth bit was used as the standard sampling procedure. However, because of its location under the animals tongue, it was expected to sample more selectively from those glands opening close to it (Phillipson & Mangan 1959).

Because the mouth saliva collection was not likely to be representative of whole saliva as saliva collected from an oesophageal fistula or at the cardia, the following points were investigated:

a) Were all the proteins which were found in the whole saliva (oesophageal saliva) found in the mouth collections?

b) Were there differences in the levels of sodium and potassium between mouth and whole saliva which might indicate the types of glands being sampled by the mouth bit?

c) Was there a significant correlation between the percentages of individual protein bands seen in the mouth samples compared to those in the whole saliva?

d) Which protein bands appeared to be secreted by the glands sampled by the mouth bit?

e) Which protein bands were secreted by glands not selectively sampled by the mouth bit?

f) How did the samples collected by the mouth bit compare with more specific mandibular gland collections?

The presence of immunochemically cross reacting proteins between mouth saliva and other secretions (and body fluids) were also investigated, these included; mandibular gland secretions, nasolabial gland secretions, and nasal secretions, and blood (as serum).

TABLE 8.1.1 A comparison of saliva collected by the mouth bit with samples collected from an oesophageal fistula

Protein band	Mouth Mean ± SEM	Oesophagus Mean ± SEM	Mouth Oesophagus Mean ± SEM	Oesophagus Mouth Mean ± SEM
1	23.5 ± 6.6	7.6 ± 1.6	20.9 ± 5.6	8.5 ± 2.2
2	6.7 ± 2.3	9.1 ± 2.6	9.1 ± 2.6	6.7 ± 0.6
3	10.3 ± 1.8	10.7 ± 0.9	11.5 ± 1.5	10.8 ± 1.0
4	3.7 ± 1.5	21.6 ± 2.0	3.3 ± 0.9	13.4 ± 0.8
5	2.3 ± 1.0	6.8 ± 0.6	2.2 ± 1.0	4.2 ± 0.2
6	2.3 ± 0.9	11.3 ± 1.3	2.3 ± 0.6	8.5 ± 1.1
7	19.5 ± 3.6	5.1 ± 0.6	19.5 ± 3.8	8.7 ± 2.3
7A	1.3 ± 0.2	3.1 ± 0.5	1.5 ± 0.4	2.3 ± 0.2
8	19.0 ± 2.3	6.3 ± 0.9	17.9 ± 2.5	10.6 ± 1.6
9	8.3 ± 1.0	8.3 ± 1.4	8.4 ± 1.1	11.5 ± 1.0
10	2.1 ± 0.5	4.6 ± 0.5	1.8 ± 0.5	6.8 ± 0.7
Total protein µg/ml	1793 ± 212	240 ± 52	2306 ± 292	513 ± 148
Flow rate ml/min	2.6 ± 1.0	22.9 ± 5.9	2.2 ± 0.9	19.1 ± 2.5
(Na) m mol/l	23.7 ± 4.5	150.2 ± 5.6	36.5 ± 8.7	142.7 ± 6.4
(K) m mol/l	13.6 ± 1.9	4.8 ± 0.2	16.4 ± 1.4	5.8 ± 0.6

Three cows were used in this one day trial. Each cow was sampled twice (i.e. morning and afternoon) for each treatment. Each mean represents an average of six results.
Individual electrophoretic bands were expressed as percentages.

8.1

MOUTH VERSUS OESOPHAGEAL SALIVA COLLECTIONS

Three cows each fitted with an oesophageal fistula were used in this one day trial. Each cow was sampled twice (i.e. morning and afternoon) for each treatment.

The treatments consisted of collecting saliva over 5 min periods by three procedures:

(a) Mouth collection by the standard bit (mouth sample)

(b) Oesophageal collection only (oesophageal sample)

(c) Standard bit collection from the mouth (mouth/oesophageal sample), while the oesophageal collection (oesophageal/mouth sample) was being performed.

The cows had been starved overnight prior to these collections, but were fed immediately after the morning collections had been performed, thus there was at least 3 h between the morning feed and the afternoon sample.

The parameters measured in this trial were the individual protein bands expressed as percentages, the total protein measured by the Lowry method, saliva flow rate in ml/min, and the sodium and potassium concentrations expressed as mmol/l.

Results

Results are tabulated (means $\pm$ SEM) in Table 8.1.1.

Bands 2, 3, 7A and probably 9, did not differ significantly between the mouth and oesophageal collections. Bands 1, 7, and 8 were increased in the mouth compared to the oesophageal collections, while bands 4, 6 and 10 (and possibly 5) were decreased in the mouth compared to the oesophageal collections.

The individual protein band values from the mouth/oesophageal collection were plotted against the oesophageal/mouth collection results. All bands except 1 and 7 gave correlations not significantly different from zero, but correlation coefficients for percent of bands 1 and 7 found in the mouth compared to that measured in the oesophagus were 0.742 and 0.804 respectively. Thus the probability that these correlations could have arisen by chance was around the 10% level.

The average flow rate of saliva from the oesophagus was 9 times that of saliva from the mouth, while the protein results were 7.5 times higher in the mouth saliva compared to the oesophagus when treatment (a) was compared to treatment (b). However, in treatment (c) where both mouth and oesophageal collections were done simultaneously the protein values for both mouth and oesophageal collections had increased but the protein concentration in the mouth samples were still 4.5 times higher than that seen in the oesophagus (Table 8.1.1).

Sodium and potassium results were markedly different between mouth and oesophageal collection. The sodium concentration in the mouth collections being only 15-25% of that found in the oesophageal collection whereas the potassium concentrations in the mouth were 2-3 fold that found in the oesophagus (Table 8.1.1).

Discussion

The major limitation of these results was that the protein bands were expressed as percentages of the total. However the absolute amounts of an individual protein (mg/ml) can only be calculated when the concentration of the total protein entering the polyacrylamide gel can be calculated.

These results indicated that the mouth saliva samples collected by the standard bit was selective for glands other than the parotid. The large increase in protein concentration in the mouth collection and the low flow rate suggested that the bit sampled predominantly from the mucous glands or mixed salivary glands as opposed to the serous glands. However this was best illustrated by the large difference between sodium and potassium values from the mouth collections as compared to those from the oesophagus, which according to the sodium and potassium results of Phillipson & Mangan (1959), indicated that a large proportion of the mouth saliva was secreted by the mandibular gland.

Bands 1, 7 and 8 were all increased in the mouth compared with the oesophageal collections, indicating that these proteins were probably secreted (and possibly synthesized) by the glands which were more slectively sampled from, by the mouth bit. Bands 4, 6 and 10 (and possibly 5) were decreased in the mouth saliva, suggesting they were secreted by glands other than those which were selectively sampled by the mouth bit.

From this comparison of mouth and oesophageal saliva collections it was concluded that all the anodal proteins (as determined by 7.5% PAGE) seen in the whole saliva collected from the oesophagus were also represented in the mouth collections, but the proportion of the individual proteins differed.

8.2

COLLECTION OF SECRETIONS FROM THE MANDIBULAR, NASAL AND NASOLABIAL GLANDS OF COWS AND THEIR COMPARISON WITH THE MOUTH SALIVA COLLECTED BY THE STANDARD BIT

8.2.a

Preliminary samplings to compare mandibular and nasal secretions with mouth saliva

Cow 2100 was used for this first mandibular saliva collection. Less than 1 ml of saliva was collected from this animal when sampled by means of the rubber suction cup which fitted over the opening of Whartons duct. The collection procedure was described in Chapter 2.1.e. Electrophoretic results indicated that the proportion of individual protein bands seen in this collection were very similar to the proportions seen in the mouth collection which was performed immediately after the mandibular collection. That is, there were no protein band proportions which differed greatly from the standard bit collections of mouth saliva. The results from this collection can be seen in Table 8.2.1.

Nasal swabs were collected from cow 2100 after both the mandibular and mouth saliva samples had been collected. The electrophoretic results for this nasal collection (Table 8.2.1) showed the presence of high levels of bands 1 and 8, 21.3 and 27.6% respectively. Band 6 was sharply resolved, but was only 3.7% of the total protein entering the gel. There were proteins present in the bands 2-5 region but these could not be resolved as individual bands. In this collection there was almost no band 7 or 7A and very little if any post band 8 protein.

Table 8.2.1 Comparison of Mandibular Gland and Nasal Collections from cow 2100, with Mouth Collections

Bands	1	2	3	4	5	6	7	7A	8	9	10
Mandibular	29.5	13.6	10.5	10.4	3.3	9.6	6.3	1.8	10.4	3.8	1.0
Nasal	21.3	←unresolved(44.7)→				3.7	←1.3→		27.6	←1.4→	
Mouth	33.0	9.0	14.7	11.3	3.5	6.5	10.3	2.5	5.5	2.5	1.3

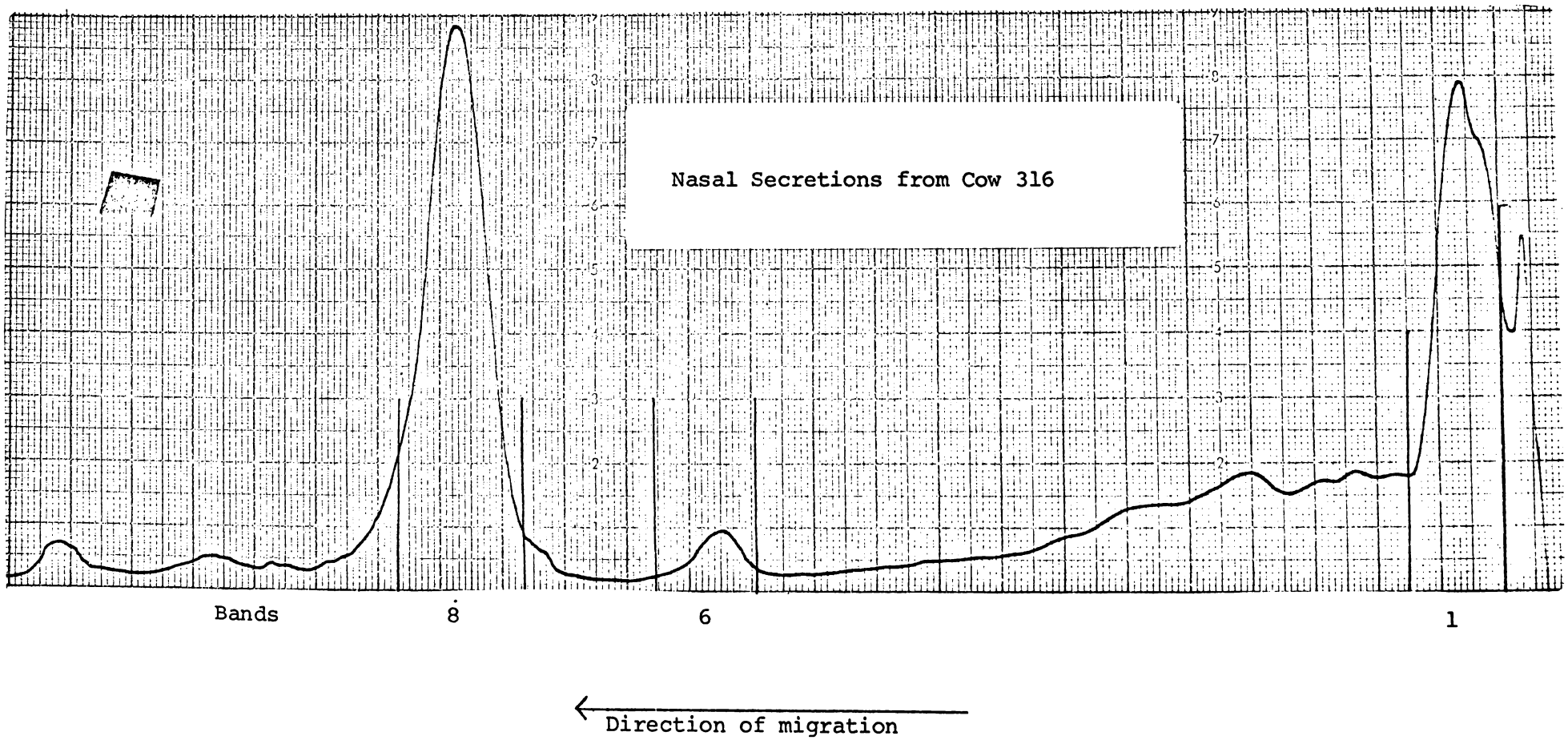


FIGURE 8.2.1 An electrophoretic scan of nasal secretions from cow 316 showing the analytical separation of protein bands 1, 6 and 8 by PAGE.

Nasal collection from the 1973 progeny as yearlings

Nasal swabs were taken from the twelve 1973 progeny as yearlings. These results showed that bands 1 and 8 were present in the highest proportion, with band 6 being sharply resolved. Bands 2 and 3 were present in all these samples in higher proportions than band 6, but in most gels these proteins were not clearly resolved. The band 2 - 5 region made up 30 - 45% of the total protein present in these samples (Figure 8.2.1).

8.2.b

A comparison of the secretions from the mandibular, nasal, and nasolabial glands with mouth saliva, by electrophoresis and immunoelectrophoresis

A further experiment using two HS and two LS animals was carried out to compare mandibular, nasal, and nasolabial gland secretions with the saliva collected by the mouth bit. The purpose of this experiment was to determine the relationship between mouth saliva and saliva collected directly from the mandibular gland. Furthermore it was of some importance to this study to determine whether or not any of the proteins secreted by the nasal and nasolabial glands were the same as those detected in the mouth saliva. Cows tend to lick the end of their nose and put their tongues into their nostrils at frequent intervals, and therefore it would be expected that at certain times mouth saliva will contain some secretions from the nose and nasolabial glands.

In the first part of this experiment the protein bands were studied by PAGE and the proportion of the individual bands compared between the different sampling sites. In the second part of the experiment all the samples including serum from these animals were run on immunoelectrophoresis using whole saliva antiserum.

Materials and Methods

Four animals, two HS (305 and 307) and two LS (312 and 316) were used for this experiment. Secretions from the mandibular, nasal, and nasolabial glands were collected as well as mouth saliva. A serum sample was also collected from each animal at the time of sampling from the various secretion sites. The details of the sampling procedures, PAGE and immunoelectrophoresis were given in Chapter 2.

TABLE 8.2.2 A comparison of the proportions of individual protein bands, determined by PAGE, for saliva (collected by the mouth bit) compared with mandibular, nasal, and nasolabial gland secretions, collected from four, three year old cows

Sample	Protein band	1	2	3	4	5	6	7	7A	8	9	10	11
Mouth													
Mean		23.4	7.7	11.4	12.9	4.9	5.5	4.3	1.8	16.3	6.4	2.7	3.3
SEM		2.9	0.9	2.0	0.6	0.5	0.9	1.3	0.3	2.5	0.5	0.4	1.5
Mandibular													
Mean		34.8	5.6	11.8	10.2	2.8	7.8	2.4	1.1	11.4	6.7	2.0	3.7
SEM		5.8	1.8	1.3	1.0	0.9	2.1	0.3	0.2	2.0	1.8	0.3	0.6
Nasal													
Mean		22.7	← 31.5 (unresolved) →				4.6	1.8	-	29.4	← (10.0) (unresolved) →		
SEM		2.2					0.8	0.2	-	1.3			
Nasolabial													
Mean (approximately)		9.1	← 23.1 (unresolved) →							50.6	← 17.3 (unresolved) →		

Results

PAGE results comparing proteins from the different secretions

Results for the electrophoretic studies are presented in Table 8.2.2

Mandibular collection: As for the original mandibular collection, the percent protein for the individual bands found in these four saliva collections from the mandibular gland did not differ significantly from the levels seen in the mouth saliva collected by the standard bit.

Nasal secretions: The nasal secretions were similar to previous collections, bands 1 and 8 being most concentrated (22.7 and 29.4% respectively). Band 6 was sharply resolved but contributed only 4.6% of the total protein. The band 2 - 5 region was not clearly resolved but collectively represented 28-39% of the total protein entering the gel.

Nasolabial gland secretions: Band 8 was the main band present in the nasolabial secretions, having a mean value of 55.6% of the total protein. The only other protein band of considerable proportion was a protein which migrated in the band 1 region.

The results of the immunoelectrophoresis using antiserum prepared against bovine salivary proteins are shown in Table 8.2.3, where the precipitin arcs seen when mouth saliva, mandibular, nasal, and nasolabial gland ;secretions, and serum are compared.

Mouth saliva from all four animals had the same precipitin arcs present, these were 2A, 2B, and 4C, however the mandibular saliva differed slightly between the four animals, but all had the 2A and 2B precipitin arcs present. Cow 305 had a 3B precipitin arc, while cows 312 and 316 each had a 3-4B arc and 307 had a 3-4C arc.

Nasal samples from the four animals were similar, all having 2B and 3-4C precipitin arcs, but cow 307 had what appeared to be an additional precipitin arc in the 3B region, while the nasolabial samples from animals 305 and 312 both had 2B and 3B precipitin arcs. Cow 305 also had a 4C arc while 312 had a 2-4C arc. The other two cows 307 and 316 each ahd a 2-3B precipitin arc.

Serum samples from all four animals showed two precipitin arcs, these were 2-3B and 0-3C.

TABLE 8.2.3 A comparison of the precipitin arcs seen when saliva (collected by the mouth bit) and mandibular, nasal and nasolabial gland secretions, from four, three year old cows were run on immunoelectrophoresis using bovine salivary protein antiserum

		2	3	4
Cow 305				
Mouth	A		2-3A	
	B	2B		
	C			4C
Serum	A			
	B	2-3B		
	C		0-3C	
Mandibular	A	2A		
	B	2B	3B**	
	C			
Nasal	A			
	B	2B		
	C			3-4C
Nasolabial	A			
	B	2B**	3B**	
	C			4C**
Cow 307				
Mouth	A	2A		
	B	2B		
	C			4C
Serum	A			
	B		2-3B	
	C		0-3C	
Mandibular	A	2A		
	B	2B		
	C			3-4C
Nasal	A			
	B	2B	3B	
	C			3-4C
Nasolabial	A			
	B		2-3B	
	C			
Cow 312				
Mouth	A		2-3A	
	B	2B		
	C			4C
Serum	A			
	B	2B		
	C		0-3C	
Mandibular	A	2A		
	B	2B		3-4B*
	C			
Nasal	A			
	B	2B		
	C			3-4C
Nasolabial	A			
	B	2B**	3B**	
	C			2-4C
Cow 316				
Mouth	A		2-3A	
	B	2B		
	C			4C
Serum	A			
	B		2-3B	
	C		1-3C	
Mandibular	A	2A		
	B	2B		3-4B
	C			
Nasal	A			
	B	2B		
	C			3-4C
Nasolabial	A			
	B		2-3B*	
	C			

* faint ** very faint

Discussion

The proportion of the individual protein bands seen in the mouth saliva and saliva collected directly from the mandibular gland were very similar. This supports the conclusion drawn in Chapter 8.1, that mouth saliva collected from under the tongue by the standard bit, contains a large proportion of secretions from the mandibular glands.

In both experiments the nasal secretions were shown to have a high proportion of both band 1 and band 8. These proteins appeared immunochemically similar to the band 1 and band 8 proteins seen in mouth saliva. However it was not possible from this data to determine what proportion (if any) of these proteins found in the mouth saliva were derived from the nasal glands, because both these protein bands were shown to be secreted by the mandibular glands.

Band 6 protein was identified in the nasal secretions from all animals tested. This protein had been demonstrated (Chapter 7.1.e) to be partly or wholly composed of a protein which cross-precipitated with bovine serum albumin antiserum. It was concluded that albumin was present in the nasal secretions, but because of the low proportion present it was unlikely to influence the levels of albumin detected in the mouth saliva, but these results indicated that more albumin would enter the rumen than would be suggested from the studies on mouth saliva.

The main protein secreted by the nasolabial glands was band 8 ($\bar{x}$ = 55.6%), but on immunoelectrophoresis using whole saliva antiserum no precipitin arc corresponding to band 8 protein was detected. It was concluded from these studies that the band 8 protein from the nasolabial gland was immunochemically distinct from the main band 8 protein seen in mouth saliva. The nasolabial glands of cows and buffaloes have been shown to secrete an amylase which does not appear to be secreted by the salivary glands (Majeed et al. 1970). Amylase was detected in a very low concentration in the saliva from cows used in this study, and therefore it would be of interest to test for amylase activity in the band 8 nasolabial protein.

The band 1 protein present in the nasolabial secretions cross-precipitated with whole saliva antiserum and was probably due to immunoglobulin molecules, but this will be further investigated using specific antiserum against the immunoglobulins.

SUMMARY

Mouth saliva collected by the standard bit collection was largely composed of mandibular secretions, or secretions of similar composition. All the anodal proteins of bovine saliva detected in the oesophageal saliva samples were detected in the saliva collected by the mouth bit sampling procedure. Bands 7 and 8 appeared to be secreted by either the mandibular glands or glands in close proximity, whereas band 10 was produced by glands not selectively sampled by the mouth bit. Bands 4 and 6 were present in the mandibular collections, 10.2 and 7.8% respectively, but were increased in proportion in saliva collected from the oesophagus. This would suggest that these proteins are secreted by the mandibular glands, and other glands not selectively sampled by the mouth bit.

Bands 1 and 8 were the predominant protein bands present in the secretions from the nasal glands, and these were immunochemically related to the bands 1 and 8 seen in the mouth saliva. However from this data it was not possible to determine whether or not these two protein bands contributed to the levels of bands 1 and 8 in the mouth saliva. The secretions from the nasolabial glands contained some band 1 protein and this was immunochemically similar to the band 1 seen in mouth saliva. However the band 8 protein present in the secretions from the nasolabial glands was immunochemically distinct from the band 8 protein in the mouth saliva (as determined by immunoelectrophoresis).

The secretions from the nasal glands contained some band 6 protein, some of which was albumin, however the band 6 protein from these secretions was present in a low proportion and was therefore unlikely to influence the levels in the mouth saliva, but more albumin would enter the rumen than would be suggested by the results calculated from the proportion of albumin present in the mouth saliva.

CHAPTER 9

DISCUSSION OF RESULTS AND GENERAL DISCUSSION

9.1
A SUMMARY AND DISCUSSION OF RESULTS ON SALIVA FLOW AND THE SALIVARY PROTEINS

The work reported in this thesis constitutes the first systematic study of the lower molecular weight salivary proteins in relation to bloat susceptibility in cattle. The study of these proteins paralleled the breeding programme selecting for two lines of animals, those of high (HS), and those of low (LS) susceptibility to bloat. This physiological genetics investigation of bloat susceptibility in cattle relied on studying the variation amongst animals in their susceptibility to the disorder, along with the variation in the salivary proteins and saliva flow.

Relationships of the salivary proteins and saliva flow with bloat

From the preliminary studies on the Group 1 and 2 animals the protein bands 4, 6 and 7 along with saliva flow appeared to show consistent relationships with bloat susceptibility. The proportion of bands 4 and 6 were positively correlated with bloat, while band 7 and saliva flow were negatively correlated with bloat. These latter two parameters were themselves positively correlated with each other. Both bands 6 and 7 were related to the individual cow variance in bloat grade.

In general, from the study of the Group 1 and 2 animals and the progeny group animals, band 4 and band 6 were higher and band 7 lower in the HS compared with the LS animals. The LS animals in both Groups 1 and 2 had higher saliva flow rates than did the HS animals. This was also the case for the 1973 progeny, but for the 1974 progeny the LS animals did not have a significantly higher flow rate than the HS animals until two years of age.

Bloat grade at the time of sampling is not necessarily comparable to bloat susceptibility. The bloat susceptibility of an animal is the average bloat grade over a number of grading periods, as opposed to a single bloat grade recorded at the time of sampling. As well as having LS and HS cows (plus a gradient between these two extreme classifications), within either LS or HS groups there is a range from low to high variance animals.

In effect a low variance animal will have a consistent bloat grade over a number of grading periods when pastured on bloat potent pasture, while a high variance animal, under the identical conditions may range from a low to a high bloat grade.

When saliva samples were collected from the Group 2 animals directly after they came off 'bloat potent' pasture, as opposed to standing' in the yard for two hours after milking, there was an increase in the proportion of band 4 and possibly (but not significant at the 5% level) an increase in the proportion of band 6. This observation was further investigated by three repeat Clover/Grass Pasture Type Trials using the 1973 progeny. These trials were designed to investigate whether the increase in bands 4 and 6 were due to the different sampling regime, (immediately off pasture) or due to the bloat potency of the pasture. The results from these trials demonstrated that there was a significant ($P<0.05$) environmental correlation of band 4 and bloat grade (at the time of sampling). The levels of band 4 were higher on bloat potent pasture regardless of whether the pasture was clover or grass dominant, and the band 4 levels were increased in bloated animals regardless of their bloat group, (HS or LS). There was a positive phenotypic correlation of band 6 and bloat grade, but no environmental correlation. There was also a positive environmental correlation of band 6 protein and saliva flow ($P<0.01$), but this was caused by an unknown environmental factor unrelated to that causing bloat, as was the environmental correlation between band 7 and the total protein.

General studies on the lower molecular weight proteins in bovine saliva

The existance of statistical relationships between the salivary proteins and bloat susceptibility differences amongst animals does not establish that these proteins are involved in determining differences in susceptibility. However, the work on all groups of animals studied gave similar results which indicated that these relationships were worth investigating experimentally. Furthermore the HS and LS 1975 F_2 progeny which showed significant bloat grade differences between the two groups of animals, also showed differences in salivary protein bands (bands 4, 6 and 7) in line with the results from the statistical studies.

If the salivary proteins are involved in determining bloat susceptibility differences amongst animals, then a knowledge of the biochemical properties and their physiological role in the animal's metabolism

is of some importance to the study of bloat susceptibility. The biological process, which results in a proportion of animals bloating when a herd of cows are grazed on bloat potent pasture, will only be explained when the role of the salivary proteins are understood.

In Part 2 of this thesis preliminary work investigating the properties of these proteins was reported. In the following section the properties of the salivary proteins, classified as individual electrophoretic bands, are discussed with respect to the biochemistry of the proteins and their relationship to bloat.

Band 1 protein

Band 1 was one of the major protein bands in the mouth saliva and the studies on the Group 1 animals indicated that there was some genetic or congenital determination in the levels of this protein band. There was a tendency for the proportion of band 1 protein to increase with increasing age but this was accompanied by a decrease in total protein, which suggests that the concentration of band 1 remained much the same while the other proteins were decreased in concentration. The proportion of band 1 also varied considerably between the different saliva sampling trials. In the three repeat Clover/Grass Pasture Type Trials the levels of band 1 were considerably higher in the second repeat than the other two trials. In the Time of Sampling Trials, where saliva was collected at four different intervals after feeding, there was no time effect on band 1 but there was a day effect and a day x sire interaction. That is, band 1 showed a marked increase in the HS cows only, on the second day of each trial. This consistent change in the proportion of band 1, despite the 18 months interval between the repeat trials, indicated that there must have been some specific effect acting on the HS animals.

Electrophoretic studies comparing the levels of band 1 in mouth saliva with secretions from the mandibular glands of the same animals demonstrated a very similar proportion of this protein in both methods of saliva collection. However the proportion of band 1 protein was considerably less in saliva collected from the oesophagus compared with mouth saliva. This would suggest that band 1 protein is predominantly secreted by the mandibular glands and possibly other glands selectively sampled by the mouth bit, e.g. sublingual glands. This is in contrast to the levels of

bands 2 and 3 which were in a similar proportion in either the mouth or oesophageal collections.

The salivary glands of cattle and sheep have been shown to secrete immunoglobulins (Chapter 1.2), the predominant immunoglobulin present being secretory-IgA and this protein appears to be synthesised by the salivary glands (Mach & Pahud 1971; Watson & Lascelles 1973). Some IgG , IgG_2 and IgM is also present in the saliva but very little appears to be synthesised in the salivary glands (Strober et al. 1970; Mach & Pahud 1971; Watson & Lascelles 1973).

The electrophoretic mobilities on PAGE of the band 1 and 2 proteins were very similar to the mobilities of the purified IgG proteins. Furthermore when mouth saliva was incubated with β-mercaptoethanol the proportion of band 1 protein was decreased, indicating that the band 1 protein contained disulphide bonds which were reduced by the sulphydryl reagent.

Crossed electroimmunodiffusion studies of mouth saliva run against bovine serum protein antiserum showed a faint precipitin rocket in the band 1 protein region. When tested with bovine IgA antiserum there was a definite precipitin rocket corresponding to the band 1 protein. Mouth saliva cross reacted with antiserum prepared against the heavy and light chains of IgG, but it did not cross react with the antiserum prepared against the heavy chains of human IgA. Crossed electroimmunodiffusion studies using whole saliva antiserum demonstrated one protein in the boundary of band 1 and another in the centre of the band 1 region. There was also a clearly defined precipitin rocket in the band 2 protein region, but the band 3 region was very complex containing at least five different precipitin rockets.

A semi-purified sample of band 1 protein, designated band 1-2-3 complex was prepared. This preparation contained mostly band 1 protein but there was also a small proportion of bands 2 and 3. When run on thin layer gel chromatography (Sephadex G-150) a molecular weight of greater than 150 000 was obtained. On SDS-electrophoresis this band 1-2-3 complex gave a series of protein bands with molecular weights which could be accounted for by the subunits of secretory-IgA and IgG.

These results suggest that the mouth saliva used in these studies contain immunoglobulins (some of which is IgA) and these immunoglobulin molecules are confined to the proteins at the cathode end of the polyacrylamide gel.

Binkley & Wiseman (1975) suggested that the secretory component of secretory immunoglobulins might be the same as γ-glutamyl transpeptidase. In this work γ-glutamyl transpeptidase activity was demonstrated in the mouth saliva and this activity was shown to be present in the purified band 1-2-3 complex. However further work would be required to resolve whether or not this enzymic activity was due to, or associated with the secretory component in saliva. Labib et al. (1976) has recently produced evidence which indicates the γ-glutamyl transpeptidase activity can be separated from the secretory component.

Band 4 protein

Jones (pers.comm.; Clarke et al. 1974) was unable to detect band 4 protein in the saliva collected from the mouth of cows. However, in this work using immunoelectrophoresis with monospecific band 4 antiserum band 4 protein was shown to be present in the mouth saliva samples collected from the 20 Group 1 animals. There was a lower proportion of band 4 protein present in the mouth saliva, compared with saliva collected from oesophagus (Chapter 8.1) indicating that this protein is predominantly secreted by glands other than those selectively sampled by the mouth bit. Recent studies by W.T. Jones & M. Gurnsey (pers.comm.) would suggest that this protein might be secreted from the palatine glands.

Crossed electroimmunodiffusion studies using whole saliva antisera showed one major precipitin rocket corresponding to the band 4 protein, plus a very broad based precipitin rocket which peaked in this region. There was some indication from the PAS staining reaction that some of the mucin molecule entered the 7.5% polyacrylamide gel, and this mucin was responsible for much of the PAS positive staining reaction in the bands 1-5 region. If mucin was entering these gels then this broad based precipitin rocket seen in these studies might be due to the mucin, but further experimentation would be necessary to test this hypothesis.

Band 6 protein

Band 6 protein in saliva was shown to have the same electrophoretic mobility as bovine serum albumin, and the existance of a protein in this region which cross-reacted with bovine serum albumin antiserum was demonstrated by immunoelectrophoresis. A semi-purified sample of band 6 protein, when run on SDS-electrophoresis, had one peak corresponding in molecular

weight to the bovine serum albumin control protein. These results suggested that at least part of the band 6 protein was composed of albumin.

In an electroimmunodiffusion experiment where band 6 protein, dissected from polyacrylamide gels, was run against bovine serum albumin antiserum, and whole saliva antiserum (devoid of anti-albumin activity), an additional protein other than the protein which cross-reacted with serum albumin antiserum was detected in the band 6 region. This result, which suggested that there were two or more proteins in the band 6 region, was confirmed by the crossed electroimmunodiffusion studies using the whole saliva antisera.

Albumin had been shown to be present in ovine saliva (Watson & Lascelles 1973), and the results from this work indicated that albumin was present in the mouth saliva collected in these studies, and it migrated in the region designated band 6, along with another unknown protein. The relationship of albumin to band 6 protein was further investigated by quantitating the albumin levels in saliva by an electroimmunoassay. Saliva samples from the Pasture Type Trials and the September 1976 collection were quantitated for albumin and the results indicated that there was probably a chemical relationship between band 6 and albumin. There was a high correlation between albumin and total protein within cows, but not between cows. Thus an individual cow when it had a higher total protein concentration in the saliva it would most probably have a higher albumin concentration, but this did not apply between animals. When tested for a relationship with bloat there was an HS/LS sire group difference for salivary albumin in the 1974 animals and these sire groups also differed in their bloat susceptibility. However, there was no significant relationship obtained with salivary albumin and bloat potency, nor was there between animal relationships with average bloat, which is in contrast to the band 6 results.

Band 7 protein

Statistically bands 7 and 8 tended to be negatively correlated, and this relationship might have a chemical basis. This was demonstrated by incubating saliva and band 7-8-9 complex with neuraminidase, where after a short period of incubation the proportion of band 7 decreased and the proportion of band 8 increased. Prolonged incubation periods reduced the amount of band 8 protein while increasing the proportion of band 9. A simple explanation for the statistical relationship between bands 7 and 8 might be

that these proteins differ only by the number of sialic acid residues present. However, if this were the case then neuraminidase would be expected to reduce rather than increase the electrophoretic mobility of band 7 protein and therefore there would appear to be some additional factor involved such as the removal of a positively charged prosthetic group.

The proportion of bands 7 and 8 in the mouth saliva and saliva collected directly from the mandibular gland were very similar, whereas their proportion in the saliva collected from the oesophagus was considerably less. This would suggest that the mandibular gland secretes bands 7 and 8 protein. Nasal secretions were particularily rich in an immunochemically similar band 8 protein, with virtually no band 7 protein being detected. However, secretions from the nasolabial gland also had a protein which electrophoretically migrated in the band 8 position, but this protein appeared immunochemically distinct, as determined by immunoelectrophoresis. The band 8 protein from the nasolabial gland would appear to be chemically different from the major band 8 protein seen in the mouth saliva.

Statistically band 7 protein was positively related to bands 3 and 5, and these three proteins were positively related to saliva flow; however, band 7 appeared to be the key protein in this relationship. When salivation was stimulated by the oral administration of acetic acid, saliva flow and the proportion (and concentration) of band 7 was increased (Chapters 3.4 and 4.5). Protein bands 1, 7 and 8 appear to be predominantly secreted by the mandibular gland. The mucoprotein molecule in bovine saliva is also secreted by the mandibular gland (Lyttleton 1964; Gottschalk et al. 1972). However when salivation was stimulated by acetic acid, the results of assaying total protein suggested that the proportion of mucoprotein molecule was not increased, and band 8 protein was unaffected, while the band 1 protein levels tended to decrease. Therefore the stimulation of protein secretion by the oral administration of acetic acid would appear to be specific for one protein molecule secreted from the mandibular gland.

A partial purification procedure for band 7 was developed, resulting in semi-purified band 7 protein which contained some band 8 and some band 9.. This semi-purified protein was designated band 7-8-9 complex and similar preparations were obtained from repeated purifications using different batches of saliva. Molecular weight determinations on this protein complex using thin layer gel chromatography gave a molecular weight of 65 300

(SEM 2 800) SDS-electrophoresis indicated that the band 7-8-9 complex was composed of three subunits which had molecular weights of approximately 26 000, 22 500 and 17 500. The sum of these three subunit molecular weights corresponded to the total molecular weight for the band 7-8-9 complex as determined by thin layer gel chromatography. Whether or not the band 7 protein is composed of three subunits cannot be concluded from the present data, but further purification of the band 7-8-9 complex using preparative isoelectric focusing should provide more information on the relationship of the three subunits seen on SDS-electrophoresis with the protein bands seen on PAGE. Crossed electroimmunodiffusion studies using whole saliva antserum showed one major precipitin rocket in the band 7 region and this rocket spanned bands 7, 7A and 8 indicating that these proteins might have similar antigenic properties. However, there was an additional small precipitin rocket in the band 8 region.

9.2
GENERAL DISCUSSION

From the evidence in the literature it was apparent that there were bloat susceptibility differences amongst cows as judged by their response to feeding on 'bloat potent' pasture; furthermore the differences seemed to have some genetic component. Much of the evidence indicated that saliva flow or the salivary proteins might constitute all or part of the animal differences in bloat susceptibility.

The importance of the results from this study of the lower molecular weight salivary proteins and saliva flow, in relation to the small amount of data in the literature on the bovine salivary proteins, can be evaluated on the basis of the contribution made to:

a) Defining which proteins might be worthy of further investigation with respect to bloat susceptibility in cattle.

b) Demonstrating a difference in saliva flow rates between HS and LS animals.

c) Demonstrating statistically that certain environmental factors influence the level of these salivary proteins.

d) The identification and purification of the salivary proteins.

e) The basic statistical and biochemical information on saliva flow and the salivary proteins and their inter-relationships.

Factors affecting salivation

The nervous control of salivation in mammals was reviewed by Emmelin (1967) and discussed in Chapter 1.2 of this thesis. The studies reviewed by Emmelin concentrate on the type of nervous control (para-sympathetic or sympathetic) but do not investigate the types of stimuli which evoke salivation. Evidence reviewed in Chapter 1.2 demonstrated that different feeds evoke different degrees of salivation in ruminants. Bartley (1976) suggested that feeds which do not induce large quantities of saliva to be secreted, are those with either a low dry matter content (such as lush pasture), or those eaten rapidly such as ground or pelleted hay or grain concentrates.

Previous work on factors effecting salivation has concentrated on measuring the volume and ionic composition of saliva, but the studies described in this thesis place the emphasis on the individual salivary proteins. Studies on the reflex control of salivary secretion as reviewed by Kay (1966) and described in Chapter 1.2, along with studies on conditioned reflexes (Pavlovian type experiments); for example psychic stimulation by the sight or smell of food, as reported by Denton (1957b), might be worth re-investigating with respect to the individual salivary proteins. Recent studies on the physiology and biochemistry of the taste buds (Bowman et al. 1972; Lum & Henkin 1976) along with recent findings on taste modifier proteins (Cagan 1973) would suggest that it might be worth investigating the importance of the taste buds in stimulating secretion from specific salivary glands. The fact that certain individuals differ in their ability to taste certain compounds (Strickberger 1968) would suggest that the taste buds might possibly play a role in determining animal differences in the amount of individual salivary proteins secreted when a group of animals are fed the same pasture.

Saliva flow and some factors influencing the flow rate

The results from this study measuring the saliva flow, as collected by the mouth bit, indicated that LS animals had a higher saliva flow than did the HS animals. These results were repeatedly demonstrated for the Group 1, Group 2 and 1973 animals; for the 1974 (Friesian cross) animals the higher saliva flow rate for the LS animals was established by two years of age. Thus the higher saliva flow rate in LS animals compared with HS animals was consistent for the four groups of animals studied.

Saliva flow was in general shown to be positively correlated with the proportion of band 7 protein, and negatively correlated with bands 4 and 6. In the trials designed to stimulate saliva flow by the oral administration of acetic acid (Chapters 3.2 and 4.5), the only protein band shown to increase in porportion (or concentration) was band 7. This result would support the hypothesis that the positive correlation between band 7 and saliva flow might have some biological significance.

In a grazing situation other factors appear to influence the saliva flow, as was shown by the positive environmental correlation between band 6 and saliva flow in the Clover/Grass Pasture Type Trials. The results from this series of trials demonstrated that some environmental component,

unrelated to bloat grade, was stimulating saliva flow and the proportion of band 6 protein.

It can be concluded from these studies on the saliva flow rates, of saliva collected by the mouth bit, that the positive correlation between saliva flow and band 7 is probably a meaningful biological relationship, and that LS animals have a higher flow rate than HS animals. However, it would appear that under a grazing situation other factors are involved which can influence these saliva flow rates.

Induction of protein synthesis and secretion of salivary proteins

Squires (1953) demonstrated differences in amylase levels in human saliva depending on the dietary regime of the individual. McIntosh (1975) suggested that the synthesis of a specific protein (or proteins) might be induced by components in bloat potent pasture. If HS and LS animals differed in their response to this environmental stimulus, then this would be reflected in the levels of the specific salivary protein(s) amongst animals after feeding on bloat potent pasture. Results from the work covered in this thesis demonstrated that the three salivary proteins which appeared important in determining an animal's susceptibility to bloat could all be stimulated by environmental components, but the stimulating factors were different for each of these proteins. The band 4 protein was shown to be stimulated by a component in the pasture related to the bloat potency. This environmental correlation between bloat grade and band 4 would suggest that there might be a component in the 'bloat potent' pasture which could induce protein synthesis and secretion of this salivary protein. The results from the Clover/Grass Trials gave no indication to suggest differences between HS and LS animals in their response to this stimulus, but the animals used in these trials did not show significant HS/LS bloat grade differences.

The possible influence of rumen pressure on the salivary proteins

Pressure in the rumen or oesophagus has been shown to cause changes in the quantity and composition of saliva secreted by ruminants, depending upon which pressure receptors respond to the stimulus (Phillipson & Reid 1958; Kay & Phillipson 1959). Therefore it is possible that the increased proportion of band 4 protein, seen in cows which have recently bloated, is a result of the increased rumen pressure rather than a direct effect of the component in the 'bloat potent' pasture. However, it should be possible

to test this hypothesis by collecting saliva from cows which have been feeding on 'bloat potent' clover prior to the onset of bloat. An increase in the band 4 protein, prior to the animal bloating would indicate that the rumen pressure was not the important factor.

Identification of plant components responsible for increased Band 4 secretion

The experimental work in this thesis gives no indication as to how band 4 protein was related to bloat susceptibility in cattle, but the fact that this relationship was consistently demonstrated, opens the field for further experimentation. By studying the proteins in saliva collected from animals fed on pasture which did not induce bloat and 'bloat potent' pastures (different grasses and legumes in their bloating and non-bloat states, and plant extracts) in conjunction with studies on the plant constituents, some insight should be gained into which factors in the plant (including the cytoplasmic proteins) might be important in determining bloat potency of the pasture. Thus the study, by a carefully designed experimental plan, of the induction,synthesis and secretion of the specific salivary proteins by plant components, might provide the tool for identifying the elusive plant components responsible for the bloat potency of pasture. However, this approach makes the assumption that band 4 protein is actively involved in determining whether or not an animal will bloat. The role of band 4 will only be understood when it can be biochemically identified and its physiological role established.

Band 6 protein, salivary and serum albumin and their relationship to bloat

Band 6 protein, which was shown to be phenotypically correlated with bloat grade, was capable of being stimulated by an environmental component unrelated to bloat (Chapter 4.2), and this stimulus also evoked an increase in saliva flow. This environmental effect on band 6 protein was independent of the acetic acid stimulation of saliva flow which caused a specific increase in secretion (and presumably synthesis) of the band 7 protein.

There were two protein components demonstrated in the band 6 region one of which was albumin. The concentration of salivary albumin in the 1974 progeny was higher in the HS compared with the LS animals, as were the bloat grades, but there were no significant relationships shown between

bloat grade and albumin concentrations from the data obtained from the Clover Grass Pasture Type Trials. Whether or not albumin is important in determining an animal's bloat susceptibility will be further investigated, as will the second protein detected in the band 6 region. Albumin could be involved in the condition of bloat merely by its ability to produce a protein foam, as it has been shown to be degraded in the rumen more slowly than many other proteins (Annison 1956). Albumin has been shown to be present in a high concentration in the fluid drained from rumen foam from a steer which had been stabbed because of acute bloat (McIntosh 1975). Furthermore Boda et al. (1957) was able to produce bloat in cows by giving 4 l of egg white through the rumen fistula, indicating that bloat or a 'pseudo-bloat' condition could be induced by proteins with known foaming properties.

An explanation for the differences in albumin concentration in saliva amongst animals could be as a result of a factor which allowed more serum proteins to enter the saliva. Kallikrein through its action on α_2-globulins (kininogens) produce vasodilatory polypeptides known as kinins. Kinins lower the blood pressure and increase tissue permeability, thus more albumin might enter the saliva when more kallikrein has been released in the salivary glands. In man, kallikrein is known to be released in the salivary glands in response to stimulation of the parasympathetic nerves (Ganong 1975). Work in this thesis has demonstrated the presence of a kallikrein like enzyme (Trautschold & Werle 1965) by showing that there was an enzyme in whole saliva capable of hydrolysing benzoyl-l-arginine ethyl ester. At least some of this enzymic activity was detected in the band 7-8-9 complex, but whether this enzymic activity was due to the main proteins in this preparation, or a contaminant protein will be resolved when these band 7, 8 and 9 proteins have been further purified by preparative isoelectric focusing.

As a result of the relationships of band 6 protein with bloat grade and bloat susceptibility, and the presence of albumin in the band 6 region in saliva, the levels of serum albumin were measured. Most of the albumin in saliva is believed to be derived from the serum (Watson & Lascelles 1973), and therefore it was thought that higher levels of serum albumin might allow more albumin to transude into saliva, but no relationship was demonstrated between serum and salivary albumin levels in this study. However, the relationship between bloat susceptibility and the serum albumin/haematocrit ratio indicated that some factors in plasma relating to either albumin or

plasma volume could play some role in influencing an animal's susceptibility to bloat, and might warrant a more detailed investigation.

A salivary protein which appears important in breeding LS animals

The negative relationship between band 7 protein and bloat, and the relationship of this protein with variance in bloat grade, along with the positive relationship between band 7 and saliva flow, would indicate that this protein might be of major importance in selecting animals of low susceptibility to bloat. Band 7 protein might act as an antifoaming agent, such that the animal factors important in the bloat foam might be a balance between the amount of band 7 protein and the mucoprotein present. Alternatively if band 7 protein was a kallikrein like enzyme, then the increased salivation without necessarily increasing the amount of mucoprotein presnet might prevent the bloat foam from forming if the dilution effect, or some antifoaming properties of saliva were important. The oral administration of acetic acid to cows produced an increase in the proportion and concentration of band 7 protein, along with a large increase in saliva flow. If certain plant constituents had a similar effect to that seen for acetic acid and HS and LS animals differed in their response to the stimulus this might account for some of the susceptibility differences amongst animals. Thus LS animals might be cows that do not produce high levels of bands 4 and 6 protein but are capable of producing a higher saliva flow with a higher concentration of band 7 protein; this hypothesis is supported by the studies on the salivary proteins from the HS and LS F_2 progeny (Cockrem 1976). The physiological basis, or the mechanics of how these factors integrate with the formation of the stable foam formed in the rumen of bloated cows forms part of the next stage planned for the research on bloat in cattle.

Immunoglobulins in saliva

In addition to the proteins discussed above, band 1 protein, which appeared to be partially or wholly composed of immunoglobulins, might warrant a more detailed study. It has been shown that antibodies can be formed against rumen bacteria and that these antibodies can be detected in the saliva (Bartley et al. 1975; Horacek et al. 1977). Furthermore Bartley et al. (1975) has suggested that immunoglobulins might play some role in preventing the build up of Streptococci chains in the rumen which could be an important factor in feedlot bloat in determining whether or not bloat develops.

The importance of the immunoglobulins with respect to defence against microorganisms, and their possible role in regulating the types and numbers of rumen microorganisms could also be of importance to pasture bloat and other aspects of animal husbandry. Results from this study demonstrated that the levels of band 1 protein in saliva varied markedly between different sampling but the reason for this variation was not explainable from the available data.

The conclusions drawn from this study on the possible involvement of the salivary proteins in determining bloat susceptibility in cattle, were that at least some of the lower molecular weight proteins were worthy of a more detailed investigation. The electrophoretic bands 4, 6 and 7 add the saliva flow, all showed relationships with bloat susceptibility; protein band 7 and saliva flow appeared negatively related and might have some 'anti-bloating' properties, while protein bands 4 and 6 were both positively related to bloat susceptibility, and tended to be increased in proportion when animals bloated. Biochemical and immunochemical studies on these proteins gave some indication as to the properties and possible identity of some of the protein bands.

The final section of this thesis describes in outline the areas of future research, relevant to the findings of this study, which are likely to contribute to the understanding of bloat susceptibility in cattle.

9.3
FUTURE RESEARCH ON BLOAT SUSCEPTIBILITY IN CATTLE RELEVANT TO THE FINDINGS OF THIS INVESTIGATION

Most of the work discussed in this thesis involved collecting saliva samples by means of the mouth bit. Results from a comparative study between saliva collected by the mouth bit and saliva collected from the oesophagus were reported in Chapter 8.1. Consistent results were obtained by this mouth bit procedure as described earlier, indicating two points; (1) that saliva flow and the salivary proteins might be involved in determining bloat susceptibility, and (2) that the mouth bit might be a suitable method of collection for future studies on large numbers of animals. However, in the next stage of research it will be of considerable importance to understand the relationship between the saliva collected by the mouth bit and the saliva entering the rumen. Collecting saliva from an oesophageal fistula would appear to be the most suitable means of collecting 'whole saliva' from a small number of animals, and therefore a series of studies collecting saliva by both methods are planned. Because of the comparative ease of saliva collection from an animal with an oesophageal fistula it would be possible, using a number of fistulated animals, to look more closely at some of the environmental factors which affected bands 4, 6 and 7, including repeat Clover/Grass Pasture Type Trials and further Acetic Acid Stimulation Trials.

Ideally a specific means of quantitating the salivary proteins not influenced by dye binding differences would give the most information when comparing saliva collected by the mouth bit with that collected from an oesophageal fistula, but this is not possible on the basis of the present knowledge of these proteins. Therefore the method of choice for evaluating the saliva samples collected by the two different methods would be by the corrected integration method (Method D) described in Chapter 4.5. Ultimately however, preparing antibodies against the individual proteins and quantitating by an electroimmunoassay (Chapter 2.3.d) would be the method of choice.

The preparation of monospecific antisera for quantitating these proteins would necessitate purifying the individual proteins. It might be possible to prepare monospecific antisera by dissecting the proteins from the analytical polyacrylamide gels and using these as the antigens. However if it is to be understood how the individual protein bands

4, 6 and 7 are involved in 'determining' bloat susceptibility, then complete purification of the proteins will be necessary. A partial purification procedure for band 7 was described in Chapter 5. Developing methods for complete purification of these proteins would serve two major functions; (i) it would provide a source of purified antigen for the production of monospecific antisera; and (ii) the individual proteins could be biochemically characterised leading ultimately to a physiological explanation for the role of these proteins in bloat susceptibility.

A knowledge of which glands secrete the salivary proteins important to bloat susceptibility could be important for at least two reasons; (a) it might be possible to design a more suitable bit for collecting saliva samples from large numbers of animals; (b) a knowledge of the glands involved in secreting these proteins might be useful if more detailed physiological studies are necessary to explain the biological basis for these protein differences. Possible sights of secretion of some of these proteins were discussed in Chapter 8, however when monospecific antisera to these proteins are available then it should be possible to determine, by histochemical procedures, from which glands the individual proteins are secreted.

Work reported in this thesis demonstrated that certain environmental stimuli of unknown nature could affect the levels of some of the protein bands in saliva, and in some instances saliva flow. A more comprehensive study of feeding grasses and legumes under bloating and non-bloating conditions and their effects on the saliva flow and the salivary proteins should be of benefit to the study of bloat susceptibility. This type of study could include Pasture Type Trials to compare legumes with a high tannin content, which are thought not to cause bloat (Jones & Lyttleton 1971), with 'bloat potent' red clover. A further sophistication of this work could involve large scale fractionation of plant constituents from 'bloat potent' pasture and pasture not causing bloat for stall feeding experiments on cattle. Mechanical de-juicing of plants is already being carried out at Ruakura Animal Research Station (Bryant 1976), and therefore the equipment necessary for the first stages of this work is already available.

A more direct study of the effect of altering the concentration of the salivary proteins could be achieved through the use of pharmacological agents (ganglionic blocking and stimulating agents). This approach to the

study of the salivary proteins would allow the concentration of these proteins to be artificially varied and it would then be possible to detect whether or not there was an altered response when animals were fed on bloat potent pasture. Furthermore this type of study could provide some information on the neurophysiology of secretion of these specific proteins.

Although it is known that in vitro protein foams can be formed from proteins found in the rumen, and that a stable foam in the rumen is a characteristic of pasture bloat, it is not known whether HS and LS animals differ in the proportion or concentration of these factors involved in the foam formation (Chapter 1.3). Work on the foaming properties of rumen liquor has recently been reported by Jones & Lyttleton (1973), but the hypothesis, that the foaming property of rumen liquor differs between HS and LS animals, has not been tested. Whether or not differences can be detected in the physical properties of foams generated in vitro from rumen liquor is of fundamental importance to the study of bloat susceptibility. However this will only be resolved by experimentation based on a correctly designed experimental plan. This would involve testing the rumen contents from HS and LS animals on 'bloat potent' pasture and pasture not causing bloat (control), in a cross-over design experiment where animals tested on 'bloat potent' pasture were subsequently tested on the control pasture and vice versa.

The points discussed above outline some areas of research which would be profitable to follow if the role of the salivary proteins in relation to bloat susceptibility is to be understood. As bloat is one of the major metabolic disorders in cattle in New Zealand, the research project investigating breeding HS and LS cattle should be continued in conjunction with the study of the salivary proteins. Ultimately, by understanding the physiological role of these proteins, it might be possible to establish a selection index for low susceptible (LS) animals and thereby considerably reduce the incidence of bloat in cattle.

APPENDIX A ELECTROPHORESIS

The techniques of analytical electrophoresis are widely used in the study of the biological and medical sciences, either as qualitative or a quantitative procedures. The resolving power of electrophoresis was enhanced with the development of the polyacrylamide gel technique pioneeered by Ornstein (1964), and Davis (1964) for anionic proteins, and Reisfeld et al. (1962) for the cationic proteins.

The two most common approaches to electrophoresis are: (a)Searching for the presence or absence of 'allelic proteins' as used in biochemical genetic studies. Often in this approach the study is qualitative and only concerned with the presence or absence of particular 'alleles', but the resolving power of electrophoresis is important and hence polyacrylamide gel electrophoresis is commonly used as the separating media (Azen 1973, Merritt et al. 1973). (b) Quantitating the serum proteins as routinely used in clinical biochemistry. In clinical biochemistry the quantitation of the electrophoretic bands is most important and separating the individual proteins is less important. The proteins are broadly classified as albumin, α_1-, α_2-, β-, and γ-globulins, but the proportion of each electrophoretic region is accurately quantitated and therefore cellulose acetate, with a lower resolving power than polyacrylamide gel, is commonly used for this work (Wybenga 1974). Accurate quantitation of the electrophoretic regions allows increases or decreases (outside two standard deviations) in the proteins in the electrophoretic region to be used as a guide to the diagnosis and/or progress of a disease, as well as to give information to suggest which proteins might warrant a more detailed investigation (Lynch 1971, Rutledge 1971).

The physiological genetics approach to the study of proteins as used in this project utilized some of the properties from both the above approaches. The high resolving power polyacrylamide gel electrophoresis technique was employed to separate the maximum number of protein bands; these individual electrophoretic bands were stained with the quantitative dye Coomassie Brilliant Blue R250 (Fazekas de St Groth et al. 1963). The accuracy of quantitating the individual bands was an important aspect of this work as is the case in clinical biochemistry. However, this approach differs from either of the others in that individual samples from

individual animals were less important. A large number of samplings on different groups of animals, classified as either HS or LS were used in the physiological genetics study of the salivary proteins. Furthermore when looking at the effect of cows grazing different pastures on the levels of proteins in saliva a series of repeat trials were performed and it was the consistency of the results which was important. Thus data from the physiological genetics approach (as opposed to the biochemical genetics or clinical biochemistry approach), were analysed statistically to determine effects and relationships. The physiological genetics approach results in identifying the areas and direction of research which warrant a more detailed investigation (McIntosh & Cockrem 1977).

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TABLE B Correlations and regressions for Quickscan (y) and Densicord (x). July samples.
Note: Quickscan run with variable gain.

A. For individual animals

Band 1	n = 12	y = 1.35x - 0.56	Range values 10 - 60%	r = 0.97
Band 4	n = 12	y = 0.5x + 0.29	Range values 0 - 10%	r = 0.69

B. Means HS cows, means LS cows for each band covering full range 0 - 40%

n = 24 Linear. y = 1.2x - 1.2 r = 0.93

APPENDIX B COMPARISON BETWEEN THE DENSICORD AND QUICKSCAN DENSITOMETER

At the start of this project the only densitometer available was the Densicord (Photovolt Corp. New York City, N.Y. USA) which had a fixed gain setting and thus integrations between gels were comparable, and as indicated previously, were a measure of the protein concentration entering the gel matrix and the concentration within individual bands. However because of the time taken to scan the gels and the non-linearity at the highest protein band concentrations a Quickscan densitometer (Model Flur-vis, Helena Laboratories, Beaumont, Texas, USA) was purchased and used for all the work on the 1973, 1974 and 1975 progeny. A comparison was made between the two instruments using the saliva samples collected from the Group 2 animals in May and July 1974.

The gels from the May 1974 collection were scanned by both the Densicord and Quickscan at constant gain, however owing to a loss of senstivity of the Quickscan when used at constant gain, it was therefore used at variable gain in July 1974.

Comparison of densitometers

Band 1 peaks in both May and July were higher on the Quickscan, which emphasised that the Densicord was not linear at the higher peaks. The higher values for the main bands meant that other bands were slightly less as percentages on the Quickscan. However the correlation between the two machines was good, the relevant results are given in Table B. The relationships are linear but not equal. They pass through the origin but high values are increased by the extended range of the quickscan, while low values, (e.g. band 4) are decreased. This is effectively a change of scale and not of order so that conclusions from the two machines are unlikely to differ.

The linearity of band 1 (indicated by R^2 of 0.94) between the two machines at high percentages suggests that the stain intensity is within the capacity of the machines.

APPENDIX C The analysis of variance (split plot design) and mean squares of band percentages, total protein, and saliva flow for the repeat Clover/Grass Pasture Type Trials (Chapter 4.2)

	Source	d.f	Saliva Flow	Protein	Band 1	Band 2	Band 3	Band 4
Blocks	Sires (S)	1	111.5022**	428429	385.9568	17.3068	11.5200	10.5305
	Cows within sires (CWS)	10	8.7229	570855	271.7605	5.3792	37.3431	40.4632
Whole Plot	Repeats (R)	2	4.6677	370876	1989.4593***	46.8039	79.5350**	147.5872**
	R x S	2	1.2227	134194	93.3260	8.7339	13.8817	0.1672
	R x CWS	20	3.1000	391994	108.3076	4.8362	11.2783	16.3882
Sub-plot	Periods (P)	1	10.7339+	991701*	56.3568	49.1701*	4.8050	16.5313
	P x S	1	3.6450	12116	139.7235	1.0035	2.8006	0.2813
	A = P x CWS	10	2.2675	210427	98.9520	5.3918	17.7879	7.8689
Subdivide remaining interactions								
	Feeds (F)	1	0.6050	161501	2.6068	0.7001	52.7022	44.9668+
	F x S	1	0.4050	5796	266.4201+	0.0401	0.1089	4.4501+
	P x R	2	10.0093+	252336	70.5209	29.5089*	2.3116	24.5517
	P x R x S	2	0.4304	379478	91.4193	2.2639	2.3472	12.2450
	B = Residual	18@	4.1510	142412	73.5168	7.2781	10.3934	13.2079
Pooled sub-plot error								
	A + B	28@	3.4534	167603	82.9273	6.5795	13.2028	11.2305

Band 5	Band 6	Band 7	Band 8	Band 9	Band 10	Band 11	Bloat
0.8235	1.6806	26.1606	74.4200	42.7813	0.0113	1.1339	0.2222
6.5308	7.0471	68.8025	17.5404	38.1078	4.3670	8.8997	0.5444
26.1752**	43.4254***	129.1888**	20.3739	38.6217	2.3318	67.6077	0.7604
1.2601	1.6684	39.8477	27.9617	25.5950	1.3379	3.1435	0.3576
3.4235	3.0432	16.7026	23.9916	17.2466	5.8168	6.1959	0.5300
8.4375+	14.5800*	15.1250	9.1022	0.0001	0.9113	16.8200	2.7222**
3.5113	0.5689	2.2756	67.7200	12.5835	10.3513	3.3800	0.5556
3.4397	1.4701	15.9384	23.6152	5.3571	4.6511	1.8816	0.6222
0.0401	0.9339	2.4200	0.6050	13.4335	1.1001	0.1442	2.3472**
6.6613	0.2939	4.8050	45.7606	3.2513	0.1513	0.3200	1.1256+
5.2276	5.0113	66.3338	149.3272+	20.4406	8.5679	10.7129	1.3785 *
0.4908	2.4335	3.8926	49.5217	12.5272	2.8379	7.7254	0.3368
2.2850	3.5655	8.5157	55.3011	15.9829	3.7420	7.1891	0.1822
2.7127	2.7898	11.2649	43.5656	12.0474	4.0787	5.2234	0.3452

APPENDIX C

Footnote: @ 17 and 27 d.f. actually used for A + B because of cow that died

P + <0.1, * <0.05, ** <0.01, *** <0.001

Whole plot error = R x CWS

All sub-plot terms tested against A + B error term

A = P x CWS not tested

APPENDIX D The analysis of variance (split plot design) and mean squares of band percentages, total protein, and saliva flow for the repeat Time of Sampling Trials (Chapter 4.3)

Source	d.f.	Saliva Flow	Protein	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6
Sires (S)	1	130.200 *	1955555*	680.003	36.138*	4.043	31.282	0.050	12.470
Error 1	10	16.950	381771	210.465	6.507	7.231	26.704	4.892	6.617
Time (T)	3	0.292	290757	17.140	1.134	12.211	3.594	2.382	8.250*
T x S	3	6.375	227467	100.697	2.012	5.777	1.419	2.521	1.371
Error 2	30	2.637	136452	107.532	3.482	14.958	7.321	3.314	1.583
Days (D)	1	1.260	968619**	1382.443***	25.938***	43.605	110.940***	1.927	29.040
D x S	1	0.027	56212	849.065***	1.788	94.605**	14.416*	16.667*	0.327
Error 3	28	2.780	109598	35.516	2.740	11.090	3.367	2.886	0.647
R x S	1	7.820	13090	45.238	8.343	0.038	36.326	10.010	21.661*
Error 4	10	6.398	230187	174.945	13.363	13.750	12.910	5.899	3.773
Repeats (R)	1	10.402	1338829*	119.483	28.275	1.788	327.082***	11.900	56.427***
R x T	3	4.815	118397	257.420	12.656	27.315	22.241	12.031	9.165
R x S x T	3	1.534	51641	96.247	5.732	0.837	12.109	4.403	4.224
Error 5	40	3.446	246563	155.137	7.538	15.253	10.549	3.768	3.791
Total	95	6.042	244063	145.214	6.404	13.426	14.590	4.053	4.472

Band 7	Band 8	Band 9	Band 10	Band 11
25.834	168.805*	7.537	2.975	21.660
137.118	26.755	25.545	5.079	8.226
16.400	49.987	20.855	8.723	14.007
36.235	12.581	1.042	1.073	4.376
30.393	17.914	7.085	3.068	5.453
0.400	46.065	2.313	6.355	0.282
134.900*	0.030	2.130	4.905	0.167
27.375	17.547	7.433	2.885	5.826
0.015	8.343	28.058	0.618	23.404
99.331	17.992	10.344	1.976	7.216
528.282***	0.753	37.375*	18.113*	329.300***
23.968	99.898***	19.110	0.832	4.484
21.420	28.062	2.926	6.399	6.490
47.992	14.806	8.726	2.688	4.441
53.167	22.597	10.756	3.401	9.326

APPENDIX D

Footnote: P + <0.1, * <0.05, **<<0.01, *** <0.001

Error 1: Cows within sires
Error 2: Residual of terms above (including sires and Error 1, equivalent to analysis with Repeats summed
Error 3: Breakdown of Error 2
Error 4: Equivalent to cows within Repeats x Sires
Error 5: Overall residual
Total - All other terms

APPENDIX E Means from the least squares estimates and analysis of variance with model containing all interactions (if significant), or main effects only if no significant interaction (Chapter 4.4.a)

	(a) Year Born		(b) Sire		(c) Sex		5% Difference@	Significant Interaction
	1974	1975	HS	LS	Female	Male		
Number of animals	21	18	21	18	20	19		
Saliva flow								
9 months	2.75	1.91	2.74	1.93	2.56	2.11	1.32	nil
1 year	2.44	1.86	2.20	2.10	1.66*	2.63	0.85	nil
Protein								
9 months	1260**	751	1087	924	964	1048	364	ab*
1 year	964	890	881	1047	805	1093	328	nil
Band 1: 9 months	23.5	29.0	28.1	24.5	29.6	23.0	7.2	nil
1 year	22.2	27.3	24.7	24.8	27.4	22.1	6.65	nil
Band 4: 9 months	8.9	8.3	9.2	7.9	8.8	8.4	2.65	nil
1 year	10.2	8.1	9.6	8.7	8.2	10.1	2.71	nil
Band 6: 9 months	3.9	4.4	3.8	4.4	4.0	4.3	1.04	ac*
1 year	4.7	3.7	3.6	4.8	3.6	4.8	1.22	nil
Band 7: 9 months	12.5***	5.3	7.2*	10.5	8.4	9.4	3.3	nil
1 year	9.5	9.1	8.1	10.5	9.9	8.7	3.8	nil

Sub-class means for interactions

	Protein (9 mths)		Band 6		Saliva flow (1 yr)	
	HS	LS	Male	Female	HS	LS
1974	1548	992	3.1	4.7	2.07	2.80
1975	626	876	5.0	3.9	2.33	1.39

Bloat Grades

	Six months Interaction**		One year Not significant	
	HS	LS	HS	LS
1974	2.44	1.66	1.55	1.28
1975	0.46	0.50	1.17	1.00
5% Difference	0.26		0.35	

P * <0.05, ** <0.01, *** <0.001 @ Difference required for significance at the 5% level

Saliva flow ml/min ; Total protein µg/ml

APPENDIX F Means for bloat grades from nine months to two years of age, for the 1973, 1974, and 1975 animals from the Anova least squares or equal sub-class; showing main effects and interactions (Chapter 4.4.b)

Year Born		n	BLOAT GRADE 9 months	1 year	18 months	2 years
1973		12	0.81)	1.94)	0.79)	0.82
1974		12	1.93)***	1.59)**	0.99)*	1.31
1975		8	0.63)	0.92)	-	-
HS		17	1.33	1.45	1.1	1.37
LS		15	0.91	1.51	0.68	0.77
Mean		32	1.13	1.48	0.90	1.06
5% Difference@			0.36	0.63	0.41	0.42
Interactions						
1973	HS	6	1.2	1.93	0.92	0.78
	LS	6	0.4	1.95	0.67	0.85
1974	HS	6	2.3	1.58	1.28	1.93
	LS	6	1.5	1.60	0.70	0.70
1975	HS	5	0.5	0.84	(0.23)	-
	LS	3	0.8	1.0	(0.28)	-
P			<0.05	ns	ns	<0.01

n = number of animals

@ = difference required for significance at the 5% level, based on the year born. This 5% difference is slightly larger than would be required for HS/LS susceptibility group differences.

P * <0.05, ** <0.01, *** <0.001

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