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AN *IN VITRO* STUDY OF THE REPLICATION, MORPHOLOGY AND DNA BASE COMPOSITION OF *MYCOPLASMA OVIPNEUMONIAE*

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requirements for the degree of
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ABSTRACT

Mycoplasma ovipneumoniae can almost invariably be isolated from the lungs of sheep with chronic pneumonia, which is a prevalent disease in New Zealand hoggets. At Massey University, a study is in progress to establish the part, if any, played by *M. ovipneumoniae* in the pathogenesis of the disease. This thesis represents an *in vitro* investigation of some properties of *M. ovipneumoniae*. It was undertaken as part of the larger study, and is presented in that context.

To establish a method for the production of high titre exponential phase inocula for use in disease transmission experiments, the growth of *M. ovipneumoniae* in FM4 broth was studied. It was found that a maximum titre of 1.0 to 3.0 x 10^9 CFU/ml was produced regardless of the inoculum size or degree of aeration. The organism had a minimum division time of 1.7 hr; had no stationary phase and in the late death phase was inactivated with a half-life of about 0.5 hr.

The organism was stored at -70° with little loss in titre (less than two-fold) over an 18 month period.

Shaking cultures became sufficiently turbid during growth to allow meaningful measurements to be made using an SP20 spectrophotometer. In defined conditions, viz. when a shaking culture is in the exponential phase and contains 2.0 to 10.0 x 10^8 CFU/ml, the viable cell count can be estimated from turbidity measurements.

Electron microscopy of *M. ovipneumoniae* showed that the cells are roughly spherical, 400 to 700nm in diameter, probably replicate by binary fission, contain ribosomes and fibrils of deoxyribonucleic acid, and are bounded by a trilaminar membrane bearing projections 12nm long. No specialized structural feature such as the attachment sites found in *M. pneumoniae* was detected.

The New Zealand isolate of *M. ovipneumoniae* was morphologically indistinguishable from the standard *M. ovipneumoniae* strain isolated in Australia.

Although the above description could be applied to many mycoplasma species, it should be noted that the

average cell diameter of M. ovipneumoniae (about 550nm) is larger than that found for most but not all species of mycoplasma.

The base composition of the DNA of M. ovipneumoniae determined by the thermal denaturation and buoyant density studies was 28.1% GC and 28.0% GC respectively. This relatively low GC content falls within the accepted range for mycoplasma species (23 - 40% GC) and within the much narrower range (26.8 - 28.5% GC) of glycolytic mycoplasmas causing respiratory disease in domestic animals.

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INTRODUCTION

Sheep are obviously an important domestic animal species, especially in New Zealand, and it is generally recognized that chronic pneumonia is one of the most prevalent diseases of sheep in this country. It is surprising, therefore, that the disease has received little attention until recently, and because it has not yet been studied intensively, the cause or causes of the disease have not so far been unequivocally established.

Although it could be argued that any investigation of the disease should avoid preconceived ideas, in practice it was found necessary, in this laboratory, to limit initial investigations to an area which appeared to be the one most likely to give positive results. For this reason, clues to the etiology of the disease were looked for by examining what was established for similar diseases in both domestic and laboratory animals.

Analogous diseases (reviewed in a later section) in animals, such as goats, cattle, swine and rodents have been shown to have, at least in part a mycoplasmal etiology. For this reason an investigation was undertaken (Clarke *et al.*, 1974) to isolate and identify mycoplasmas from the respiratory tract of normal and pneumonic sheep in New Zealand. Subsequently, two species of mycoplasma were isolated, one of which required arginine for growth and was identified as *M. arginini*. The other was a glycolytic mycoplasma which was found, by gel precipitin tests, to be related to an organism isolated three years earlier in Australia (St. George *et al.*, 1971) and later designated *Mycoplasma ovipneumoniae*. (Carmichael *et al.*, 1972).

The Australian workers (St. George *et al.*, 1971) claimed to have transmitted chronic pneumonia experimentally to sheep using broth cultures of *M. ovipneumoniae*, but attempts to repeat this in New Zealand (Alley & Clarke, personal communication) have either failed, or caused a disease not necessarily identical to the natural disease, in a low proportion of inoculated animals. However, the disease could be transmitted (Alley,

personal communication) experimentally by inoculating sheep intranasally with an aerosol of diseased lung homogenate.

There are several possible reasons for the failure to transmit chronic pneumonia by inoculating an *in vitro* culture of *M. ovipneumoniae* intranasally into lambs, e.g. the immune status of the lamb, or the size of droplet. However, since whole lung homogenate was successfully used to transmit the disease under similar conditions and using similar animals, it seems likely that other reasons must be sought, e.g. *M. ovipneumoniae* may not be the primary cause of the disease and is only a secondary invader, or it may be a primary invader, but only when in association with another organism.

The question of whether or not *M. ovipneumoniae* is a primary cause of chronic pneumonia in sheep can in principle be decided by intranasal inoculation of sheep or lambs which are known to be susceptible to the disease, but a technical difficulty arises when preparing the inoculum, which ideally should contain a high titre of actively growing organisms. This difficulty is due to the fact that mycoplasmas have little or no stationary phase, and die rapidly after the maximum titre has been reached. In practice, growth of mycoplasmas is indicated by a pH change of the medium, which, depending on the circumstances, may only be visible at, or even after the maximum titre has been reached. Consequently the preparation of a high titre inoculum of actively growing mycoplasmas requires a detailed study of the growth of the organisms. One section of the present study examines the growth and subsequent death of *M. ovipneumoniae*.

Further clues to the significance of *M. ovipneumoniae* with respect to chronic pneumonia in sheep could be sought by electron microscopic examination of infected sheep. However, a useful preliminary for *in vivo* electron microscopy is a detailed examination of the morphology of the organism which is best achieved using *M. ovipneumoniae* cultured *in vitro*.

Bearing in mind that mycoplasmas die rapidly following growth, it is surprising that many morphological studies of these organisms conducted using *in vitro* cultures have used mycoplasmas grown for an arbitrary period not determined from growth curve data (Anderson *et al.*,1965; Nelson *et al.*, 1965; Domermuth *et al.*, 1964 (i); Domermuth *et al.*, 1964 (ii)). However, this approach is likely to give a mixture of some living organisms among an excess of dead ones. For this reason, the present electron microscopic study was preceded by growth studies so that the phase of growth or death of the culture was known. The morphology of both New Zealand and Australian strains of *M. ovipneumoniae* are investigated in this thesis.

During the course of the present work, it became apparent that several basic properties of *M. ovipneumoniae* have not been previously determined. Among these properties was the base composition of the organism, a character of taxonomic importance. Thus, melting temperature and buoyant density studies of DNA isolated from *M. ovipneumoniae* were undertaken so as to determine its GC/AT ratio.

The following historical review examines previous investigations of mycoplasmas and mycoplasmal infections with particular reference to organisms found in the respiratory tract.

Introductions to each section of the experimental work are included at the beginning of the appropriate section.

b) HISTORICAL OVERVIEW:

a) Mycoplasmas : General Aspects

Contagious bovine pleuropneumonia was first recognized as a specific disease in the early eighteenth century. Although not highly lethal, it causes a significant mortality rate and serious economic losses. A century after its initial recognition, it was demonstrated that subcutaneous inoculation of a drop of the fluid present in the interlobular connective tissue of infected lungs produced an infection which spread through the subcutaneous tissue and contiguous areas to the lung and pleura. (see Sharp, 1970). Some animals died, but others recovered and were subsequently immune to further inoculation and natural infection.

Early attempts to isolate the causative agent failed until Nocard and Roux (1898) propagated the organism by inoculating bouillon broth with fluid from infected lung. The inoculated broth was placed in a collodion sac and inserted into the peritoneal cavity of a rabbit. When the sac was removed several days later, the inoculated broth was slightly cloudy, and the opalescence could be transmitted serially by subculturing to fresh broth in collodion sacs. After serial passage the opalescence retained its ability to produce the typical lesions of pleuropneumonia in cattle. Control broths remained clear. The same workers, in the course of their investigations, propagated the organism *in vitro* in serum enriched peptone broth, and subsequently (Dujardin-Beaumetz, 1900) it was cultured on solid media, leading to the first description of the classical fried-egg appearance of colonies which is now known to represent the colonial morphology characteristic of many mycoplasmas.

The organism of pleuropneumonia, (ultimately designated *Mycoplasma mycoides var. mycoides*,) although isolated on cell-free medium was still referred to as a "virus" and it occupied a unique position in taxonomy for 25 years, until *M. agalactiae* was cultured in serum broth inoculated with the joint fluid, milk and draining lymph nodes of sheep with contagious agalactia (Bridrē and

Donatien, 1923, 1925). Subsequently, several organisms with the cellular and colonial morphology of the bovine pleuropneumonia organism were isolated from a variety of sources and the lack of an acceptable classification scheme led to their being called "pleuropneumonia-like organisms" or PPLO.

Laidlaw and Elford (1936) recovered from sewage the first "mycoplasma" isolated from other than an animal source. This organism was also the first "mycoplasma" isolated which did not require serum which is a source of sterols for growth. Although it was named M. laidlawii at the time, it is now called Acholeplasma laidlawii, because, by the current definition, mycoplasmas, unlike acholeplasmas require sterols.

In 1956, a previously unrecognized group of mycoplasmas was isolated from the genitournary tract of men with non-gonococcal urethritis (Shepard, 1956). These organisms, designated T-strains differ from other mycoplasmas in that they require urea for growth, and have an optimum of pH 6.0 (Smith, 1971). They produce tiny (T) colonies on conventional mycoplasma media, are microaerophilic and do not ferment carbohydrates.

Mycoplasmas are now divided into three main groups, with respect to growth requirements:

(i) Those which ferment carbohydrates (Fermentative mycoplasmas). Most pathogenic species, are included in this group.

(ii) Those which derive their energy from the conversion of arginine to citrulline and ornithine via the arginine dihydrolase pathway. (Non-fermentative mycoplasmas) e.g. M. arginini.

(iii) Mycoplasmas requiring urea for growth. Although at present called T-strains, they may soon be classified as the genus Ureaplasma within the Mycoplasmatales.

b) Pathogenic Mycoplasmas in General

Mycoplasmas have been shown to cause, or are suspected of causing a wide variety of diseases in various organs of many animals, including man. These pathogenic mycoplasmas display a high degree of specificity of both the species parasitized and the organ or tissue colonised or invaded. They may affect the central nervous system, joints, pleura, peritoneum, upper and lower respiratory tract and genitourinary system. A list of pathogenic mycoplasmas and the diseases they cause is presented in Table I.

c) Mycoplasmas Involved in Diseases of the Respiratory Tract.

Although, as can be seen from Table I, mycoplasmas can, and do infect many sites in the body, the most prevalent type of infection caused by mycoplasmas are those of the upper and lower respiratory tract (Smith, 1971).

Before discussion various aspects of mycoplasmal infections of the respiratory tract, it is relevant to note that different designations have been used: thus mycoplasmas can be responsible for conditions variously referred to as "viral" pneumonia; atypical pneumonia; enzootic pneumonia; chronic respiratory disease and chronic pneumonia.

Clearly "viral" pneumonia should not be used to describe mycoplasmal infections. The terms "atypical" or "chronic" are used in practice to distinguish the pneumonia from the "typical acute" form of the disease caused by bacteria. Since in many animals, including sheep, "atypical" pneumonia is much more common than "typical" pneumonia, the term chronic pneumonia gives a more reasonable representation of the disease. Some pathological and epidemiological differences between the acute and chronic forms of lung disease in sheep are dealt with in a subsequent section. The term enzootic pneumonia can be applied to any pneumonia caused by organisms endemic in a population: thus it

TABLE I Diseases caused by mycoplasmas in a variety of hosts.

HOST	DISEASE	MYCOPLASMA SPECIES
Man	Primary atypical pneumonia	M. pneumoniae
	Urogenital tract infections, possibly causing infertility and non gonococcal urethritis)	T-strains
Cattle	Contagious bovine pleuropneumonia	M. mycoides var mycoides
	Mastitis	M.bovigenitalium
	Mastitis and arthritis	M.bovimastitidis
	Mastitis	T-strains
	Possibly chronic pneumonia of calves	M.dispar
Sheep & Goats	Contagious caprine pleuropneumonia	M. mycoides var capri
	Mastitis, contagious agalactia	M. agalactiae
Swine	Enzootic pneumonia	M. suipneumoniae (hyopneumoniae)
	Polyarthritis	M. hyosynoviae
	Arthritis and polyserositis	M. hyorhinis
Rodents	Rolling disease of rats and mice	M. neurolyticum
	Infectious catarrh and possibly pneumonia of mice and rats	M. pulmonis
	Arthritis of rats and mice	M. arthritidis
Fowl	Chronic respiratory disease	M. gallisepticum
	Infectious sinusitis	
	Cerebral polyarteritis of turkeys	
	Infectious synovitis & chronic respiratory disease	M. synoviae
	Air sac disease of turkeys	M. meleagridis
Cats	Conjunctivitis	M. felis

Adapted and extended from Hayflick, L. (ed) 'The Mycoplasmatales and L-phase of Bacteria' Appleton, New York, 1969, & Sharp J.T. (ed) 'The Role of Mycoplasmas and L-forms of Bacteria in Disease' Charles C. Thomas, 1970.

can be either acute or chronic. In practice however, the term is applied mainly to a particular chronic disease in swine caused by M. hyopneumoniae. Chronic respiratory disease can be applied to conditions of the upper and/or lower respiratory tract. Since this thesis is concerned only with infection of the lung, the term chronic pneumonia is used.

It is by no means simple to define the etiological role of a certain mycoplasma species in the production of chronic pneumonia in an animal. Although (as will be shown in a later section) it has been established that M. ovipneumoniae is involved in the chronic pneumonia syndrome in sheep, its exact role is uncertain. To illustrate that the presence of a mycoplasma in a pneumonic lung does not in itself establish a primary etiological role for the organism, four disease complexes are considered below. Any one of them might be an appropriate model system for M. ovipneumoniae infection in sheep, but the significance of the mycoplasma differs in each case. Respiratory diseases in man, swine, domestic fowl and rodents are discussed.

(i) Primary Atypical Pneumonia in Man.

Primary atypical pneumonia was the name given to a syndrome which was recognised as an infectious disease but which could not be associated with pathogenic microorganisms by the laboratory methods available in the late 1930's (Scadding, 1937). Patients with the disease frequently developed cold agglutinins (antibodies which agglutinate human O group erythrocytes at 0-4°, but not at 37°) during the course of the illness. It was believed at that time that this syndrome was caused by a specific virus (see Grayston, Foy and Kenney, 1969). However an agent was ultimately isolated (the "Eaton" agent) which could pass through 180-250nm pore size filters and could be serially passaged in embryonated eggs without discernable effect on the embryo. The passaged agent, when inoculated intranasally into hamsters and and cotton rats produced pneumonia. (Eaton,

Meiklejohn and van Herrick, 1944). This agent was, nevertheless, not accepted as the cause of primary atypical pneumonia in man until Liu, Eaton and Heyl (1959) demonstrated that epidemics of cold agglutinin-positive pneumonia were associated with the Eaton agent, by detecting significant antibody rises to the agent in paired sera. Marmion and Goodburn (1961) then showed that the Eaton agent was susceptible to chlorotetracycline, streptomycin and gold salts, and so could not be a virus but was likely to be a mycoplasma. This was confirmed by Chanock, Hauflick and Barile (1962) who cultivated the agent on cell-free agar medium, and subsequently demonstrated that the colonies were centreless and mulberry-like; rather than the fried-egg type typical of most mycoplasma colonies on solid media. The agent was named Mycoplasma pneumoniae by Chanock et al. (1963).

Koch's postulates relating M. pneumoniae to primary atypical pneumonia in man were fulfilled when, in a series of volunteer studies (Chanock, Steinberg and Purcell, 1970), 32 of 84 individuals free of growth-inhibiting antibody inoculated with cultured organisms developed a febrile respiratory illness, whereas only 3 of 71 volunteers with pre-existing growth-inhibiting antibody became ill. The finding that less than half the inoculated volunteers developed discernable respiratory tract illness should be interpreted in the light of the finding that natural infection with M. pneumoniae produces a spectrum of effects ranging from inapparent infection to bronchitis and clinical pneumonia (Grayston et al., 1969).

M. pneumoniae is one of the few mycoplasmas for which the mechanism of pathogenesis has been established. In cultured hamster trachea and fetal human trachea, M. pneumoniae interferes with normal ciliary activity. The toxicity involves a combination of cell-attachment and cell lysis mediated by the release of peroxide because the parasite has an attachment structure reminiscent of a phage tail, which can come into intimate contact with the host cell, and thus allow mediators of cell injury to pass directly to their target (Clyde, 1973).

From the above, it can be seen that M. pneumoniae has been established as the etiological agent of primary atypical pneumonia. This clear situation however is the exception rather than the rule when linking mycoplasmas with specific diseases.

(ii) Enzootic Pneumonia of Swine.

Enzootic pneumonia of swine is a chronic disease which has been estimated to affect 40 to 50 percent of all swine (Switzer, 1969). The severity and incidence of the disease has been shown to be increased by swine ascarid larvae infections and influenced by stress and environmental factors (Ross, 1973).

The etiological agent was propagated on cell-free media and shown to have the properties of a mycoplasma by Marē and Switzer (1965) who proposed the name M. hyopneumoniae for the organism, and by Goodwin, Pomeroy and Whittlestone (1965) who proposed the name M. suipneumoniae. Both groups of workers continue to use different names for the mycoplasma, and the nomenclature has not been resolved in the eighth edition of 'Bergey's Manual of Determinative Bacteriology', although the organisms have been shown to be strains of the same species (Goodwin, Pomeroy and Whittlestone, 1967).

Both Marē and Switzer (1965) and Goodwin et al. (1965) used pure cultures of the organism to produce pneumonia in SPF pigs on intranasal inoculation, although they had to inoculate the pigs on more than one occasion in the course of the experiment. (Betts, 1971). Since then, however, Hodges, Betts and Jennings (1969) have produced extensive pneumonia, indistinguishable from the natural disease, in gnotobiotic pigs by a single inoculation with a pure culture of M. hyopneumoniae. Clearly then M. hyopneumoniae is a cause of Enzootic Pneumonia in pigs but is it the sole cause?

Pneumonias caused by M. hyopneumoniae are commonly complicated with M. hyorhinis, a mycoplasma which has been isolated from both the upper and lower respiratory tract in swine with pneumonia and the upper respiratory tract of normal swine. (L'Ecuyer, Switzer and Roberts,

1961). Polyserositis and arthritis have been consistently produced experimentally with broth cultures of the organism (Ross, 1973), but there is some debate as to the role of M. hyorhinis in enzootic pneumonia. Many workers have been unable to produce respiratory disease by intranasal inoculation of M. hyorhinis in SPF pigs (Betts, 1969). However, Czechoslovakian workers Gois, Valicek and Sovadina (1971) using gnotobiotic pigs have produced rhinitis and a pneumonia with lesions similar to those of 'natural' enzootic pneumonia. In Britain, a mild transient pneumonia in gnotobiotic pigs has been reported (Poland, 1969) as the result of intranasal inoculation with broth cultures of M. hyorhinis, but the lesions were unlike the extensive pneumonia reported by Gois et al. (1971). When one of the Czechoslovakian strains isolated by Gois et al. was inoculated into gnotobiotic pigs in Britain, extensive pneumonia was produced in three of nine pigs (Poland, 1969). Baskerville (1972) and Baskerville and Wright (1973) produced enzootic pneumonia in pigs with a pneumonic lung homogenate from which only M. hyorhinis could be cultured. However M. hyopneumoniae has particularly fastidious nutritional requirements whilst M. hyorhinis is relatively easy to grow, so the apparent absence of organisms other than M. hyorhinis may have been due to technical difficulties.

It is concluded that M. hyopneumoniae is a cause of enzootic pneumonia in swine but there is some uncertainty as to whether it is the only cause of the disease with M. hyorhinis playing the role of a secondary invader, or alternatively if M. hyorhinis like M. hyopneumoniae can cause the disease on its own.

(iii) Air-Sac Disease in domestic Fowl.

Chronic respiratory disease in chickens is a relatively mild disease with a slow spread, long incubation period and long course. Exudate from the respiratory tract of infected chickens was inoculated into embryonated eggs and the infected yolk was seeded into heart-infusion broth enriched with horse serum by M arkham and Wong (1952). After passaging to remove possible viral

agents, the cultures were inoculated into embryonated eggs, and when the yolk from infected embryos was inoculated into the sinuses of turkeys sinusitis resulted. During propagation on cell-free media it was demonstrated that the organism was a mycoplasma, and was subsequently designated M. gallisepticum (Edward and Kanarek, 1960).

Thus, M. gallisepticum was shown to be the cause of this mild chronic respiratory disease. However, in the early 1950's, outbreaks of a much more severe respiratory disease were reported. This disease became known as "air-sac" disease or complicated chronic respiratory disease. E.coli was shown to be the most frequent complicating organism (Wasserman, 1954) and the disease was first produced experimentally by infecting chickens with combinations of E. coli plus M. gallisepticum, plus either infectious bronchitis virus or Newcastle disease virus (Gross, 1961). Fabricant and Levine (1962) inoculated fowl intranasally with combinations of M. gallisepticum, E. coli and infectious bronchitis virus and produced lesions indistinguishable from that of the natural field disease. In the course of the experiment, it was shown that E. coli does not readily invade the lower respiratory tract unless it has been previously infected with M. gallisepticum. A higher percentage of deaths and lesions produced in experimentally inoculated chickens occurred if, prior to E. coli infection, the birds were inoculated with both M. gallisepticum and infectious bronchitis virus.

Live virus vaccination with either infectious bronchitis or Newcastle disease virus, or natural infection with these viruses activates chronic respiratory disease in domestic hens and accelerates the spread of M. gallisepticum through a flock, especially when the birds are kept in close contact, as is the case in the modern poultry industry (Fabricant, 1969).

The resulting mild infection renders the lower respiratory tract susceptible to invasion by the ubiquitous E. coli and the birds subsequently develop the

severe 'air-sac' disease.

Although 'air-sac disease' in the fowl requires the presence of M. gallisepticum, without the complicating factors E. coli and a virus, only a relatively mild infection of the respiratory tract results. The situation with respect to M. ovipneumoniae and chronic respiratory disease of sheep is likely to be different, but the avian disease highlights the problems inherent in assigning an etiological role to an agent without taking many possible factors into account.

(iv) Chronic pneumonia of rodents

Initial studies of the etiology of chronic pneumonia of rodents were complicated by the isolation of the mycoplasma, later named M. pulmonis (Edward and Freundt, 1956), from rats and mice both with and without the lesions of respiratory diseases. The mycoplasma was isolated by:

1) Klieneberger and Steabben (1937) who designated the organism L3, from the lung lesions of rats with chronic bronchopneumonia.

2) Nelson (1937), from mice with infectious catarrh. He termed the agents "coccobacilliform bodies".

3) Edward (1940), from the lungs of normal mice.

4) Klieneberger-Nobel and Cheng (1955) from the nasopharynx of weanling rats.

They also showed that caesarean-derived rats of the same age were free of mycoplasmas.

It is now generally accepted that most strains of M. pulmonis are capable of causing upper respiratory tract infection, i.e. infectious catarrh. However the conclusion that it is the cause, or even a cause of chronic pneumonia in rodents is not universally accepted. Thus, Nelson (1967) regards the chronic respiratory disease syndrome in rats and mice as a complex of two independent diseases: infectious catarrh caused by M. pulmonis, and enzootic bronchietasis which he alleges is of viral origin, although the putative virus has not been characterized. Andrewes and Glover (1946) also ascribed the etiology of chronic pneumonia in laboratory mice to a virus which they called "gray-lung virus". The infection

is latent, but can appear after serial passage of lung material. The agent was later shown to be susceptible to tetracyclines and sodium aurothiomalate but not penicillin, so it cannot be a virus. Its fine structure, moreover, suggests that it is a mycoplasma (Tully, 1969).

In spite of such reports, some workers still maintain that M. pulmonis is the sole cause of chronic pneumonia in rodents: thus according to Cassell, Lendsey, Overcash and Baker (1973) three groups of workers, using pure cultures of M. pulmonis have produced lesions in SPF rats identical with those produced by placing the rats in contact with conventional animals with spontaneous chronic respiratory disease. All lesions were suppressed by oral administration of tetracycline. They admit however, that although all of the rat isolates tested produced upper respiratory tract infections, 28 days after inoculation, significant lower respiratory tract infection had been produced only occasionally and inconsistently. They also found that pulmonary clearance of M. pulmonis in the rat is much more efficient than that in the mouse, and believe that this relatively high clearance efficiency is important in explaining the difficulties of producing lower respiratory tract infection with M. pulmonis in the rat. They further suggest that impaired bronchial clearance due to a variety of natural stimuli might precipitate active lung disease.

In contrast to the above workers, Gay(1967) and Gay et al., (1972) produced chronicpneumonia in ten out of ten neonatal SPF rats by inoculating them intranasally with infected lung homogenate. From this lung homogenate he could isolate M. pulmonis and S. moniliformis, but when he attempted to produce experimental disease with an aerosol of these organisms, either individually or in combination, he had no success. However, he consistently produced a chronic pneumonia, histologically indistinguishable from the natural disease by inoculating neonatal SPF rats with lung material from a 4-week old conventional rat. No bacteria or mycoplasmas could be cultivated from the inoculum but mice inoculated with control lung

material from SPF rats remained healthy.

Attempts to culture the causative agent from the lung homogenate that caused this chronic pneumonia were unsuccessful, even though a wide variety of cell-free media and tissue culture systems were used. Electron microscopic inspection of the alveoli of the infected lungs, however, showed a large number of mycoplasma-like organisms. These organisms were predominantly narrow, elongated organisms which commonly occurred in parallel groups. When Gay et al. (1972) compared the lesions produced by this rat pneumonia agent, the "gray-lung virus" and the "enzootic bronchiectasis virus" of Nelson, he could find no difference macroscopically, and identical mycoplasma-like organisms could be seen in the lung lesions produced by each of the three agents. No virus-like particles were seen. Consequently he believed that chronic pneumonia of rodents is caused by a mycoplasma that has not yet been propagated.

Although rodents, being laboratory animals lend themselves to the study of the etiology of diseases affecting them it is obvious from the preceding section that the cause or causes of chronic pneumonia of rodents is still in dispute - and from the point of view of using chronic pneumonia in rodents as a model for chronic pneumonia in sheep, it is important to note that although M. pulmonis can be recovered from all natural cases of chronic pneumonia in rodents, it may not be the primary cause of the disease.

(d) Chronic Pneumonia of Sheep

Chronic pneumonia of sheep is a low mortality, high morbidity disease characterized by inadequate weight gain and exercise intolerance, and is thus of considerable economic importance. In New Zealand it has been estimated to affect 70% to 80% of lambs in some groups of animals, and although mortalities are usually low, the mortality rate can reach 15% in bad seasons (Smith, 1970 ; Davis 1970).

The chronic disease, unlike the acute form which can affect sheep of any age is prevalent only in lambs of three to ten months (hence the name "hogget pneumonia"). Acute pneumonias of sheep are typically caused by bacteria, e.g. P. haemolytica, while in the chronic form of the disease, bacteria is either absent or present in such low concentrations as to be inadequate to account for the disease (Alley, 1975). Furthermore, if the isolated bacteria are inoculated intranasally into susceptible animals, usually no lesions are produced, but occasionally a typical acute pneumonia appears. Acute pneumonia is characterized by rapid onset and short duration, and the chronic form by a long incubation period and slow progression. In practice, however, the two forms of the disease are distinguished by macroscopic and microscopic examination of the lesions of the lung. The acute form of pneumonia in sheep is characterized by intense congestion and varying degrees of red or grey consolidation of the ventral portion of one or both lungs. A cellular exudate composed of neutrophils, macrophages and detached alveolar epithelial cells with which many bacteria are closely associated, is present in the lungs. The alveoli are filled with large macrophages and small focal areas of neutrophil infiltration appear. In the "atypica'" or chronic lung disease, the macroscopic lesions vary from dark-red to grey areas of consoliation of the lung, to narrow branching bands of collapse in the anterior lobes of both lungs. The alveoli are infiltrated with macrophages and both lymphocytes and macrophages are present in the alveolar septa. The major factor underlying the pathological differences between acute and

chronic pneumonia is the degree and rapidity of destruction and damage to the alveolar epithelium, which is universal in the acute form of the disease, but less severe and more localized in the chronic form (Alley, 1975).

Since the earliest report of the isolation of a mycoplasma from the lungs of sheep (Grieg, 1955), refinements in techniques, and the realization that many mycoplasmas have complex nutritional requirements have led to reports of mycoplasma isolations from sheep in a large number of countries : Turkey (Dunusan and Dogyer, 1955; Cottew, Watson, Arisoy, Erdag and Buckley, 1968); Israel (Nobel, 1958); U.S.A. (Boidin, Cordy and Adler, 1958; Hamdy, Pounden and Ferguson, 1959; Barber and Fabricant, 1961); U.S.S.R. (Farzaliev, Khalimbekov, Dandamaeu and Aliev, 1962); Britain (Mackay, Nisbet and Foggie, 1963; Mackay, 1966; Mackay and Nisbet, 1966); Italy (Dieana and Cereto, 1967); Kenya (Krauss and Wandera, 1970); Australia (Cottew, 1971; St George et al., 1971; Carmichael et al., 1972; Sullivan, St George and Horsfall, 1973(i)) and New Zealand (Clarke et al., 1974: Alley et al 1975). Early attempts to produce chronic pneumonia with these isolated mycoplasmas were unsuccessful (Boidin et al 1958: Hamdy et al., 1959: Farzaliev et al., 1962; Hamdy and Pounden, 1959). The significance of these results is unclear, as it has been recognized that there are at least three species of "mycoplasmas" associated with sheep:

1) An arginine-requiring mycoplasma which does not ferment glucose, gives fried-egg shaped colonies and has been shown to be M. arginini (Clarke et al., 1974 Cottew, 1971; Carmichael et al., 1972; Krauss and Wandera, 1970).
2) A acholeplasma which does not require sterols for growth and has been identified as A. laidlawii (Krauss and Wandera, 1970).
3) A glycolytic mycoplasma that forms centreless colonies on solid media and was first reportedly isolated by Barber and Fabricant (1961) on a medium containing yeast hydrolyzate and horse serum.

This last organism now designated M. ovipneumoniae is generally believed (Carmichael et al., 1972; Clarke et al., 1974: Jones et al., 1976) to be the most significant from the pathological point of view, so an increasing amount of attention has been centred on it. This work is reviewed below:

Studies of M. ovipneumoniae in countries other than New Zealand:

A glycolytic mycoplasma was isolated by St George, Sullivan, Love and Horsfall (1971) from the lung of a sheep with chronic pneumonia. It did not grow on standard mycoplasma media, but was cytopathic in bovine testis cell cultures and could be propagated on Hanks medium plus lactalbumin hydrolysate, yeast extract and fetal calf serum. Colonies of the organism on solid media did not show the fried-egg shape reported for most mycoplasmas, but the centreless, 'lacy' or 'vacuolated' appearance typical of M. pneumoniae. The agent passed through a filter of pore size 220nm, sufficient to retain bacteria, and grew in the presence of thallium acetate, streptomycin and penicillin. It was chloroform-sensitive, and when examined by impression smears stained by the Dienes method, showed the morphology of mycoplasmas.

Carmichael, St George, Sullivan and Horsfall(1972) isolated a strain of mycoplasma, which they termed biotype Y-98, from sheep in a Queensland flock with a high incidence of chronic interstitial pneumonia. The Y-98 biotype was found to occur with the highest frequency in the nasal cavities, trachea and bronchi of pneumonic lambs, although on two occasions it was isolated from the nasal swabs of healthy adult sheep. The organism had centreless colonies on solid media, fermented glucose with production of acid, and had a marked hemolytic activity for ovine erythrocytes. It was serologically different from all other mycoplasmas isolated from the flocks studied, and was found to be antigenically unrelated to twelve additional ovine and caprine serotypes by metabolic inhibition, growth inhibition and immunodiffusion tests.

Since these properties indicated that it was a distinct species, Carmichael *et al* (1972) proposed the name *M. ovipneumoniae*.

Glycolytic mycoplasmas isolated from the respiratory tract of apparently healthy sheep, pneumonic sheep and sheep with pulmonary adenomatosis in Scotland (Jones *et al*., 1976) were shown by polyacrylamide gel electrophoresis, gel precipitin tests, metabolic and growth inhibition tests to be related to the Queensland Y-98 strain and could thus be classified as one species, viz. *M. ovipneumoniae*. Growth and metabolic inhibition tests showed, however, that intraspecific differences occurred with apparent polarization of strains from sheep with or without pulmonary adenomatosis. Jones *et al*. (1976) also pointed out that the serological relationship of *M. ovipneumoniae* to other members of the Mycoplasmatales had been insufficiently investigated and tested their strains, and the Y-98 strain against 40 hyperimmune sera to 33 named mycoplasma species and subspecies and six serogroups of bovine or caprine origin. They confirmed that the strains of fermenting mycoplasmas from the ovine respiratory tract were a distinct species and consequently approved the name, *M. ovipneumoniae*.

Strains of *M. ovipneumoniae* isolated from pneumonic lung have also been reported in Victoria (Furlong and Cottew, 1973; Cottew, 1971); New Zealand (Clarke *et al*., 1974); U.S.A. (St George and Carmichael, 1975); England (Leach *et al*., 1976), and Canada and Hungary (St George, 1976).

The Y-98 strain isolated in Queensland by Carmichael *et al*., (1971) was the only strain of *M. ovipneumoniae* reported by name in their paper, and has since been widely distributed and studied. Subsequently, Leach Cottew, Andrews and Powell (1976) proposed that it be the type strain of *M. ovipneumoniae* and deposited the strain in the National Collection of Type Cultures as NCTC 10151.

M. ovipneumoniae has been widely isolated from the lung lesions of lambs with chronic pneumonia and only inconsistently from apparently healthy sheep, and then from the nasal cavities. In order to establish any causal

relationship between chronic pneumonia and the mycoplasma, several workers have attempted to transmit the disease experimentally using a broth culture of M. ovipneumoniae, thus:

St George et al. (1971) isolated a mycoplasma (subsequently shown to be M. ovipneumoniae (Carmichael et al., 1972)) from the lung of a pneumonic sheep, propagated it in vitro for six subcultures, then investigated its ability to produce chronic pneumonia in caesarean derived and conventional lambs following intratracheal or intranasal inoculation. These were preliminary experiments, and poorly controlled because at least one lamb inoculated with sterile medium died. However, they produced evidence, which while not conclusive, indicated that the mycoplasma could play an important role in sheep pneumonia.

Sullivan, St George and Horsfall (1973(ii)) inoculated day-old lambs intranasally, or intravenously with M. ovipneumoniae and produced a proliferative interstitial pneumonia. The authors claimed that the disease produced closely resembled the chronic pneumonia seen in field cases. Similar lambs put in contact with the inoculated lambs also developed a chronic pneumonia. However, in no case was M. ovipneumoniae recovered from infected lungs, and this failure to recover the mycoplasma is difficult to account for if M. ovipneumoniae is indeed the cause of chronic pneumonia in sheep.

Foggie, Jones and Buxton (1976) inoculated SPF lambs intrabronchially with M. ovipneumoniae and produced small, discrete lesions in three out of six lambs. However, the lesions were milder than those of the natural disease. They recovered M. ovipneumoniae from the lungs of all infected animals, but not from the controls. SPF lambs in contact with infected animals, became infected with M. ovipneumoniae but the organism was recovered only from the upper respiratory tract, and none of the animals developed pneumonia.

We conclude that the role of M. ovipneumoniae in natural cases of chronic pneumonia in sheep remains to be clarified, as not all infected sheep get pneumonia,

and more significantly, the disease produced experimentally may not be identical to that seen in field cases.

Studies of M. ovipneumoniae in New Zealand.

Sheep play an important role in the economy of New Zealand, and chronic pneumonia has been estimated to affect 70% to 80% of lambs in many flocks (Smith, 1970). Nevertheless, until 1974, little or no effort had been made in this country to establish theetiology of the disease.

In an initial study undertaken to elucidate the etiology of chronic pneumonia in sheep, Clarke, Brown and Alley (1974) recovered a number of mycoplasmas from the respiratory tract of sheep with or without chronic pneumonia. The isolates could be divided into strains which fermented arginine and produced typical "fried-egg" shaped colonies on 1% agar, and glycolytic strains which produced "vacuolated" or "lacy" centreless colonies. The arginine-fermenting mycoplasma was identified as M. arginini, whereas the glycolytic mycoplasma was found to be indistinguishable from the Australian Y-98 strain isolated by St George et al (1971).

Alley, Quinlan and Clarke (1975) in a survey of the prevalence of M. ovipneumoniae in New Zealand sheep, recovered the mycoplasma from all of sixty pneumonic lungs sampled. The organism was found to be present in pneumonic lungs at a titre of 10^6 - 10^7 organisms per gram. However, the mycoplasma was also isolated from ten of forty normal lungs, although in only two cases did the titre exceed 10^3 organisms per gram. This low titre of mycoplasmas apparently present in normal lung could have been due to contamination of the lung by meat inspectors who handled the tissue before it could be sampled in the survey.

Although this high titre in pneumonic lungs, and 100% recovery rate from these lungs is suggestive that M. ovipneumoniae causes chronic pneumonia in sheep in New Zealand, it is possible that the mycoplasma is only an efficient secondary invader, so experiments to attempt

to transmit the disease experimentally to colostrum-deprived sheep using broth culture of M. ovipneumoniae were performed (Alley, personal communication). An aerosol of pneumonic lung homogenate consistently caused lesions in 60% to 70% of sheep inoculated, and the lesions produced were typical of the field disease. However, when an aerosol of M. ovipneumoniae was used as inoculum, lesions were produced only once in three attempts and with the exception of one lamb, they were not identical with those seen in natural disease. Several reasons could be advanced for these inconsistent results, but a fundamental necessity for consistent disease production is obviously the use of a consistent inoculum of micro-organisms. However, in all cases the mycoplasmas used for inoculation, were propagated until the day after a pH change was visible (Clarke, personal communication), and this can lead to large variations in the inoculum, as mycoplasmas die rapidly after reaching maximum titre. The in vitro studies of the growth of M. ovipneumoniae, reported in a later section, were undertaken so that high titre inocula could consistently be used to attempt to transmit the disease in sheep.

A parallel approach to transmission experiments investigating the role of M. ovipneumoniae in chronic pneumonia of sheep is to establish the presence of the mycoplasma in the lung by techniques such as fluorescent antibody studies or electron microscopy. Unfortunately, electron microscope studies do not usually distinguish between mycoplasmas, with the exception of the few mycoplasmas that have a characteristic feature, such as the blebs of M. gallisepticum. An in vitro electron microscope study of M. ovipneumoniae was nevertheless undertaken in order to elucidate its morphology, as this has not yet been reported. The electron microscopy was undertaken in conjunction with the growth experiments, so that the stage of growth of the mycoplasmas seen in electron micrographs was known.

Mycoplasmas have a low percentage of guanine plus cytosine in their DNA, thus, the base compositions reported for the DNA of all mycoplasmas except M.

pneumoniae, fall within the relatively narrow range of 23% to 35% GC. As the base composition of *M. ovipneumoniae* had not been unequivocally established, investigations were undertaken to find the melting temperature and buoyant density of the DNA, so that the base composition of *M. ovipneumoniae* could be determined by these two methods.

In summary therefore, the experiments reported in this thesis were undertaken as an *in vitro* study of *M. ovipneumoniae*. This study is thus part of the investigation being made at Massey University into the link between *M. ovipneumoniae* and chronic pneumonia of sheep.

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1. SOURCE OF MICROORGANISMS USED

The work in this thesis was primarily concerned with Mycoplasma ovipneumoniae strain 5, which was isolated in 1973 by Mr J.K. Clarke from the lung of a lamb with chronic pneumonia. Where not otherwise stated, the designation M. ovipneumoniae refers to this strain.

For the purposes of comparison, a strain of M. ovipneumoniae isolated from the respiratory tract of sheep in Australia was obtained from Dr T.D. St. George. It was supplied in the form of a lyophilized culture. Where this strain was used it is specifically referred to in the text.

E. coli K12. A strain of this reference organism was obtained from the culture collection of the Department of Microbiology and Genetics, Massey University.

H. influenzae, strain Rd. This is the classical transforming strain. It was obtained from Dr R.M. Herriott, Department of Biochemistry, John Hopkins University, Baltimore, U.S.A. The organism was originally obtained from Dr H. Alexander.

2. MYCOPLASMA MEDIA USED

a) FM4 Broth

The medium used throughout for the propagation of mycoplasma was based on the fourth of several formulations described by Frey, Hanson and Anderson (1968). It is referred to as FM4. The basic formula is as follows:

NaCl	5.0g
KCl	0.4g
$MgSO_4\ 7H_2O$	0.2g
$NaHPO_4\ 12H_2O$	4.03g
KH_2PO_4	0.1g
Glucose	10.0g
Albimi Peptone CS	10.0g
Albimi Yeast Autolysate	5.0g
NAD	0.1g
L-Cysteine HCl	0.1g
Eagles Vitamin Soln x 100 (see below)	25.0ml
0.4% phenol red	2.5ml
Penicillin	10^6 units
Thallium acetate	0.5g
1.0M NaOH (approx 6ml) to pH	7.8
Distilled water to	1000 .0ml

The basal medium was supplemented with 150ml of unheated pig serum before use.

Notes on preparation:

i) The thallium acetate was dissolved in 10ml of water and added dropwise to the medium to avoid the formation of a precipitate.

ii) The medium was clarified by filtration through non-sterile 0.45µm and 0.22µm pore size filters and subsequently sterilized by filtration through a sterile 0.22µm filter.

iii) The vitamin supplement used - Eagles Vitamin Solution x 100 - was as follows:-

D-Biotin	20mg	myo-Inositol	40mg
Calcium pantothenate	20mg	Niacinamide	20mg
Choline chloride	20mg	Pyridoxine	20mg
Folic Acid	20mg	Thiamine HCl	20mg
Riboflavin	2.0mg	Distilled water to	200ml

The solution was sterilized by filtration through a 0.22µm membrane filter.

b) FM4 Agar

To make solid media, 1.9g of Difco agar was added to 42ml BHI (Difco) broth, autoclaved, and cooled to about 50°. This was added to 200ml of FM4 broth, which was being shaken continuously in a 46° waterbath.

Approximately 5ml aliquots were then distributed into 50mm plastic petri dishes with tight fitting lids. The solidified media was either used immediately or stored at 4° for not more than a week.

3. CLONING OF M. OVIPNEUMONIAE

A universal bottle containing approximately 10ml of FM4 broth was inoculated with about 1ml of a culture of M. ovipneumoniae strain 5 which had been stored at -70°.

When the colour of the phenol red indicator in the medium changed from red to orange/yellow indicating that growth of culture had occurred, serial 10-fold dilutions were made in FM4 broth and 0.05ml aliquots were plated on FM4 agar then incubated at 37° for 3 to 4 days.

A plate with well isolated colonies was selected, and a sterile pipette was used to remove a colony plus a plug of agar. This plug was used to inoculate a second universal bottle containing 10ml of FM4 broth, and the process repeated. Following the 3rd clonal isolation, the colony was grown in the 10ml FM4 broth in a universal bottle, which in turn was used to inoculate a conical flask containing 100ml FM4. At late exponential phase growth, the culture was distributed in bijou bottles in approximately 2ml aliquots, and stored at -70°

4. ASSAY OF COLONY FORMING UNITS

a) Diluent

Serial 10-fold dilutions of mycoplasmas were initially made by transferring 0.2ml of the culture into 1.8ml aliquots of FM4 broth but following a comparative investigation (see Results) BHI (Difco) broth was substituted for FM4 broth.

b) Plating

Using an automatic pipette with a disposable tip, 0.05ml samples of appropriate dilutions were plated onto the surface of FM4 agar medium in 50mm plastic petri dishes with tight-fitting lids. This had the advantage that it was not necessary to keep the plates in a humidified box to prevent drying of the agar medium.

c) Incubation and Counting

The plates were incubated at 37° for 72 to 96 hours, and the colonies counted using a plate microscope.

5. GROWTH CURVE

a) Preparation of inoculum

Following preliminary experiments, mycoplasmas in the logarithmic growth phase were stored at -70° in aliquots. The mycoplasmas in these aliquots were used to inoculate FM4 broth cultures in the following growth curve experiments.

b) Size of inoculum

Since it is generally assumed that in some circumstances the inoculum size can affect the initial growth rate of mycoplasmas, two inoculation sizes differing by tenfold were used, corresponding to the addition of either 1.0ml or 0.1ml of stock culture to 9.0 or 9.9ml respectively of FM4 broth in 100ml side arm flasks. The medium was pre-heated to 37°. Two replicate cultures were prepared for each size of inoculum.

c) Incubation and sampling

The cultures were incubated at 37° in 100ml side arm flasks, stoppered with cotton wool plugs. One flask of each inoculum size was left standing, and the other

replicate flask was shaken continuously on a rotary shaker at 100rpm.

At intervals (see Results) 0.2ml samples of each culture were removed and titrated as previously described to determine the number of colony forming units. The optical density at 640nm measured at the same times by placing the side arms of the flasks in a Bausch and Lomb SP20 spectrophotometer.

6. ELECTRON MICROSCOPY

a) Propagation of mycoplasmas

Replicate 100ml volumes of FM4 broth in 500ml conical flasks plugged with cotton wool were inoculated with 1.0ml of M. ovipneumoniae culture stored at -70° at peak viability. The flasks were shaken continuously at 37° on a rotary shaker.

At intervals (see Results) a 100ml aliquot was centrifuged at 14,500g for 30 minutes at 4°. The supernatant fluid was discarded and the pellet fixed and embedded.

b) Embedding in Araldite Resin

The cells were fixed in 3% gluteraldehyde in phosphate buffer (see Appendix) at 4° for 2 hours, washed with 1% sucrose in phosphate buffer for 2 hours and post-fixed in 1% osmium tetroxide (in 0.1M phosphate buffer pH 7.2) at 4° for 2 hours.

The post-fixed cells were centrifuged at 14,500g for 30 minutes and the pellet obtained was cut into pieces that would easily fit into gelatin capsules. These pieces were dehydrated and infiltrated as follows:

(i) 50% ethanol for 15 minutes
(ii) 70% ethanol for 15 minutes
(iii) 95% ethanol for 15 minutes
(iv) 100% ethanol for 30 minutes twice
(v) propylene oxide for 30 minutes twice
(vi) a 1:1 mixture of araldite (see Appendix) and propylene oxide for one hour
(vii) araldite overnight

The cells were then embedded in gelatin capsules filled with araldite. The araldite was polymerized in an oven at 60° for 48 hours.

Note: This conventional method of preparing cells for sectioning for electron microscopy was tedious and took a longer time than was desirable, especially as a large number of cell samples had to be processed. Consequently, a more rapid technique using Spurr's resin was used, after comparison with the conventional procedure (see Results), to ensure that the rapid method gave acceptable results.

c) Embedding in Spurr's Resin (Rapid Technique)

M. ovipneumoniae cells were propagated as previously described, and the pellet obtained when the mycoplasmas were centrifuged at 14,500g for 30 min. was resuspended in Karnovsky's fixative (see Appendix) and fixed at 4° for 1 hour. The cell suspension was then centrifuged, the supernatant discarded, and the cells re-suspended in 1% osmium tetroxide (see Appendix) for 1 hour.

After post-fixation, the pellet of cells was very dark in colour, and it was possible to remove the pellet either whole, or as a few large fragments. These were cut into pieces to fit into the gelatin capsules used for embedding. The pieces were dehydrated and in-filtrated as follows:

(i)	2 x 70% acetone for 3 minutes
(ii)	3 x 100% acetone for 5 minutes
(iii)	1:1 mixture of acetone and Spurr's resin (see Appendix) for 10 minutes
(iv)	2 x 100% Spurr's resin for 5 minutes

The fragments were then placed on the surface of resin-filled gelatin capsules, and allowed to sink to the bottom of the capsule.

Various temperatures and times of polymeriz-ation were initially tried, and the best results were obtained when the resin was polymerized in an oven at 75° overnight (18 hours approx.) This time and temperature was therefore used routinely except where otherwise stated.

d) Sectioning, Staining and Examination

The gelatin capsule was peeled off the polymerized block and the face of the block was cut flat using a razor blade. A 'mesa' was cut in the face of the block using an LKB 11800 pyramitome. Thin sections were cut from the 'mesa' with an LKB Huxley ultra-microtome, and were floated off the edge of the knife into a 'boat' containing a1% solution of Teepol in distilled water. Gold and silver coloured sections were picked up with an electron microscope grid dipped in a sellotape/chloroform mixture.

The sections were stained in uranyl acetate (see Appendix) for 15 minutes; washed in 50% ethanol followed by distilled water and stained again in lead citrate (see Appendix) for 15 minutes. The grids were then washed in several changes of distilled water and dried.

The stained sections were examined in a Philips EM200 electron microscope; photomicrographs of mycoplasmas were made and printed to give a final magnification of 55,000X.

e) Negative Staining

Cultured cells were sedimented at 14,500g for 30 minutes, washed using 0.1M phosphate buffer (pH 7.2), resuspended in distilled water, and diluted with water so that the suspension looked slightly turbid.

A drop of the suspension was placed on a glass slide, and a Formvar-coated EM grid was placed, Formvar side down, on the drop. After a few minutes, the grid was removed, blotted dry by touching its edge with blotting paper, and placed Formvar side down on a drop of sodium phosphotungstate (pH 7.0) for a few seconds. The grid was then removed, blotted gently and allowed to dry. The negatively stained cells were examined as described for sections.

f) Australian Strain of M. ovipneumoniae

The Australian strain of M. ovipneumoniae was prepared for electron microscopy of sections and negatively stained cells as described for the New Zealand strain.

7. DNA STUDIES

a) Purification of M. ovipneumoniae DNA

Replicate cultures of M. ovipneumoniae were grown to late exponential growth phase in two 500ml conical flasks plugged with cotton wool and containing 100ml of FM4 broth. Each culture was used to inoculate 2.0 1. of FM4 broth in a 5.0 1. conical flask which was incubated on a rotary shaker at 37°.

The mycoplasmas were deposited by centrifugation at 14,500g for 30 minutes, washed once with 0.15M saline plus 0.1M EDTA (disodium salt) and resuspended in 10ml of the same solution. The cells were lysed by the addition of sufficient 25% sodium dodecyl sulphate to give a final concentration of 2% sodium dodecyl sulphate in the cell suspension, and subsequent immersion in a 60° waterbath for 10 minutes.

The DNA was purified by the following adaption of the Sevag process:

(i) The salt concentration of the lysate was raised to 2.0M by the addition of NaCl.

(ii) An equal volume of a 9:1 mixture of chloroform and octanol was shaken vigourously for 3 to 4 hours with the lysate to precipitate the protein.

(iii) The emulsion was centrifuged at 600rpm for 15 minutes.

(iv) The top aqueous layer was removed using a pasteur pipette. This viscous layer contained most of the DNA.

(v) 3.0ml of 2.0M NaCl was added to the interface gel, shaken and re-centrifuged to extract as much as possible of the remaining DNA. The top layer was removed and pooled with the first supernatant if it was detectably viscous. Otherwise it was discarded.

(vi) The supernatant was cooled in an ice bath, then an equal volume of cold 95% ethanol was layered on the surface. The precipitated DNA was wound around a glass rod, drained of as much ethanol as was possible, and resuspended in 3ml of 0.02M saline containing 3µg of ribonuclease. The beaker was covered with parafilm, and the DNA dissolved at 4° using a magnetic stirrer.

(vii) 0.3g of NaCl was added to give a 2.0M salt concentration, then a half volume of a 9:1 mixture of chloroform and octanol was added

and the mixture shaken for 0.5 to 1 hour.

(viii) The emulsion was centrifuged, and the aqueous layer taken as before and cooled in an ice bath. 95% ethanol was layered on top and a glass rod was used to wind up the precipitated DNA, which was resuspended in 0.15M saline and dissolved by stirring overnight at 4°.

The purity of the DNA was assessed by noting the absorbance at 260nm and 230nm and the concentration of the DNA was estimated on the basis that for a DNA concentration of 1.0mg/ml, $E_{260}=22$.

b) Extraction of E. coli K12 DNA

A 200ml overnight culture of E. coli K12 in nutrient broth was deposited by centrifugation. The cells were lysed and the DNA purified as described for mycoplasmas.

c) Extraction of H. influenzae strain Rd DNA

This organism was propagated in 200ml BHI (Difco) broth, supplemented with 4µg/ml NAD and 10µg/ml of hemin. The inoculated broth was shaken overnight at 37° and processed as described for mycoplasmas.

d) Melting Temperature of DNA

All determinations of melting temperature of DNA samples were carried out using a Unicam SP 1800 Ultraviolet Spectrophotometer equipped with a heated cell block connected to a SP876 Series 2 Temperature Programme Controller.

Following preliminary experiments, the following technique was used routinely: DNA in standard saline citrate (0.15M NaCl plus 0.015M trisodium citrate SSC) was diluted to give an extinction coefficient of 1.2 - 1.6 at 260nm, which corresponds to a DNA concentration of 55 - 75µg/ml. Approximately 3.0ml of this solution was placed in a UV grade silica cuvette which had a path length of 10mm. Stoppers were used to prevent evaporation of solvents at elevated temperatures. The reference cell used contained an adenine solution in SSC previously diluted to give the same absorbance as the DNA sample.

These cells were placed in the metallic block in

the cell compartment. The temperature of the block was regulated using the set point dial on the Temperature Programme Controller which is connected to a heater within the block.

The cell temperature probe was placed in a third cell filled with SSC. This cell was placed in the cell block in sample position 3, as recommended by the SP 876 technical manual. The probe registered the sample temperature on the dial of the Temperature Programme Controller. The nominal cell probe temperature had previously been calibrated against a Griffin and George Ltd Standard Thermometer, which had been compared with the standard thermometers of the British Standards Institution Testing and Approvals Centre.

The temperature of the solutions was then raised to 25°, and the absorbance of the DNA solution zeroed against the adenine solution.

The temperature was then slowly raised to about ten degrees below the expected melting point of the DNA solution. After the needle on the absorbance dial had stabilized at a constant reading for not less than five minutes at the set temperature, the absorbance was noted and the set point dial raised by 1°. The new absorbance was noted when the reading stabilized and this procedure was repeated at 1° intervals until the absorbance no longer increased with increase in temperature.

e) Determination of GC Content of DNA From Melting Point Data

A graph was drawn of absorbance versus temperature (see Results) and the melting temperature was estimated from the graph. This temperature was then corrected by a factor determined during the standardization of the temperature probe.

The equation of DeLey (1970) was used to calculate the base composition of the DNA after melting in SSC;, viz:

$$\%GC = \frac{Tm - 69.37}{0.41} \times \frac{100}{1}$$

Melting temperatures were determinated at least 3 times each for the DNA of *E. coli* K12, *H. influenzae* strain Rd and *M. ovipneumoniae*.

f) Buoyant Density of DNA

Optical grade CsCl was made up to a density of slightly greater than 1.710g.cm^{-3} in 0.02M tris buffer, pH 8.5. To this solution approximately 0.5µg of E. coli K12 DNA was added plus 0.5µg of M. ovipneumoniae DNA, extracted as previously described. The refractive index of this solution was determined in an Atago Abbe Refractometer at 25°, and related to the CsCl density using the following equation of Schildkraut, Marmur and Doty, (1962):

$$p^{25.0°} = 10.8601.N_d^{25.0°} - 13.4974$$

where p = buoyant density

N_d = refractive index

It is desirable that the CsCl solution have approximately the same density as the E. coli K12 DNA, which is known to have a buoyant density of 1.710g.cm^{-3}, so that after ultracentrifugation the E. coli DNA bands near to the centre of the gradient. From the above relationship, it was determined that CsCl with a density of 1.710g.cm^{-3} has a refractive index of 1.400. Hence the CsCl and DNA solution was diluted in small steps until it had a refractive index of 1.400.

Approximately 0.75ml of this solution was placed in a cell containing a plastic centrepiece and centrifuged in a Spinco model E analytical ultracentrifuge at a nominal 44,000 rev/min at 25°. After 20 hr of ultracentrifugation, ultraviolet absorption photographs were made on Kodak commercial film. Tracings of the negative were made on a Joyce Recording Microdensitometer, and the buoyant density of the M. ovipneumoniae DNA was calculated as follows:

g) Determination of Base Composition from Buoyant Density Data

The buoyant density of the M. ovipneumoniae DNA was calculated, with reference to the position of the standard DNA, using the equation of Schildkraut et al. (1962):

$$P = P_o + 4.2w^2 (r^2 - r_o^2) \times 10^{-10} g.cm^{-3}$$

Where P = density of M. ovipneumoniae DNA

P_o = density of E. coli K12 DNA

w = speed of rotation in radians sec^{-1}
r = distance of *M. ovipneumoniae* DNA from the centre of rotation
r_o = distance of *E. coli K12* DNA from the centre of rotation

r and r_o were determined from the position of the absorption peaks on the Joyce microdensitometer tracing, in association with standard instrument data. The density of *E. coli* K12 DNA (P_o) was taken as $1.710 g.cm^{-3}$ as recommended by Schildkraut *et al*. (1962).

From the density value obtained from the above relationship, the mole fraction of guanine plus cyosine (%GC) was determined for *M. ovipneumoniae* DNA using the following equation of Schildkraut *et al*. (1962) :

$$\% GC = \frac{P - 1.660}{0.098}$$

EXPERIMENTAL AND RESULTS

1. GROWTH STUDIES
   a) Reproducibility of assay of *M. ovipneumoniae*
   b) Use of BHI broth as diluent in viability assays
   c) Maintenance of viability of *M. ovipneumoniae* at -70°
   d) Growth curve of *M. ovipneumoniae*
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2. ELECTRON MICROSCOPY
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3. DNA STUDIES
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1) GROWTH STUDIES

a) Reproducibility of assay of M. ovipneumoniae

M. ovipneumoniae is a fastidious mycoplasma which cannot be serially propagated in media used for mycoplasmas with less complicated growth requirements, e.g. PPLO broth supplemented with serum. Moreover, it is well known that the more fastidious mycoplasmas can vary markedly in their growth on different batches of the 'same' medium. This is particularly true when solid medium is used, because slight differences in the degree of hydration of the agar surface appear to determine, in the case of some organisms, whether or not a colony develops (Switzer, 1969). For that reason, it was necessary to find if different batches of FM4 agar gave reproducible results when used to assay M. ovipneumoniae.

Three separate batches of FM4 agar were made and each was used twice to assay aliquots of M. ovipneumoniae stored at -70° over a short period. The results are recorded in Table II, and the typical "vacuolated" or "lacy" centreless colonies of M ovipneumoniae are shown in Fig. 1.

TABLE II: Titres of aliquots of M. ovipneumoniae stored at -70° assayed for colony forming units using different batches of FM4 agar.

FM4 Batch	Assay	Titre
1	A	2.1×10^8 (CFU/ml)
	B	1.9×10^8 (CFU/ml)
2	A	1.8×10^8 (CFU/ml)
	B	1.8×10^8 (CFU/ml)
3	A	2.0×10^8 (CFU/ml)
	B	2.2×10^8 (CFU/ml)

It is concluded that assay of M. ovipneumoniae by this method, provides a reproducible system even when different batches of medium are used.

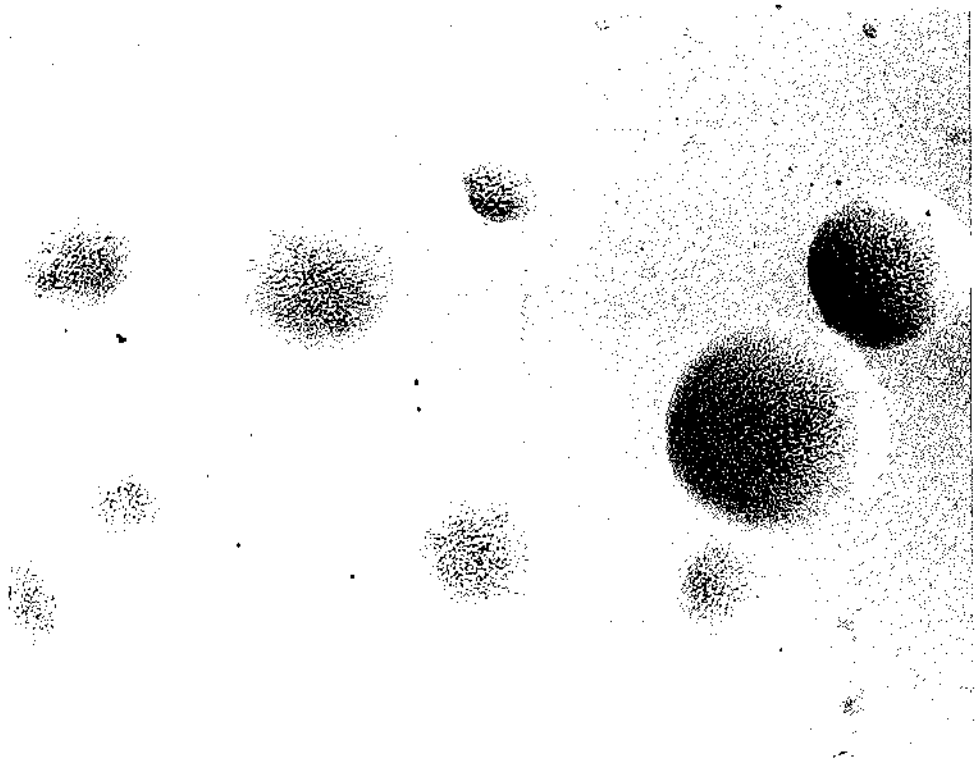


Figure 1: Colonies of M. ovipneumoniae on 1% agar. Note the typical centreless "lacy or "vacuolated" appearance of the colonies. x120.

b) Use of BHI broth as diluent in viability assays

FM4 medium was used for growing M. ovipneumoniae in broth culture, and as the base for solid medium. FM4 was also initially used as diluent before plating onto solid medium in viable count assays. However, FM4 is relatively expensive and inconvenient to make up because it cannot be sterilized by autoclaving. Therefore, experiments were performed to find if BHI (Difco) broth could be substituted for FM4.

The results of two experiments are shown in Table III:

TABLE III: Titres of aliquots of M. ovipneumoniae assayed by colony counts using either FM4 or BHI broth as a diluent.

	Diluent	Titre
Experiment 1	FM4	2.0×10^8 (CFU/ml)
	BHI	1.8×10^8 (CFU/ml)
Experiment 2	FM4	1.8×10^8 (CFU/ml)
	BHI	2.2×10^8 (CFU/ml)

As no significant difference was shown between titres obtained using different diluents, BHI broth was used in subsequent viable count assays.

c) Maintenance of viability of M. ovipneumoniae at -70°

Initially, one of the reasons for undertaking a study of the growth curve of M. ovipneumoniae was to be able to reproducibly provide high titre inocula for disease transmission experiments. If a reproducible growth curve was obtained, then using the same conditions and the same inoculum concentration, the viability of the growing culture at any time could be determined by reference to standardised growth curve data.

This approach obviously requires that a source of M. ovipneumoniae of known titre be available as the inoculum for the culture. This source could be provided if M. ovipneumoniae is stable at -70°, or even if it dropped off in viability at a predictable rate. To investigate the stability of M. ovipneumoniae at -70°, a batch culture

in the logarithmic stage of growth was divided into aliquots, stored at -70° and assayed at intervals. The results are shown in Table IV.

TABLE IV: Titres of aliquots of M. ovipneumoniae stored at -70° for varying periods.

Date of Assay	Interval (months)	Titre (CFU/ml)
March 1975	0	2.0×10^8
June 1975	3	1.8×10^8
July 1975	4	1.9×10^8
November 1975	8	7.8×10^7
September 1976	18	1.2×10^8

The loss of viability over a period of 18 months was less than twofold, which although small would normally be regarded as significant. However, since the fall is small, it would be necessary to assay the aliquots over a much longer period to accurately determine the rate of viability loss.

It must be noted that the apparent viability actually increased between November 1975 and September 1976. This result was probably due to the use of a suboptimal batch of medium used for the November 1975 assay.

It is concluded that M. ovipneumoniae is stable over long periods at -70°, and the loss of titre over eighteen months, even if it occurs, can be neglected for most practical purposes.

d) Growth curve of M. ovipneumoniae

The growth curve of M. ovipneumoniae in FM4 broth culture was investigated by following the change, with time, in the number of colony-forming units per ml of culture.

This change in viable count was assayed at intervals for four cultures, each differing in inoculum size or degree of aeration at 37° as follows:

(i) Shaking, inoculated to give an initial density of 2.0×10^7 CFU/ml

(ii) Shaking, inoculated to give an initial density of 2.0×10^6 CFU/ml

(iii) Stationary, inoculated to give an initial density of 2.0×10^7 CFU/ml

(iv) Stationary, inoculated to give an initial density of 2.0×10^6 CFU/ml

This experiment was repeated three times, and gave consistent results on each occasion. A typical result for each of the four cases is shown in Figs. 2 and 3, and from these curves, the following points were noted:

1) The mean generation times were calculated from the slope at the maximum rate of growth. The results for each of the four culture conditions are shown in Table V.

TABLE V Generation time of *M. ovipneumoniae* grown in FM4 broth culture under different conditions.

	Generation times (hr)			
Inoculum size (CFU/ml)	Expt 1	Expt.2	Expt.3	Mean
Standing culture:				
2×10^7	2.2	2.0	2.3	2.1
2×10^6	1.7	N.D.	1.9	1.8
Shaking culture:				
2×10^7	2.3	2.0	2.3	2.2
2×10^6	1.7	N.D.	1.7	1.7

N.D. = Not Done

2) No lag phase, defined as an initial period during which no increase in viable count occurs, was exhibited by any culture. Nevertheless, the initial growth rate of the stationary cultures was less than that achieved by the cultures later. This phenomenon was not observed in shaking cultures.

The maximum growth rate of the stationary cultures was not significantly different from that of the shaking cultures.

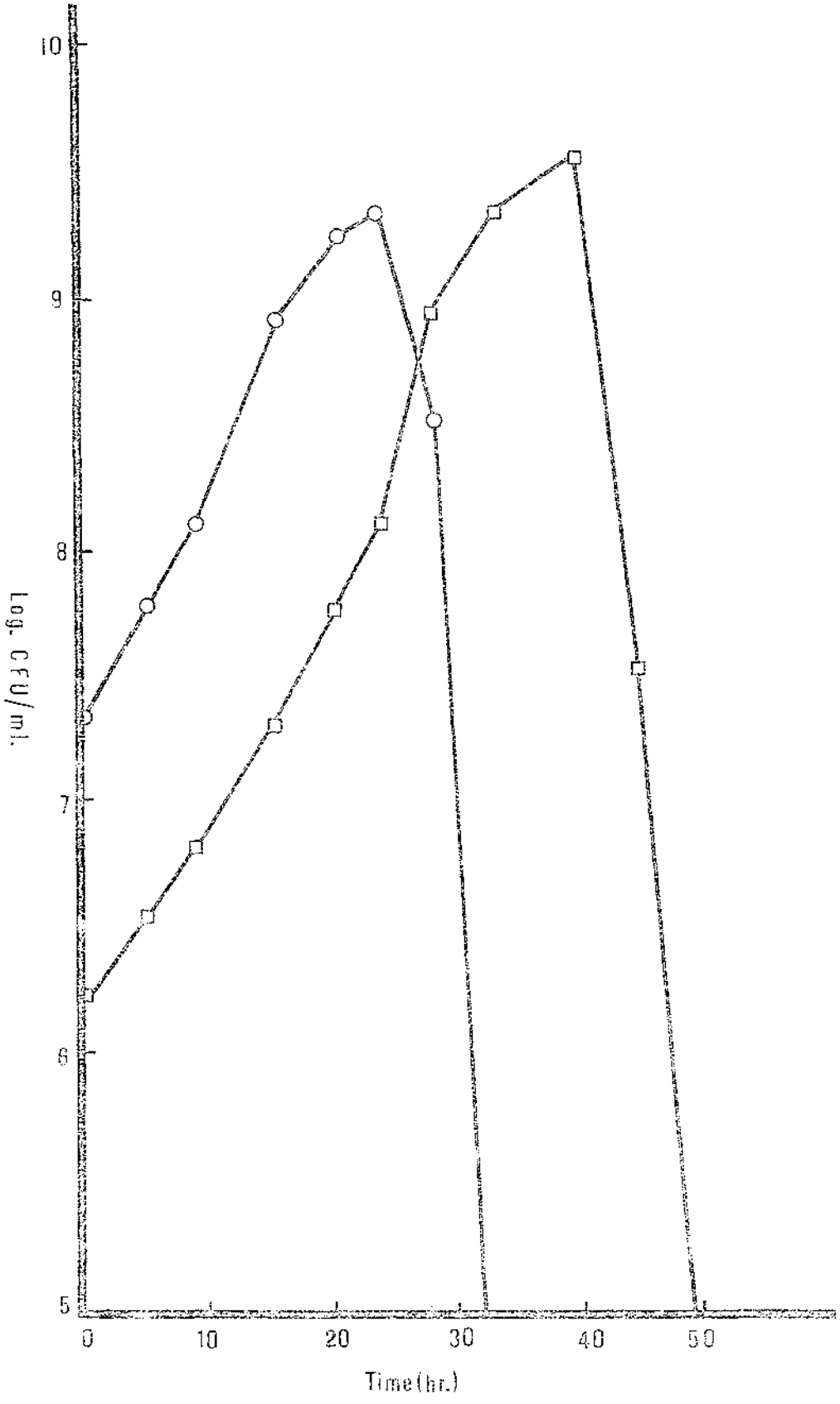


Figure 2: Growth of M. ovipneumoniae in two shaking cultures differing by tenfold in inoculum size. Note that there is no lag or stationary phase and that the culture dies rapidly following the peak titre.

Figure 3: Growth of M. ovipneumoniae in two stationary cultures differing by ten-fold in inoculum size. Note that although there is no absolute lag phase, the initial growth rate is less than the maximum. Apart from this point, the growth curve and peak titre do not differ significantly from those for shaking cultures (Fig.2).

3) The maximum population sizes reached in each of three separate experiments is shown in Table VI.

TABLE VI: Maximum population sizes reached in growth curves of cultures of M. ovipneumoniae with different inoculum sizes in three separate experiments.

	Maximum titre x 10^9 (CFU/ml)			
Condition of Aeration	Experiment: 1	2	3	Mean
Shaking	2.5	2.1	1.1	1.9
Stationary	3.0	2.1	1.0	2.0

4) The half life of M. ovipneumoniae in the death phase of the culture is shown in Table VII.

TABLE VII: Half life of M. ovipneumoniae at the maximum rate of decline recorded in the population.

		Half life of culture (hr)			
Condition of Aeration	Inoculum size (CFU/ml)	Expt: 1	2	3	Mean
Standing culture	2 x 10^7	0.5	0.6	0.4	0.45
	2 x 10^6	0.65	N.D	0.45	0.55
Shaking culture	2 x 10^7	0.5	0.6	0.5	0.53
	2 x 10^6	0.7	N.D	0.45	0.57

N.D. = Not Done

5) A decline in population occurred immediately after maximum titre had been reached without a detectable stationary phase. The maximum rate of decline occurred five hours after maximum titre had been reached. Ten hours after the peak population density, the culture was "sterile", in the sense that 0.05ml plated on FM4 agar produced no colonies, i.e. "sterility" is defined as <20 CFU/ml.

e) Optical density changes related to time and viable count.

When the growth curve studies were undertaken, apart from ascertaining basic information regarding the minimum division time, maximum titre attained, death rate, etc., it was intended that the graphs could be used for

estimating the viable count of mycoplasmas in a culture growing under defined conditions for a defined period. However, as the time when maximum titre is reached is clearly dependent on the number of cells used to inoculate the culture, growth curves would have to be established for each stock culture before the viable count of mycoplasmas could be known at any time.

When this work was undertaken, the classical method for instant estimation of viable count of bacteria seemed inapplicable because mycoplasmas usually do not give a turbidity large enough to measure reproducibly. However, in the course of the investigation, it became clear that cultures of M. ovipneumoniae in FM4 broth did indeed produce a consistently measurable turbidity. In view of these observations, experiments were performed to measure turbidity and viable counts at various time intervals, and then relate turbidity to viable count.

The relationship between change in optical density with time of incubation is shown in Figs 4 and 5, and the relationship between optical density and viable count is illustrated in Fig. 6.

From Figs. 4, 5, and 6, and macroscopic examination, the following points were noted:

(1) The maximum optical density reached by shaking cultures was consistently two and a half times greater than the maximum optical density reached by stationary cultures. Furthermore, macroscopic examination of the cultures demonstrated that in contrast to the stationary cultures, the turbidity of shaking cultures was of a floccular nature.

(2) The attainment of maximum optical density coincides with the maximum viable count and with the initiation of the death phase.

(3) It is important to note, however, that no significant decrease in optical density occurred during the death phase so that even when the cultures became sterile, the optical density was still close to the maximum determined.

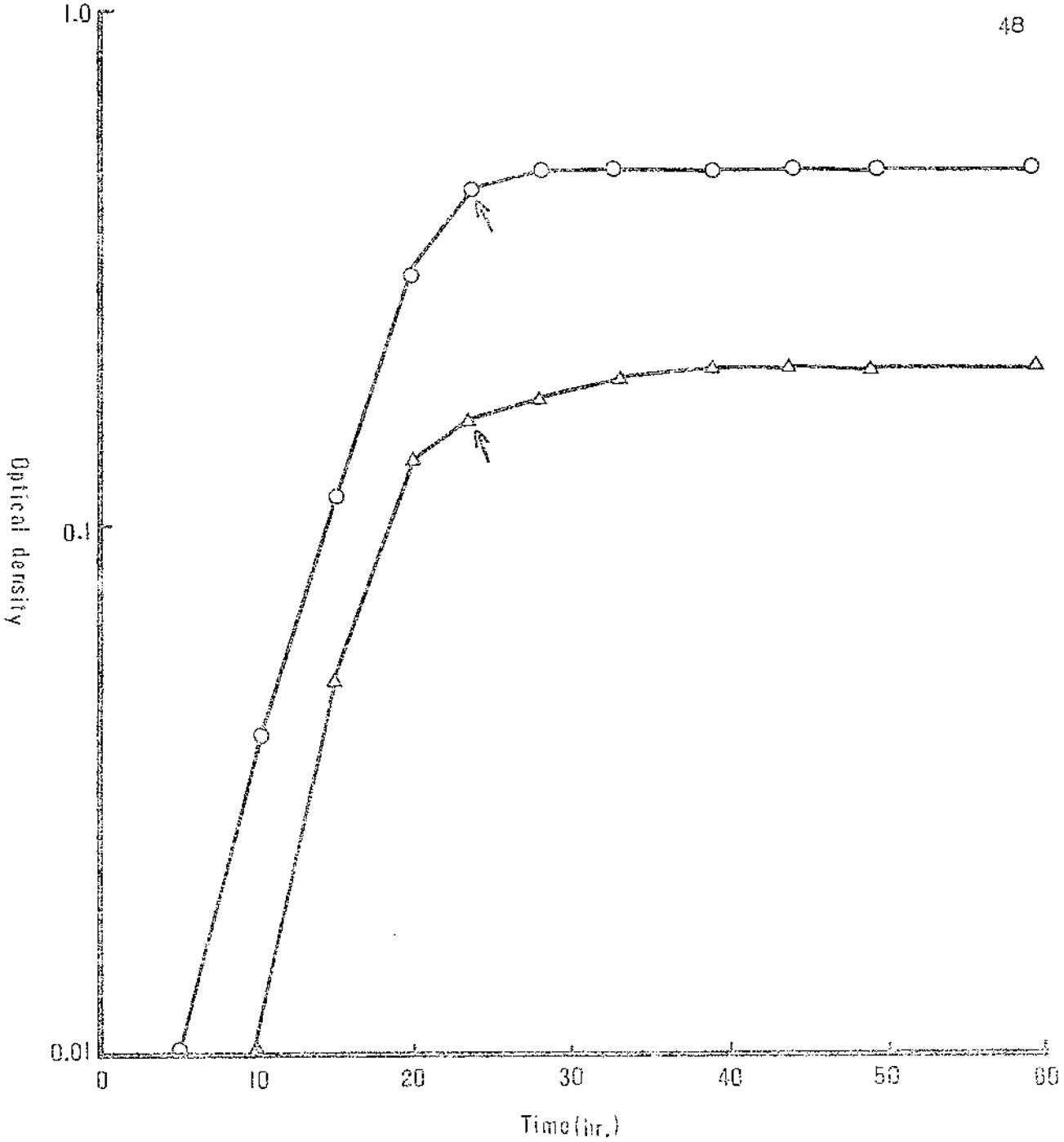


Figure 4: Increase in optical density with time of shaking (circles) and stationary (triangles) cultures of M. ovipneumoniae. Note that the shaking cultures reach a turbidity 2 to 3 times greater than that reached by stationary cultures. Nevertheless, as is shown in Figs. 2 and 3, the maximum colony count is the same for both. The time when peak titres were obtained are indicated by arrows.

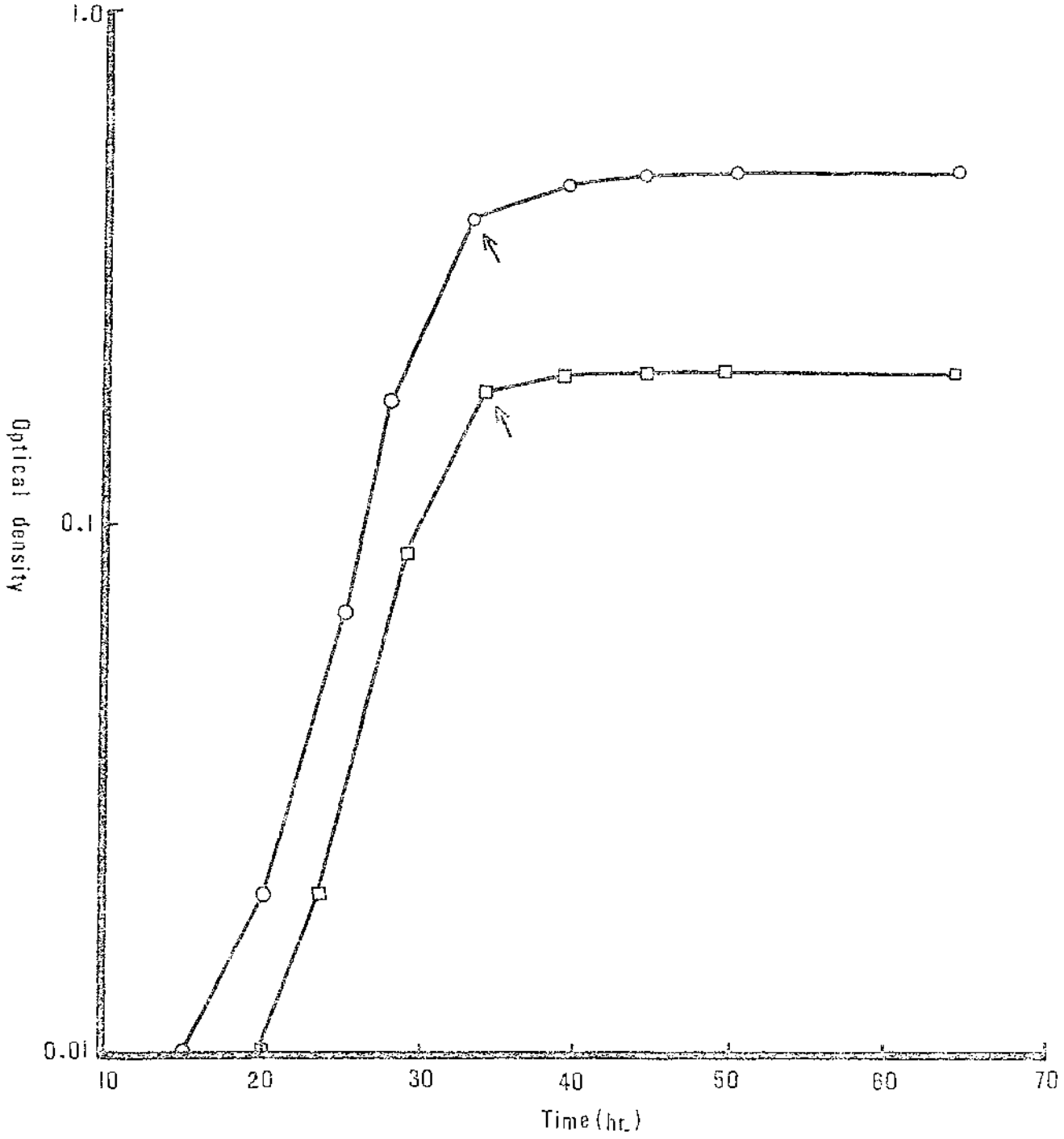
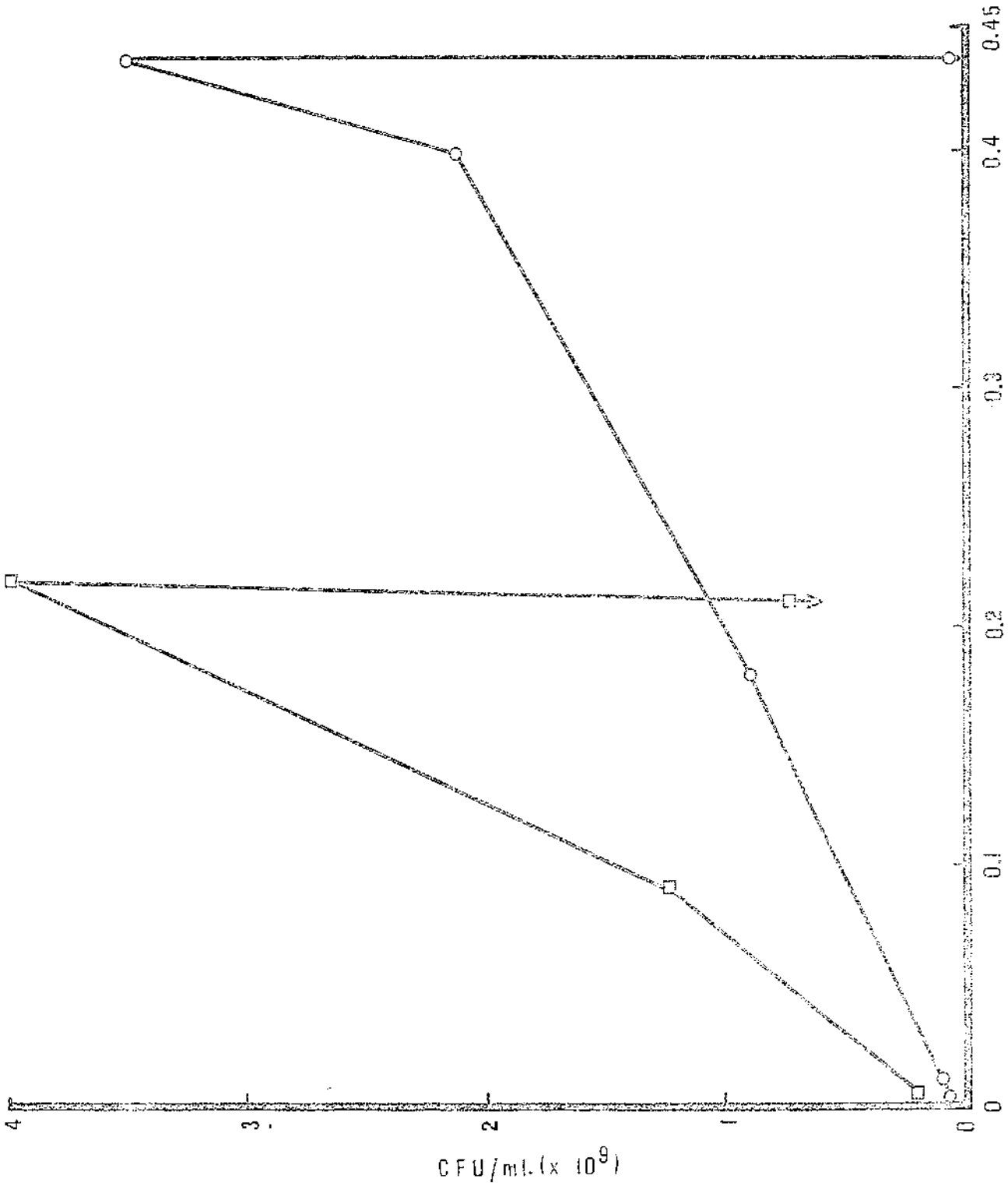


Figure 5: Increase in optical density with time of shaking (circles) and stationary (squares) cultures of M. ovipneumoniae using an inoculum size tenfold less than that shown in Fig.4. Note that the results are almost identical except that the increase in turbidity is delayed 10hr. The time when peak titres were obtained are indicated by arrows.

Figure 6: The relationship of the optical density of shaking (circles) and stationary (squares) cultures of M. ovipneumoniae to the number of colony forming units per ml. Note the higher optical density reached by shaking cultures. In both cultures, as the maximum optical density is reached, the viable count diminishes rapidly.



2) ELECTRON MICROSCOPY

a) Thin Sections

Cultures of M. ovipneumoniae in FM4 were selected at three stages of growth for sectioning:

(i) Logarithmic phase viz. 26hr after inoculation in the conditions used.

(ii) Death phase (48 hr) note: since death is rapid less than 0.1% of the original titre of viable cells remain.

(iii) Point of maximum titre (36 hr). note: as there is no detectable stationary phase, this also corresponds to the initiation of the death phase.

Logarithmic phase cells: These were for the most part roughly spherical in shape (Fig. 7). However, oval or elongated forms (Fig.7) were commonly seen. The great majority of cells measured 400 to 700nm in diameter, although some cells were seen, albeit rarely, which measured up to 1500nm in diameter (Fig. 8).

The cells were limited by a typical trilaminar membrane 8 to 10nm thick consisting of two electron dense zones separated by an electron lucent area. In most preparations the position of this unit membrane was detectable only by the electron lucent zone because the inner lamina was obscured by the cell contents and the outer dense zone appeared to be continuous with electron dense material on the cell surface (see below).

Outside the trilaminar membrane, and in close contact with it there was an electron dense zone with striations about 12nm in length, vertical to the cell surface (Fig. 9 and 10, arrows).

The unit membrane enclosed a cytoplasm which contained many ribosomes. Within the cytoplasm an electron lucent area containing thin fibrils was often visible (Figs. 9, 10, and 13). Such areas are usually presumed to represent the nuclear zone (Robertson, Gomersall and Gill, 1975). Dumb-bell shaped cells were frequently seen (Figs. 7, 20 and 21). Neither filamentous forms, nor intact cells within other cells were observed. (See Discussion: Electron microscopy).

Cells were commonly observed in pairs or small

groups in which areas of the membranes of individual cells were in close contact. (Figs. 10, 11, 12, and 13).
Death phase cells: The main reason for studying the morphology of dead cells was to avoid interpreting degenerate forms as part of the life cycle of the mycoplasmas. The main morphological features of the death phase cells were that the cell membrane of most cells was often ruptured or indistinct in parts and the ribosomes were aggregated in clumps and associated with the inner surface of the plasma membrane (Fig. 14, 15, and 16).
Cells harvested at maximum titre: Cells harvested at this stage represent the juncture between rapid growth, and even more rapid death. Most cells in such cultures were intact. Their ribosomes, however, were often partially aggregated, leaving large electron-lucent areas. The cytoplasm was often separated from the outer electron dense layers by a relatively thick electron-lucent zone (Fig.17).

b) Negative Staining

Negative staining was undertaken in the hope that the nature of the outer surface of the cells of M. ovipneumoniae could be better resolved than in the thin sections Small, individual projections, which were vertical to the cell surface could be seen. In some areas the projections showed a tight, pallisade appearance, and in others they appeared more sparse (Fig. 18 and 19).
Note: Efforts to increase the resolution of the surface projections by examining cells fixed with Karnovsky's fixative and washed with 0.1M phosphate buffer, pH 7.2 gave diminished resolution when compared to unfixed cells.

c) Morphology of Australian isolate of M. ovipneumoniae

The morphology of the Australian Y-98 biotype of M. ovipneumoniae was examined at the same stages of growth and under the same conditions as was that of the New Zealand strain.

It was morphologically indistinguishable from the New Zealand strain.

Figure 7: Exponential phase culture of M. ovi-pneumoniae showing two roughly spherical cells, and one dumb-bell shaped form, which may represent a stage in binary fission. Arrows indicate typical tri-laminar cell membrane. x73,000

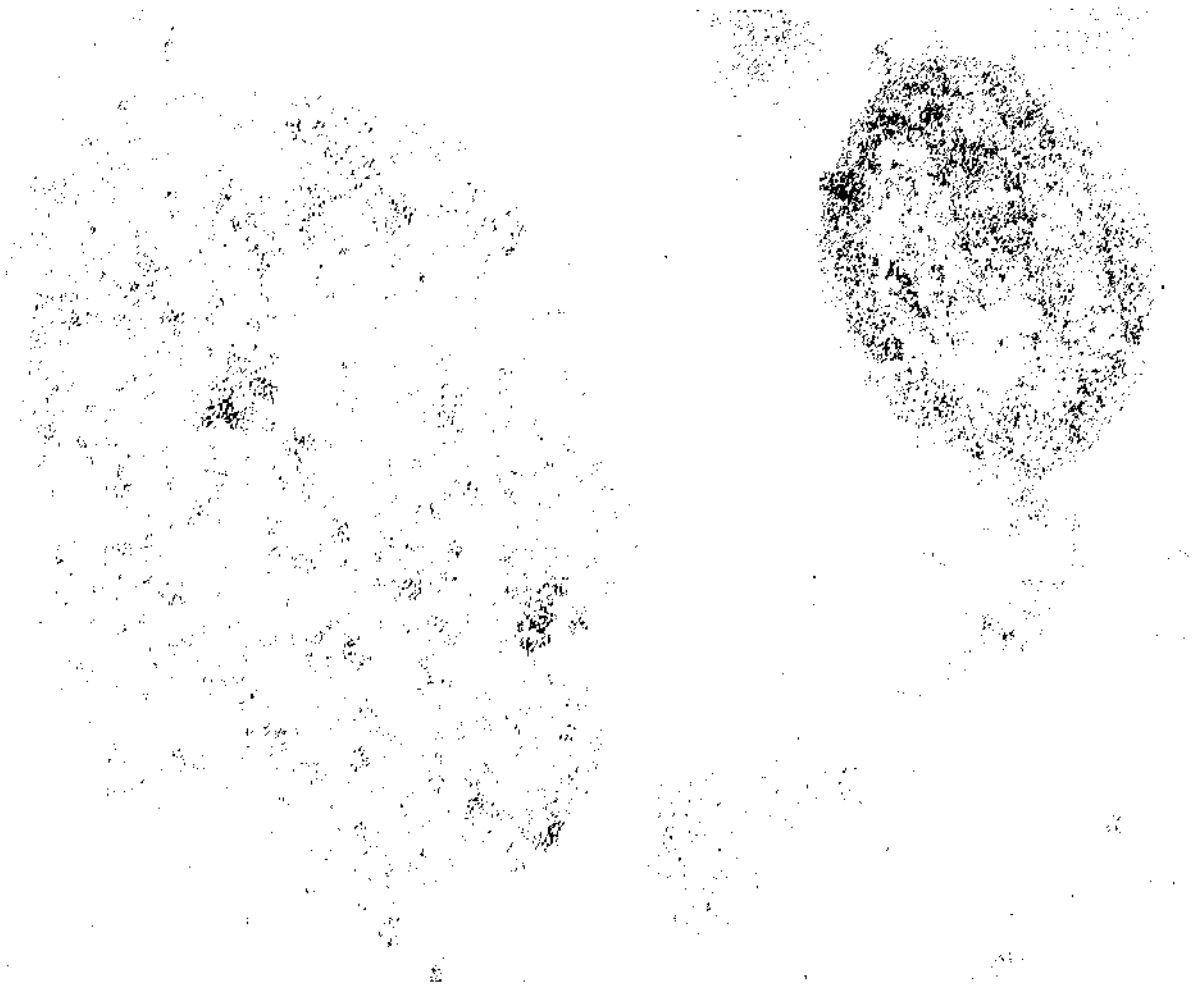


Figure 8: Two cells of M. ovipneumoniae from an exponential phase culture. The larger cell measured 1100nm x 1500nm and is the largest cell seen in this study. x73,000.

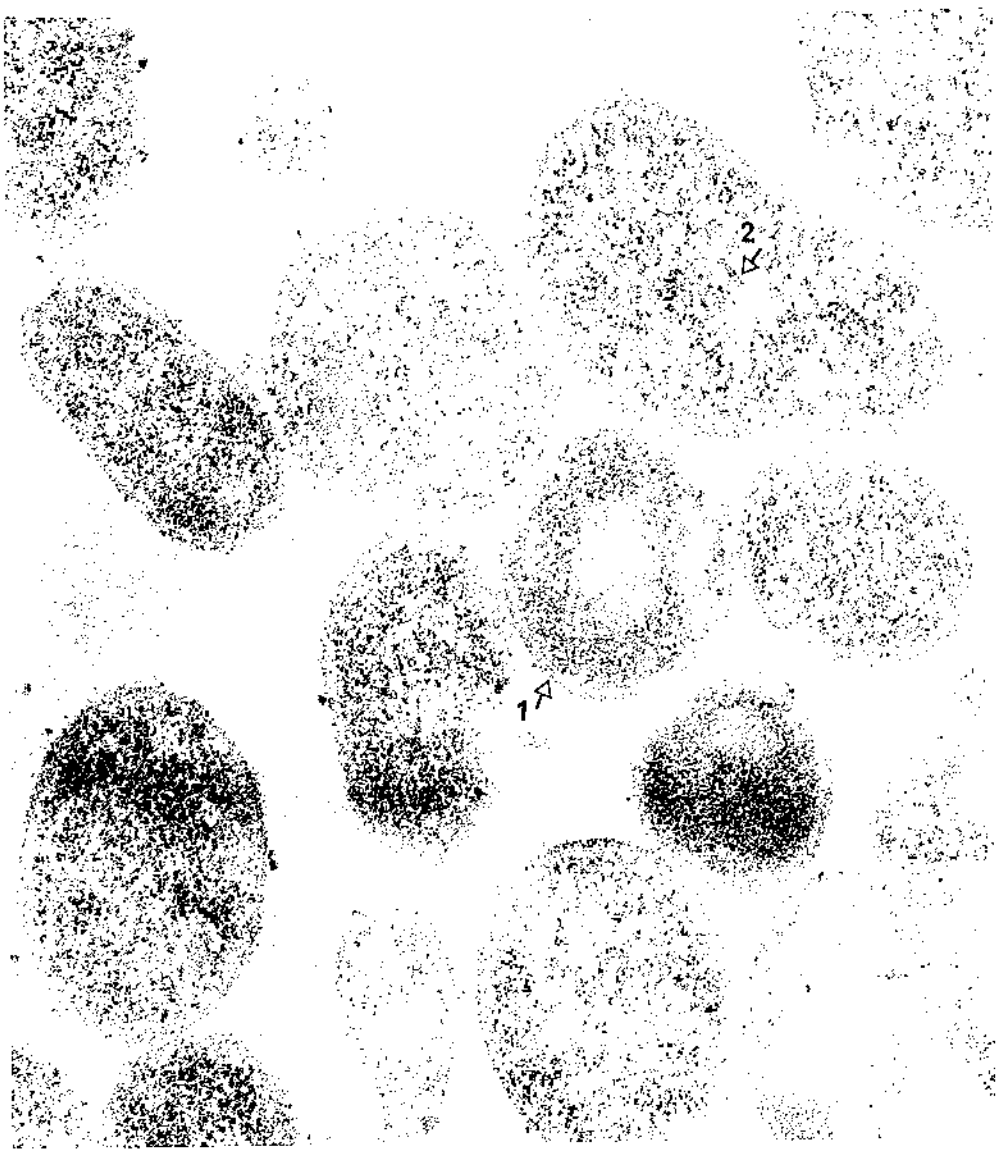


Figure 9: An exponential phase culture of M. ovipneumoniae. Note the striated appearance of the outer surface of the cell (arrow 1). This is apparently due to the presence of projections 12nm long orientated vertically to the membrane. The fibrillar structure seen in the cell cytoplasm (arrow 2) is generally assumed to represent DNA. x73,000.

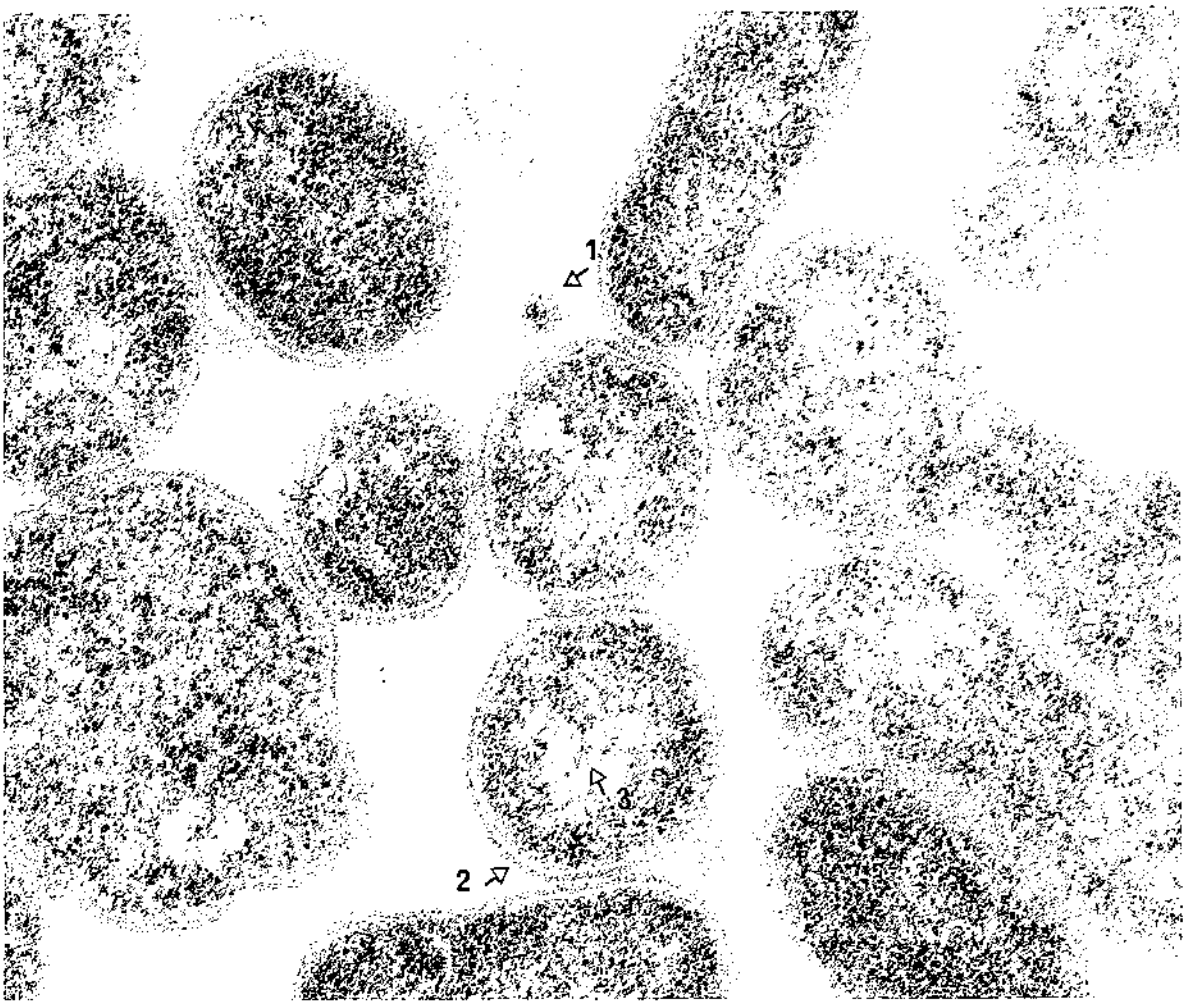


Figure 10: An exponential phase culture of M. ovipneumoniae. Note the small body 100nm in diameter (arrow 1), the material resembling projections on the cell surface (arrow 2) and the fibrillar structure generally interpreted as being DNA (arrow 3). Relatively large areas of the cell surface are in close contact.
x73,000.

Figure 11: Three cells of M. ovipneumoniae which appear to be aggregated. x73,000.

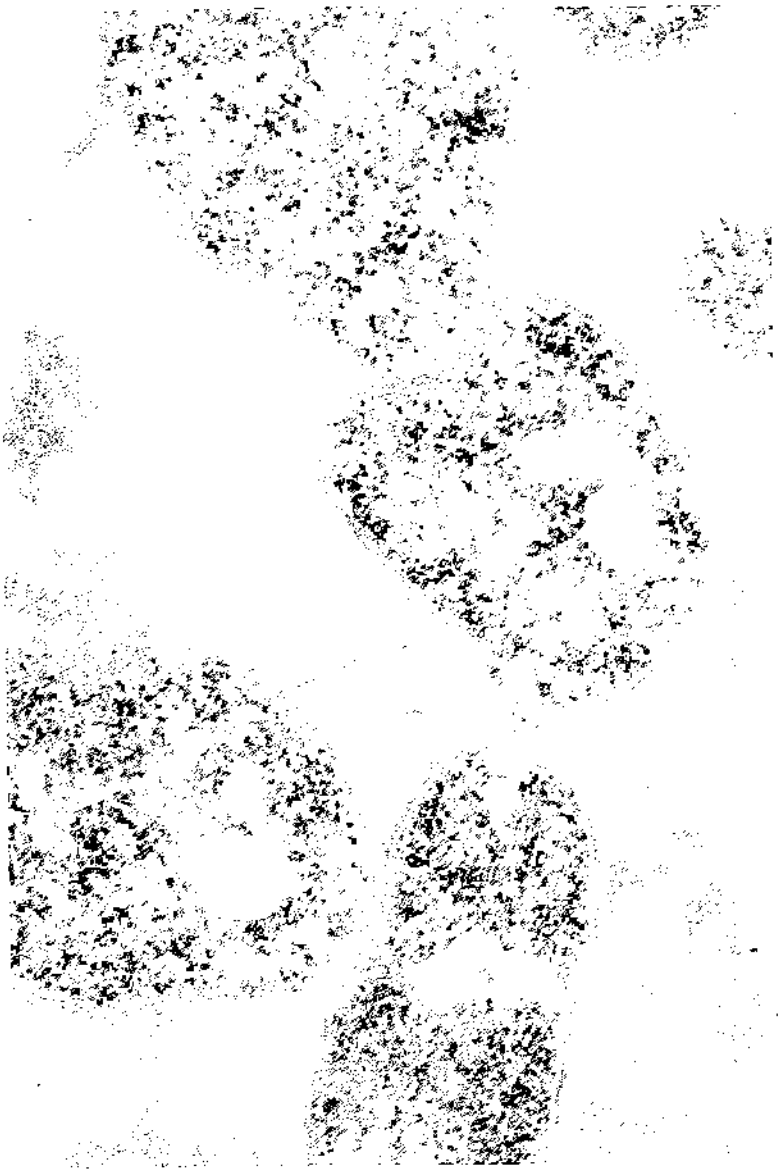


Figure 12: Culture of M. ovipneumoniae showing part of a clump of aggregated cells x73,000.

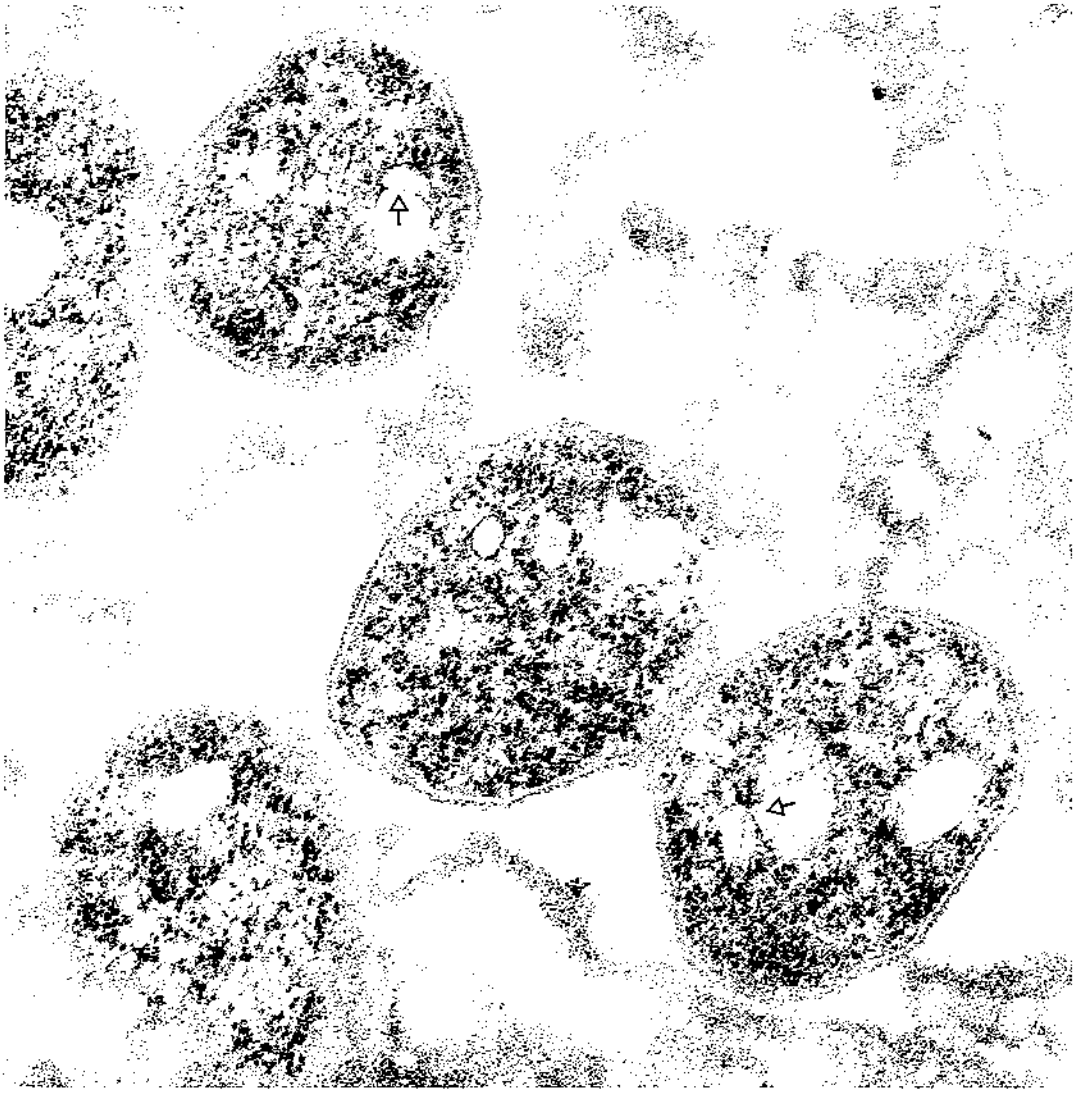
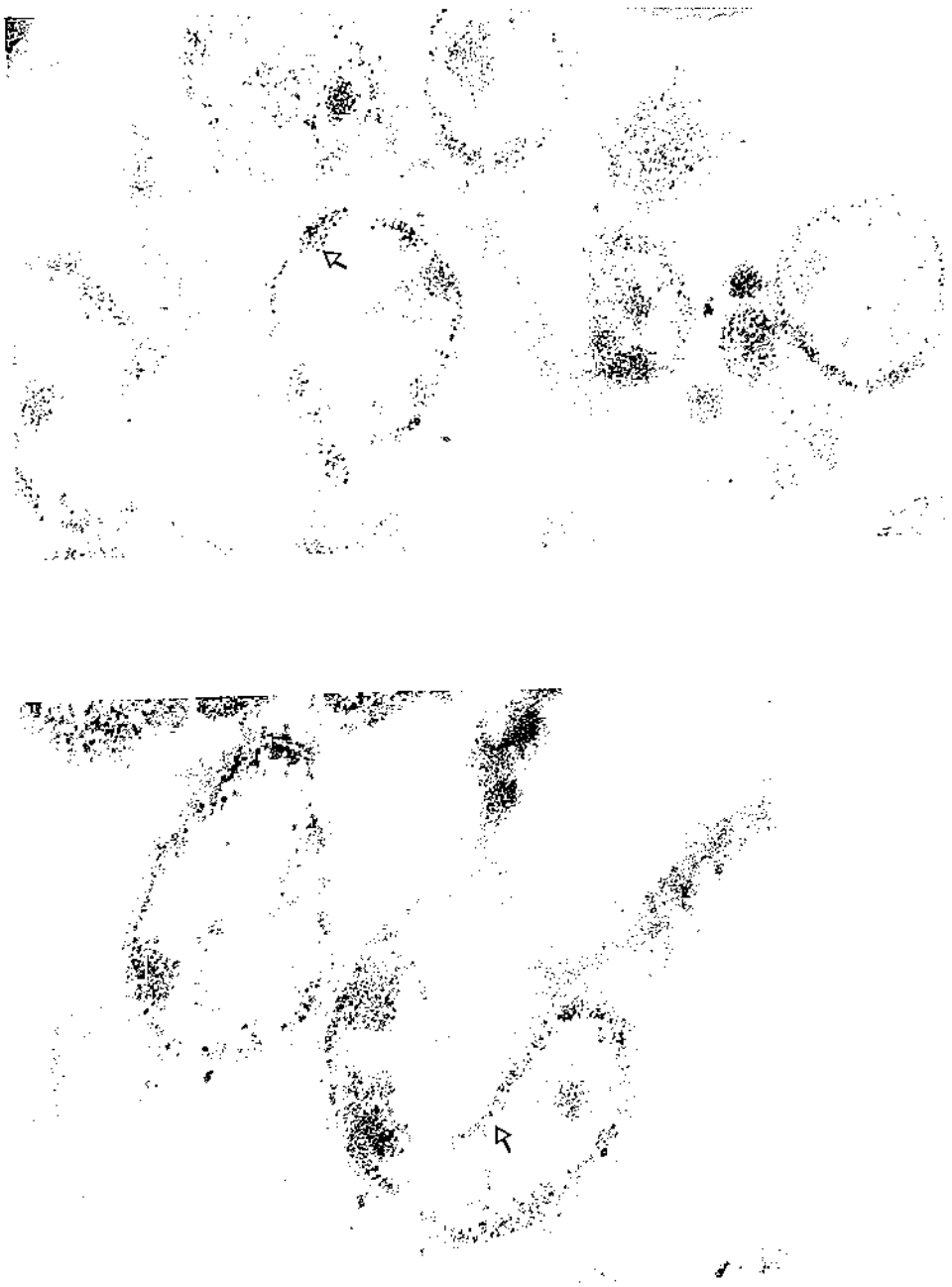


Figure 13: Aggregated cells of M. ovipneumoniae Arrows indicate the fibrillar structures generally interpreted as being DNA. x73,000.



Figures 14 & 15: Death phase culture of M. ovipneumoniae. Note that all cells have large electron-lucent zones which suggest that parts of the cytoplasm have been lost, and an apparently intact cell (bottom figure) in which the ribosomes have aggregated and are in close contact with the inside surface of the cell membrane. Many of these aggregates remain attached to the cell membrane, even after cell lysis (arrows) x55,000.

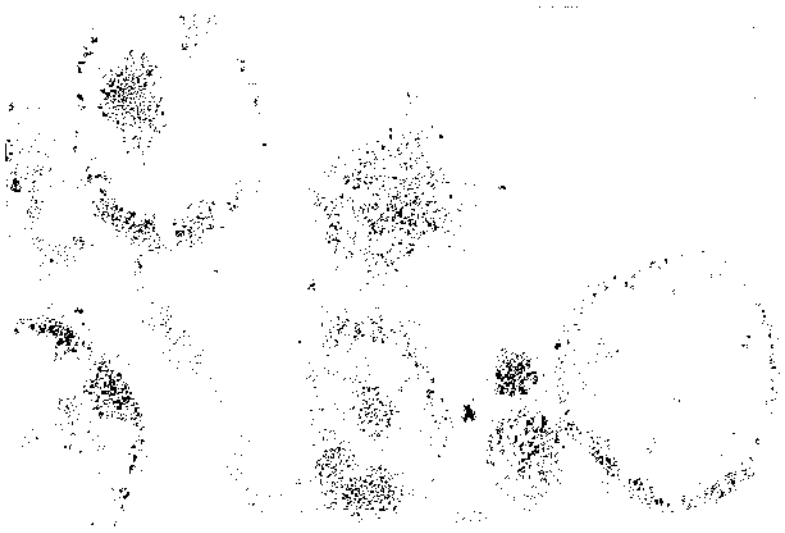


Figure 16: Death phase culture of M. ovipneumoniae x55,000.

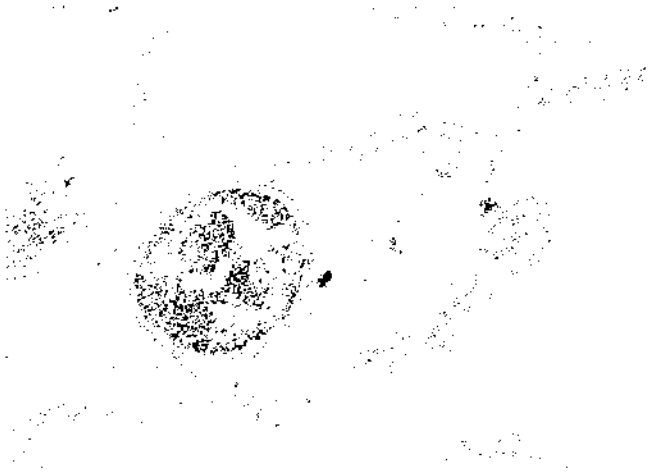


Figure 17: Cell of M. ovipneumoniae harvested at point of maximum titre. Note the larger proportion of electron lucent area than is present in exponential phase cells and the tendency for the ribosomes to aggregate. x55,000.

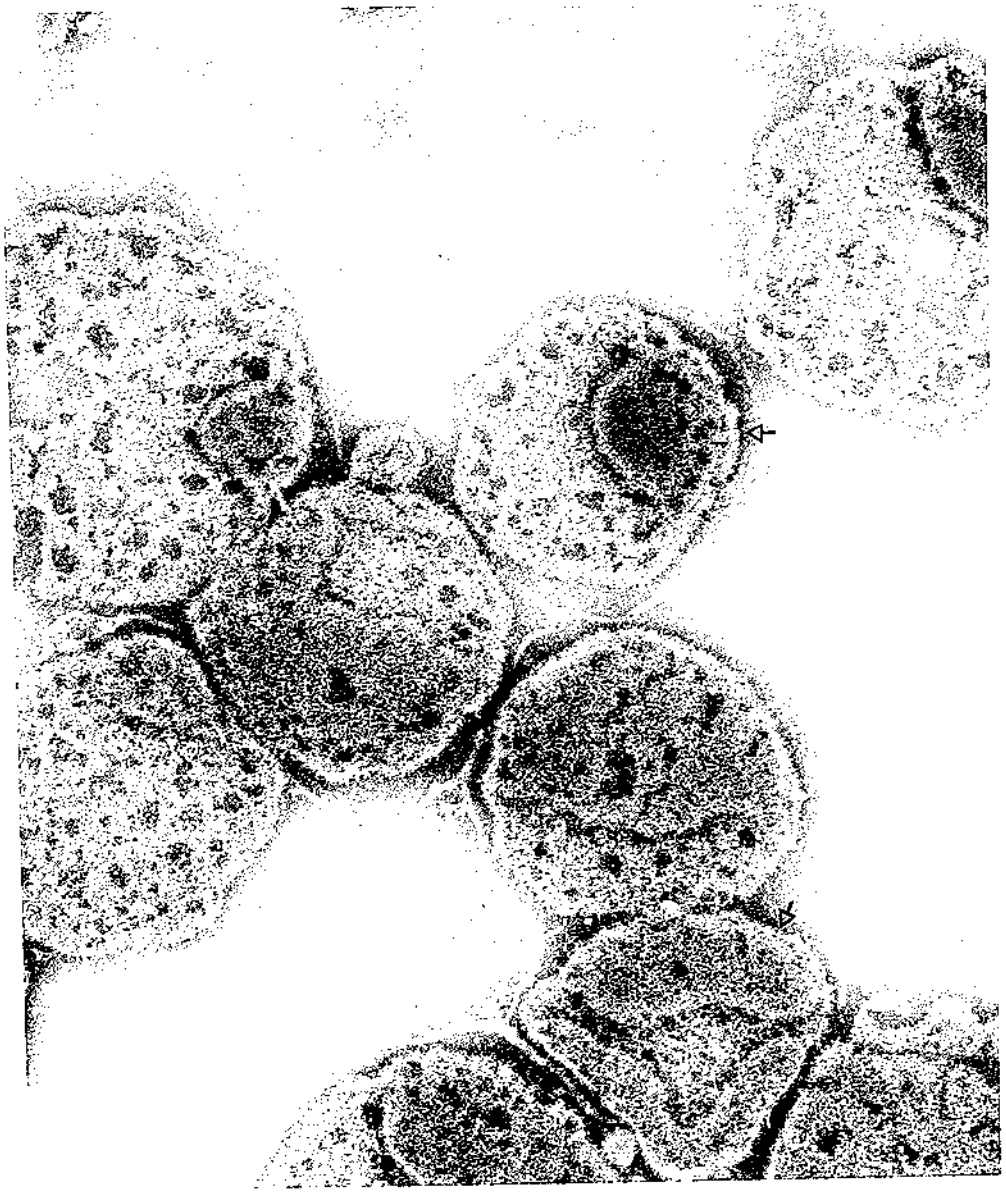


Figure 18: Negatively stained, unfixed cells of M. ovipneumoniae from an exponential phase culture. Arrows indicate the striated appearance of the cell surface, possibly due to the presence of 12nm long projections. x73,000.

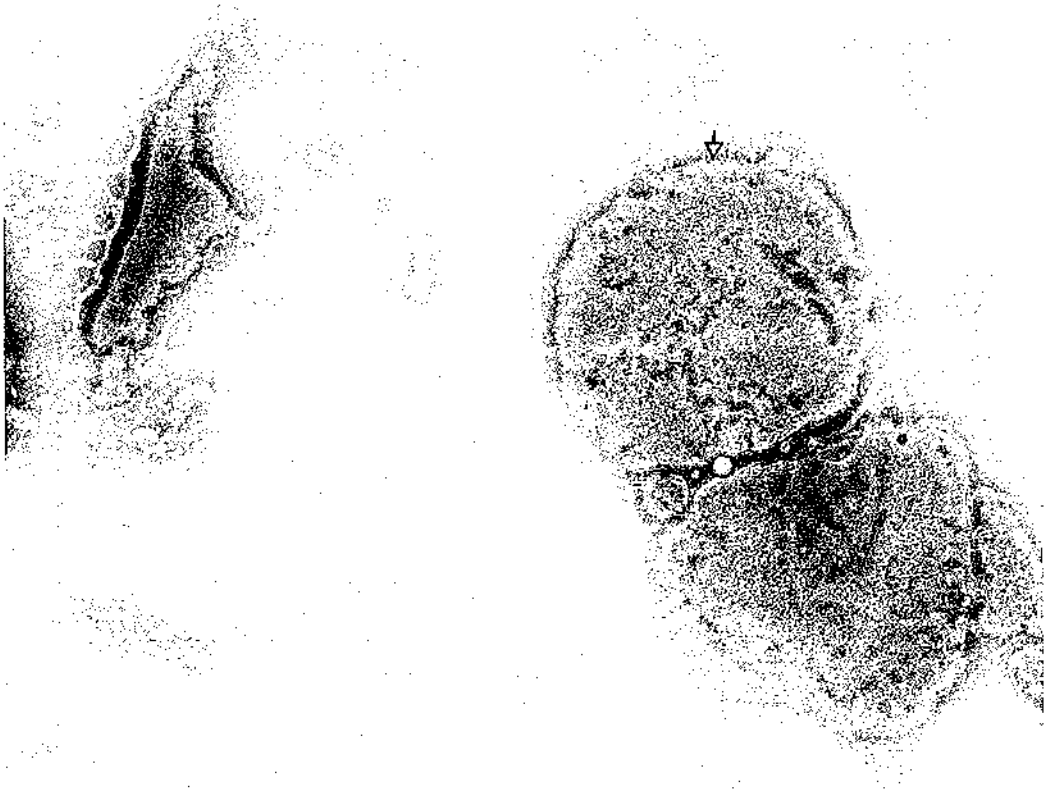


Figure 19: Negatively stained, unfixed cells of M. ovipneumoniae from an exponential phase culture. Arrows indicate the striated appearance of the cell surface, possibly due to the presence of 12nm long projections x73,000.

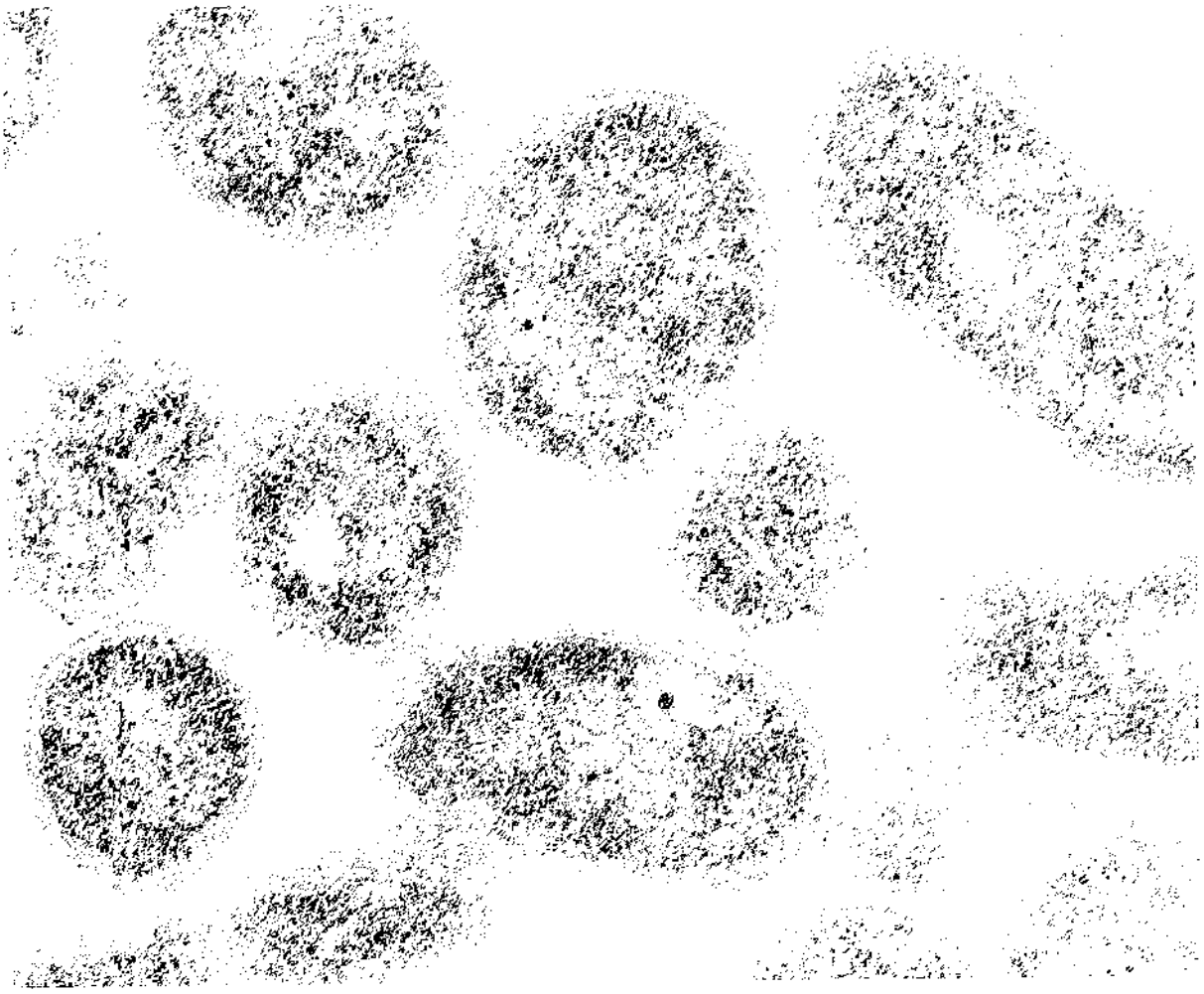


Figure 20: Exponential phase culture of M. ovipneumoniae. Note the cell in top right hand corner which may be at an early stage of binary fission. x73,000.

Figure 21: Exponential phase culture of M. ovipneumoniae. Note the dumb-bell shaped form. x73,000.

3) DNA STUDIES

Although one report of the GC content of a mycoplasma now believed to be a strain of M. ovipneumoniae has appeared in the literature (see Leach et al. 1976) the GC content of the DNA of a well characterised strain of M. ovipneumoniae has not been reported. Hence, the base composition of the New Zealand strain of M. ovipneumoniae was determined in this study. Two approaches were used: determination of the melting temperature of the DNA. and its buoyant density in CsCl.

To establish the validity of the melting temperature studies, determinations of the base composition of two controls were made: E. coli K12, because it is a well characterized organism, and H. influenzae Rd because it, like mycoplasmas, has a relatively low GC content.

a) Purity of Extracted DNA

DNA was extracted from M. ovipneumoniae, E. coli K12 and H. influenzae Rd, and its purity investigated by measuring the ratio of the extinction coefficients at 260nm and 230mm ($E_{260/230}$) in a UV spectrophotometer. An $E_{260/230}$ of 2.4 was taken as absolute purity, but DNA with an $E_{260/230}$ of greater than 2.0 was regarded as being sufficiently pure for these investigations. The results were as follows:

E. coli K12	:	2.18
H. influenzae Rd	:	2.26
M. ovipneumoniae	:	2.36

The extracted DNA was sufficiently pure for use in the DNA studies undertaken.

b) Thermal Denaturation Studies:

Thermal denaturation of DNA was studied by recording the increase in extinction coefficient with rise in temperature in a UV spectrophotometer.

Most workers who use this method to determine the base composition of DNA, denatured the DNA in 0.1 x SSC buffer (Bowie, Grigor, Dunckly, Loutit and Loutit, 1972; Mandel et al., 1970: Green and Dick, 1972) The main advantage of using a dilute buffer is that the melting temperature of the DNA is lower in low salt concentrations, and in instruments not specifically designed to accurately

maintain temperatures in the range 80° to 100°, lowering of the temperature necessary to melt the DNA by 15° to 20° makes the experiment technically more accurate.

Thus, 0.1 xSSC buffer was used in preliminary experiments in this study. However, the reproducibility of the results was less than acceptable and the source of variation was ultimately traced to small differences in salt concentration in different preparations of DNA.

The GC content of DNA denatured in SSC buffer of concentration "x" can be calculated from the following equation (Mandel et al., 1970):

$$GC = \left[Tm_{SSC_x} - 16.3 \log \left(\frac{SSCx}{SSC_{0.1}}\right) \right] x_{50}\frac{1}{.2} - 0.990$$

It can be seen from the equation that small absolute differences in salt concentration are important at low salt concentrations but the same absolute error is much less significant at higher salt concentrations. Consequently, all subsequent determinations were performed using SSC. This gave more reproducible results. It is relevant to note that the equipment available in this laboratory for thermal denuration experiments had been specifically designed so that high temperatures could be accurately maintained and measured.

The melting profiles of the DNA (see Fig. 22) of E. coli K12 and H. influenzae Rd was determined three times in SSC, and that of the DNA of M. ovipneumoniae four times and the melting temperature determined from these profiles in the following way (as described by Knittel, Black, Sandine and Fraser, 1968).:

The parts of the curve where the extinction coefficient was constant (i.e. before and after thermal denaturation occurred) were extrapolated. A line was drawn through the part of the curve of maximum gradient, and the temperatures at which this line intersected the two lines of no gradient were taken. The midpoint between these two temperatures was the melting temperature.

The base compositions were determined from the melting temperatures by the equation of DeLey (1970):

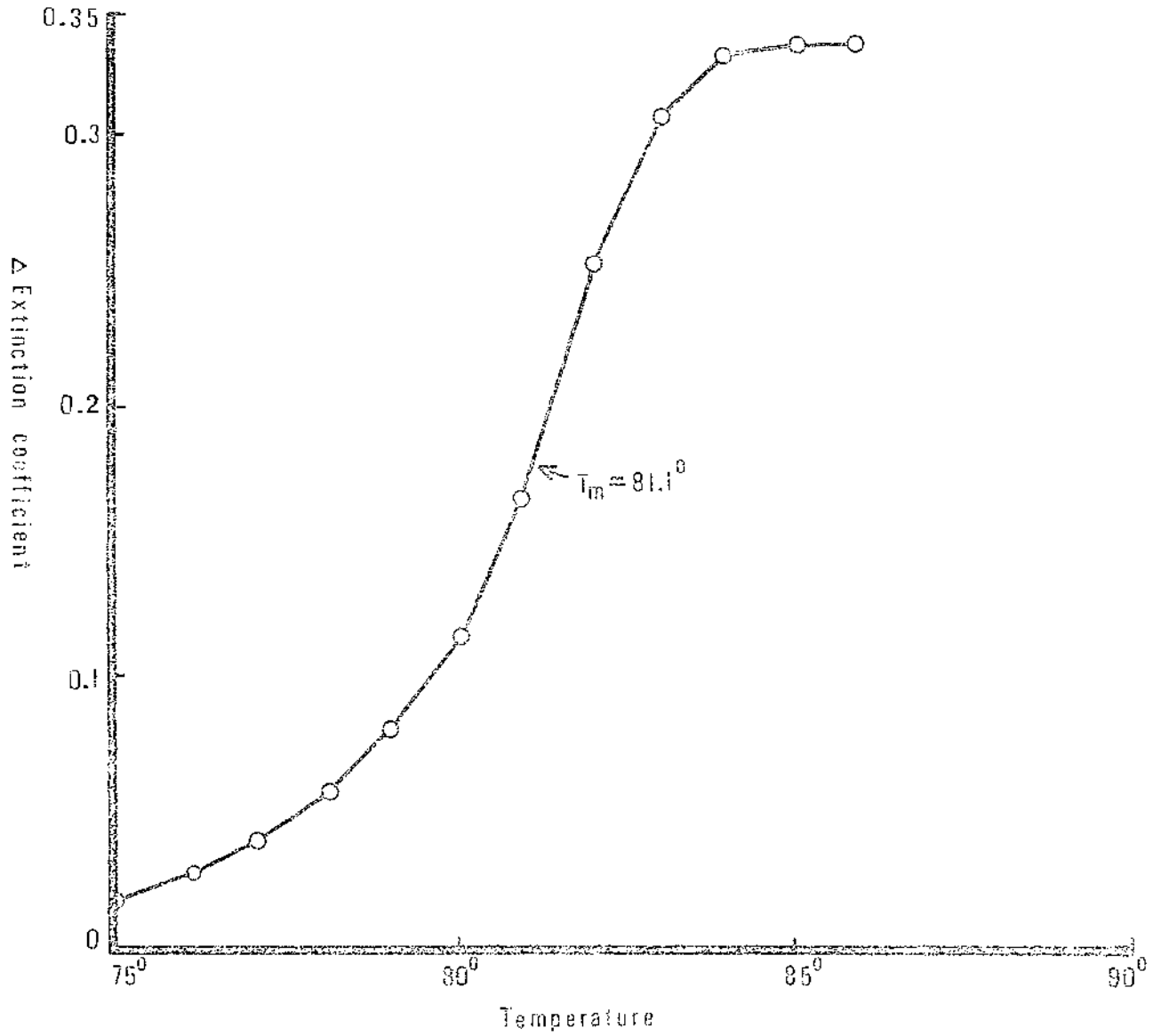


Figure 22: Melting profile of the DNA of M. ovipneumoniae obtained by recording the change in extinction coefficient of the DNA in SSC as the temperature of the solution increased. The melting temperature, viz. the temperature corresponding to a 50% increase in extinction coefficient, obtained in this determination is marked with an arrow.

$$\% \text{ GC} = \frac{T_m - 69.37}{0.41} \times \frac{100}{1}$$

T_m = Melting temperature

The melting temperatures obtained, and the base composition determined from them are shown in Table VIII.

TABLE VIII Melting temperatures and base compositions determined for the DNA of *E. coli* K12, *H. influenzae* Rd and *M. ovipneumoniae*.

Organism	Expt.	T_m	Base Composition (%GC)	Reported Base Comp.
E. coli K12	1	90.7°	52.0	
	2	89.8°	50.0	
	3	90.1°	50.6	
	Mean:	90.2°	50.9	51.0%GC
H. influenzae Rd	1	85.5°	39.3	
	2	85.4°	39.1	
	3	85.3°	38.9	
	Mean:	85.4°	39.1	39.2%GC
M. ovipneumoniae	1	80.7°	27.6	
	2	80.8°	27.9	
	3	81.1°	28.6	
	4	81.0°	28.4	
	Mean:	80.9°	28.1	

c) Buoyant Density Studies

The buoyant density of the DNA of *M. ovipneumoniae* was determined by reference to that of the DNA of the recommended control *E. coli* K12, using the method of Schildkraut *et al.* (1962).

Ultraviolet photographs of the DNA absorbtion in CsCl (Fig.23) were traced using a Joyce Recording Microdensitometer. This tracing (Fig. 24) was analyzed in the following manner to determine the buoyant density of the *M. ovipneumoniae* DNA.

On the tracing, the distance between the reference edges of the cell = 14.35cm.

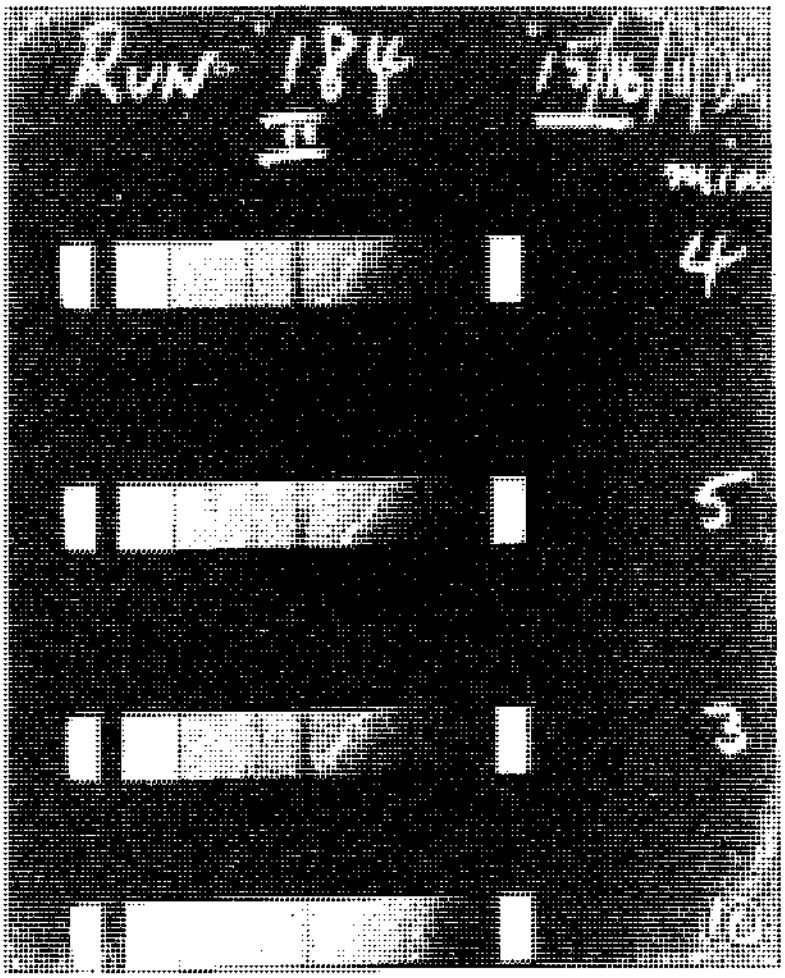


Figure 23: UV absorption photographs of bands of E. coli K12 DNA and M. ovipneumoniae DNA at equilibrium during ultracentrifugation in a CsCl gradient. The right hand band is E. coli K12 DNA and the left band is M. ovipneumoniae DNA. The spot between the bands is an artifact (dust speck). The time in minutes figures refers to the exposure time of the four photographs of the same gradient.

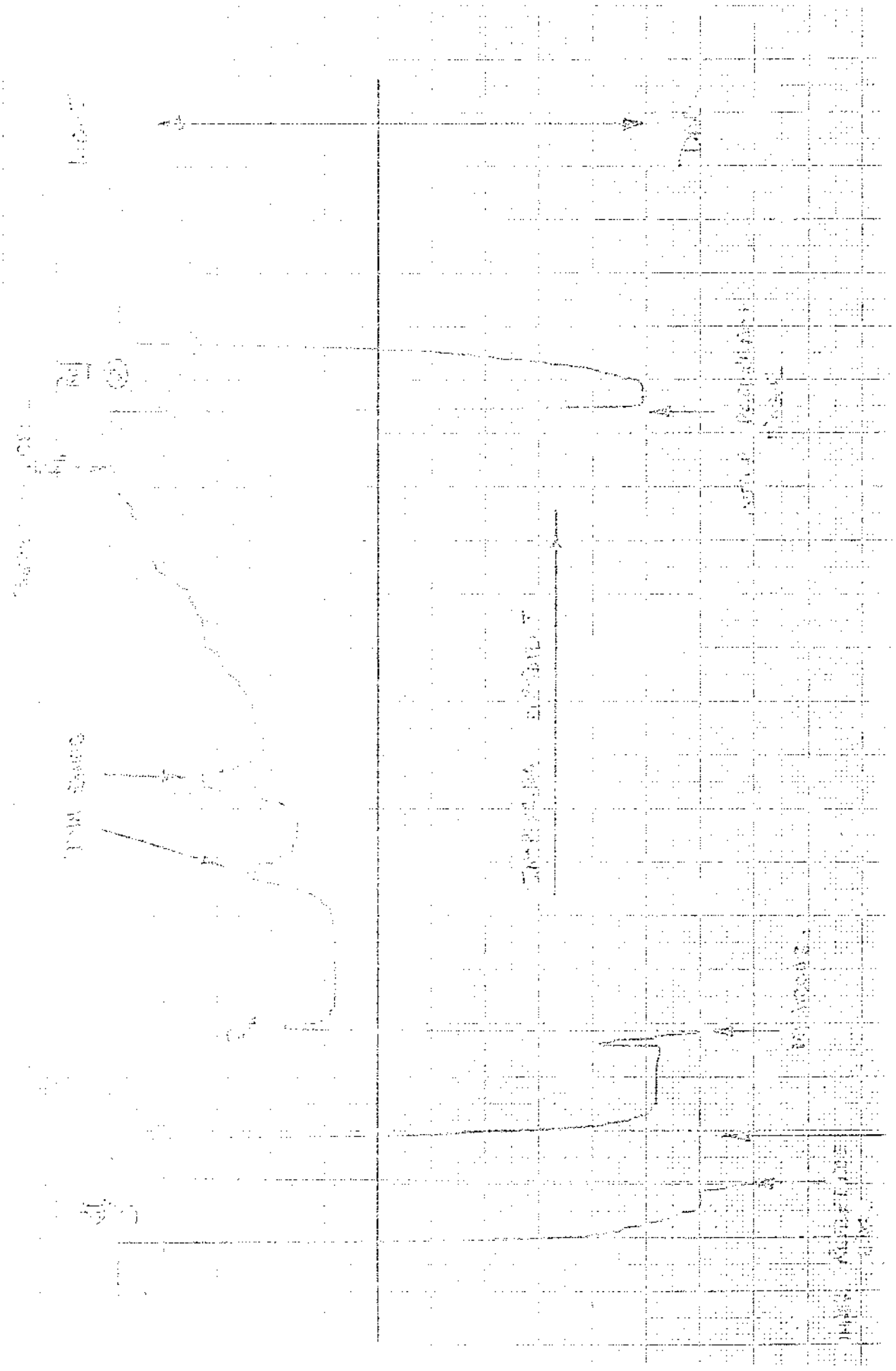


Figure 24: Microdensitometer tracing of UV absorption photographs (Fig.23) showing the positions of the DNA of E. coli K12 (right peak) and M. ovipneumoniae (left peak) at equilibrium in a CsCl gradient during ultracentrifugation.

This represents a distance, in the cell of 1.60cm. Therefore, the magnification factor was $\frac{1.60}{14.35}$.

The actual distance of the inner reference edge of the cell from the centre of rotation = 5.71cm.

The distance on the tracing of the E. coli K12 DNA from the inner reference edge of the cell = 7.45cm, so the actual distance of the E. coli K12 DNA from the centre of rotation (r_o)=(7.45 $\times \frac{1.60}{14.35}$) + 5.71cm = 6.541cm.

Similarly, the distance of the M. ovipneumoniae DNA from the centre of rotation (r) = (5.70 $\times \frac{1.60}{14.35}$ +5.71cm

= 6.346cm.

The average speed of rotation calculated from the revolution counter readings over 5hr = 44,229r.p.m. Thus speed of rotation (w) = 44,229 $\times \frac{2\pi}{60}$

rad. sec^{-1} = 2.145×10^7 rad. sec^{-1}.

Thus: $w = 2.145 \times 10^7$ rad. sec^{-1}

$r_o = 6.541$cm

$r = 6.346$cm

The buoyant density (P_o) of the E. coli K12 DNA is known to be 1.710 $g.cm^{-3}$.

So, from the equation of Schildkraut et al.(1962):

$$P = P_o + 4.2\ w^2\ (r^2 - r_o^2) \times 10^{-10} g.cm^{-3}$$

$$P = 1.710 + 4.2 \times 2.145 \times 10^7\ (6.346^2 - 6.541^2) \times 10^{-10} g.cm^{-3} = 1.6874 g.cm^{-3}$$

Using this figure, the base composition of the DNA can be determined as follows:

$$\% GC = \frac{P - 1.660}{0.098} \times \frac{100}{1}$$

$$= \frac{1.6874 - 1.660}{0.098} \times \frac{100}{1}$$

$$= 27.96$$

$$= 28.0$$

DISCUSSION

1) GROWTH STUDIES

   (a) Change in Colony Forming Units with Time

   (b) Change in Optical Density with Time and Number of Colony Forming Units

2) ELECTRON MICROSCOPY

   (a) Cells observed in Exponential Phase Cultures

   (b) Cells in Aged Cultures

   (c) Electron Microscopy of Australian Strain of M. ovipneumoniae

3) BASE COMPOSITION

1) GROWTH STUDIES

a) Change in Colony Forming Units with Time

The growth curves obtained by propagating M. ovipneumoniae in FM4 broth are shown in Figs 2 and 3. This pattern of growth, in general, conforms to that described for other mycoplasmas (Low and Eaton, 1965; Razin and Cosenza, 1966). However, it is generally assumed by most workers (Furness, 1968) that mycoplasmas, like bacteria have a lag phase before cell division occurs. In contrast to this, M. ovipneumoniae grown under shaking conditions showed no lag phase. This could be accounted for by the fact that the stock culture used to inoculate the medium had been harvested while undergoing exponential growth. Cells in stationary cultures also had no absolute lag phase but did not achieve the maximum growth rate for ten hours. However, the maximum growth rate achieved was the same as that of shaking cultures.

M. ovipneumoniae does not grow anaerobically (Clarke, personal communication). It is surprising therefore, that the mean minimum generation times of cells in both shaking and stationary cultures are the same. However, the experimental conditions used were such that 10ml of culture was placed in a 100ml conical flask. This allowed for a large liquid-air interface and a shallow culture so that oxygen may have diffused into the culture at a rate sufficient to avoid oxygen depletion becoming a factor limiting the growth rate.

There was a significant difference in generation times of cultures grown with different inoculum sizes, viz. 1.7hr for cultures with a small inoculum size, and 2.3hr for cultures with a tenfold larger inoculum. This was probably due to the presence of inhibitory substances in the relatively large inoculum, although the dilution effect of adding one volume of depleted medium to nine volumes of fresh FM4 obviously involves a slight dilution of nutrients which could also slow the growth rate.

Does the measurement of a mean generation time have any validity? There is general acceptance of the fact that the generation time of a microorganism decreases as

a better quality of medium is used to propagate it. However, cells grown in 'ideal' conditions and media will ultimately divide at a rate limited by the replicative mechanisms of the cell. This means that if the growth rate is determined using different media and conditions, it is the minimum division time which most nearly reflects the 'ideal' minimum. In the absence of a detailed knowledge of the physiology and metabolic requirements of the organism, there is no guarantee that the estimated minimum generation time represents even an approximation of the theoretical minimum. This particularly applies to microorganisms such as mycoplasmas with complex growth requirements. So could the minimum generation time of 1.7hr estimated for M. ovipneumoniae be representative of the 'ideal' minimum, or is it a gross overestimate? Clues as to the validity of the estimate can be obtained by comparing this figure to that obtained for other mycoplasmas, and by comparing the quality and complexity of the medium used to grow M. ovipneumoniae with other mycoplasma media. The medium used was FM4, which is a highly complex medium containing serum, yeast autolysate, peptones, many vitamins and NAD. Clarke et al. (1974), using this medium, isolated M. ovipneumoniae from 100% of pneumonic lungs sampled, whereas other workers, using other media (Carmichael et al., 1972; St George et al., 1975; Leach et al., 1976) achieved a lower recovery rate. Comparison between the generation time of 1.7 hr determined for M. ovipneumoniae and generation times determined for other mycoplasmas are made in Table IX.

TABLE IX: Generation times determined for some members of the Mycoplasmatales

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

Species(cont'd)	Mean generation time (hr) cont'd
M. pneumoniae	19 (est)
A. laidlawii	1.8
M. orale	3.3
Goat mycoplasma strain Y	1.6 - 1.7
M. hominis	1.6

From Smith (1971) and Robertson et al., (1975).

The longer generation times reported in the above table are probably the result of the inadequate nutritional quality of the culture medium in which the cells were propagated. The generation time of 1.7hr determined for M. ovipneumoniae in these experiments is otherwise remarkably similar to that determined for five of the other mycoplasmas listed in Table IX. It is therefore likely that the theoretical minimum generation time of M. ovipneumoniae is not much less than the observed time of 1.7 hr.

The maximum yield of mycoplasmas in broth cultures varies with the organism under test and the medium used. However, Thomas (1973) states that, in general, the maximum yield obtainable is about 5.0×10^9 CFU/ml. Other workers investigating specific mycoplasmas report significantly lower maximum titres: thus Robertson et al., (1975) report a maximum titre of 4.0×10^8 CFU/ml for M. hominis; Morton and Roberts (1967) 2×10^8 CFU/ml for M. arthritidis; Furness (1968) 1.2×10^8 CFU/ml for M. orale; Furness, Pipes and McMurtrey (1968) 4.1×10^7 CFU/ml for M. meleagridis.

The maximum population density of 3.0×10^9CFU/ml attained in the present study therefore represents a higher yield than that determined in most studies, and is of the same order of magnitude as the absolute maximum of 5.0×10^9CFU/ml reported by Thomas (1973). The cells of M. ovipneumoniae are somewhat larger than those of most mycoplasmas (see Discussion: Electron Microscopy). Thus it might be expected that a lower maximum viable count would be obtained for M. ovipneumoniae than for M. hominis or other, smaller mycoplasmas. However, as

can be seen by comparison with values reported by other workers for other species with smaller cells, in the present study, M. ovipneumoniae was grown to higher titres. This suggests that the medium used (FM4) is a highly satisfactory growth medium for M. ovipneumoniae even though it was developed for a different but fastidious mycoplasma viz. M. gallisepticum.

In any one experiment where aliquots of the same batch of FM4 medium were inoculated with M. ovipneumoniae and then incubated either stationary, or with shaking, the same maximum titre was reached in both cases. This result, however, contrasts with the turbidity data shown in Figs 4 and 5, in which the maximum optical density reached by shaking cultures was approximately 2.5 times greater than that reached by standing cultures. The two most probable explanations for this phenomenon are that either the cells grown in aerated conditions are, on the average, larger than those grown in partly anaerobic conditions, or that cells in shaking cultures aggregate, so that when plated, aggregates of several viable mycoplasmas give rise to only one colony. This latter explanation is supported by macroscopic examination of turbid shaking cultures, which, unlike the standing cultures, appeared to be flocculated.

The conclusion thatturbid shaking cultures of M. ovipneumoniae contain aggregations of cells is supported by electron microscopic examination which frequently shows cells with areas of their surface in close contact (Figs. 10,11,12 and 13). The areas of close contact do not arise from centrifugation before fixing because in sectioned cell pellets, the cells were very loosely packed together (Figs. 11 and 13).

The aggregates seen in thin sections usually consist of two or three cells so that the ratio of viable cells to colony-forming units would be 2 to 3:1 which is consistent with the observation that the unflocculated stationary cultures produce the same number of colony-forming units as the shaking cultures, although their turbidity is about 2.5 times lower. It is relevant to note that

Furness (1968) using mild sonication to break aggregates of cells found that following sonication, the number of colony-forming units increased at least twofold.

Morton and Roberts (1967) compared shaking and stationary cultures of a mycoplasma and found also that the maximum titres of shaking and stationary cultures were the same. They however, do not provide any turbidity data, and do not comment on this phenomenon.

Mycoplasmas die very rapidly after reaching maximum titre. In the present study, the maximum death rate was reached five hours after maximum titre was attained, and a further five hours later no colonies were formed when 0.05ml of the culture was plated onto solid media. The half-life of the cells during this period was calculated from the maximum (i.e. final) slope of the death phase curve, so the average half-life of cells which had just reached maximum titre would be longer than that determined. The half-life calculated was 0.45 - 0.57hr. This corresponds to a drop in titre of approximately 10^2CFU/ml per hour over the latter five hours of death. Maniloff and Morowitz (1972) report that cultures of mycoplasmas can drop in titre by a factor of 10^2 to 10^4CFU/ml per hour.

The reason for the rapid death of mycoplasmas is usually ascribed to production of acid by the organism and the sensitivity of mycoplasmas to pH changes. (Smith, 1971; Low and Eaton, 1965). Robertson et al., (1976) determined a half-life of 9 hr for a culture of M. hominis growing in PPLO broth modified by the addition of HEPES buffer.

This long half life in well buffered medium is consistent with the suggestion that a low pH causes the rapid death of many mycoplasmas.

b) Change in Optical Density with Time and Number of Colony Forming Units.

The growth of mycoplasma species has often been measured by optical density readings (Smith, 1956; Butler and Knight, 1960; Robertson et al., 1976), but there seems general agreement that the sensitivity of this method is poor, mainly because mycoplasmas, being small cells, produce little turbidity during growth

(Smith 1971). (In Fig. 6 it can be seen that no significant turbidity is produced in a growing culture of M. ovipneumoniae until the titre reaches 10^7 to 10^8CFU/ml). Other problems which have been reported by workers quantitating growth using optical density data include the need for optimalgrowth conditions, as most species tend to clump under suboptimal growth conditions rending turbidity measurements less useful (Smith, 1971). Furthermore, in mycoplasma media which contain serum, precipitates may be produced by either the organisms themselves, or the temperature of incubation, and these precipitates could obviously interfere with optical density measurements (Wolf and Marcus, 1969).

In the present study, data relating turbidity to number of colony forming units present in the culture, were collected and expressed graphically (Fig. 6) and, in spite of potential problems, gave consistent results: thus, it is possible, albeit within fairly narrow limits and in defined conditions to use optical density to estimate the colony count of shaking (and within even more narrow limits of stationary) cultures of M. ovipneumoniae.

Because the size of M. ovipneumoniae is small when compared to an average bacterium, a measurable turbidity is not achieved until the viable count reaches 10^8CFU/ml. Further more, the measurements must be made while the culture is actively growing. This latter point is particularly important as the optical density of a culture of M. ovipneumoniae, unlike that of cultures of some other mycoplasmas e.g. M. hominis (Rottem and Greenberg, 1975), does not significantly diminish during the death phase. Even within the limits stated, the results have at least one important application because they allow reproducible production of high titre growing cultures which can be used for disease transmission experiments. This was a major objective of the present study.

It was initially assumed, because of the results reported for many other mycoplasmas (Smith, 1971) that it would probably not be possible to use turbidity data

to estimate the viable cell count of a culture of M. ovipneumoniae. In practice, however it did turn out to be possible, and the achievement of a measurable turbidity could be attributed to a combination of three factors which may be absent from many studies: viz. FM4 appears to be a particularly effective medium for the culture of M. ovipneumoniae; M. ovipneumoniae cells are larger than many other mycoplasmas, and thus a measurable optical density is achieved by a lower number of cells than for other species and finally aeration of the culture markedly increases the maximum optical density attained.

2) ELECTRON MICROSCOPY

a) Cells Observed in Exponential Phase Cultures

Morphology:

The main conclusion reached from examination of the electron micrographs of thin sectioned and negatively stained preparations of M. ovipneumoniae cells is that they are approximately spherical, 400 to 700nm in diameter, and limited by a trilaminar membrane whose outer surface bears projections approximately 12nm long, and which surrounds a cytoplasm containing ribosomes and fibrils of deoxyribonucleic acid. This description is not basically different from that described for many other mycoplasmas (Razin, 1969; Stanbridge, 1976).

It was initially thought possible that electron microscopic examination might have detected some distinguishing feature of M. ovipneumoniae which would allow it to be differentiated from other mycoplasmas in electron micrographs of sheep lung, because such features have been demonstrated on a few other mycoplasmas, e.g. the "blebs" of M. gallisepticum and the "terminal structures" of M. pneumoniae both of which seem to be involved in attaching the mycoplasmas to surfaces.

Although M. ovipneumoniae has no unique morphological features, the average cell diameter seen was larger than that reported (Razin, 1969; Smith, 1971) for most other mycoplasmas. This relatively large cell size was predictable even before electron microscopic studies had been performed,because M. ovipneumoniae produced centreless colonies on 1% agar (Fig.1). Centreless colonies are produced by larger mycoplasmas, such as M. dispar (Gourlay and Leach, 1970) which has the same cell diameter as M. ovipneumoniae, because they cannot penetrate the interstices of the agar gel, and thus fail to give rise to the core of the fried-egg colony form typical of most mycoplasmas (Smith, 1971).

Although most mycoplasmas are reported to have a smaller cell diameter than M. ovipneumoniae, it is important to note that on in vivo examination of sheep lung, smaller than average cells of M. ovipneumoniae would have the same cell diameter as larger than average cells

of other species. Hence, species could not be distinguished on the basis of size.

A second feature of the morphology of M. ovipneumoniae demonstrated in these studies, which has been reported for some but not all mycoplasmas, was the presence on the outer surface of the cell membrane of 12nm long projections, similar to those reported during studies of M. gallisepticum, M. pulmonis and T-strains (Black, Birch-Anderson and Freundt, 1972). However, it is possible that such material is present on all or most mycoplasmas, but the material is hard to resolve, and hence is dismissed (possibly correctly!) by some workers (Maniloff and Morowitz, 1972; Stanbridge, 1976) as adsorbed medium proteins. It has been postulated by other workers, however, that the projections are an integral part of the mycoplasmal cell: thus, Black et al. (1972) suggested that they could be reinforcing material for the delicate limiting membrane of the cell. A more likely explanation is that the projections are surface sites on the mycoplasma which can intereact with mammalian cells (Stanbridge, 1971; Black et al. 1972). Whitescarver and Furness (1975) have postulated that the projections (which they call pili) on T-strains have a pathogenic significance.

Replication:

A number of theories concerning the mode of replication of mycoplasmas have been postulated:

(a) liberation of "elementary bodies" following the rupture of large single cells (Klienberger - Nobel, 1962).

(b) fragmentation of filaments (Razin and Cosenza, 1966; Tanaka and Woods, 1970; Biberfield and Biberfield, 1970)

(c) budding of small bodies from single cells (Furness and De Maggio, 1973; Whitescarver and Furness, 1975)

(d) binary fission (Whitescarver and Furness, 1975; Robertson et al, 1976).

Postulate (a) suggests that in thin sections of a growing culture of M. ovipneumoniae, some large cells containing elementary bodies ready to be released, should

be observed. At no stage in the present study were bodies of this sort seen.

Replication by fragmentation of filaments has been observed by phase contrast microscopy for some mycoplasma species such as M. mycoides var mycoides (Razin and Cosenza, 1966) grown in liquid medium. In the present study, M. ovipneumoniae growing in liquid culture and observed by thin section electron microscopy demonstrated no features analogous to filaments.

Replication by budding is usually said to occur when disproportionate amounts of cytoplasm are divided between the two daughter cells. Hence, it may be difficult to distinguish between budding and binary fission as the amounts of cytoplasm divided between the two cells become more equal. In the electron micrographs of exponentially growing cells of M. ovipneumoniae, no dividing cells were seen in which the two potential daughter cells were of markedly different sizes.

It is now generally accepted, and has been shown by time lapse movies of single cells in exponential growth, that binary fission is the predominant mode of replication of mycoplasmas (Stanbridge, 1976). Cells of M. ovipneumoniae whose morphology was consistent with that of cells undergoing binary fission were observed in the present study (Fig. 7,20 and 21).

In summary, no morphological forms suggestive of replication by either rupture of large bodies or fragmentation of filaments were seen, whereas dumbell shaped forms consistent with organisms undergoing binary fission were commonly detected. It is concluded, therefore, that the most likely mode of replication of M. ovipneumoniae growing in the conditions described is by binary fission.

b) Cells in Aged Culture

In these studies, electron microscopy of cells in aged cultures was performed so that the morphology of dead and dying cells would be known. Hence, cells of this morphology would not be mistaken for living cells and included in a life cycle of M. ovipneumoniae. In many studies (Anderson and Barile, 1965; Nelson and Lyons, 1965) bizarre and aberrant forms of mycoplasma have been

observed in cultures of indeterminate age and life cycles have been postulated to accommodate these abnormal organisms.

In this study, however, the morphology of dead cells was not the abnormal pleomorphic type seen by many workers (Maniloff, 1970; Freundt, 1969). Instead it appeared that cells lost most of their electron staining material before lysis, then lysed. Similar observations were made by Rottem, Pfendt and Hayflick (1966) during thin section examination of T-strains. Although they do not report the stage of growth of the culture examined, they note that 'ghosts' could be obtained from cultures of pH 8.0, but not pH 7.3. According to Smith (1971) cultures of T-strains at pH 8.0 are non-viable. Rottem et al. (1966) attribute the appearance of the 'ghost' forms to the toxic effect of accumulation of ammonia produced in the medium by the cells themselves. Although T-strains and M. ovipneumoniae have fundamental differences in metabolism, it is probable that the cells typically seen in death phase cultures of M. ovipneumoniae are the result of the accumulation of toxic cell products which have a degenerative effect on the cell.

c) Electron Microscopy of Australian Strain of M. ovipneumoniae

M. ovipneumoniae was originally isolated from the respiratory tract of pneumonic sheep in Australia (St George et al., 1971). Three years later, Clarke et al. (1974) isolated a similar mycoplasma from the respiratory tract of pneumonic sheep in New Zealand.

Although it is difficult to establish that two organisms are identical, Clarke et al. (1974) showed that the New Zealand strain 5, and the Australian Y-98 biotype had the same colonial morphology, animal source and were serologically related as shown by gel precipitin tests. In the present study, electron microscropy of thin sectioned and negatively stained preparations of both New Zealand and Australian isolates was performed and it was found that the two organisms could not be distinguished. Thus morphology is one further property which the Y-98 isolate

and strain 5 have in common, which in turn supports the conclusion of Clarke *et al* (1974) that strain 5 represents a New Zealand isolate of *M. ovipneumoniae*.

3) BASE COMPOSITION:

The base composition of the DNA of M. ovipneumoniae was determined in this study by two independent methods; thermal denaturation and buoyant density in a CsCl gradient, and the GC contents estimated by these methods were 28.1% GC and 28.0% respectively. These values fall within the range reported for all mycoplasmas (23 to 40% GC) and also within the range 27 to 29% GC reported (before the species M. ovipneumoniae was recognised) for a strain of mycoplasma now believed to be a member of that species (see Leach et al., 1976).

Ocassionally cell wall defective mutants of bacteria (L-forms) can be isolated and confused with mycoplasmas, but their DNA retains the GC content of the parent organism and this can distinguish them from true mycoplasmas. Since the organism under study is an aerobe with a GC content of about 28% which is lower than that reported for any aerobic bacteria (Neimark, 1970); it can not be an L-form.

Given that similar base compositions are usually indicative of close relationships between organisms, it is interesting to compare the GC content of M. ovipneumoniae with those reported for other glycolytic mycoplasmas which have been found to have a pathogenic significance in the respiratory tract of domestic animals. This comparison is made in Table X.

TABLE X: Base compositions reported for those species of mycoplasmas known to have a pathogenic significance in the respiratory tract of domestic animals.

Species	Base Composition(%GC)	Reference
M. pneumoniae	39.9	Williams et al 1969
M. dispar	28.5	Buchanan and Gibbons, 1974
M. ovipneumoniae	28.0	
M. pulmonis	27.9	Williams et al 1969
M. hyorhinis	27.8	McGee et al., 1967

Species	Base composition(%GC)	Reference
M. mycoides var capri	27.6	Neimark, 1970
M. mycoides var mycoides	26.8	Neimark, 1967

M. pneumoniae has been shown to be an unusual mycoplasma in that its DNA has a base composition of 40%GC whereas that of all other mycoplasmas are included in the range 23-35%GC (Neimark, 1970).

It is interesting to note, that apart from M. pneumoniae the base composition of the DNA of the other mycoplasmas pathogenic for the respiratory tract and M. ovipneumoniae fall within the narrower range 26.8 to 28.5%GC.

Taking into account this similarity in base composition, and the fact that all the above mycoplasmas are glycolytic, it can be speculated that mycoplasmas pathogenic for the respiratory tract of animals form a relatively homogeneous sub-group within the Mycoplasmatales.

APPENDIX :

Karnovsky's fixative (modified)

$Na_2HPO_4.\ 12H_2O$	12.55g
KH_2PO_4	2.05g
paraformaldehyde	10.0g
gluteraldehyde (25% solution)	60ml

The paraformaldehyde (10.0g in 420ml distilled water) is heated to 60 to 70°, then 1.0N NaOH is added dropwise until the solution clears. The buffer salts and gluteraldehyde are then added.

Store at 4°.

Spurr's Resin

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

Araldite Resin

Epoxy resin	CY 212	6.0ml
Hardener	HY 964	6.5ml
Accelerator	DY 064	0.19ml

The components were thoroughly stirred and the mixture was then heated at 60° to remove air bubbles.

Osmium tetroxide

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

Uranyl acetate

The stain was made by adding uranyl acetate crystals to 50% ethanol until saturation point was reached, then shaking intermittently for 5 to 15 minutes.

0.1M Phosphate buffer pH 7.2

$Na_2HPO_4.12H_2O$	2.51g
KH_2PO_4	0.41g
Distilled water	100.0ml

Lead citrate

$Pb(NO_3)_2$	1.33g
Trisodium citrate	1.76g
Distilled water	30ml, then 50ml

The ingredients were shaken for 30min. The solution was cleared by the addition of 8ml 1.0N NaOH. Dilution was made to give a final volume of 50ml which was filtered and stored at 4°.

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