STUDIES ON *Mycoplasma ovipneumoniae* IN NEW ZEALAND SHEEP: EPIDEMIOLOGY AND COMPARISON OF ISOLATES

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY AT MASSEY UNIVERSITY, NEW ZEALAND.

GEORGE IONAS

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ABSTRACT

As part of a larger study to examine the role of *M. ovipneumoniae* in chronic non-progressive pneumonia (CNP) of sheep, the colonisation of the respiratory tract by mycoplasmas was examined in two flocks of lambs over a nine month period.

In both farms *M. ovipneumoniae* was detected in the ewes at the time of first swabbing of the lambs. The two flocks of lambs differed in the time when the nasal cavity first became colonised by *M. ovipneumoniae*: thus in Flock 2 *M. ovipneumoniae* was detected in the nasal cavity relatively early and also became disseminated throughout the flock some months earlier than occurred in Flock 1. Nevertheless, *M. ovipneumoniae* was widespread in both flocks of lambs by March i.e. at or before peak seasonal prevalence of CNP. At slaughter in May, Flock 2 (colonised early) had a much higher prevalence of CNP than Flock 1.

These findings are consistant with the hypothesis that *M. ovipneumoniae* colonises the nasal tract of lambs and subsequently invades the lung possibly in response to the stress of exposure to hot dry weather.

The second part of this thesis is concerned with the adaptation of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to distinguish strains of *M. ovipneumoniae* with the ultimate objective of comparing *M. ovipneumoniae* strains isolated from pneumonic lungs to nasal isolates and isolates from apparently normal lungs.

Isolates from different sources were heterogeneous when examined by SDS-PAGE, but comparisons were made difficult

because of the excessive number of protein bands. In response to this problem fractions of *M. ovipneumoniae* were examined. Membrane preparations conserved the unique protein bands which in principle allow discrimination between strains, but because the number of protein bands was still excessive we examined surface proteins by labelling intact cells with Fluorescein isothiocyanate (FITC). This approach still gave gels with too many protein bands for convenient comparisons to be made, but had the advantage of allowing the identification of surface proteins, some of which were unique to individual isolates.

This encouraged us to combine SDS-PAGE with a classic immunological approach to strain identification i.e. we investigated the possibility of excising protein bands from gels for subsequent use as immunising antigens. One common protein band was excised, it was found to be antigenic and the antisera crossreacted with a single line of identity in gel precipititin tests with all the strains tested. While within the time limit available we have examined only one common protein band, the result suggests that the excision of individual strain-specific protein bands from SDS-PAGE for use as immunising antigens will provide strain-specific antisera which should allow the development of a simple approach to strain identification.

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INTRODUCTION

In New Zealand chronic non-progressive pneumonia (CNP) typically occurs in 6 to 9 month old sheep and reaches its maximum prevalence during the late summer and autumn months and is believed to cost the country about $26,000,000 per annum. *M. ovipneumoniae* can be isolated from almost 100% of the CNP lesions but its relationship to the disease is not clear. A plausible hypothesis is that the organism colonises the nasal cavity and, given the appropriate conditions, virulent strains can subsequently invade the lungs. The 'appropriate conditions' may be a combination of the presence of potentially pathogenic bacteria plus hot dry weather, because the prevalance of CNP in the autumn is higher following hot dry summer weather and bacteria, usually, but not exclusively, *P. haemolytica* can be recovered from a high proportion of pneumonic lungs. However, we are concerned here only with the possible role of *M. ovipneumoniae* in CNP, so initial experiments are directed towards investigating the following in two flocks:

(i) The age at which the nasal cavity of lambs is colonised by *M. ovipneumoniae*. Note: In particular it is of interest to find if the nose is colonised before or after the peak prevalence of CNP.

(ii) The time for which nasal carriage persists.

(iii) As a further contribution to elucidate the epidemiology of *M. ovipneumoniae* in sheep and with a view to ultimately comparing nasal and pneumonic lung isolates, the lungs of lambs from the same two flocks were examined at slaughter for the presence of *M. ovipneumoniae*.

Having obtained nasal and pneumonic lung isolates the second part of this thesis was undertaken to investigate possible means of identifying and classifying these and other isolates.

Two techniques were considered *viz* RE-analysis of DNA and SDS-PAGE examination of protein. The former technique was applied by Mrs A.J. Mew in parallel with the present work which examines different isolates of *M. ovipneumoniae* by SDS-PAGE.

Two contrasting approaches were taken to the use of SDS-PAGE gels as an aid to classification of isolates

(i) To directly compare gels prepared from different isolates or fractions (e.g. membranes) of isolates and

(ii) To use SDS-PAGE gels to identify protein bands which were either common or not common to all isolates.

These proteins could be purified and used as antigens to produce non cross-reacting (group specific) or cross-reacting (species specific) antisera.

Both these approaches led us to attempt to fractionate mycoplasmas and to identify protein bands which were common to all isolates and more importantly to attempt to identify protein bands which were not common to all isolates.

CHAPTER 1

HISTORICAL REVIEW

1.1 Nomenclature of the Disease

In New Zealand, lambs born in the spring are typically slaughtered in the summer and autumn months. A significant proportion of such lambs (over 50% in flocks from some farms Alley - personal communication) have chronic lung lesions characterised by the presence of macrophages and lymphocytes. These lesions can readily be distinguished from acute (bacterial) pneumonia which elicites a neutrophil response. They can also be differentiated from progressive pneumonia of sheep because they fail to progress and are ultimately resolved. With these characteristics in mind, a veterinary pathologist colleague, designated the disease as chronic non-progressive pneumonia (CNP) of sheep (Alley, 1975a). We embrace this description but it is necessary to mention that similar if not identical diseases have been assigned a bewildering number of different names:

Hogget pneumonia
Summer pneumonia
Enzootic pneumonia
Atypical pneumonia
Proliferative exudative pneumonia
Proliferative interstitial pneumonia
Sheep pulmonary adenomatosis
Chronic non-progressive pneumonia

These names have some merit but can also be criticised. The evidence that these designations were applied to the disease we refer to as CNP is based on the study of the pathology of these diseases. Thus the detailed arguments

fall outside the scope of this thesis but were fully considered Alley (1975a). However, we indicate below why we have selected CNP as the most appropriate designation and in later parts of this thesis designations used by other authors have been "translated" as CNP.

1.1.1 Hogget Pneumonia

CNP is indeed a prevalent disease of 6 to 8 month old lambs (i.e. hoggets). However, it is not the only type of pneumonia seen in such animals hence the name is ambiguous.

1.1.2 Summer Pneumonia

This disignation was derived from the observation that in Australia CNP reaches its peak prevalence in lambs slaughtered in the summer months (St. George *et al.*, 1972).

It can be criticised on 2 grounds: it is not the only pneumonia to occur in summer even in Australia, and in New Zealand the disease reaches its peak prevalence not in high summer but in the late summer and autumn months.

1.1.3 Enzootic Pneumonia

This designation correctly implies that the disease in enzootic in New Zealand (Kirton *et al.*, 1976). However, there are other causes of pneumonia in New Zealand sheep which could be designated "enzootic" e.g. *P. haemolytica* can cause acute bacterial pneumonia in New Zealand sheep and while this is indubitably an "enzootic" pneumonia it is readily distinguishable from CNP. Hence the unqualified use of the term enzootic pneumonia would lead to ambiguities.

1.1.4 Atypical Pneumonia

"Atypical pneumonia" was derived from human pathology and is used in contrast to "typical" pneumonia which is due to bacteria. As applied to lambs (Stamp and Nisbet, 1963)

"atypical" is unsatisfactory since the "atypical" lesions outnumber the "typical" by about 2 orders of magnitude. Furthermore, "atypical" pneumonia in humans has come to be applied mainly if not exclusively to disease caused by *M. pneumoniae*, whereas in sheep the aetiology of "atypical" pneumonia has not been unequivocally established.

1.1.5 Proliferative Exudative Pneumonia

This designation was applied by Gilmour (1979) to lesions produced by inoculating a homogenate of lungs from sheep with "atypical pneumonia" into the bronchi of normal sheep. It is a discriptive term for the lesions produced and left open the question as to whether or not the lesion represented experimental transmission of atypical pneumonia.

1.1.6 Proliferative Interstitial Pneumonia

This descriptive name has merit but could also be applied to the disease caused by progressive pneumonia virus (PPV) i.e. it does not distinguish between chronic lesions which are, or are not progressive.

1.1.7 Sheep Pulmonary Adenomatosis

This term is particularly confusing because it has sometimes been used to indicate a disease which is probably identical to CNP, but more recently it has come to be applied to a disease of sheep transmitted by a virus (Mackay and Nisbet, 1966; Wandera, 1970). It follows that this term is not now a synomym from CNP.

1.2 Economic Importance of Chronic Non-Progressive Pneumonia

Although CNP is usually subclinical and on its own is rarely fatal it is important economically for two reasons: -

(1) It causes the formation of pleural adhesions in some lambs, which may lead to downgrading, or in severe cases, to rejection of carcasses for export.

(2) It diminishes weight gain in lambs.

The first point is widely accepted and can be measured in financial terms. The latter is almost impossible to quantitate though it is not difficult to believe that a lamb with pneumonia will be less thrifty than one not so afflicted.

1.2.1 Diminished Weight Gain

St. George *et al.*, (1971) and Carmichael *et al.*, (1972) states but cited no evidence that CNP caused inadequate weight gain in lambs.

A study in New Zealand of the affect of CNP on weight gain was undertaken by Kirton *et al.*, (1976). These workers examined 3243 lamb carcasses at slaughter over a 5 year period. They found that 60% of lambs had CNP. However, the average carcass weight of lambs with CNP was significantly higher than normal lambs. This unexpected result did not lead Kirton *et al.*, (1976) to conclude that CNP increased weight gain, rather they suggested that CNP did not affect weight gain.

An alternative hypothesis to explain Kirton's data was proposed by Harris and Alley (1977). They suggested that the carcass weight difference should not be ignored and pointed out that most lambs contract CNP during their first summer and that the disease resolves over a period of several months. Thus most of the lambs without pneumonic lesions at slaughter could represent lambs which became infected with CNP earlier in the year followed by total recovery before slaughter. While the pneumonic sheep (which were heavier) had probably contracted the disease immediate-

ly prior to slaughter and were thus infected for a shorter period and probably at a less critical time in their life. These considerations allowed Harris and Alley (1977) to conclude that Kirton *et al.*, (1976) findings were consistant with the hypothesis that CNP has an adverse effect on the weight gain of lambs in New Zealand and hence is of economic importance.

1.2.2 Pleural Adhesions

Not all cases of CNP resolve without sequelae. Thus some lambs develop plural adhesions and the carcasses of such animals are diverted from the main killing chains of the abattoir to the "detain rail". Here the affected pleura is stripped from the carcasses and re-examined by Meat Inspectors, prior to railing back into the mainstream of production.

816,700 (3.2%) of lambs killed in New Zealand during the 1974/75 season had pleural adhesions (Dysart, 1976) so the problem of dealing with large numbers of carcasses on a short detaining rails and the disruption of throughput of carcasses in such a labour-intensive industry is expensive though hard to quantify. However an even more severe problem arises:

Carcasses with pleurisy are not accepted in the United States, Canada and Western Germany. So when slaughtered sheep of all ages are included over 2 million such carcasses in the 1974/5 season were excluded from these markets. The United Kingdom will accept lamb and mutton which have minimal pleural lesions (Dysart, 1976) but reject carcasses with more widespread damage.

It has been estimated that $1.8 million is lost in revenue due to pleurisy in lambs in the 1974/5 season (Dysart, 1976). Due to a combination of inflation and the continued upgrading of standards for export carcasses, the present

figure is about $26 million (Alley - personal communication). Clearly, it is desirable to prevent the development of pleurisy.

1.3 Epidemiology of *M. ovipneumoniae*

Mycoplasmas are well known to have the ability to colonise the respiratory tract of a variety of species of animals. But nevertheless, no single description encompasses the relationships of the organism to the diseases with which they are associated.

The host-parasite relationship falls into one of three major categories:

A) The organism is the cause of the disease e.g. pleuropneumonia of cattle (*M. mycoides*) and atypical pneumonia of humans (*M. pneumoniae*).

B) The mycoplasma plays no essential role in the pathogenesis of the disease but colonises a lesion produced by other organisms e.g. *M. arginini*.

C) The organism plays an important role in the pathogenesis of the disease but for the lesions to be produced, an organism or organisms other than mycoplasma must be present e.g. chronic respiratory disease in poultry. In this case the upper respiratory tract is colonised by *M. gallisepticum* and this when followed by infection with infectious bronchitis virus causes lesions in the lower respiratory tract. These allow colonisation by *Escherichia coli*. Partial resolution of the lesion occurs when the bird develops immunity to infectious bronchitis virus. However, this leaves a residual joint colonisation of the lower respiratory tract with *M. gallisepticum* and *Escherichia coli*, in which the latter organism is responsible for the mortalities.

When sheep get CNP, *M. ovipneumoniae* can almost invariably be isolated from the lung (Alley and Clarke, 1980), but this finding does not help us to assign *M. ovipneumoniae* to a particular one of the above 3 categories.

Epidemiological data on *M. ovipneumoniae* although not necessarily decisive could nevertheless be useful in understanding the relationship of *M. ovipneumoniae* to chronic pneumonia e.g. if *M. ovipneumoniae* is highly pathogenic with considerable potential to invade the lung, it may be expected that the peak prevalence of CNP would coincide with or follow soon after nasal colonisation. In an opposite hypothesis i.e. if *M. ovipneumoniae* colonises prexisting lesions, then *M. ovipneumoniae* might become disseminated in the flock at a time following the peak prevalence of CNP.

The ultimate aim of the project of which this thesis is a part, is to understand the relationship of *M. ovipneumoniae* to CNP. Clearly, it is worthwhile to undertake a study to establish the time of colonisation of the nasal tract of lambs by *M. ovipneumoniae* to find; for how long the organism persists in the nose and finally to find if more than one strain can colonise a flock or an individual animal. No such study has yet been undertaken either in New Zealand or abroad.

1.4 Transmission of Chronic Non-progressive Pneumonia

The experimental transmission of CNP by intratracheal inoculation of ground pneumonic lung was described by St. George *et al.*, (1971). A cytopathic agent was isolated from pneumonic lungs of lambs using cultures of bovine testes. The cytopathic agent was then propagated in cell-free liquid medium but grew poorly on solid media. Following serial passage in liquid culture the organism grew on solid media and gave colonies typical of mycoplasmas.

The organism also had other characteristics that were typical of the genus *Mycoplasma, viz.*, it passed through a 0.22µm pore diameter filter, it grew in the presence of thallium acetate, methylene blue, streptomycin and penicillin. The organism was named *M. ovipneumoniae* (St. George) *et al.*, 1971) and lambs inoculated intratracheally with this organism, developed pneumonia which the authors claimed was similar to the field disease.

In further experiments Sullivan *et al.*, (1973) isolated *M. ovipneumoniae* from the lung of a pneumonic sheep and inoculated one-day-old Marino lambs either intravenously or by an intranasal aerosol. Similar lambs were placed in contact with the intranasally inoculated lambs. Post-mortem examination of in-contact lambs showed that they had developed "mild proliferative interstitial pneumonia". Sullivan *et al.*, (1973) could not reisolate the organism from the lungs of the experimental animals. They attributed this failure to technical factors. Jones *et al.*, (1978) in a series of experiments, inoculated 6 to 8 month-old-lambs with either a pneumonic lung-tissue suspension or with a mixture of *M. ovipneumoniae*, *M. arginini* and *P. haemolytica* isolated from the original lung suspension. Both the lung suspension and the mixtures of microorganisms transmitted the disease. *M. ovipneumoniae* was recovered from all experimental animals but *P. haemolytica* and *M. arginini* were recovered less consistently.

In New Zealand Alley and Clarke (1979) inoculated 20, five-month-old conventionally reared lambs with pneumonic lung homogenate using an intranasal aerosol. 17 (85%) of the lambs developed CNP indistinguishable from field cases. In a parallel experiment a further 20 lambs were inoculated intranasally with a culture of *M. ovipneumoniae*. 4 (20%) of the experimental lambs showed lesions which were less severe than the natural disease. This results suggests that *M. ovipneumoniae* though involved in CNP may not on

its own be capable of causing the lesions seen in the more severe field cases.

In further experiments (Alley and Clarke - unpublished data) in which sheep inoculated with pneumonic lung were treated with penicillin, mi[illegible]lesions were produced and *M. ovipneumoniae* was recovered from them. They concluded that a penicillin sensitive organism *viz.*, a bacteria plays a role in the pathogenesis of CNP. The latter experiment may cast doubt on the necessity for the presence of *M. ovipneumoniae* in developing CNP lesions. However, lung homogenates treated with digitonin (Brian, 1980), which inactivates mycoplasmas but not bacteria failed to transmit the disease and recently Jones *et al.*, (1982) produced CNP using intratracheal inoculation of *M. ovipneumoniae* alone although they also state that "*P. haemolytica* should be considered as an important exacerbating factor of the mobidity and of the severity of chronic ovine pneumonia".

1.5 Distinguishing Strains of *M. ovipneumoniae*

Suggestive evidence that *M. ovipneumoniae* plays a major role in CNP has been outlined above. However, *M. ovipneumoniae* cannot be routinely isolated and used to consistantly transmit CNP to a high proportion of lambs using the natural route of infection (i.e. intranasal inoculation). While other explanations are not excluded it is possible that this may be due to differences in *M. ovipneumoniae* isolates. For that reason we now consider the methods by which individual isolates of any one species of mycoplasma may be distinguished from other isolates of the species.

1.5.1 Serology

The subcommittee on the Taxonomy of Mycoplasmatales (1972) has recommended procedures to be used in classifying new species of Mycoplasma. These procedures are based on

cultural, biochemical and antigenic characters. The first two of these are used to identify species but serological tests may examine either interspecies or intraspecies differences.

A test which examines total proteins i.e. double immunodiffusion or the complement fixation test is useful in examining interspecies similarities and difference e.g. using gel precipitation tests cross-reactions may or may not occur between mycoplasma species (Ball and Todd, 1978). However, we are concerned with intraspecies differences hence the serological tests most likely to be useful are limited to metabolic inhibition and growth inhibition since these are relatively strain specific (Ball and Todd, 1978) and give little or no reaction between species.

Only one group of workers (Jones *et al.*, 1976) has compared strains of *M. ovipneumoniae* by metabolic inhibition and growth inhibition tests. They concluded that, although all strains are related to some extent, isolates tended to be divided into two major groups: those associated with CNP and those isolated from normal lungs. However, since the groups overlapped the absence of clear serotypes inhibited progress at that time.

Workers in this laboratory (Clarke - personal communication) found that isolates of *M. ovipneumoniae* in New Zealand are all serologically related but not identical. This confirms the conclusion of Jones *et al.*, (1976), but unlike Jones and co-workers Clarke found no consistant distinction between strains derived from pneumonic lungs and other isolates.

It is possible that strain or serotype specific antisera could be produced e.g. by cross-adsorption or by monoclonal antibody technqiues. Another possible approach would be

to immunise animals with purified membrane proteins thus producing a polyclonal but single-antigen-specific antiserum. This thesis makes a contribution to the latter goal by attempting to identify and purify membrane proteins.

1.5.2 Restriction Endonucleas Analysis

Restriction endonuclease analysis (REA) has been used to identify strains of viruses (Skare *et al*., 1975) and bacteria (Marshall *et al*., 1981) and has been extensively used in this laboratory (Mew, 1982) to examine *M. ovipneumoniae* strains. It was found that the REA patterns of independent isolates of *M. ovipneumoniae* (independent isolates in the sense that they were derived from different flocks) gave REA patterns that were distinguishable and indeed showed no detectable similarity. It was concluded that REA is a powerful method for distinguishing between strains but since the number of different patterns appeared to be limitless this method could not be used to assign isolates to a manageable number of groups.

1.5.3 SDS-PAGE

Fowler *et al*., (1963) were first to suggest that the electrophoretic separation of proteins of pleuropneumonia-like organisms, as mycoplasmas were then called, could be used as a method for identifying the species of isolates. Razin and Rottem (1967), were the first workers to apply the technique to several different mycoplasmas and they concluded that isolates of different species gave patterns which are totally or at least substantially unrelated. In contrast different isolates of one species gave identical or at least closely similar patterns. These conclusions have been confirmed and extended to many different mycoplasmas by subsequent workers (Razin, 1968; Zola *et al*., 1970; Daniels and Meddins, 1973; Forshaw, 1972; Jones *et al*., 1976; Asa *et al*., 1979).

Jones *et al*. (1976) applied the technique to isolates of *M. ovipneumoniae* and concluded that there was "a very close resemblance between all 10 strains". Jones *et al*. (1976) stressed the similarity of patterns and did not comment on differences which could be observed in his illustrations. These differences prompted us to examine New Zealand isolates of *M. ovipneumoniae* in an attempt to find if this technique could be used to assign isolates to a limited number of groups (see Chapter 3).

1.6 Biochemical Dissection of Mycoplasmas

In the course of this present investigation it became clear that a major difficulty in the use of SDS-PAGE in distinguishing between strains of any species of mycoplasma including *M. ovipneumoniae* is that even when differences occur, the majority of the protein bands are common to all isolates and the total number of bands is too large for convenient comparisons to be made. For these reasons we became interested in examining selected fractions of mycoplasmas, with a view to selecting a cell fraction that minimised the number of bands while retaining most if not all those bands which vary between isolates.

Other workers have not used this approach in a comprehensive fashion but nevertheless some have examined selected fractions of mycoplasmas especially membrane preparations (Amar *et al*., 1974; Archer and Rodwell, 1982).

1.6.1 Separation of Internal and Membrane Proteins

One approach to fractionating mycoplasmas is to separate the membrane from the cytoplasmic proteins. This requires that the organisms are first lysed and various techniques have been used for this purpose. Each has advantages and disadvantages eg:

A) Lysing by ultrasound may leave variable proportions of the mycoplasmas unlysed, also when the organisms are lysed the membranes may be broken into minute fragments which do not sediment and hence cannot be efficiently separated from the cytoplasmic fraction by differential centrifugation (Kahane, 1969).

B) Osmotic lysis is a gentle method for the isolation of mycoplasma membranes. However, the sensitivity of mycoplasmas to osmotic shock varies with the species, the growth phase of the culture, the pH, the salt concentration and the temperature. Therefore, the optimum conditions for lysis has got to be established for the mycoplasma under study, however, even in optimal conditions, not all cells are necessarily lysed (Razin 1963 and 1964).

C) Freezing and thawing of mycoplasma suspensions is ineffective, only 31-52% of cells were lysed after 10 freezing and thawing cycles (Hollingdale, 1969).

D) Digitonin lyses mycoplasmas because the membrane contains steroids. This method was used e.g. to lyse *M. hominis* (Rottem and Razin, 1972).

E) Carbonate bicarbonate buffer pH 9.2 to 10.2 lyses mycoplasmas (Goel, 1973). Membranes prepared from cells lysed by this method were able to retain their antigenicity and immunogenicity. However, glycolytic mycoplasmas harvested at or below pH 6.0 were found by the above author to be resistant to lysis at pH 10.0 (*M. ovipneumoniae* at the end of log phase of growth is approximately pH 6.5).

F) Dicyclohexylcabodiimide (DCCD) lyses mycoplasmas (Shirvan *et al.*, 1982). It blocks ATPase activity and interferes with the regulation of the cells metabolism. The use of DCCD as a toxic agent to

lyse mycoplasmas has the advantage that the cells are lysed in an isotonic solution of monovalent ions and does not fragment the membrane or interfere with its lipid composition. Also Shirvan *et al*., (1982) cut thin sections of mycoplasma cells lysed by DCCD and showed that the lysed cells were empty. This implies that membrane fractions would contain little if any cytoplasmic contaminants.

This latter method was selected for use in this present work (see Chapter 4.1), in which we compare membrane and total protein in *M. ovipneumoniae*.

1.6.2 Identification of Surface Proteins by Labelling

In the course of the present investigations it became clear that membranes like total proteins contained too many proteins to allow easy comparisons of isolates, hence we now consider an examination of surface proteins only.

The labelling of surface proteins with either fluorescent or radioactive probes has been well documented with other cells and microorganisms (Maddy, 1964; Dockter, 1979; Schmidt-Ullrich *et al*., 1973). However, the use of such compounds on mycoplasmas has not been described. Dockter (1979) labelled the surface proteins of intact human erythrocytes using 3-azido (2,7)-naphthalene disulfonate (ANDS) and Maddy (1964) with 4-acetamido, 4'-isothiocyanostilbene -2,2' disulphonic acid which is a fluorescent probe. Both of these compounds are claimed by the above authors to be unable to cross membranes and thus must label the surface only. Other photochemical probes e.g. diazonium salts (Amar *et al*., 1974) have failed to selectively label outer membrane proteins because it entered the cells.

The essential properties of a reagent to be used to selectively label proteins on the membrane surface are:

(i) It must not pass through the membrane.

(ii) The reagent must react with the membrane under physiological conditions of temperature, pH and tonicity without disrupting the cell.

(iii) The label must be detectable in small amounts i.e. in practice fluorescent or radioactive compounds are used.

(iv) The label should react non-selectively with all surface proteins.

It is not entirely clear if any compound fulfills these criteria and in practice another criteria i.e. availability is critical. This point is further developed in section 4.2.

1.6.3 Detection of Adsorbed Media Constituents

Mycoplasmas may adsorb media proteins onto their surface (Jordan and Kulasegaran, 1968; Yaguzhinskaya, 1976; Nicolet *et al* ., 1980). Therefore, protein detected by fluorescent labelling of the mycoplasmal surface may include adsorbed proteins.

If however mycoplasmas are propagated in medium containing radioactive amino acids, the mycoplasma protein (but not the media proteins) will become radioactive. Hence, the adsorbed surface proteins can be identified by comparing a coomassie-blue and fluorogram preparation of the same gel because adsorbed medium constituents will be represented by those protein bands which are visible by coomassie-blue staining but are not visible in the fluorogram.

CHAPTER 2

A STUDY OF THE COLONISATION OF THE RESPIRATORY TRACT OF LAMBS BY MYCOPLASMAS

2.1 Introduction

The aetiology of chronic non-progressive pneumonia (CNP) is uncertain but the following represents a plausable working hypothesis: *M. ovipneumoniae* colonises the nasal track of lambs some time before the peak prevalence of CNP i.e. before March-April and subsequently invades the lungs. This either causes or significantly contributes to the lesions of CNP. This hypothesis unlike some others, [which explain CNP as sequential virus and bacterial infections (Hore *et al* ., 1968; Davies *et al* ., 1977; Thurley *et al* ., 1977; Davies *et al* ., 1980; Davies 1980; Davies *et al* ., 1981)], requires that colonisation of lambs by *M. ovipneumoniae* occurs before the peak prevalence of CNP. However, nasal colonisation of sheep by *M. ovipneumoniae* has not been investigated either here or overseas, so it is desirable to undertake such a study, preferably on a country-wide basis so that different breeds and different geographic areas could be compared. Practical considerations however limited us to an examination of 2 flocks of different breeds in contrasting locations in the Manawatu region.

Using lambs from these two flocks we investigate the following:

(i) The age at which the upper respiratory tract of lambs becomes colonised by *M. ovipneumoniae;*

(ii) The persistance of *M. ovipneumoniae* in the nasal tract following colonisation;

(iii) The relationship, if any, between the time of nasal colonisation and the presence of CNP lesions at

slaughter;

(iv) For the sake of completeness, the time of colonisation of the nasal cavity of lambs by *M. arginini* was also investigated.

It is not possible to sequentially sample lungs for the presence of mycoplasmas, however the lungs of the lambs of both flocks were collected at slaughter, examined for CNP lesions and tested for the presence of *M. ovipneumoniae* and *M. arginini*. This provided isolates from the lung which can ultimately be compared and contrasted with the nasal isolates both by restriction endonuclease anlaysis (initiated by Mrs A.J. Mew) and by SDS-PAGE (see Chapter 3).

2.2 Materials and Methods

Mycoplasma Media

FM4 and FM4A broth are the fourth of several formulations of Frey *et al.* (1968).

The formula used is as follows: -

NaCl	5.0g
KCl	0.4g
$MgSO_4.7H_2O$	0.2g
$Na_2HPO_4.12H_2O$	4.03g
KH_2PO_4	0.1g
Glucose	10.0g
Peptone CS (Albimi)	10.0g
Yeast autolysate (Albimi)	5.0g
NAD	0.1g
L-Cysteine HCl	0.1g
Eagles Vitamin Solution (x100)	25.0ml

0.4% Phenol red	2.5ml
Penicillin	10^6 units (600mg)
Thallium acetate	0.5g
Deionised water to	1000.0 ml

This solution was stored in 300ml aliquots and stored at 4°C until required.

Additional factors to take note of: -

A) The thallium acetate was dissolved in 10ml of deionised water and added to the medium dropwise to prevent precipitation.

B) The basal medium was supplemented with 150ml of swine serum.

C) For glycolytic mycoplasmas, i.e. glucose fermenting mycoplasmas the media was adjusted to pH 7.8 with 1.0M NaOH.

D) For arginine requiring mycoplasmas, $10gL^{-1}$ of arginine was added and the media adjusted to pH 7.0 with 1.0M HCl. This modified medium was referred to as FM4A medium.

E) The complete medium was clarified by filtration through non-sterile 5.0µm, 0.45µm and 0.2µm pore-size filters, and sterilised by filtration through a sterile 0.2µm filter.

F) The formulation of Eagles Vitamin Solution (x 100) is as follows: -

Calcium pentothenate	20mg
D-biotin	20mg
Calcium chloride	20mg
Folic acid	20mg
Riboflavin	2.0mg

Myo-Inositol	40mg
Niacinamide (Nicotinamide)	20mg
Pyridoxine	20mg
Thiamin-HCl	20mg
Distilled water to	200ml

This solution was stored in 25ml aliquots at -20°C until required.

FM4 Agar and FM4A Agar

The formulation used was as follows: -

BHI (Difco)	0.74g
Agar (Davis)	1.0 g
Distilled water to	20.0 ml

This was autoclaved at 121°C for 15 minutes, allowed to cool to approximately 50°C and added to 80ml of FM4 or FM4A preheated to 46°C.

This medium was mixed thoroughly by inversion to stop frothing and 4ml aliquots were pipetted into Falcon (1006) 50 x 9mm petri dishes with tight fitting lids. The plates were left to solidify, placed in a 37°C incubator for 15 minutes to dry excess moisture and stored at 4°C.

Lambs Used for Survey

Two flocks of lambs were examined for the colonisation of the nasal cavity by *M. ovipneumoniae* and *M. arginini*. One flock contained 28 Perendale lambs, located adjacent to the Pahiatua Track (hill country in the Manawatu) and is referred to as flock 1. The other fock contained 25 Suffolk lambs, located on the Manawatu plains *viz.*, at Massey University Sheep Unit No. 1, and is referred to as flock 2.

Lambs in both flocks were first swabbed immediately after weaning (at 45-50 days old) and then at monthly intervals for approximately 7 months. When approximately 9 months old the lambs from both flocks were slaughtered at the local abattoir and their lungs were collected for further examination.

Collection and Treatment of Specimens

The swabs used were 150mm in length. They were inserted 100mm into one nostril and rotated clockwise and anticlockwise to ensure good contact with the nasal surface. The swab was then retracted, broken off into a bijou bottle containing 3ml of FM4 medium and shaken. Two 0.3ml aliquots were inoculated into bottles containing 2.7ml of FM4 and FM4A medium. The two inoculated bottles were incubated at 37°C and the original specimen bottle was discarded.

Lung specimens were collected from the abattoir and placed in individual plastic bags to prevent cross-contamination. The lungs were labelled and photgraphed to record the lesions.

Mycoplasma Isolation from the Lung

Lung specimens were aseptically cut with scissors into small pieces and fragments were placed in bottles containing 3ml of FM4 medium to give an approximately 20% suspension. This was shaken and two 0.3ml aliquots were transferred into bottles containing 2.7ml of FM4 and FM4A medium respectively. The original lung suspension was discarded and the inoculated media was incubated at 37°C.

Processing of Specimens

The inoculated media was examined daily for a pH (colour) change, i.e. for FM4 from red to yellow and for FM4A from

yellow to red. When this was observed 0.3ml of the culture was transferred to a fresh bottle containing 2.7ml of the same medium. If no colour change was observed after 7 days 0.3ml of medium was passaged. The samples were incubated for a further 7 days and if still negative, the sample was frozen at -70°C except for a 0.05ml aliquot which was spotted onto FM4 or FM4A agar and incubated at 37°C for 7 days.

The plates were examined for colonies after 3, 5 and 7 days using a plate microscope at 88 x magnification. Plates which showed no colonies and the associated frozen aliquot were discarded.

Isolates were further passaged in liquid medium because some strains gave colonies which were too small to be seen clearly. If both centred and centreless colonies i.e. *M. ovipneumoniae* and *M. arginini* were present in the same culture, they were separated by propagating them respectively in the presence of 10% *M. ovipneumoniae* or *M. arginini* antisera.

Cloning of Isolates

Tenfold serial dilutions of *M. ovipneumoniae* isolates were prepared in FM4 broth and 0.05ml of each dilution was transferred to petri dishes containing FM4 agar. These were incubated at 37°C and examined using a plate microscope after 3 to 7 days for the presence of colonies. Isolated colonies near the limiting dilution were selected. Using a pasteur pipette and bulb the colonies were removed along with a plug of agar and transferred to 2.7ml of FM4 medium. The suspension was mixed thoroughly and incubated at 37°C until a pH (colour) was observed. 10ml of FM4 media was then inoculated and following a pH (colour) change aliquots of 1ml were frozen at -70°C.

Antiserum Production

M. ovipneumoniae (strain 5) was propagated for eight passages in "modified" FM4 medium in which the swine serum and animal peptone were replaced by bovine serum and phytone peptone (BBL) respectively. When the final passage culture exhibited a change from pH 7.8 to approximately pH 6.8, aliquots were stored at -70°C.

10ml of *M. ovipneumoniae* (strain 5) was then propagated in 150ml of "modified" medium and incubated at 37°C on a shaker at 125rpm. After a pH change was observed 0.05ml of sample was spotted on BHI plates to act as a check on the absence of bacteria and a further 0.05ml of sample was spotted on FM4 agar to check that the culture produced confluent growth, typical of *M. ovipneumoniae*.

The remaining suspension of *M. ovipneumoniae* was deposited at 14,600g for 30 minutes, resuspended in 0.15M saline and centrifuged. The pellet was diluted with 0.15M saline to give an approximate 10% suspension and stored at -20°C until required as an antigen for rabbit inoculation. This procedure was repeated with *M. ovipneumoniae* strains 1 and 10.

Antigen Preparation for Gel Precipitation Tests

An aliquot of *M. ovipneumoniae* (strain 5) stored at -70°C was inoculated into 150ml of FM4 media and incubated at 37°C until it reached pH 6.8. The culture was then centrifuged at 14,600g for 30 minutes. The resulting deposit was washed twice in PBS and resuspended in PBS to make an approximately 20% suspension. The cells were sonically disrupted as 20kc/s using 8 x 15 second bursts with a MSE 100 watt ultrasonic disintegrator and Triton-X 100 was added to a final concentration of 2%. This antigen was stored at -20°C. The procedure was repeated with *M. ovipneumoniae* strains 1 and 10.

Agar Preparation for Gel Precipitation Test

The formulation was as follows: -

Noble agar	3.0g
NaCl	16.0g
Distilled water to	200 ml

This was autoclaved at 121°C for 15 minutes and 20ml was added to each standard petri dish. The petri dishes were placed at 37°C for 30 minutes to dry excess moisture on the agar surface and wells were cut using a template.

2.3 Results

(A) Nasal Carriage of Mycoplasmas by Lambs

(i) Flock 1 - *M. ovipneumoniae*

The results of the isolation of *M. ovipneumoniae* and *M. arginini* from (Flock 1) were recorded in Table I. Typical colonies of both species of isolates are shown in Figures 4, 5, 6 and 7.

Using this data the cummulative totals i.e. the total number of lambs that carried *M. ovipneumoniae* on at least one occasion was calculated and the results were shown graphically in Figure 1.

(ii) Flock 2 - *M. ovipneumoniae*

The results from (Flock 2) were recorded in Table II.

Using this data the cummulative totals i.e. the total number of lambs that carried *M. ovipneumoniae* on at least one occasion was calculated and the results were shown graphically in Figure 2.

(iii) Flock 1 and 2 - *M. arginini*

The recovery of *M. arginini* from both flocks of lambs is included in Tables 1 and 2. Note: that the flocks differ markedly i.e. only 1 isolate of *M. arginini* was recovered from Flock 1.

The cummulative totals i.e. the total number of lambs that carried *M. arginini* in each of the two flocks (Flocks 1 and 2) on at least one occasion was calculated and the results were shown graphically in Figures 3a and b.

(B) Recovery of Mycoplasmas from the Lungs of Lambs at Slaughter

The lungs of lambs from both flocks were examined after slaughter, the results are included in tables 1 and 2 and are shown graphically in Figure 1 (Flock 1) and Figure 2 (Flock 2).

(C) Proportion of Lambs in Both Flocks Exhibiting CNP Lesions

The lungs of lambs in both flocks were examined at slaughter from CNP lesions, the results are recorded in Table IV. A lesion typical of CNP but more severe than the average lesion seen in this survey is illustrated in Figure 8.

(D) Recovery of Mycoplasmas from the Ewes

The ewes corresponding to the lambs in both flocks were swabbed once when the lambs were weaned. The recovery of mycoplasmas is recorded in Table III.

TABLE I

Recovery of *M. ovipneumoniae* or *M. arginini* from:

(i) The nasal cavity of Perendale lambs swabbed at approximately monthly intervals. Note: the swabbing began at weaning and ended one day before slaughter.

(ii) The lungs at slaughter.

V = *M. ovipneumoniae* isolated

A = *M. arginini* isolated

\- = no mycoplasmas isolated.

FLOCK 1

Swab Series	1	2	3	4	5	6	7	8	9	Lungs
Approximate age of lambs (days)	50	77	102	119	149	184	210	240	266	267
Lamb No 17	-	-	-	-	-	-	-	V	-	-
18	-	-	-	-	-	-	-	V	V	V
19	-	-	-	-	-	-	-	-	-	V
20	-	-	-	-	-	-	-	-	V	V
21	-	-	-	-	-	-	-	-	-	V
22	-	-	-	-	-	-	-	-	-	V
23	-	-	-	-	-	-	-	V	-	V
24	-	-	-	-	-	-	-	V	V	V
25	-	-	-	-	-	-	-	-	V	V
26	-	-	-	-	-	V	V	V	V&A	-
27	-	-	-	-	-	-	-	V	V	V
28	-	-	-	-	-	-	-	-	-	V
29	-	-	-	-	-	-	-	-	-	V
30	-	-	-	-	-	-	-	V	V	V
31	-	-	-	-	V	-	-	-	-	V
32	-	-	-	-	-	-	-	V	V	V
33	-	-	-	-	-	-	-	V	V	V
35	-	-	-	-	-	-	-	-	V	V
36	-	-	-	-	-	-	-	-	-	V
38	-	-	-	-	-	-	-	-	-	V
39	-	-	-	-	-	-	-	V	V	V
40	-	-	-	-	-	-	-	-	V	V
42	-	-	-	-	-	-	-	-	V	V
43	-	-	-	-	-	V	V	V	-	V
44	-	-	-	-	-	V	-	-	-	V
45	-	-	-	-	-	-	-	V	V	V
46	-	-	-	-	-	-	V	V	V	V
48	-	-	-	-	-	-	-	V	V	-

FIGURE 1

(i) The (cummulative) proportion of Perendale lambs which showed nasal carriage of *M. ovipneumoniae* on at least one occasion. The 28 lambs were examined on 9 occasions. The times of swabbing when *M. ovipneumoniae* was not recovered are denoted by (↓)

(ii) The proportion of lambs from which *M. ovipneumoniae* was recovered from the lungs at slaughter (hatched bars at right of diagram).

Lambs in Flock 1

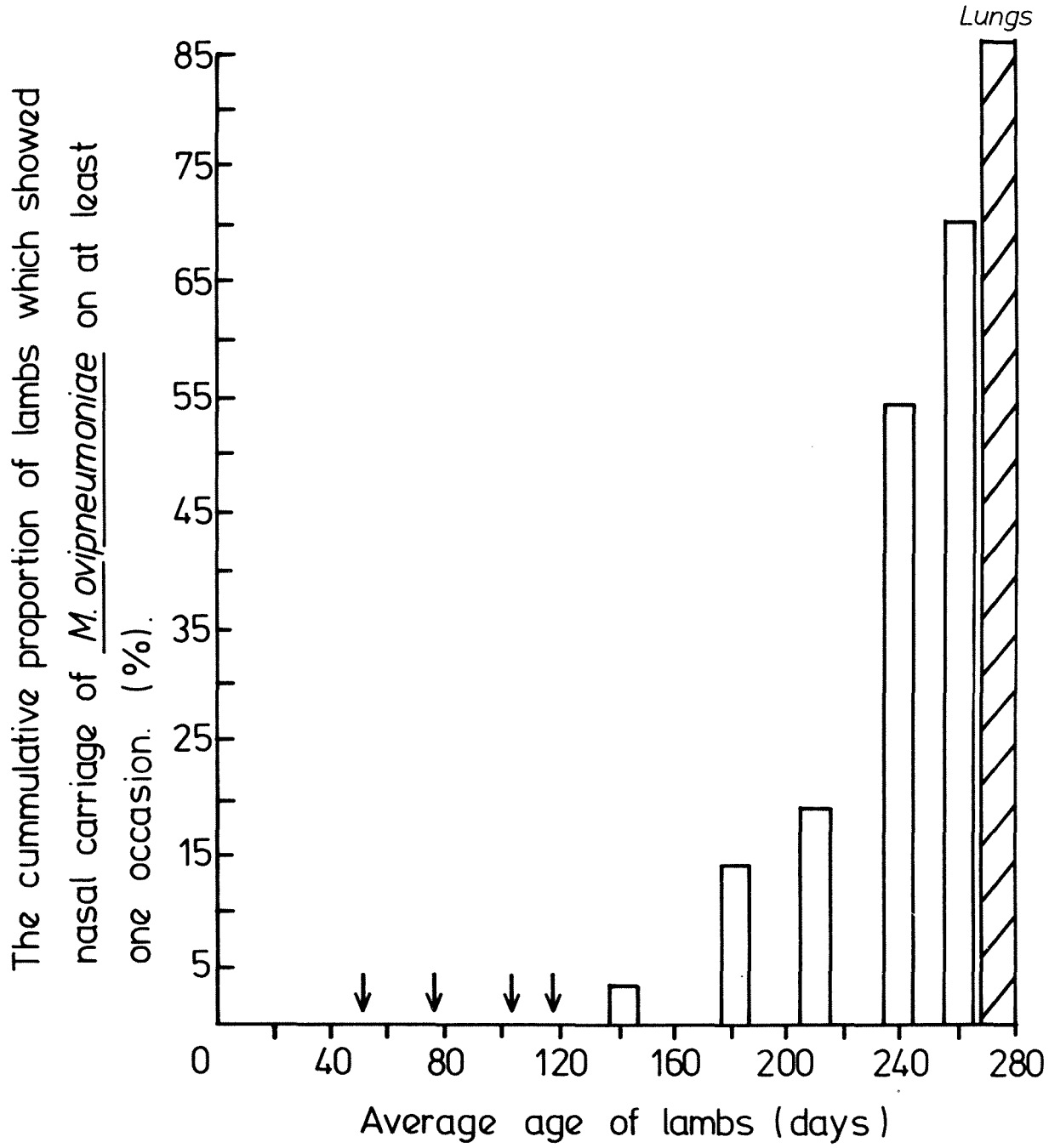


TABLE II

Recovery of *M. ovipneumoniae* or *M. arginini* from:

(i) The nasal cavity of Suffolk lambs swabbed at approximately monthly intervals. Note: the swabbing began at weaning and ended two days before slaughter.

(ii) The lungs at slaughter.

V = *M. ovipneumoniae* isolated

A = *M. arginini* isolated

- = no mycoplasmas isolated

FLOCK 2

Swab Series	1	2	3	4	5	6	7	8	Lung
Approximate age of lambs (days)	45	71	93	114	142	177	207	225	227
Lamb No. M1	-	-	-	-	-	-	V	A	V
M3	-	-	V	V	-	V&A	V	-	-
M4	-	-	-	-	-	-	V&A	A	V
M5	-	-	A	-	A	-	-	-	V
M6	-	-	-	-	-	-	-	-	V
M7	-	-	-	-	-	-	A	A	V
M8	-	-	-	-	-	-	V	-	V
M9	-	-	-	-	-	-	A	A	V&A
M10	-	-	-	-	-	-	V&A	A	V
M11	-	-	-	-	-	-	V	A	V
M12	-	-	-	V	-	V	V&A	V&A	V
M14	-	-	-	V	-	V	V	V	V
M15	-	-	V	-	-	-	-	-	-
M16	-	-	-	-	-	-	V	A	V&A
M18	-	-	-	-	-	-	V	A	V
M19	-	-	-	-	-	-	V&A	A	V&A
M20	-	-	-	-	-	-	A	A	V
M21	-	-	V	V	-	-	-	-	V
M26	-	-	V	-	-	-	-	V	-
M27	-	-	-	A	-	-	V	A	-
M28	-	-	-	V	-	-	V	V	V
M29	-	-	-	-	-	-	A	-	V
M30	A	-	-	-	-	-	A	A	V
M31	-	V	V	-	-	-	V	V	-
M32	-	-	-	-	-	-	V	-	V

FIGURE 2

(i) The (cummulative) proportion of Suffolk lambs which showed nasal carriage of *M. ovipneumoniae* on at least one occasion. The 25 lambs were examined on 8 occasions. The time of swabbing when *M. ovipneumoniae* was not recovered are denoted by (↓).

(ii) The proportion of lambs from which *M. ovipneumoniae* was recovered from the lungs at slaughter (hatched bars at right of diagram).

Lambs in Flock 2

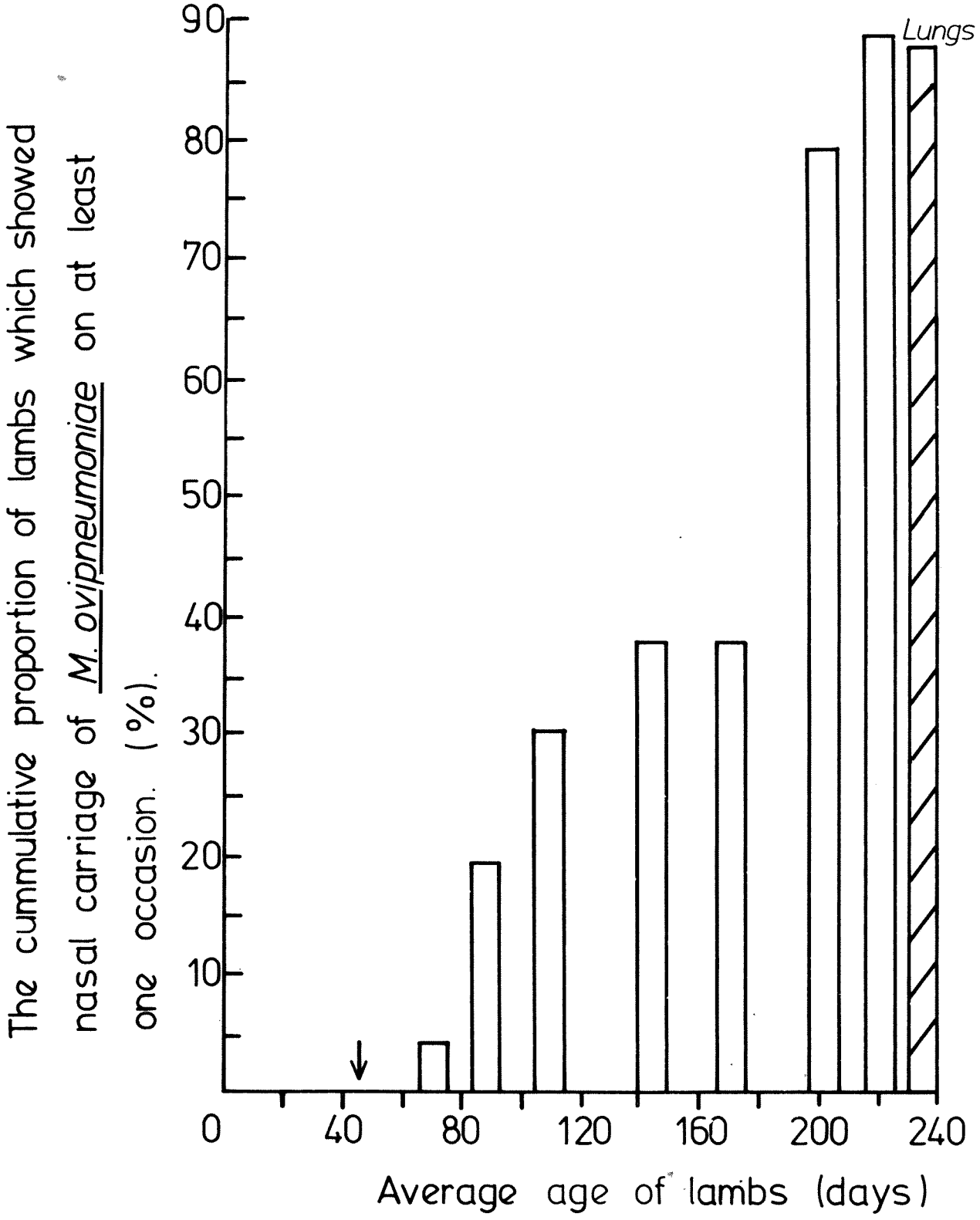
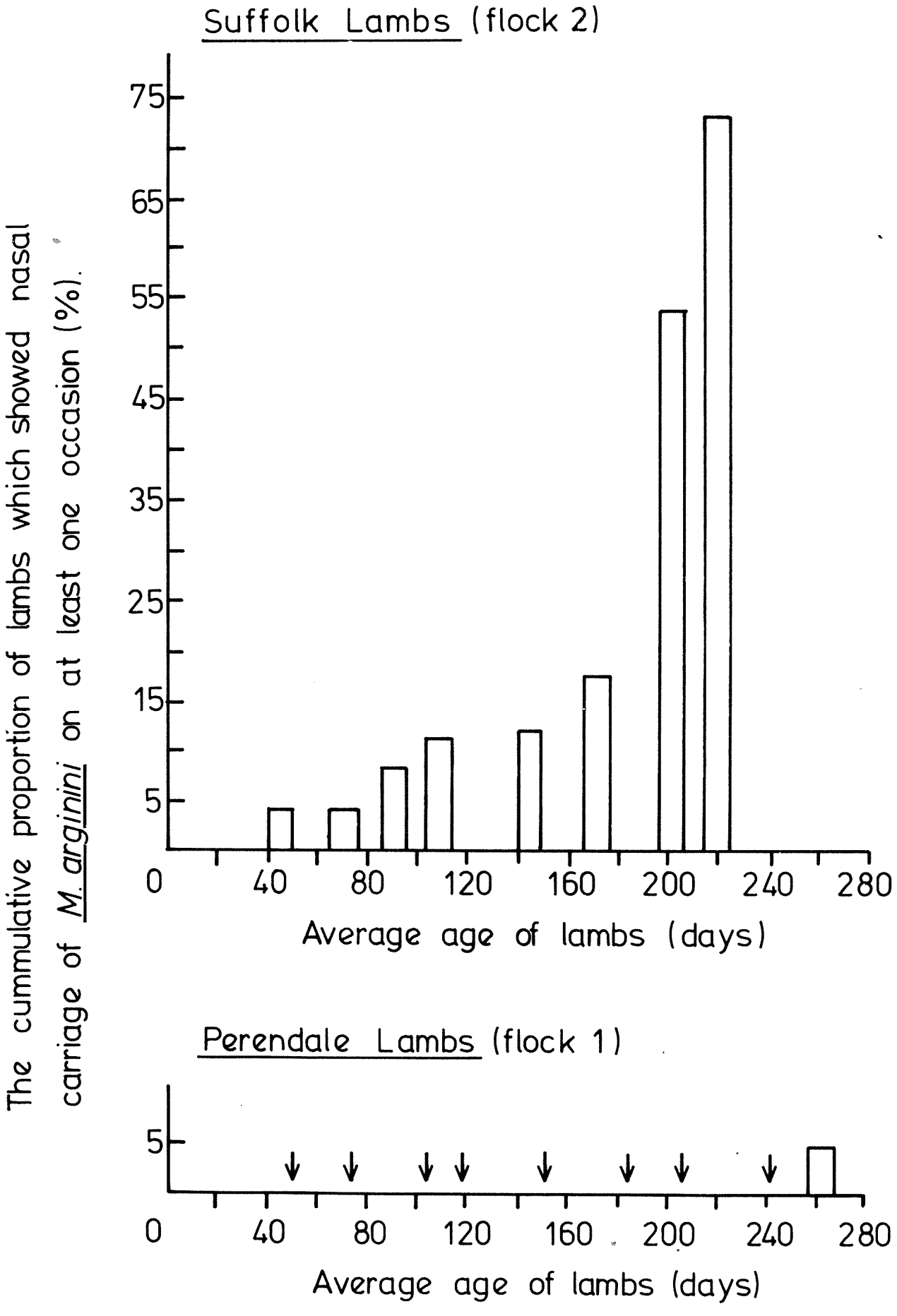


FIGURE 3a

The cummulative proportion of Suffolk lambs (Flock 2) from which *M. arginini* was recovered from the nasal cavity.

FIGURE 3b

The cummulative proportion of Perendale lambs (Flock 1) from which *M. arginini* was recovered from the nasal cavity. The time of swabbing when *M. arginini* was not recovered are denoted by (↓).


Suffolk Lambs (flock 2)
The cummulative proportion of lambs which showed nasal carriage of M. arginini on at least one occasion (%).
75
65
55
45
35
25
15
5
0
40
80
120
160
200
240
280
Average age of lambs (days)
Perendale Lambs (flock 1)
5
0
40
80
120
160
200
240
280
Average age of lambs (days)

	Date	Number of Ewes Swabbed	Positives *M. ovipneumoniae*	Positives *M. arginini*
Flock				
1	6/10/80	20	1 (5%)	1 (5%)
2	7/10/80	20	3 (15%)	3 (15%)

TABLE III

The nasal carriage of *M. ovipneumoniae* and *M. arginini* of flock 1 (Perendale) and flock 2 (Suffolk) ewes at the time of weaning of their lambs. Note: these ewes are the dams of the lambs studied in this survey.

	CNP Lesions	
Flock 1	9/28	(32.0%)
Flock 2	16/25	(64.0%)

TABLE IV

The prevalence of lung lesions in:

(i) Flock 1 (Perendale lambs) located adjacent to the Pahiatua Tract; and

(ii) Flock 2 (Suffolk lambs) located at Massey University Sheep Unit No. 1.

FIGURE 4

M. ovipneumoniae (strain 5), 7 days growth on FM4 agar. The colonies are centreless and have a "lacey" or "vacuolated" appearance. Photographed by oblique light (x 56).

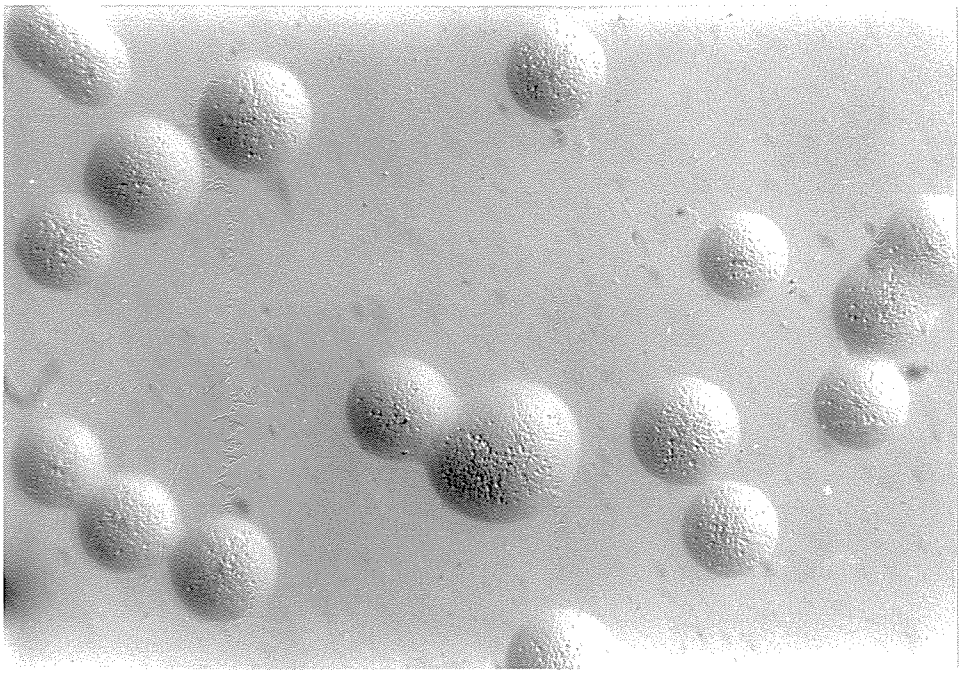


FIGURE 5

M. arginini (isolate 37E), 7 days growth on FM4A agar. The colonies have the typical "fried-egg" appearance of many mycoplasmas, due to the organism growing into the agar. Photgraphed by oblique light (x 56).

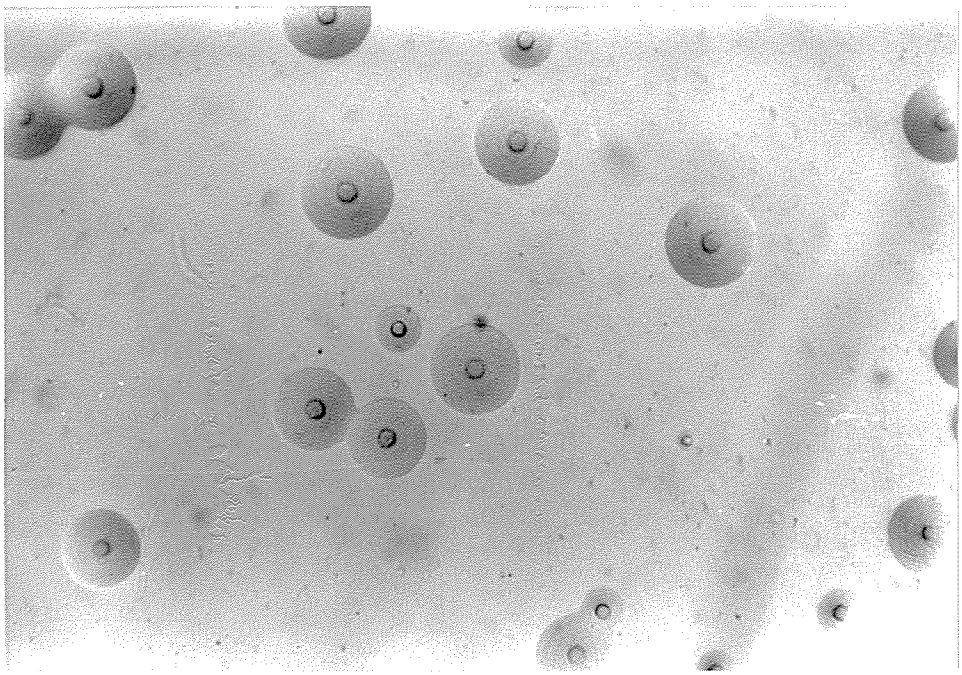


FIGURE 6

Colonies of *M. arginini* at 7 days growth, viewed with transmitted light. Note: the growth at the edge of the inoculation drop (x 56).

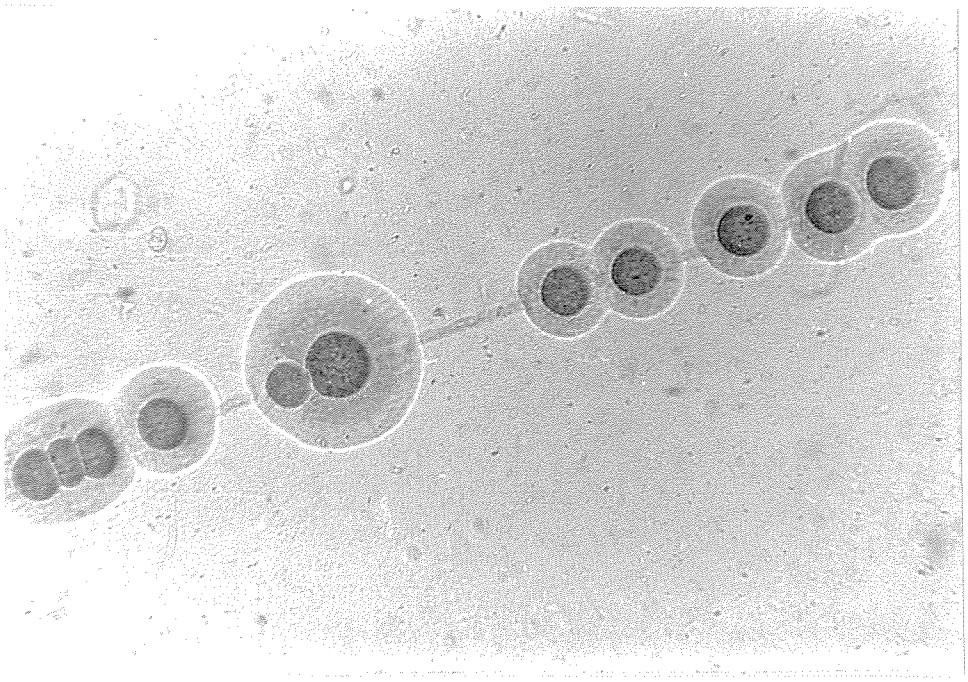


FIGURE 7

Colonies of *M. ovipneumoniae* exhibiting enharnced growth beside a cotton fibre (this was frequently seen and was reproduceably observed with a proportion of isolates). Magnification x 56.

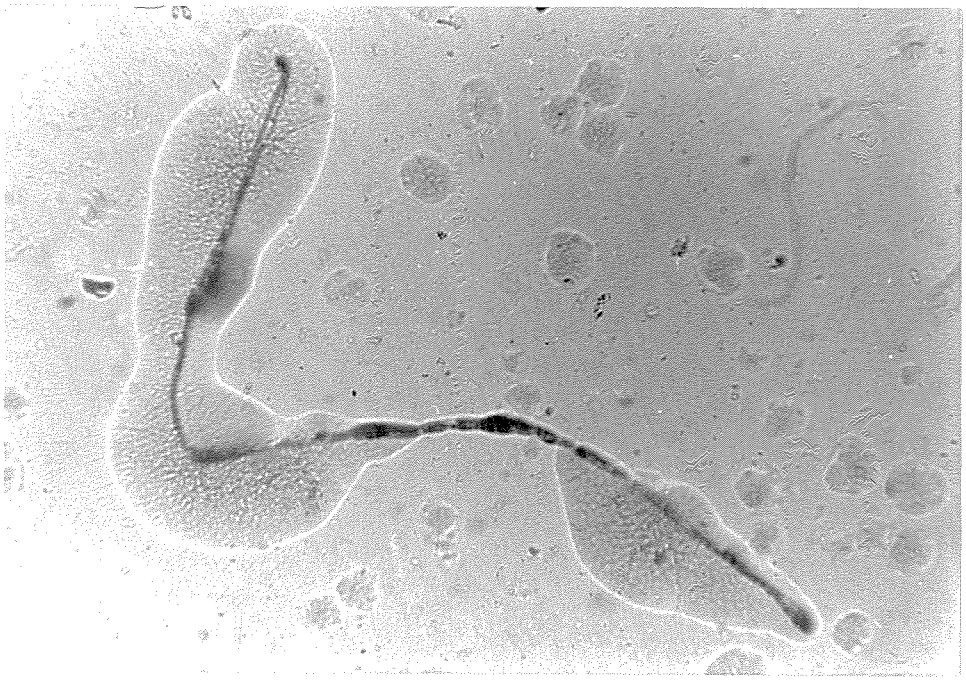
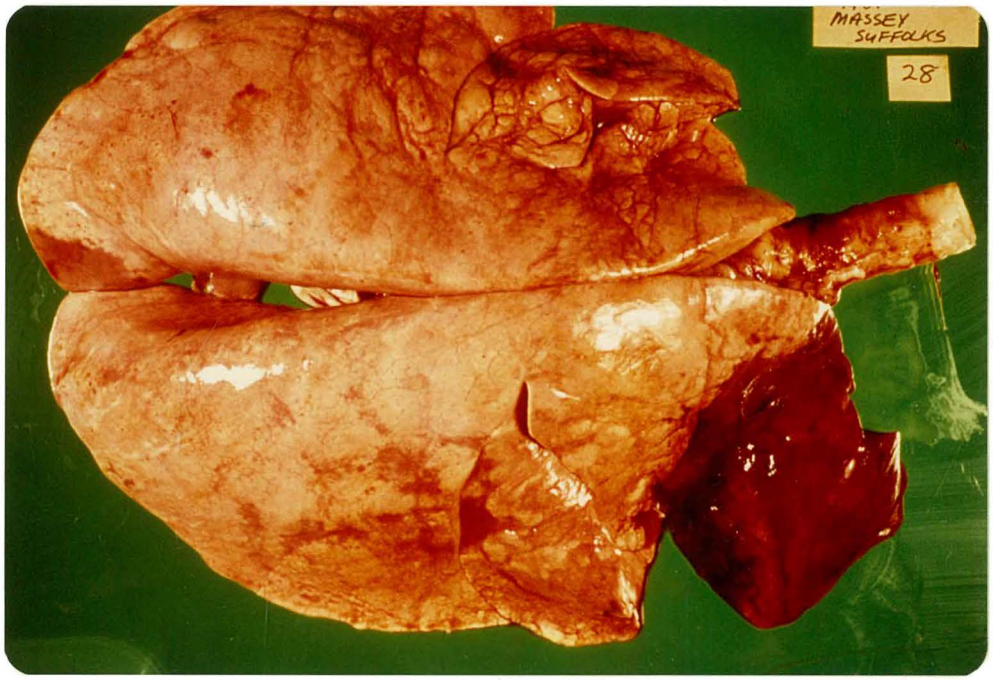


FIGURE 8

CNP lesions consisting of dull red consolidation on the right cardiac lobe in a $8\frac{1}{2}$ month old lamb. Lesions of this size are described in this thesis as "moderate".


MASSEY
SUFFOLKS
28

2.4 Discussion

Identification of Isolates

All the glycolytic mycoplasmas isolated from the respiratory track of sheep and lambs in this study gave centreless "lacy" colonies. In an earlier study (Clarke *et al*, 1974) examined a large number of such isolates using immunofluorecence and found that all were *M. ovipneumoniae*. In the present investigation over 50% of isolates of glycolytic mycoplasmas examined by gel precipitation tests and all gave a line of identity with antigen prepared from a standard strain of *M. ovipneumoniae*. We concluded that all the glycolytic mycoplasmas isolated were strains of *M. ovipneumoniae*.

Time of Colonisation of the Nasal Cavity

The time of colonisation of the nasal cavity of lambs in the two flocks by *M. ovipneumoniae* showed clear differences. Thus:

A) *M. ovipneumoniae* was recovered at least once between weaning and slaughter from a high proportion of lambs from both flocks (67.0% and 87.0% for Flocks 1 and 2 respectively) as shown in figures 1 and 2.

B) Colonisation of the nasal tract of lambs in Flocks 1 and 2 was first detected in mid-January and early November respectively. *M. ovipneumoniae* became disseminated in lambs of Flock 2 earlier than lambs in Flock 1 (compare Figures 1 and 2).

C) 32.0% (Flock 1) and 64.0% (Flock 2) of lambs had CNP (Table IV). *M. ovipneumoniae* was recovered from the lungs of 87.0% (Flock 1) and 88.0% (Flock 2) of lambs. The organism was recovered from the lungs of all lambs with CNP but was also detected in a proportion of apparently normal lungs.

D) The flock in which *M. ovipneumoniae* became disseminated most rapidly had the higher prevalance of CNP (Flock 2).

The Source of Infection of Lambs

The organism was detected in the nasal cavity of ewes (5% and 15% in Flocks 1 and 2 respectively) at the time of weaning of the lambs, but was not detected in the lambs at this time. Nevertheless, since *M. ovipneumoniae* can be maintained in the nasal cavity of older sheep, this is an obvious potential source of infection of lambs.

The Persistence of *M. ovipneumoniae*

Lambs which had *M. ovipneumoniae* in their nasal cavity on one occasion may or may not still have the organism present 3 weeks later. This is further considered in the general discussion (Chapter 5).

Isolation of *M. arginini*

M. arginini was detected in at least one of the ewes in both flocks at the first swabbing. At this time the lambs were approximately 2 months old (Table III).

M. arginini was found only once in the nasal cavity of lambs in Flock 1, which contrasts with Flock 2, where 74.0% (Figure 3) of the lambs had *M. arginini* in their nasal cavity on at least one occasion. Hence in Flock 2 unlike Flock 1, *M. arginini* colonised the nasal tract relatively early and became disseminated through the flock. Both *M. arginini* and *M. ovipneumoniae* were simultaneously present in the nasal cavity at least once in 24.0% of lambs in Flock 2. This implies that *M. arginini* and *M. ovipneumoniae*

do not mutally exclude each other. Nevertheless, compared to *M. ovipneumoniae*, *M. arginini* was disseminated later in the season and in Flock 2 tended to colonise the nasal cavity of lambs after *M. ovipneumoniae* was lost (Table II).

M. arginini was found only infrequently in lungs i.e. 0% (Table I) in Flock 1 and 12.0% (Table II) in Flock 2. This contrasts with *M. ovipneumoniae* which was present in about 80.0% of the lungs of both flocks. Clearly, *M. arginini* has less potential than *M. ovipneumoniae* to colonise the lungs of lambs.

CHAPTER 3

COMPARISON OF *M. ovipneumoniae* ISOLATES BY SDS-PAGE

3.1 Estimation of Total Protein of *M. ovipneumoniae* by the Use of a Protein-Dye Binding Technique

3.1.1 Introduction

To ensure that a consistant amount of protein is applied to the polyacrylamide gels it is necessary to have a method of assay. Protein if pure can be assayed by absorbance at 280nm or if pure and dry can be assayed by weight. Normally however, proteins to be assayed are in solution, but are mixed with other UV absorbing materials e.g. nucleic acid and in that case they are assayed by the Lowry *et al* (1951) method, however this method is slow and is not highly sensitive.

A relative novel technique has been described by Bradford (1976), this method is a more rapid and sensitive than Lowry's method. But, (like Lowry's method) is susceptable to interference by various agents (Bradford 1976).

One potential disadvantage of this method is that SDS (used in SDS-PAGE) interferes with the assay. However, mycoplasma lysed for PAGE (see subsequent sections) can be solubilised by the addition of a small aliquot (see materials and methods of Section 3.2.3c) of 0.2M NaOH.

This section examines the validity of Bradford's technique when applied to the present problems and proposes a modification in which the protein is solubilised by the addition of 0.2M NaOH.

3.1.2 Materials and Methods

a) Preparation of Coomassie-Blue Reagent

Coomassie-Blue G-250	100mg
95% ethanol	50ml
85% (w/v) phosphoric acid	100ml
Distilled water to	1000ml

The ethanol and phosphoric acid were mixed. The dye was dissolved in the mixture and diluted to 1000ml with distilled water. The resulting solution was filtered through two layers of Whatman No 1 filter paper and stored at room temperature in a dark bottle.

b) Preparation of Standard Curves for Protein Solutions

(i) (Bradford's technique) - A range of bovine serum albumin (BSA) solutions from 0 to 100 µg/0.1ml were prepared in 0.15M NaCl. To a 0.1ml aliquot of each dilution 5mls of Coomassie-Blue reagent was added and the contents mixed by inversion.

(ii) (Modification of Bradford's technique) - A range of bovine serum albumin (BSA) solutions from 0 to 100µg/ 0.1ml were prepared in 0.2M NaOH and were heated to 100°C for 3 minutes to simulate the treatment of *M. ovipneumoniae* proteins (see Section 3.2.2).
The solutions were cooled to room temperature and to a 0.1ml aliquot of each dilution 5mls of Coomassie-Blue reagent was added and the contents mixed by inversion.

In both Bradford's technique (i) and in the modified technique (ii) the samples were placed in 4ml glass cuvettes and their absorbances at 595nm were measured using a SP 1800 spectrophotometer.

Protein standards in 0.15M NaCl and 0.2M NaOH were each assayed in triplicate for the first two batches of Coomassie-Blue protein reagent. Subsequent batches of Coomassie-Blue protein reagents were assayed once, with BSA solubilised in 0.2M NaOH and heated at 100°C for 3 minutes (Modification to Bradford's technique), as this method was used to solubilise lysed mycoplasmas for protein determination.

Technical Note on Washing

The protein-dye complex tends to adhere to the surface of the glass cuvettes (this problem is increased with quarts or plastic) and is resistant to removal by normal washing procedures. To overcome this problem a solution of 5% laboratory detergent (Alconox ^{TM}) was prepared, the glass cuvettes were soaked for 10 minutes, followed by several rinses in distilled water and one in acetone. This procedure removed all traces of the protein dye-complex. Alternatively the cuvettes were placed in 0.1M HCl for 2 hours followed by the above rising procedure.

3.1.3 Results

Two standard curves were prepared using both assay techniques i.e. Bradford's technique and the modified technique.

The results from both assays are compared in Figure 9.

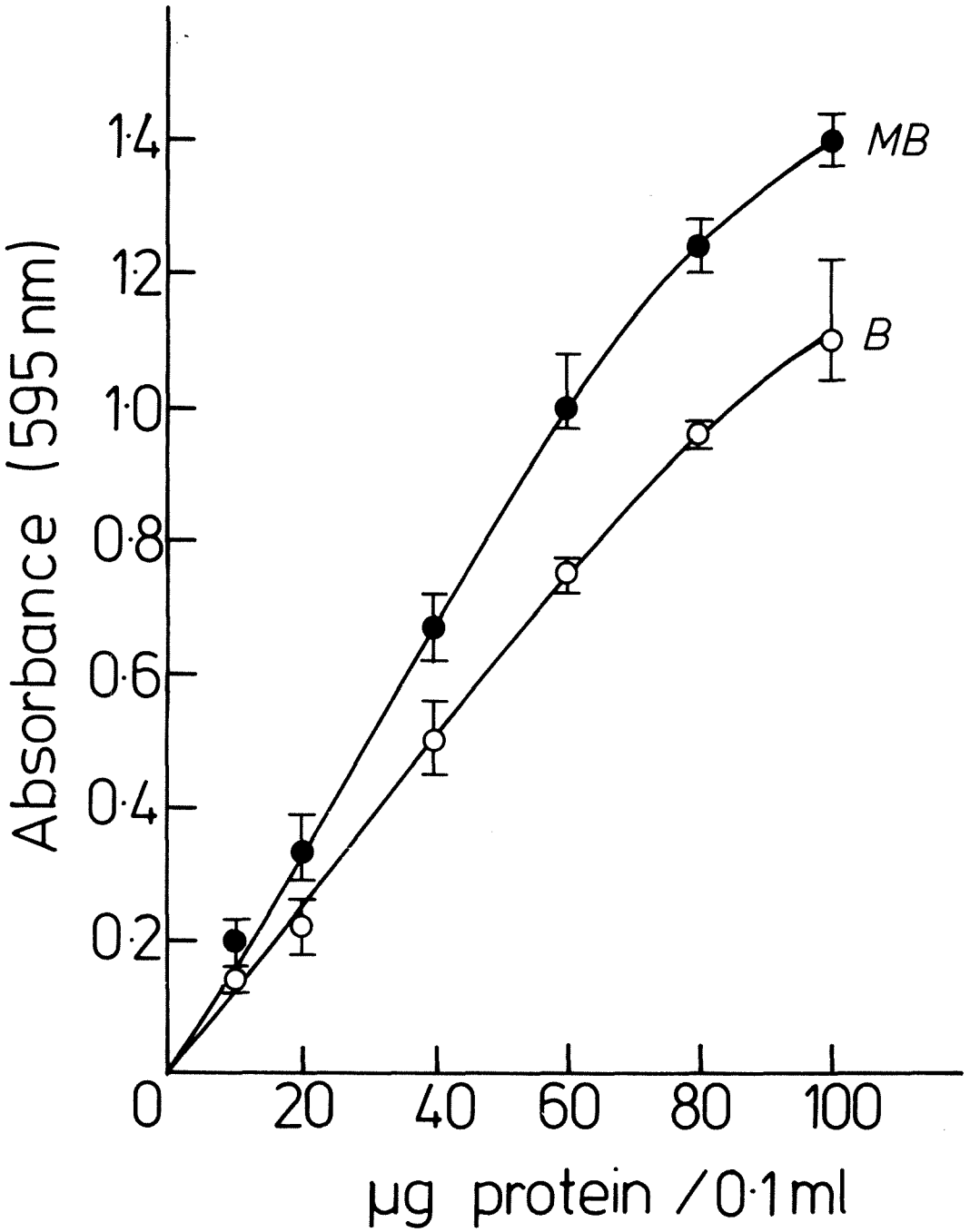
FIGURE 9

Standard curves for protein assay:

(i) Bradford's technique (B)

(ii) Modified Bradford technique (MB)

The limits are derived from triplicate assays. Note that the modified technique is the more sensitive.


Absorbance (595 nm)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
20
40
60
80
100
µg protein / 0·1 ml
MB
B

3.1.4 Discussion

Both Bradford's method and the modification to his technique i.e. solubilising protein in 0.2M NaOH gave reproducable standard curves (Figure 9) which were linear up to approximately 80 µg of protein/0.1ml. It is interesting to note that this modification increased the senstivity of the assay although this was not the original intention.

In subsequent experiments to determine the protein concentration of mycoplasmas, these were lysed by 0.2M NaOH, and dilutions were prepared so that one or more fell within the 20 to 80µg/0.1ml range.

3.2 Selection of a Gel System and Protein Concentration for Maximum Resolution

3.2.1 Introduction

Polyacrylamide gel electrophoresis (PAGE) has been used to examine the protein of microorganisms, including mycoplasmas for taxonomic purposes (Razin and Rottem, 1967; Razin, 1968; Forshaw, 1972; Daniels and Meddins, 1972; Amar *et al.* 1973; Gois *et al.* 1974; and Asa *et al.* 1979).

The purpose of this section is to find if this approach is useful for distinguishing and classifying isolates of *M. ovipneumoniae*. The most widely used system is the SDS-discontinuous buffer system developed by Laemmli (1970) and it is basically this technique which is applied here. However, for our purposes it is necessary to optimise the gel concentration; the amount of protein added per tack and the running conditions. Gradient and non-gradient gels were also compared.

3.2.2 Materials and Methods

General: The method used was basically the SDS-discontinuous system used by Laemmli (1970). However, since a number of technical modifications were made and a succession of minor difficulties were encounted and overcome, the technique used here is recorded in detail.

(A) The Gel System

(i) Gel Apparatus

A vertical slab gel system was used. The gel solution was polymerised between two glass plates and then placed vertically between an upper (negative electrode) and lower (positive electrode) reservoirs containing the appropriate buffers (see Figure 10).

(ii) Preparation of the Glass Plates

Two rectangular pieces of plate glass measuring 5.5 x 170 x 130mm were cut. From one of these plates a section was removed (see Figure 11) to allow contact between the upper reservoir buffer and the gel.

To ensure a high degree of cleanliness, after use each glass plate was washed with detergent (5% Alconox®) and thoroughly rinsed with distilled water. After 3 to 5 uses the glass plates were immersed overnight in chromic acid (5g sodium dichromate dissolved in 5ml distilled water and added slowly with stirring to 100ml of concentrated H_2SO_4). The spacers and stacking gel combs were also cleaned with 5% Alconox®, rinsed in water and dried.

(iii) Assembly of Apparatus

Spacers i.e. perspex strips were placed in position (see Figure 12). A glass plate with a cut out segment was placed flat on the bench with the cut out portion of glass furtherest away. A small "thread" of petroleum jelly was placed along the base and sides of the glass plate approximately 2mm from the edge. The spacers (i.e. 135 x 12 x 1.5mm) were pressed into place. First the base spacers and then the side spacers, making sure that no sealant spread beyond the spacers. Another "thread" of petroleum jelly was placed on top of the positioned spacers, approximately 2mm from the edge. The second glass plate was lowered on to the spacers and pressed firmly into position to give a good seal thus avoiding leakage. The two glass plates were then clamped together on a perspex stand (see Figure 12) with two "bulldog" clips on each side. They were then ready for the gel solution to be added.

(iv) Preparation of Stock Solution for Polyacrylamide Gels

(a) Running gel

Acrylamide	30g
Methylene bis acrylamide	0.5g
Distilled water to	100ml

(b) Stacking gel

Acrylamide	30g
Methylene bis acrylamide	1.6g
Distilled water to	100ml

To prepare acrylamide solutions, the acrylamide was added to 70ml distilled water and stirred magnetically for 30 minutes or until the solution returned to room temperature. Methylene bis acrylamide was dissolved in this solution which was then made up to 100mls with distilled water and filtered through a single layer of Whatman No 1 filter paper.

2a) Lower Tris buffer (1.5M Tris HCl, pH 8.8, 0.4% SDS)

Trizma base	18.17g
10% solution of SDS in distilled water	4ml
12N HCl to	pH 8.8
Distilled water to	100ml

2b) Upper Tris buffer (0.5M Tris HCl, pH 6.8, 0.4% SDS)

Trizma base	6.06g
10% solution of SDS in distilled water	4.0ml
12N HCl to	pH 6.8
Distilled water to	100ml

For both the above solutions the Trizma base was added to 70ml distilled water and the reaction adjusted to the required pH with HCl. The SDS was then

added and the solution diluted to 100ml with distilled water. The pH of the final solutions was checked and adjusted if necessary. These buffers are 4 x the normal working concentration.

3) Ammonium persulfate

Ammonium persulfate	0.1g
Distilled water to	1.0ml

A fresh solution was prepared daily.

4) Tris-glycine reservoir buffer (pH 8.3)

Trizma base	6.07g
Glycine	28.8g
SDS	2.0g
Distilled water to	2000.0ml

5) SDS sample buffer

β-mercaptoethanol	10.0ml
SDS	6.0g
Upper Tris buffer	25.0ml
Distilled water to	100.0ml

This buffer solution is 4x the working concentration and was diluted 4 fold with the protein sample.

6) Bromophenol Blue tracking dye (x 10)

Bromophenol blue	0.05g
Glycerol	40.0ml
Distilled water to	50.0ml

The tracking dye was diluted 10 fold in the protein sample immediately before boiling.

Note: The glycerol increased the density of the sample so that it went to, and remained at, the base of the well following the addition of the sample.

7) Isopropanol stain

Isopropanal alcohol	250ml
Glacial acetic acid	100ml
Coomassie brilliant blue R-250	0.4g
Distilled water to	1000ml

8) Storage

The above solutions were stored at 4°C except for the SDS sample buffer, tracking dye and isopropanol stain which were stored at room temperature.

(v) Preparation of Running Gels

For non-gradient gels i.e. 5, 7.5, 10, 15 and 20% acrylamide, the stock solutions (at room temperature) were added to a conical flask in the order listed in Table V. After each addition, the ingredients were thoroughly mixed by rotational shaking to avoid bubble formation. This mixing is critical to ensure even polymerisation. Immediately after preparation, the yet unpolymerised solution was poured between the glass plates which were positioned at an approximately 45° angle to the the vertical until the desired column length (110mm) of acrylamide was reached. This column of acrylamide was carefully and immediately overlayed with distilled water (with the glass plates at the vertical) so that a flat upper surface was formed (see Figure 13). The water layer prevents direct contact between the unpolymerised gel and the air i.e. gave the relatively anaerobic conditions required to allow complete polymerisation. Polymerisation took approximately one hour at room temperature. However, at this stage the gel (still overlayed with water) was normally left at room temperature overnight or at 4°C over the weekend.

	Running Gel (pH 8.8) 30% Acrylamide 0.5% Methylene bis acrylamide					Stacking Gel (pH 6.8) 30% Acrylamide 1.6% methylenebis acrylamide
Final Acrylamide concentration	5%	7.5%	10%	15%	20%	4.5%
Lower Tris buffer pH 8.8	5.0	5.0	5.0	5.0	5.0	-
Upper Tris buffer pH 6.8	-	-	-	-	-	2.5
Acrylamide	3.4	5.0	6.7	10.0	13.0	1.5
Distilled water	11.6	10.0	8.3	-	-	6.0
40% glycerol	-	-	-	5.0	1.65	-
Ammonium persulfate[1]	0.5	0.5	0.5	0.05	0.04	0.03
TEMED[2]	0.01	0.01	0.01	0.05	0.01	0.01
Total volume (approx) ml	20.0	20.0	20.0	20.0	20.0	10.0

TABLE V

Recipe for gel preparation using the SDS-discontinuous buffer system.

The above volumes are for 11.5 x 11.0 x 0.15cm gels with an approximate volume of 19.0ml.

[1] Fresh 10% solution in distilled water.

[2] N,N,N^1,N^1 - Tetramethylethylenediamine.

(vi) Gradient Gels

For gradient gels, two solutions were prepared of different acrylamide concentrations (see Table V) depending on the gradient required. 9.5ml of each of the solutions were poured into two conical reservoirs of the gradient mixer (see Figure 14). The most concentrated acrylamide solution was placed in the reservoir closest to the peristaltic pump. Then air was bubbled through this solution to ensure rapid mixing of the two preparations as they were pumped from one reservoir at a flow rate of 7.5ml/min through a 0.5mm (inside diameter) plastic tube. The other end of this tube was inserted between the glass plates until the reservoirs were empty and the column length was approximately 110mm. The tube was then carefully withdrawn from between the glass plates and the acrylamide was overlayed with water and allowed to polymerise as described above for non-gradient gels.

(vii) Preparation and Addition of the Stacking Gel

The stacking gel solutions, at room temperature, were mixed in the order listed in Table V. The distilled water was decanted from the running gel and 1 to 2ml of unpolymerised stacking gel solution was poured on to the running gel. The gel was rocked several times and the unpolymerised stacking gel was decanted. This washing process allowed the next aliquot of stacking gel to adhere to the running gel. Fresh stacking gel was added to fill the remaining upper surface. The comb (to cast wells) was then pushed between the plates into the stacking gel (see Figure 15) which was then left to polymerise. This usually occured in 15 to 20 minutes although a longer period of polymerisation was no disadvantage. The comb was then removed and each well rinsed with Tris-glycine buffer pH 8.3 immediately prior to use.

(viii) Preparation and Application of the Protein Samples

As described in Section 3.2.2c.

(ix) PAGE of Protein

The two glass plates with the polymerised gel sandwiched in between was taken from the gel stand and the bottom spacer was carefully removed, to allow contact between the gel and buffer at the anode, without disturbing the side spacers. This gel assembly was then clamped with "bulldog" clips to the electrophoresis apparatus (see Figure 16) which contained a "thread" of petroleum jelly placed horizontally half way between the two buffer reservoirs and another "thread" of petroleum jelly around just below the cut portion of the top reservoir. This insured that the current went through the gel. The upper and lower reservoirs were filled with Tris-glycine buffer pH 8.3 and air bubbles trapped at the base of the gel plates were displaced using a syringe, containing Tris-glycine buffer fitted with a bent needle.

Protein samples usually ranging from 5 µl to 50 µl (depending on the protein concentration) were added to the wells. Larger volumes caused the protein track to widen slightly. The protein samples were electrophoresed through the stacking gel using a current of 10 milliamps. When the tracking dye in the sample reached the stacking-gel running-gel interface the current was increased to 15 milliamps and maintained until the tracking dye was approximately 5mm from the bottom edge of the running gel. Electrophoresis of the dye through the stacking gel (a distance of 10mm) took approximately 1 to $1\frac{1}{2}$ hours. It took a further $4\frac{1}{2}$ hours to traverse the 110mm of the running gel.

(x) Staining the Gel

After electrophoresis the gel assembly was removed from the electrophoresis apparatus by removing the clips and running a scalpel blade between the gel apparatus and the glass plates. The side spacers were carefully removed from between the glass plates so as not to damage the gel and the plates levered apart by inserting the tip of a spatula up one side of the gel assembly. Once the plates were apart, the stacking gel was dislodged from the running gel by running a spatula through the interface. At this stage a reference mark (a small triangle cut from the bottom left corner) was made on the gel so that the orientation of sample loading could not be mistaken. The glass plate with the gel adhering to it was held gel-down over a container of approximately 200ml isopropanol stain (see preparations of stock solutions for polyacrylamide gels). Using a spatula a corner of the gel was separated from the glass plate. The gel slowly peeled of the glass surface by gravity and was left in the stain for 12 hours. This time was occasionally shortened to 2 hours by continually agitating the gel in the stain.

(xi) Destaining the Gel

The isopropanol stain was decanted and the gel rinsed for 2 to 3 minutes with 200mls of 10% (w/v) acetic acid to remove any stain which had precipitated. Fresh 10% acetic acid was then added and replaced every 2 hours while the gel was continuously agitated. This was continued until the background was clear. The acetic acid was then decanted and replaced with 200ml of distilled water and agitated for 2 hours. This procedure was repeated until the acetic acid was no longer detected by smell.

Note: The acetic acid was removed from the gel before photography because of its corrosive effect on photographic equipment. Also its removal was necessary before drying the gel since the boiling point of acetic acid (118°C) slowed the drying process.

(xii) Gel Drying

Permanent records of gels were made by drying and/or photography. To vacuum-dry the gel it was removed from the distilled water tank and placed with the cut corner on the right hand side on a glass surface. Whatman No 1 filter paper approximately 1cm larger than the gel was placed on the gel's surface making sure that any air bubbles were expelled from between the two surfaces. The gel firmly adhered to the filter paper and was lifted from the glass surface still attached to the paper. A thin layer of Glad-wrap™ was placed over the other gel surface thus sandwiching the gel. It was ensured that the plastic wrapping was not creased and had no air bubbles entrapped. The gel was inverted on to a rubber sheet approximately 4mm thick and 50% larger than the gel in length and width. Thick blotting paper up to 5 sheets was added on to the gel followed by a very fine wire mesh and finally another piece of rubber sheet which was similar in dimensions to the first except with a valve in the center where the vacuum was applied (see Figure 17). The two rubber sheets were pressed together at the edges with "bulldog" clips to form a seal and a vacuum was applied (12-14 torr) using a water vacuum pump.

To stop the gel from drying too rapidly and hence cracking, the entire gel drying apparatus was placed over a container of boiling water and left for 1 hour, then removed from the heat and left on the

bench for a further 1½ hours with the vacuum still applied until the gel reached room temperature. The gel drying apparatus was then opened and the Gladwrap™ was peeled from the gel's surface. The dried gel now stuck to the Whatman No 1 filter paper was trimmed and taped into a book for future reference.

(xiii) Gel Photography

Selected gels were photographed on an illumination box with an opal-white screen. The gel was wetted and placed in position avoiding trapped air bubbles. All Coomassie-Blue stained gels were photographed using a standard procedure which initial investigation showed was optimum for our purpose. The aperture, exposure time etc was kept constant and 5" x 4" Tri-X (Kodak™) film was used.

FIGURE 10

The apparatus used for SDS-PAGE electrophoresis of mycoplasma proteins.

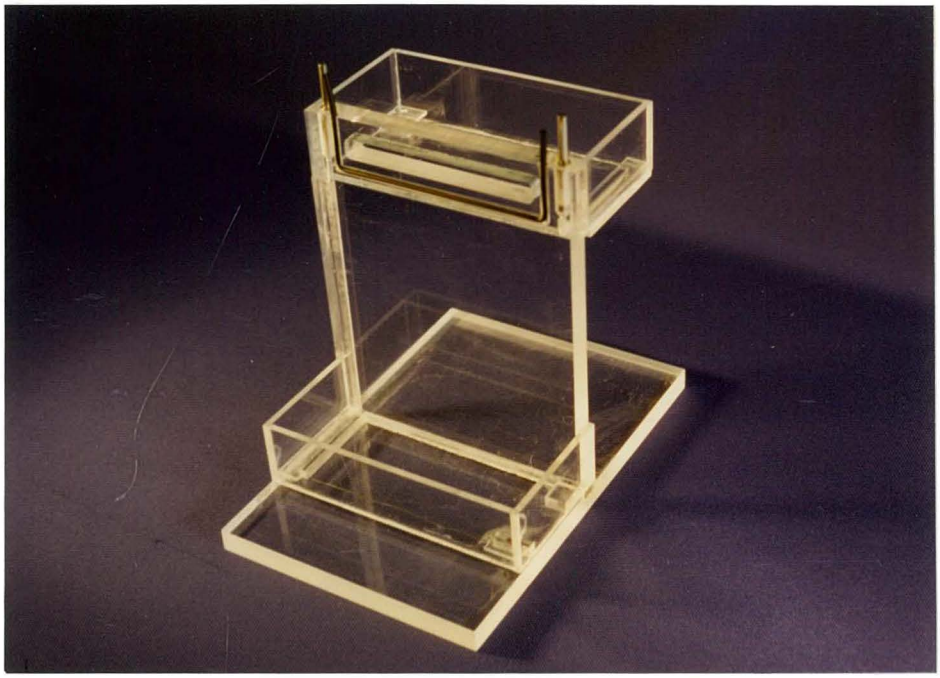


FIGURE 11:

Two rectangular pieces of plate glass measuring 5.5 x 170 x 130 mm were cut (Not shown). From one of these plates a section of glass was removed from one end.

FIGURE 11:

Two rectangular pieces of plate glass measuring 5.5 x 170 x 130 mm were cut (Not shown). From one of these plates a section of glass was removed from one end.

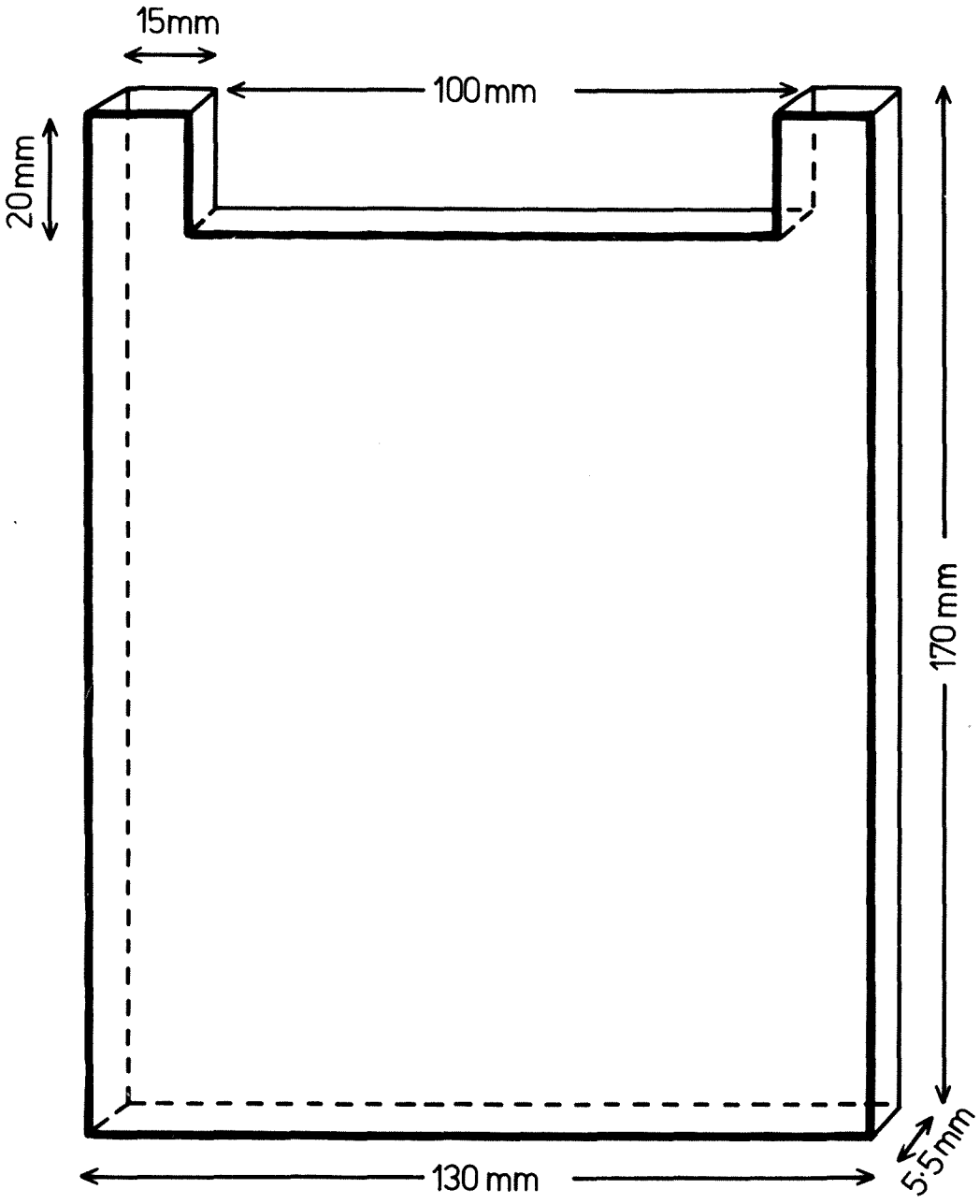

15mm
100mm
20mm
170mm
130 mm
5·5mm

FIGURE 12

Assembly of the glass plates. A "thread" of petroleum jelly was placed around the side and lower edges of one of the glass plates and the perspex spacers were pressed on to the threads. A second "thread" was positioned on to the spacers and the notched glass plate was pressed into position. The glass "sandwich" was clamped to the stand and was then ready for the pouring of the unpolymerised gel solution.

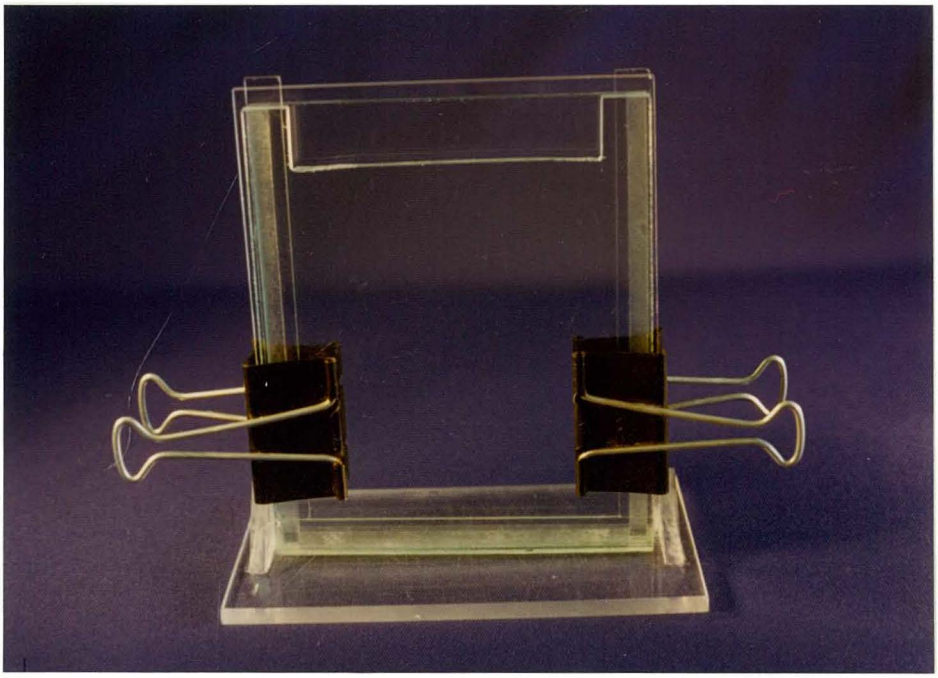


FIGURE 13

Pouring the gel. Unpolymerised acrylamide was poured between the two glass plates (positioned at approximately 45° to the vertical) until the desired column length (110 mm) of acrylamide was reached. The plates were positioned vertically and a layer, approximately 5 mm high, of distilled water was poured on top of the gel. Note that there is a visible acrylamide/water interface after polymerisation of the gel is complete.

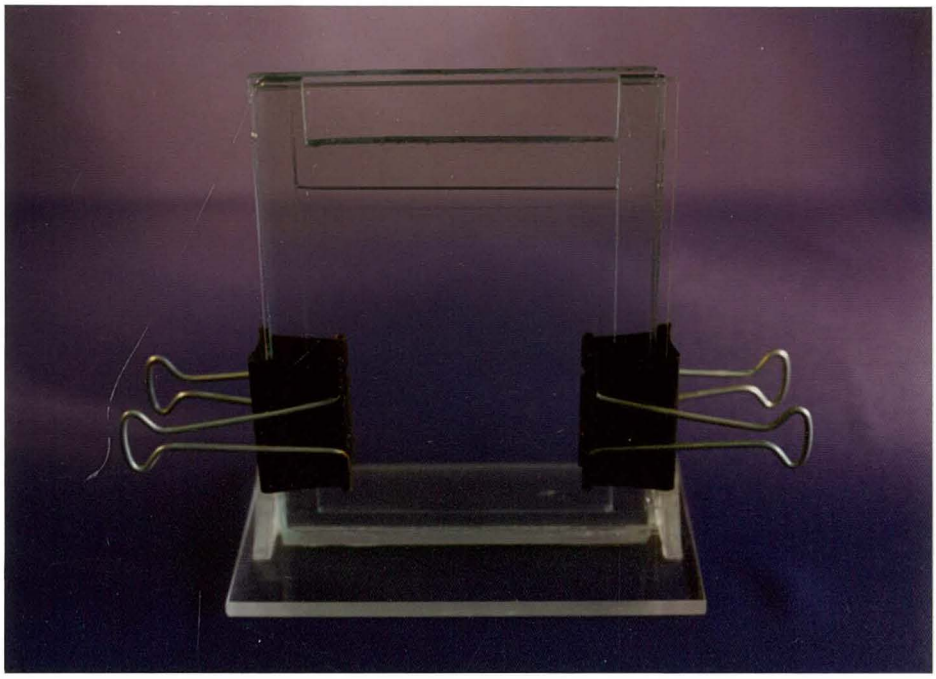


FIGURE 14

Preparation of gradient gels. Solutions of two different acrylamide concentrations are placed in the conical reservoirs. The higher acrylamide concentration is next to the peristaltic pump and the valve is opened. When pumping begins a stream of air is used to mix the two concentrations as they are drawn from the reservoirs. The mixture is pumped between the two glass plates until the desired gel height is attained.

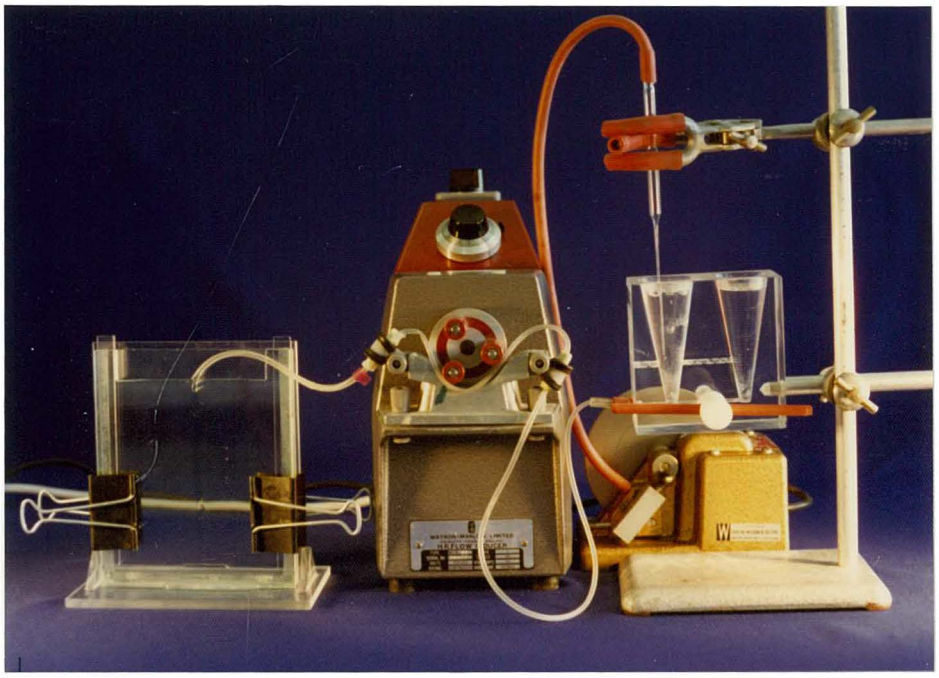


FIGURE 15

Stacking gel and wells. Following the polymerisation of the running gel, the stacking gel was poured until it reached the top of the notched glass plate. Before polymerisation occurred a "comb" with 10 teeth was positioned between the glass plated and pushed into the stacking gel to mould wells capable of containing up to 50 µl of sample. After polymerisation was complete the comb was removed.

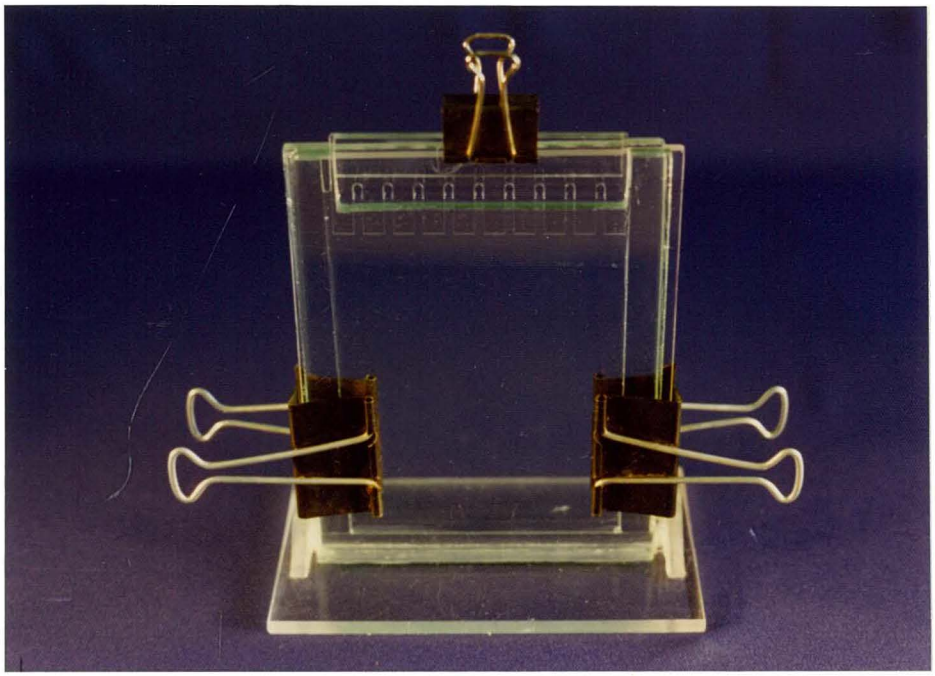


FIGURE 16

Assembly of the gel apparatus. The glass plates were removed from the gel pouring stand. The bottom perspex spacer was removed and the plates clamp on to the electrophoresis stand. "Threads" of petroleum jelly were placed around the notched glass plate to stop leakage or short circuits.

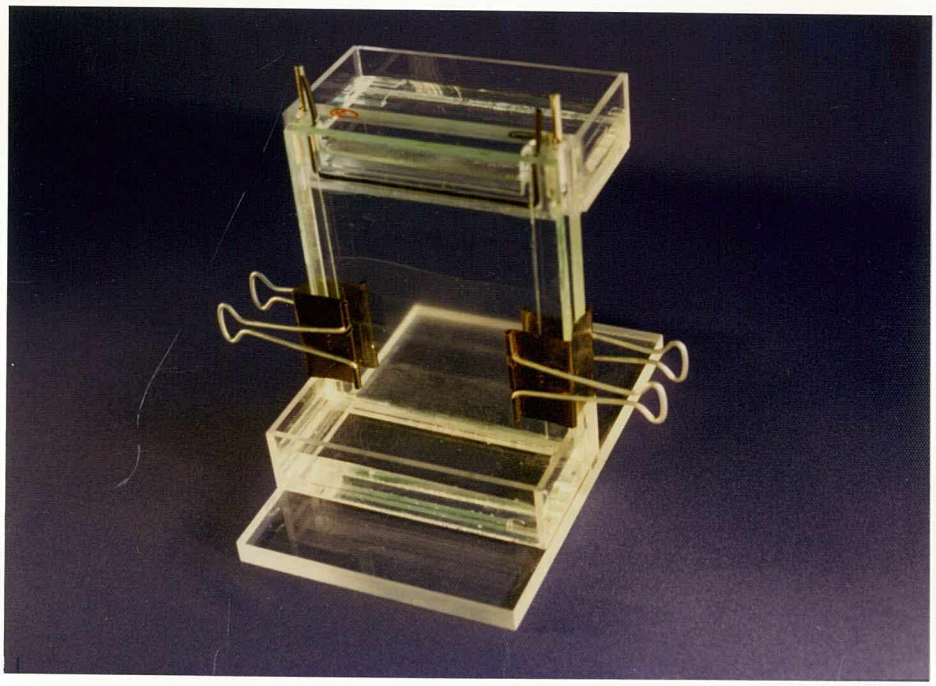


FIGURE 17

Gel drying apparatus. The gels were dried to prepare permanent (non-photographic) records and for fluorography. This system was composed of:

A) A sheet of silicone rubber, 300mm square by 4mm thick.

B) A layer of plastic (Gladwrap^{TM}).

C) The gel.

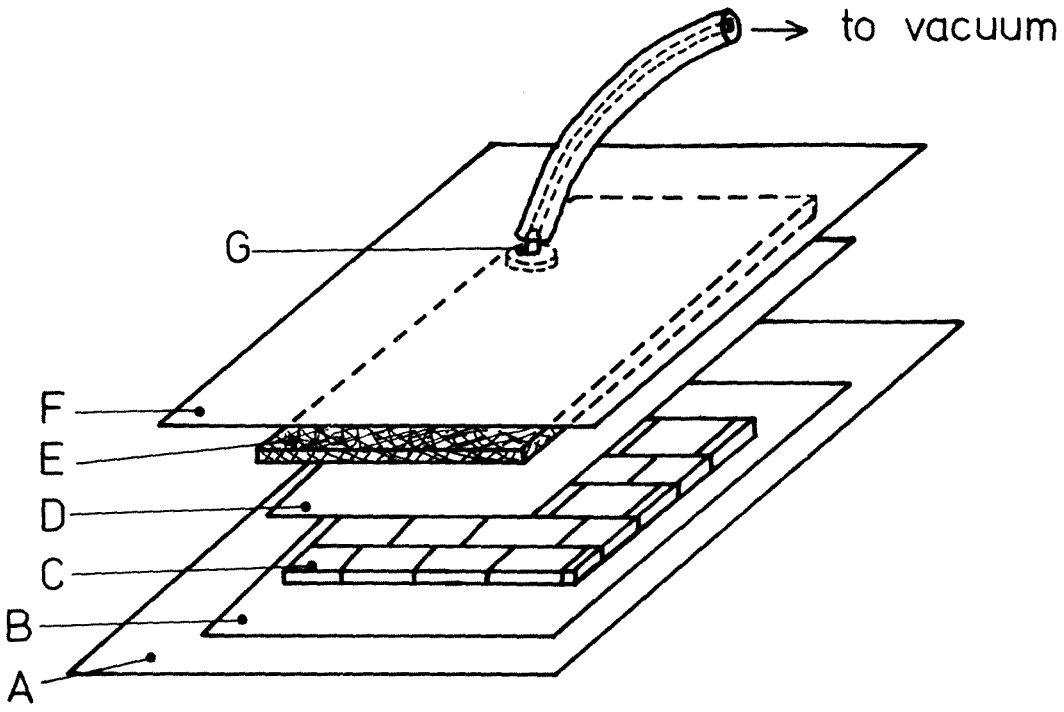
D) A sheet of Whatman No 1 filter paper (10mm larger than the gel).

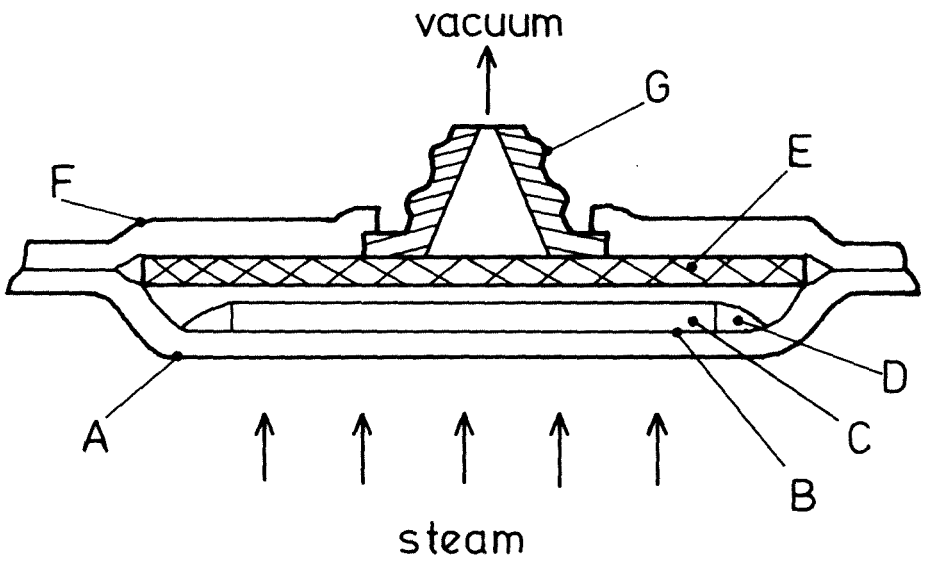
E) 6 sheets of Whatman No 1 filter paper with a wire grid on top for rigidity.

F) A sheet of silicone rubber, 300mm square by 4mm thick.

G) A nozzle positioned in the centre of the silicone rubber sheeth to apply a vacuum.

The apparatus was closed at the edges with bulldog clips and placed over a container of boiling water.


to vacuum
G
F
E
D
C
B
A


vacuum
G
E
F
D
C
A
B
steam

(B) Origin of Strain

M. ovipneumoniae strain 5 was used. It was isolated in 1973 from a sheep in New Zealand.

(C) Preparation and Application of the Protein Samples

1) Extraction of Total Proteins

A 10ml aliquot of *M. ovipneumoniae* (Strain 5) stored at -70°C was inoculated into 300ml of FM4 media and incubated at 37°C on a rotary shaker at 125rpm until the reaction reach pH 6.8. The culture was then centrifuged at 14,600g for 10 minutes. the resulting deposit was washed twice in 0.15M PBS and resuspended to give an approximate 10% suspension. This was placed in an ice bath and sonically disrupted at 20kc/s using 8 x 15 second bursts with a MSE 100 watt ultrasonic disintegrator. The lysed suspension was frozen at -70°C until required for protein assay.

2) Protein Assay

A 0.1ml aliquot of this 10% suspension of disrupted cells was diluted 1/3, 1/10, 1/30, 1/100 in 0.2M NaOH to give a final volume of 0.1ml. These solutions were heated at 100°C for 3 minutes to solubilise the protein. The solutions were cooled to room temperature and to each 0.1ml aliquot, 5ml of Coomassie-Blue reagent was added and the contents was mixed. Each sample was placed in a glass cuvette and its adsorbance at 595nm was measured using a SP 1800 spectrophotometer. The protein content was determined from a standard curve (see Section 3.1).

3) SDS-Solubilization of Protein Samples

To a 0.1ml aliquot of the original protein sample, 25µl of SDS-sample buffer (4x concentrated) and 12.5µl Bromophenol Blue (10x concentrated) was added. The contents were mixed using a Vortex and heated to 100°C for 3 minutes to denature and solubilise the protien. The solution was cooled to room temperature.

4) Application of Samples to the Gel

The wells were filled with Tris-glycine reservoir buffer pH 8.3. Volumes varying between 5 and 50µl of SDS-solubilised protein in Bromophenol Blue tracking dye containing 0.8% glycerol were added to wells using a micropipette. The glycerol ensured that the sample settled to the bottom of the wells.

(D) Gel Concentrations

Non-gradient gels of the following concentrations 5.0, 7.5, 10.0, 15.0% and a linear gradient gel of 7.5 to 15.0% acrylamide were prepared as described in Table V.

3.2.3 Results

A series of different amounts of proteins extracted from *M. ovipneumoniae* were electrophoresed on 5, 7.5, 10 and 15% gels, the results are shown in Figures 18, 19, 20 and 21 respectively. This was repeated using a 7.5 to 15% linear gradient. This result is shown in Figure 22.

FIGURE 18

Total protein of *M. ovipneumoniae* (strain 5) electrophoresed through a 5% acrylamide gel. Protein loadings for tracks 1 to 8 are 215; 172; 128; 107; 86; 64; 43; 21.5 µg/track. To establish the optimum gel concentration and protein loading for our purposes this figure was compared with Figure 19 (7.5% gel), Figure 20 (10% gel), Figure 21 (15% gel) and Figure 22 (7.5 - 15% gradient gel).

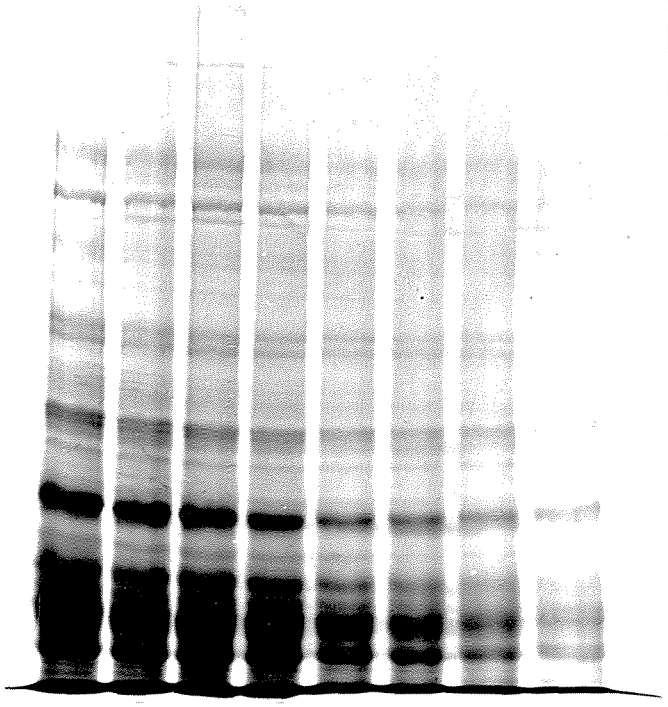


FIGURE 19

Total protein of *M. ovipneumoniae* (strain 5) electrophoresed through a 7.5 % acrylamide gel. Protein loadings for tracks 1 to 8 are 215; 172; 128; 107; 86; 64; 43; 21.5µg/track. To establish the optimum gel concentration and protein loading for our purposes this figure was compared with Figure 18 (5 % gel), Figure 20 (10 % gel), Figure 21 (15% gel) and Figure 22 (7.5-15% gradient gel).

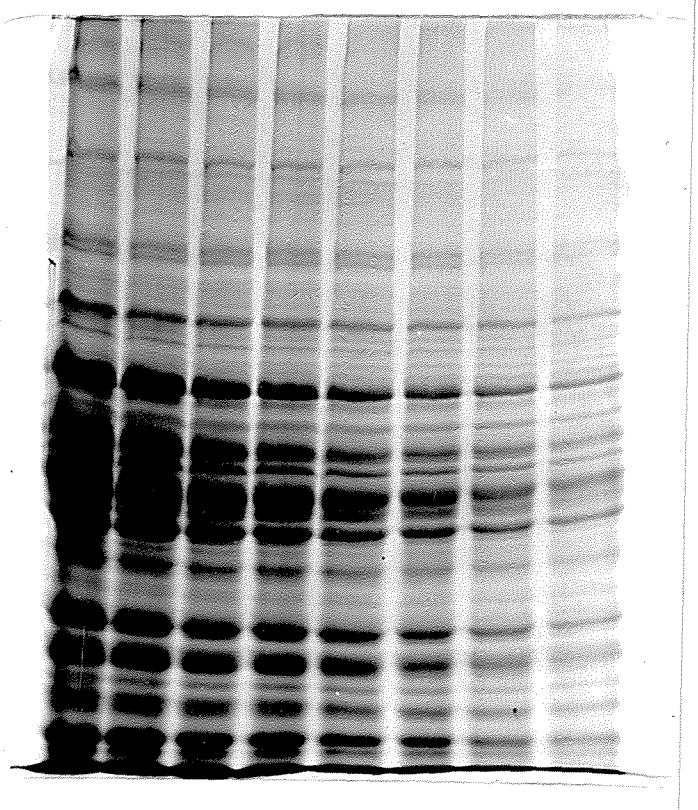


FIGURE 20

Total protein of *M. ovipneumoniae* (strain 5) electrophoresed through a 10 % acrylamide gel. Protein loadings for tracks 1 to 8 are 215; 172; 128; 107; 86; 64; 43; 21.5µg/track. To establish the optimum gel concentration and protein loading for our purposes this figure was compared with Figure 18 (5 % gel), Figure 19 (7.5 % gel), Figure 21 (15 % gel) and Figure 22 (7.5-15% gradient gel).

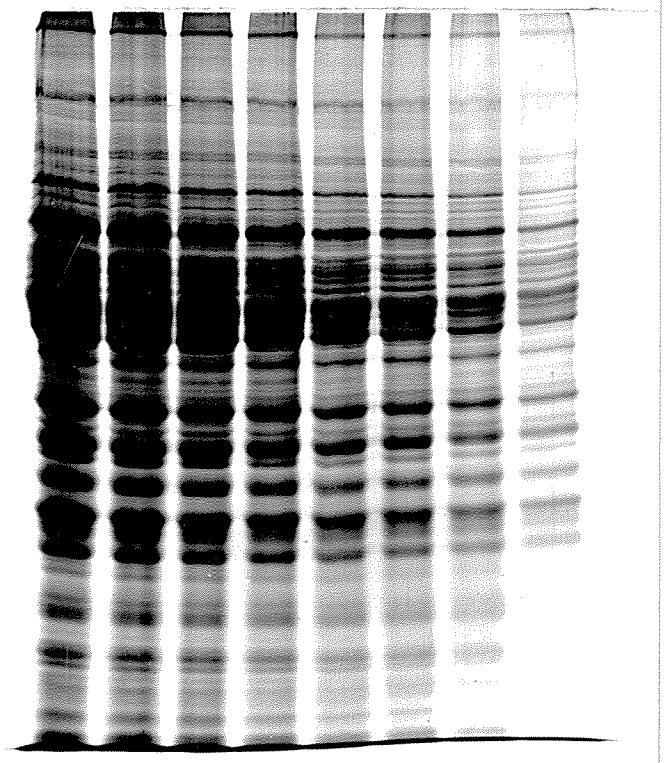
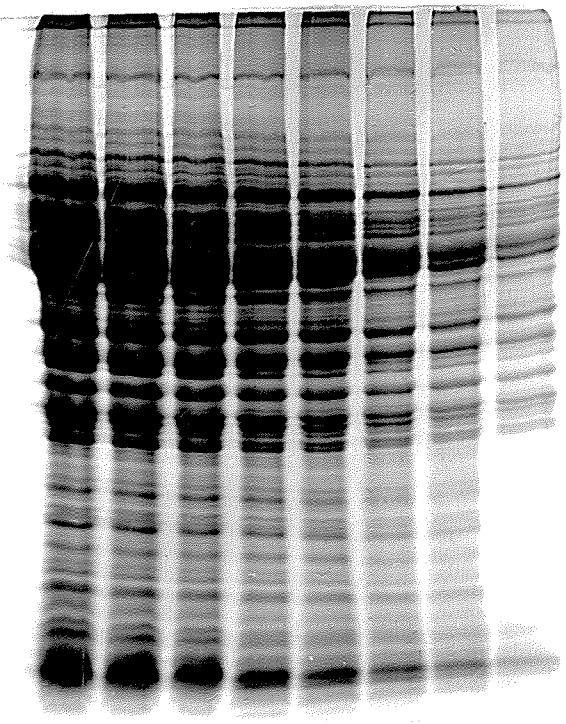


FIGURE 21

Total protein of *M. ovipneumoniae* (strain 5) electrophoresed through a 15 % acrylamide gel. Protein loadings for tracks 1 to 8 are 215; 172; 128; 107; 86; 64; 43; 21.5µg/track. To establish the optimum gel concentration and protein loading for our purposes this figure was compared with Figure 18 (5 % gel), Figure 19 (7.5 % gel), Figure 20 (10% gel) and Figure 22 (7.5-15% gradient gel).

FIGURE 22

Total protein of M. ovipneumoniae (strain 5) electrophoresed through a 7.5-15% acrylamide gradient gel. Protein loadings for tracks 1 to 8 are 215; 172; 128; 107, 86; 64; 43; 21.5 µg/track. To establish the optimum gel concentration and protein loading for our purposes this figure was compared with Figure 18 (5% gel), Figure 19 (7.5% gel), Figure 20 (10% gel), and Figure 21 (15% gel).



3.2.4 Discussion

Optimum gel concentration

Acrylamide gel concentrations of 5% and 7.5% (Figure 18 and Figure 19) gave good separation of the high molecular weight proteins but, gave poor resolution of the low molecular weight bands. The reverse was true of the 15% acrylamide gel (Figure 21). For linear gels the best compromise was obtained using a 10% acrylamide gel (Figure 20) which gave a reasonably even distribution of protein bands over the entire gel track. The 7.5% to 15% linear gradient gel (Figure 22) also gave good resolution of high and low molecular weight protein bands. However in repeated experiments (not shown) the reuslts obtained using gradient gels was less reproducible than those obtained with 10% linear gels. So since the gradient gel had no advantage as compared to a 10% linear gel, the latter was used in subsequent sections in the separation of *M. ovipneumoniae* proteins.

Optimum protein load

Different amounts of *M. ovipneumoniae* total protein ranging from 215 to 21.5 µg/track were loaded on to all the gels shown (Figures 18, 19, 20, 21 and 22). However, since 10% linear gels were chosen for routine protein separation, it is the optimum loading of this gel which is critical. Tracks 1 to 4 (Figure 20) indicates an excessive protein load since the major bands are so wide that they obscure adjacent bands. Tracks 7 and 8 contained less protein and some individual proteins present in low amounts did not produce visible bands. Tracks 5 and 6 loaded with 86 and 64 µg of protein respectively produced the best compromise.

From the above we decided to use a 10% linear gel with 80 µg of protein per track as the standard method of separating *M. ovipneumoniae* proteins.

3.3 Reproducibility of SDS-PAGE Separation of Proteins from Replicate Culture of an *M. ovipneumoniae* Strain

3.3.1 Introduction

Subsequent sections of this thesis are concerned in differences in SDS-PAGE patterns between *M. ovipneumoniae* isolates. However, before different isolates are examined it is necessary to show that the SDS-PAGE patterns obtained from a replicate culture of a single isolate do not vary in their band pattern. The reproducibility of such patterns are examined in this section.

3.3.2 Materials and Methods

Preparation and Application of Protein Samples

8 replicate cultures of *M. ovipneumoniae* strain 5 were prepared and protein extracted from each as described earlier (Section 3.2.2).

Preparation of 10% Acryamide Gel

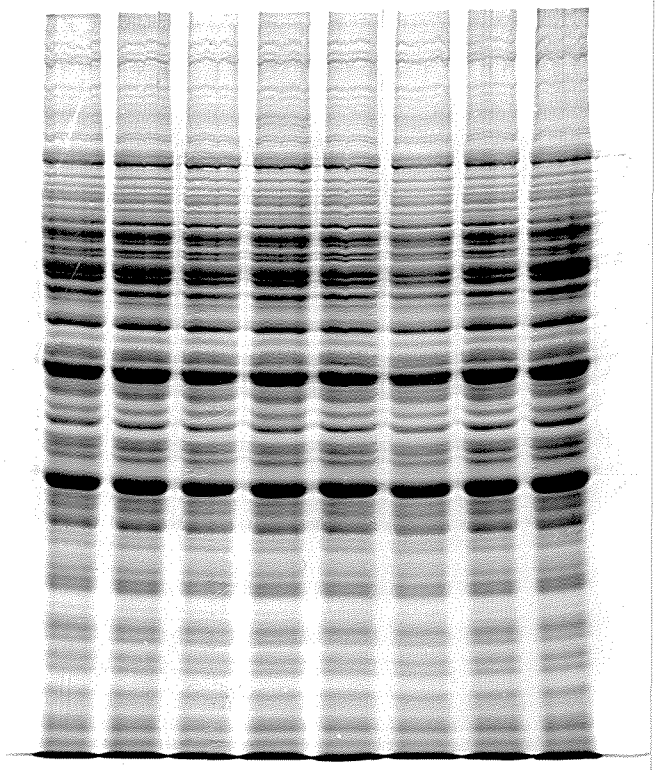
This procedure is described in section 3.2.2.

3.3.3 Results

8 total protein extracts from *M. ovipneumoniae* strain 5 were electrophoresed through a 10% acrylamide gel. All tracks 1 to 8 contained 80 µg of protein, the results are shown in Figure 23.

FIGURE 23

Reproducibility of protein separation on a 10% SDS-PAGE gel. *M. ovipneumoniae* (strain 5) was propagated as 8 separate cultures which were independently processed and electrophoresed. Each track was loaded with 60µg of protein.



3.3.4 Discussion

The results shown in Figure 23 indicate that the electrophoretic patterns of total proteins extracted from *M. ovipneumoniae* strain 5 are reproducable.

3.4 The Effect of Trichloroacetic Acid Precipitation and Sonic Disruption on the Electrophoretic Banding Pattern of *M. ovipneumoniae*

3.4.1 Introduction

In typical experiments described in this thesis, *M. ovipneumoniae* cell proteins were solubilised by the addition of SDS to a sonicated suspension. However, some experiments (involving surface labelling by FITC or radioactive labelling of total protein) require that the cells be precipitated with 20% TCA before these proteins are solubilised.

This section examines the possible effects of this treatment on the SDS-PAGE banding pattern.

3.4.2 Materials and Methods

Protein Extraction by TCA Precipitation

300ml of a log phase culture of *M. ovipneumoniae* was centrifuged at 14,600g for 10 minutes and the resulting deposit was washed twice in 0.15M PBS and resuspended in 0.15M PBS to give an approximate 10% suspension. To this suspension 5 times the volume of 20% TCA was added and the mixture was left on ice for 1 hour. The resulting precipitate was centrifuged at 14,600g for 20 minutes, the supernatant was discarded, the pellet was resuspended in 5% TCA and then washed in diethyl ether to remove the TCA. The precipitated protein was dried by a stream of air; weighed and resuspended in SDS-sample buffer to give a final concentration of 5.5mg/ml.

Solubilisation of TCA Precipitated Protein

M. ovipneumoniae proteins precipitated with TCA did not readily redissolve in SDS-sample buffer. To facilitate solubilisation the dried pellet was broken and ground with

a glass rod. To this a few drops of 10% $NaHCO_3$ solution was added and the grinding continued until a paste was formed. Then SDS-sample buffer was added to give a protein concentration of 5.5mg/ml.

From this a 100µl aliquot was taken. 25µl of SDS-sample buffer and 12.5µl of Broghenol Blue tracking dye was added and the mixture heated at 100°C in a waterbath for 3 minutes. The sample was cooled and an aliquot was added to a SDS-PAGE gel.

Sonic Disruption of *M. ovipneumoniae* Cells

This procedure is described in Section 3.2.2.

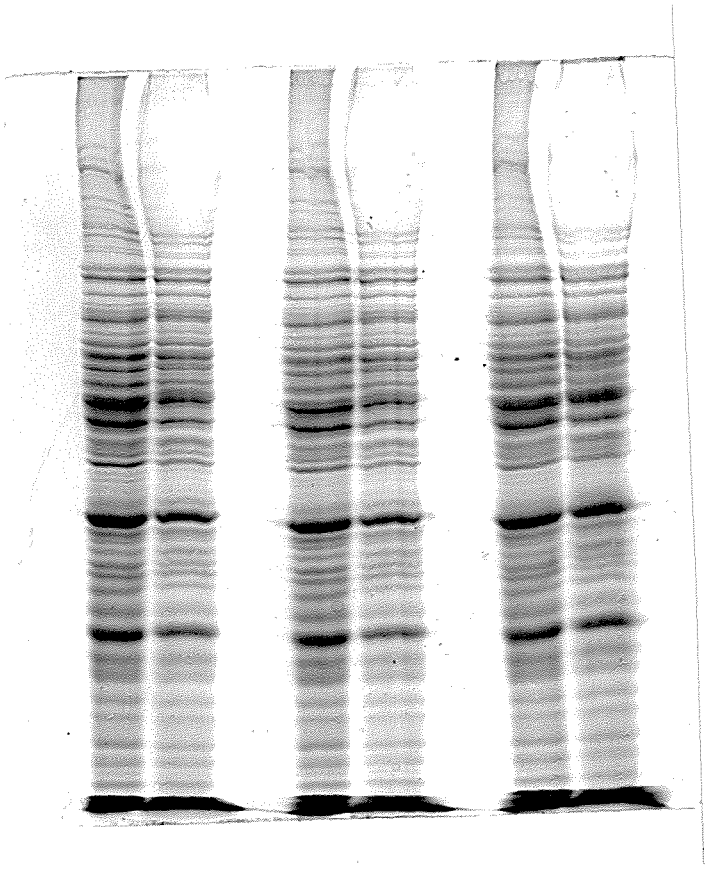
3.4.3 Results

M. ovipneumoniae protein solubilised by SDS with or without TCA precipitation was applied in pairs to a 10% SDS-PAGE gel. Three pairs were examined and the results are shown in Figure 24. No significant difference in the electrophoretic banding patterns was detected. An increase in the track width of the TCA precipitated sample of high molecular weight protein was noted. This was a constant finding but the reason was not investigated.

FIGURE 24

Some experiments require that protein be precipitated by TCA. This figure investigates any affect which this step may have on subsequent protein separations by electrophoresis. Tracks 1, 3 and 5 by standard preparation. Tracks 2, 4 and 6 protein precipitated by TCA.

No significant difference in the electrophoretic banding patterns is detected, except that a local increase in the track width of the TCA precipitated sample was constantly found.



3.4.4 Discussion

The electrophoretic banding pattern of *M. ovipneumoniae* protein is not significantly changed following precipitation with TCA.

3.5 Comparison of Different Isolates of *M. ovipneumoniae* by SDS-PAGE

3.5.1 Introduction

Strains of *M. ovipneumoniae* can be distinguished by serology (Jones *et al*, 1976) although extensive cross-reactions did not allow isolates to be assigned to well defined groups. In contrast, examination of independent* isolates of *M. ovipneumoniae* by REA (Mew, Thesis 1982) gave patterns which were totally different i.e. gave no common bands and the number of different patterns was large or possibly unlimited. However, an examination of 12 isolates (related in the sense that they were all derived from lambs on one farm) by REA revealed patterns which fell into 3 groups. REA is therefore a powerful tool for identifying individual strains but the multiplicity of different patterns obtained when independent isolates were examined did not allow the isolates to be assigned to a limited number of groups.

Since neither serology nor RE-analysis facilitated the grouping of *M. ovipneumoniae* isolates, this section examines the possibility of using SDS-PAGE for this purpose.

3.5.2 Materials and Methods

Origin of Isolates

12 nasal isolates of *M. ovipneumoniae* (obtained from lambs at Massey University Sheep Unit No 1 and designated 'M) were examined (see Table VI and Table VII). These belong to 3 REA groups (Mew, Thesis 1982).

A further 8 "independent isolates" of *M. ovipneumoniae* were examined. These included both nasal and lung isolates, obtained from sheep in New Zealand on different farms, over a period of several years. One Australian strain (see Table VIII) was also examined.

---

* The term "independent isolates" is used later in this thesis. It has the meaning defined above.

Track No.	Isolate	REA Group	Origin of Isolate
1	M3 (4)	1	Nasal cavity
2	M14(6)	1	Nasal cavity
3	M12(6)	3	Nasal cavity
4	M12(8)	3	Nasal cavity
5	M 4(7)	4	Nasal cavity
6	M 8(7)	4	Nasal cavity

Table VI 6 isolates of *M. ovipneumoniae* from one flock. These consist of 3 pairs. The isolates within a pair are indistinguishable by REA. The isolates of different pairs were substantially or totally different by REA. ( ) indicates the swab number. The above isolates are shown in Figure 25.

Track No.	Isolate	REA Group	Origin of Isolate
1	M3 (6)	1a	Nasal cavity
2	M14(4)	1a	Nasal cavity
3	M12(7)	3a	Nasal cavity
4	M14(7)	3b	Nasal cavity
5	M1 (8)	4a	Nasal cavity
6	M3 (7)	4a	Nasal cavity

Table VII. 6 isolates of *M. ovipneumoniae* from one flock. These consist of 3 pairs. The isolates within a pair are indistinguishable by REA. The isolates of different pairs were substantially or totally different by REA. ( ) indicates the swab number. The above isolates are shown in Figure 26.

Track No.	Strain	Origin of Strain	Year Isolated
1	1	Lung	1973
2	2	Lung	1973
3	4	Lung	1973
4	5	Lung	1973
5	10	Lung (Australia)	1973
6	37E	Nasal cavity	1980
7	MPP74	Nasal cavity	1981
8	L3/C3	Lung	1980

Table VIII. 8 independent isolates of *M. ovipneumoniae*, examined by SDS-PAGE (see Figure 27).

3.5.3 Results

The results are shown in Figures 25, 26 and 27 and the following points were noted:

(i) 50 to 60 protein bands were visible on each track and most of these bands were common to all isolates.

(ii) Not withstanding (i) above all the independent isolates (Figure 27) had several unique bands. In principle this allowed each independent isolate to be differentiated from all the others examined.

(iii) Isolates related in the sense that they were derived from lambs at one farm over a short period but unrelated in the sense that they had different REA patterns, could be distinguished by SDS-PAGE although the variation seen was less than that seen with isolates derived from different farms (i.e. independent isolates).

(iv) Isolates from one farm showing the same REA patterns were also examined. In some cases no differences were detected. However, in others small but unequivocal band differences were seen by SDS-PAGE (see Figures 25 and 26).

FIGURE 25

Six isolates from the nasal tract of lambs of one flock were examined by *EcoRI* restriction endonuclease analysis. Within a group the REA patterns were identical or nearly identical. Whereas the REA patterns of different groups showed no similarity.

These isolates are examined by SDS-PAGE.

	Tract Number (left to right)					
	1	2	3	4	5	6
REA group	1	1	3	3	4	4

Note the following:

(i) Differences can be seen between the SDS-PAGE bands of different REA groups. These differences are smaller than those seen when isolates from different farms were examined (Figure 27).

(ii) Within one REA group differences may, or may not, be detected i.e. tracks 5 and 6 are identical. Whereas tracks 1 and 2 (see arrows) are not identical. A small difference (in the centre of the gel) is also seen when tracks 3 and 4 were compared.

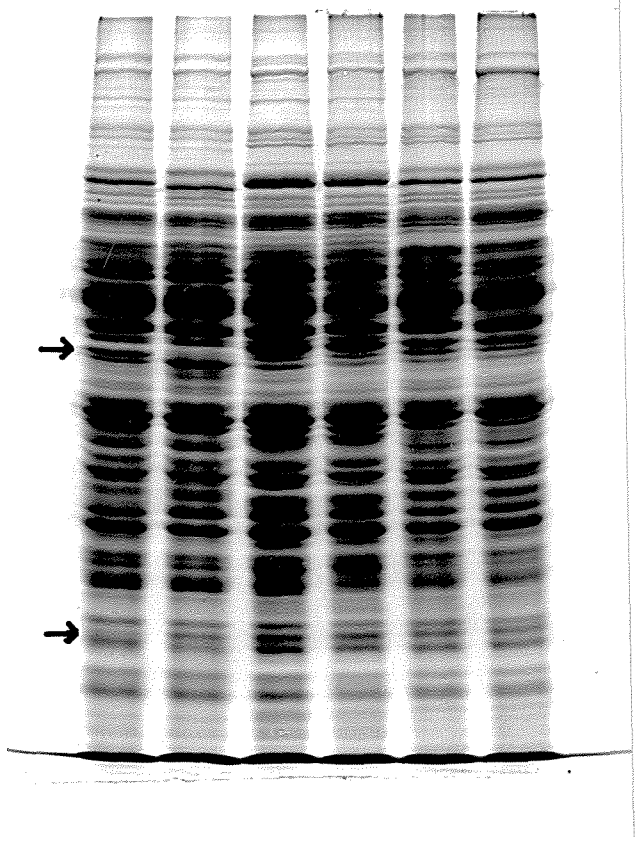


FIGURE 26

This is an extension of the results shown in Figure 25. Three pairs of isolates giving identical REA patterns within the pairs are examined by SDS-PAGE. Note that small differences were occassionally detected (e.g. Tracks 1 and 2 - see arrows) between 2 isolates even when the REA patterns appeared to be identical.

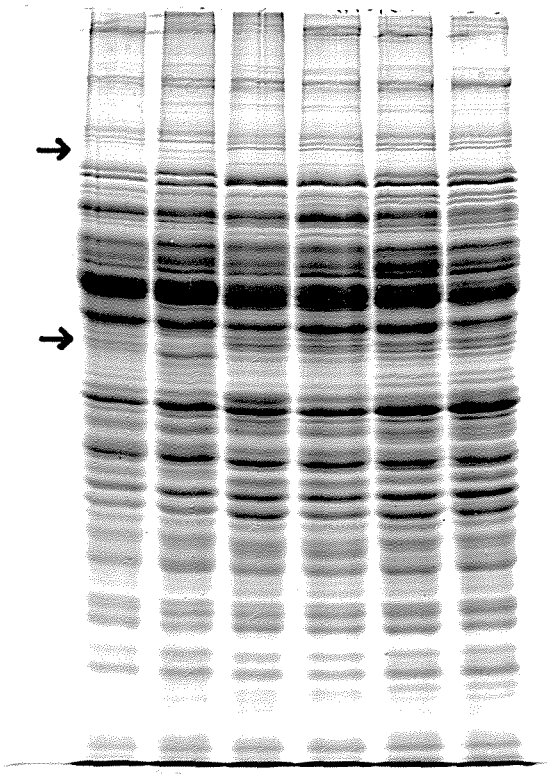
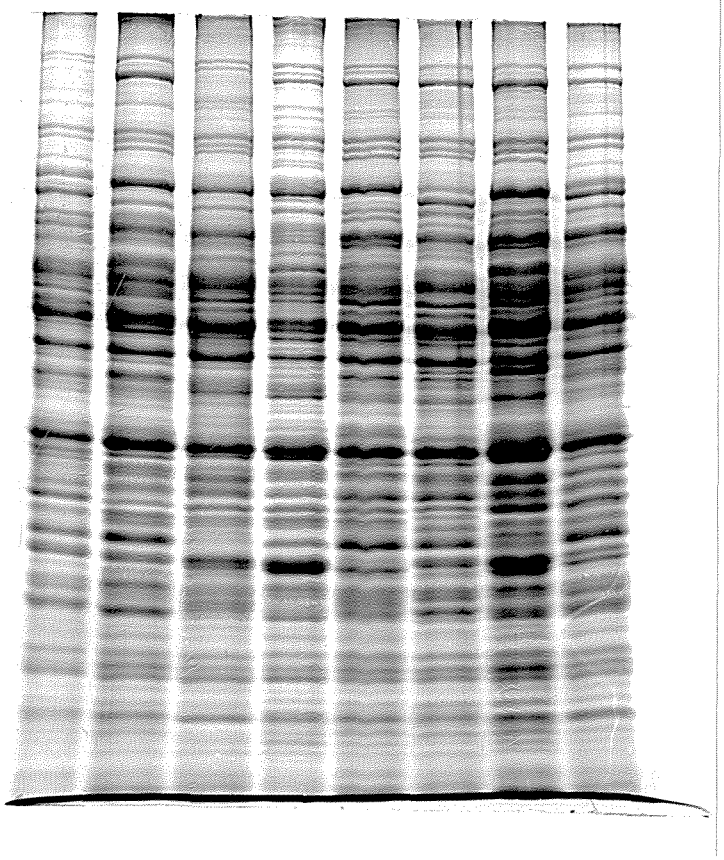


FIGURE 27

The total protein of eight isolates of *M. ovipneumoniae* all derived from sheep on different farms examined by SDS-PAGE.

Note that although many common bands are visible especially among the high molecular weight proteins, no 2 isolates give identical banding patterns i.e. each track is unique.



3.5.4 Discussion

SDS-PAGE examination of independent isolates, or of isolates from one flock, which differ by REA, show a limited number of variable bands against a background of a much larger number of constant bands. We concluded that if SDS-PAGE of proteins is to play a role in assigning *M. ovipneumoniae* isolates to groups, some modification or extension of this technique must be introduced. The following possibilities were considered.

(i) The removal from the gel of individual variable bands to be used as immunizing antigens to produce discriminating antisera.

(ii) The biochemical "dissection" of *M. ovipneumoniae*, followed by an examination of a specific "fraction" of these total proteins e.g. the membrane proteins. This might conserve most variable bands but eliminate some of the background constant bands.

The first approach might be handicapped since these antigens were boiled in SDS before electrophoresis. This may, or may not destroy their antigenicity. This led us to initially adopt the second approach i.e. we attempt to assign protein bands to particular sites in or on the mycoplasmal cell.

These themes are pursued in the next chapter.

CHAPTER 4

FRACTIONATION OF *M. ovipneumoniae*

4.1 Separation of Internal Proteins from Membrane Proteins

4.1.1 Introduction

SDS-PAGE examination of electrophoretic banding patterns of *M. ovipneumoniae* proteins in the previous chapter led us to conclude that although, theoretically, enough variation in protein bands were detected to allow isolates to be distinguished and possibly classified, in practice the comparisons were made difficult due to the presence of a large number of common bands. One approach to overcome this problem is to examine the proteins in a "fraction" of the organism. Ideally the fraction examined should exclude many of the common bands while retaining most of the variable bands.

This approach would be facilitated if bands could be assigned to different 'sites' in or on the mycoplasma i.e.:

A) Internal proteins
B) Membrane proteins (which could be subdivided into;
   (i) protein exposed, or
   (ii) not exposed to the surface).
C) Adsorbed surface proteins, derived from the medium (these might in some circumstances appear to be membrane proteins).

In this section we attempt to biochemically "dissect" *M. ovipneumoniae* and assign its proteins to one of the above categories. The first stage in this dissection was to separate the internal proteins from the membrane-associated proteins and to examine both fractions by SDS-

PAGE. This requires the availability of a method for lysing mycoplasmas which does not depend on major structural damage to the membrane such as is caused i.e. by SDS.

Many methods have been used to lyse mycoplasmas, some of these e.g. lysis by ultrasound (Kahane, 1969), osmotic shock (Rottem and Razin 1967), or freezing and thawing (Hollingdale, 1969), vary in their ability to lyse mycoplasmas of different species and also do not give consistant results even with one species - especially if the "age' of the culture is varied. Other more efficient methods include: lysis by digitonin (Rottem and Razin, 1972), lysis in carbonate-bicarbonate buffer at pH 9.2 to 10.5 (Goel, 1972) and the use of Dicyclohexylcarbodiimide (DCCD) (Shirvan *et al.*, 1981). The latter authors showed that DCCD lysis leaves membranes substantially intact. Since this is a necessary prerequisite for separating the membrane, it was selected for the present work. However, since mycoplasmas may differ in their response to DCCD we initially examine the effect of DCCD on *M. ovipneumoniae*.

Hence, this section is concerned with the following:

(A) The use of DCCD to lyse *M. ovipneumoniae*

(B) The separation of *M. ovipneumoniae* membranes from internal proteins liberated following cell lysis and

(C) Examination of membrane and soluble proteins by SDS-PAGE.

4.1.2 Materials and Methods

Preparation of *M. ovipneumoniae* Cells

300 ml of FM4 media in a 1 litre conical flask was inoculated with *M. ovipneumoniae* strain 5 and incubated at 37°C until the reaction indicated a colour change of pH 6.8.

The cells were collected by centrifugation at 14,600g for 10 minutes and washed twice in 2 x 40ml sterile 0.25M NaCl containing 50mM Tris buffer pH 7.4. The wet weight of the resultant pellet was estimated and resuspended in the above buffer (preheated to 37°C), to give a suspension of approximately 12mg/ml.

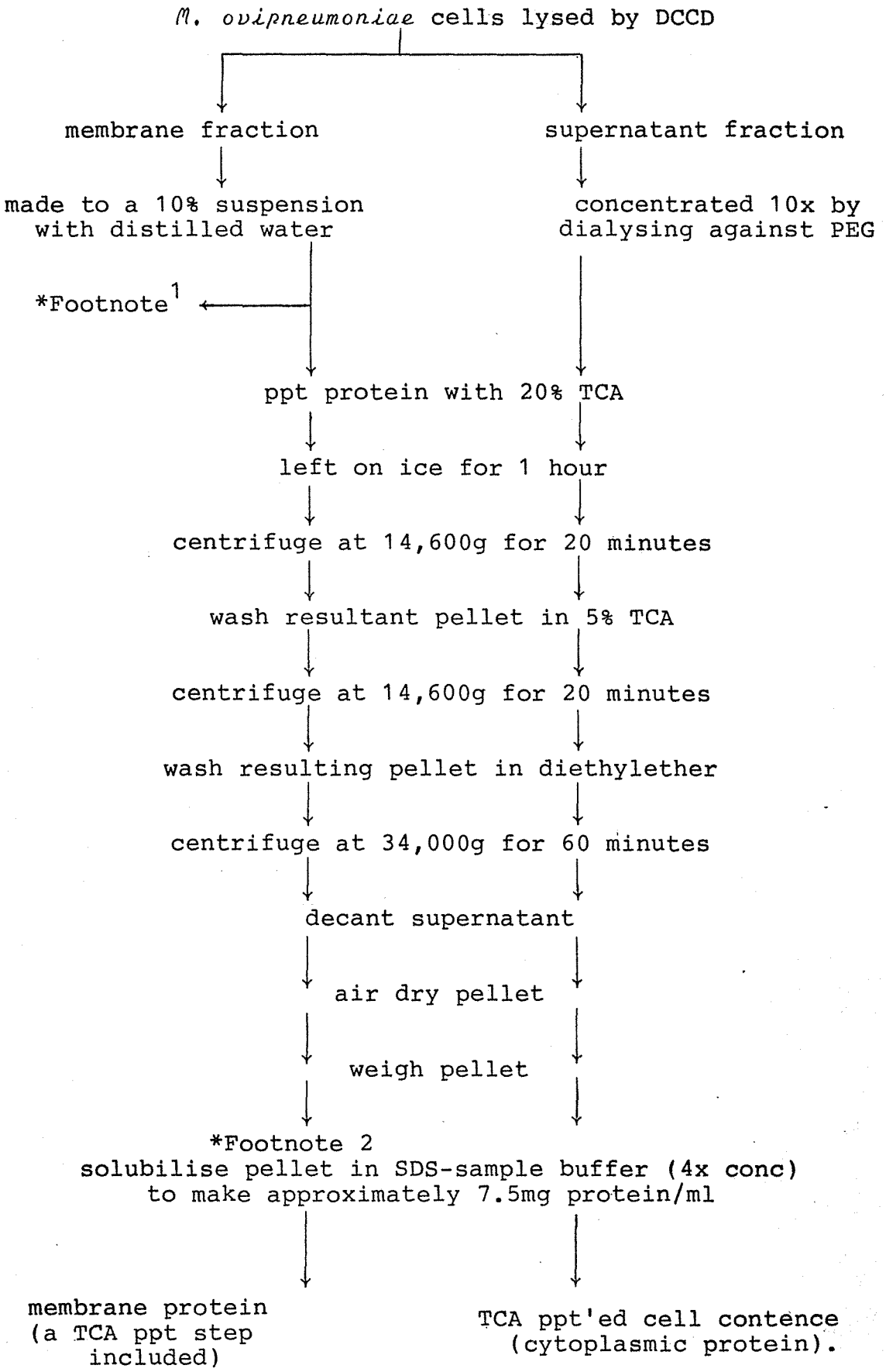
DCCD Lysis of *M. ovipneumoniae* Cells

To the above cell suspension an equal volume of 0.25M NaCl solution containing 50mM Tris buffer (pH 7.4) and 100µM DCCD was added (to give a final concentration of 6mg of cells/ml and 50µM DCCD). The suspension was shaken in a conical flask at 125rpm on a rotary shaker at 37°C and the optical density at 500nm was measured at 15 minute intervals using a Baush and Lomb spectrophotometer. An aliquot was taken at intervals and assayed for viable cells by preparing 10-fold dilutions in FM4 media. These were incubated at 37°C for 7 days and the titre was taken as the reciprocal of the lowest dilution which produced a colour change.

Preparation of Lysed *M. ovipneumoniae* Cells

Following 6 hours incubation of the cells with DCCD, the salt concentration was increased to 0.5M NaCl. *M. ovipneumoniae* membranes were collected by centrifugation at 34,000g for 30 minutes and the supernatant was decanted.

The pellet (containing the membranes and membrane associated proteins) and the supernatant fraction (containing cytoplasmic proteins) were prepared as follows:


M. ovipneumoniae cells lysed by DCCD
membrane fraction
supernatant fraction
made to a 10% suspension with distilled water
concentrated 10x by dialysing against PEG
*Footnote 1
ppt protein with 20% TCA
left on ice for 1 hour
centrifuge at 14,600g for 20 minutes
wash resultant pellet in 5% TCA
centrifuge at 14,600g for 20 minutes
wash resulting pellet in diethylether
centrifuge at 34,000g for 60 minutes
decant supernatant
air dry pellet
weigh pellet
*Footnote 2
solubilise pellet in SDS-sample buffer (4x conc) to make approximately 7.5mg protein/ml
membrane protein (a TCA ppt step included)
TCA ppt'ed cell contence (cytoplasmic protein).

The samples were prepared for SDS-PAGE examination in a 10% acrylamide gel as described in Section 3.2.

*Footnote[1]: An aliquot from the membrane was treated as described except that the TCA precipitation was omitted.

*Footnote[2]: It was not possible to directly solubilise the dried pellets in SDS-sample buffer so, the following modification was used:

the pellets were ground to a powder using a glass rod and the minimum possible volume of 0.12M (10mg/ml) sodium bicarbonate was added to the powder to allow it to be turned into a paste before the addition of SDS-sample buffer.

Preparation of Total *M. ovipneumoniae* Proteins

The preparation of total proteins from *M. ovipneumoniae* strain 5 is similar as described in Section 3.12.

4.1.3 Results

Titre of Viable Cells: this was assayed at time intervals after the addition of DCCD, the results are shown in figure 28.

Optical Density Change: a decrease in the optical density of DCCD treated cells was recorded at 15 minute intervals, the results are shown in figure 29.

SDS-PAGE Examination of Fractions: the associated proteins and internal proteins were compared with total protein (Figure 30) using a 10% acrylamide gel.

The electrophoretic banding pattern of membrane proteins before and after TCA precipitation remained substantially unaltered (Figure 30).

The main finding from this investigation is that most of the total proteins appeared in the membrane fraction. Initial experiments used 0.25M NaCl buffer to wash the membrane fraction. This may have been inadequate to break ionic bonds, so the salt concentration of the buffer was increased to 0.5M. This made no detectable difference to the number of electrophoretic bands. We conclude that a high proportion of the total proteins of *M. ovipneumoniae* are associated with the membrane.

The internal proteins as prepared, represent less than 10% of the total protein. For comparisons sake, the protein loading in the tracks was equalised. It is clear that fractionation has occured, but comparison between strains showed that no variable bands were associated with this fraction.

FIGURE 28:

Inactivation of *M. ovipneumoniae* (strain 5) by Dicyclohexylcarbodiimide. Note that no viable cells were detected ater 120 minutes.

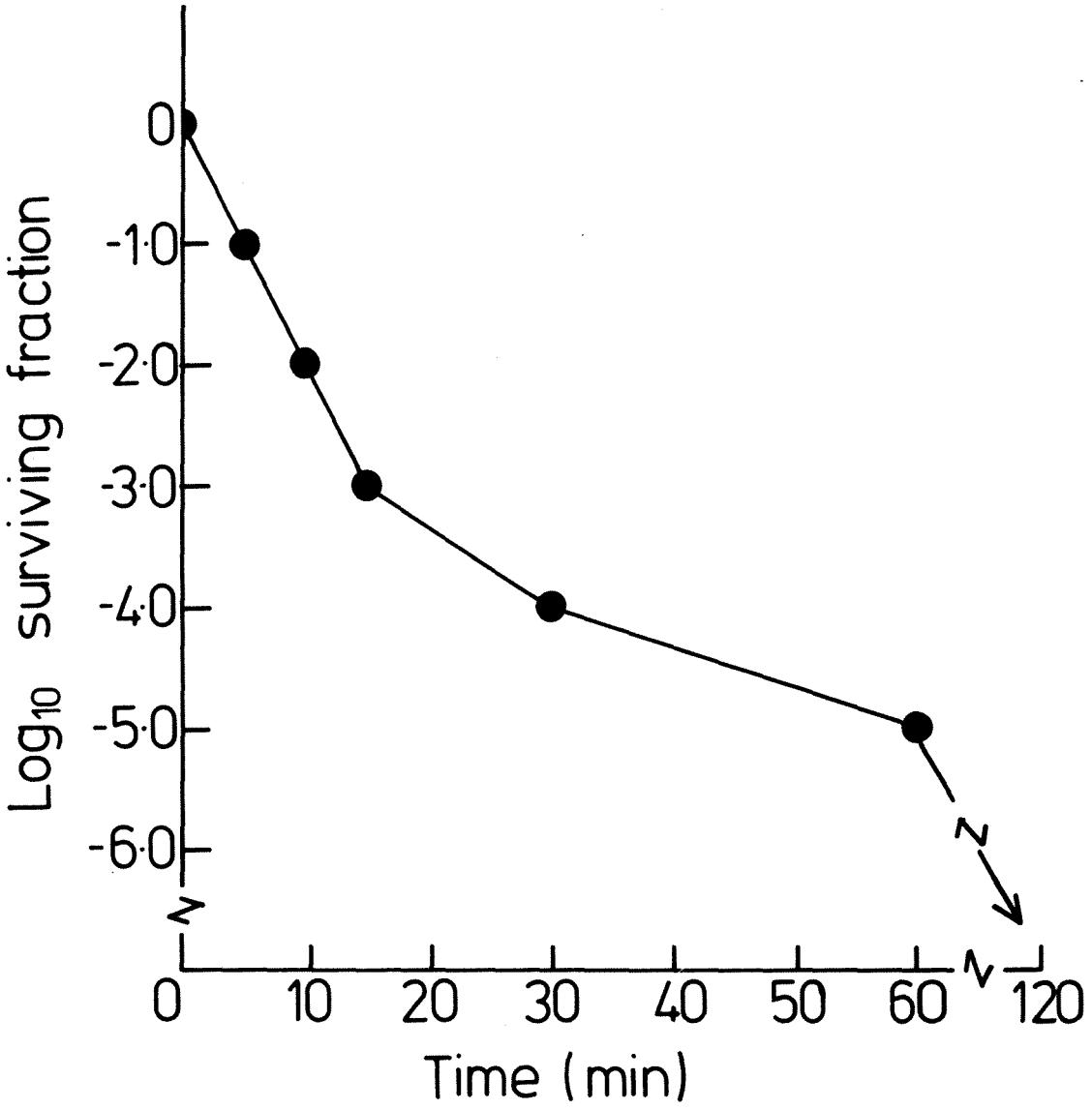

Log10 surviving fraction
0
-1·0
-2·0
-3·0
-4·0
-5·0
-6·0
0
10
20
30
40
50
60
120
Time (min)

FIGURE 29:

The decrease in the optical density of *M. ovipneumoniae* (strain 5) following the addition of Dicyclohexylcarbodiimide.

Note: A 74.6% decrease occurred after $5\frac{1}{2}$ hours. This increased only slightly (to 79.1%) after 15 hours.

No viable organisms were detected after 120 minutes (see arrow).

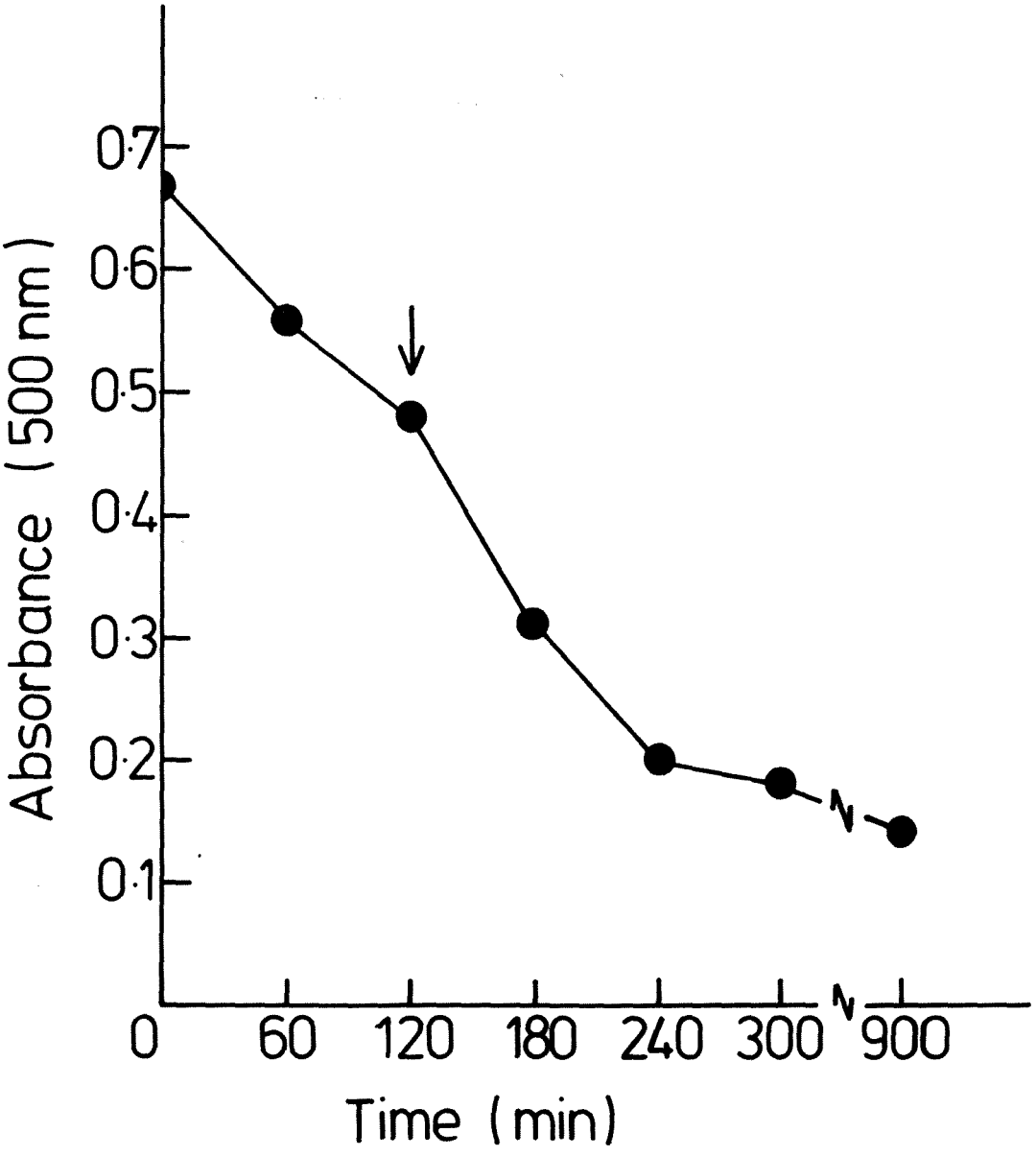
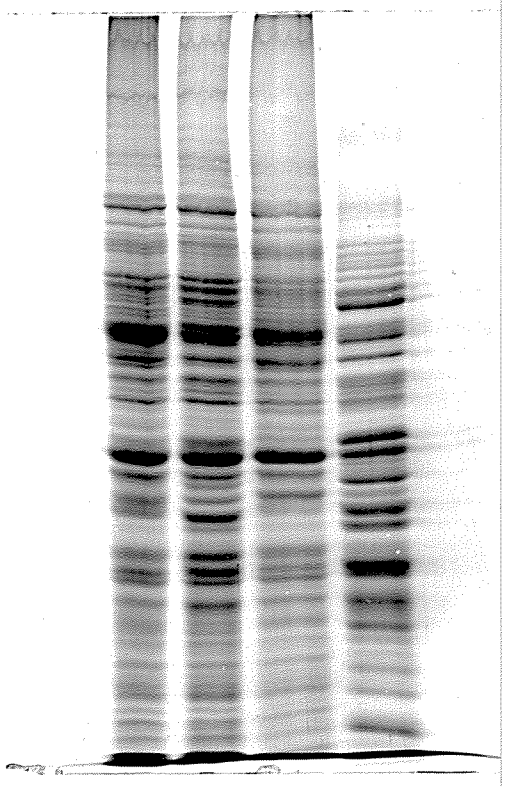

Absorbance (500 nm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
60
120
180
240
300
900
Time (min)

FIGURE 30:

A comparison of total protein, membrane-associated protein and cytoplasmic proteins of *M. ovipneumoniae* (strain 5).

Track 1; membrane fraction.
Track 2; total protein
Track 3; membrane protein precipitated with TCA.
Track 4; cytoplasmic protein precipitated with TCA

Note that the number of protein bands in the membrane fraction (Track 1) is not greatly diminished, compared to the total protein bands (Track 2). Track 4 shows many protein bands not visible on track 2. This is because the track loadings are equalised for protein. Since about 90% of the protein was in the membrane fraction, the protein in track 4 represents about 10 times the cell-equivalent loading of track 2.



4.1.4 Discussion

DCCD lyses mycoplasmas because it inhibits ATPase activity and hence interferes with the regulation of the cells volume (Shirvan *et al*., 1981). The same authors have shown with *M. gallisepticum* that a substantial drop in the optical density occurs following the addition of DCCD. They examined thin sections of DCCD lysed mycoplasma cells and concluded that the mycoplasma membrane was still structurally intact, although the cell content had been lost.

DCCD-induced lysis of *M. ovipneumoniae* cells by the same conditions produced a 75% drop in the optical density over a $5\frac{1}{2}$ hour period (Figure 29). An examination of the viability of *M. ovipneumoniae* cells (Figure 28) indicated that approximately 10^{10} cells were rendered non-viable after 120 minutes following the addition of DCCD. Clearly, DCCD lyses *M. ovipneumoniae* in the conditions used by Shirvan *et al*., (1981) for *M. gallisepticum*.

SDS-PAGE examination of the resulting fractions following DCCD - induced lysis i.e. membrane associated protein and cytoplasmic protein (Figure 30), indicate that most of the proteins are associated with the membrane even though these were washed with a high salt buffer. We conclude that for the purposes of classification and identification of *M. ovipneumoniae* isolates, the examination of membranes fractions has little or no advantage compared to using total protein. On the other hand the bands associated with the internal proteins did not include the variable bands. Hence, in the next section we attempt to further separate membrane proteins.

4.2 The Use of Fluorescein Isothiocyanate to Preferentially Label Surface Proteins of *M. ovipneumoniae*

4.2.1 Introduction

In the previous section (4.1) we found that most protein bands, including the variable bands, were associated with the membrane.

In this section, to further subdivide the membrane fraction, fluorescein isothiocyanate (FITC) is used to preferentially label surface proteins. Since some penetration of the cell membrane by FITC may occur with time, the intact mycoplasmas were exposed to FITC for a short period only.

4.2.2 Materials and Methods

Propagation of *M. ovipneumoniae*

1,200ml of FM4 media inoculated with *M. ovipneumoniae* (strain 5) was incubated at 37°C on a rotary shaker at 125rpm until the culture reached pH 6.8. The culture was centrifuged at 14,600g for 10 minutes and the resulting deposit was washed twice in 0.15M PBS and resuspended in Tris buffer (pH 8.2) to give an approximate 10% suspension. Four equal aliquots were taken and designated as A, B, C and D.

Preparation of the Fluorescent Label

FITC (isomer 1) was dissolved in Tris buffer (pH 8.2) to a concnetration of 5.0mg/ml.

Labelling Total Protein with FITC

Two of the 4 suspensions were placed in an ice bath and sonically disrupted at 20kc/s using 8 x 15 second bursts with a MSE 100 watt ultrasonic disintegrator. The lysed cell suspensions were placed in a waterbath at 37°C until

they reached this temperature. 0.2ml of FITC was then added to both. The FITC label was mixed thoroughly and placed in a 37°C waterbath.

Aliquot (A) was allowed to react with the FITC for 3 minutes and aliquot (B) for 8 hours. In both aliquots the reactions were stopped by the addition of a 1/3 volume of 20% TCA (giving a final concentration of 5% TCA). The protein/TCA mixture was placed in an ice bath for 1 hour to allow complete precipitation of the proteins.

Labelling Surface Proteins with FITC

The remaining two suspensions of intact mycoplasma cells were preheated to 37°C in a waterbath. 0.2ml of FITC at 37°C was added to 2.0ml of both suspensions (giving a final concentration of FITC of 0.5mg/ml). The reaction was terminated after 3 minutes in aliquot (C) by the addition of 1/3 volume of 20% TCA and the protein/TCA mixture was placed in an ice bath for 1 hour to allow complete precipitation of the proteins.

The the case of the last aliquot (D), the cells were washed free of FITC as follows before the addition of TCA:
the cell suspension/label mixture was diluted with 10x its volume of prewarmed Tris buffer (pH 8.2), centrifuged at 14,600g for 10 minutes and the resulting deposit was extensively washed in the same buffer until no FITC was visible when the supernatant was exposed to UV light. The pellet was resuspended in Tris buffer pH 8.2 to make a 10% suspension, precipitated with 20% TCA and left on ice for 1 hour.

Aliquot designation	Cell Treatment	Exposure to label	Termination of label	Intended labelling
A	Lysed cells	3 min	TCA	Total protein
B	Lysed cells	8 hours	TCA	Total protein
C	Intact Cells	3 min	TCA	Surface protein
D	Intact Cells	3 min	Dilution/ washing (TCA added later)	Surface protein

TABLE IX: SUMMARY OF THE LABELLING PROCEDURES

Extraction of Proteins

All 4 aliquots (A, B, C and D) were centrifuged at 14,600g for 20 minutes, washed with 5% TCA and then with diethyl-ether to remove residual TCA. The resulting pellets were dried, weighed and dissolved in 6% SDS-sample buffer to make a final protein concentration of approximately 7.5mg/ml.

Preparation of Samples

The 4 samples were equalised for emitted fluorescence (not for protein content) and applied to 10% and 15% SDS-PAGE gels using the standard technique (see section 3.2).

Detection of Fluorescent Protein Bands

Following electrophoresis the gels were placed over a UV light source (240nm) and the emitted light was recorded on Tri-X film (Kodak ^{TM}). A yellow filter was used to absorb the exciting UV light.

4.2.3 Results

Labelling of Total Proteins

Total protein i.e. cell lysates, labelled with FITC for 3 minutes or for 8 hours gave identical results (Figure 31; Tracks 1 and 2, Figure 32; Tracks 1 and 2), but with one exception i.e. the aliquot labelled for 3 minutes showed a low molecular weight protein (Figure 32; band-d) in the 15% gel.

Labelling of Surface Proteins

The two labelling procedures (i.e. labelled cells precipitated with TCA before or after washing) gave near identical

results when compared in 10% gels (Figure 31) or 15% gels (Figure 32). However, one difference is seen i.e. a band (Figure 32-d) a low molecular weight protein, is seen just above the dye front on the 15% gel.

Comparison of Surface Labelling with Total Protein

When intact and lysed mycoplasmas were labelled with FITC, many bands were common to both preparations, but some bands (Figure 31; band-c) clearly visible in the total protein are only weakly stained in the "surface protein" preparation. The reverse is true of other bands i.e. some bands e.g. Figure 31-a and b are clearly brighter in "surface protein" preparation than in the total protein.

FIGURE 31:

The detection of surface proteins of *M. ovipneumoniae* (strain 5) by FITC labelling.

Track 1; total protein labelled for 3 minutes. The reaction was terminated by the addition of TCA.
Track 2; total protein labelled for 8 hours. The reaction was terminated by the addition of TCA.
Track 3; intact mycoplasma cells labelled for 3 minutes. The reaction was terminated by the addition of TCA.
Track 4; intact mycoplasma cells labelled for 3 minutes. The reaction was terminated by diluting the label/protein mixture in buffer.

The tracks were equalised for fluorescence (not for protein). Surface proteins are detected by an increase in their brightness in tracks 3 and 4 relative to tracks 1 and 2. The converse is true for internal proteins.

While equivocal results were obtained for many protein bands we conclude:

(i) Bands a and b represent surface proteins.

(ii) Band c represents an internal protein.

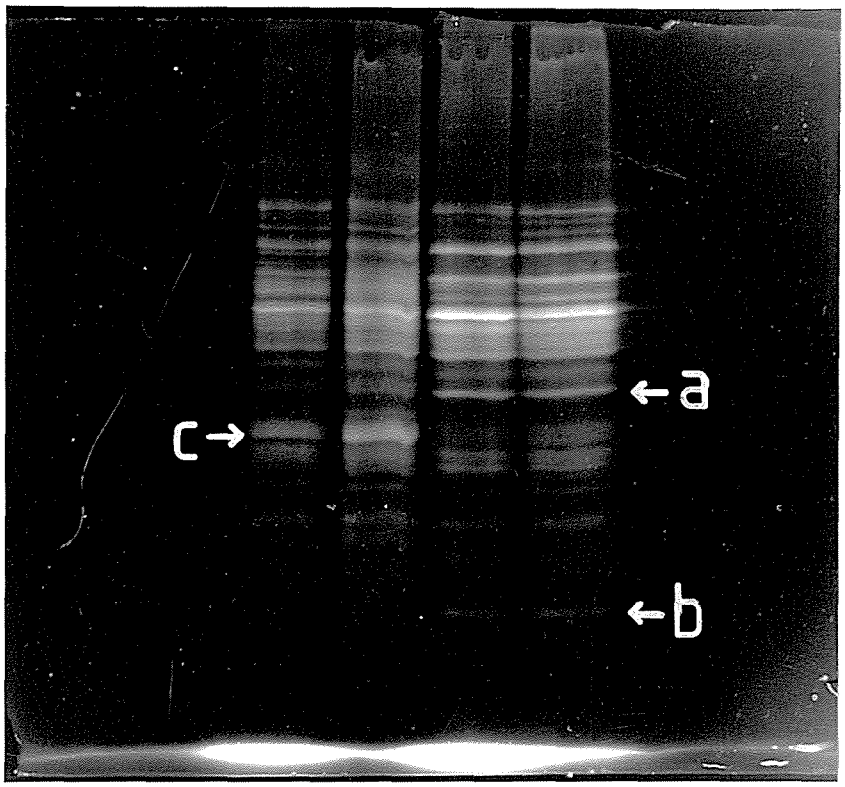

a
c
b

FIGURE 32:

This is a repeat of the experiment shown in Figure 31, except that a 15% acrylamide gel (instead of the usual 10% acrylamide gel) was used.

Track 1; total protein labelled for 3 minutes. The reaction was terminated by the addition of TCA.

Track 2; total protein labelled for 8 hours. The reaction was terminated by the addition of TCA.

Track 3; intack mycoplasma cells labelled for 3 minutes. The reaction was terminated by the addition of TCA.

Track 4; intact mycoplasma cells labelled for 3 minutes. The reaction was terminated by diluting the label/protein mixture in buffer.

Bands a, b (surface proteins) and c (internal protein) are labelled as in Figure 31.

Attention is drawn to band-d.

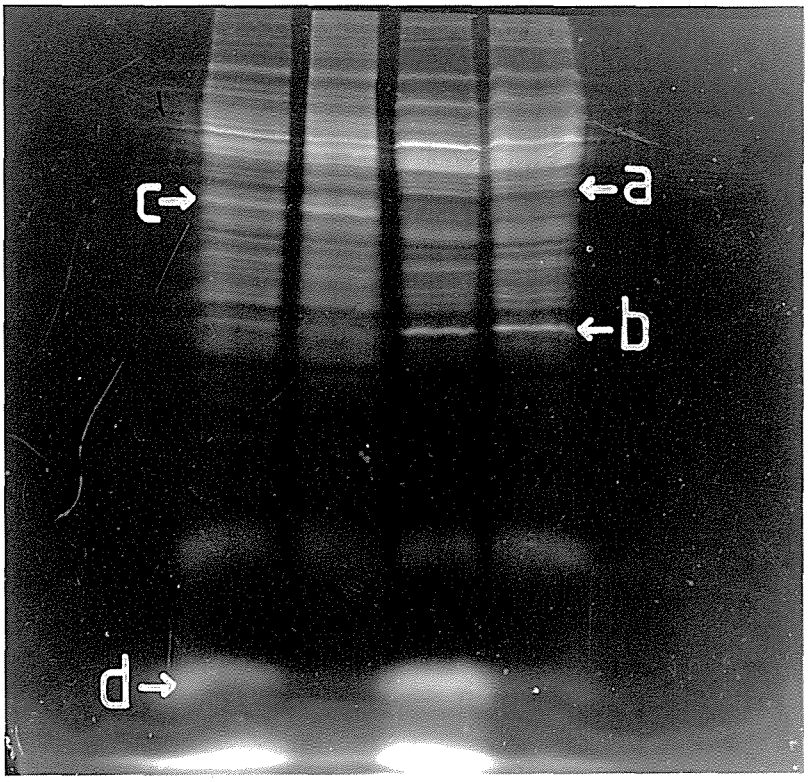
Note:

(i) The protein is strongly labelled in intact cells in 3 minutes (Track 3).

(ii) When labelled as in (i) and followed by washing the protein band is almost totally lost.

We conclude that it represents a surface protein which is readily removed by washing.

The leading band represents free FITC.


c
a
b
d

4.5.4 Discussion

If intact cells are exposed to FITC and this does not penetrate the membrane, only surface proteins will be labelled and only some of the total protein bands will appear in the fluorogram. Maddy, (1964) suggests that isothiocyanates do not penetrate the membrane. If however, FITC did freely penetrate the cell then all the bands visible in the total protein preparation will appear in the "surface labelled" preparation and they should have the same relative density.

Comparisons of Figures 31 and 32 show clearly that not all the total protein bands are seen on the surface labelled preparation. This shows that differential staining by FITC has occurred. However, the relatively large number of bands seen on the fluorograms implies either that the surface of *M. ovipneumoniae* has many proteins or that some penetration of FITC has occured. Further work e.g. with 3-azido-(2,7)-naphthalene disulfonate (ANDS) which is reputed not to penetrate membranes (Dockter, 1979) would be useful.

Despite uncertainty in the case of some protein bands, others can be clearly identified as surface proteins e.g. figure 31a and b, because they are visible in labelled intact cell preparations, but are barely visible in the total labelled preparations. The converse is true of figure 31 (band-c).

An unexpected result was obtained with band-d (Figure 32). This band is obviously brighter in the total protein labelled for 3 minutes (Track 1) than in the total protein labelled for 8 hours (Track 2). Since both preparations are lysed and the total fluorescence was equalised, it seems reasonable to suppose that this band represents a low molecular weight protein which has a high affinity

for FITC e.g. a protein rich in exposed ε-NH_2-Lysine groups. This may allow the reaction to be completed in a short period of time (i.e. 3 minutes), whereas other proteins continue to take up the label over a 8 hour period, thus reducing the relative density of band-d in track 2.

Band-d also appears as the leading band (apart from free FITC) in the "surface labelled" preparation immediately precipitated by TCA (Track 3), but is barely visible in the surface labelled preparation which was washed before being TCA precipitated (Track 4).

In summary, this protein moves with the dye front (i.e. is low molecular weight), is strongly labelled when intact cells are exposed to FITC for 3 minutes (Figure 32; track 3), but is lost from such cells if they are washed before precipitating the protein with TCA (Figure 32; track 4). We conclude that it is a low molecular weight surface protein which is only weakly bound to the membrane. We originally thought it likely that this protein (or polypeptide) represented adsorbed medium constituents e.g. from the peptone. This point is returned to in section 4.4.

4.3 A Comparison of 3 Strains of *M. ovipneumoniae* by Labelling Intact Cells with FITC

4.3.1 Introduction

In an attempt to identify surface components which are either common to all, or many strains, or are strain specific, we compare three strains of *M. ovipneumoniae* by surface labelling with FITC.

Note: The possibility of some surface proteins being adsorbed medium constituents is considered in this section.

4.3.2 Materials and Methods

The labelling of intact *M. ovipneumoniae* strains is described in Section 4.2.2 (aliquot D).

M. ovipneumoniae Strains Used

M. ovipneumoniae strains 1, 5 and 10 were compared. These strains were selected because they differed quantitatively in growth inhibition tests using rabbit antisera (Clarke-unpublished).

4.3.3 Results

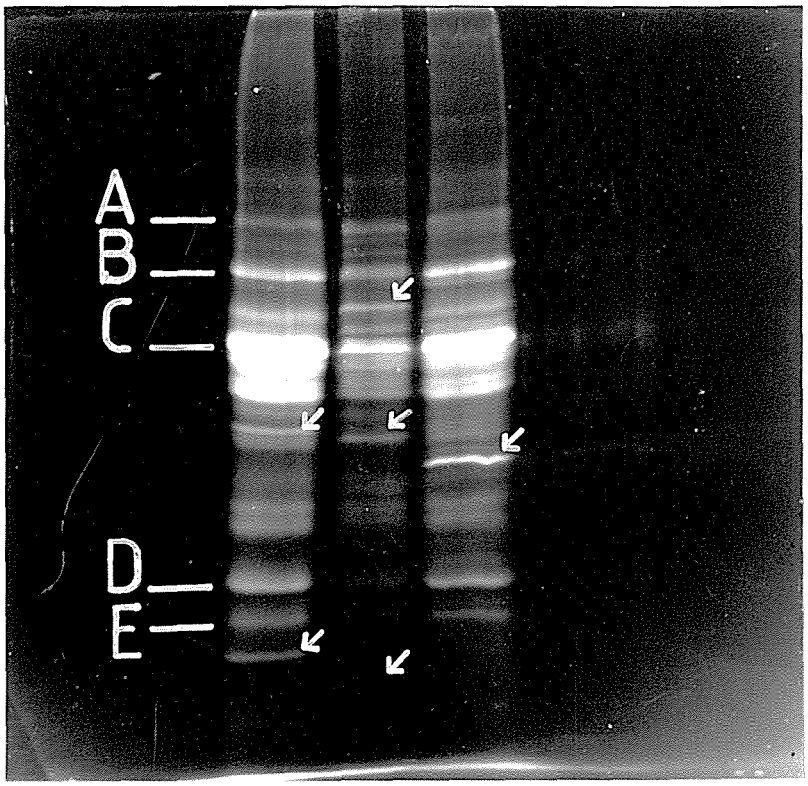
All 3 *M. ovipneumoniae* strains were exposed as intact cells to FITC, lysed and electrophoresed through a 10% SDS-PAGE gel. The gel was exposed to UV light and the fluorescing bands were photographed (Figure 33).

FIGURE 33:

A comparison of the surface proteins of 3 strains of *M. ovipneumoniae*. Intact cells were labelled with FITC.

Track 1; *M. ovipneumoniae* (strain 1)
Track 2; *M. ovipneumoniae* (strain 5)
Track 3; *M. ovipneumoniae* (strain 10)

Note that while a large number of common bands are present i.e. bands A, B, C, D and E. Bands which are unique to 1 of the 3 strains can also be identified (see arrows).


A
B
C
D
E

4.3.4 Discussion

Three strains of *M. ovipneumoniae* surface-labelled by FITC are compared in Figure 33. Note that:

(i) Common protein bands are seen in all 3 strains (i.e. bands A, B, C, D and E). The common bands could either be species specific proteins or adsorbed medium constituents.

(ii) Variable protein bands (see arrows Figure 33) unique to one of the three strains.

These observations could be useful in strain identification using either of 2 approaches:

(i) Compare surface labelled strains directly with SDS-PAGE. This has an advantage over an examination of total protein, in that the total number of bands are reduced, but unique protein bands are conserved.

(ii) Individual (unique) protein bands could be excised and used to prepare antisera which would probably be strain specific. Alternatively if a common protein band was used as an antigen, it would be expected that a species-specific antisera would be produced.

A possible complication i.e. the adsorbtion of medium constituents to the cell surface is considered in the next section.

4.4 Detection of Medium Constituents Adsorbed to the Surface of *M. ovipneumoniae*

4.4.1 Introduction

Mycoplasmas are known to adsorb medium constituents onto their surface (Jordan and Kulasegaram, 1968; Yaguzhinskaya 1975; Nicolet *et al*., 1980). This is a potential hazard in studies designed to identify surface antigens of mycoplasmas.

We originally became concerned with the problem of adsorbed medium constituents following the use of fluorescein isothiocyanate to label membrane surface proteins (see Section 4.2). In particular the leading (low molecular weight) protein band was strongly labelled when intact cells were exposed to FITC (Figure 32), but following washing of the cells, the labelled band was greatly diminished. It seemed reasonable to support that this band represented low molecular weight constituents of the medium i.e. peptides (from the peptone).

In this section to investigate the adsorption of media constituents, *M. ovipneumoniae* (strain 5) was grown in the presence of ^{14}C-protein hydrolyate. The labelled cellular proteins were subjected to SDS-PAGE, stained with Coomassie-blue to record total protein content, which will include adsorbed media constituents, and then a fluorogram was prepared from the same gel which (since media protein are not labelled) will detect only mycoplasma proteins. Comparisons within the same gel examined for radioactive protein bands and total protein bands are made to find if any of the total protein bands are non-radioactive and thus represent media constituents.

It is also possible, by slicing the gel, to assay the proportion of the total protein present in any band.

4.4.2 Materials and Methods

Propagation of *M. ovipneumoniae*

A 1ml aliquot of *M. ovipneumoniae* (strain 5) stored at -70°C was inoculated into 10ml of FM4 media and incubated at 37°C until a colour change was observed (pH 6.8) indicating that the culutre was in late log phase of growth. The culture was centrifuged at 14,600g for 10 minutes, the pellet washed in 20mls FM4, from which the amino acids and serum were omitted, and resuspended in 0.5ml of the above media.

^{14}C-Labelling of *M. ovipneumoniae*

0.1ml of ^{14}C-protein hydrolysate (Amersham) was added to the 0.5ml mycoplasma suspension and incubated at 37°C for 6 hours i.e. approximately one generation of growth. The pH of the mycoplasma suspension was adjusted at hourly intervals to pH 7.8 by the addition of 0.2M NaOH. After 6 hours the cell suspension was centrifuged at 14,600g for 10 minutes, washed and resuspended in 2ml FM4 media with the amino acids and serum omitted. The labelled mycoplasma proteins were precipitated by the adding 2.0ml of 20% TCA and left on ice for 1 hour. The precipitate was collected by centrifugation at 14,600g for 20 minutes, washed with 5% TCA, then with diethylether to remove residual TCA present in the pellet. The pellet was air dried and dissolved in 100µl SDS-sample buffer (4x concentrated).

Detection of Radioactivity in the Sample

A 5µl aliquot of labelled mycoplasma protein solubilised in SDS-sample buffer was added to a vial containing 10ml of liquid scintillation fluid and the incorporated label was measured with a LS 7000 scintillation counter.

Preparation of Scintillation Fluid

2,5 diphenyloxazole (PPO)	4g
2,2' paraphenylene bis 5 phenyl-oxazole (POPOP)	100mg
Toluene	667ml
Triton X-100	333ml

Preparation and Application of Sample to a 10% Acrylamide Gel

This procedure is described in Section (3.2.2).

Detection of Labelled Cellular Proteins - Fluorography

200,000 cpm/track of ^{14}C labelled cellular proteins were electrophoresed in a 10% acrylamide gel and the gel was stained with Coomassie-blue (see Section 3.2.2).

The gel was dehydrated by soaking it in 20x its own volume in dimethyl sulphoxide (DMSO) for 30 minutes and reimmersed in fresh DMSO for a further 30 minutes. The gel was then immersed in 4x its own volume of 20% (w/w) 2,5 diphenyloxazole (PPO) in DMSO 22.2% (w/v) for 3 hours, then immersed in distilled water for 1 hour to remove the DMSO and precipitate the PPO in the gel.

The gel was dried under vacuum (see Section 3.2.2) and placed over a x-ray film (RP royal "X-Omat" S Kodak ^{TM}) in total darkness. The gel and film were sandwiched together in a x-ray film holder, placed in a light proof box which was then placed in a -70°C freezer for 9 hours.

The film was developed in total darkness using D19 (Kodak^{TM}) developer for 5 minutes and then placed in fixer for a further 5 minutes.

Estimation of the Leading Protein Band as a Proportion of Total *M. ovipneumoniae* Protein

Following the preparation of the fluorogram, the ^{14}C-labelled protein track was excised from the remaining gel and sequentially segmented into 25 x 5mm segments. The first segment contained only the leading band. Each segment was placed in a scintillation counter vial containing 10ml of NCS^{TM}-solubiliser and incubated at 37°C for 24 hours on a rotary shaker at 125rpm. Each vial was assayed for radioactivity using a LS 7000 scintillation counter.

Preparation of NCS-Solubiliser

2,5 diphenyloxazole (PPO)	1.40g
2,2' paraphenylene bis 5 phenyl-oxazole (POPOP)	0.021g
NCS^{TM}-Solubiliser	14.3ml
Toluene	375.1ml

4.4.3 Results

A track stained by Coomassie-blue is shown in Figure 34a. Using the same track, a fluorogram of the radioactive proteins was prepared and is shown in Figure 34b.

The leading band represented 17.5% of the total radioactivity on the track i.e. it represents 17.5% of the total protein of *M. ovipneumoniae*.

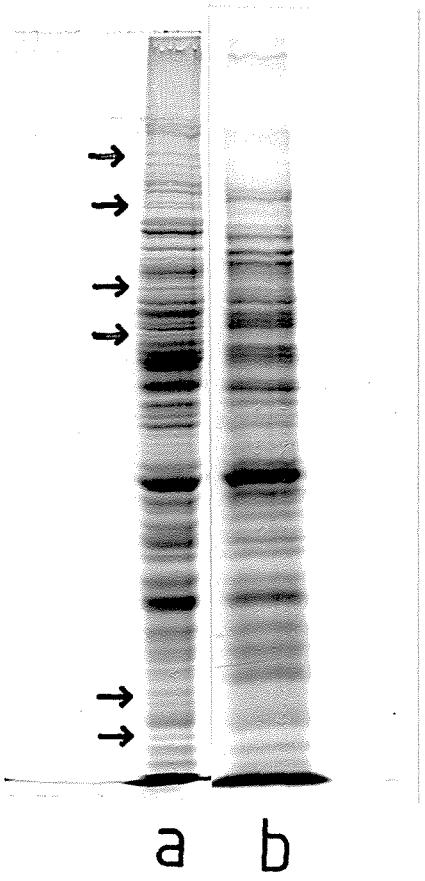
FIGURE 34

Detection of medium constituents adsorbed to the surface of *M. ovipneumoniae*.

Track A; total protein (including adsorbed medium constituents) of *M. ovipneumoniae* (strain 5), electrophoresed through a 10% acrylamide gel and stained with Coomassie-blue.

Track B; *M. ovipneumoniae* (strain 5) grown in the presence of ^{14}C-amino acids. The fluorogram detects mycoplasma specified proteins, but not medium constituents.

A comparison of tracks indicates that a number of protein bands in track A represent adsorbed medium constituents, (see arrows).


a
b

4.4.4 Discussion

When propogated in the presence of radioactive amino acids all mycoplasma proteins will be labelled. Proteins adsorbed from the medium however, will not be labelled, so these may be identified as bands present on the Coomassie-blue stained preparation but absent from the fluorgram.

Several such bands were identified (see arrows) however, all of them were minor bands and (so far as comparison between different gels allow) we believe do not correspond to those bands identified in an earlier section (see Section 4.3) as major surface components.

An unexpected but unequivocal finding is that the leading band of low molecular weight protein, which is present on the surface of the mycoplasma (see Section 4.3) and was originally assumed to be peptide adsorbed from the peptone of the medium, is in fact highly radioactive and is thus a mycoplasma specified protein. This low molecular weight protein represented a major band in the fluorogram (Figure 34b) and accounts for 17.5% fo the total mycoplasmal specified protein. The significants of this protein is considered in the next section.

4.5 Determination of the Molecular Weight and Antigenicity of a Low Molecular Weight Protein

4.5.1 Introduction

An ultimate objective of this research programme of which this thesis represents a part, is to produce antisera to strain-specific and species-specific antigens on the mycoplasma surface. Such antisera would be useful in strain or species identification. The simplest approach to this is to exise a protein band from the gel and use it to immunise animals. As a first step to this end, in this section we exise the leading band which is a major common band (presumably species-specific) readily separable from other bands. However, this band is unique in the sense that it is the lowest molecular weight protein of *M. ovipneumoniae* and since the threshold molecular weight for antigenicity is in the region of 1,500 to 4,000 (De Weck, 1974), it may not be antigenic. The possibility a non-antigenic surface protein (or polypeptide) seemed to have exciting implications, so this section asks three questions.

(i) What is the molecular weight of the leading protein band?
(ii) Is it antigenic? and
(iii) If antigenic, is it species-specific?

4.5.2 Materials and Methods

(A) Molecular Weight Determination of a Major Surface Protein

A 10% acrylamide gel was prepared as described in Section 3.2. However, to improve the resolution of the low molecular weight bands the Tris-glycine

reservoir buffer (normally pH 8.3) was adjusted to pH 9.5.

(i) Preparation of *M. ovipneumoniae* Sample

M. ovipneumoniae (strain 5) was propagated on FM4 media as described in section 3.2.

(ii) Preparation of the Molecular Weight Markers

5mg of Bacitracin (1411 Mr) and a polypeptide of known sequence with a molecular weight of 2872 Mr (a fragment from Bovine Adrenal Preproenkephalin, residues 119 to 141) were solubilised in 1ml of 6% SDS-sample buffer.

A 100µl aliquot from each of the two molecular weight markers was seperately added to 25 µl SDS-sample buffer and 12.5 µl Bromophenol-blue tracker dye, giving a concentration of 3.6mg/ml. Both mixtures were heated to 100°C for 3 minutes and cooled. 3.6 µl was then added to each well (final loading of 1.0µg/track).

(B) Is the Low Molecular Weight Protein of *M. ovipneumoniae* antigenic?

A 10% acrylamide gel was prepared using the same modification as above. The gel was also increased in thickness from 1.5 to 4.5 mm and the comb (for casting wells) was omitted.

(i) Antisera Production

M. ovipneumoniae (strain 5) was propogated in FM4 media and electrophoresed through a 10% acrylamide gel as described in Section 3.2. Following electrophoresis the low molecular weight protein was excised from the gel, chopped into fragments and lyophilised. The

dried protein-acrylamide mixture was ground to a fine powder and suspended in 5mM $NaHCO_3$ plus 0.1% SDS to make a 10% suspension. This was used as the immunising antgen.

(ii) Immunisation Procedure

1.0ml aliquots of the low molecular weight protein-acrylamide mixture was injected intra-peritionally into a Guinea-pig at weekly intervals for 6 weeks. The Guinea-pig was bleed and the serum collected by centrifugation.

(iii) Gel Precipitation Tests

This procedure is described in Section 2.2.

4.5.3 Results

The two molecular weight markers Bacitracin (1411 Mr) and the polypeptide (2872 Mr) are shown in Figure 35, tracks 1 and 2. *M. ovipneumoniae* of 2 loadings are shown in tracks 3 and 4. The leading protein of *M. ovipneumoniae* falls between these two molecular weight markers and hence has a molecular weight of approximately 2500 Mr.

In a gel precipitation test (Figure 36), antisera prepared against the low molecular weight protein (centre well) gave a single gel precipitation line when tested against an antigen containing lysed *M. ovipneumoniae* cells (in 2 peripheral wells).

In further tests the antibody cross-reacted with 2 other strains of *M. ovipneumoniae* (Figure 37). A single line of identity is seen.

M. ovipneumoniae antigen (Figure 38, centre well) when tested against serum from rabbits immunised with whole organisms (well 2) showed many gel precipitation lines. Antiserum (well 1) from the low molecular weight protein however, gave only one single line. This line did not appear to be continuous with any of the "total protein" lines i.e. the low molecular weight protein is not a major antigen.

FIGURE 35:

Determination of the molecular weight of the leading protein band.

Track 1; Bacitracin (1411 Mr).

Track 2; a polypeptide of known sequence (2872 Mr) - see text.

Track 3; total protein of *M. ovipneumoniae* (strain 5) at the usual loading of 80µg/track.

Track 4; total protein of *M. ovipneumoniae* (strain 5) at a loading of 20µg/track.

Note that band-d (Track 4) is approximately midway between the two molecular weight markers. The molecular weight of band-d is estimated to be approximately 2500 Mr.

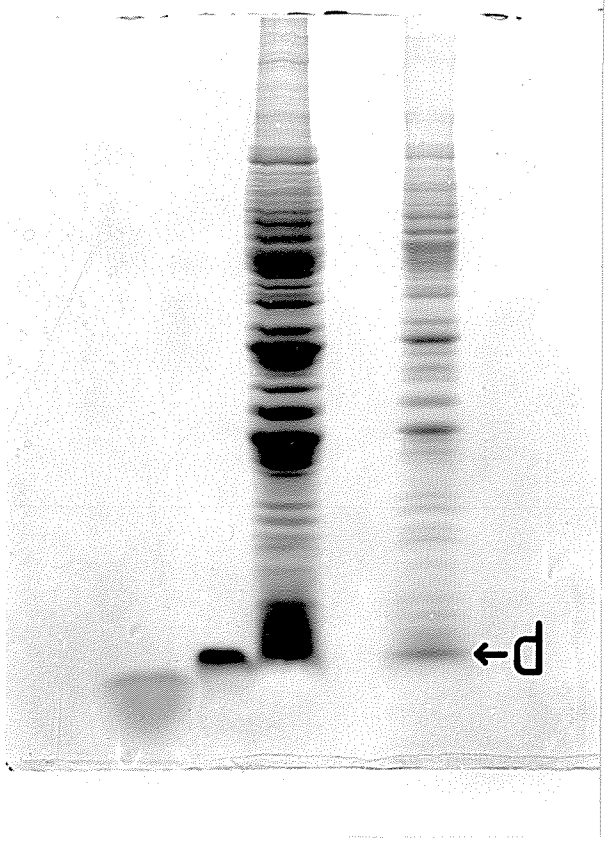

←d

FIGURE 36:

Gel precipitation test for antibody to low molecular weight protein.

Wells 1 and 2; lysed cell suspension of *M. ovipneumoniae* (strain 5).

Wells, 3, 4, 5 and 6; medium controls.

Centre well; serum from a Guinea-pig injected with the low molecular weight protein band excised from a 10% acrylamide gel.

Note a single line of precipitation between the antigen (wells 1 and 2) and the serum tested (centre well). This implies that the low molecular weight protein is antigenic. The single precipitation line suggests that it is a single antigen rather than a heterogeneous mixture of partly degraded *M. ovipneumoniae* proteins.

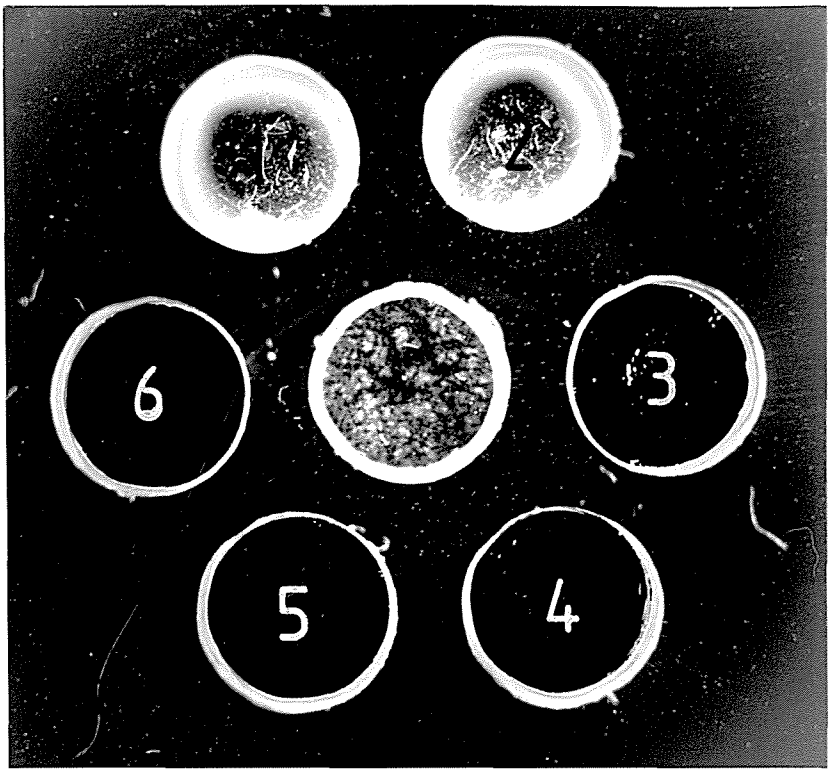

6
3
5
4

FIGURE 37:

Gel precipitation test of 3 *M. ovipneumoniae* strains against antibody to the low molecular weight protein.

Well 1; lysed cell suspension of *M. ovipneumoniae* (strain 1).

Well 2; lysed cell suspension of *M. ovipneumoniae* (strain 5).

Well 3; lysed cell suspension of *M. ovipneumoniae* (strain 10).

Wells 4, 5 and 6; medium controls.

Centre well; antiserum to low molecular weight protein.

A single line of identity is seen with all 3 *M. ovipneumoniae* strains. This implies that a species-specific antiserum has been produced i.e. the low molecular weight protein is a common antigen of (probably) all strains of *M. ovipneumoniae*.

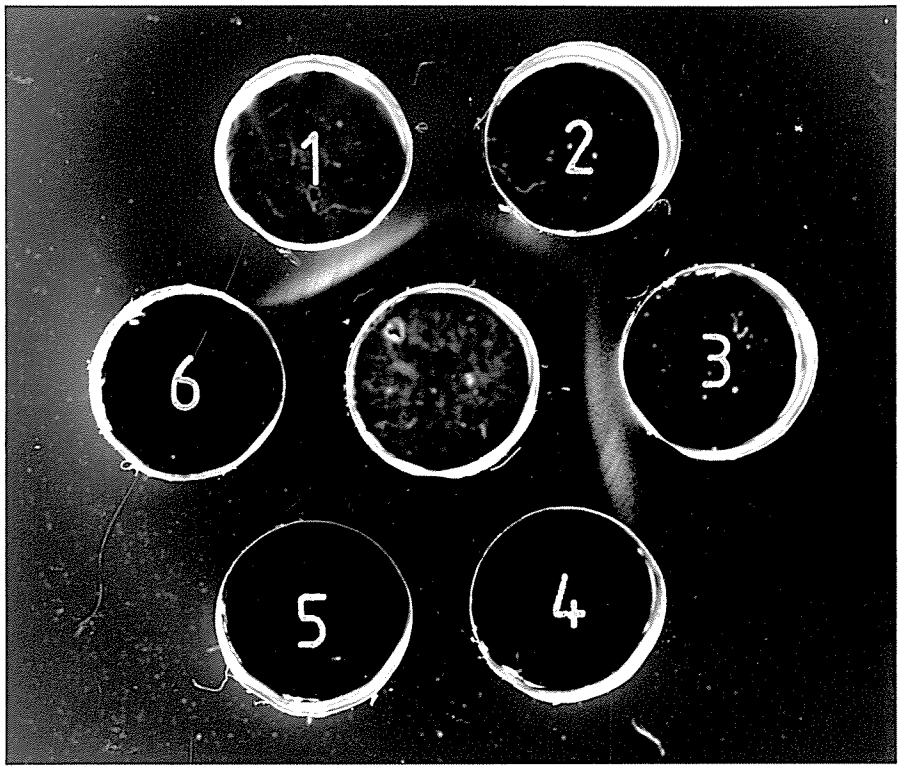

1
2
6
3
5
4

FIGURE 38:

Gel precipitation test to determine if the low molecular weight protein is a major antigen or not.

Well 1; antiserum to low molecular weight protein.

Well 2; Serum from rabbits immunised with whole *M. ovipneumoniae* (strain 5) organisms.

Wells 4, 4, 5 and 6; medium controls.

Centre well; lysed cell suspension of *M. ovipneumoniae* (strain 5).

The single precipitation line between wells 1 and the centre well was not continuous with the multiple precipitation of well 2. This implies that the low molecular weight protein is not a major antigen.


2
3
1
4
6
5

4.5.5 Discussion

The leading protein band of *M. ovipneumoniae* (strain 5) has an approximate molecular weight of 2500 (Figure 35), which is near the limit of antigenicity (De Weck, 1974). However, the protein in antigenic and antisera to it cross-reacts with other strains of *M. ovipneumoniae* in gel precipitation tests (Figure 37). This implies that this protein is species-specific.

Although this protein represents approximately 17.5% of the total protein (see Section 4.4) of *M. ovipneumoniae* (strain 5), it does not appear as a major antigen (Figure 38) in gel precipitation tests. This point is returned to in the general discussion.

CHAPTER 5

GENERAL DISCUSSION

About a decade ago it was established that *M. ovipneumoniae* could be recovered from the lungs of almost all sheep with CNP; from some apparently normal lungs and from the nasal cavity of some sheep with or without CNP. A relationship between *M. ovipneumoniae* and the economically important disease CNP is acknowledged by all workers (Cottew, 1971; St. George *et al.*, 1971; Carmichael *et al.*, 1972; Furlong and Cottew, 1973; Sullivan *et al.*, 1973; Clarke *et al.*, 1974; Alley *et al.*, 1975), although there is continuing discussion as to whether the organism is the sole pathogen; acts in consort with other microorganisms to produce CNP lesions (Jones *et al.*, 1978) or colonises lesions produced by other agents.

Clearly, if *M. ovipneumoniae* is the primary pathogen it must become disseminated in the flock, not later than the time of peak prevalence of CNP. Our results (Figures 1 and 2), clearly show that this is indeed the case.

The time of colonisation of the nasal cavity of flock 2 was earlier than in flock 1 and the prevalence of CNP in flock 2 was higher than in flock 1. This suggests that the colonisation of the nasal cavity by *M. ovipneumoniae* preceeds the colonisation of the lung and is consistant with the hypothesis that *M. ovipneumoniae* first colonises the nasal tract and later, probably in response to respiratory stress, subsequently invades the lung. The hypothesised stress factor is most likely to be hot dry weather, since following such weather the prevalence of CNP increases (Alley - personal communication).

It could of course be argued that the strains of *M. ovipneumoniae* colonising the lungs do not, or even cannot, colonise the nasal cavity. To investigate this possibility Mrs A.J. Mew (M.Sc. Thesis, 1982) examined some of our nasal and lung isolates from flock 2 and found by restriction endonuclease analysis that the lung isolates of *M. ovipneumoniae* from flock 2 were almost all identical in their REA pattern, whereas the nasal isolates fell into 7 distinctly different groups. One of these 7 "nasal" groups was indistinguishable from 18 of the 20 lung isolates.

One interpretation of this result is that only a minority of *M. ovipneumoniae* strains can invade the lung. This, if true is a potentially important point if a vaccine is ultimately produced, so clearly, it would be useful to find a way of classifying and identifying new isolates from both the nasal cavity and the lungs.

Initial RE-analysis seemed ideal for this purpose (Mew, 1982), but it soon became evident that when more than one flock was involved, a bewilderingly large number of different "strains" could be identified by RE-analysis i.e. the technique is too discriminating for the purpose intended. A further disadvantage of REA is that the patterns used to discriminate between strains have no obvious correlation with antigenicity or immunity. For these reasons the second part of this thesis aims to develop a method of distinguishing and classifying isolates of *M. ovipneumoniae*. The approach taken is to use SDS-PAGE gels.

Our initial experiments were concerned with the selection of a suitable gel and we were able to show that a linear 10% gel with a loading of 80 µg/track was the optimum for this purpose (compare Figures 18, 19, 20, 21 and 22). Using such gels it is indeed possible to distinguish

isolates of *M. ovipneumoniae* by SDS-PAGE (Figures 25, 26 and 27), but the number of protein bands was too large for convenient comparisons to be made. Two contrasting (but complementary) approaches were made in response to this problem;

(i) To "fractionate" *M. ovipneumoniae* in the hope that a fraction could be found which conserved most of the strain-specific (variable) bands but markedly reduced the total number of common bands;

(ii) To examine the possibility of excising individual protein bands from the gel and to use these as antigens to produce antisera directed against a single *M. ovipneumoniae* antigen.

Fractionation of the Mycoplasmas

One strain of *M. ovipneumoniae* was used to find if protein bands from different fractions could be assigned to different "sites". Initially the internal proteins were separated from the membrane-associated proteins and both fractions were examined by SDS-PAGE. This required a method of cell lysis which caused no major stuctural damage to the membrane. DCCD-induced lysis served this purpose.

An examination of both the soluble internal and the membrane associated proteins by SDS-PAGE (Figure 30), suggested that most of the mycoplasma proteins were associated with the membrane fraction. Therefore, for the purposes of classification and identification of *M. ovipneumoniae* isolates, the examination of the membrane fraction had little or no advantage compared to using total proteins. On the other hand this fraction (unlike the internal protein fraction) included the variable bands which in principle would allow strain distinction to be made. Hence, further fractionation of these membrane-associated proteins was undertaken to further

examine surface proteins.

Surface Labelling of Mycoplasmas

To subdivide the membrane fraction, FITC (isothiocanates are reputed to be impermeable to membranes, Maddy, 1964) was used to preferentially label the surface proteins of intact mycoplasmas and some of the proteins have been assigned as surface proteins (Figures 31 and 32). However, relatively large numbers of protein bands were seen on the fluorogram even when intact cells were labelled, this implies that:

(i) The surface of *M. ovipneumoniae* has many proteins or

(ii) That some penetration of FITC through the membrane has occurred or

(iii) That some of the surface labelled proteins are adsorbed medium constituents.

In a further attempt to identify surface components which are either common to all strains or are strain specific, 3 intact *M. ovipneumoniae* strains were labelled with FITC for a short period i.e. 3 minutes. Comparisons of these 3 strains (Figure 33) allowed us to assign protein bands to 2 categories:

(i) Those unique to one of the 3 strains (probably strain-specific antigens).

(ii) Those common to all 3 strains. These latter could be:
(a) Species-specific antigens or
(b) Adsorbed media constituents

As pointed out above, strains could in principle be categorised by comparison of bands following surface labelling, but this approach (even though it involves fewer bands than total protein) still has too many bands for convenient comparisons to be made, hence at this stage of our investigation, we concluded that the excision of bands for use as antigens to produce strain-specific antiserum was more likely to be a practical possibility. To test the practicality of this possibility, a band common to all (tested) strains was excised and used as an immunising antigen. However, it was first necessary to ensure that this or any other band ultimately selected was not an adsorbed medium constituent.

Detection of Medium Constituents

To identify medium constituents the organism was propagated in the presence of radioactive amino acids. An examination of the fluorogram in association with Coomassie-blue stain, allows medium constituents to be identified as these will stain with Coomassie-blue but will not appear in the fluorogram.

Unexpectedly the low molecular weight leading protein band was radioactive i.e. is not adsorbed peptone and accounts for 17.5% of the total cellular protein (see section 4.4).

The interesting possibility arose, later shown to be incorrect, that this protein (or polypeptide) might, because of its size, be a non-antigenic mycoplasma product. To investigate its possible antigenicity its molecular weight was determined by comparisons with markers to be approximately 2,500 (Figure 35).

Low Molecular Weight Protein

This protein is, in terms of its molecular weight, near the threshold of antigenicity (De Weck, 1974). To test its antigenicity it was excised from the gel and used to immunise a Guinea-Pig. Antisera was indeed produced as shown by gel precipitation tests. In preliminary experiments (not recorded here) we obtained no evidence that this antibody inhibited the growth of the organism. Thus this protein is of very low molecular weight; is synthesised in relatively massive amounts (17.5% of total protein); is found at the surface of *M. ovipneumoniae*; is easily removed by washing; is antigenic, but the antibody does not appear to inhibit growth of the mycoplasma. Its immunological significance is therefore unclear, but obviously antibody directed against this could have the potential to fix complement, opsonise etc. Yet if the antigen/antibody complex became detached from the cell surface, these activities would not only be valueless, but could even be counter productive in terms of eradication of *M. ovipneumoniae* from the lungs. The role of this protein in the pathogenesis of CNP therefore must be further investigated.

As suggested above we concluded that SDS-PAGE separation of the total proteins or of any fraction of *M. ovipneumoniae* does not in itself provide a convenient approach to classifying *M. ovipneumoniae*. We have however shown that when many isolates are examined they show many common bands and a smaller number of unique bands. This remained true even when the surface proteins only are examined.

We believe that this opens up a potentially powerful way of classifying strains of *M. ovipneumoniae*, since individual common bands can following excission from a gel, be used to produce a species-specific antisera and more importantly that the unique bands may be used

as antigens to produce an antisera which could discriminate between groups of isolates of *M. ovipneumoniae*.

While both the above statements are plausable, the time available has limited our work to an examination of a species specific protein band i.e. the production of a species-specific antiserum. The next step would clearly be to repeat this with an unique band. Our demonstration that at least one excised band (see Section 4.5) allows the production of antisera directed against a single *M. ovipneumoniae* protein, suggests that this approach can be successfully applied to strain-specific anitgens.

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