A STUDY OF MYCOPLASMAS OF THE OVINE LUNG AND THEIR RELATIONSHIP TO CHRONIC NON-PROGRESSIVE PNEUMONIA OF SHEEP IN NEW ZEALAND

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Peter Norman Brian

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ABSTRACT

The relationship of mycoplasmas to diseases of the lower respiratory tract in a variety of animals was reviewed and investigations were undertaken to determine the role of micro-organisms, with particular reference to mycoplasmas, in the aetiology of ovine chronic non-progressive pneumonia (CNP).

A survey of the prevalence of mycoplasmas in pneumonic sheep lungs revealed that Mycoplasma ovipneumoniae was present in 98% of the lungs tested, whereas Mycoplasma arginini was present in 4%. Ureaplasmas were not detected in any lungs.

To facilitate further investigations into the significance of M. arginini in ovine CNP, the in vitro growth of the organism was investigated and its ultrastructure was determined and compared with that of M. ovipneumoniae. Although ultrastructural differences between M. arginini and M. ovipneumoniae were found, these would probably not allow all cells of each of the two species to be unequivocally identified in thin sections of lung material.

M. ovipneumoniae, M. arginini and parainfluenza type 3 virus were shown to be sensitive to digitonin when suspended in either conventional laboratory medium, or in lung homogenate. Furthermore, treatment of pneumonic lung homogenate with 10 mg/cm^3 digitonin destroyed its ability to transmit ovine CNP. Viruses (in particular P.13 virus) were not detected in aliquots of the pool of lung homogenate used to transmit CNP so it is likely that the necessary digitonin-sensitive component is a mycoplasma. Since M. arginini has a consistently low prevalence in pneumonic lesions, whereas M. ovipneumoniae is found in the vast majority of such lesions, it was concluded that M. ovipneumoniae is responsible for initiating primary lesions of the disease. This however does not imply that M. ovipneumoniae on its own is capable of causing lesions comparable in severity to the fully developed "field" cases.

The inactivation of M. ovipneumoniae by formalin, with a view to making a vaccine, was investigated.

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INTRODUCTION

Chronic non-progressive pneumonia is a prevalent and economically important disease of sheep in New Zealand, but until recently little attention has been focused on the disease. A study is now in progress at Massey University to establish the aetiology of the disease, and this thesis is presented as part of that study.

Sheep outside New Zealand (e.g. in Australia and Scotland) get a disease similar in pathology to CNP in New Zealand. The disease in Australia has been attributed to M. ovipneumoniae alone (St. George et al., 1971; Carmichael et al., 1972; Sullivan et al., 1973b), whereas in Scotland the disease has been attributed to a mixture of organisms including M. ovipneumoniae, M. arginini, and Pasteurella haemolytica (Jones et al., 1978) and possibly parainfluenze type 3 (PI3) virus (Jones et al., 1979). It remains to be proven which, if either, of these conclusions applies to sheep in New Zealand because diseases with a similar pathology do not necessarily have an identical aetiology.

Diseases of other animals with clinical and pathological features in common with ovine CNP include pleuropneumonia of cattle, enzootic pneumonia of swine, chronic respiratory disease (CRD) of poultry, CRD of rodents and "atypical" pneumonia of man. These diseases all have a common feature, i.e. mycoplasmas are involved in their (sometimes complex) aetiology. It appears plausible, therefore, that mycoplasmas (M. ovipneumoniae in particular) play some role in ovine CNP in New Zealand.

Workers in New Zealand (Alley and Clarke, 1979: Alley and Clarke, in the press) found that CNP could be transmitted using whole lung homogenate. This lung homogenate invariably contained bacteria (mostly P. haemolytica), and mycoplasmas (mostly M. ovipneumoniae). They also found that the transmitted disease was partially, but not totally, suppressed by penicillin. This implies that an organism with a cell wall (i.e. a bacterium) is involved in the development of lesions. However, inoculation of sheep with P. haemolytica usually failed to produce any disease (Alley, personal communication); and overseas workers who inoculated 4 to 5 month old lambs

intrabronchially with large numbers of P. haemolytica produced an acute bacterial pneumonia, which is both clinically and pathologically distinguishable from CNP (Biberstein et al., 1971). Inoculation of pure cultures of M. ovipneumoniae, on the other hand, produced in a minority of inoculated animals, minimal lesions which were markedly less severe than typical field cases of the disease (Alley and Clarke, 1979). Taken together, these results suggest that a mixture of bacteria (P. haemolytica) and mycoplasmas (M. ovipneumoniae) may be needed to produce the field disease. However, other New Zealand workers have stressed the importance of viruses (in particular PI3 virus) as a cause of pneumonia in sheep (Davies et al., 1977).

The purpose of the present work is to further investigate the aetiology of ovine CNP, and in particular to examine the role of mycoplasmas in the disease.

Earlier surveys were conducted over a limited period on the prevalence of M. ovipneumoniae and M. arginini in pneumonic lungs of New Zealand sheep by Alley et al. (1975) and it was thought appropriate that a further survey should be conducted. Since ureaplasmas have subsequently been shown to be capable of colonising the ovine lung (Jones et al., 1979) methods for detecting ureaplasmas were included.

M. arginini is present in some lesions of ovine CNP, and was present in some pools of pneumonic lung homogenate used to transmit the disease by Alley and Clarke. An assessment of the role of M. arginini in the disease is therefore appropriate, so the in vitro growth characteristics and the ultrastructure of the organism were examined to facilitate future work on its role, if any, in ovine CNP.

Despite much circumstantial evidence, the failure of mycoplasmas to produce typical field cases of the disease in any country does raise at least some doubts as to their significance. Therefore, in order to confirm their significance, the ability of pneumonic lung homogenate to transmit the disease was investigated when mycoplasmas were specifically inactivated by digitonin. Pools of lung homogenate known to transmit CNP were also examined for cytopathic agents,

in particular PI3 virus.

The above investigations gave further evidence for the central role of M. ovipneumoniae in ovine CNP, so in the final section of this thesis the inactivation of the organism by formalin is investigated with a view to developing a killed vaccine.

CHAPTER 1 Review of Ovine Chronic Non-progressive Pneumonia

1.1. Definition and description of the disease

In New Zealand chronic non-progressive pneumonia (CNP) is prevalent in sheep aged from 3 to 10 months, and is sometimes referred to as "hogget pneumonia". The disease elicits few clinical signs except in severe cases where coughing and loss of condition may be observed. It has been estimated to affect up to 70 to 80% of some groups of lambs at slaughter in the early autumn months i.e. March, April, May (Davis, 1970; Smith, 1970). Mortalities as a direct result of the disease are low and resolution normally occurs in late autumn (Alley, 1975a).

The macroscopic lung lesions of ovine CNP vary from dull-red to grey areas of consolidation in the cranio-ventral lobes of both lungs. In addition narrow branching bands of collapse are sometimes present. On microscopic examination the disease is characterised by alveolar collapse, infiltration of alveolar spaces with macrophages and variable numbers of neutrophils, lymphoid and bronchiolar epithelial cell hyperplasia, and the presence of both lymphocytes and macrophages in the alveolar septa (Alley, 1975a).

1.2 Distinction from progressive viral pneumonia

It is relevant to distinguish ovine CNP from the pneumonias produced by viruses of the Lentivirinae subfamily[1] i.e. maedi, visna, and progressive pneumonia virus (PPV) of sheep. These agents produce a progressive disease which is slow to develop. Thus maedi has an incubation period which may last several years and clinical signs are seldom seen in sheep less than four years old. After the onset of signs, life expectancy is a matter of a few months to one year (Sigurdsson, 1954; Andrewes and Pereira, 1972; Fuccillo et al., 1974; Hasse, 1975; Dawson et al., 1979).

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[1] This classification is from Fenner, F. (1976). The classification and nomenclature of viruses. Virology 71: 371-378.

In contrast, ovine CNP affects only sheep aged from 3 to 10 months and transmission experiments performed by Alley and Clarke (1979) produced the disease 2 to 3 weeks after inoculation. Unlike progressive viral pneumonias, CNP usually resolves in late autumn and mortalities are low (Alley, 1975a).

These criteria distinguish progressive viral pneumonia of sheep from CNP of lambs in New Zealand. It is also relevant that extensive virological investigations of pneumonic sheep in New Zealand have been undertaken and resulted in the isolation of parainfluenza type 3 (PI3) virus (Carter and Hunter, 1970) and adenoviruses (Thurley et al., 1977). However visna, maedi and PPV of sheep were not detected in association with CNP and indeed no agent of the Lentivirinae subfamily has been recovered from sheep in New Zealand, nor is there any clinical or pathological evidence to suggest that the pneumotropic (PPV and maedi) or the neurotropic (visna) viruses of this group are present in sheep in New Zealand up until the present time.

1.3 Distinction from bacterial pneumonia

Bacterial pneumonia of sheep in New Zealand is an acute disease. It was reported by Salisbury (1957) and Downey (1957) as "Southland Pneumonia". The majority of cases of acute bacterial pneumonia occur in the early summer months (unlike CNP which peaks in early autumn) and sheep of all ages are affected (in contrast to CNP which affects sheep under 1 year of age). Bacterial pneumonia is typified by a sudden onset of signs and rapid progression usually leading to death about 12 hours later. Pasteurella haemolytica in high concentrations is almost invariably associated with acute pneumonic lesions (Downey, 1957).

Bacterial pneumonia can also be clearly distinguished from CNP on the basis of their different pathological lesions. The gross pathology of acute bacterial pneumonia is characterised by intense congestion and varying degrees of red or grey consolidation of the ventral portion of one or both lungs. Histopathological examination reveals a cellular exudate consisting of neutrophils, macrophages and detached alveolar epithelial cells. Large numbers of bacteria

are present in the lesions. Destruction and damage to the alveolar epithelium is universal whereas in the chronic non-progressive disease this damage is localised and less severe (Alley, 1975a).

The histopathology of CNP is characterised by proliferation of lymphoid and epithelial tissue, with exudation of macrophages and to a lesser extent neutrophils, which contrasts with the large number of neutrophils seen in bacterial pneumonia (Alley, 1975a).

1.4 Diseases of sheep with a similar pathology to ovine CNP

Atypical pneumonia

The macroscopic lesions of the lungs of sheep with "atypical pneumonia" were described as pneumonic consolidation affecting the apical and cardiac lobes of the lung and occasionally the anterior borders of the diaphragmatic lobes. The colour of the consolidated areas varied between grey and pink. Lymphoid hyperplasia, infiltration of the alveolar septa with lymphocytes and macrophages, and alveolar collapse were the main features of atypical pneumonia (Stamp and Nisbet, 1963).

Proliferative interstitial pneumonia

This disease is typified by septal and bronchiolar eptithelial cell hyperplasia and proliferation, with only minor infiltration by lymphocytes and neutrophils. High morbidity, low mortality, exercise intolerance and inadequate weight gain of sheep are the main clinical features of the disease (St. George et al., 1971; Carmichael et al., 1972; Sullivan et al., 1973a; Sullivan et al., 1973b; St George and Carmichael, 1975; Friis et al., 1976.).

Summer pneumonia

It was reported that "summer pneumonia" affected sheep in the 6 to 12 months age range in Australia. The disease exhibited a high morbidity and a low mortality. Consolidation of the lungs, which could involve the whole or part of a lobe was common, but occasionally small (1 cm^2) lesions were observed. The colour of the lesions varied from reddish-purple to grey. Microscopically, the lesions revealed

areas of collapse, proliferation of the alveolar epithelium, and lymphoid hyperplasia (St. George, 1972).

Proliferative exudative pneumonia

The principal histological features of this disease are lymphoid hyperplasia, bronchiolar hyperplasia, polymorph and macrophage exudation, giant cells, hayline scar formation, and alveolar collapse (Jones et al., 1978; Jones et al., 1979). These workers stated that proliferative exudative pneumonia had a close resemblance to the "atypical pneumonia" described by Stamp and Nisbet (1963).

1.5 Comparison of ovine CNP with the above diseases

Although CNP of sheep in New Zealand can be distinguished from both acute bacterial pneumonia and progressive viral pneumonia, a basic difficulty arises when comparing the disease with similar pneumonias of sheep in overseas countries i.e. the aetiology of CNP has not been unequivocally established and indeed this thesis is part of a study aimed at investigating its aetiology. Furthermore, the aetiology of apparently similar diseases of overseas sheep is likewise under investigation (Foggie et al., 1976; Jones et al., 1978; Jones et al., 1979). So while it is possible to compare CNP of sheep in New Zealand with similar diseases elsewhere, the similarity must presently be based on the pathology produced. In the absence of an established aetiology, the number of descriptive terms for similar diseases has multiplied, but on the basis of their pathology and epidemiology it is likely that the above diseases (viz atypical pneumonia, proliferative interstitial pneumonia, summer pneumonia, and proliferative exudative pneumonia) will ultimately be shown to have a similar aetiology to CNP of sheep in New Zealand.

In any case, it is convenient for the sake of brevity in discussion to treat these diseases as being similar, if not identical to each other. Nevertheless it must be clearly understood that this assumption does not yet have the ultimate justification i.e. a proven identical aetiology.

1.6 Investigations into the aetiology of ovine CNP

1.6.1 The role of viruses

Parainfluenza type 3 virus has been isolated from the respiratory tract of sheep. In Scotland, Hore (1966) took nasal swabs from intensively housed lambs which had a nasal discharge, and thirteen isolates of PI3 were obtained. In Canada, Ditchfield (1966) isolated PI3 from sheep with acute "Pasteurella pneumonia".

Hore (1970) suggested that PI3 virus may be implicated in sheep pneumonia, but severe clinical illness or persistent lesions could not be attributed to the virus alone. St. George and Liefman (1972), in an 11 month investigation of respiratory disease in a flock of Australian lambs, failed to isolate PI3 from pneumonic animals. Serology indicated that PI3 virus infection had occurred in the flock 7 months prior to the occurrence of clinical pneumonia, and they also found that an asymptomatic pneumonia was present in the flock from when the lambs were approximately 1 month old. It was concluded that PI3 did not appear to be the cause of chronic pneumonia in the flock studied and they stated that PI3 virus appeared to produce a mild pneumonia which was superimposed for a short time on the existing pneumonia.

Attempts have been made to produce pneumonia in lambs using PI3 virus inocula, but success has been limited (Davies *et al.*, 1977). Sharp *et al.* (1978) reported that specific pathogen free (SPF) lambs inoculated with PI3 virus alone, developed a transient respiratory disease which resolved after 9 days.

Concurrent infection of lambs with PI3 virus and *Pasteurella haemolytica* produced an acute pneumonia (Sharp *et al.*, 1978). Similar work in New Zealand was carried out by Davies *et al.* (1977) but the pathology of the disease produced was not described fully.

Although Jones *et al.* (1979) failed to isolate PI3 virus from lambs with proliferative exudative pneumonia, they did show that PI3 virus infection had occurred in most lambs in their flock as indicated by serology, and they suggested that the establishment of

Mycoplasma ovipneumoniae in the lower respiratory tract of lambs may be facilitated by PI3 virus infection. Clearly the role of PI3 virus in the aetiology of ovine CNP merits further investigation, but at present there is no unequivocal evidence that the presence of PI3 virus is a necessary prerequisite for the development of the disease.

Adenoviruses were isolated from the respiratory tract of sheep in New Zealand (Thurley et al., 1977) but no evidence which associates adenoviruses with ovine CNP was presented.

1.6.2 The role of bacteria

An investigation of the bacteria in the respiratory tract of 5 to 10 month old lambs with CNP was carried out in New Zealand by Alley (1957b). P. haemolytica was the predominant organism isolated from both the normal and pneumonic respiratory tract. It was most frequently isolated from the nasal cavity of pneumonic sheep (78%) but this frequency was only slightly higher than the isolation rate from the nasal cavity of normal sheep (73%). P. haemolytica was isolated in pure culture from 45% of pneumonic lungs, and combined with Neisseria catarrhalis in a further 14%. Only 6% of lung material from normal sheep yielded P. haemolytica.

Neisseria catarrhalis was the second most frequently isolated organism, being recovered from 22% of pneumonic lungs (mostly in combination with P. haemolytica), but only 1.6% of normal lungs.

P. haemolytica or N. catarrhalis were not isolated from every pneumonic lung studied and it was suggested that the bacteria normally resident in the nasal cavity of sheep have the ability to colonise the lungs of sheep with CNP, but they are not essential for initiating the disease process.

Jones et al. (1979) found low numbers of P. haemolytica in some cases of proliferative exudative pneumonia in housed sheep in Scotland and also suggested that P. haemolytica was only a secondary invader in the disease. They noted the later appearance of P. haemolytica compared with M. ovipneumoniae in the lungs of lambs.

Alley and Clarke (1979) demonstrated the ability of homogenised pneumonic lung tissue to produce lesions of CNP when inoculated intranasally into conventionally reared lambs. These lesions were identical to the field disease. In later work (Alley and Clarke, in the press) they found that treating the sheep with penicillin for a period of 2 weeks from the time of inoculation with lung homogenate until slaughter stopped bacteria colonising the lungs, and signficantly reduced the severity of the lesions produced. However, minimal lesions still developed. Sheep treated with high doses of Ronidazole (which stopped the colonisation of lungs by both bacteria and M. ovipneumoniae) did not develop any pneumonic lesions. These workers concluded that M. ovipneumoniae could colonise the lungs of lambs and produce a relatively mild pneumonia and that bacteria as secondary invaders made the disease more severe i.e. it required both M. ovipneumoniae and bacteria to produce a disease typical of the more severe field cases of ovine CNP.

1.6.3 The role of chlamydiae

Chlamydiae have been isolated from sheep with pneumonia. These isolations have as yet only been made in the Northern Hemisphere (McKercher, 1952; Boiden et al., 1958; Hamdy et al., 1959; Stevenson and Robinson, 1970).

Intratracheal inoculation of lambs with chlamydiae by Dungworth and Cordy (1962) produced an extensive, but mild form of pneumonia. The lesions resolved in 13 to 20 days following inoculation, which is in contrast to the more chronic nature of ovine CNP. Stevenson and Robinson (1970) also inoculated lambs intratracheally with chlamydiae and produced pneumonic lesions similar to "atypical pneumonia" as described by Stamp and Nisbet, which is similar to CNP. The disease ran its course in only 9 days after which resolution occurred. Both groups of workers described the presence of chlamydial "elementary bodies" in sections of pneumonic lung. These "elementary bodies" have not been reported as being present in pneumonic lesions from sheep with CNP in New Zealand.

The short duration, and the presence of "elementary bodies" associated with chlamydial pneumonia distinguish it from ovine CNP.

1.6.4 The role of mycoplasmas in pneumonia of animals, including sheep

(a) Primary atypical pneumonia in man

Primary atypical pneumonia in man was recognised in the 1930's as an infectious disease, but could not be associated with a specific agent at that time (Scadding, 1937). Patients with this disease often developed cold agglutinins (antibodies which agglutinated human group O erythrocytes at 0 to 4°C, but not at 37°C). It was first thought that a virus was responsible for the disease. An agent (the "Eaton agent") was eventually isolated which could pass through a 180 to 250 nm pore size filter, and could be serially passaged in embryonated eggs. This agent produced pneumonia in hamsters and cotton rats when inoculated intranasally (Eaton et al., 1944). Subsequently it was shown by measuring antibody titre rises to the agent in paired sera that epidemics of cold agglutinin positive pneumonia were associated with the "Eaton agent" (Liu et al., 1959).

The likelihood that the "Eaton agent" was a mycoplasma was suggested by Marmion and Goodburn (1961) who demonstrated that the agent was sensitive to chlortetracycline, gold salts, and streptomycin; and also that the agent morphologically resembled a mycoplasma. The agent was grown on cell-free agar medium by Chanock et al., (1962) and was identified as a member of the genus Mycoplasma on the basis of the morphologic and staining properities of the organism, and also its requirement for serum. The majority of colonies on agar were granular and centreless, unlike the typical centred colonies of most mycoplasmas. The agent was named Mycoplasma pneumoniae by Chanock et al., (1963).

Final proof that M. pneumoniae was the aetiological agent of primary atypical pneumonia of man was provided by the experimental inoculation of the organism into human volunteers who did not have pre-existing inhibitory antibody. Inoculation produced respiratory disease, a rise in antibody to the organism, and the development of

cold agglutinins in a significant portion of the inoculated persons (Chanock et al., 1970).

(b) Bovine pleuropneumonia

Bovine pleuropneumonia was recognised in Europe in the 18th century and by the mid 19th century reports of the disease had been made in many parts of the world. It was carried from Europe to Africa, America and Australia with the transport of cattle.

Bovine pleuropneumonia causes great economic losses in areas where it is established. In Australia, the disease is still present in the North and rigid control measures are employed in "clean" areas to keep them free from pleuropneumonia. These measures include the slaughter of all diseased and serologically positive cattle, immunisation of all healthy cattle, follow up of contacts of diseased animals, and notification of the disease (Klieneberger-Nobel, 1962; Cottew and Leach, 1969).

The organism causing bovine pleuropneumonia was discovered by Nocard and Roux in 1898, who grew the agent in a collodian sac inside the peritoneal cavity of a rabbit. Subsequently it was found that peptone broth enriched with serum would support growth of the pleuropneumonia organism, and growth on solid medium gave centred colonies which are now known to be typical of many mycoplasmas (Lloyd and Trethewie, 1970). The organism of bovine pleuropneumonia was used as the type species for a new genus Mycoplasma in 1956 and was named Mycoplasma mycoides var mycoides (Edward and Freundt, 1956).

Although M. mycoides was generally accepted as being the cause of bovine pleuropneumonia, production of experimental disease in cattle using pure cultures of the organism was not easily achieved. Other non-living agents were shown to be necessary as well as the mycoplasma when cattle were inoculated by a variety of methods (viz aerosol inoculation, intrabronchial intubation, and intravenous injection). These agents (chopped agar, blood clots, macerated lung tissue, or lung exudate) appeared to assist in the process of infection (Lloyd and Trethewie, 1970).

Vaccines against bovine pleuropneumonia generally contain live *M. mycoides* organisms and appear to have a long term effect. Only limited success has been achieved using dead vaccines (Cottew and Leach, 1969; Lloyd and Trethewie, 1970).

(c) Enzootic pneumonia of swine

Enzootic pneumonia of swine (chronic porcine pneumonia) is a major problem in many countries, especially when pigs are housed in large numbers. Similar to ovine CNP, the disease shows a low mortality and a high morbidity with up to 50% of pigs slaughtered for human consumption having gross pneumonic lesions. Growth retardation and decreased efficiency of food utilisation are economically important effects of the disease (Whittlestone, 1972; Ross, 1973).

The causative agent was grown on cell-free medium and was shown to have the properties of a mycoplasma by Maré and Switzer (1965) who proposed the name *Mycoplasma hyopneumoniae* and by Goodwin *et al.*, (1965) who proposed the name *M. suipneumoniae*. Both organisms were subsequently shown to be strains of the same species (Goodwin *et al.*, 1967) but confusion about the names still exists in the 8th edition of *Bergey's Manual of Determinative Bacteriology*.

Both groups of workers demonstrated the ability of intranasal inoculation of the organism to produce the disease in pneumonia-free pigs. However more than one inoculation was needed to produce the disease.

Gnotobiotic pigs inoculated with a single dose of a pure culture of *M. hyopneumoniae* developed extensive pneumonia similar to the natural disease (Hodges *et al.*, 1969). These experimental findings indicate that *M. hyopneumoniae* (*suipneumoniae*) is a cause of enzootic pneumonia (Betts, 1971), but is it the sole cause?

M. hyorhinis has also been associated with pneumonia in swine. L'Ecuyer *et al.*, (1961) and Friis (1971) reported that pneumonias caused by *M. hyopneumoniae* are frequently complicated by *M. hyorhinis* infection. Experimental inoculation of pigs with *M. hyorhinis* by Gois *et al.* (1971) produced rhinitis and pneumonia similar to

enzootic pneumonia. Other workers confirmed this and Betts (1971) concluded that some strains of M. hyorhinis are pulmonary pathogens capable of producing pneumonia similar to enzootic pneumonia.

M. hyosynoviae and M. flocculare were isolated from the porcine respiratory tract by Ross and Karmon (1972) and Friis (1972) respectively. The significance of these in porcine pneumonia, however, has not been reported (Whittlestone, 1973).

In summary, therefore, it may be said that M. hyopneumoniae (suipneumoniae) and some strains of M. hyorhinis are pathogens capable of causing enzootic pneumonia of swine.

(d) Air-sac disease of poultry

Chronic respiratory disease of poultry is a mild disease with a slow spread, long incubation period, and a slow course (Fabricant, 1969). The causative agent was grown in embryonated eggs and the yolk from these eggs produced sinusitis when inoculated into the sinus of turkeys (Markham and Wong, 1952). Subsequently the organism was grown on cell-free medium and shown to be a mycoplasma. The name Mycoplasma gallisepticum was proposed by Edward and Kanarek (1960).

Outbreaks of a more severe respiratory disease with high mortalities (air-sac disease) were subsequently reported. Wasserman et al. (1954) reported that Escherichia coli was isolated from infected birds in a high number of cases: isolations of mycoplasmas, infectious bronchitis virus (IBV), and Newcastle disease virus (NDV) were noted also. Air-sac disease was reproduced experimentally by inoculating hens with M. gallisepticum plus IBV or NDV, followed by an aerosol of E. coli (Gross, 1961).

Fabricant and Levine (1962) inoculated hens with M. gallisepticum, E. coli, and IBV in several combinations and produced lesions indistinguishable from field cases. Birds inoculated with all three agents developed the most severe disease, and birds inoculated with M. gallisepticum plus E. coli developed a slightly less severe disease. An important conclusion drawn from these experiments was that E. coli did not readily invade the lower respiratory tract of hens unless

previous infection with M. gallisepticum had occurred. This is supported by the fact that air-sac disease is almost completely absent from flocks of M. gallisepticum-free chickens (Fabricant, 1969).

Because of intensive poultry management nowadays involving large numbers of birds in close contact, even a small number of infected chickens can serve as a reservoir of M. gallisepticum which is spread by contact infection. This contact spread can be accelerated by virus infection, or live virus vaccination with NDV or IBV (Fabricant, 1969).

It is seen therefore that respiratory disease of poultry has a complex aetiology, M. gallisepticum alone may cause a mild chronic respiratory disease, but when complicated by a viral and an E. coli infection severe air-sac disease results.

(e) Chronic respiratory disease of rodents

Chronic respiratory disease (CRD) of rats and mice is indigenous in many colonies of laboratory rodents. Several groups of workers isolated mycoplasmas from the respiratory tracts of pneumonic mice and Edward (1954) reported that these mouse pneumonia strains were similar to mycoplasmas isolated from rats with CRD. The organism was named Mycoplasma pulmonis by Edward and Freundt (1956). This mycoplasma was also isolated from the respiratory tract of normal rodents (Edward, 1940; Klieneberger-Nobel and Cheng, 1955).

Over the years there has been considerable confusion about the aetiological agent of CRD. It is generally accepted that M. pulmonis causes upper respiratory tract disease in rats i.e. infectious catarrh (Cassell et al., 1973), but the role of M. pulmonis as a cause of chronic lower respiratory tract disease is less certain. Nelson (1967) proposed that chronic respiratory disease of rats was the result of two diseases: infections catarrh produced by M. pulmonis, and a bronchiectasis caused by a "virus". This "virus", however, has not been characterised. A viral aetiology was also proposed for chronic murine respiratory disease by Andrewes and Glover (1945), who called the agent the "grey lung virus".

Cassell et al. (1973) cite three groups of workers who successfully reproduced chronic respiratory disease using M. pulmonis inoculated into pathogen-free rats, however significant lower respiratory tract infection was produced inconsistently in their own laboratory.

Production of experimental respiratory disease in mice has been more successful. M. pulmonis was shown to be pathogenic for mice and intranasal inoculation produced upper and lower respiratory tract disease which was occasionally fatal if a large dose of the organism was administered (Cassell et al., 1973). Mice given lower doses of the organism showed lesions closely related to naturally occurring respiratory mycoplasma infection.

It was postulated that the reason behind the limited success of producing lower respiratory tract infection in rats (unlike mice) using M. pulmonis is that rats have a more efficient pulmonary clearance system. This is supported by the fact that chemicals which interfere with pulmonary clearance in the rat enhance lower respiratory tract lesions due to M. pulmonis (Cassell et al., 1973).

Another group of workers have a contrary view of the aetiology of CRD of rodents. Gay et al., (1972) attempted to isolate micro-organisms from the lungs of conventional rats, and to induce chronic pneumonia in specific pathogen free (SPF) rats. They transmitted chronic pneumonia to SPF rats and to laboratory mice, using pneumonic lung homogenate from which no organisms could be cultivated on the wide variety of media and tissue cultures used. However, mycoplasmas could be seen in stained impression smears of lung material from the experimentally infected mice. Ultrastructurally, a mycoplasma-like organism was seen, and this was morphologically identical to the "viral" cause of bronchiectasis of Nelson and the "grey-lung virus" of Andrewes and Glover. The disease in mice was suppressed by anti-mycoplasmal agents. M. pulmonis and Streptobacillus moniliformis (both as a bacterium and an L-form) could be isolated from old conventional rats, but the disease could not be reproduced by inoculating cultures of these organisms, either singly or in combination, into rats. It was concluded that M. pulmonis and S. moniliformis were opportunistic secondary invaders. These workers also concluded that

another agent, closely resembling a mycoplasma morphologically and biologically was the cause of chronic pneumonia in rodents, and it was probably highly fastidious as it could not be grown on conventional tissue cultures and inanimate media.

So it is seen that the cause of chronic pneumonia in rodents is still in dispute. It should be noted that although M. pulmonis can be isolated from most cases of the natural disease, it may not be the primary aetiological agent of chronic pneumonia of rodents.

(f) Summary of the role of mycoplasmas in the above diseases

The role of mycoplasmas in pneumonia of animals other than sheep is varied. Mycoplasmas can range from being the primary pathogen, as in atypical pneumonia of man, and contagious bovine pleuropneumonia; to being part of a sequence of infection in a disease of complex aetiology, as in air-sac disease of fowl; to being implicated in pneumonia with an as yet unresolved aetiology such as CRD of rodents.

(g) Chronic non-progressive pneumonias of sheep

The experimental transmission of ovine pneumonia by the intratracheal inoculation of pneumonic lung tissue suspension was described by St. George et al. (1971). The natural disease and experimental disease were described as a proliferative interstitial pneumonia. An agent was isolated in cell cultures and eventually grown in non-living liquid medium, but it did not grow well on solid medium. When the agent was eventually grown on solid medium the colonies resembled those of members of the genus Mycoplasma. Other properties typical of mycoplasmas were also noted viz the organism passed through a 0.22 µm pore size filter; it grew in the presence of thallium, streptomycin and penicillin; and did not grow in the presence of tylosin. Young lambs inoculated with pure cultures of the organism developed pneumonia similar to the field disease.

Three species of mycoplasma were isolated from sheep in Australia by Carmichael et al. (1972). One was identified as Mycoplasma arginini and it was found most commonly in the pharynx of normal and pneumonic sheep.

M. arginini formed centred colonies on solid medium. The species typified by isolate 2-D (a ureaplasma) was isolated only from the genital tract of sheep. The species typified by isolate Y-98 was similar to the organism isolated by St. George et al. (1971) and was most commonly isolated from pneumonic lungs, frequently in pure culture. This organism formed "vacuolated" centreless colonies on solid medium, fermented glucose, and produced acid in liquid medium. The name Mycoplasma ovipneumoniae was proposed. It was postulated that M. ovipneumoniae had an important aetiological role in chronic interstitial pneumonia of sheep. M. arginini was thought to be a normal inhabitant of the upper respiratory tract of sheep, and this was supported by Foggie and Angus (1972) who reported that M. arginini was not pathogenic for SPF sheep when inoculated intranasally and intratracheally.

The above data indicate that M. ovipneumoniae may be the primary lung pathogen in chronic sheep pneumonia. Sullivan et al. (1973b) exposed lambs to M. ovipneumoniae by either intravenous injection, an aerosol inoculum, or contact with infected animals and produced pneumonia similar to the less severe field cases of proliferative interstitial pneumonia. They could not, however, reisolate the organisms from the lungs of experimental animals and this was attributed to mycoplasmacidal factors in the lung being released when the tissue was ground in the isolation procedure.

Subsequently M. ovipneumoniae has been isolated from sheep with chronic pneumonia in New Zealand (Clarke et al., 1974), the United States (St. George and Carmichael, 1975), Britain (Leach et al., 1976; Jones et al., 1976) and Iceland (Friis et al., 1976).

Transmission experiments using pneumonic lung homogenate and/or M. ovipneumoniae have been reported by various workers. Foggie et al. (1976) inoculated SPF lambs endobronchially with M. ovipneumoniae, but only produced mild lesions of pneumonia in a small number of lambs. No definite conclusions were reached about the pathogenicity of these strains of M. ovipneumoniae for SPF lambs. Jones et al. (1978) inoculated homogenised pneumonic lung suspension containing M. ovipneumoniae, M. arginini and P. haemolytica endobronchially

into 6 to 7 month old sheep and produced proliferative exudative pneumonia similar to field cases. Inocula consisting of cloned cultures of these same organisms produced similar results. M. ovipneumoniae was recovered from all experimental animals but P. haemolytica and M. arginini were recovered less consistently, indicating that M. ovipneumoniae was probably the primary pathogen in the disease. Alley and Clarke (1979) transmitted CNP to conventionally reared lambs with an intranasal inoculum of pneumonic lung homogenate. The disease produced was indistinguishable from field cases. However, when M. ovipneumoniae alone was inoculated intranasally only 20% of the experimental animals showed pneumonic lesions, and these were relatively mild.

Although the results of the above experiments are not conclusive, they indicate that M. ovipneumoniae is important, and is probably the major pathogen in chronic non-progressive forms of sheep pneumonia. The evidence shows that M. arginini is a normal inhabitant of the ovine upper respiratory tract. Jones et al. (1978) suggested that M. arginini was associated with pleurisy and death in pneumonic sheep, but in a later paper (Jones et al., 1979) stated that M. arginini was unimportant in the disease. The isolation rate of M. arginini from pneumonic sheep lungs is considerably lower than the isolation rate of M. ovipneumoniae (Carmichael et al., 1972; Alley et al., 1975; Jones et al., 1978) which also lends support to the suggestion that M. arginini is not a primary pathogen in chronic ovine pneumonia.

Ureaplasmas were isolated from the lower respiratory tract of sheep by Jones et al. (1979) but were considered unimportant in proliferative exudative pneumonia.

1.7 Summary of evidence suggesting that ovine CNP has a complex aetiology

There has been accumulating evidence that ovine CNP may have a complex aetiology. Hore (1970), in reference to "atypical" pneumonia of sheep in Australia, suggested that PI3 virus may be one of the factors which alters the susceptibility of sheep to infection with more persistent respiratory pathogens, because infection with the virus

caused damage to the epithelium of the smaller air passages. It was also inferred that PI3 virus could act as a respiratory pathogen, but severe clinical illness or persistent pulmonary lesions could not be attributed to the virus alone.

Sullivan et al. (1973a) made sequential clinical, pathological and microbiological investigations in a flock of lambs naturally affected with proliferative interstitial pneumonia. Their results indicated that the lambs became infected with M. ovipneumoniae soon after birth, and this infection was complicated at a later stage by secondary bacterial invasion, producing a chronic pneumonia. No evidence of PI3 infection was found.

Jones et al. (1978) inoculated sheep endobronchially with homogenates of pneumonic lung from naturally occurring cases of proliferative exudative pneumonia. One homogenate contained P. haemolytica, M. ovipneumoniae and E. coli; and inoculated sheep developed clinical disease and lesions indistinguishable from the natural disease. Another homogenate contained P. haemolytica, M. ovipneumoniae and M. arginini. Sheep inoculated with this homogenate, and sheep inoculated with mixed cultures of the three organisms developed clinical respiratory disease and lesions indistinguisable from field cases of the disease. Some deaths occurred. M. ovipneumoniae was recovered from all animals, but P. haemolytica and M. arginini were recovered less consistently. These workers suggested that the aetiology of proliferative exudative pneumonia (atypical pneumonia) may commonly involve the combination of mycoplasmas and P. haemolytica.

In New Zealand, Alley and Clarke (1977) studied the correlation of the numbers of bacteria and the numbers of M. ovipneumoniae with the histopathological lesions of ovine CNP. They found that high titres of M. ovipneumoniae were associated with chronic proliferative changes in the lung. More severe lesions (i.e. greater neutrophil exudation into the alveoli, and increased proliferative changes) were associated with high titres of both M. ovipneumoniae and bacteria. Further work (Alley and Clarke, 1979) demonstrated the ability of a homogenate of pooled pneumonic lungs to produce the disease following intranasal inoculation into conventionally-reared lambs. Treating lambs with

penicillin (thereby stopping bacteria colonising the lungs) was found to reduce the severity of the disease produced (Alley and Clarke, in the press), but mild lesions were still present from which M. ovipneumoniae could be isolated. These mild lesions resembled the lesions produced when M. ovipneumoniae alone was inoculated intranasally into lambs. Treating lambs with a large dose of Ronidazole stopped the colonisation of lungs by both bacteria and M. ovipneumoniae after intranasal inoculation of pneumonic lung homogenate, and no lesions of pneumonia were detected (Alley and Clark, in the press). The conclusion drawn from these data is that M. ovipneumoniae is capable of colonising the lungs of lambs, and produces mild pneumonic lesions. Subsequent bacterial invasion increases the severity of these lesions.

Jones et al. (1979) isolated a number of organisms from the respiratory tracts of a flock of housed sheep affected by a proliferative exudative pneumonia viz M. ovipneumoniae, M. arginini, M. conjunctivae, Acholesplasma laidlawii, ureaplasmas and P. haemolytica. M. ovipneumoniae and P. haemolytica were isolated from all cases of proliferative exudative pneumonia. No viruses were isolated, although PI3 virus infection had occurred in the flock as indicated by serology. Although these sheep were in a housed environment (in contrast to the outdoor conditions of New Zealand sheep) the importance of both M. ovipneumoniae and P. haemolytica as aetiological agents of proliferative exudative pneumonia is noted. Because P. haemolytica could only be isolated in low numbers from some cases it was suggested that these bacteria are secondary invaders. It was also suggested that PI3 virus may have enhanced the establishment of mycoplasmas in the lungs, but there was no proof of this. M. arginini, M. conjunctivae, A. laidlawii, and ureaplasmas were not associated with any specific pathological changes in the lungs and their role in the disease was considered negligible.

The above data clearly indicate that ovine CNP may have a complex aetiology. M. ovipneumoniae and P. haemolytica are suspected as major pathogens; M. ovipneumoniae colonises the lungs, and P. haemolytica is probably a secondary invader increasing the severity of the lesions. PI3 virus is suspected by some workers of enhancing the establishment of M. ovipneumoniae in the lungs. This present investigation attempts to clarify further the aetiology of ovine CNP.

1.8 Economic importance of the disease

It has been reported that chronic ovine pneumonia resulted in inadequate weight gain of sheep (St. George *et al.*, 1971; Carmichael *et al.*, 1972). Kirton *et al.* (1976) however, who assessed the effect of chronic pneumonia on the growth rate of sheep in New Zealand got unexpected results. They divided lambs into groups on the basis of the presence or absence of pneumonic lesions at slaughter and compared the carcass weights of the groups. They found that on the average, the carcasses of pneumonic sheep were heavier than the carcasses of sheep without pneumonia. Kirton *et al.* (1976) were obviously unwilling to conclude that pneumonic lesions increased the growth rate so they merely concluded that pneumonia could not be shown to reduce the average carcass weight at slaughter.

In marked, and perhaps amusing contrast, Harris and Alley (1976) felt that the statistically significant differences in carcass weight between pneumonic and non-pneumonic sheep should not be ignored, and indeed argued that Kirton's results could be reconciled with the conclusion that chronic ovine pneumonia does diminish growth rate. This opposed explanation depends upon the observation that most lambs get CNP at some time during the first year of life, and the disease resolves over a period of several months. Thus most of the lambs without pneumonia at slaughter described by Kirton *et al.* (1976) probably represented lambs which became infected with CNP at a sufficiently early stage of life to allow resolution of the disease to occur before slaughter. On the other hand, the pneumonic sheep (heavier) had probably contracted the disease more recently and hence had been affected by it for a shorter and less critical period of their life. So, on the basis of this hypothesis, it could be concluded that CNP has an adverse effect on weight gain of sheep in New Zealand and hence be of economic importance.

Although ovine CNP is characterised by a high morbidity and low mortality, severe cases can extend to involve the visceral pleura, and often secondary pleurisy develops (Dysart, 1976). Carcasses with pleural adhesions are required to be diverted from the main killing chains of freezing works to "detain rails" where the pleural

adhesions are cut out and the carcass is re-examined by a meat inspector. When a heavily infected flock is being slaughtered the detain rail often becomes full, and the killing chain is required to stop until the carcasses are cleared. A direct result of this is the loss of productive man-hours at freezing works.

Even more significantly, carcasses which have had pleural adhesions removed are unacceptable for several major overseas markets viz the U.S.A., Canada, and West Germany, hence the carcasses are down-graded. A figure of $1.8 million was the estimated loss to the New Zealand farmer from pleurisy in lambs in the 1974/5 season (Dysart, 1976). This figure is undoubtedly a gross underestimate for the current season because of a combination of inflation and the continued upgrading of meat hygiene standards for export carcasses.

In conclusion, although the effects of ovine CNP are difficult to measure directly in economic terms it is apparent that the disease is economically important to New Zealand, which relies on lamb exports for a major source of its income.

CHAPTER 2 Survey of the prevalence of mycoplasmas in pneumonic sheep lungs with particular reference to ureaplasmas

2.1 Introduction

Non-progressive pneumonia of sheep is a chronic disease, and by analogy with other chronic respiratory diseases of animals (see Chapter 1) it is likely that mycoplasmas are involved in its aetiology.

Based on serological evidence and high isolation rates, ureaplasmas (T-strains) are considered to play an important role in pneumonia of calves (Shimizu et al., 1975): and indeed experimental pneumonia has been produced in calves by endobronchial inoculation with ureaplasmas (Gourlay and Thomas, 1970). Recently (Jones et al., 1979) ureaplasmas were isolated from the lower respiratory tract of housed sheep in Scotland, but their role in ovine pneumonia was considered negligible.

Over 500 isolations of mycoplasmas from pneumonic and normal sheep lungs have been made during several surveys in New Zealand (Clarke et al., 1974; Clarke, unpublished results). All isolates gave either centred colonies on solid medium and were identified as M. arginini, or gave centreless "lacy" or "vacuolated" colonies and were identified as M. ovipneumoniae. Identification was made using acetone-fixed impression smears stained by immunofluorescent techniques. However, these workers did not employ techniques for isolating ureaplasmas, and since it is now known that ureaplasmas can indeed invade the ovine lung the present investigation undertook a further survey of mycoplasmas in pneumonic sheep lungs which included an isolation procedure which could detect ureaplasmas if they were present.

2.2 Materials and methods

2.2.1 Mycoplasma media

See appendix.

2.2.2 Source of mycoplasmas used to test isolation techniques

(a) Ureaplasmas

The ureaplasma strain in this study was of human origin and was obtained from Dr N. Markham, National Health Institute, Wellington. The organism was recovered during routine isolation procedures undertaken for diagnostic purposes.

(b) M. ovipneumoniae and M. arginini

See appendix.

2.2.3 Test of the ability of U9 medium to support the growth of ureaplasmas

A ureaplasma (described above) from the human genito-urinary tract was used to test the ability of U9 medium to support its growth. 0.3 cm^3 of a broth culture of the ureaplasma stored at -70°C was inoculated into 2.7 cm^3 of U9 broth. This was incubated at 37°C and observed for a pH change. When a pH change occurred, 0.3 cm^3 of the culture was removed and inoculated into another 2.7 cm^3 of U9 broth. This was performed serially more than 10 times. At intervals, 0.05 cm^3 of culture was spotted on solid U9 medium and plates were incubated at 37°C for 2 to 4 days. Typical "tiny" colonies (microcolonies) were seen using a plate microscope at 40x magnification (see figs 1 and 2).

2.2.4 Isolation of mycoplasmas from lung specimens

Four groups of 12 pneumonic lungs were collected randomly from a local freezing works at approximately weekly intervals during April and May. Due to hygiene regulations all the lungs were handled by a meat inspector prior to collection. Pneumonic lesions were aseptically cut off each lung and transported back to the laboratory in sterile petri-dishes. The samples were then cut into small pieces with a scalpel and placed in 3 cm^3 of BHI broth (Difco) to give an approximately 10% suspension. The lung suspension was shaken at intervals for 20 minutes at room temperature. After the solid material had settled, 0.3 cm^3 of the supernatant was added to 2.7 cm^3 each of FM4 broth, FM4A broth, and U9 broth; a further 1:10 dilution of each was then made. The bottles

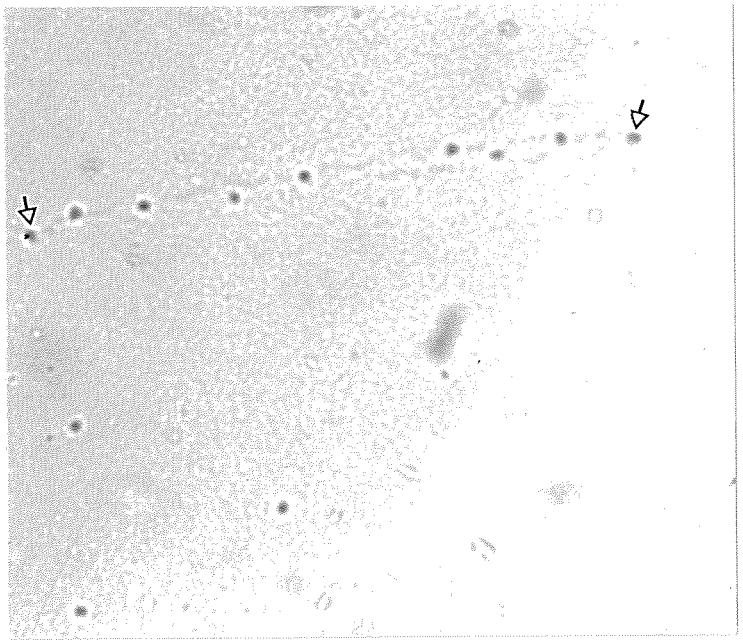


FIGURE 1 Colonies of ureaplasmas on U9 agar (unstained, x 97)

These microcolonies have a diameter of 15-20 µm and are seen as refractile bodies. This is typical of most ureaplasmas. The line of colonies between the two outermost ones (arrowed) are aligned along the edge of the original inoculum.

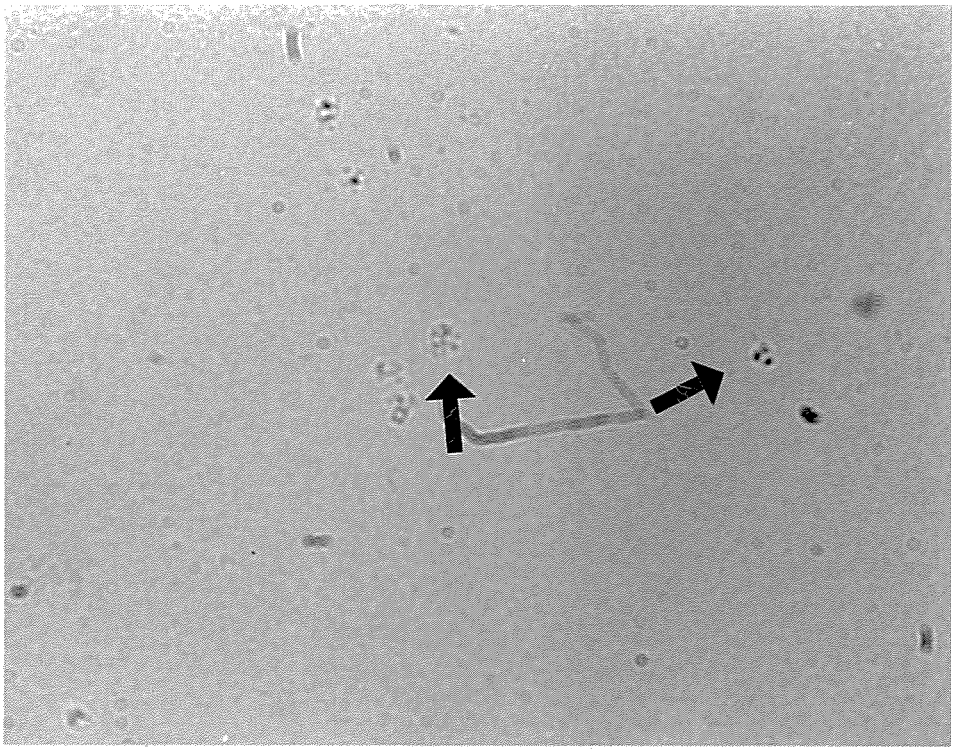


FIGURE 2 Colonies of ureaplasmas on U9 agar (unstained, x 240)

These are similar colonies to those in fig. 1, but are more highly magnified. Note that the colonies do not have the "fried-egg" appearance typical of many mycoplasmas.

were incubated at 37°C for 5 days or until growth occurred as indicated by a colour change in the pH indicator. If growth occurred a 1/10 dilution was made into fresh liquid medium and 0.05 cm^3 was spotted on the equivalent solid medium. If the original specimens showed no growth after 5 days a 1/10 dilution of each bottle was made into fresh liquid medium, and if there was no growth after incubation for a further 4 days 0.05 cm^3 was spotted on solid medium. Plates were examined for colonies after 5 days incubation at 37°C.

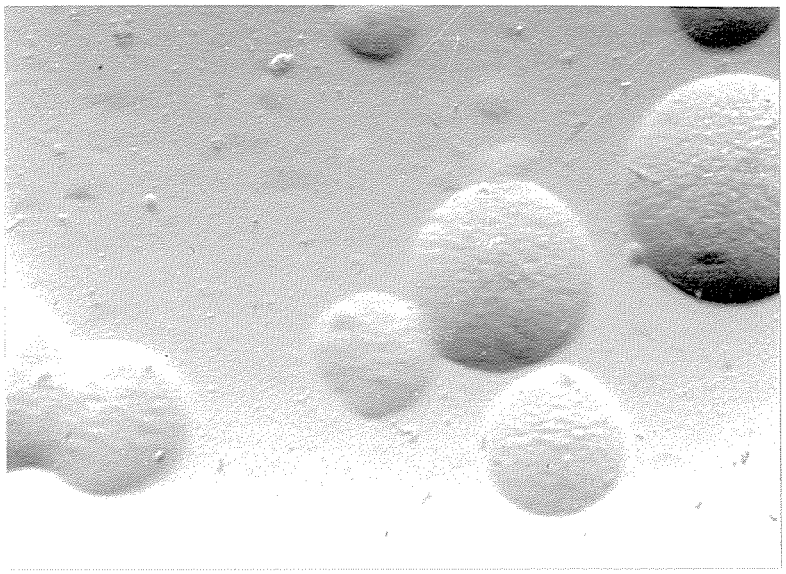
2.2.5 Attempted isolation of ureaplasmas from pneumonic lung homogenate known to transmit ovine CNP

The pneumonic lung material, homogenised in phosphate buffered saline (PBS), used here was from the same pool of pneumonic lungs which was used to transmit CNP as described in Chapter 4. This pool was stored at -70°C until required.

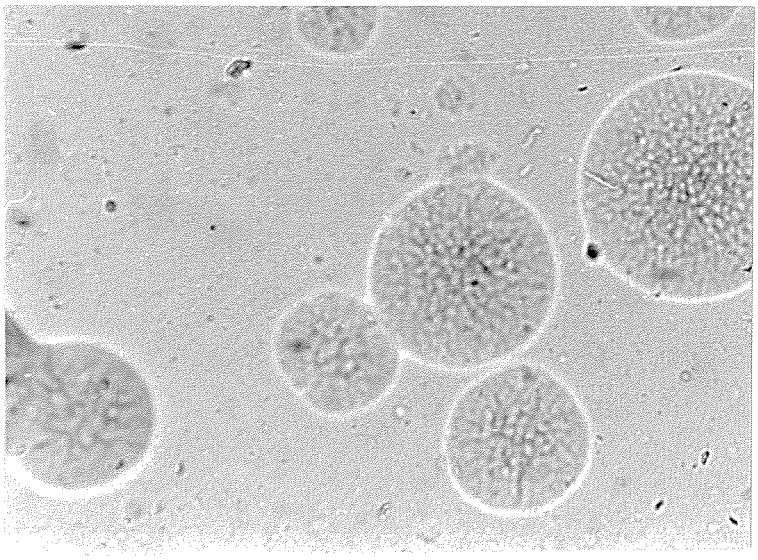
A bottle containing 2.7 cm^3 of U9 broth was inoculated with 0.3 cm^3 of pneumonic lung homogenate and a further two serial 10-fold dilutions of this were made. The bottles were incubated at 37°C until growth occurred as indicated by a colour change, or for 5 days. If there was no growth after 5 days, 0.3 cm^3 was removed from each bottle and added to 2.7 cm^3 of fresh U9 broth. If there was no growth in the fresh broth after a further 4 days 0.05 cm^3 of the broth was spotted on U9 agar. Plates were examined after incubation at 37°C for 2 to 4 days.

2.2.6 Identification of mycoplasmas

A large number of isolations of mycoplasmas from pneumonic sheep lungs performed previously in this laboratory yielded only two species of mycoplasmas which had different colony types. The mycoplasma which fermented glucose (hence changing FM4 medium from red to yellow) and always gave centreless "lacy" or "vacuolated" colonies on solid medium (see fig. 3) was identified serologically as M. ovipneumoniae. The mycoplasma which hydrolysed arginine (hence changing FM4A medium from orange to purple) and always gave centred colonies on solid medium (see fig. 4) was serologically identified as M. arginini. The present investigation therefore relied on the appropriate pH change and colony type for identification of the isolates.



3(a)



3(b)

FIGURE 3 Colonies of M. ovipneumoniae on 1% agar viewed with (a) oblique, and (b) transmitted light (x 62)

The colonies are centreless and have a "lacy" or "vacuolated" appearance.

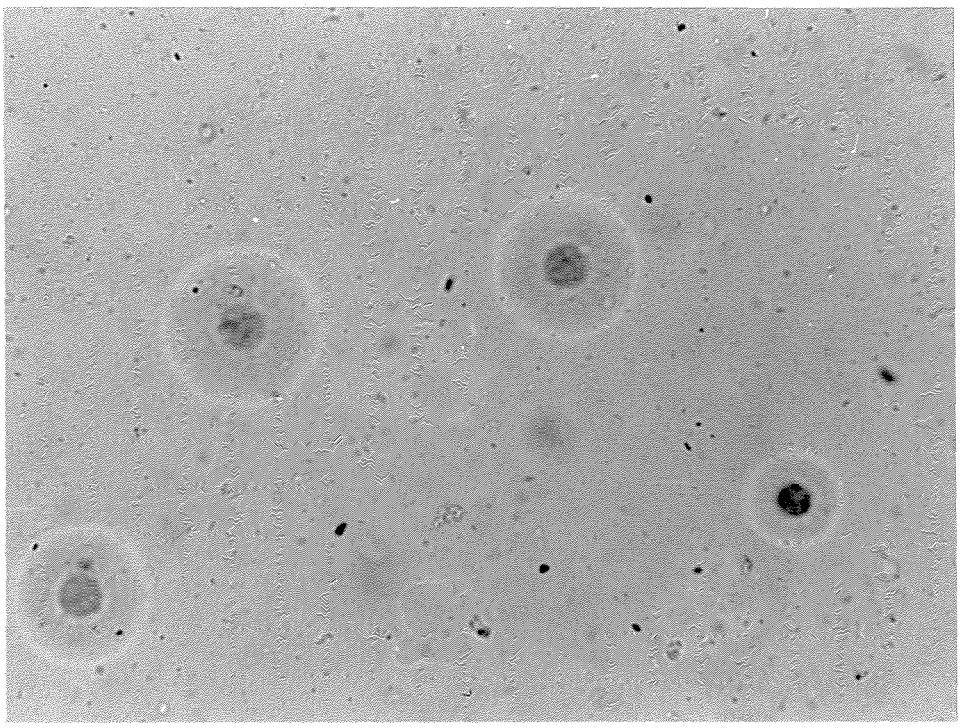


FIGURE 4 Colonies of M. arginini on 1% agar viewed with transmitted light (x 47)

The colonies have the typical "fried-egg" appearance of many mycoplasmas. This is due to the organisms growing into the agar in this central portion.

2.3 Results

The results of the mycoplasma isolations from the 48 lungs examined are summarised in Table I.

TABLE I Isolation of mycoplasmas from pneumonic sheep lungs

Organism	Number of lungs examined	Number of lungs giving positive isolation	Proportion of lungs giving positive isolation
M. ovipneumoniae	48	47	98%
M. arginini	48	2	4%
Ureaplasmas	48	0	0%

It should be noted that nearly all of the pneumonic lungs were positive for M. ovipneumoniae and only a small proportion positive for M. arginini. There were no ureaplasmas isolated from any of the lungs, as indicated by the appropriate colour change in liquid medium, or the presence of microcolonies on solid medium. Nor were there any ureaplasmas isolated from the pneumonic lung homogenate known to transmit ovine CNP.

2.4 Discussion

The main observation of this study was that ureaplasmas could not be isolated from any of 48 pneumonic lungs collected from different flocks of sheep over a period of approximately one month in the Manawatu region. Nor were ureaplasmas isolated from pooled pneumonic lung homogenate which was known to transmit CNP when inoculated intranasally into sheep. It is concluded that ureaplasmas are not common inhabitants of the lungs of sheep with CNP, and that they are not necessary for experimental transmission of the disease.

It is relevant to emphasise that the U9 medium used in this study was shown to be capable of supporting the growth of a human ureaplasma strain, and that the medium was developed for the detection and identification of ureaplasmas from clinical material (Shepard and

Lunceford, 1970). It is also relevant that U9 medium is essentially the same as the medium used by Jones et al. (1979) who isolated ureaplasmas from the lower respiratory tract of sheep in Scotland (i.e. the pH of the medium was 6.0, and it contained serum and urea).

A survey of the prevalence of M. ovipneumoniae and M. arginini in pneumonic sheep lungs in New Zealand was performed by Alley et al. (1975) who found that M. ovipneumoniae was present in 79% and 100% of pneumonic lungs in two samplings and M. arginini was recovered from 32% of pneumonic lungs. A comparison with the present 98% recovery rate for M. ovipneumoniae and 4% for M. arginini from pneumonic lungs suggests that M. ovipneumoniae is consistently present in a high proportion of pneumonic lungs, whereas the isolation rate of M. arginini varies, but is lower than that of M. ovipneumoniae.

These data, combined with the failure to isolate ureaplasmas from pneumonic sheep lungs, suggest that if indeed there is a mycoplasmal aetiology in ovine CNP, then M. ovipneumoniae is most likely to be the organism implicated. In contrast,ureaplasmas are not essential for the development of the lesions. The role of M. arginini is not clear: its absence from most lesions suggests that it is not a primary pathogen, but its presence in at least a minority of lesions in all surveys does suggest the possibility that it may exacerbate existing lesions. The characteristics of this organism are therefore examined and reported in the next chapter.

CHAPTER 3 An in vitro investigation of the growth and ultrastructure of M. arginini, and the comparison of the ultrastructure of M. arginini with that of M. ovipneumoniae

3.1 Introduction

Mycoplasma arginini has been frequently isolated from the respiratory tract of normal and pneumonic sheep (Carmichael et al., 1972; Foggie and Angus, 1972; Clarke et al., 1974; Alley et al., 1975: Jones et al., 1978; El Mahi and Nayil, 1978). However its role, if any, in the pathogenesis of ovine CNP is uncertain.

In Scotland, Foggie and Angus (1972) demonstrated that the strain of M. arginini they used in transmission experiments was not pathogenic for SPF lambs. Jones et al. (1978) inoculated 6 to 7 month old lambs with a mixture of P. haemolytica, M. ovipneumoniae and M. arginini and concluded that P. haemolytica and mycoplasmas may commonly be the aetiological agents involved in proliferative exudative pneumonia (similar to ovine CNP). They noted that the recovery of M. arginini from experimental animals was inconsistent, but stated that M. arginini infection of sheep lung was closely associated with both the development of pleurisy and an increased probability of death. Subsequently, however, the same group of workers (Jones et al., 1979) concluded that the presence of M. arginini was unimportant in the disease.

In New Zealand, Alley et al. (1975) and Alley (1975b) undertook microbiological surveys of lungs of lambs at slaughter which showed that M. ovipneumoniae could be isolated from lesions of CNP at all stages of development, whereas bacteria and M. arginini could be found consistently only in lesions of a substantial size or duration.

The above data indicate that while it is unlikely that M. arginini is a primary pathogen in ovine CNP, the organism may well be important as a secondary invader, and since the aetiology of the disease is thought to be complex it is important to establish its role, if any, in the pathogenesis of the disease.

An investigation into the significance of M. arginini in CNP of sheep in New Zealand could be facilitated both by transmission experiments using a local isolate alone, and in association with other organisms; and by electron microscopic examination of pneumonic lungs, especially if M. ovipneumoniae and M. arginini could be distinguished in thin section. These objectives require that a high titre inoculum of M. arginini in the logarithmic growth phase be available, and that its ultrastructure be established. Since M. arginini is a ubiquitous organism it is surprising that this information is not apparently available; thus the 8th edition of Bergey's Manual of Determinative Bacteriology states that the morphology of M. arginini is "poorly defined".

Previous workers (Major et al., 1979) reported the in vitro growth and ultrastructure of M. ovipneumoniae.

The following investigation was undertaken as an initial in vitro study to be ultimately followed by an in vivo investigation. The objectives were:

i) To provide data which will enable high titre inocula in the logarithmic phase of growth to be obtained consistently for future transmission experiments to assess the role of M. arginini as a pulmonary pathogen of sheep.

ii) To determine, in defined conditions, at what stage of growth the logarithmic, stationary, and death phases occur so that the ultrastructure of M. arginini can be determined at each of these phases.

iii) To compare the ultrastructure of M. arginini with that described for M. ovipneumoniae by Major et al. (1979).

3.2 Growth curve of M. arginini

3.2.1 Materials and methods

(a) Source of M. arginini

See appendix.

(b) FM4A medium

See appendix.

(c) Growth curve of M. arginini

M. arginini was propagated in FM4A medium and frozen in aliquots at -70°C at a titre of 1.5 x 10^8 colony forming units (CFU)/cm^3.

A 100 cm^3 conical flask containing 19.8 cm^3 of FM4A broth prewarmed to 37°C was inoculated with 0.2 cm^3 of M. arginini culture from -70°C. The flask was fitted with a screw cap to prevent excessive evaporation of the medium which was noted in preliminary experiments. The culture was incubated at 37°C on a rotary shaker at 125 r.p.m. The viable count was estimated every 3 hours by removing 0.2 cm^3 of the culture and serially diluting it in FM4A broth. A 0.05 cm^3 volume from each dilution was spotted on FM4A agar and plates were incubated at 37°C for 5 days. Colonies were counted using a plate microscope.

3.2.2 Results

The growth curve of M. arginini was repeated 3 times and gave consistent results. A typical result is seen in figure 5. The following points were noted:

i) During the first 6 hours of incubation there was an approximately 10-fold decline in titre of the organism.

ii) After this initial drop there was a rapid increase in titre (the logarithmic phase) to a maximum of approximately 1.2 x 10^9 CFU/cm^3 after 18 hours.

iii) This was followed by a period of 12 hours during which the titre changed by less than a factor of 2-fold (stationary phase) after which the titre dropped off rapidly (death phase) to a titre of 6.4 x 10^4 CFU/cm^3 after 60 hours incubation.

iv) The mean generation time calculated from the slope of the graph at the maximum rate of growth was approximately 0.5 hours.

v) The half-life of the organisms in the death phase was approximately 2.5 hours.

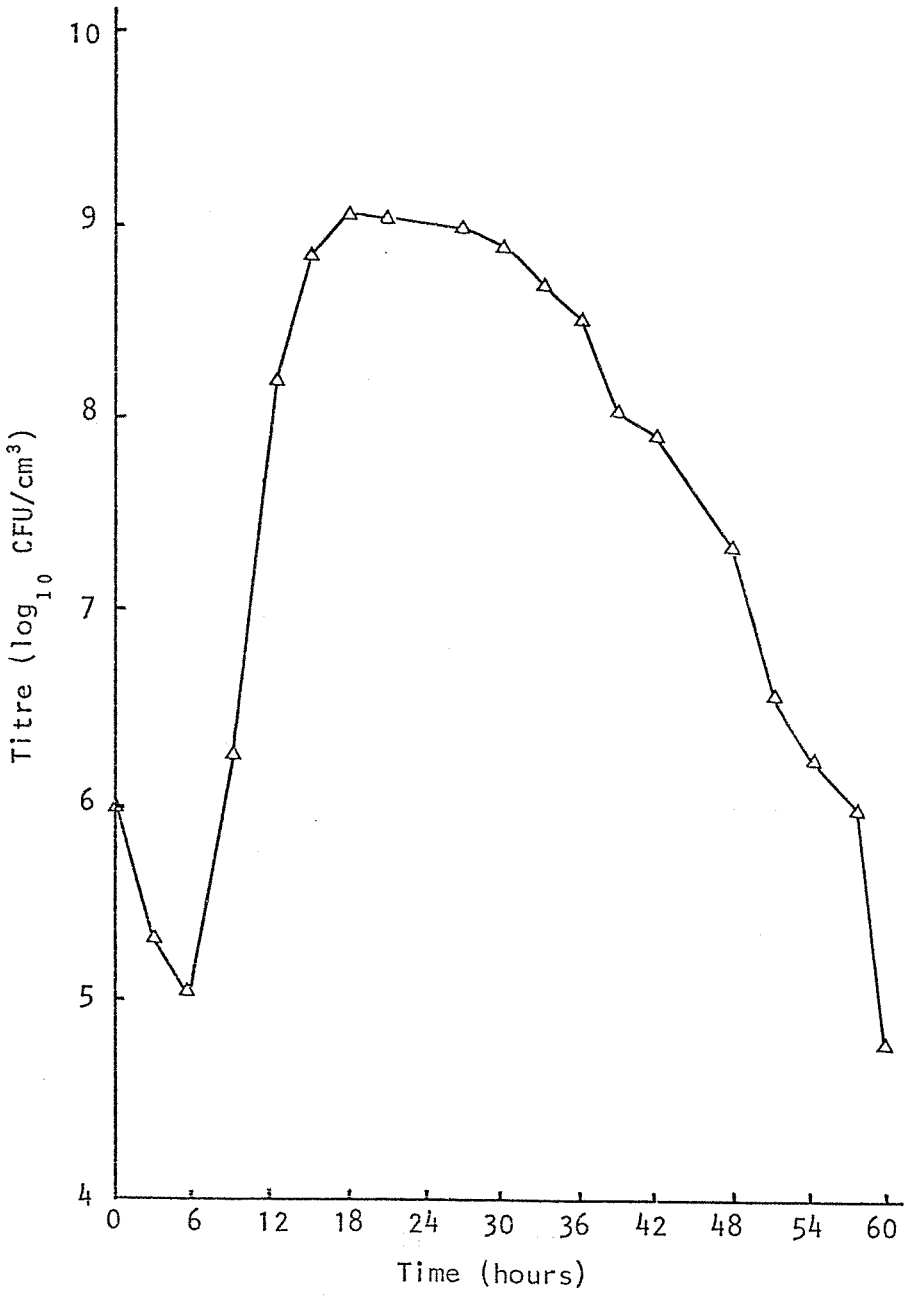


FIGURE 5: The growth curve of M. arginini

3.3 Ultrastructure of *M. arginini* and *M. ovipneumoniae*

3.3.1 Materials and Methods

(a) Reagents for electron microscopy

Karnovsky's fixative (modified)

$Na_2HPO_4.12H_2O$	12.55 g
KH_2PO_4	2.05 g
paraformaldehyde	10.0 g
gluteraldehyde (25% solution)	60.0 cm^3

The paraformaldehyde (10.0 g in 420 cm^3 distilled water) was heated to 60 to 70°C, then 1.0N NaOH added dropwise until the solution cleared. The buffer salts and gluteraldehyde were then added. The solution was stored at 4°C.

0.1M Phosphate buffer pH 7.2

$Na_2HPO_4.12H_2O$	2.51 g
KH_2PO_4	0.41 g
Distilled water to	100.0 cm^3

Osmium tetroxide

Osmium tetroxide was made up as a 2% aqueous solution, and kept in a dark bottle at 4°C. Before use it was diluted with an equal volume of 0.1M phosphate buffer pH 7.2.

Spurr's resin

ERL 4206	1.25 g
DER 736	1.0 g
NSA	3.25 g
S-1	0.05 g

The components were thoroughly mixed before use.

Uranyl acetate

The stain was made by adding uranyl acetate powder to 50% ethanol until saturation was reached, and then shaking intermittently for 10 to 15 minutes. The stain was filtered into a brown bottle and stored at 4°C.

Lead citrate stain

Lead citrate	0.025 g
Distilled water	10.0 cm^3
10N NaOH	0.1 cm^3

Shake until dissolved.

(b) Propagation of M. arginini

Replicate 500 cm^3 conical flasks containing 99 cm^3 of prewarmed FM4A broth were inoculated with 1 cm^3 of a culture of M. arginini stored at -70°C in the exponential phase. The flasks were incubated at 37°C on a rotary shaker at 125 r.p.m.

At intervals corresponding to the log, stationary and death phases of growth (i.e. 12 hours, 25 hours, and 50 hours respectively) 100 cm^3 of the culture was removed and centrifuged at 14,500 g for 30 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded and the pellets were fixed and embedded as below.

(c) Fixation and embedding of M. arginini

M. arginini was propagated and centrifuged as described above. The pellets were resuspended in modified Karnovsky's fixative for 1 hour at 4°C. The cell suspension was centrifuged, washed once with 0.1M phosphate butter pH 7.2, and centrifuged again. The pellet of cells was resuspended in 1% osmium tetroxide for 1 hour at 4°C. The post-fixed cells were centrifuged and the pellet was washed with 0.1M phosphate buffer pH 7.2. The pellet was removed, cut into small pieces, dehydrated and infiltrated as follows:

i) 1 x 50% acetone for 5 minutes
ii) 2 x 70% acetone for 5 minutes each
iii) 3 x 100% acetone for 10 minutes each
iv) 1:1 mixture of acetone and Spurr's resin for 20 minutes
v) 2 x 100% Spurr's resin for 10 minutes each.

The fragments were then placed on the surface of resin-filled gelatin capsules and allowed to sink to the bottom. The capsules were polymerised by heating in an oven at 70°C for 24 hours.

(d) Sectioning, staining, and examination of specimens

The gelatin capsule was removed from the polymerised block. The face of the block was cut flat and a "mesa" was cut on the face using an LKB 11800 pyramitome. Thin sections were cut off the mesa using an LKB-Huxley ultramicrotome and were floated off the knife edge into a "boat" filled with a 0.25% solution of teepol in distilled water. Gold and silver coloured sections were picked up with copper electron-microscope grids dipped in a sellotape-chloroform mixture.

The sections were stained in uranyl acetate for 5 minutes, washed in 50% ethanol for 30 seconds and then washed thoroughly in distilled water. They were then stained in lead citrate for 5 minutes, washed thoroughly in distilled water, and air dried.

Stained sections were examined in a Philips EM 200 electron microscope and photomicrographs of the mycoplasmas were taken.

(e) Electron microscopy of M. ovipneumoniae

M. ovipneumoniae was propagated in FM4 broth as described for M. arginini previously and a 100 cm^3 sample was removed in the logarithmic phase of growth (Major et al., 1979). These mycoplasmas were prepared for electron microscopy by the same techniques used for M. arginini.

3.3.2 Results

(a) Ultrastructure of M. arginini

Log phase: The cells were roughly spherical in shape although slightly elongated or dumb-bell shaped forms were observed (figs 6, 7 and 8). They usually measured 200 to 500 nm in diameter and were limited by a trilaminar membrane approximately 12 nm thick. There was no evidence that this membrane bore surface projections or was surrounded by capsular material. The cytoplasm was uniformly electron dense and granular. No budding or filamentous forms were seen.

Stationary phase: These resembled the log phase cells in most respects but fewer elongated and dumb-bell shaped cells were observed, and the cytoplasm of many cells was less electron dense than was observed with the log phase cells (figs. 9, 10 and 11).

Death phase: The cells varied greatly in size. The largest cell seen (fig. 12) measured about 750 nm in diameter. A few were obviously lysed and most others, although apparently intact had a relatively electron-lucent cytoplasm suggesting that some of the cell contents may have leaked (figs 13 and 14). The smallest "cells" in the death phase cultures were roughly spherical and measured 110 to 150 nm in diameter. They apparently formed by "pinching-off" from large cells (fig. 13). This "pinching-off" process frequently appeared to be incomplete in that pairs or short chains of "mini-cells" were seen (fig. 12).

(b) Ultrastructure of M. ovipneumoniae

The ultrastructure of M. ovipneumoniae was described by Major et al. (1979). However the techinques used differed in some respects from those used to examine M. arginini in the present study. Since one object of the present investigation was to find if individual cells of M. ovipneumoniae and M. arginini can be distinguished in thin section it was desirable to examine both organisms using identical techniques.

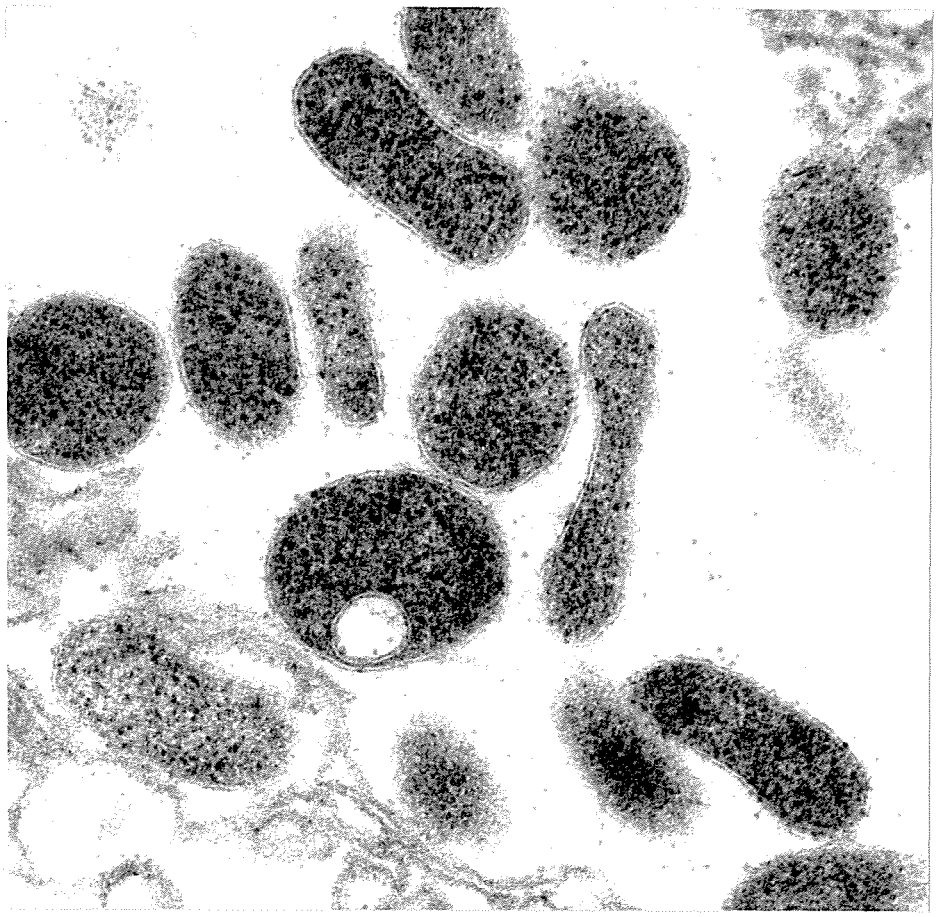


FIGURE 6 Exponential phase culture of M. arginini (x 61,000)

Note the presence of both spherical and elongated cells; and the electron-dense, granular cytoplasm. The cells are limited by a trilaminar membrane which does not bear surface projections, although a slight "fuzziness" can be seen outside the membrane of some cells.

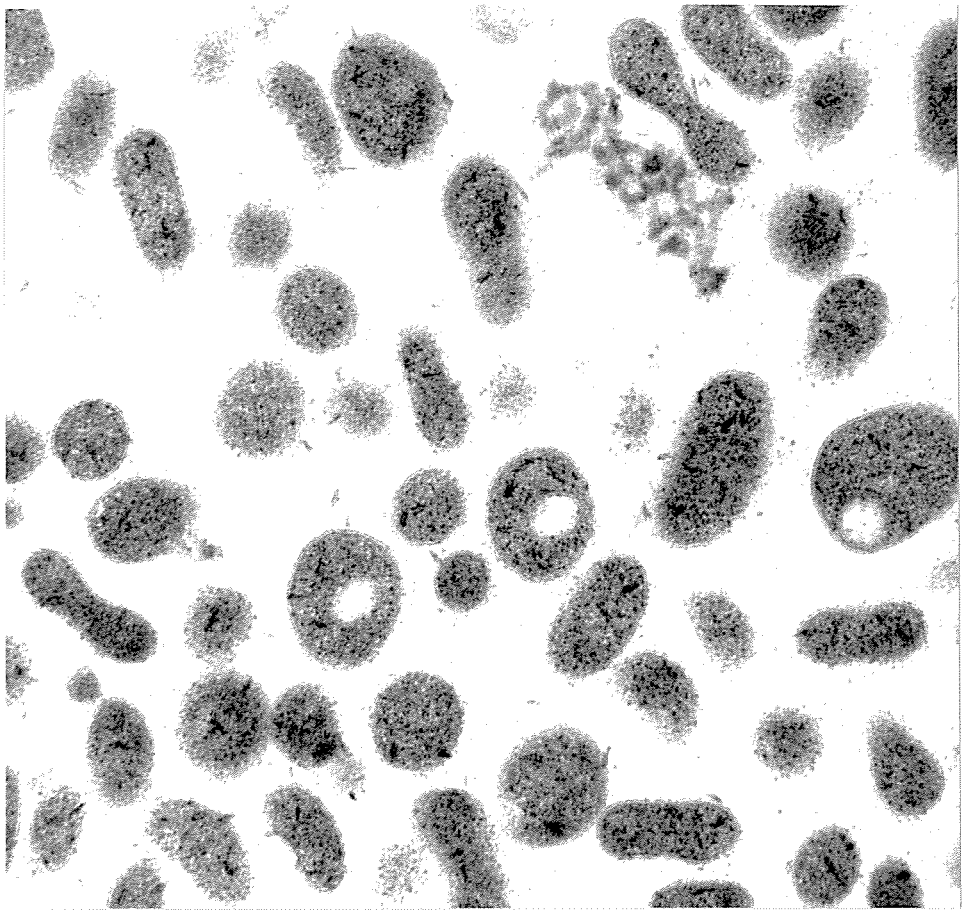


FIGURE 7 Exponential phase culture of M. arginini (x 36,000)

Spherical, elongated and dumb-bell shaped cells are present. The presence of dumb-bell shaped cells in such cultures implies that the organisms replicate by binary fission.

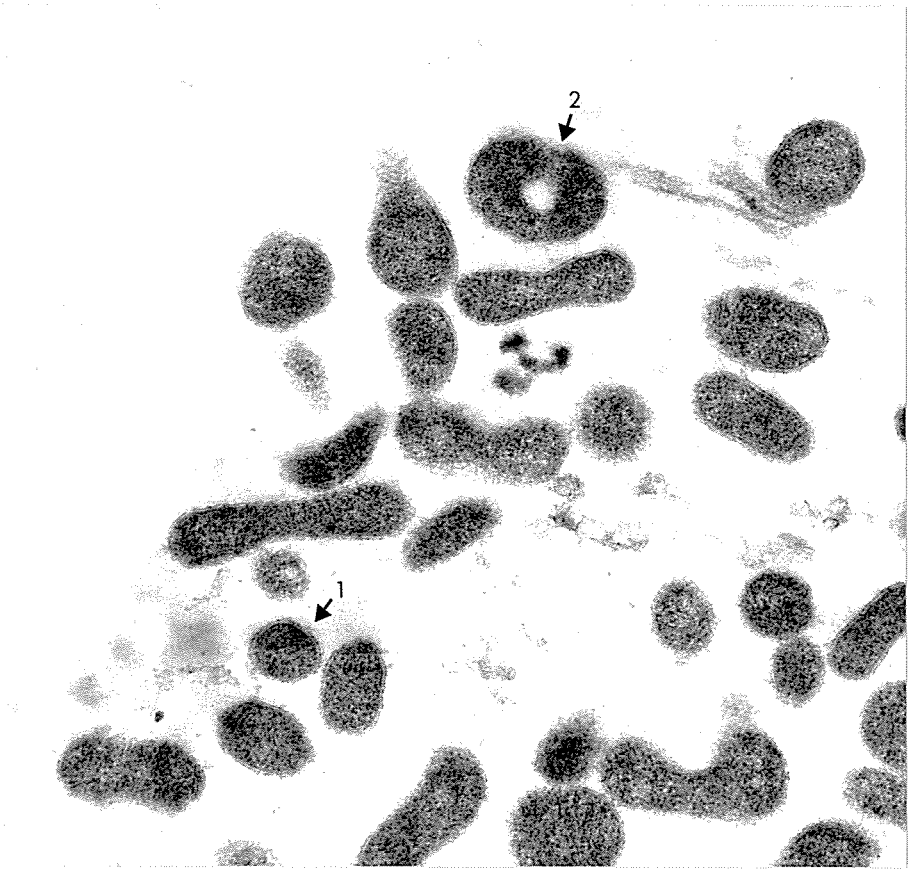


FIGURE 8 Exponential phase culture of M. arginini (x 34,000)

The cell arrowed (1) measures 260 nm in diameter and the cell arrowed (2) measures 550 nm x 380 nm. Several dumb-bell shaped cells are seen.

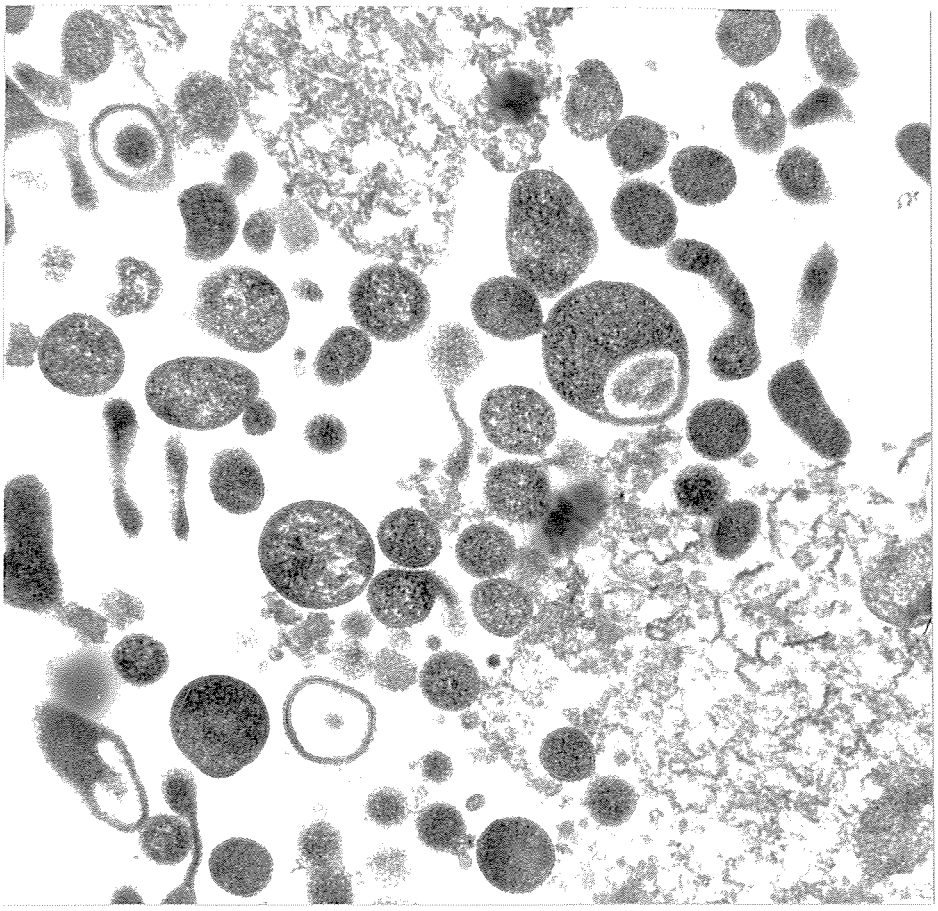


FIGURE 9 Stationary phase culture of M. arginini (x 25,000)

Note that there are fewer elongated and dumb-bell shaped cells than in the exponential phase.

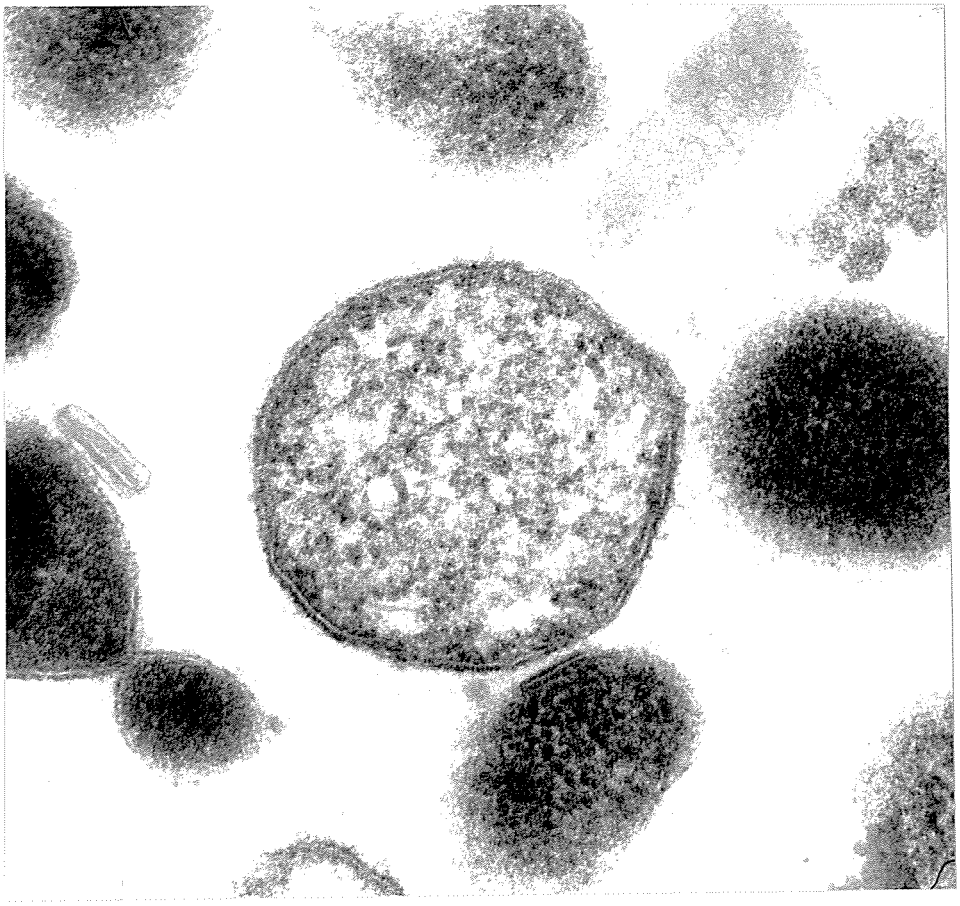


FIGURE 10 Stationary phase cell of M. arginini (x 98,000)

The cytoplasm of this cell is less electron-dense than exponential phase cells.

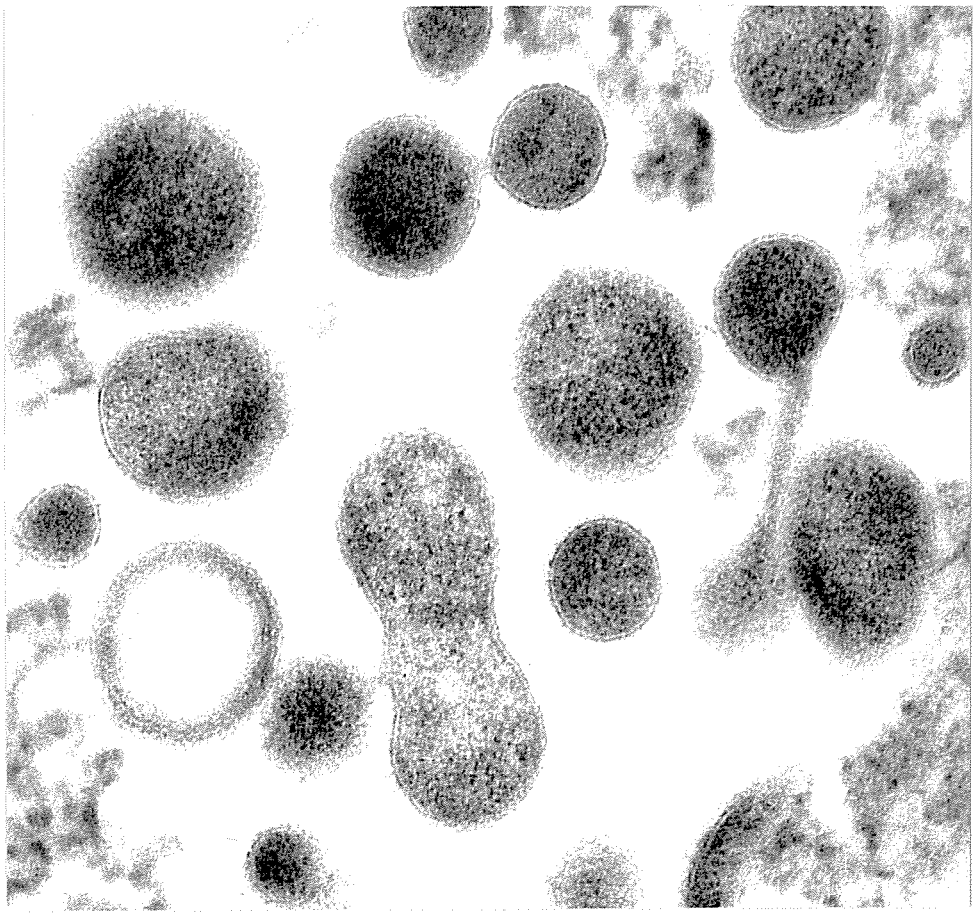


FIGURE 11 Stationary phase cells of M. arginini (x 61,000)

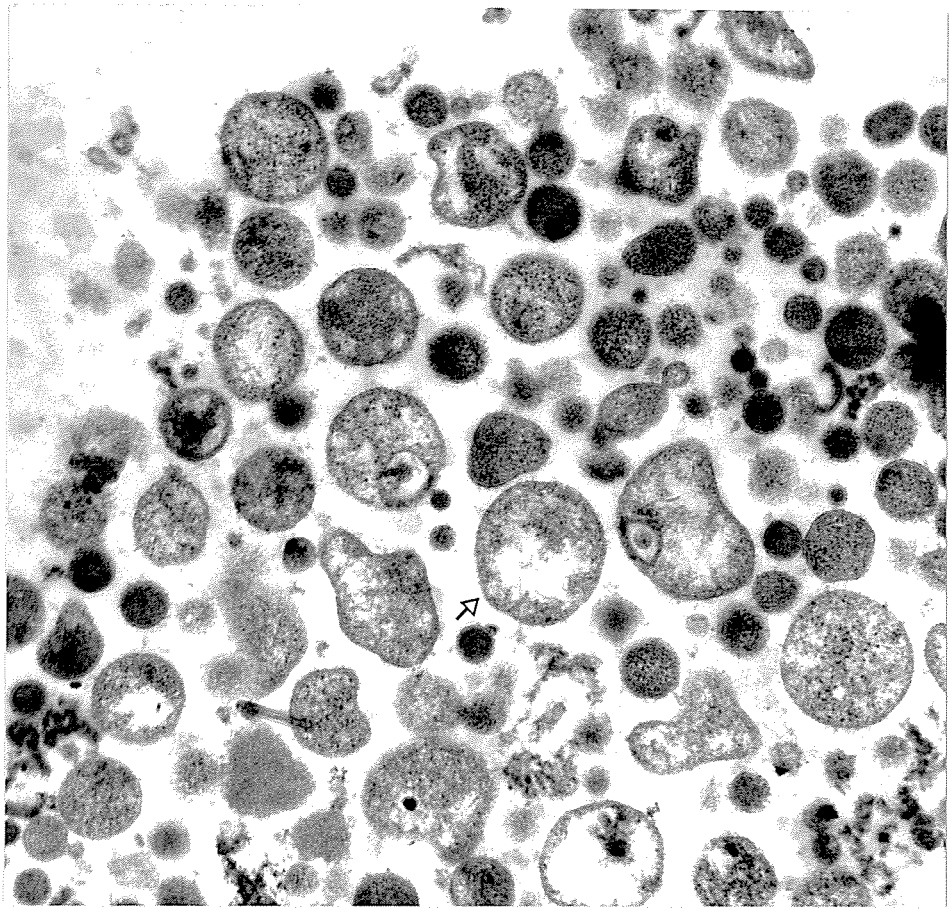


FIGURE 12 Death phase culture of M. arginini (x 25,000)

Note the size variation in the cells, and the electron-lucent cytoplasm of many cells. The arrowed cell measures approximately 750 nm in diameter.

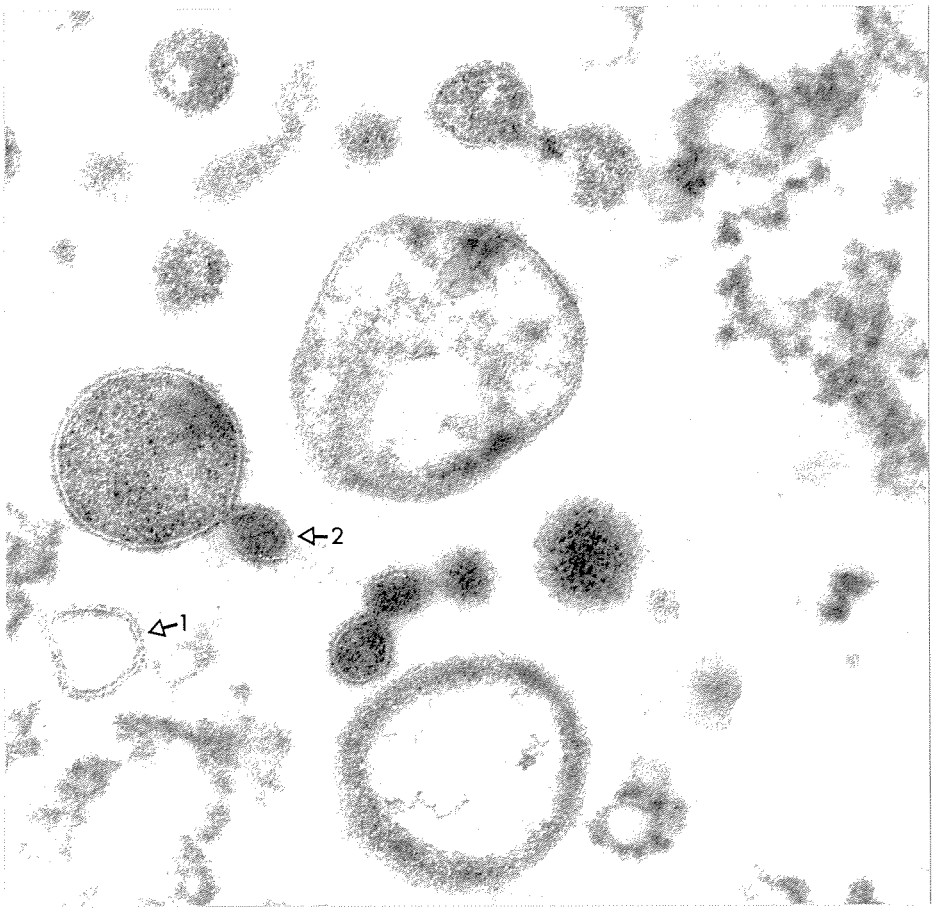


FIGURE 13 Death phase cells of M. arginini (x 61,000)

The presence of membrane fragments (1) suggests that many cells have lysed. Note the "mini-cell" (2) which is apparently "pinching-off" from a larger cell.

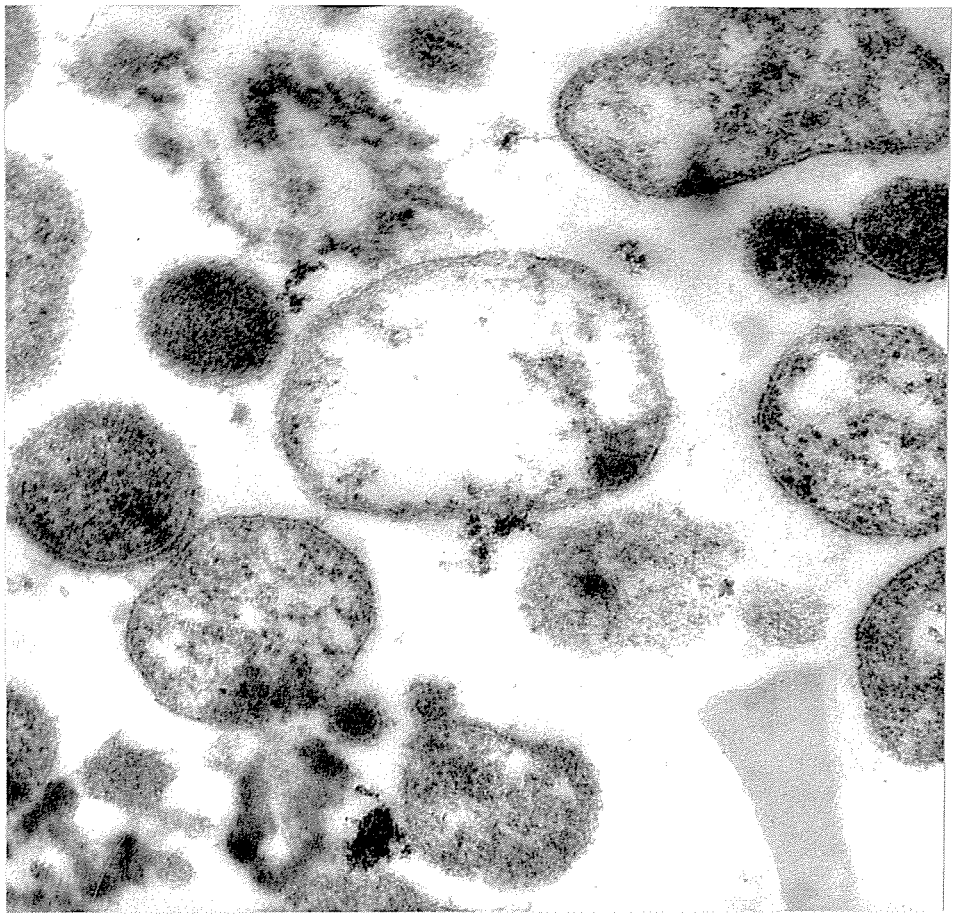


FIGURE 14 Death phase cells of M. arginini (x 69,000)

Note the electron-lucent cytoplasm of several of the cells. The largest cell in this group measures approximately 720 nm x 500 nm.

The observed ultrastructure of M. ovipneumoniae (figs 15 to 18), however, did not differ in the present study from that described by Major et al. (1979). The main features of the ultrastructure of M. ovipneumoniae are listed below:

i) The cells were predominantly spherical, but elongated and dumb-bell forms were also seen.

ii) The cells were usually between 500 and 800 nm in diameter, and were limited by a trilaminar membrane whose outer surface was covered by projections approximately 12 nm in length.

3.4 Discussion

3.4.1 Growth curve of M. arginini

An idealised growth curve typical of many mycoplasmas was described by Smith (1971). It consisted of a short lag phase in which the titre of organisms remained constant, followed by a period of exponential growth to the maximum titre, then, without a significant stationary phase, a period of exponential death immediately occurred.

The growth curve of the M. arginini strain used in this study differed from the idealised growth curve of Smith in two features: a marked fall in the initial titre occurred, and a stationary phase was noted.

The initial decline in titre is unusual, but not unique: thus Low and Eaton (1965) and Boatman and Kenny (1970) reported a similar phenomenon in M. pneumoniae and M. felis respectively, but no explanations were offered. An hypothesis to account for this phenomenon is that when M. arginini is taken from storage at -70°C, its metabolic and/or physical state is such that the organism survives better on solid medium than in liquid medium. Further studies, however, would be needed to investigate this.

It is interesting to note that the maximum generation time of 0.5 hours calculated for M. arginini in the present study approaches that of E. coli which is one of the most rapidly growing organisms, and in rich medium has a generation time of approximately 20 minutes (Clancy, 1977).

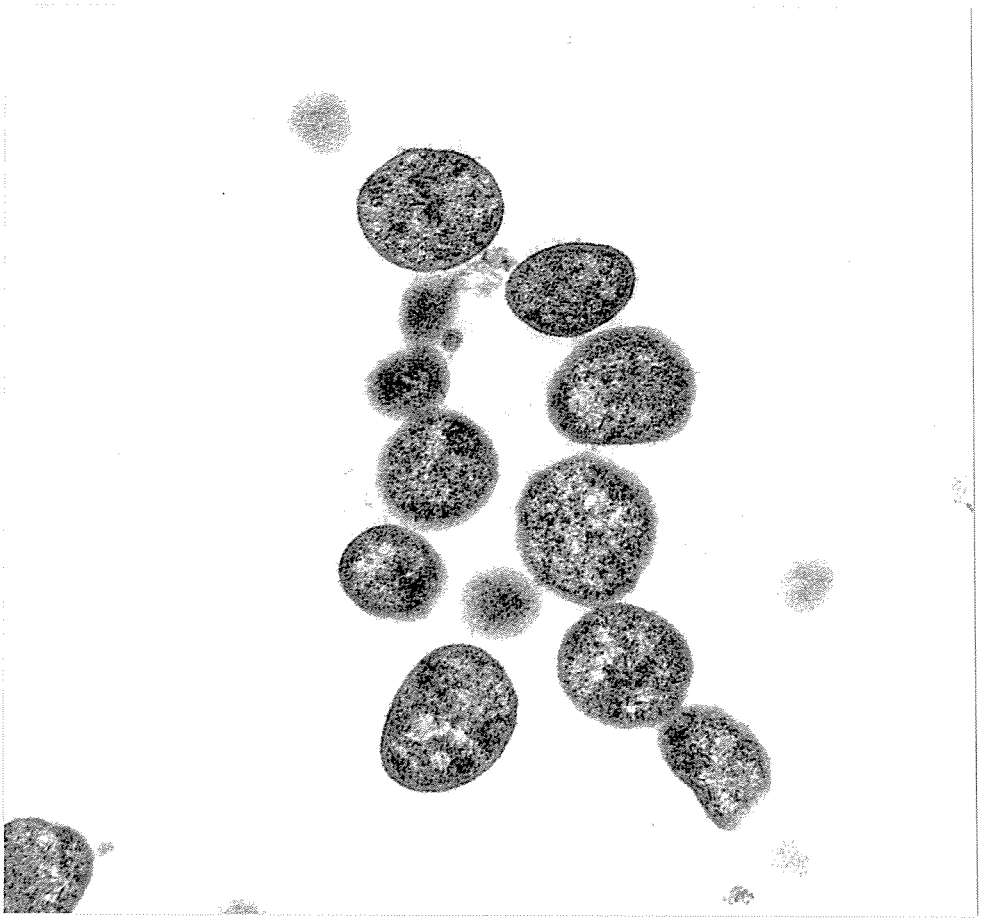


FIGURE 15 Exponential phase M. ovipneumoniae cells (x 26,000)

The cells are predominantly spherical to ovoid in shape and have an electron-dense, granular cytoplasm.

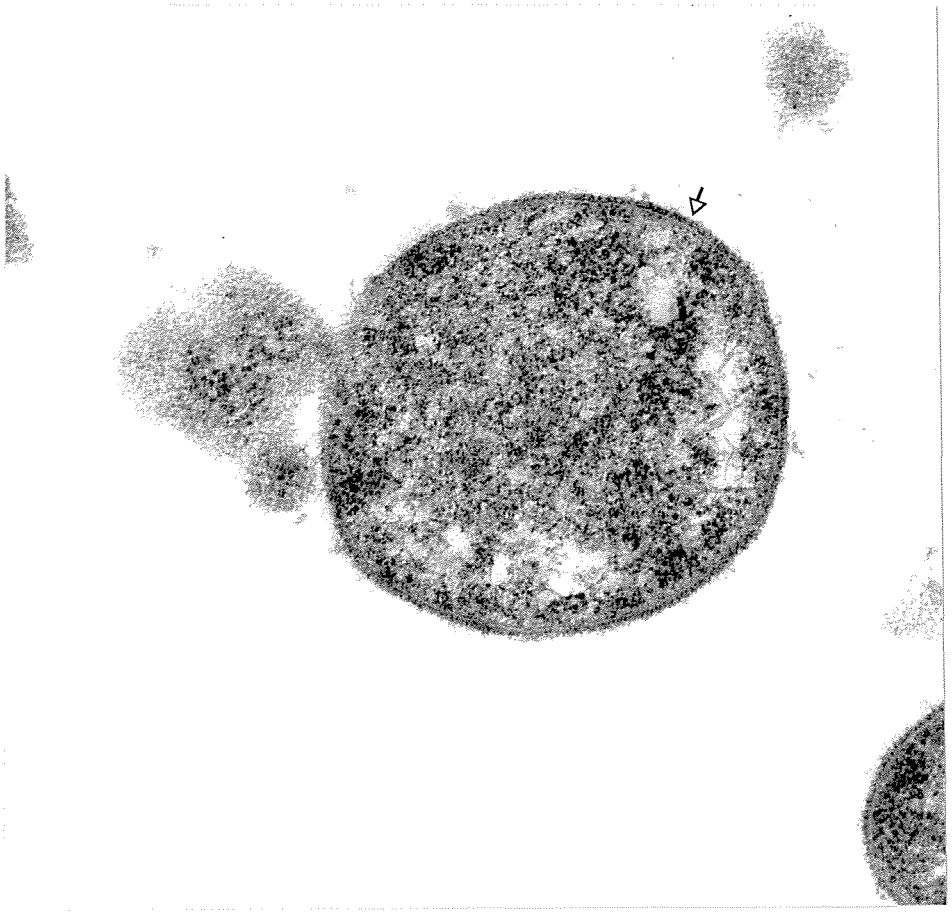


FIGURE 16 Exponential phase M. ovipneumoniae cell (x 68,000)

Surface projections about 12 nm in length outside the limiting membrane can be seen (arrowed). This is a large cell measuring about 900 nm in diameter.

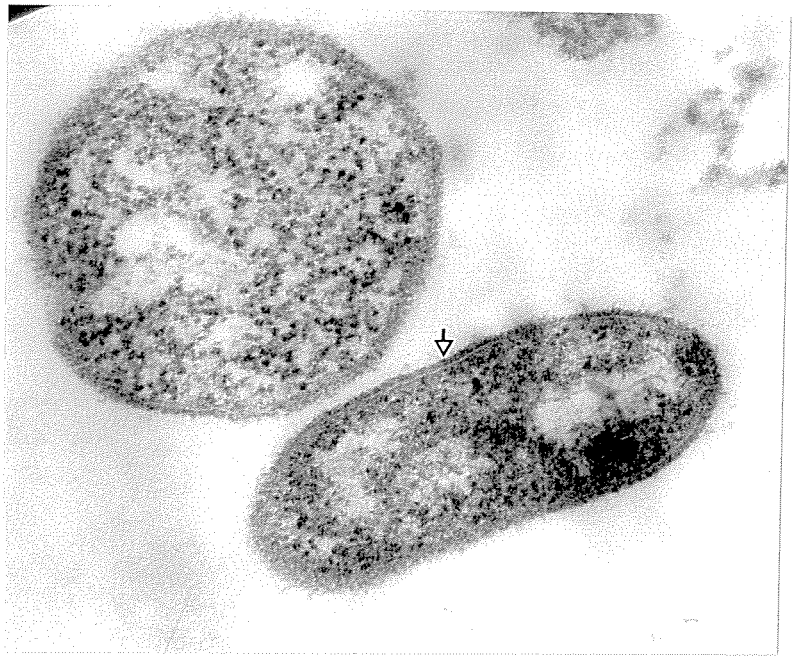


Figure 17

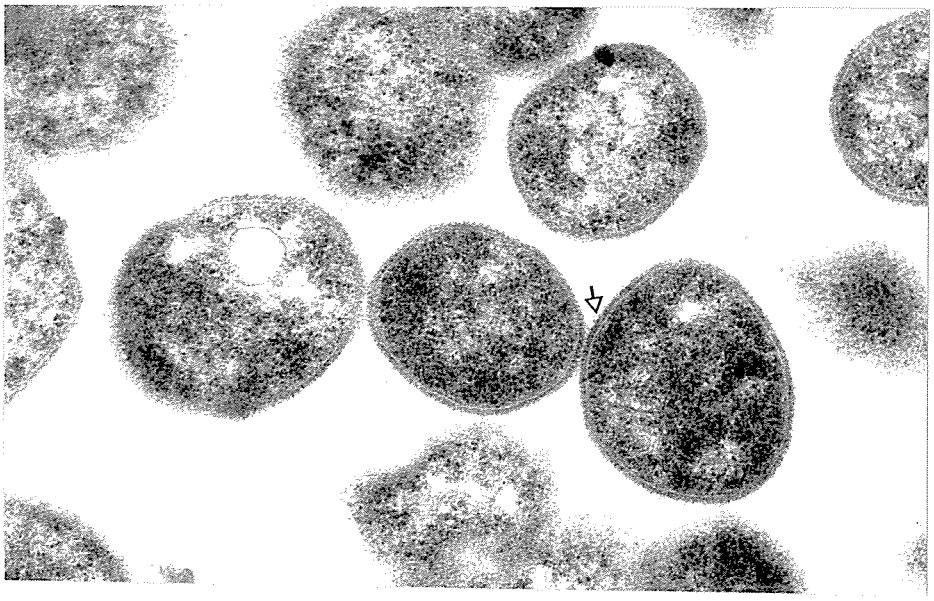


Figure 18

FIGURES 17 & 18 Exponential phase M. ovipneumoniae cells (Fig. 17 x 64,000; Fig. 18 x 42,000)

Surface projections can be seen outside the membrane of the cells (arrows).

It is generally accepted that the generation time of micro-organisms decreases as the quality of the medium used for propagation improves. As compared with the generation times of other mycoplasmas (see table II), M. arginini propagated in FM4A medium has a remarkably short generation time. It is concluded that FM4A medium is a particularly satisfactory medium for growing M. arginini, and this is not surprising as the original FM4 medium was developed for the isolation of a fastidious mycoplasma viz M. gallisepticum.

TABLE II Generation times determined for some mycoplasmas

Species	Mean generation time (hr)
M. arthritidis	3.3
M. arthritidis	1.7 - 2.4
M. gallisepticum	0.8 - 1.0
M. felis	1.0 - 1.5
M. ovipneumoniae	1.7*
M. pneumoniae	6
Ureaplasmas	≃1 (est)
M. arginini	0.5**

Adapted from Smith, P.F. (1971)

* Ref: Major et al. (1979).

** From the results of the present investigation.

It was stated by Thomas (1973) that the maximum titre attainable by mycoplasmas in broth culture is about 5×10^9 CFU/cm^3 and the maximum titre of about 1.2×10^9 CFU/cm^3 reported here is another indication that FM4A medium is highly satisfactory for the growth of M. arginini.

One of the major reasons the growth curve of M. arginini was studied was to obtain in defined conditions, reproducible data which will allow high titre logarithmic phase inocula of the organism to be prepared in future transmission experiments. Such inocula may be obtained by harvesting the mycoplasmas after 12 to 15 hours incubation in the same conditions described for the growth curve. Most cells at this time should be in the logarithmic phase and the titre should be approximately 2 to 7×10^8 CFU/cm^3.

The results of future transmission experiments using such inocula may assist in determining the aetiological role of M. arginini in ovine CNP. These further experiments, however, lie outside the scope of the present thesis.

3.4.2 Ultrastructure of M. arginini and M. ovipneumoniae

The ultrastructure of both organisms essentially fits the general description of the ultrastructure of the genus Mycoplasma in the 8th edition of Bergey's Manual of Determinative Bacteriology (1974). However, the average sizes of both organisms are somewhat larger than the 125 to 250 nm diameter reported in Bergey.

The ultrastructure of M. arginini was examined in all 3 phases of growth to avoid the possibility of mistaking degenerate forms for part of the life cycle of the mycoplasma. The presence of many dumb-bell shaped cells in the logarithmic growth phase, less in the stationary phase, and their absence in the death phase indicates that the organisms divide by binary fission.

The typical trilaminar membrane of mycoplasmas according to Bergey is 7 to 10 nm thick, although Maniloff (1972) stated that the thickness of mycoplasma membranes falls into 2 size ranges, 7 to 8 nm and 10 to 12 nm. So the 12 nm membrane reported here is within reported findings, although the figure is relatively high. There is an obvious lack of well defined extracellular structures such as surface projections which are present, for example, on M. ovipneumoniae (Major et al., 1979), or capsular material which usually extends 10 to 25 nm outside the membrane (Black et al., 1972; Green and Hanson, 1973; Howard and Gourlay, 1974). However, in some electron-micrographs a slight "fuzziness" can be seen outside the cell membrane. This "fuzziness" is a frequent observation with mycoplasmas and has been attributed to material adsorbed from the growth medium, or highly polymerised material excreted from the cells, like the galactan of M. mycoides (Razin, 1973) and this could account for the relatively high measurement for the thickness of the membrane.

The membrane bounded "vacuole-like" structures seen in some *M. arginini* cells (figs 6, 7 and 8) are generally interpreted as being sections through deep "cup-like" invaginations of the cell membrane, and indeed serial sections of cells has confirmed this for *M. felis* cells (Razin, 1973; Boatman and Kenny, 1970).

The small bodies which appeared to "pinch off" from *M. arginini* cells are unlikely to be associated with any replicative process because they were absent from the logarithmic growth phase. They occurred only in the death phase, where the viability of the culture was decreasing.

The basic purpose of examining the ultrastructure of *M. arginini* was to complement the work done on the ultrastructure of *M. ovipneumoniae* and to determine if there were any major ultrastructural differences between the two so that eventually electron microscopic examination of pneumonic lung tissue might be performed and be able to distinguish between the two mycoplasmas *in vivo*.

There are two major ultrastructural differences between *M. ovipneumoniae* and *M. arginini*; *viz* i) their size, and ii) the presence of surface projections on *M. ovipneumoniae*.

i) *Size*: *M. ovipneumoniae* cells are usually between 500 and 800 nm in diameter; thus they are generally larger than *M. arginini* cells, which usually measure 200 to 500 nm in diameter. Hence, the smallest *M. ovipneumoniae* cell is about the same size as the largest *M. arginini* cell, and therefore distinguishing between the two organisms on the basis of size would not be possible.

ii) *Surface projections*: *M. ovipneumoniae* cells bear projections about 12 nm in length outside the trilaminar membrane. They show up clearly in some thin sections (figs 17 and 18) and negatively stained preparations (Major *et al.*, 1979). No such structures are visible associated with *M. arginini* cells. In principle this would seem to be a possible way of recognising individual cells of the two species *in vitro* and perhaps *in vivo*. However, not every *M. ovipneumoniae* cell is sufficiently well resolved to allow the surface projections to be seen, and

therefore it would probably not be possible to ensure that every cell could be identified as *M. ovipneumoniae* or *M. arginini* on the basis of the presence or absence of surface projections *in vitro*.

It can be concluded that there is no method of distinguishing between *M. ovipneumoniae* and *M. arginini* *in vitro* on the basis of consistently obvious ultrastructural differences, and this would probably be the case *in vivo* also. The distinction between the two mycoplasmas *in vivo* will probably have to be made using immunofluorescent methods such as ferritin or peroxidase labelled specific antibody.

CHAPTER 4 The effect of digitonin on the ability of pneumonic lung homogenate to transmit ovine CNP

4.1 Introduction

Many organisms (see Review, chapter 1) have been suggested as being the cause, or causes of ovine CNP. These organisms include PI3 virus, M. ovipneumoniae, M. arginini, and bacteria (especially P. haemolytica).

Since mycoplasmas (in particular M. ovipneumoniae) are consistently found in association with lesions of ovine CNP (Clarke et al., 1974; Alley et al., 1975; Alley & Clarke, 1979) they must either cause the primary lesions, or colonise lesions initiated by some other organism (e.g. P. haemolytica or PI3 virus). If the latter is true then the selective removal of mycoplasmas from pneumonic lung homogenate should not diminish its ability to initiate the disease.

Digitonin is a steroid with three glucose molecules attached. It binds to cholesterol forming a very stable molecular complex, and thus inactivates mycoplasmas (but not bacteria) because mycoplasma cell membranes contain cholesterol.

This section reports the effect of selectively removing mycoplasmas from pneumonic lung homogenate by treatment with digitonin, on the ability of the homogenate to transmit ovine CNP. Necessary preliminary investigations were undertaken (a) to confirm that bacteria remain unaffected by digitonin treatment, (b) to determine the minimum inhibitory concentration (MIC) of digitonin on mycoplasmas in broth culture and the mycoplasmacidal concentration in lung homogenate, and (c) to determine a suitable concentration of digitonin to use for eliminating mycoplasmas from lung homogenate for transmission experiments.

4.2 Materials and methods

4.2.1 Mycoplasma media and source of micro-organisms

See appendix.

4.2.2 Digitonin (Sigma)

Digitonin is only sparingly soluble in water, so unless otherwise stated, suspensions were warmed to 37°C and shaken vigorously to disperse the digitonin.

4.2.3 Lung homogenate for preliminary digitonin experiments

Lungs from lambs with CNP were collected from a local freezing works and pneumonic lesions were removed and pooled. The pooled lesions were added to an approximately equal volume of sterile nutrient broth (Difco) and homogenised in a blender. The homogenate was passed through a fine (0.25 mm) sieve and stored in aliquots at -70°C.

4.2.4 Lung homogenate for transmission experiments

Lungs from lambs with CNP were collected from a local freezing works and pneumonic lesions were removed. A small portion of each lesion was taken, pooled, homogenised in PBS, sieved as above and stored in aliquots at -70°C. This material was used for virology (see Chapter 5).

The remaining lesions were pooled, homogenised in sterile nutrient broth and sieved. Two equal aliquots of this homogenate were removed and one was treated with digitonin. The treated and untreated aliquots were used to inoculate 2 groups of lambs (details are given subsequently).

4.2.5 MIC of digitonin for M. ovipneumoniae and M. arginini in broth culture

Digitonin was suspended in 2.7 cm^3 volumes of FM4 broth at concentrations of 200, 100, 50 and 25 $\mu g/cm^3$. M. ovipneumoniae was grown to late exponential phase in FM4 broth and a 0.3 cm^3 volume of culture was added to each digitonin dilution. Bottles were incubated at 37°C for 10 days and observed for a pH change indicating growth. The same procedure was used for M. arginini in FM4A broth.

4.2.6 Minimum mycoplasmacidal concentration of digitonin for *M. ovipneumoniae* and *M. arginini* in lung homogenate

The lung homogenate used (see 4.2.3) was found in preliminary experiments not to contain *M. arginini*, so 0.05 cm^3 of *M. arginini* culture was added to 4.95 cm^3 of lung homogenate to give a final titre of about 10^5 CFU/cm^3.

The lung homogenate was divided into five 0.9 cm^3 aliquots and 0.1 cm^3 of digitonin suspended in FM4 broth was added to four aliquots to give final digitonin concentrations of 10, 5, 1 and 0.1 mg/cm^3. 0.1 cm^3 of FM4 broth was added to 0.9 cm^3 of lung homogenate as a control. These were shaken intermittently at room temperature (approximately 20°C) for 30 minutes. Each bottle was then assayed for *M. ovipneumoniae* and *M. arginini* by removing two 0.2 cm^3 aliquots of lung homogenate and making serial 10-fold dilutions in FM4 and FM4A broth. A 0.05 cm^3 volume from each dilution was spotted on the equivalent solid medium. Plates were incubated at 37°C for 3 to 5 days and colonies were counted using a plate microscope.

4.2.7 The effect of 10 mg/cm^3 digitonin on bacteria and mycoplasmas in pneumonic lung homogenate

Two 1.8 cm^3 aliquots of lung homogenate were dispensed in bijou bottles and 0.2 cm^3 of *M. arginini* culture was added to each bijou to give a final titre of about 10^5 CFU/cm^3. Digitonin was added to one of the bijous to give a final concentration of 10 mg/cm^3, and the bijou was shaken to disperse the digitonin. The other bijou was left as a control.

The two bijous were shaken intermittently at room temperature (approximately 20°C) for 30 minutes. They were then assayed for *M. ovipneumoniae* and *M. arginini* as described in 4.2.6. Bacteria were assayed by removing 0.2 cm^3 of lung homogenate, making serial 10-fold dilutions in BHI broth, and plating 0.1 cm^3 of each dilution on BHI agar. Mycoplasma plates were incubated at 37°C for 3 to 5 days and colonies counted using a plate microscope. BHI plates were incubated at 37°C for 1 to 2 days and colonies counted. Bijous containing dilutions of lung homogenate in FM4 and FM4A broth were incubated at 37°C for 10 days and observed for a colour change.

4.2.8 Mycoplasma isolations from nasal swabs

Nasal swabs from experimental lambs were broken off into 3 cm^3 aliquots of FM4 broth. These were shaken intermittently for 10 minutes and a 0.3 cm^3 aliquot was inoculated into 2.7 cm^3 each of FM4 broth and FM4A broth. Bottles were incubated at 37°C until a pH change (indicating growth) occurred, or for 7 days. If a pH change occurred, 0.05 cm^3 of the culture was spotted on the equivalent agar medium. If there was no growth in the original specimen after 7 days, 0.3 cm^3 from the bottle was transferred to fresh liquid medium and incubated at 37°C for another 7 days or until a pH change occurred, at which time 0.05 cm^3 was spotted on the equivalent solid medium. Plates were incubated at 37°C for 5 to 7 days and examined for colonies using a plate microscope.

4.2.9 Assay of pneumonic lung homogenate (for transmission experiment) for micro-organisms before and after digitonin treatment

Lung homogenate was prepared (see 4.2.4) and divided into 2 equal aliquots. Digitonin was added at a concentration of 10 mg/cm^3 to one of the aliquots which was then shaken intermittently for 30 minutes at room temperature (approximately 20°C). The lung homogenate was assayed for M. ovipneumoniae, M. arginini, and bacteria before and after treatment with digitonin. Mycoplasmas were assayed by quantal titration of the lung homogenate (using 4 replicate serial 10-fold dilutions in broth) and bacteria were assayed by surface counts on 10% sheep-blood agar (serial 10 -fold dilutions of lung homogenate prepared in BHI broth were inoculated onto blood agar plates using 4 x 0.02 cm^3 volumes from each dilution).

4.2.10 Inoculation of lambs

Lambs were given 15 cm^3 of the appropriate inoculum as an aerosol through the nasal cavity using a nebulising spray gun (Alley and Clarke, 1979).

4.2.11 Titration of mycoplasmas in lung tissue

0.3 g of lung tissue was ground with sterile sand using a mortar and pestle, and 2.7 cm^3 of FM4 (or FM4A) broth was added. This was

regarded as a 10^{-1} dilution. Serial 10-fold dilutions were made from 10^{-2} to 10^{-9}. The dilutions were incubated at 37°C for 10 days.

The end-point was regarded as the highest dilution showing the appropriate colour change, and 0.05 cm^3 from this dilution was spotted on FM4 (or FM4A) agar to confirm the colour change was due to mycoplasmas. The agar plates were examined for colonies after 4 to 5 days incubation at 37°C.

4.3 Results

4.3.1 MIC of digitonin for M. ovipneumoniae and M. arginini in broth culture

This was determined by the methods described in 4.2.5. The experiment was repeated several times and gave consistent results. A typical result is summarised in Table III.

TABLE III The effect of digitonin on the growth of M. ovipneumoniae and M. arginini

Digitonin concentration ($\mu g/cm^3$)	M. ovipneumoniae	M. arginini
200	-	-
100	+	-
50	+	+
25	+	+

+ = growth of mycoplasmas

- = no growth of mycoplasmas

The MIC of digitonin for M. ovipneumoniae in broth culture was 200 $\mu g/cm^3$ and the MIC of digitonin for M. arginini in broth culture was 100 $\mu g/cm^3$.

4.3.2 Minimum mycoplasmacidal concentration of digitonin for *M. ovipneumoniae* and *M. arginini* in lung homogenate

This was determined by the methods described in section 4.2.6.

A typical result is seen in Table IV.

TABLE IV Titre of *M. ovipneumoniae* and *M. arginini* in pneumonic lung homogenate after treatment with varying concentrations of digitonin

Digitonin concentration (mg/cm^3)	Titre of *M. ovipneumoniae*	Titre of *M. arginini*
0 (control)	6.0×10^5 CFU/cm^3	2.6×10^5 CFU/cm^3
0.1	7.2×10^5 "	1.5×10^5 "
1.0	8.0×10^4 "	1.8×10^3 "
5.0	none detected*	none detected
10.0	none detected	none detected

*None detected = No colonies detected by removing 0.2 cm^3 of lung homogenate, making serial 10-fold dilutions in FM4 broth, plating 0.05 cm^3 from each dilution on solid medium, and incubating at 37°C for 3 to 5 days.

The minimum mycoplasmacidal concentration of digitonin for both *M. ovipneumoniae* and *M. arginini* in pneumonic lung homogenate was 5.0 mg/cm^3.

4.3.3 The effect of 10 mg/cm^3 digitonin on bacteria and mycoplasmas in pneumonic lung homogenate

Since 10 mg/cm^3 digitonin was ultimately used to inactivate mycoplasmas in lung homogenate (based on the results obtained in section 4.3.2) it was necessary to establish that the bacteria in the lung homogenate were not significantly affected by this concentration of digitonin and to confirm that the mycoplasmas were inactivated. Bacteria and mycoplasmas were assayed in lung homogenate with and without digitonin treatment as described in 4.2.7. The experiment

was repeated three times with consistent results. One result is summarised in Table V.

TABLE V Titre of bacteria and mycoplasmas in control, and digitonin (10 mg/cm^3) treated pneumonic lung homogenate

Organisms	Titre of organism (CFU/cm^3)	
	control lung homogenate	digitonin treated lung homogenate
M. ovipneumoniae	6.2×10^4	none detected*
M. arginini	1.9×10^5	none detected
Bacteria	$1.21 \pm 0.22 \times 10^4$	$1.02 \pm 0.20 \times 10^4$

*None detected = No colonies detected by removing 0.2 cm^3 of lung homogenate, making serial 10-fold dilutions in FM4 broth, plating 0.05 cm^3 from each dilution on FM4 (or FM4A) agar and incubating plates at 37°C for 3 to 5 days. Furthermore, incubation of dilutions did not produce a colour change.

4.3.4 Preliminary screening of lambs to detect nasal carriers of mycoplasmas

Sixty, conventionally reared, 5 month old lambs in healthy condition were drafted from a flock and divided into three groups of 20. Nasal swabs were taken from each lamb and tested for mycoplasmas as described in 4.2.8. The results are summarised in Table VI.

TABLE VI Prevalence of nasal carriage of mycoplasmas

Group	M. ovipneumoniae	M. arginini
A	4*/20**	0*/20**
B	4/20	0/20
C	4/20	0/20

* Number of nasal swabs positive

** Number of nasal swabs tested

Because of the possibility of (i) the lambs with positive nasal swabs already having a lower respiratory tract infection with M. ovipneumoniae, or (ii) washing M. ovipneumoniae down into the lungs with the aerosol inoculum, the lambs which were nasal carriers of the organism were omitted from each group.

4.3.5 Assay of pneumonic lung homogenate (for transmission experiment) for micro-organisms before and after digitonin treatment

The micro-organisms present in the pneumonic lung homogenate were assayed before and after treatment with 10 mg/cm^3 digitonin (see 4.2.9) and the results are seen in Table VII.

TABLE VII Titres of bacteria and mycoplasmas in pneumonic lung homogenate before and after treatment with 10 mg/cm^3 digitonin

Organism	Titre	
	Before digitonin treatment	After digitonin treatment
M. ovipneumoniae	$10^{7.0}$ ccu_{50}^{*}/cm^3	none detected**
M. arginini	$10^{7.2}$ ccu_{50}/cm^3	none detected
Bacteria	$4.6 \pm 1.5 \times 10^5$ CFU/cm^3	$3.4 \pm 0.6 \times 10^5$ CFU/cm^3

* CCU = colour changing units

** none detected = No colour change in the pH indicator of any of the serial 10-fold dilutions.

4.3.6 Transmission of ovine CNP by pneumonic lung homogenate with and without digitonin treatment

One group of lambs (group A) was inoculated with sterile nutrient broth; each lamb received 15 cm^3 as an aerosol inoculum (see 4.2.10). A second group of lambs (group B) was inoculated with digitonin-treated pneumonic lung homogenate (see 4.2.4). The third group of lambs (group C) was inoculated with untreated pneumonic lung homogenate. After inoculation the three groups of lambs were run separately for 17 days and were then slaughtered at a local freezing works. One lamb in group

C died before slaughter, but the cause of death was unrelated to the experiment.

After slaughter, the lungs were collected individually in plastic bags and the nature and distribution of lesions was recorded. Due to hygiene regulations, all lungs were handled by a meat inspector prior to collection. Samples of lesion were taken from each pneumonic lung, and where no evidence of pneumonia was apparent a sample from the right cranial lobe of each lung was taken. Each sample was assayed for the presence of M. ovipneumoniae and M. arginini by the method described in chapter 2 (2.2.4), and an aliquot of each sample was frozen at -70°C. All those giving a positive isolation for M. ovipneumoniae and M. arginini were titrated as described previously (4.2.11). These positive lungs were titrated for M. ovipneumoniae starting at a dilution of 10^{-2} (i.e. a 1/10 dilution of 10% lung suspension). Only those lungs which were positive in this assay were accepted as true positives i.e. those lungs which initially gave a positive isolation but were negative at the 10^{-2} dilution were regarded as false positives and were assumed to represent low level contamination of the lungs due to handling by meat inspectors.

In group A (negative controls - inoculated with sterile nutrient broth) only one lung showed any signs of pneumonia, and this was confined to small areas of collapse. All the lungs were negative for both M. ovipneumoniae and M. arginini.

In group B (inoculated with digitonin-treated lung homogenate) 4 lungs were found to have small areas of collapse, and all of these were negative for M. ovipneumoniae. Only one lung out of the remaining 12 with no macroscopic pneumonic lesions was positive for M. ovipneumoniae, and the titre was 10^{2} organisms/gram of tissue. M. arginini was not recovered from any of the lungs in this group.

In group C (positive controls - inoculated with untreated pneumonic lung homogenate) 13 of the 15 lungs showed signs of pneumonia: 4 lungs had small areas of collapse, 4 lungs had mild pneumonia, and 5 lungs had moderate to severe pneumonia. 10 out of 15 lungs in this group (67%) were positive for M. ovipneumoniae and the geometric

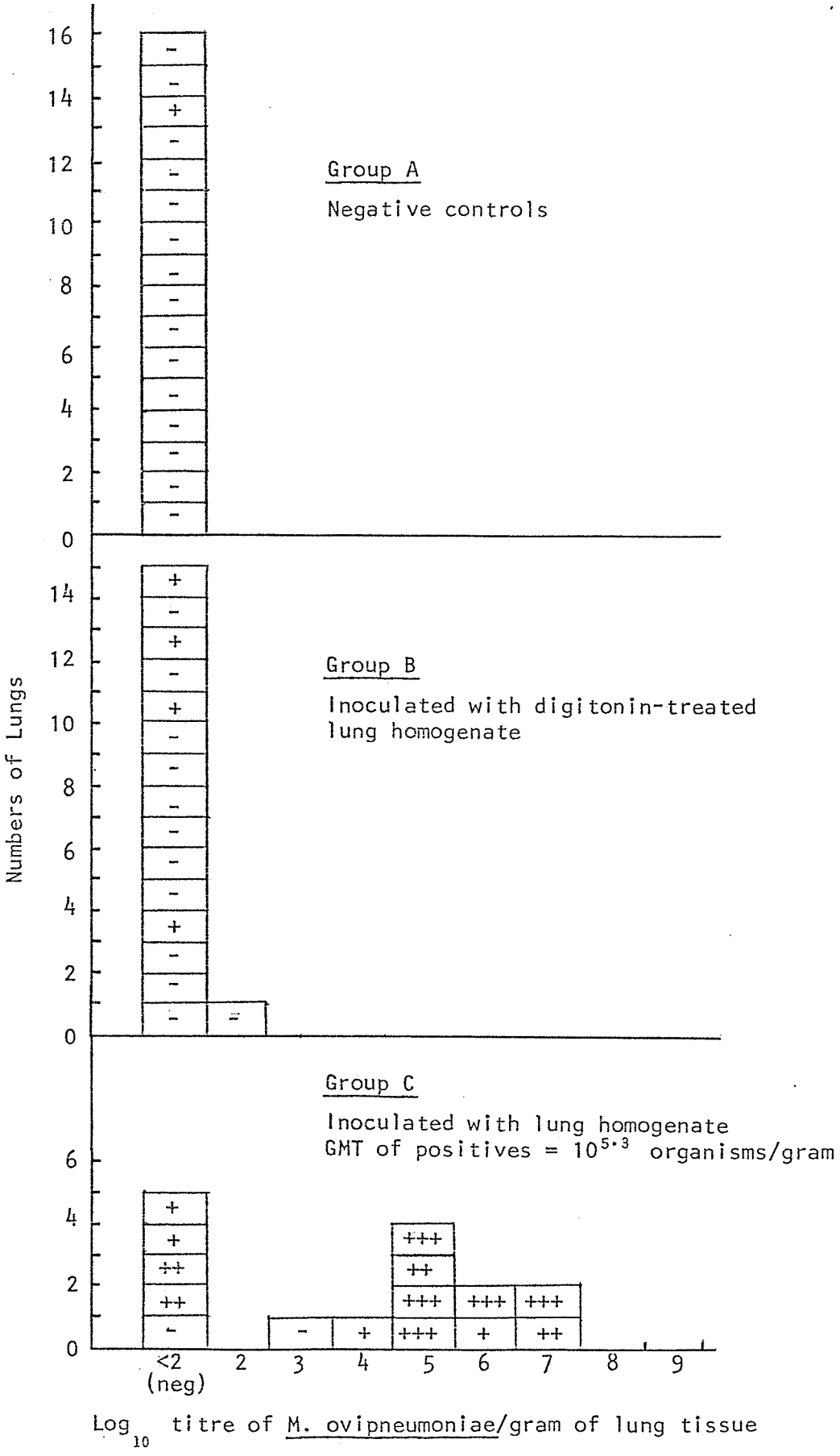
mean titre (GMT) was $10^{5.3}$ organisms/gram. M. arginini was recovered from 2 lungs in this group at titres of 10^8 and 10^9 organisms/gram, and both of these lungs were also positive for M. ovipneumoniae: one lung had moderate to severe pneumonia and the other had mild pneumonia.

Figure 19 summarises the results.

FIGURE 19: Transmission experiment: pathology of the lungs and recovery of mycoplasmas after slaughter

Key to the severity of pneumonic lesions:

- = nil
+ = small areas of collapse
++ = mild pneumonia
+++ = moderate to severe pneumonia


Numbers of Lungs
Group A
Negative controls
Group B
Inoculated with digitonin-treated lung homogenate
Group C
Inoculated with lung homogenate
GMT of positives = $10^{5.3}$ organisms/gram
<2 (neg)
2
3
4
5
6
7
8
9
Log10 titre of M. ovipneumoniae/gram of lung tissue

4.4 Discussion

4.4.1 Elimination of mycoplasmas from lung homogenate by digitonin treatment

Digitonin at concentrations of 10-15 $\mu g/cm^3$ is reported to lyse mycoplasmas in medium without serum (8th edition of Bergey's Manual of Determinative Bacteriology, 1974). Since digitonin acts by complexing cholesterol, clearly the concentration required to kill or inhibit mycoplasmas will vary with the cholesterol content of the medium e.g. serum or homogenised lung will alter the concentration of digitonin required. Thus it was not surprising that it required 0.2 and 0.1 mg/cm^3 of digitonin respectively to inhibit M. ovipneumoniae and M. arginini in FM4 and FM4A broth.

Preliminary investigation showed that a concentration of 5 mg/cm^3 of digitonin was required to eliminate mycoplasmas from pneumonic lung homogenate (4.3.2), so for the transmission experiment a concentration of 10 mg/cm^3 digitonin was arbitrarily chosen. This ensured an excess of digitonin. A difficulty arose because the high concentration of digitonin used (10 mg/cm^3) meant that the initial dilutions for assaying the mycoplasmas still contained inhibitory concentrations of digitonin (at a 1/10 dilution for M. ovipneumoniae and up to a 1/100 dilution for M. arginini). This difficulty did not prevent detection of the initial fall in viability of the mycoplasmas i.e. from $>10^4/cm^3$ to $10^2/cm^3$ in the case of M. ovipneumoniae or from $>10^5/cm^3$ to $10^3/cm^3$ in the case of M. arginini (4.3.3). This represented at least a 99% kill in both cases. The lung homogenate pool actually used in the transmission experiment contained higher initial levels of both M. ovipneumoniae and M. arginini, and since no mycoplasmas were detected after digitonin treatment the fall in titre represented at least a 10^5-fold drop in the case of M. ovipneumoniae and a $>10^4$-fold drop in the case of M. arginini (4.3.5).

Preliminary experiments (not recorded) showed that the kill occurred rapidly ($\leq$2.5 minutes). Since an excess of digitonin was used and 30 minutes was allowed for it to act, it is reasonable to assume that the mycoplasmas were totally eliminated from the pneumonic lung

homogenate used in the transmission experiment. The concentration of digitonin used (10 mg/cm^3) had no significant effect on bacteria in the lung homogenate (see Table VII).

4.4.2 Transmission experiment

This experiment was conducted in conventional lambs, which may or may not already have been infected with M. ovipneumoniae. It is assumed that M. ovipneumoniae infection is initiated in the nasal tract, and may subsequently lead to infection of the lower respiratory tract. For that reason those lambs which were nasal carriers of M. ovipneumoniae were eliminated from all 3 groups.

With these sheep eliminated, none of the 16 negative controls (group A) showed mild or severe lesions of CNP. Some sheep in this and the other groups showed small areas of collapse in the lungs. Since the significance, if any, of these minimal lesions is uncertain they will be considered as negative in all 3 groups.

In marked contrast to group A, 9 out of the 15 lambs in the "positive controls" (group C-inoculated with untreated pneumonic lung homogenate) showed mild to severe pneumonia. Clearly group C differs from group A ($X^2 = 10.67$, $p < 0.005$)[1] so it is concluded that untreated pneumonic lung homogenate successfully transmitted ovine CNP.

Those sheep in group B (inoculated with digitonin-treated lung homogenate) failed to develop significant pneumonic lesions i.e. they developed neither mild, nor moderate to severe pneumonia. In this respect the lambs in this group were identical to the negative controls, but differed from those sheep inoculated with untreated pneumonic lung homogenate ($x^2 = 10.67$, $p < 0.005$).

It is concluded that the treatment of pneumonic lung homogenate with 10 mg/cm^3 digitonin inactivates a component which is essential for the transmission of ovine CNP.

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[1] Chi squared test (Snedecor and Cochran, 1967).

CHAPTER 5 Sensitivity of PI3 virus to digitonin and an investigation of the presence or absence of PI3 virus in pneumonic lung homogenate known to transmit ovine CNP

5.1 Introduction

The previous chapter concludes that digitonin treatment of pneumonic lung homogenate destroys its ability to transmit ovine CNP. This implies that an organism limited by a cholesterol-containing membrane initiates the lesions. While this strongly supports the idea that the disease is initiated by a mycoplasma it is also possible that an enveloped virus is responsible. If indeed a virus is responsible the prime "suspect" would be PI3 virus. A role for this virus has been suggested by Jones et al. (1979) who stated that proliferative exudative pneumonia (similar to ovine CNP) may be caused by sequential infection with PI3 virus, mycoplasmas, and bacteria, in that order.

This section examines the sensitivity of PI3 virus to digitonin and subsequently investigates the presence or absence of the virus from aliquots of pneumonic lung homogenate known to transmit the disease.

5.2 Materials and Methods

5.2.1 Cell culture media

Antibiotic trypsin-versene (ATV)

Trypsin (Difco 1:250)	0.5 g
Versene (EDTA)	0.2 g
NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 g
$NaHCO_3$	0.58 g
Penicillin	2×10^5 units
Streptomycin	0.1 g
Phenol red	0.02 g
Distilled water to	1000 cm^3

The solution was sterilised through a 0.2 µm pore size filter and stored in aliquots at -20°C. ATV was warmed to 37°C before use.

PSK antiobiotic solution

Penicillin	10^6 units
Streptomycin	1.0 g
Kanamycin	1.0 g
Phosphate buffered saline to	100 cm^3

The solution was sterilised through a 0.2 µm pore size filter and stored in aliquots at -20°C. 1.0 cm^3 was added to 100 cm^3 of Eagles MEM.

Strong antibiotics (for virus isolation)

Penicillin	600,000 units
Streptomycin	0.3 g
Kanamycin	0.1 g
PBS to	10.0 cm^3

The solution was sterilised through a 0.2 µm pore size filter and stored at -20°C. 1.0 cm^3 was added to 100 cm^3 of Eagles MEM. Final concentrations in MEM were: penicillin 600 units/cm^3, streptomycin 300 µg/cm^3, and kanamycin 100 µg/cm^3.

Eagles minimal essential medium (MEM)

Eagles MEM dried powder	10.0 g
Deionised water	950 cm^3
4.4% $NaHCO_3$ solution	50 cm^3

The medium was sterilised through a 0.2 µm pore size filter and stored in 100 cm^3 aliquots at 4°C. Before use 1.0 cm^3 of antibiotic solution (PSK) was added. MEM was supplemented with 10% sterile foetal calf serum (FCS)and used for propagating MDBK and primary ovine kidney cells.

Serum free maintenance medium (MEM + 0.5% bovine serum albumin (BSA))[1]

0.5 g of BSA was added to 100 cm^3 of MEM and the medium was sterilised through a 0.2 µm pore size filter.

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[1] Horner et al. (1973) conducted a survey of 347 foetal calf sera in Australia and found that non-specific inhibitors of PI3 virus were present in every sample tested. Because of the possibility that the FCS used for propagating cell cultures in the present study might contain similar inhibitors, it was replaced by 0.5% BSA when propagating or attempting to isolate PI3 virus.

Phosphate buffered saline (PBS)

See appendix.

5.2.2 Source of MDBK cells

These were obtained from Dr A. Robinson, Department of Veterinary Pathology and Public Health, Massey University.

5.2.3 Source of PI3 virus

This was obtained from Dr G. Burgess, Department of Veterinary Pathology and Public Health, Massey University. It was a laboratory adapted strain of bovine origin.

5.2.4 Maintenance of MDBK cells and the preparation of cell monolayers in microtiter plates

MDBK cells were maintained as monolayers in 75 cm^2 "Falcon" tissue culture flasks. After 4 to 7 days incubation at 37°C the growth medium was removed and the monolayer of cells was washed with 5.0 cm^3 of PBS. The PBS was removed then 1.0 cm^3 of ATV was added and the flask was incubated at 37°C until the cells were detached from the plastic. The cells were resuspended and diluted in MEM + 10% FCS to a concentration of 10^5 cells/cm^3. 20 cm^3 of the cells was used to seed another 75 cm^2 flask. 0.2 cm^3 of MDBK cells in MEM + 10% FCS (10^5 cells/cm^3) was added to each well of a "Cooke" microtiter plate. The plate was covered and incubated at 37°C in an atmosphere of 5% CO_2 until monolayers of cells were formed in the wells. The medium was removed, the cells washed with PBS and 0.2 cm^3 of MEM + 0.5% BSA was added to each well. The cells were incubated for another 24 hours, and then used for PI3 virus experiments.

5.2.5 Pneumonic lung homogenate

Samples of pneumonic lung to be used for virus isolation were homogenised in PBS as described in 4.2.4.

Note i) The lung homogenate used for inoculating sheep was homogenised in nutrient broth, but preliminary experiments revealed that nutrient broth contained some factor which was toxic for tissue culture cells, so the lung homogenate for virology was homogenised in PBS.

ii) Homogenates of these lungs had the ability to transmit ovine CNP. (See previous chapter, sections 4.3.6 and 4.4.2).

5.2.6 Assay of PI3 virus by haemadsorption

Guinea pig blood was collected and stored at $4^{o}C$ in Alsever's solution (see appendix). Erythrocytes were washed twice in PBS and resuspended as a 0.4% suspension in PBS.

i) Haemadsorption in microtiter plates

The medium from the cell monolayers was removed and the cells were washed twice with PBS. 0.05 cm^3 of a 0.4% suspension of guinea pig erythrocytes in PBS was added to each well, and the plate was placed at $4^{o}C$ for 30 minutes. The cells were then washed 3 times with cold ($4^{o}C$) PBS and examined microscopically.

ii) Haemadsorption in 35 mm petri-dishes

The medium from the cell monolayers was removed and the cells were washed twice with PBS. 0.5 cm^3 of a 0.4% suspension of guinea pig erythrocytes in PBS was added to each dish and the dish was placed at $4^{o}C$ for 30 minutes. The cells were then washed 3 times with cold PBS and examined microscopically.

5.2.7 Primary ovine kidney (POK) cells

A vial of primary ovine kidney cells (passage 3) was obtained from Dr A. Robinson, Department of Veterinary Pathology and Public Health, Massey University. These cells were maintained in Eagles MEM + 10% FCS for several passages as monolayers in 25 cm^2 "Falcon" tissue culture flasks. The cells were suspended using ATV and diluted in MEM + 10% FCS to 10^5 cells/cm^3. 3 cm^3 aliquots of the suspension were added to 35 mm plastic petri-dishes which were incubated at $37^{o}C$ in an atmosphere of 5% CO_2 until monolayers had formed. 24 hours before the cells were used to attempt to isolate PI3 virus the cells were washed with PBS, and 3 cm^3 of MEM + 0.5% BSA was added to each dish.

5.2.8 Sensitivity of PI3 virus to digitonin

An arbitrary concentration of 1.0 mg/cm^3 was used to test the sensitivity of PI3 virus to digitonin.

PI3 virus in MEM + 0.5% BSA was taken from storage at - 70°C and divided into two 0.45 cm^3 aliquots. 0.05 cm^3 of 10 mg/cm^3 digitonin in PBS (warmed to 37°C and shaken to disperse the digitonin) was added to one, and 0.05 cm^3 of PBS was added to the other. These were left at room temperature (approximately 20°C) for 15 minutes with intermittent shaking. Serial 10-fold dilutions in serum-free MEM of both samples were performed and 0.05 cm^3 of each dilution was added to each of 4 wells of a microtiter plate containing washed monolayers of MDBK cells. The cells were incubated at 37°C in 5% CO_2 for one hour to facilitate virus adsorption, then 0.15 cm^3 of MEM + 0.5% BSA was added to each well. Plates were incubated at 37°C in 5% CO_2 for 4 days and tested for the presence of PI3 virus by haemadsorption (see 5.2.6).

5.2.9 Sensitivity of PI3 virus in lung homogenate to 10 mg/cm^3 digitonin

0.2 cm^3 aliquots of PI3 virus stored at -70°C were added to each of two 1.8 cm^3 aliquots of pneumonic lung homogenate. 0.02 g of digitonin was added to one of the lung homogenate/virus mixtures and the other was left as a control. The bottles were left at room temperature (approximately 20°C) for 30 minutes with intermittent shaking. Each sample was then centrifuged at 12,000 g for 10 minutes in a Sorvall RC2-B refrigerated centrifuge. Serial 10-fold dilutions of the supernatants in serum-free MEM were made, and 0.05 cm^3 of each dilution was added to each of 4 wells of a microtiter plate containing washed monolayers of MDBK cells. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 1 hour to facilitate virus adsorption, and then 0.15 cm^3 of MEM + 0.5% BSA was added to each well. Plates were incubated at 37°C in an atmosphere of 5% CO_2 for 4 days and then tested for the presence of PI3 virus by haemadsorption (see 5.2.6).

5.2.10 Attempted isolation of PI3 virus from pneumonic lung homogenate known to transmit ovine CNP[2]

Pneumonic lung homogenate was taken from storage at -70°C and quickly

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[2] The isolation procedure is a modification of that used by Carter and Hunter (1970) who were successful in isolating PI3 virus from sheep in New Zealand.

thawed. It was then centrifuged at 3000 g for 15 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge. 2.0 cm^3 of the supernatant was mixed with an equal volume of Eagles MEM + 0.5 BSA + strong antibiotics and incubated at 22°C for 30 minutes. POK cells in 35 mm plastic petri-dishes (see 5.2.7) were washed once with PBS. 0.5 cm^3 of the lung homogenate/MEM mixture was added to each of two cell monolayers.

For a negative control a 1:1 mixture of PBS:MEM + 0.5% BSA + strong antibiotics was incubated at 22°C for 1 hour, and 0.5 cm^3 was added to a monolayer of POK cells.

For a positive control 0.5 cm^3 of a 1/100 dilution (in serum-free MEM) of PI3 virus stored at -70°C (titre = $10^{6.3}$ $TCID_{50}/cm^3$) was added to 4.5 cm^3 of lung homogenate, which was then centrifuged, mixed with an equal volume of MEM + 0.5% BSA + strong antibiotics, incubated, and inoculated onto POK cells as above.

Cell cultures were incubated at 37°C for 1 hour in an atmosphere of 5% CO_2 to facilitate virus adsorption, then 2.5 cm^3 of MEM + 0.5% BSA + strong antibiotics was added to each dish. After 4 to 6 days incubation at 37°C in an atmosphere of 5% CO_2 the cells were examined for a cytopathic effect (CPE) and tested for the presence of PI3 virus by haemadsorption (see 5.2.6).

5.3 Results

5.3.1 Sensitivity of PI3 virus to digitonin

The effect of 1.0 mg/cm^3 digitonin at room temperature over an arbitrary period of 15 minutes was determined by the methods described in 5.2.8. The experiment was repeated twice with consistent results. The findings are summarised in Table VIII.

TABLE VIII The effect of 1.0 mg/cm^3 digitonin on PI3 virus

	Titre of PI3 virus
Virus + PBS (control)	$10^{6.0}$ $TCID_{50}^{**}$/0.05 cm^3
Virus + digitonin	$<10^{2.0}$ $TCID_{50}$/0.05 cm^{3*}

** TCID = Tissue culture infectious dose.

* The digitonin concentration in the wells containing the 10^{-1} dilution of the virus/digitonin mixture was toxic for the MDBK cells, so the haemadsorption test started at the 10^{-2} dilution.

These results showed that there is at least a 10,000-fold drop in titre of PI3 virus (i.e. a 99.99% kill) after exposure to 1.0 mg/cm^3 digitonin for 15 minutes at room temperature.

5.3.2 Sensitivity of PI3 virus in lung homogenate to 10 mg/cm^3 digitonin

This experiment was performed using the methods described in 5.2.9, and was repeated several times with consistent results. A typical result is summarised in Table IX.

TABLE IX The effect of 10 mg/cm^3 digitonin on PI3 virus in pneumonic lung homogenate

	Titre of PI3 virus
Control lung homogenate (virus added)	$10^{4.5}$ $TCID_{50}$/0.05 cm^3
Lung homogenate + 10 mg/cm^3 digitonin	$<10^{2.0}$ $TCID_{50}$/0.05 cm^{3*}

* The digitonin concentration in the wells containing the 10^{-1} dilution of the lung homogenate/digitonin mixture was toxic to the MDBK cells so the haemadsorption test started at the 10^{-2} dilution.

More than a 300-fold drop in titre of PI3 virus could be detected (i.e. > 99% kill) in lung homogenate treated with 10 mg/cm^3 digitonin.

5.3.3 Attempted isolation of PI3 virus from pneumonic lung homogenate known to transmit ovine CNP

This was carried out by the methods described in 5.2.10. The procedure was performed twice on each of two aliquots of a pool of pneumonic lung homogenate and the results were the same in all cases. Table X summarises the findings.

TABLE X Recovery of PI3 virus from pneumonic lung homogenate

	Recovery of PI3 virus
Negative control (PBS)	-
Lung homogenate (aliquot 1)	-
Lung homogenate (aliquot 2)	-
Positive control (Lung homogenate + added PI3)	+

- = no haemadsorption of guinea pig erythrocytes

+ = haemadsorption of guinea pig erythrocytes

No PI3 virus was isolated from the pneumonic lung homogenate using primary ovine kidney cells. Nor was any evidence of a CPE observed. The technique was adequate to reisolate PI3 virus from lung homogenate following the addition of a low concentration of the virus.

5.4 Discussion

In the investigation of the sensitivity of PI3 virus to 1.0 mg/cm^3 digitonin, the initial dilution contained an amount of digitonin toxic for the MDBK cells. Therefore, it was not possible to determine whether haemadsorption occurred at the 10^{-1} dilution. However a 4-log drop in titre (99.99% kill) was still detected, and it is reasonable to assume that all the virus was inactivated, i.e. that 1.0 mg/cm^3 digitonin for 15 minutes at room temperature inactivates PI3 virus. A similar situation arose when determining the effect of 10 mg/cm^3 digitonin on PI3 virus in lung homogenate. Although, for technical reasons, only a 2.5-log drop in titre (>99% kill) could be detected, it is probable that all the PI3 virus was

inactivated because the time of exposure to digitonin was increased to 30 minutes, and a high concentration (10 mg/cm^3) of digitonin was used. This concentration is far in excess of the solubility of digitonin and thus ensured that the lung suspension was permanently saturated by the compound.

It is not unexpected that PI3 virus is sensitive to digitonin since it is an enveloped mammalian virus, and the envelope originates from the membrane of the host cell and therefore contains cholesterol (which complexes with digitonin). This observation underlines the importance of establishing the presence or absence of PI3 virus in the lung homogenate used to transmit ovine CNP.

PI3 virus was not detected in two aliquots of pneumonic lung homogenate known to transmit CNP. The observation that PI3 virus added to the pneumonic lung homogenate could be reisolated from it by the techniques described, indicates that these techniques were adequate to detect the virus. It is also relevant to note that subsequent to this work, PI3 virus was isolated from the lungs of a sheep with acute "Pasteurella" pneumonia at this university using the same cell line and identical techniques (Dr A. Robinson, personal communication).

It is concluded that PI3 virus was not present in the samples of pneumonic lung homogenate tested. Because aliquots of the same homogenate were capable of transmitting ovine CNP, it is also concluded that a digitonin sensitive organism other than PI3 virus is required for the initiation of the disease.

While this limited investigation was concerned mainly with PI3 virus it is relevant to note that no agent capable of producing a CPE in POK cells in the conditions used was detected.

CHAPTER 6 Preliminary investigations into the production of a vaccine against M. ovipneumoniae

6.1 Introduction

The results of Chapters 2, 4 and 5 give support to the hypothesis that M. ovipneumoniae is the primary pathogen involved in the aetiology of ovine CNP. It would therefore be appropriate to make a vaccine against M. ovipneumoniae and test its protective effect in an experimental flock of lambs. If it were demonstrated that the vaccine prevented the initiation of CNP in sheep challenged with an aerosol inoculum of pneumonic lung homogenate, then this would provide strong evidence that M. ovipneumoniae was the primary cause of the disease, and hence, that other micro-organisms (viz M. arginini, P. haemolytica and other bacteria) found in pneumonic lungs were secondary invaders.

Sullivan et al. (1973b) reported that they produced proliferative interstitial pneumonia in 1 day old lambs with an intravenous injection of live M. ovipneumoniae culture. It was therefore decided to make a vaccine containing formalin killed M. ovipneumoniae organisms to circumvent the possibility that injecting live M. ovipneumoniae intramuscularly could lead to the development of pneumonia in 4 to 6 month old lambs which will be used for testing the vaccine.

This chapter reports preliminary work to establish a method for producing a formalin killed M. ovipneumoniae vaccine. The follow-on work involving the inoculation of lambs with the vaccine and their subsequent challenge with pneumonic lung homogenate lies outside the scope of this thesis.

6.2 Materials and Methods

6.2.1 Source of M. ovipneumoniae

See appendix.

6.2.2 Modified FM4 medium

Modified FM4 medium consisted of FM4 medium (see appendix) with the following changes:

(a) the yeast autolysate (Albimi) and thallium acetate were omitted

(b) the 150 cm^3 of pig serum was replaced by 150 cm^3 of bovine serum

(c) the 10.0 g of peptone-CS (Albimi) was replaced by 10.0 g of phytone (BBL).

Note i) The thallium acetate was omitted because it is toxic to animals

ii) The yeast autolysate was omitted and the pig serum and phytone were changed to avoid cross-reactions with M. ovipneumoniae propagated in standard FM4 medium which will be used subsequently in assaying the immune response of sheep to the mycoplasmas.

M. ovipneumoniae was grown for 6-passages in modified FM4 broth, then 1.0 cm^3 aliquots were frozen at -70°C (at a titre of 9×10^7 CFU/cm^3). These aliquots were used to inoculate modified FM4 broth in the following inactivation experiments.

6.2.3 Preliminary experiment to determine the appropriate strength of formalin to use for inactivating M. ovipneumoniae

1.0 cm^3 of M. ovipneumoniae culture (grown in modified FM4) was added to 99.0 cm^3 of modified FM4 broth (pre-warmed to 37°C) in a 500 cm^3 side-arm flask fitted with a Bausch and Lomb Spectronic-20 tube. The culture was incubated at 37°C on a rotary shaker at 125 r.p.m. until an optical density of 0.10 was reached (read on a Bausch and Lomb Spectronic-20 spectrophotometer at 640 nm) corresponding to a viable count of about 6×10^8 CFU/ml in the late logarithmic phase (Major et al., 1979). The time taken to reach this optical density was about 26 hours. The organisms were harvested by centrifugation at 14,500 g for 30 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge, and the pellet was resuspended in 10 cm^3 of sterile saline (see appendix). This gave a nominal 10-fold increase in mycoplasma concentration which was desirable for future use as a vaccine.

The resuspended cells were divided into 10 x 0.9 cm^3 aliquots. 0.1 cm^3 of formalin (adjusted to pH 7.2 and diluted with sterile saline) was added to each 0.9 cm^3 of cells to give final concentrations of 1/25, 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400 formalin. 0.1 cm^3 of sterile saline was added to 0.9 cm^3 of resuspended cells as a control. The organisms were left at room temperature (approximately 20°C) for 15 minutes, then a 1/10 and 1/100 dilution of each was made into standard FM4 broth. These dilutions were incubated at 37°C for 5 to 10 days and growth of M. ovipneumoniae (as indicated by the development of turbidity and a colour change in the pH indicator from red to yellow) was noted.

6.2.4 Inactivation of M. ovipneumoniae with formalin for vaccine production

M. ovipneumoniae was grown in modified FM4 to an optical density of 0.10 at 640 nm, centrifuged and resuspended in 10 cm^3 of sterile saline as described in 6.2.3.

The resuspended cells were divided into 2 x 4.5 cm^3 aliquots. 0.5 cm^3 of 1/10 formalin (adjusted to pH 7.2 and diluted in sterile saline) was added to one of the aliquots to give a final concentration of 1/100 formalin. 0.5 cm^3 of sterile saline was added to the other 4.5 cm^3 aliquot as a control.

The mycoplasmas were shaken intermittently at room temperature (approximately 20°C). At times 0, 2, 5, 10, 20 and 30 minutes, a 0.05 cm^3 aliquot of the formalin treated mycoplasmas was removed and added to 4.95 cm^3 of standard FM4 broth. After this initial 10^{-2} dilution, serial 10-fold dilutions were made in standard FM4 broth. The control mycoplasmas were assayed the same way at times 0 and 30 minutes. Bottles were incubated at 37°C for 10 days and growth of M. ovipneumoniae (as indicated by the development of turbidity and a colour change in the pH indicator of the FM4 broth from red to yellow) was noted.

6.3 Results

6.3.1 The strength of formalin needed to inactivate M. ovipneumoniae

This was determined by the method described in 6.2.3. The experiment was repeated twice with consistent results. One set of results is summarised in Table XI.

TABLE XI Inactivation of M. ovipneumoniae by varying strengths of formalin

Dilution of Formalin (final)	Growth of M. ovipneumoniae
Control (no formalin)	+
1/25	-
1/50	-
1/100	-
1/200	+
1/400	+
1/800	+
1/1600	+
1/3200	+
1/6400	+

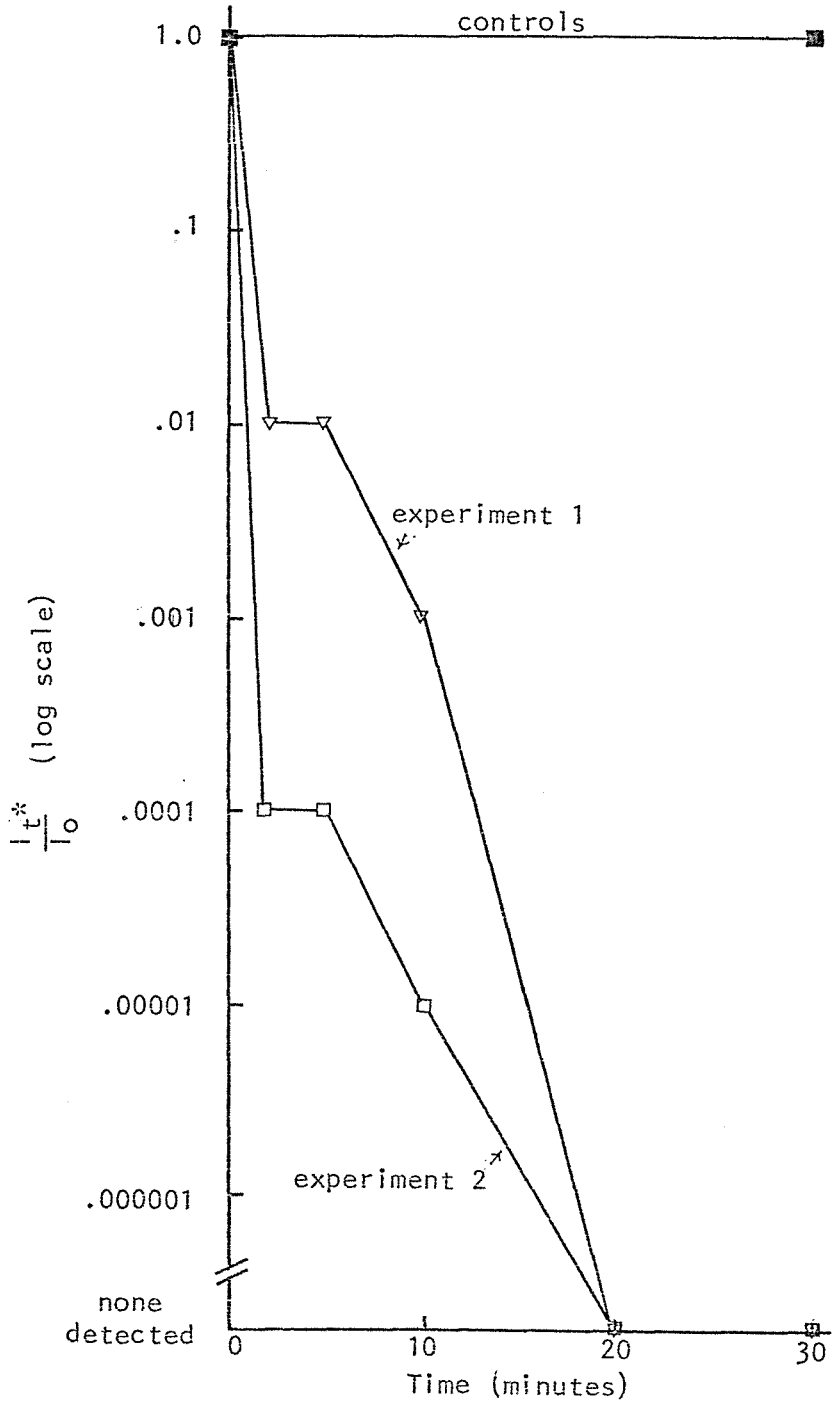
+ = colour change from red to yellow in FM4 broth

- = no colour change in FM4 broth

The minimum strength of formalin required to inactivate M. ovipneumoniae in the conditions used was a 1/100 final dilution of formalin (i.e. 1%)

6.3.2 Inactivation of M. ovipneumoniae with formalin for vaccine production

Based on 6.3.1, a 1/100 (final) dilution of formalin was used for inactivating M. ovipneumoniae. M. ovipneumoniae cells suspended in sterile saline were treated with a 1/100 (final) dilution of formalin and assayed as described in 6.2.4. The experiment was performed twice. The results are seen in figure 20. In both experiments there were no mycoplasmas detected at 20 and 30 minutes in the formalin treated samples. The titre of control (untreated) mycoplasmas remained constant for the 30 minute time interval.



* I_t = Titre of M. ovipneumoniae at time t

I_o = Titre of M. ovipneumoniae at time o

FIGURE 20: Inactivation of M. ovipneumoniae with 1/100 (final) formalin

6.4 Discussion

The purpose of this experiment was to determine a suitable procedure for producing a formalin inactivated *M. ovipneumoniae* vaccine. The concentration of 1% formalin shown here to be the minimum concentration needed to inactivate *M. ovipneumoniae* is in agreement with the values of 1 to 8% stated by Gaughran and Borick (1977) in the *CRC Handbook of Microbiology* as the concentration of formalin needed to inactivate vegetative bacteria.

The fact that no viable mycoplasmas were detected after 20 minutes treatment with 1% formalin suggests that for experimental purposes a suitable vaccine containing killed *M. ovipneumoniae* organisms could be made using the conditions described in this experiment and treating the organisms with 1% (final) formalin for 30 minutes.

CHAPTER 7 General Discussion

The research reported in this thesis is part of a programme undertaken to establish the role of mycoplasmas in the aetiology of ovine chronic non-progressive pneumonia. Studies by Clarke et al. (1974) and Alley et al. (1975) resulted in the isolation of two mycoplasma species (viz. M. ovipneumoniae and M. arginini) from lambs with CNP, but these workers did not employ methods which would demonstrate the presence of ureaplasmas. Since Jones et al. (1979) showed that ureaplasmas could colonise the ovine lower respiratory tract it was important to determine the presence or absence of ureaplasmas in the lungs of sheep with CNP in New Zealand. The failure to isolate ureaplasmas from pneumonic sheep lungs (including material known to transmit the disease) in the present study suggests that ureaplasmas, even if they are ultimately shown to be present in sheep in New Zealand, are not a necessary component in the aetiology of ovine CNP.

Advantage was taken of the material collected for the ureaplasma survey to reassess the prevalence of M. ovipneumoniae and M. arginini in pneumonic sheep lungs. These results show that the prevalence of M. ovipneumoniae (98%) confirms the results of Alley et al. (1975) who recovered M. ovipneumoniae from 79% and 100% of pneumonic lungs tested in two samplings. The present investigation revealed that M. arginini was present in only 4% of the pneumonic lungs, which may be contrasted with the results of Alley et al. (1975) who recovered M. arginini from 32% of pneumonic lungs tested.

Since the lung is reported to have no normal flora (Isenberg and Painter, 1974) and there are many examples of mycoplasmas pathogenic for the lower respiratory tract (see chapter 1, 1.6.4) it could hardly be doubted that the presence of M. ovipneumoniae and/or M. arginini in lungs of lambs with CNP is pathologically significant. However, the relatively low and variable presence of M. arginini suggests that its role in the pathogenesis of the disease is probably confined to exacerbating pre-existing lesions. M. arginini is well known to infect a large range of animals (Bergey's Manual of Determinative Bacteriology, 1974) but in no case has its role as a primary pathogen been demonstrated. This, however, does not rule out

a synergistic role for the organism in the development of the lesions of ovine CNP. Indeed, Jones et al. (1978) suggested that the organisms may be crucial in the development of pleurisy, which is probably the most economically significant aspect of the disease.

To facilitate future investigations into the significance of M. arginini, its ultrastructure (hitherto unreported) was established and contrasted with that of M. ovipneumoniae. M. arginini measured 200 to 500 nm in diameter, had an electron-dense cytoplasm, and the presence of dumb-bell shaped forms (mainly in the exponential growth phase) implied that the organism divided by binary fission. The size range of M. arginini (200 to 500 nm) was smaller than that of M. ovipneumoniae which measured 500 to 800 nm in diameter, and unlike M. ovipneumoniae the limiting trilaminar membrane of M. arginini did not appear to bear surface projections. However, distinction between the two organisms in thin sections of, say, lung material would be difficult because the two size ranges overlapped, and the resolution of individual M. ovipneumoniae cells was not consistently adequate to demonstrate these surface projections. A distinction between the two organisms in thin sections would therefore require further work involving immunological labelling (e.g. using ferritin or peroxidase-conjugated specific antibody).

Investigation of the in vitro growth characteristics of M. arginini, while undertaken mainly with a view to production of a challenge inoculum for sheep and to determine the ultrastructure of the organism at different growth stages, provided some interesting contrasts with the growth characteristics of M. ovipneumoniae (which is representative of the growth characteristics of many other mycoplasmas) reported by Major et al. (1979). Thus, the initial fall in titre seen in the growth curve of M. arginini is not seen in the growth curves of M. ovipneumoniae and many other mycoplasmas. The generation time of M. arginini (0.5 hours) is less than that reported for M. ovipneumoniae (1.7 hours) and other mycoplasmas. This is probably a reflection of the highly complex nature of FM4A medium which was used for the growth of M. arginini. More surprisingly, there is a marked stationary phase in the growth of M. arginini which is not usually seen with other mycoplasmas (Smith, 1971).

In conclusion, it is likely that M. arginini may play a secondary role in the development of ovine CNP and it is expected that the growth characteristics and ultrastructure of the organism reported here will facilitate further studies which must be carried out if the role of M. arginini in the disease is to be unequivocally established.

The consistently high prevalence of M. ovipneumoniae in pneumonic lung homogenate demonstrated both in this and earlier work suggests that this organism plays a major role in ovine CNP. Even if this is admitted it does not distinguish between a primary role of the organism initiating lesions or a secondary role of the organism exacerbating pre-existing lesions produced by some other organism e.g. a virus, as has been suggested by Hore (1970) and Jones et al., (1979) who implicated PI3 virus; or a bacterium (including chlamydiae). However the demonstration (chapter 5) that digitonin treatment of pneumonic lung homogenate destroyed its ability to produce lesions of CNP implies that these lesions are initiated by an organism limited by a cholesterol-containing membrane. Such an organism could obviously be a mycoplasma since by definition these contain cholesterol in their membranes, but could also be an enveloped virus (e.g. PI3).

The ability of pneumonic lung homogenate to consistently produce lesions of CNP in inoculated sheep implies that all the necessary infectious components are present in the inoculum. Hence it is particularly significant that examination of the pneumonic lung homogenate used to transmit the disease in the present investigation failed to demonstrate the presence of viruses using a standard technique; and in particular failed to isolate PI3 virus from these homogenates, although I was successful in reisolating PI3 virus following its addition at a low titre to the pneumonic lung homogenate.

If, therefore, ovine CNP can be consistenly transmitted with homogenates of pneumonic lung in the apparent absence of viruses, and in the specific absence of PI3 virus, the ability of digitonin to prevent such transmission supports the conclusion that M. ovipneumoniae (which is consistently present in the inoculum, and was shown to

be digitonin sensitive) is responsible for the initiation of the primary lesions of the disease. It is relevant to note here that although M. arginini is usually (but not always) present in pools of pneumonic lung, following administration of such a pool as an aerosol inoculum to susceptible lambs and examining early lesions, the organism is recovered from only a low proportion (22% in the present investigation) of lungs with mild to severe pneumonic lesions.

Some observations concerning ovine CNP from the present work, together with earlier work done in New Zealand are presented below:

i) CNP can be transmitted using pneumonic lung homogenate as an intranasal inoculum (Alley and Clarke, 1979)

ii) Bacteria (especially P. haemolytica) are consistently present in such inocula (Alley, personal communication)

iii) Penicillin has the ability to markedly diminish the severity of (but not totally prevent the development of) lesions transmitted by pneumonic lung homogenate. (Alley and Clarke, in the press).

iv) P. haemolytica on its own, when administered intranasally into sheep, usually produces no lesions (Alley, personal communication).

Note: Overseas workers who inoculated large numbers of P. haemolytica intrabronchially into 4 to 5 month old lambs produced acute pneumonia which can be distinguished from CNP. (Biberstein et al., 1971).

v) M. ovipneumoniae is consistently present in inocula of pneumonic lung homogenate, and also in a high proportion of lesions of CNP at all stages of development, which contrasts with the low prevalence of M. arginini (Alley and Clarke, 1979; present investigation).

vi) M. ovipneumoniae (inoculated intranasally as a broth culture) can colonise the ovine lung and produce minimal lesions in some animals. (Alley and Clarke, 1979).

Note: The colonisation of the lung by M. ovipneumoniae appears to be less efficient when the organism is present in pure culture than when present in lung homogenate.

vii) A digitonin sensitive component is necessary for the transmission of CNP.

Note: This component could either be a mycoplasma, or an enveloped virus (present investigation).

viii) Viruses, in particular PI3 virus, are apparently absent from lung homogenates which transmit the disease (present investigation).

These observations are consistent with the following general hypothesis: Ovine CNP is initiated by M. ovipneumoniae which initially infects the upper respiratory tract of sheep. This organism can then colonise the lungs of a high proportion of lambs causing minimal lesions which require the presence of a bacterium (probably P. haemolytica) to give the fully developed field cases. While it seems unlikely that M. arginini is a necessary component in the disease, its ability to grow in the ovine lung, often to a high titre (e.g. 10^8 to 10^9 organisms/gram of tissue reported in chapter 4), strongly suggests that the organism can have an exacerbating effect on pre-existing lesions in some cases. At the present state of investigation there is no evidence that a virus is a necessary component in the development of ovine CNP, although the scientific difficulty of proving a negative must be borne in mind.

It is difficult to compare the above hypothesis in detail with the hypotheses of other workers. Some say that ovine CNP is of complex aetiology not only in the sense that more than one organism may be required to cause the disease, but also in the sense that different combinations of organisms may produce the same disease as defined pathologically. (Jones et al., 1978; Jones et al., 1979).

Some authors are more specific: thus St George et al. (1971), Carmichael et al. (1972), and Sullivan et al. (1973b) suggested that M. ovipneumoniae alone was responsible for the disease, but they only produced mild macroscopic lesions in approximately 50% of animals inoculated with M. ovipneumoniae. Other workers have confirmed the results of St George et al. (1971), Carmichael et al. (1972), and Sullivan et al. (1973b) only to the extent of producing minimal lesions in low numbers of animals (Foggie et al., 1976; Alley and

Clarke, 1979). Dr G. Cottew (personal communication to Clarke) has failed to produce pneumonic lesions in lambs using M. ovipneumoniae inocula.

Jones et al. (1978) stated that mixed cultures of M. ovipneumoniae, M. arginini and Pasteurella haemolytica produced lesions in 6 to 7 month old lambs identical to the field disease, but a year later (Jones et al., 1979) stated that M. arginini was unimportant in the disease, and that PI3 virus may be necessary to initiate lesions.

Davies et al. (1977) suggested the importance of PI3 virus and P. haemolytica in pneumonia of sheep. Their suggestion was based on the experimental production of pneumonia in lambs inoculated with PI3 virus, followed by P. haemolytica. However, they did not give a pathological description of the disease produced, and indeed, Sharp et al. (1978) performed similar experiments (i.e. concurrent infection of lambs with PI3 virus and P. haemolytica) and produced an acute pneumonia.

The present hypothesis that M. ovipneumoniae initiates lesions of CNP which are made more severe by the invasion of bacteria (P. haemolytica especially), is more specific than those of most of the above workers. The evidence that M. ovipneumoniae initiates the disease is strong. Nevertheless it will have to be unequivocally established, and the most direct approach is to inoculate lambs with mixtures of organisms including all possible permutations of M. ovipneumoniae, M. arginini, PI3 virus, and bacteria (especially P. haemolytica). The study of M. arginini reported here will make a contribution to this approach.

A parallel approach which the present investigation also anticipates is the production of M. ovipneumoniae vaccines both inactivated and, possibly, living. Thus if it could be shown that immunisation with M. ovipneumoniae alone prevents the initiation of CNP lesions in lambs after challenge with pneumonic lung homogenate, this would not only strongly support the central role of M. ovipneumoniae in CNP, but could hopefully be developed to the point where routine vaccination of severely affected flocks against CNP could prevent the disease and minimise the economic loss which the disease causes in New Zealand.

APPENDIX

Source of mycoplasmas

(a) M. ovipneumoniae strain 5

This strain was isolated in 1973 from the lungs of a lamb with CNP by Dr J.K. Clarke, and was cloned after isolation. At the commencement of this present work an isolated colony was taken with a plug of agar from an FM4 agar plate, propagated in FM4 broth, and the organism was passaged 3 times at limit dilution. It was then grown to late exponential phase in FM4 broth and stored in aliquots at -70°C. In a gel precipitin test, this organism showed lines of identity with the original Australian isolate.

(b) M. arginini

The strain used was isolated from a pneumonic sheep lung in 1973 by Dr J.K. Clarke. It was identified serologically as Mycoplasma arginini by Microbiological Associates, U.S.A. A culture of this organism derived from a single colony was used in the present work. The organism was stored in aliquots at -70°C.

Mycoplasma media

(a) U9 medium for ureaplasmas

The composition was as follows:

Base:

Peptone CS (Albimi)	0.75 g
Glucose	0.08 g
KH_2PO_4	0.02 g
Deionised water to	100 cm^3
2N HCl to	pH 5.5

The base was autoclaved at 121°C for 15 minutes.

Complete medium:

Base	95 cm^3
Unheated horse serum	4 cm^3 (filter sterilised)
10% Urea solution*	0.5 cm^3 (filter sterilised)
1% phenol red	0.1 cm^3 (autoclaved)
Penicillin (10^5 units/cm^3)	1.0 cm^3 (filter sterilised)

The final pH of the medium was 6.0 $\pm$ 0.2. The medium was stored at 4°C until use.

* Ultrapure was used.

The growth of ureaplasmas in liquid medium was indicated by a colour change in the medium from yellow to purple.

U9 agar:

Base	95 cm^3
Agar	1.0 g

The mixture was autoclaved at 121°C for 15 minutes, then allowed to cool to about 46°C. Horse serum, 10% urea, 1% phenol red, and penicillin were added as above. 4 cm^3 volumes were pipetted into 50 mm plastic petri-dishes with tight fitting lids. Plates were stored at 4°C until use.

(b) FM4 (and FM4A) broth

This medium is the fourth of several formulations of Frey, Hanson and Anderson (1968). The formula is:

NaCl	5.0 g
KCl	0.4 g
$MgSO_4.7H_2O$	0.2 g
$Na_2HPO_4.12H_2O$	4.03 g
KH_2PO_4	0.1 g
Glucose	10.0 g
Peptone CS (Albimi)	10.0 g
Yeast autolysate (Albimi)	5.0 g
NAD	0.1 g

/...

L-cysteine HCl	0.1 g
Eagles vitamin solution (x 100)	25 cm^3
0.4% phenol red	2.5 cm^3
Penicillin	10^6 units
Thallium acetate	0.5 g
Deionised water to	1000.0 cm^3

Note: i) The thallium acetate was dissolved in 10 cm^3 deionised water and added to the medium dropwise to prevent precipitation.

ii) The basal medium was supplemented with 150 cm^3 of pig serum.

iii) For glucose fermenting mycoplasmas the pH was adjusted to pH 7.8 with 1.0N NaOH.

iv) For arginine requiring mycoplasmas, 10 g of arginine was added and the pH was adjusted to 7.0 with 1.0N HCl. This modification was referred to as FM4A medium.

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

vi) The formula for Eagle's vitamin solution (x 100) is:

D-biotin	20 mg
Calcium pantothenate	20 mg
Choline chloride	20 mg
Folic acid	20 mg
Riboflavin	2.0 mg
myo-Inositol	40 mg
Niacinamide	20 mg
Pyridoxine	20 mg
Thiamine-HCl	20 mg
Distilled water to	100 cm^3

The solution was stored in 25 cm^3 aliquots at -20^oC until use.

Growth of M. ovipneumoniae in FM4 broth was indicated by a colour change in the pH indicator from red to yellow, and growth of M. arginini in FM4A broth was indicated by a colour change in the pH indicator from orange to purple.

FM4 agar and FM4A agar

BHI (Difco)	0.74 g
Agar	1.0 g
Deionised water to	20.0 cm^3

The BHI-agar was autoclaved at 121°C for 15 minutes. It was then cooled to 50°C and 80 cm^3 of FM4 (or FM4A) at 45°C was added. The medium was mixed thoroughly and 4 cm^3 volumes were pipetted into 50 mm plastic petri-dishes with tight fitting lids. The solidified medium was used immediately, or stored at 4°C for not more than 2 weeks.

Phosphate buffered saline (PBS)

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water to	1000.0 cm^3

The solution was autoclaved at 121°C for 15 minutes. Final pH = 7.2 to 7.4.

Alsever's solution (modified)

Glucose	20.5 g
Tri-sodium citrate	8.0 g
NaCl	4.2 g
Citric acid	0.55 g
Distilled water to	1000.0 cm^3

The solution was autoclaved at 121°C for 15 minutes, and stored at 4°C. Final pH = 6.1.

Saline (Physiological saline)

NaCl	8.5 g
Distilled water to	1000.0 cm^3

The solution was autoclaved at 121°C for 15 minutes.

BIBLIOGRAPHY

ALLEY, M.R. (1975a) The pathogenesis of pneumonia in sheep. Thesis, Ph.D., Massey University.

ALLEY, M.R. (1975b) The bacterial flora of the respiratory tract of normal and pneumonic sheep. New Zealand Veterinary Journal 23:113-118.

ALLEY, M.R. & CLARKE, J.K. (1977) The influence of microorganisms on the severity of lesions in chronic ovine pneumonia. New Zealand Veterinary Journal 25:200-203.

ALLEY, M.R. & CLARKE, J.K. (1979) The experimental transmission of chronic non-progressive pneumonia. New Zealand Veterinary Journal 27:217-220.

ALLEY, M.R., QUINLAN, J.R. & CLARKE, J.K. (1975) The prevalence of Mycoplasma ovipneumoniae and Mycoplasma arginini in the respiratory tract of sheep. New Zealand Veterinary Journal 23:137-141.

ANDREWES, C.H. & GLOVER, R.E. (1945) Grey lung virus: An agent pathogenic for mice and other rodents. British Journal of Experimental Pathology 26:379-387.

ANDREWES, C. & PEREIRA, H.G. (1972) Viruses of Vertebrates 3rd ed. Bailliere Tindall.

BETTS, A.O. (1971) The role of mycoplasmas in diseases of pigs. Proceedings of the Royal Society of Medicine 64:34-35.

BIBERSTEIN, E.L., SHREEVE, B.J., ANGUS, K.W. & THOMSON, D.A. (1971) Experimental pneumonia of sheep. Clinical, microbiological and pathological responses to infection with Myxovirus parainfluenzae 3 and Pasteurella haemolytica. Journal of Comparative Pathology 81:339-351.

BLACK, F.T., BIRCH-ANDERSON, A. & FRENDT, E.A. (1972) Morphology and ultrastructure of human T-mycoplasmas. Journal of Bacteriology 111:254-259.

BOATMAN, E.S. & KENNY, G.E. (1970) Three dimensional morphology, ultrastructure, and replication of Mycoplasma felis. Journal of Bacteriology 101:262-277.

BOIDEN, A.G., CORDY, D.R. & ADLER, H.E. (1958) A pleuropneumonia-like organism and a virus in ovine pneumonia in California. Cornell Veterinarian 48:410.

BUCHANAN, R.E. & GIBBONS, N.E. eds (1974) Bergey's Manual of Determinative Bacteriology 8th edition. Williams and Watkins.

CARMICHAEL, L.E., ST GEORGE, T.D., SULLIVAN, N.D. & HORSFALL, N. (1972) Isolation, propagation and characterisation studies of an ovine Mycoplasma responsible for proliferative interstitial pneumonia. Cornell Veterinarian 62:654-679.

CARTER, M.E. & HUNTER, R. (1970) Isolation of parainfluenza type 3 virus from sheep in New Zealand. New Zealand Veterinary Journal 18:266-267.

CASSELL, G.H., LINDSEY, J.R., OVERCASH, R.C. & BAKER, H.J. (1973) Murine mycoplasma respiratory disease. Annals of the New York Academy of Sciences 225:395-412.

CHANOCK, R.M. DIENES, L., EDWARD, D.G., FREUNDT, E.A., HAYFLICK, L., HERS, J.F., JENSEN, K.E., LIU, C., MARMION, B.P., MORTON, H.E., MUFSON, M.A., SMITH, P.F., SOMERSON, N.L. & TAYLOR-ROBINSON, D. (1963) Mycoplasma pneumoniae: Proposed nomenclature for atypical pneumonia organism (Eaton agent). Science 140:662-663.

CHANOCK, R.M., HAYFLICK, L. & BARILE, M.F. (1962). Growth on artificial medium of the agent assoicated with atypical pneumonia, and its identification as PPLO. Proceedings of the National Academy of Science, U.S.A. 48:41-49.

CHANOCK, R.M., STEINBERG, P. & PURCELL, R.H. (1970) Mycoplasmas in human respiratory disease. Chapter 6 In Sharp, J.T. (ed.) The Role of Mycoplasmas and L-forms of Bacteria in Disease. Charles C. Thomas.

CLANCY, C.F. (1977) Enterobacteriaceae. In Laskin, A. & Lechevalier, H.A. (eds) CRC Handbook of Microbiology. 2nd ed. Vol. I. CRC Press.

CLARKE, J.K., BROWN, V.G. & ALLEY, M.R. (1974). Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. New Zealand Veterinary Journal 22:117-121.

COTTEW, G.S. & LEACH, R.H. (1969) Mycoplasmas of cattle, sheep and goats. Chapter 19 In Hayflick, L. (ed.) The Mycoplasmatales and the L-phase of Bacteria. Appleton.

DAVIES, D.H., DUNGWORTH, D.L., HUMPHREYS, S. & JOHNSON, A.J. (1977). Concurrent infection of lambs with parainfluenza virus type 3 and Pasteurella haemolytica. New Zealand Veterinary Journal 25: 263-265.

DAVIS, G.B. (1970) Personal communication cited by Alley, M.R. (1975a).

DAWSON, M., CHASEY, D., KING, A.A., FLOWERS, M.J., DAY, R.H., LUCAS, M.H. & ROBERTS, D.H. (1979) The demonstration of maedi/visna virus in sheep in Great Britain. Veterinary Record 105:220-223.

DITCHFIELD, J. (1966). Isolation of parainfluenza type 3 from Canadian sheep. Veterinary Record 79:773.

DOWNEY, N.E. (1957) A preliminary investigation into the aetiology of enzootic pneumonia of sheep ("Southland pneumonia"). New Zealand Veterinary Journal 5:128-133.

DUNGWORTH, D.L. & CORDY, D.R. (1962) The pathogenesis of ovine pneumonia.(I) Isolation of a virus of the PLV group. Journal of Comparative Pathology 72:49-70.

DYSART, T.H. (1976) Enzootic pneumonia - its effect on the meat industry. pp. 94-100. In Proceedings of the New Zealand Veterinary Association Sheep Society. 6th seminar. Massey University.

EATON, M.D., MEIKLEJOHN, G. & VAN HERRICK, W. (1944). Studies on the aetiology of primary atypical pneumonia: a filterable agent transmissible to cotton rats, hamsters and chick embryos. Journal of Experimental Medicine 79:649-668.

EDWARD, D.G. (1940) The occurrence in normal mice of pleuropneumonia-like organisms capable of producing pneumonia. Journal of Pathology and Bacteriology 50:409-418.

EDWARD, D.G. (1954) The PPLO group of organisms: A review, together with some new observations. Journal of General Microbiology 10:27.

EDWARD, D.G. & FREUNDT, E.A. (1956) The classification and nomenclature of organisms of the pleuropneumonia group. Journal of General Microbiology 14:197-207.

EDWARD, D.G. & KANAREK, A.D. (1960) Organisms of the pleuropneumonia group of avian origin: their classification into species. Annals of the New York Academy of Sciences 79:696-702.

EL MAHI, M.M. & NAYIL, A.A. (1978) Isolation of mycoplasmas from pneumonic sheep lungs in the Sudan. Research in Veterinary Science 24:314-317.

FABRICANT, J. (1969) Avian mycoplasmas. Chapter 22 In Hayflick, L. (ed.) The Mycoplasmatales and the L-phase of Bacteria. Appleton.

FABRICANT, J. & LEVINE, P.P. (1962) Experimental production of complicated respiratory disease infection ("air-sac" disease). Avian diseases 6:13-23.

FOGGIE, A. & ANGUS, K.W. (1972) Observations on the distribution of Mycoplasma arginini as a respiratory tract infection in sheep and its pathogenicity for specific pathogen free lambs. Veterinary Record 90:312-313.

FOGGIE, A., JONES, G.E. & BUXTON, D. (1976) The experimental infection of specific pathogen free lambs with Mycoplasma ovipneumoniae. Research in Veterinary Science 21:28-35.

FREY, M.C., HANSON, R.P. & ANDERSON, D.P. (1968) A medium for the isolation of avian mycoplasmas. American Journal of Veterinary Research 29:2163-2179.

FRIIS, N.F. (1971) Mycoplasmas cultivated from the respiratory tract of Danish pigs. Acta Veterinaria Scandinavica 12:68-79.

FRIIS, N.F. (1972) Isolation and characterisation of a new porcine mycoplasma. Acta Veterinaria Scandinavica 13:284-286.

FRIIS, N.F. PALSSON, P.A. & PETURSSON, G. (1976). Mycoplasma ovinpneumoniae demonstrated in Icelandic sheep. Acta Veterinaria Scandinavica 17:255-257.

FUCCILLO, D.A. KURENT, J.E. & SEVER, J.L. (1974) Slow virus diseases. Annual Review of Microbiology 28:231-264.

GAUGHRAN, E.R.L. & BORICK, P.R. (1977) Sterilisation, disinfection and antisepsis. In Laskin, A. & Lechevalier, H.A. (eds) CRC Handbook of Microbiology 2d ed. Vol II. CRC Press.

GAY, F.W. MAGUIRE, M.E. & BASKERVILLE, A. (1972) Etiology of chronic pneumonia in rats and a study of the experimental disease in mice. Infection and Immunity 6:83-91.

GOIS, M., POSPISIL, Z., CERNY, M. & MRVA, M. (1971) Production of pneumonia after intransal inoculation of gnotobiotic piglets with three strains of Mycoplasma hyorhinis. Journal of Comparative Pathology 81:401-411.

GOODWIN, R.F.W., POMEROY, A.P. & WHITTLESTONE, P. (1965) Production of enzootic pneumonia in pigs with a mycoplasma. Veterinary Record 77:1247-1249.

GOODWIN, R.F.W, POMEROY, A.P. & WHITTLESTONE, P. (1967) Characterisation of Mycoplasma suipneumoniae: a mycoplasma causing enzootic pneumonia of pigs. Journal of Hygiene (Cambridge) 65:85-96.

GOURLAY, R.N. & THOMAS, L.H. (1970) The experimental production of pneumonia in calves by the endobronchial inoculation of T-mycoplasmas. Journal of Comparative Pathology 80:585-594.

GREEN, F. & HANSON, R.P. (1973) Ultrastructure and capsule of Mycoplasma meleagridis. Journal of Bacteriology 116:1011-1018.

GROSS, W.B. (1961) The development of "air sac disease". Avian diseases 5:431-439.

HAASE, A.T. (1975) The slow virus infection caused by visna virus. Current Topics in Microbiology and Immunology 72:101-156.

HAMDY, A.H. POUNDEN, W.D. & FERGUSON, L.C. (1959) Microbial agents associated with pneumonia in slaughtered lambs. American Journal of Veterinary Research 20:87-90.

HARRIS, R.E. & ALLEY, M.R. (1977) Pneumonia in sheep: does it affect weight gain? New Zealand Veterinary Journal 25:108.

HODGES, R.T., BETTS, A.O. & JENNINGS, A.R. (1969). Production of pneumonia in gnotobiotic pigs with pure cultures of Mycoplasma hyopneumoniae. Veterinary Record 84:268-273.

HORE, D.E. (1966) Isolation of ovine strains of parainfluenza virus serologically related to type 3. Veterinary Record 79:466-467.

HORE, D.E. (1970) Pneumonia in sheep. Australian Veterinary Journal 46:169-172.

HORNER, G.W., JOHNSON, R.H., DENNETT, D.P. & LANE, W.R. (1973) A serological study of bovine foetal immunoglobulins. Australian Veterinary Journal 49:324-329.

HOWARD, C.J. & GOURLAY, R.N. (1974) A electron-microscopic examination of certain bovine mycoplasmas stained with ruthenium red and the demonstration of a capsule of Mycoplasma dispar. Journal of General Microbiology 83:393-398.

ISENBERG, H.D. & PAINTER, B.G. (1974) Indigenous and pathogenic microorganisms of man. Chapter 5 In Lenette, E.H., Spaulding, E.H. & Truant, J.P. (eds) Manual of Clinical Microbiology 2nd ed. American Society for Microbiology.

JONES, G.E., BUXTON, D. & HARKER, D.B. (1979) Respiratory infections in housed sheep, with particular reference to mycoplasmas. Veterinary Microbiology 4:47-59.

JONES, G.E., FOGGIE, A., MOULD, D.L. & LIVITT, S. (1976). The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. Journal of Medical Microbiology 9:39-52.

JONES, G.E., GILMOUR, J.S. & RAE, A. (1978) Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasmas isolated from them. Journal of Comparative Pathology 88:85-96.

KIRTON, A.H., O'HARA, P.J., SHORTRIDGE, E.H. & CORDES, D.O. (1976). Seasonal incidence of enzootic pneumonia and its effect on the growth of lambs. New Zealand Veterinary Journal 24:59-64.

KLIENEBERGER-NOBEL, E. (1962) Pleuropneumonia-like organisms (PPLO), Mycoplasma-taceae. Academic Press inc.

KLIENEBERGER-NOBEL, E. & CHENG, K. (1955). On the association of the pleuropneumonia-like L3 organism with experimentally produced bronchiectasis in rats. Journal of Pathology and Bacteriology 70: 245-246.

LEACH, R.H., COTTEW, G.S., ANDREWS, B.E. & POWELL, D.G. (1976) Atypical mycoplasmas from sheep in Great Britain and Australia identified as Mycoplasma ovipneumoniae. Veterinary Record 98:377-379.

L'ECUYER, C., SWITZER, W.P. & ROBERTS, E.D. (1961) Microbiologic survey of pneumonic and normal swine lungs. American Journal of Veterinary Research 27:1020-1025.

LIU, C., EATON, M.D. & HEYL, J. (1959) Studies in primary atypical pneumonia: (II) Observation concerning development and immunological characteristics of antibody in patients. Journal of Experimental Medicine 109:545-556.

LLOYD, L.C. & TRETHEWIE, E.R. (1970) Contagious bovine pleuropneumonia. Chapter 9 In Sharp, J.T. (ed.) The Role of Mycoplasmas and L-forms of Bacteria in Disease. Charles C. Thomas.

LOW, I.E. & EATON, M.D. (1965) Replication of Mycoplasma pneumoniae in broth culture. Journal of Bacteriology 89:725-728.

MAJOR, R.M., CLARKE, J.K. & ALLEY, M.R. (1979). An in vitro study of the replication of Mycoplasma ovipneumoniae. New Zealand Veterinary Journal 27:58-61.

MANILOFF, J. (1972) Cytology of the mycoplasmas. In Pathogenic Mycoplasmas; A ciba foundation symposium. Associated Scientific Publishers.

MARÉ, C.J. & SWITZER, W.J. (1965) New Species: Mycoplasma hypopneumoniae A causative agent of virus pig pneumonia. Veterinary Medicine 60:841-846.

MARKHAM, F.S. & WONG, S.C. (1952). Pleuropneumonia-like organisms in the aetiology of turkey sinusitis and chronic respiratory disease in chickens. Poultry Science 31:902-904.

MARMION, B. & GOODBURN, G. (1961) Effect of an organic gold salt on Eaton's primary atypical pneumonia agent, and other observations. Nature 189:247-248.

MCKERCHER, D.G. (1952) A virus possibly related to the psittacosis-lymphogranuloma-pneumonitis group causing pneumonia in sheep. Science 115:543-544.

NELSON, J.G. (1967) Pathological response of Swiss and Princeton mice to M. pulmonis. Annals of the New York Academy of Sciences 143:778-783.

NOCARD, E. & ROUX, E.R. (1898). Le microbe de las péripneumonie. Annales de l'Institut Pasteur (Paris) 12:240-262. Cited by Lloyd and Trethewie (1970).

RAZIN, S. (1973) Physiology of mycoplasmas. Advances in Microbial Physiology 10:1-80.

ROSS, R.F. (1973) Pathogenicity of swine mycoplasmas. Annals of the New York Academy of Sciences 225:347-368.

ROSS, R.F. & KARMON, J.A. (1972) Heterogeneity among strains of Mycoplasma granularum and identification of Mycoplasma hyosynoviae sp. n. Journal of Bacteriology 103:707-713.

SALISBURY, R.M. (1957) Enzootic pneumonia of sheep in New Zealand. New Zealand Veterinary Journal 5:124-127.

SCADDING, J.G. (1937) British Medical Journal ii:956 Cited In Spencer, H. (1968) Pathology of the Lung (excluding pulmonary tuberculosis). 2nd ed. London.

SHARP, J.M., GILMOUR, N.J.L., THOMSON, D.A. & RUSHTON, B. (1978) Experimental infection of specific pathogen-free lambs with parainfluenza virus type 3 and Pasteurella haemolytica. Journal of Comparative Pathology 88:237-243.

SHEPARD, M.C. & LUNCEFORD, C.D. (1970) Urease colour test medium U-9 for the detection and identification of "T" mycoplasmas in clinical material. Applied Microbiology 20:539-543.

SHIMIZU, T., NOSAKA, D. & NAKAMURA, N. (1975) Significance of T-mycoplasmas in enzootic calf pneumonia. Japanese Journal of Veterinary Science 37:121-131.

SIGURDSSON, B. (1954) Rida, a chronic encephalitis of sheep. With general remarks on infections which develop slowly, and some of their characterisitcs. British Veterinary Journal 110:341-354.

SMITH, B.L. (1970) Personal communication cited by Alley, M.R. (1975a).

SMITH, P.F. (1971) The Biology of Mycoplasmas. Academic Press.

SNEDECOR, G.W. & COCHRAN, W.G. (1967) Statistical Methods 6th ed. Iowa State University Press.

STAMP, J.T. & NISBET, D.I. (1963) Pneumonia of sheep. Journal of Comparative Pathology 73:319-328.

STEVENSON, R.G. & ROBINSON, G. (1970) The pathology of pneumonia in young lambs inoculated with Bedsonia. Research in Veterinary Science 11:469-474.

ST GEORGE, T.D. (1972) Investigations of respiratory disease of sheep in Australia. Australian Veterinary Journal 48:318-322.

ST GEORGE, T.D. & CARMICHAEL, L.E. (1975) Isolation of Mycoplasma ovipneumoniae from sheep with chronic pneumonia. Veterinary Record 97:205-206.

ST GEORGE, T.D. & LIEFMAN, C.E. (1972) A field experiment to assess the role of parainfluenza type 3 virus in pneumonia in a flock of sheep in Victoria. Australian Journal of Biological Science 25:319-325.

ST GEORGE, T.D., SULLIVAN, N.D., LOVE, J.A. & HORSFALL, N. (1971) Experimental transmission of pneumonia in sheep with a mycoplasma isolated from pneumonic sheep lung. Australian Veterinary Journal 47:282-283.

SULLIVAN, N.D., ST GEORGE, T.D. & HORSFALL, N. (1973a) A proliferative interstitial pneumonia of sheep associated with mycoplasma infection. 1. Natural history of the disease in a flock. Australian Veterinary Journal 49:57-62.

SULLIVAN, N.D., ST GEORGE, T.D. & HORSFALL, N. (1973b) A proliferative interstitial pneumonia of sheep associated with mycoplasma infection. 2. The experimental exposure of young lambs to infection. Australian Veterinary Journal 49:63-68.

THOMAS, L. (1973) Mycoplasmas and L-forms. Chapter 40 In Davis et al. (eds) Microbiology 2nd ed. Harper and Row.

THURLEY, D.C., BOYES, B.W., DAVIES, D.H., WILKINS, M.F., O'CONNELL, E. & HUMPHREY, S. (1977) Subclinical pneumonia in lambs. New Zealand Veterinary Journal 25:173-176

WASSERMAN, B., YATES, U.J. & FREY, D.E. (1954) On so-called air-sac infection. Poultry Science 33:622-623.

WHITTLESTONE, P. (1972) The role of mycoplasmas in the production of pneumonia in the pig. In Pathogenic Mycoplasmas. A ciba foundation symposium. Associated Scientific Publishers.

WHITTLESTONE, P. (1973) Enzootic pneumonia of pigs (EPP). Advances in Veterinary Science and Comparative Medicine 17:1-55.