# STUDIES ON MYCOPLASMAS OF THE RESPIRATORY TRACT OF SHEEP

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## DECLARATION

The work embodied in this thesis was part of a larger, multi-disciplinary project concerned with investigations into respiratory diseases of sheep, and many of the findings were obtained in collaboration with my colleagues at the Moredun Institute. However, most of the work presented, the initiation of all aspects, the interpretation of results and composition of the thesis were performed by myself.

CONTENTS

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Finally/

Finally, I wish to dedicate this thesis to my wife and family, without whose prompting, understanding and sacrifice it might never have been undertaken or completed.

## SUMMARY

The rôles of mycoplasmas in pneumonias of sheep were investigated by survey and by pathogenicity experiments in specific pathogen free (SPF) and conventionally reared lambs.

Glycolytic mycoplasmas isolated from sheep in Scotland were found, by comparison with the Queensland Y98 strain using biochemical and serological tests, to be classifiable as Mycoplasma ovipneumoniae. Two serological tests indicated considerable intraspecific differences among strains. The ultrastructural morphology of M. ovipneumoniae was found to be typical of mycoplasmas.

M. ovipneumoniae and Mycoplasma arginini were the most commonly recovered mycoplasmas in a survey of housed sheep. Ureaplasmas, hitherto not reported to occur in the ovine respiratory tract, were also isolated. M. ovipneumoniae was recovered from the lungs of most lambs necropsied: its presence with Pasteurella haemolytica was consistently associated with proliferative exudative (P.E.) pneumonia, the incidence of which increased with age. M. arginini recoveries were not correlated with any specific histopathological changes.

Endobronchial inoculation with M. ovipneumoniae induced lung changes in only a proportion of SPF lambs, although the lungs of almost all animals became colonised. Lesions were similar to but milder than respiratory mycoplasmoses of other species of animals. In-contact transmission produced nasal but not pulmonary infection, and no lung lesions. Attempts to enhance the pathogenic

effects of M. ovipneumoniae by sensitization through prior infection were unsuccessful.

Endobronchial inoculation of SPF lambs with M. arginini produced neither lung colonisation nor lesions, although the upper respiratory tract became infected.

The effects of endobronchial administration of lung homogenates, which were prepared from naturally-occurring P.E. pneumonia and which contained principally M. ovipneumoniae and P. haemolytica, were simulated by mixed cloned cultures of M. ovipneumoniae, M. arginini and P. haemolytica administered to conventionally reared sheep by the same route. Clinical signs and P.E. pneumonia were produced in almost all animals. M. ovipneumoniae was the only consistently recovered organism. P. haemolytica did not establish in the lungs of sheep unless combined with M. ovipneumoniae. This combination produced lesions of P.E. pneumonia. However, P.E. pneumonia was also observed in some animals inoculated with M. ovipneumoniae alone.

## ABBREVIATIONS USED IN TEXT

| | |
|---|---|
| 1A | Agar medium used initially for the cultivation of non-glycolytic mycoplasmas |
| AB | Arginine-supplemented broth used for the cultivation of arginine-utilizing mycoplasmas |
| AB(-) | AB without ampicillin and thallous acetate |
| AGDD | Agar gel double diffusion |
| a.p.d. | Average pore diameter |
| Ba | 7% sheep blood agar |
| BSS | Hanks Balanced Salt Solution |
| CAM | Chorio-allantoic membrane |
| CBPP | Contagious bovine pleuropneumonia |
| CCPP | Contagious caprine pleuropneumonia |
| ccu | Colour changing units |
| Ch 3, Ch 4 | Broth cultures of M. ovipneumoniae strain 5759 (Experiments 3 and 4) |
| cfu | Colony forming units |
| CLS | Consolidated lung lesion score |
| CTC | Control tissue culture (Experiment 3) |
| d.o. | Days old |
| d.p.e. | Days post exposure |
| d.p.i. | Days post infection |
| d.w. | Distilled water |
| EA, EB | Media similar to OA, OB, but incorporating Eagles MEM instead of Medium 199 (Chapter 3) |
| e.b. | Endobronchial |
| GI | Growth inhibition |
| h | Hours |

| | |
|---|---|
| HAI | Haemagglutination inhibition |
| HEPES | N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid buffer |
| Ig | Immunoglobulin |
| IHA | Indirect haemagglutination |
| i.m. | Intramuscular |
| i.n. | Intranasal |
| i.p. | Intraperitoneal |
| i.t. | Intratracheal |
| ITC | Infective tissue culture (Experiment 3) |
| i.v. | Intravenous |
| LH | Lung homogenate (Experiment 4) |
| LRT | Lower respiratory tract |
| LS | Lung homogenate suspension (Experiments 6 and 7) |
| MEM | Eagles Minimal Essential Medium |
| MI | Metabolism inhibition |
| min | Minutes |
| m.o. | Months old |
| MS | Microorganism suspension (Experiment 7) |
| MTM | Mycoplasma transport medium |
| NB2 | Nutrient broth |
| OA, OB | Agar and broth media used for the cultivation of glycolytic mycoplasmas |
| OB(-) | OB without ampicillin and thallous acetate |
| orgs | Organisms |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline (pH 7.2) |

| | |
|---|---|
| P.E. | Proliferative exudative (pneumonia) |
| PI3 | Parainfluenza virus type 3 |
| PM | Partial medium (Chapter 3) |
| RBC | Red blood cells |
| RBG, RBA | Media based on OB and AB respectively, but with rabbit serum and rabbit meat broth replacing swine serum and brain-heart infusion (Chapter 3) |
| RH | Relative humidity |
| SC | Seed culture of M. ovipneumoniae, strain 5759 (Experiments 3 and 4) |
| s.c. | Subcutaneous |
| Sen 4 | Broth culture of M. ovipneumoniae strain 5759 (Experiment 4) |
| SPA | Sheep pulmonary adenomatosis |
| SPF | Specific pathogen free |
| SPS | Sodium polyanethol sulfonate |
| TA, TB | Media used for the cultivation of ureaplasmas |
| TCM | Tissue culture medium (Experiment 3) |
| TSM | Medium used for the determination of specific substrate requirement (Chapter 3) |
| v | Volume |
| w | Weight |
| w.o. | Weeks old |
| w.p.i. | Weeks post inoculation |

# CHAPTER 1

## REVIEW OF THE LITERATURE

### INTRODUCTION

The common respiratory diseases of sheep in the United Kingdom are parasitic pneumonia, sheep pulmonary adenomatosis (SPA) or jaagsiekte, "enzootic" pneumonia and "atypical" pneumonia (Poynter and Selway, 1966; Stamp and Nisbet, 1963). The damage caused by lungworms may possibly predispose to opportunist microbial invasion, but in these circumstances pulmonary presence of mycoplasmas or bacteria is coincidental or secondary to infection with the helminths. The aetiology of SPA, although presently in doubt, almost certainly does not involve mycoplasmas as primary agents (Mackay and Nisbet, 1966; Krauss and Wandera, 1970; Wandera, 1971; Perk, Hod and Nobel, 1971; Perk and Hod, 1971), despite the several reports of mycoplasma recovery from cases of SPA (Nobel, 1958; Mackay, Nisbet and Foggie, 1963; Deiana and Ceretto, 1967; Krauss and Wandera, 1970). The aetiologies of enzootic and atypical pneumonia have not yet been fully elucidated but may be multiple and possess factors in common, including the involvement of mycoplasmas. Part 1 of the literature review is thus restricted to clinico-pathological descriptions of enzootic and atypical pneumonia, and a consideration of the microorganisms, excluding mycoplasmas, which may be implicated in these two diseases. Part 2 of the review deals with the reports of mycoplasma isolations from sheep.

## PART 1 ENZOOTIC AND ATYPICAL PNEUMONIAS OF SHEEP - THE DISEASES AND ASSOCIATED BACTERIA AND VIRUSES

Reviews of the pulmonary diseases of sheep have been made by Stevens (1957), Shirlaw (1959), Stamp and Nisbet (1963), Dungworth (1963), Stevenson (1968, 1969), Hore (1968, 1970), Manktelow (1970), Nayil (1971), St. George and Sullivan (1973) and Jensen (1974).

### ENZOOTIC PNEUMONIA (Synonyms: acute necrotizing or exudative pneumonia, shipping fever, pasteurellosis).

#### Epidemiology and clinical signs

All ages of sheep may be affected; the highest incidence is stated by Jensen (1974) to be in lambs of 5-7 months old (m.o.) although the outbreaks observed by Montgomerie, Bosworth and Glover (1938) involved mainly adult animals. Sudden deaths with little or no prodromal symptoms often occur, but coughing, fever, nasal discharge, dyspnoea, diarrhoea and depression with subsequent unthriftiness of affected lambs and reduced milk production in ewes may be observed in animals which survive for longer periods (O'Gairbhidhe, 1965; Harris, 1974).

#### Necropsy findings

Consolidated lesions are generally found in the apical, cardiac and anterior portions of the diaphragmatic lobes and the lobar and lobular septa are prominent and opalescent. In acute cases, the pleurae may be thickened or fibrinous, with frequent development of pleural adhesions. The thorax contains a straw-coloured fluid, and petechiae and echymoses are found over the

serosal surfaces, particularly of the heart and lungs. The involvement of joints is reported in the U.S.A. (Jensen, 1974), and some changes may also be found in the liver and kidneys (e.g. Montgomerie et al., 1938).

The histopathological changes in the lungs vary from area to area according to the stage of inflammatory process. The alveoli of consolidated areas contain numerous elongated ("oat-shaped") cells within a structureless exudate, and extensive necrotic areas are often present. The alveoli surrounding the main lesions contain large macrophages. Small focal accumulations of neutrophils are occasionally present.

## ATYPICAL PNEUMONIA

### Epidemiology and clinical signs

Stamp and Nisbet (1963) reported this disease as being a frequent cause of loss in young housed sheep but Gilmour and Brotherston (1963) found that the disease per se caused little appreciable change in the condition of the animal in the absence of secondary bacterial invasion. Clinical symptoms are mild and may easily be missed: fever, coughing, tachypnoea and abnormal auscultatory sounds may be present, but dullness, inappetance and dyspnoea are not observed (Gilmour and Brotherston, 1963).

### Necropsy findings

Dark red, pink or grey areas of consolidation or narrow bands of collapse are generally found in the apical, cardiac and,

occasionally, in the anterior borders of the diaphragmatic lobes. The cut surface of the lesions may show the air passages to be surrounded by grey-coloured "cuffs". Pleural adhesions are often present.

Stamp and Nisbet (1963) described the occurrence of two types of histological lesion. Lymphoid hyperplasia, in which the bronchi and bronchioles are surrounded by lymphoid cell cuffs, is mainly confined to the apical lobes. Lymphocytes, plasma cells and reticulo-endothelial cells are present, and surrounding alveoli contain limited numbers of macrophages. Uncomplicated lesions of this description were found to be relatively rare.

Interstitial pneumonia was found in about 90% of lungs. In early lesions there is infiltration of alveolar septa with lymphocytes and macrophages, which leads to partial obliteration of alveolar spaces. Bronchial and bronchiolar damage consisting of fibrous tissue ingrowths were invariably found in the cases examined by Stamp and Nisbet (1963), but Stevenson (1969) found this to be an inconstant feature in cases examined by him over a four year period. The air passages show varying degrees of obstruction and epithelial destruction, and surrounding alveoli are filled with large macrophages. As the disease progresses, macrophage numbers increase and hyaline or myxomatous scars appear in the walls of many bronchi and bronchioles. Peribronchiolar and perivascular lymphocytic cuffing was also a constant feature in advanced lesions examined by Stamp and Nisbet, although Stevenson is less definite concerning this point. Focal alveolar

epithelialization occurs, particularly around affected bronchi and bronchioles, and extensive interstitial fibrosis of epithelialized areas occasionally follows.

## THE BACTERIA AND VIRUSES POSSIBLY IMPLICATED IN ENZOOTIC AND ATYPICAL PNEUMONIAS OF SHEEP

The microorganisms associated with sheep pneumonias have been reviewed by Stevenson (1968, 1969), Dungworth (1963) and St. George and Sullivan (1973).

### Bacteria

The bacteria considered to be primary lung pathogens of sheep are *Pasteurella* spp. *Mycobacterium* spp., *Pseudomonas pseudomallei* (Smith, cited by Stevenson, 1969) and *Streptococcus zooepidemicus* (Stevenson, 1974), of which all but the pasteurellae cause a specific sporadic pneumonia and need not be considered further.

#### *Pasteurella* spp.

Two species, *Pasteurella multocida* and *Pasteurella haemolytica* may be involved in pneumonic conditions of sheep (Hudson, 1959).

#### *P. multocida*

There are no confirmed reports of the isolation of this organism from sheep in the United Kingdom, though strains similar to or identified as *P. multocida* have been recovered from sheep in several other countries (e.g. Hudson, 1959).

P. haemolytica

The literature relating to P. haemolytica has recently been reviewed by Thompson (1973). P. haemolytica comprises two biotypes (Smith, 1959, 1961), of which biotype A (arabinose fermenters) has been shown to comprise nine serotypes, viz. 1,2,5,6,7,8,9,11 and 12 and biotype T (trehalose fermenters) three serotypes, viz. 3,4 and 10 (Biberstein and Gills, 1962; Biberstein and Thompson, 1966). There are, in addition, untypable strains in which the specific soluble polysaccharide antigens found in the classifiable strains are deficient or non-existent (Aarsleff, Biberstein, Shreeve and Thompson, 1970). Smith (1959, 1961) noted that biotype A strains were associated with enzootic pneumonia of lambs and sheep, and were almost always the cause of a septicaemia of lambs occurring within the first few weeks of life. In contrast, biotype T strains were associated with a septicaemic disease of older lambs. Stamp and Nisbet (1963) also noted a frequent but not invariable association of P. haemolytica with atypical pneumonia, and Downey (1957) made the same observation regarding chronic lung lesions in lambs at slaughter.

Biotype A strains: Field and abattoir surveys have shown that normal sheep may harbour P. haemolytica biotype A strains in the nasal cavity and tonsillar tissue; the incidence of carriers shows a seasonal fluctuation which is unrelated to health status (Biberstein, Shreeve and Thompson, 1970; Gilmour,

Thompson and Fraser, 1974). In outbreaks of pneumonia, the incidence of nasal carriers rises and a narrowing of the serotype spectrum within the flock occurs (Biberstein and Thompson, 1966; Shreeve, Biberstein and Thompson, 1972).

Experimental pathogenicity studies involving inoculation of P. haemolytica biotype A strains into sheep and lambs by the respiratory route have shown that lesions typical of those observed in natural cases of enzootic pneumonia can be produced, but the dose required is generally of the order of $10^8$ organisms or greater (Table 1.1). Only by the administration of aerosols of P. haemolytica to specific pathogen free (SPF) lambs have lower doses been successful in producing pulmonary lesions and death (Gilmour, Thompson, Smith and Angus, 1975). Biberstein, Nisbet and Thompson (1967) were also able to produce lung lesions similar to enzootic pneumonia by the intratracheal (i.t.) inoculation of large doses of Staphylococcus aureus. These authors observed that "the most impressive feature of the attempts to produce pneumonic changes by means of bacterial infection was the phenomenal tolerance displayed by the ovine lung to the introduction of enormous numbers of bacteria by the most rigorous exposure techniques". In contrast, the intraperitoneal (i.p.) or intravenous (i.v.) inoculation of even low doses of biotype A strains caused death of young lambs, although young adult sheep were highly resistant to challenge by the same methods (Smith, 1960a).

Biotype T strains: Surveys have shown that 2.6-4.0% of normal sheep carry biotype T strains in the nasal cavity (Biberstein and Thompson, 1966; Gilmour et al., 1974),

Table 1.1 Experimental Infection of Sheep with *P. haemolytica* Biotype A by the Respiratory Route

| Author(s) | Experimental animals* | Dose (total count, expressed as powers of $\log_{10}$) | Route | Proportion of animals dying or with lung lesions |
|---|---|---|---|---|
| Smith, G.R. (1960) | Lambs | 8.8 | i.t. | 1/1 |
| " | " | 10.1-10.4 | " | 2/2 |
| Smith, G.R. (1964) | Adult | 10.8-11.25 | " | 6/6 |
| " | " | 9.9 | " | 5/6 |
| " | " | 8.9 | " | 2/4 |
| " | " | 7.9 | " | 0/4 |
| Biberstein *et al.* (1967) | " | 9.8 | " | 2/3 |
| Biberstein *et al.* (1971) | Lambs | 8.7 - 9.6 | " | 6/9 |
| Biberstein and Thompson (1966) | Adult | 9.5 | n.p.s. | 0/2 |
| | " | -** | aer. | 0/2 |
| Gilmour *et al.* (1975) | SPF Lambs | 4.8 | aer. | 5/10*** |

i.t. - intratracheal
n.p.s. - nasopharyngeal swabbing
aer. - aerosol
* - conventionally-reared unless otherwise stated
** - animals exposed for 15 min to 25 ml of culture containing $10^{9.5}$ orgs/ml
*** - figures derived from unvaccinated group only: 4 of these were treated with hog gastric mucin 5 d after aerosolization

but that 61% harbour these strains in tonsillar tissue (Gilmour et al., 1974). All cases of septicaemia in lambs over 3 m.o. yield biotype T strains, which also comprise 22% of pasteurellae recovered from pneumonic lungs (Biberstein and Thompson, 1966).

The natural septicaemic disease of older lambs with which biotype T strains are associated has been reproduced by the i.v. inoculation of cultures and lung homogenates containing the organism (Stamp, Watt and Thomlinson, 1955 (see Smith, 1959, 1960b); Smith, 1960b). However, doses in excess of $10^9$ organisms were required to produce an effect, and the lethal dose of a heat-killed broth culture was only a low multiple of that of a living culture. The i.p. inoculation of graded doses produced death in only one of a pair of lambs receiving the highest dose administered of $14 \times 10^7$ organisms (Smith, 1960a). The i.t. inoculation by Stamp et al. (1955) of lung suspensions or plate culture washings produced no effect in sheep, but Biberstein and Thompson (1966) produced pneumonia in four of 16 sheep by the i.t. inoculation of $1525\text{-}12{,}000 \times 10^6$ organisms; the histopathology of the lesions was indistinguishable from that caused by biotype A strains.

Chlamydiae

General reviews of chlamydiae and chlamydiosis have been made by Page (1966, 1968), Meyer (1967), Tamarin (1969), Storz (1971) and Schachter, Storz, Tarizzo and Bögel (1973), and Stevenson (1968, 1969) has reviewed the specific association of chlamydiae with ovine pneumonias.

The present classification of the order Chlamydiales (Page, 1974) provides one family, the Chlamydiaceae, which contains one genus, the Chlamydia. Two species are recognised, Chlamydia trachomatis and Chlamydia psittaci, of which the latter may be involved in pneumonitis, polyarthritis, placentitis, conjunctivitis, meningitis and intestinal infections of sheep and other animals.

Serological differences have been demonstrated among pneumonitis, arthritis and abortion strains of ovine origin, and serological homogeneity found between abortion and faecal strains, between polyarthritis and conjunctivitis strains and between faecal and pneumonitis strains (Frazer and Berman, 1965; Wilson, 1966; Schachter, Banks, Sugg, Sung, Storz and Meyer, 1974).

Several sero-epidemiological surveys for chlamydia antibodies in sheep have been performed (e.g. Page and Erickson, 1969; Estola and Salmela, 1970; Krauss, Wandera and Lauerman, 1971; Neuvonen and Estola, 1974), but as the tests presently available are sensitive only on the group or species-specific level, the involvement of these organisms in specific disease entities such as pneumonia cannot be inferred from positive reactions. In one large-scale microbiological survey (Ungureanu, Popovici, Hiastru, Sirbu, Fromunda and Cirstet, 1972) 10% of 2708 lungs from slaughter and pneumonic sheep yielded chlamydiae.

Storz (1971) considered that the epidemiology of chlamydial pneumonias in sheep (and cattle) "is closely interdependent with the widespread, clinically inapparent intestinal infections with

chlamydia in these animals." Under adverse conditions such as the stress of shipping, adjustment to a new environment or weaning, the latent chlamydial infections give rise to an active infection which results in pneumonia.

Pathogenicity studies involving the i.t. inoculation of ovine lung strains of chlamydia have generally shown that a clinically mild but extensive pneumonia is produced which is histopathologically similar to atypical pneumonia, and which is maximal in effect at 4-23 days post inoculation (d.p.i.) (McKercher, 1952; Boidin, Cordy and Adler, 1958; Dungworth and Cordy, 1962; Stevenson and Robinson, 1970). The negative results obtained in the experiments performed by Hamdy and Pounden (1959) may have been due to delay in the slaughter of the lambs until three weeks post inoculation (w.p.i.) (Dungworth and Cordy, 1962).

Pneumonitis of an identical form, though sometimes of shorter duration than that caused by ovine lung strains, has been produced by the i.t. inoculation into sheep of ovine abortion and faecal strains (Dungworth and Cordy, 1962), bovine (Romvary, 1963), caprine (Omori, Ishii, Harada, Ischikawa, Murase, Katada and Araumi, 1953) and turkey strains (Pierce, Moore, Carroll and Bridges, 1963). Storz (1971) states that "the interrelationships among chlamydial agents that cause different diseases in one animal species are not well understood, nor are there satisfactory answers to questions of host specificity." However, Storz (1966) has also suggested that a given antigenic make-up is associated with a specific pathogenicity, at least under natural conditions.

## Viruses

### Myxovirus

The isolation from sheep in Hungary of Shope (Influenza A) virus (Romvary, Takatsy, Barb and Farkas, 1962; Barb, Farkas, Romvary and Takatsy, 1962) has been reported. The experimental i.t. inoculation of lambs with Influenza A virus produced clinical symptoms with macroscopic and/or microscopic pulmonary lesions. The virus was recovered at 7 d.p.i.

### Paramyxovirus

Parainfluenza 3 (PI3) virus has been isolated from sheep in Scotland (Hore, 1966), Canada (Ditchfield, 1966), Australia (St. George, 1969), New Zealand (Carter and Hunter, 1970), France (Giauffret and Russo, 1972, 1974) and Hungary (Belak and Palfi, 1974a).

Serological surveys have indicated that a high proportion of sheep possess antibodies to PI3 virus. Hore (1969) found 53% and St. George (1971) 76.5% of sheep were serologically positive for the virus.

The experimental inoculation of PI3 virus into lambs (Hore and Stevenson, 1967, 1969) and a ewe (St. George, 1969) produced a mild clinical pneumonia with the development of consolidated lesions. Two lambs placed in-contact with the ewe infected by St. George (1969) showed mild clinical symptoms and virus was recovered from one, but only microscopic lesions of pneumonia were found at necropsy. Bovine strains of PI3 virus have also produced pneumonic lesions in lambs (Woods, Sibinovic and Marquis, 1965; Stevenson, 1968).

However, whether PI3 virus is the cause of pneumonic conditions of sheep under natural conditions remains doubtful. In a field experiment involving the microbiological and serological monitoring of ewes and lambs over an 11 months period, PI3 virus infection occurred which was detected serologically but not clinically, and which was unconnected with two outbreaks of pneumonia within the flock (St. George and Kiefman, 1972).

Adenovirus

Adenovirus types 1, 2 and 3 have been isolated in Northern Ireland from sheep faeces (McFerran, Nelson, McCracken and Ross, 1969; MacFerran, Nelson and Knox, 1971) and subsequent recoveries have been reported from Australia (Snowdon, 1970) and Hungary (Belak and Palfi, 1974b), where the isolations were made from the nasal cavities of sheep with respiratory and intestinal disease. A fourth adenovirus type has been isolated in Scotland from ovine faecal swabs (Sharp, McFerran and Rae, 1974). Preliminary observations indicated to McFerran et al. (1969) that adenovirus may have a rôle in the aetiology of respiratory and intestinal conditions of sheep, but this remains unsubstantiated at present.

Reovirus

The isolations from sheep faeces in Northern Ireland of reovirus subsequently identified as type 3 (McFerran et al. 1969; McFerran, Nelson and Clarke, 1973) have been followed by the recovery of reovirus type 1 from sheep with pneumonia and enteritis in Hungary (Belak and Palfi, 1974c). The field disease was

reproduced experimentally by the Hungarian workers (Belak and Palfi, 1974d), but McFerran, Baskerville and Nelson (1974) were unable to demonstrate any pathogenic effect with type 3 virus in lambs.

Respiratory syncytial virus

At present, serological evidence only is available for the infection of sheep with this virus. One survey showed that 81% of 31 sheep sera tested were positive (Berthiaume, Joncas, Boulay and Pavilanis, 1973).

## PATHOGENICITY STUDIES INVOLVING COMBINATIONS OF RESPIRATORY ORGANISMS

The pathogenic effects of chlamydia have been found by Boidin et al. (1958) and Dungworth and Cordy (1962) to be unaltered by the incidental presence or concurrent inoculation of mycoplasmas and/or pasteurellas, or by the application of "stress". In experiments which involved combinations of chlamydia with A or T biotypes of P. haemolytica, Biberstein et al. (1967) found that lesions characteristic of both agents were produced without any obvious synergism, although clinical and pathological responses in those animals inoculated with both agents was greater than in those receiving P. haemolytica alone.

In contrast, Hamdy and Pounden (1959), using P. multocida, an unidentified mycoplasma and a chlamydia strain, consistently produced pneumonia only in those animals inoculated with pasteurella and mycoplasma and subjected to "stress", and those subjected to these three factors combined with chlamydia. Inoculation of the three microorganisms without the application of stress produced

pneumonia in two of four lambs. Two forms of stressing were applied, viz. the subjection to rapid environmental temperature changes, and the injection of cortisone acetate.

Pathogenicity experiments which involved a combination of P. haemolytica biotype A strains and PI3 virus failed to show evidence of synergism between the two agents, although clinical and pathological responses were greater in animals inoculated with both organisms than in those given P. haemolytica alone: virus alone produced negligible effects (Biberstein, Shreeve, Angus and Thompson, 1971).

The experimental reproduction of enzootic pneumonia by the inoculation of sheep with suspensions of affected lungs has been attempted by several workers. Montgomerie et al. (1938) were unable to produce any effects by the inoculation of sheep with cultures of pasteurellae isolated from cases of enzootic pneumonia, but the i.v. and intra-pulmonary and/or i.t. administration of lung lesion suspensions caused severe clinical and pathological changes in four of six sheep. Similar effects were obtained in one of two sheep by the intranasal (i.n.) and i.t. inoculation of a culture of pasteurella together with suspensions of lungs from mice previously inoculated with sheep lung lesion material. Animals inoculated with mouse lung suspensions only showed no response. Downey (1957) reproduced enzootic pneumonia-like symptoms in one of 12 adult sheep by the i.v. and i.t. inoculation of lung lesion suspensions obtained from field and experimentally-produced cases of enzootic pneumonia. In addition, the i.v. and i.t. administration of an inoculum comprising chorio-allantoic membranes (CAM) from eggs

inoculated with lung suspensions combined with cultures of P. haemolytica caused rapid death and the development of lesions indistinguishable from those of enzootic pneumonia in one of 12 adult sheep. No effects were observed following inoculation of CAM or P. haemolytica cultures alone. Salisbury (1957) was unable to produce any effects in sheep by the inoculation of either cultures of a haemolytic pasteurella, macerated lung lesions or lung filtrate together with cultures of pasteurella. Montgomerie et al. (1938), Downey (1957) and Salisbury (1957) all considered that a virus might be implicated in the aetiology of enzootic pneumonia, particularly as a predisposing factor in the establishment of pasteurellosis. Boidin et al. (1958), in one of several experiments, prepared from a case of acute pneumonia a suspension of lung lesions from which mycoplasmas and pasteurellae were recovered. Half the suspension was treated with penicillin, and the treated and untreated suspensions were each given by i.t. inoculation into one lamb. Both lambs subsequently developed clinical signs of respiratory disease and showed lung consolidation and fibrinous pleurisy at autopsy. Mycoplasmas and pasteurellae were recovered from several organs including lungs of both animals.

## PART 2 THE MYCOPLASMAS OF SHEEP, WITH PARTICULAR REFERENCE TO THOSE ISOLATED FROM THE RESPIRATORY TRACT

Major developments in the field of ovine respiratory mycoplasmosis occurred during the period of these studies; this review will therefore be restricted to reports published before December 1972. The recoveries of mycoplasmas from sheep have been reviewed by Cottew and Leach (1969) and Nayil (1971).

Part of the outcome from the increased interest shown in the Mycoplasmatales in recent years has been the recognition of a much larger number of species within the Order, with a consequent expansion of the taxonomic structure. The classification of the Mycoplasmatales proposed by Edward and Freundt in 1956 listed one family, one genus and 15 species, while two families containing three genera and 44 species are listed by Freundt in the Eighth Edition of Bergey's Manual of Determinative Bacteriology (1974). Table 1.2 gives the presently accepted classification of the Mycoplasmatales according to Wittler (1973) and Freundt (1974), but incorporating an amendment to the classification of "T-mycoplasmas" following the recent proposal that these be recognised as a distinct genus within the family Mycoplasmataceae, with the generic name "Ureaplasma" (Shepard, Lunceford, Ford, Purcell, Taylor-Robinson, Razin, and Black, 1974).

The history of mycoplasma isolations from sheep reflects the advances made in mycoplasmology as a whole. By 1972, six mycoplasma species had been identified as occurring in sheep, namely Mycoplasma agalactiae subsp. agalactiae, Mycoplasma arginini, Mycoplasma ovipneumoniae, Acholeplasma laidlawii,

Table 1.2 The Taxonomy of Mycoplasmas

| Taxonomic Rank | Characters used to Circumscribe Taxa |
|---|---|
| Plant Kingdom | |
| Division I: Protophyta Sachs, 1874, Emend. Krassilnikov, 1949 | Primitive plants |
| Class IV: Mollicutes Edward and Freundt, 1967 | Procaryotic organisms bounded by a triple-layered membrane. Cells small (may be 125 nm). Highly pleomorphic - coccoid to filamentous. Gram negative. May be grown on artificial media, and may be saprophytic, parasitic or pathogenic. |
| Order I: Mycoplasmatales Freundt, 1955 | As for Class. |
| Family I: Mycoplasmataceae Freundt, 1955 | Sterol required for growth. |
| Genus I: Mycoplasma Nowak, 1929, 1949 Nom. cons. Jud. Comm. Opin. 22, 1958 | Most species utilise either glucose or arginine for energy, rarely both. Urea is not hydrolyzed. Growth generally (with some exceptions) inhibited by digitonin and 5% sodium polyanethol sulfonate (SPS). Temperature range 22-41°C, optimum 36-37°C. G + C content of DNA ranges from 23-40 moles %. Genome size $4.5 \times 10^8$ daltons. |
| Genus II: Ureaplasma Shepard, Lunceford, Ford, Purcell, Taylor-Robinson, Razin and Black, 1974. | Hydrolyze urea but do not ferment carbohydrates. Sensitive to digitonin and SPS. Optimal growth at pH $6.0 \pm 0.5$. Thallium acetate, erythromycin, 5-iodo-2-deoxyuridine, hydroxyurea and hydroxyamic acid are inhibitory to growth. G + C content of DNA ranges from 27.7 - 28.5 moles %. Genome size $4.5 \times 10^8$ daltons. |

Table 1.2 (cont'd)

| Taxonomic Rank | Characters used to Circumscribe Taxa |
|---|---|
| Family II: Acholeplasmataceae Edward and Freundt, 1970 | Sterols not required for growth. |
| Genus I: Acholeplasma | Arginine and urea not hydrolyzed. Growth not inhibited by digitonin or SPS. Temperature range 20-40°C. G + C content of DNA ranges from 30-33 moles %. Genome size 1.0 x $10^9$ daltons. |
| Genera of Uncertain Affiliation | |
| A. Spiroplasma Saglio, Lhospital, Lafleche, Dupont, Bove, Tully and Freundt, 1973. | Cholesterol or possibly other sterols required for growth. Acid produced from glucose and mannose. Centred colonies formed on solid media. Optimum temperature 32°C. Helical morphology. Genome size 1.0 x $10^9$ daltons. |
| B. Anaeroplasma Robinson and Allison, 1975. Robinson, Allison and Hartman, 1975. | Some strains require sterol, some do not. Some strains resistant to digitonin, others sensitive. Strict anaerobes. |
| C. Thermoplasma Darland, Brock, Samsonoff and Conti, 1970. | Do not require sterol. Optimum temperature for growth about 59°C, optimum pH 1 - 2 Genome size 1.0 x $10^9$ daltons. |
| Species (44 spp. in 1974) | Biochemical activities, metabolic products, respiratory pathways, enzyme systems, serological reactions. |
| Subspecies. | Chemical composition, electrophoretic patterns of cell proteins and homologies of nucleic acids. |

Mycoplasma mycoides subsp. mycoides and Mycoplasma conjunctivae. With the exception of M. agalactiae subsp. agalactiae, however, no mycoplasmas isolated from sheep before 1968 had been positively identified as recognised species, or characterised and named as new species. Thus the majority of reports concerning sheep mycoplasmas can be assessed only in isolation, or from inference of the identity of the mycoplasmas recovered by comparison with recognised species. Where possible, such comparisons have been based on the biochemical and morphological characteristics of the organisms, since these are simple and easily definable criteria. The alternative, comparison and categorisation of mycoplasmas based on the clinico-pathological descriptions of the associated disease, has been avoided where possible, since in many cases such descriptions have been inadequate: the diseases have lacked pathognomic features and may have stemmed from multiple aetiologies, and experimental evidence of pathogenicity of the isolated mycoplasmas has been questionable or non-existent. Although SPA is a well recognised and documented disease entitity (Wandera, 1971), the aetiology of the disease almost certainly does not implicate mycoplasmas. It may be supposed, therefore, that the species of mycoplasmas recovered from SPA cases are those which are commonly found in sheep populations and which are capable of secondary invasion of diseased lungs: for this reason they are considered as separate entities.

M. agalactiae subsp. agalactiae (Wroblewski) Freundt 1955

The investigations of workers in Turkey (Arisoy, Erdag, Cottew and Watson, 1967; Cottew, Watson, Arisoy, Erdag and

Buckley, 1968; Watson, Cottew, Erdag and Arisoy, 1968) showed that a poorly fermentative mycoplasma, initially termed "Type A" and later identified as M. agalactiae subsp. agalactiae, could be recovered from flocks of sheep and goats where contagious agalactia was present, but not from normal flocks. The organism was isolated from udder, joints, eyes and occasionally nasal cavity of animals. The subcutaneous (s.c.) inoculation of broth cultures of several strains into a total of 13 sheep and 64 goats produced mastitis, arthritis and, rarely, keratitis in both species. At necropsy, M. agalactiae subsp. agalactiae was recovered from several organs including lungs, but no pulmonary lesions were observed. Investigation of the pathogenicity of identified strains of M. agalactiae subsp. agalactiae by direct inoculation into the respiratory tract does not appear to have been attempted, but Cottew and Lloyd (1965) found that mycoplasmas biochemically and serologically related to M. agalactiae subsp. agalactiae induced small lung lesions in one of two sheep and one of 13 goats challenged by i.t. inoculation. These minor effects suggest that the presence of the organism in the nasal cavity of some animals in the Turkish survey was due more to secondary colonisation from infected eyes than to a predilection for this site.

Three strains, which produced little change in pH in glucose broth and were referred to as "atypical" because of this property, were isolated from lung lesions and pleural fluid by Cottew (1971a). Two of the strains were serologically related to Cottew's "Type 1" strains (see M. arginini), but the third strain did not react with antisera against either "Type 1" or "Type 2" strains (see M.

ovipneumoniae). The relationship of the three strains to other poorly fermentative species of mycoplasma was not investigated, although the isolation of organisms closely similar to M. agalactiae from sheep and goats in Australia has been reported (Cottew and Lloyd, 1965; Cottew, 1971b).

M. arginini Barile, Del Giudice, Carski, Gibbs and Morris, 1968

M. arginini is an arginine-hydrolyzing mycoplasma which is non-haemolytic and produces classical, centred colonies. Following its characterisation and naming by Barile et al. (1968), who recovered the organism from several sources including the brain of a sheep affected with scrapie, there have been a number of reports of its isolation from sheep. Leach (1970) isolated M. arginini from 10 nasal swabs taken from a flock of 30 coughing sheep, but was unable to recover the organism from 200 normal sheep. Al-Aubaidi, Taylor, Bubash and Dardiri (1972) recovered M. arginini from the lungs of pneumonic Bighorn Sheep (Ovis canadensis) and also from goats. Foggie and Angus (1972) recovered one strain of M. arginini from pneumonic lungs and 26 strains from nasal cavities of intensively-kept sheep in which there was a history of respiratory disease and conjunctivitis. Nayil (1971) isolated eight mycoplasma strains from pneumonic lungs, of which one strain ("Serotype 2") showed centred colony morphology, inability to cause haemolysis of horse erythrocytes (RBC) and a relationship by the growth inhibition test with both M. arginini and a mycoplasma isolated from a case of SPA in Kenya by Krauss (q.v.). No serological relationship could be demonstrated between "Serotype 2" and 15 other

recognised mycoplasmas by the metabolism inhibition or growth inhibition tests. Carmichael, St. George, Sullivan and Horsfall (1972) compared the nasal and pharyngeal recovery rates of mycoplasmas from one healthy flock with one in which respiratory disease was present. Mycoplasmas initially termed "Biotype 10C" and later identified as M. arginini were recovered from 42% of nasal and 53% of pharyngeal swabs taken from 38 animals in the pneumonic flock; a second sampling two months later yielded figures of 72% and 98% respectively from 46 animals. The healthy flock, from which 33 animals were sampled on a single occasion, gave recovery rates for "Biotype 10C" of 42% from nasal and 70% from pharyngeal swabs.

The characteristics of several other unidentified mycoplasma isolations from sheep are also suggestive of M. arginini. Arisoy et al. (1967) recovered 46 strains of a mycoplasma ("Type N") from 627 nasal swabs taken from normal flocks of sheep and goats and flocks in which contagious agalactia was present. "Type N" strains produced centred and centreless colonies, were weakly or non-haemolytic for sheep, horse and chicken RBC, and did not form "films and spots" on solid media. The similarity of "Type N" strains to M. arginini has also been remarked on by Foggie and Angus (1972). Cottew (1971a) classified 63 mycoplasmas isolated from sheep in Australia and Tasmania into two serological groups. "Type 1", which contained 38 strains isolated from pneumonic lungs (31 strains), nasal cavity (six strains) and brain (one strain), were found to form a single serogroup with five Turkish "Type N" strains. All 43 strains produced centred colonies, and most

caused incomplete haemolysis of sheep and chicken RBC. Surprisingly, however, 17 strains formed "films and spots".

Greig (1955) recovered mycoplasmas from the nasal cavity (12 strains from 19 sheep) and lungs (seven strains from eight sheep) of pneumonic animals, but also from lungs of normal sheep (five strains from five sheep). Similarly, Boidin et al. (1958) recovered mycoplasmas from the lungs of sheep with "enzootic pneumonia" (eight strains from 23 sheep), but also from tracheal swabs from normal sheep (13 strains from 100 sheep). However, no isolations of mycoplasmas were made from the lungs of 30 normal sheep, or from chronic lesions in the lungs of 70 lambs at slaughter. In contrast, the two mycoplasma strains isolated by workers in Ohio (Hamdy, 1958; Hamdy, Pounden and Ferguson, 1959; Hamdy and Pounden, 1959) were from pneumonic lesions in lambs at slaughter. The strains isolated by Greig, Boidin et al. and the Ohio workers were all alike in their inability to ferment carbohydrates or haemolyse RBC, and in their production of centred colonies.

Krauss and Wandera (1970) obtained 16 mycoplasma isolates from the lungs of 10 of 14 sheep with SPA. The strains were compared on the basis of morphology, some biochemical reactions and the agar gel double diffusion test. Ten stains, which were non-sugar fermenters, produced centred colonies and caused haemolysis of sheep and horse RBC, were placed in "Serogroup 1". Two strains reduced tetrazolium, a characteristic not associated with M. arginini. However, the tenuous connection of a serological relationship between a Kenyan mycoplasma isolate and M. arginini

demonstrated by Nayil (1971) suggests that at least one of the isolates of Krauss and Wandera was M. arginini.

Pathogenicity experiments performed with M. arginini or isolates resembling this species have generally been unable to demonstrate virulence for sheep. Foggie and Angus (1972) inoculated one specific pathogen free (SPF) lamb by the i.n. route with a broth culture containing $3.7 \times 10^5$ colony forming units (c.f.u.). Mild pulmonary changes only were observed, with lymphoid hyperplasia in one parabronchiolar site. M. arginini was recovered from the lungs and lymph nodes. A second SPF lamb inoculated by the i.t. route with $5.5 \times 10^6$ c.f.u. of the same strain showed a small and localized area of proliferative interstitial pneumonia, and the organism was recovered from the nasal cavity only. Watson et al. (1968) inoculated "Type N" strains into 18 goats by the s.c. route. Minor lesions only were produced, and these authors concluded that the organism was non-pathogenic. Similarly the inoculation of embryonated eggs, sheep (Hamdy and Pounden, 1959) and laboratory animals (Greig, 1955) with the respective mycoplasma strains isolated by these workers could demonstrate pathogenic effects only in eggs. The combinative effects of the mycoplasma isolates of Hamdy and Pounden with other treatments are discussed in Part 1.

Only the isolates of Boidin et al. (1958) appeared to demonstrate pathogenicity for sheep, although the results of their experiments were ambiguous. In their first experiment, nine lambs were inoculated by the i.v. or i.t. routes with broth cultures of mycoplasmas isolated from acute pneumonia: three lambs developed

arthritis and one peritonitis. At necropsy one lamb also revealed the presence of "limited" pneumonia, but this was considered to have been present before inoculation. Six animals yielded mycoplasmas from several organs. In a second experiment, four lambs were given i.t., i.v., s.c. or i.p. inoculations with a broth culture of a mycoplasma isolated from a lung lesion suspension, and three lambs were inoculated by i.t., i.v. or i.p. routes with a mycoplasma cultured from the arthritic joint of an animal from their first experiment. The inoculation of two further lambs with lung lesion suspension has been described in Part 1. Only one of the lambs inoculated with broth culture showed a reaction by the development of arthritis, and one lamb yielded mycoplasmas at a single sampling only. A third experiment, which examined the combinative effects of "virus" and other organisms including mycoplasmas, has been discussed in Part 1. A control group in this experiment, consisting of eight lambs inoculated by the i.t. route with mycoplasmas only, showed very small foci in the lungs at necropsy. The isolates of Boidin et al. were similar to those of Hamdy and Pounden (1959) and Greig (1955) in that they were lethal for embryonated eggs but had no effect in mice.

In addition to isolations from the respiratory tract and brain of sheep, M. arginini has been recovered from cases of ovine kerato-conjunctivitis (Leach, 1970; Surman, 1968; Langford, 1971; the isolates of Surman and Langford were identified retrospectively by Barile, Del Giudice and Tully, 1972). Arisoy et al. (1967) also reported the recovery of "Type N" organisms from the eyes of sheep of unstated clinical status.

## M. ovipneumoniae Carmichael, St. George, Sullivan and Horsfall 1972

M. ovipneumoniae is a glycolytic mycoplasma which causes haemolysis of ox, horse and sheep RBC, and produces granular, centreless colonies on solid media.

The characterisation and naming of this species (Carmichael et al., 1972) followed an earlier report by the same group of workers which described the isolation of a similar mycoplasma from sheep pneumonic lungs by means of bovine testis tissue cultures (St. George, Sullivan, Love and Horsfall, 1971). The cases of pneumonia examined by these Australian workers originated from two Queensland flocks which had a greater than 90% incidence of clinical pneumonia. The features of this pneumonia were high morbidity, low mortality and poor weight gains in lambs, with resultant economic loss. Clinical pneumonia in the lambs could be detected first at 5-10 weeks of age, and was most pronounced at 10-15 weeks old (w.o.). Thereafter, clinical signs generally regressed, although almost all the lambs of 16 w.o. or more revealed pneumonic lesions at slaughter. Microscopically, lung lesions consisted of a proliferative interstitial pneumonia with septal and bronchiolar epithelial cell hyperplasia and proliferation: infiltration by lymphocytes and neutrophils was slight. As previously described under M. arginini, Carmichael et al. compared one pneumonic flock with a healthy flock by nasal and pharyngeal swabbing. At the first sampling of the pneumonic flock, when 90% of animals had auscultatory evidence of respiratory disease, 89% of 38 sheep yielded M. ovipneumoniae from nasal and 21% from pharyngeal swabs. The

organism was also recovered from the eyes of one lamb suffering from keratoconjunctivitis. At the second sampling two months later, less than 50% had clinical signs of pneumonia; on this occasion, 45% of 46 sheep yielded M. ovipneumoniae from nasal and 5% from pharyngeal swabs. In contrast, the healthy flock yielded M. ovipneumoniae from 6% of nasal and from no pharyngeal swabs. The examination of 22 frozen lung specimens from the pneumonic flock yielded only eight isolates of M. ovipneumoniae. Consequently, a further abattoir sampling of 17 sheep was performed; M. ovipneumoniae was recovered from 76% of nasal swabs and 82% of bronchial swabs, but from only 57% of lung washings (cell portion) and 25% of lung suspensions. An experiment to investigate the low recovery rate from these last two samples showed that bronchial washings possessed a potent inhibitory activity against M. ovipneumoniae but not M. arginini strains. Carmichael et al. also found that 45% of the sera from 185 2 m.o. lambs in the pneumonic flock had positive or suspicious metabolism-inhibiting activity against M. ovipneumoniae, compared with 12% of 17 sheep of various ages from the healthy flock.

Prior to the naming of M. ovipneumoniae, several isolations from the respiratory tract of sheep of glycolytic mycoplasmas with fastidious growth requirements, the ability to haemolyse RBC, and a centreless colony morphology had been reported. Twenty-two of 63 strains isolated by Cottew (1971a) from lungs (19 strains) and nasal cavity (three strains) of sheep in Australia and Tasmania had these characteristics, although haemolytic ability was incomplete.

These formed a single serogroup ("Type 2"). Indirect evidence that "Type 2" strains were in fact M. ovipneumoniae is indicated by a subsequent report that they cross-reacted in the growth inhibition test with the mycoplasma strain isolated by St. George et al. (1971) (Cottew, cited by St. George et al., 1971).

The first reported isolations of mycoplasmas with the characteristics of M. ovipneumoniae were from cases of SPA in Scotland (Mackay, Nisbet and Foggie, 1963; Mackay, 1965; Mackay and Nisbet, 1966). Initially the recovery of such mycoplasmas from three cases of SPA was described. Goat spleen fibroblast tissue cultures were used for primary isolation and artificial media for subculture (Mackay et al., 1963). Subsequently, artificial media were used to isolate 25 mycoplasma strains from 30 cases of SPA, of which 21 comprised these glycolytic ("Type A") mycoplasmas (Mackay, 1965). Slide agglutination tests on sera from normal sheep and from sheep suffering from SPA showed the sera of all 20 SPA cases examined to agglutinate "Type A" antigens at dilutions greater than 1 in 4, but only 10-15% of 200 normal sheep were also positive at these dilutions (Mackay, 1965). Sera from clinical cases were also used for direct and indirect fluorescent staining of mycoplasma colonies on agar and of smears of affected lung tissue (Mackay and Nisbet, 1966). Mackay and Nisbet (1966) considered that "Type A" mycoplasmas did not have a primary aetiological role in SPA, although some lung reactions seen in the disease, in particular peribronchiolar lymphocytic and alveolar macrophage cell responses may have been due to the presence of mycoplasma. Mackay also recovered "Type A" mycoplasmas

from pneumonic but not from normal lungs, and from the upper respiratory tract of normal sheep (Mackay, cited by Cottew and Leach, 1969).

Unspecified strains of Mackay's "Type A" mycoplasmas have been compared by two groups of workers with their own mycoplasma isolates. Krauss and Wandera (1970) placed a "Type A" strain in their "Serogroup 2", together with three of their own isolates from cases of SPA (see M. arginini). However, only two of the "Serogroup 2" strains, including Mackay's "Type A" strain, were glucose fermenters. None of the strains isolated by Krauss and Wandera showed serological cross-reactivity with the T1 strain of M. mycoides subsp. mycoides or the Vom strain of M. mycoides subsp. capri. Nayil (1971), who isolated eight mycoplasmas from pneumonic sheep lungs, found complete cross-reactivity between seven strains ("Serotype 1") and Mackay's "Type A" mycoplasma by both growth inhibition and metabolism inhibition tests, but no relationship could be demonstrated between his "Serotype 1" strains and an unspecified "Serogroup 2" strain obtained from Krauss. This indicates perhaps the heterogeneity of the "Serogroup 2" of Krauss and Wandera (1970). Nayil also compared serologically his own strains and that of Mackay's with 15 other recognised mycoplasma species: no cross-reactions were noted by the growth inhibition test, but a one-way reaction between antiserum to the "Serotype 1" strains and Mycoplasma bovirhinis was demonstrated by the metabolism inhibition test.

The only pathogenicity experiments with M. ovipneumoniae or strains similar to this organism have been performed by St.

George et al.(1971). The eighth subculture of a mycoplasma isolate, which represented a theoretical $10^{12}$ dilution from the original lung material, was inoculated by the i.t. route into three caesarean-derived lambs. The dose was $10^{6.6}$ - $10^{7.6}$ organisms (orgs.). The lambs suffered an intercurrent bacterial infection during the experiment, and one control and all three test lambs died at 2 - 3 d.p.i. However, no pneumonic symptoms or pulmonary lesions were noted in the two control lambs, whereas all three test animals showed clinical signs and histopathological lesions of a proliferative interstitial pneumonia. Two of the lambs also had macroscopic lesions of pulmonary consolidation. Mycoplasmas presumably similar to those inoculated were recovered from the three test lambs. Two conventionally-reared lambs were also exposed to an aerosol of a thirteenth passage culture which represented a theoretical dilution of $10^{28}$ from the last tissue culture passage and contained $10^{6.6}$ organisms. Both lambs were placed in-contact with another lamb and with two adult sheep. At necropsy, lung consolidation was present in the one in-contact lamb, and grey changes in the lungs of one aerosolized lamb and one in-contact sheep. Histopathological lesions of proliferative interstitial pneumonia were present in the aerosolized lamb which had macroscopic lesions and in all three in-contact sheep. Mycoplasmas were recovered from three animals.

A. laidlawii (Sabin) Edward and Freundt, 1970

The isolation of A. laidlawii from sheep has been alluded to by Leach (1970). In addition, the three "Serogroup 3" strains

of Krauss and Wandera (1970) were glucose-fermenting mycoplasmas which grew at 22°C and reacted with antiserum to A. laidlawii.

M. mycoides subsp. mycoides (Borrel, Dujardin-Beaumetz, Jeantet and Jouan) Freundt, 1955

This species has been recovered from maned (Barbary) sheep (Ammotragus lervia) in a zoo in Frankfurt (Ernø, Freundt, Krogsgaard-Jensen, and Rosendal, 1972). A representative strain of the Frankfurt isolates and two other mycoplasma isolates from sheep in Iran formed part of 47 mycoplasma strains examined by Al-Aubaidi, Dardiri and Fabricant (1972). All three sheep strains were placed in a subdivision of M. mycoides subsp. mycoides termed "Group 8". Al-Aubaidi et al. (1972) also concluded that many mycoplasma strains of caprine origin which had previously been identified as M. mycoides subsp. capri belong, in fact, to "Group 8" of M. mycoides subsp. mycoides. Experimentally, several of these strains have been shown to be pathogenic for sheep (reviewed by Hudson, Cottew and Adler, 1967), but the very few reported recoveries of M. mycoides subsp. mycoides from sheep indicates that the organism is probably of minor importance in this species.

M. mycoides subsp. capri (Edward) Freundt 1955

This organism is said not to infect sheep under natural conditions (Hudson et al., 1967; Cottew and Leach, 1969), although the condition "abu nini", which is observed in Sudan and affects both sheep and goats, has been suggested by Otte (1960) to involve the causal agent of contagious caprine pleuropneumonia (CCPP). Provost (1960), however, considered that the aetiology of abu nini

was not clearcut, but listed M. mycoides subsp. capri as one of a number of possible causes. Lindley and Abdulla (1969) attempted to investigate the cause of abu nini by endobronchial (e.b.) inoculation of homogenates of contagious bovine and caprine pleuropneumonia lesions into sheep and goats, but were unable to reproduce any effects in the sheep.

Due to confusion in the identification of subspecies of M. mycoides, it is unclear as to whether the capri subspecies is experimentally pathogenic for sheep. The Cal goat strain, isolated by Cordy, Adler and Yamamoto (1955), was shown by these authors to cause fever, pneumonia, lameness and death in lambs and ewes; this strain has been shown by Hudson et al. (1967) to be serologically distinct from the mycoides subspecies and to cross-react at very low levels only with the capri subspecies.

M. conjunctivae Barile, Del Giudice and Tully, 1972

M. conjunctivae has been isolated from outbreaks of ovine keratoconjunctivitis in the U.S.A., Australia and Canada (Barile et al., 1972; Surman, 1968; Langford, 1971; the isolates of Surman and Langford were retrospectively identified by Barile et al., 1972).

Other mycoplasma isolations from sheep

The lack of biochemical and serological characterisation or the possession of unusual pathogenic properties exclude inference of the identity of other mycoplasmas isolated from sheep by comparison with known species of mycoplasmas.

An "enzootic pneumonia" of sheep occurring in Argentina, in which affected animals have bronchopneumonia, thickened pleurae and fibrinous adhesions, caused in one outbreak the death of 60 animals from a flock of 350 (Colusi, Garbini, Sequeira and Delpietro, 1964). Six strains of mycoplasmas recovered from bronchial mucous membranes of affected animals were unable to ferment carbohydrates, and possessed only feeble haemolytic ability for horse RBC, characteristics suggestive of M. arginini. However, broth cultures inoculated by a variety of routes into five sheep produced cellulitis (s.c. route), nephritis (s.c. and i.v.), generalized septicaemia (large dose i.v.), pneumonitis (i.t.) and arthritis (intra-carpal). The strains were also lethal for embryonated eggs. Antiserum prepared against a representative strain did not fix complement in the presence of M. mycoides subsp. mycoides, M. mycoides subsp. capri or M. agalactiae, but the prepared antiserum and sera obtained from their experimentally infected animals showed a positive reaction with M. mycoides subsp. mycoides by the slide agglutination test. As M. mycoides subsp. mycoides is glycolytic, in contrast to the strains isolated by Colusi et al., the significance of the findings from the slide agglutination tests is doubtful.

Three other reports of mycoplasma isolations from sheep show similarity to the findings of Colusi et al. (1964) in terms of the disease syndrome yielding the mycoplasmas and reproduced experimentally by them. Hanko and Otterlin (1955), in Norway, observed an acute septicaemic condition in 21 goats and one sheep, the predominant symptoms of which were necrotizing pneumonia, mastitis, enteritis,

splenitis and lymphadenitis. Mycoplasmas were recovered from many organs in affected animals. The inoculation of mycoplasma isolates into two goats reproduced the field syndrome, although an extended incubation period was observed. Sera from field cases but not from normal sheep fixed complement in the presence of the mycoplasma isolates. Unstated serological tests indicated to the authors that the disease was either CCPP or contagious agalactia. Pegreffi, Mura and Contini (1957), in Italy, described a pleuro-pneumonia with a subacute or chronic course, which caused mortality rates of 30-40% in adult sheep, and 85-90% in lambs. Affected animals showed tracheitis, bronchitis, pleurisy and hepatization of lungs: heart, liver, kidneys, eyes, joints and gastro-intestinal tract also had pathological changes. Cultures or filtrates of field material produced no effect in laboratory animals, but fourth or fifth passage cultures caused death in lambs, with post mortem features similar to the field disease. Mycoplasmas were recovered from experimentally infected animals. Farzaliev, Khalimbekov, Dandamaev and Aliev (1962), reported the occurrence in Russia of an acute, often fatal septicaemia which affected the viscera and occurred in lambs up to 10 d.o. Organisms identified as mycoplasmas on grounds of colony and organism morphology and staining characteristics, were recovered from lungs, heart, liver, kidney and lymph nodes of affected animals. Broth cultures of isolates produced no effects in laboratory animals and pigeons, but two lambs inoculated intrathoracically developed lesions similar to the field disease. The s.c. and i.v. inoculation of two lambs produced similar but less marked responses.

Heikkila (1956), in Angola, isolated mycoplasmas from lungs and nasal cavity of animals suffering from "pleuropneumonia". The s.c. inoculation of these strains induced hot, tender swellings in sheep, but no effect was observed following i.v. injection. Despite this, Heikkila concluded that the organisms were the causal agent of the field disease. The isolates were claimed to be culturally similar but serologically distinct from M. mycoides subsp. mycoides and M. mycoides subsp. capri, although no details of the serological tests performed were presented.

There are three reports of the isolation of mycoplasmas from pneumonic sheep in Turkey. Durusan and Doguer (1955) recovered seven strains of a centred-colony mycoplasma from ovine lungs. No pathogenic effects were observed following inoculation of the cultures into two lambs, six sheep and five goats. Aktan (1956) recovered mycoplasmas from lungs of sheep involved in two epidemics of pneumonia. The strains produced bronchopneumonia in guinea-pigs, but mild reactions only in mice and sheep: nonetheless, Aktan considered the mycoplasmas to be the causal agents of the field disease. Erdag (1972) recovered mycoplasmas of three distinct colonial morphologies from eight cases of pneumonia. These comprised three strains which were small and centred, two which were centreless and three which were granular and centred. One strain of each colony type was inoculated into one or two lambs. Mild atelectasis and emphysema were produced by each, changes considered by Erdag to be sufficient to potentiate infections with other respiratory microorganisms.

Khan (1960) recovered organisms, identified as mycoplasmas by light microscopy examination and cultural characteristics, from a pneumonic sheep in Sudan. The intramuscular (i.m.) or s.c. inoculations of a broth culture of the isolate into two sheep and a calf, and the administration of an aerosol of the culture to two further sheep caused no effects except for slight swellings at the sites of inoculation in those injected by the i.m. or s.c. route.

In the following reports of mycoplasma isolations from sheep, no pathogenicity experiments were attempted. Barber and Fabricant (1962) isolated five mycoplasma strains from lungs and two from spleen. It was not stated whether the source animals were diseased or not. Two of the strains were shown to be glycolytic. Dungworth and Cordy (1962) recovered 13 mycoplasma strains from the lungs of 28 lambs experimentally inoculated with chlamydia (see Part 1). Mycoplasmas (and pasteurellae) were regularly associated with the lung lesions which persisted in experimental animals after 13 d.p.i., and caused a slight increase in the vascular and neutrophilic responses. Alonso (1964) found a serological relationship by the agar gel double diffusion test between his mycoplasma isolates from cases of "enzootic pneumonia", Mycoplasma gallinarum and M. agalactiae. Contini (1960) studied the morphology of mycoplasmas isolated from "pleuropneumonia" by light microscopy. Krauss (1969, cited by Wandera, 1971) recovered 40 mycoplasma strains from the nasal cavity of healthy sheep in Kenya. Davies and Wandera (cited by Wandera, 1968) also recovered mycoplasmas from cases of

"pasteurella pneumonia", SPA and maedi in Kenya. Workers in Rumania initially reported the recovery of 17 mycoplasma strains from 124 pneumonic lungs (Ungureanu and Cirstet, 1969; Ungureanu, 1969). A further report of an abattoir survey and investigations of outbreaks of pneumonia claimed the isolation of mycoplasmas from 18% of 2708 pneumonic lungs (Ungureanu et al., 1972). Masalski (1971), in Bulgaria, recovered seven mycoplasma strains from the nasal cavity of 12 sheep showing copious nasal discharge. Woolf, Kradel and Bubash (1970), in the U.S.A. recovered mycoplasmas from lungs (six strains from six samples) and nasal cavity (12 strains from 22 samples) of Bighorn sheep (Ovis canadensis) suffering from pneumonia. St. George (1972) recovered 37 mycoplasma strains from 66 lung samples or nasal swabs: of three strains examined by Cottew (1971), two were classified as "Type 1" and one as "atypical". Three of the 22 mycoplasma strains isolated by Mackay (1965) from cases of SPA ("Type B") had a centred colony morphology, and did not cross-react with his "Type A" strains. Nobel (1958), in Israel, recovered mycoplasmas from two of 19 cases of SPA. Deiana and Ceretto (1967), in Italy, used artificial media and tissue cultures to isolate and culture mycoplasmas from cases of SPA. In a direct fluorescent antibody test applied to lung smears and tissue cultures infected with SPA material, four of 10 sera from clinical cases gave positive results.

Cello (1967) reported the recovery of mycoplasmas from the eyes of sheep suffering from keratoconjunctivitis. Spradbrow and Marley (1971) recovered 23 strains of mycoplasmas from the same

condition. Twenty-one of these isolates caused neither glycolysis nor alkaline change in media containing urea, but the authors considered these to be T-mycoplasmas because of their small colony size. The remaining two isolates were glycolytic and formed centred colonies.

McIlwain and Bolin (1967) recovered non-glycolytic, non-haemolytic mycoplasmas from the testes of three rams with orchitis. The isolates stained positively by the immunofluorescent technique using antiserum to Mycoplasma gallisepticum, but this identification must be regarded as dubious as M. gallisepticum is both glycolytic and haemolytic.

Aktan, Guley and Doguer (1955) recovered mycoplasmas from a contaminated batch of sheep pox vaccine which caused abortions, and from the aborted foetuses of animals treated with the vaccine.

## CONCLUSIONS FROM REVIEW OF LITERATURE, AND AIMS OF THESIS

The principal organism associated with enzootic pneumonia is P. haemolytica Biotype A, but reproduction of the disease with cultures of P. haemolytica alone has generally required very large doses. This suggests that the aetiology of enzootic pneumonia involves a predisposing factor, but neither viruses nor chlamydiae have been shown to be capable of reducing the dose level of P. haemolytica required to establish the bacterium in the lungs of conventional sheep. The aetiology of atypical pneumonia also appears to be complex. The association of P. haemolytica with the disease is stated to be variable, and although a considerable

number of strains of chlamydiae have been shown by i.t. inoculation of large doses to produce a pneumonitis which resembles atypical pneumonia in some aspects, there is as yet little direct evidence that chlamydiae are a primary cause of pneumonia in sheep. PI3 virus appears to be the most widespread of the viruses associated with the respiratory tract of sheep, and pneumonia has been produced experimentally by direct inoculation of the organism into the lungs, but its significance and involvement in naturally-occurring pneumonia requires further assessment.

The rôles of mycoplasmas in sheep pneumonias remain largely undetermined, despite the considerable volume of literature relating to the subject. In the majority of reports, the mycoplasmas isolated have been inadequately characterised, and conventionally-reared sheep of unknown susceptibility and microfloral status have been employed in pathogenicity experiments. The prevalence of recognised mycoplasma species in the respiratory tract of sheep is indicated only by the single survey of Carmichael et al. (1972), which was restricted to two flocks.

Three of the five recognised mycoplasma species isolated from the respiratory tract of sheep would appear to occur in Britain, namely M. arginini, A. laidlawii and M. ovipneumoniae. M. arginini, and organisms resembling this species, have been isolated from both normal and pneumonic flocks and also in cases of keratoconjunctivitis. The available experimental evidence indicates that M. arginini does not possess virulence for the ovine respiratory tract. The pathogenicity of A. laidlawii for

sheep has not been tested, although this saprophytic organism is generally considered to be avirulent in other animal species (Cottew and Leach, 1969). All reported lung recoveries of M. ovipneumoniae have been from sheep with respiratory disease. Furthermore, the pathogenicity of M. ovipneumoniae for the respiratory tract has been demonstrated in sheep, including caesarean-derived lambs.

The primary aim of this work was investigation of the pathogenicity of the mycoplasmas most commonly associated with sheep pneumonias in Britain. A preliminary step to these studies was the survey of mycoplasma-associated pneumonia in commercially-reared lambs and ewes.

# CHAPTER 2

## MATERIALS AND GENERAL METHODS

### MEDIA

#### Reagents and stock solution

1. Alsevers solution. Distilled water (d.w.) containing 2.05% (w/v) dextrose, 0.8% (w/v) sodium citrate and 0.42% (w/v) sodium chloride was adjusted to pH 6.1 by the addition of 10% citric acid solution, and autoclaved at 115°C for 15 min.
2. Ampicillin ("Penbritin", Beecham Animal Health, Manor Royal, Crawley, Sussex). Stock solution 100 mgs/ml of d.w.
3. Agarose (B.D.H. Chemicals Ltd., Poole, Dorset, or Miles Laboratories, Slough, Bucks).
4. L-arginine hydrochloride (hereafter referred to as arginine) (Sigma Chemical Co., St. Louis, Mo., USA). Stock solution 20% (w/v) in d.w.: sterilized by filtration.
5. Broths:
   i) Brain-heart infusion (Difco Laboratories, Detroit, Michigan, USA; or Oxoid Ltd., London, SE1). Reconstituted by the addition of 37 g of powder to 1 L d.w.: autoclaved at 121°C for 15 mins.
   ii) Hartley's digest. (Oxoid Ltd.)
   iii) Tryptose phosphate (Difco Laboratories or Oxoid Ltd.). Reconstituted by the addition of 29.5 g of powder to 1 L of d.w.: autoclaved at 121°C for 15 mins.

iv) Rabbit infusion broth. Prepared by the method of Taylor-Robinson, Somerson, Turner and Chanock (1963). To 100 g of finely minced rabbit meat is added 800 ml of d.w. The suspension is mixed well, then heated to 100°C in a Koch steamer for 30 mins. The broth is cooled, filtered through gauze then Whatmans No. 2 filter paper, following which 8 g of peptone and 4 g of sodium chloride are added. The broth is reheated to dissolve the peptone and salt, adjusted to pH 7.6 and replaced in the steamer for 30 mins. The preparation is finally autoclaved at 115°C for 30 mins.

6. Earle's Balanced Salt Solution. Prepared at the Institute according to the method of Paul (1960) but with glucose and sodium bicarbonate omitted. Sterilized by filtration.

7. Deoxyribonucleic acid (sodium salt) from calf thymus gland (Calf thymus DNA) (B.D.H. Chemicals Ltd.). Stock solution 0.2% (w/v)in BSS (q.v.): autoclaved at 115°C for 10 mins.

8. Distilled water (d.w.). Produced at the Institute by "Fi-streem 4" (Fisons Scientific Apparatus, Ltd.). Stored in borosilicate glass reservoirs: sterilized by filtration.

9. Dithiothreitol (Cleland's Reagent) (Sigma Chemical Co.). Stock solution 10% (w/v) in d.w.: sterilized by filtration.

10. Eagles Minimal Essential Medium (MEM). Constituted by the addition to BSS (96 ml) of 50X solutions of Basal Medium Eagles amino acids and vitamins (2 ml of each) (Gibco Biocult Ltd., Paisley, Renfrewshire).

11. Galactose. Stock solution 20% (w/v) in d.w.: sterilized by filtration.

12. Hanks Balanced Salt Solution (BSS). Prepared at the Institute according to the method of Paul (1960) but with glucose and sodium bicarbonate omitted. Sterilized by filtration.

13. Lactalbumin hydrolysate. Prepared as a 5% (w/v) solution in Earle's solution. Sterilized by filtration.

14. Medium 199 (Wellcome Reagents, Beckenham, Kent). Supplied as a 10X solution.

15. Phenol Red. Prepared as a 0.4% (w/v) solution in 0.0125 M NaOH. Sterilized by steaming for 2 h.

16. Phosphate buffered saline pH 7.2 (PBS). Prepared at the Institute.

17. Sera:

i) Swine )
ii) Sheep ) Prepared from blood obtained from a local abattoir.

iii) Rabbit Prepared from blood obtained from the Institute animal stock.

Sterilized by filtration and stored at -20°C.

iv) Horse )
v) Foetal calf) Obtained from Gibco Biocult Ltd.

18. Sodium bicarbonate solution. Prepared as an 8% (w/v) solution in d.w. with phenol red added.

19. 2, 3, 5-triphenyl-tetrazolium chloride solution (hereafter referred to as tetrazolium) (B.D.H. Chemicals, Ltd.). Prepared as a 1% (w/v) solution in d.w. Sterilized by filtration, and stored in the dark at 4°C.

20. Thallous acetate (B.D.H. Chemicals Ltd.). Prepared as a 10% (w/v) solution in d.w. Sterilized by filtration.

21. Urea (B.D.H. Chemicals, Ltd.). Initially prepared as a 50% (w/v) and later as a 20% (w/v) solution in d.w. Sterilized by filtration.

22. Yeast extract. Prepared by the method of Hers (Marmion, 1967). 1L of d.w. heated to 40°C is added to 1 Kg of fresh bakers yeast. The yeast is well kneaded until thoroughly mixed with the water, then heated to 80°C. Concentrated HCl (6.5 ml) is added, and the mixture is maintained at 80°C for 20-30 min, being stirred continuously. The suspension is allowed to cool and settle, clarified by centrifugation at 2000 rpm for 20 min, and then filtered through 3-4 fold Whatmans No. 2 filter paper, followed by a Seitz clarifying pad. The filtrate is adjusted to pH 4.5, and the extract finally sterilized by membrane filtration. 100 ml volumes are stored at -20°C until required.

Filtration. Unless stated otherwise, sterilization by filtration was performed using membrane filters (Millipore Corp., Bedford, Mass., USA), of 0.22 μm average pore diameter (a.p.d). According to the volume of material involved, Millipore filter holders of 25, 47, 142 or 293 mm diameter were employed.

Media components

1. Media formulations were assessed from the performance of their agar analogues, since it is generally more difficult to isolate and propagate mycoplasmas on solid medium than in broth. The criteria applied were growth and size, number and morphology of colonies.

2. Agarose. The use of agarose rather than other forms of agar was based on the reports of mycoplasma-inhibitory substances in agar (Tauraso , 1967; Carmichael et al., 1972) and the success obtained with agarose in the growth of Mycoplasma dispar (Gourlay and Leach, 1970).

3. Medium 199. This medium was preferred, though MEM was found to be equally satisfactory for laboratory-adapted strains of mycoplasmas.

4. Broths. Hartleys digest broth was initially employed, but following difficulties experienced in its acquisition a change was made to tryptose phosphate broth. Brain-heart infusion broth was later substituted for tryptose phosphate broth due to its slightly better growth-promoting properties.

5. Yeast extract. The extract prepared from fresh yeast was found to be superior to commercially-available products.

6. Sera. Swine serum was used in all basic media. This followed the findings of Carmichael et al. (1972), confirmed by us, that swine serum was superior to other sera for the growth of M. ovipneumoniae, possibly because of its

higher cholesterol content. Sera employed in all media were inactivated by heating at 56°C for 30 min to eliminate heat-labile inhibitory substances of a non-specific nature which may have been present (e.g. Coleman and Lynn, 1972), and as a safeguard against the possibility of contaminating mycoplasmas in the serum (Barile and Kern, 1971; Vogelzang and Beuvery, 1974).

7. Ampicillin. The use of ampicillin was based on the finding that ampicillin-supplemented medium yielded superior isolation rates of M. dispar compared with medium containing benzyl penicillin (Andrews, Leach, Gourlay and Howard, 1973).

Media used in isolation and propagation

The formulae of these media and of MTM are indicated in Table 2.1.

OA and OB

These were formulated for the isolation and culture of glycolytic mycoplasmas, in particular M. ovipneumoniae, but later experience showed OA to be satisfactory for all large-colony mycoplasmas encountered. OA and OB were evolved from Medium 2 of Mackay et al. (1963). The incorporation of yeast extract in the media and the substitution of agarose for Difco agar, of swine serum for horse serum, of Medium 199 for Eagles MEM, of ampicillin for penicillin G and of brain-heart infusion broth for tryptose phosphate broth were done at an early stage in the work.

Table 2.1 Formulae of growth and transport media

| Reagent | Volume in 100 ml | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1A | 0A | 0B | AB | TA1 | TB1 | TA2 | TB2 | MTM |
| D.w. | - | 45 | 54 | 49.5 | 45 | 45 | 43.2 | 43.2 | - |
| Agarose (in g) | 0.75 | 0.9 | - | - | 0.75 | - | 0.9 | - | - |
| 10X Medium 199 | - | 5.0 | 6.0 | 5.5 | 5.0 | 5.0 | 4.8 | 4.8 | - |
| Broth | 70 | 20 | 20 | 20 | 18 | 18 | 20 | 20 | 90 |
| Yeast extract | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 5 |
| Swine serum | 20 | 20 | 10 | 10 | 20 | 20 | 20 | 20 | 5 |
| Arginine (20%) | - | - | - | 5 | - | - | - | - | - |
| Urea (50%) | - | - | - | - | 2 | 2 | - | - | - |
| Urea (20%) | - | - | - | - | - | - | 0.5 | 0.5 | - |
| Dithiothreitol (10%) | - | - | - | - | - | - | 0.5 | 0.5 | - |
| Calf thymus DNA (0.2%) | - | - | - | - | - | - | 1.0 | 1.0 | - |
| Ampicillin (100 mg/ml) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Thallous acetate (10%) | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Phenol red (0.4%) | - | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | - |
| pH | 7.4-7.6 | 7.6-7.8 | 7.6-7.8 | 6.6-6.8 | 6.0-6.2 | 6.0-6.2 | 6.0-6.2 | 6.0-6.2 | 7.0-7.2 |

Final concentrations of supplements: Arginine: 1% Dithiothreitol: 0.05% Ampicillin: 1 mg/ml
Urea: TA1 and TB1: 1% Calf thymus DNA: 0.002% Thallous acetate:
TA2 and TB2: 0.1% Phenol red: 0.006% 1 in 4000

1A

This was employed as an alternative solid medium to OA, and particularly for the isolation and culture of M. arginini, but its use was discontinued when it became apparent that OA was superior.

TA and TB

These were designed for the isolation and propagation of ureaplasmas. The original formulations, TA1 and TB1 suffered from three defects:

1. A proportion of ureaplasma cultures in TB1 died out after a variable number of passages. This may have been due to medium deficiencies in certain growth requirements, or alternatively the losses may have arisen from excessive supplementary levels of urea, which would allow unrestricted metabolism by the ureaplasmas, rapid alkalinisation of the medium and therefore death of the organisms.
2. Thallous acetate has been shown to be deleterious to human ureaplasmas (Shepard, 1969). Similarly, thallous acetate at a final concentration of 1 in 4000 was found to cause a slight depression in growth titres of ovine ureaplasmas, and the supplement was accordingly omitted from TA1 and TB1. However, this exclusion caused a high proportion of cultures to be lost through contamination, generally with fungi.
3. The successful growth rate of ureaplasmas on TA1 from both positive broth cultures and specimens known to contain the organism was very low, irrespective of the gaseous environment in which the plates were incubated. This deficiency in particular led to a

search for an improved medium. The following factors were tested:

a) Variation of the levels of broth, swine serum, yeast extract, and Medium 199. No significant improvements were obtained.

b) Comparison of swine, foetal calf, horse and sheep sera. Swine serum was found to be equivalent to horse and foetal calf, and superior to sheep serum as a medium constituent.

c) Comparison of agarose with Noble agar. No major differences were noted in the successful growth rates obtained.

d) Inclusion of N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid buffer (HEPES). Manchee and Taylor-Robinson (1969) reported enhanced growth of ureaplasmas when HEPES was included in solid medium; similarly, we found that colonies became centred and generally greater in size, but no increase was obtained in the successful growth rate.

e) Use of a modification of the solid medium described by Gourlay, Brownlie and Howard (1973) for the isolation of goat ureaplasmas. The medium contained: foetal calf serum 20% (v/v); tryptone (Oxoid, Ltd.) 1.7% (w/v); neutralized soya peptone (Oxoid, Ltd.) 0.3% (w/v); yeast extract 10% (v/v); BSS 40% (v/v); 1X Medium 199 25% (v/v); ampicillin 1 mg/ml; phenol red 0.006% (w/v); and agarose 0.9% (w/v). No improvements in results were obtained with this medium.

f) Inclusion of dithiothreitol. Significantly enhanced growth of ureaplasmas was obtained by Tull, Blair, Fishman and Heatley (1975) when reducing agents, and particularly dithiothreitol, were included in solid media. Our results have corroborated

this. Growth rates and colony size both were increased by the supplementation of TA with 0.05% dithiothreitol. The addition of calf thymus DNA also appears to be beneficial, but neither the incorporation of phosphate buffers (Windsor, Edward and Trigwell, 1975) nor of $MgSO_4$ (Furness, 1973) in TA were found to improve the growth rate of ureaplasmas.

The currently employed media for ureaplasmas, TA2 and TB2 which contain dithiothreitol, thallous acetate, calf thymus DNA, and urea at a final concentration of 0.1% have improved considerably the growth rate of ureaplasmas compared with TA1 and TB1.

Other media

MTM

This was designed as a simple and cheap transport medium for samples. Its function is to maintain the viability of mycoplasmas and suppress bacterial and fungal growth during transit to the laboratory. The levels of glucose, arginine and urea were unsupplemented, to minimise excessive and harmful changes in pH by restricting the metabolism of the organisms.

RBG and RBA

These broths were used for the growth of mycoplasmas intended for the production of hyperimmune sera in rabbits. RBG was used for glycolytic mycoplasmas and RBA for arginine-hydrolyzing mycoplasmas. The media were identical to OB and AB respectively, but with rabbit serum substituted for swine serum and rabbit infusion broth as the broth source.

TRI medium

This was used in the tetrazolium reduction inhibition test for antibodies to M. ovipneumoniae. The medium contained: brain-heart infusion broth 65%; yeast extract 10%; swine serum (inactivated) 20%; tetrazolium (1% w/v solution) 5%; and ampicillin and thallous acetate as in OB. The medium was adjusted to pH 7.8 with M NaOH.

Production, handling and quality control of media

Broth media were produced in batches of 1 - 5 L and stored in 500 ml volumes at -20°C until required. Ampicillin was omitted from broths at this stage to avoid its degradation by overlong contact with proteins. Each bottle was checked during storage for bacterial sterility. Before dispensing, ampicillin was added and the pH adjusted. Volumes of 1.8 ml were dispensed into bijoux, and kept for day-to-day usage at 4°C; stocks of dispensed media were also occasionally kept for short periods at -20°C. A further sterility check was made following dispensing by the incubation of some bijoux at 37°C.

In the production of solid media, the agarose was autoclaved with d.w. (OA and TA) or broth (1A) at 115°C for 15 min, then stored at room temperature until required, or cooled to 56°C and added to the other constituents pre-heated to 56°C. Particular care was necessary not to heat the ingredients of TA2 above 56°C and not to maintain them at this temperature for too long. The thoroughly mixed constituents were poured into single vented plates

of 50 mm or 90 mm diameter (Sterilin Ltd., Teddington, Middlesex). Media were poured to a depth of 8 mm, except in OA plates used for the growth inhibition test, in which the depth employed was 5 mm. Plates were stored at 4°C: unused plates were discarded after three weeks. Freshness of preparation of TA plates was found to be particularly important in the culture of ureaplasmas.

During the initial part of the work, specific quality control measures for mycoplasma media were not performed. Latterly, however, quality surveillance of two media components, swine serum and yeast extract, has been instituted. The growth promoting properties of each new batch of these components is assayed by the titration of a second passage field isolate of M. ovipneumoniae in OB containing either substance; observation of colony growth and morphology of this strain on similarly prepared OA is also made. This more sensitive technique has shown that there is more variation in growth-promoting capacity of swine serum than had previously been supposed; similar observations regarding the variability of sera from other sources have been made by several workers (e.g. Mackay et al., 1963; Goodwin and Hurrell, 1970; Rosendal, 1976).

## ISOLATION AND CULTURING TECHNIQUES

### Isolation and titration

Tissues for examination were not ground but coarsely chopped, to minimise the release of antimycoplasmal substances (Kaklamanis, Thomas, Stavropoulos, Borman and Boshwitz, 1969). The tissue examined in tracheal samples consisted of mucosa stripped from a

5 - 7 cm piece taken from the middle third of the trachea. Single tissue samples of approximately 1 g and swabs were placed in 3 ml of MTM: pools of tissue samples of approximately 3 g were placed in 9 ml of MTM. The tissue or swab suspensions were shaken well and incubated for 1 h at 37°C, following which dried OA and TA plates were seeded from the suspending medium, and 0.2 ml volumes were inoculated into OB, AB and TB. In isolation and propagation, plates were inoculated by streaking with a platinum loop: TA plates were also stab inoculated, as this was found to increase considerably the successful growth rate of ureaplasmas, presumably through provision of microaerobic conditions.

Mycoplasmas were generally titrated in the appropriate broth as colour changing units (c.c.u.) per 0.2 ml by the serial transfer of 0.2 ml of suspension to 1.8 ml of fresh broth. With tissue and swab samples, the initial suspension in MTM was regarded as a 1 in 10 dilution: the first dilution in growth media was therefore taken as a 1 in 100 dilution of the original specimen. In filtration studies, mycoplasma titres were assayed as colony forming units (c.f.u.) per ml. Suspensions or filtrates were initially diluted in tenfold steps in broths, then pairs of OA plates were seeded with 25 or 50 µl of each broth dilution by means of micropipettes ("Finnpipette", Jencons (Scientific) Ltd., Hertfordshire, England). Colony numbers were counted after incubation for 3-7 days. The titre was calculated from the mean colony number at the dilution yielding 5-50 colonies per plate.

Culture, propagation and storage

All cultures were incubated at 37°C, and kept for three weeks before they were discarded as negative. Broth and tissue cultures were incubated aerobically, and examined daily for colour change, contamination and, in the case of tissue cultures, cytopathic changes. Broths were subcultured as soon as colour change had occurred. Fresh broth media were inoculated with one tenth of their volume of seed culture. If no colour change had been observed in the OB broth after incubation for 5-7 days, a blind subculture was made into fresh OB and onto OA plates. Contaminated broths were filtered through 0.45 μm a.p.d. membrane filters before subculture.

Solid media cultures were incubated in candle-jars containing a moisture source. Other systems tested for the maintenance of agar cultures included the use of anaerobic jars (deoxygenated with "Gaspak", produced by Becton, Dickinson and Co., Cockysville, Md., USA, or flushed through with 95% $N_2$ and 5% $CO_2$) and incubators continually flushed with 95% $N_2$ and $CO_2$, but none of these appeared to be superior to candle-jars either for large-colony mycoplasmas or ureaplasmas.

Plates were examined every 2 - 3 days with a stereo-binocular microscope. Subculture from solid media was by the excision of colony-bearing blocks of agar, which were placed in broth or smeared face-down over a fresh plate which had been dried at 37°C for approximately 30 min. Cloning, in which single colonies only were transferred, was always performed by this technique, 3 - 7 days old (d.o.) cultures with well-separated colonies being used to facilitate

colony morphology recognition and excision.

Colonies thought not to be mycoplasmas were tested for reversion to complete bacterial form by subculture onto 7% sheep blood agar plates and also, if necessary, by serial passage up to five times on OA from which bacterial inhibitors had been omitted.

Isolates and type strains of mycoplasmas were generally stored as broth cultures frozen at -70°C. Some strains were also lyophilized in 0.5 ml volumes, using 10% (w/v) powdered milk or 0.1% agarose (w/v) in the growth medium as stabilizer.

Identification

All large colony isolates were identified by the growth inhibition test (vide infra). Positive identification of ureaplasmas was based on observation of all the following criteria:

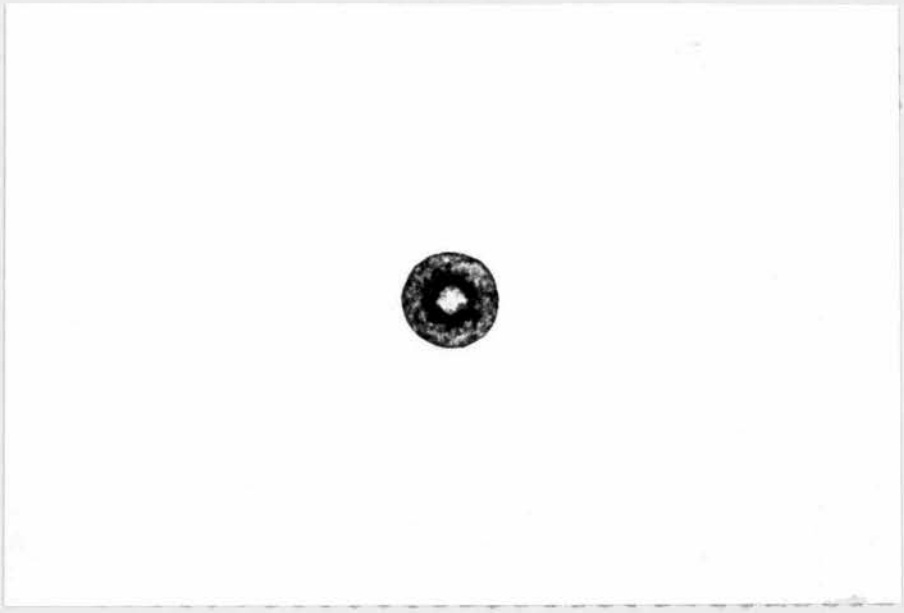
i. Alkaline change in TB through at least three passages, with no corresponding change in AB inoculated in parallel. Certain fungi were also found to be capable of causing the same effects in TB, although much less rapidly than ureaplasmas.

ii. Growth of typical ureaplasma colonies (Fig. 2.1), which on the surface of TA2 appeared brown and granular. Centred and centreless forms occurred, with diameters of up to 100 μm. Growth within the inoculation stab was frequently sheet-like in appearance (Fig. 2.2).

iii. Concomitant alkaline changes in TA2 with the presence of ureaplasma colonies.

Fig. 2.1 Centred ureaplasma colony on agar surface. X 60

Fig. 2.2 Ureaplasma growth in plane of inoculation stab. Dark brown, confluent, sometimes sheet-like colonies are seen. X 15



## PREPARATION OF CONCENTRATED ANTIGEN SUSPENSIONS

Broth media used in the production of antigen concentrates were prefiltered through 0.22 µm a.p.d. membrane filters before inoculation with cultures.

### For hyperimmune serum production

Hyperimmune sera intended for use in characterisation procedures were produced from mycoplasmas pre-passaged at least four times in RBG or RBA, then grown in bulk volumes of these media. Where the antisera produced were intended solely for routine diagnostic purposes, the organisms were grown in OB or AB. The final cultures were incubated for 1 - 4 days, centrifuged at 40,000 g for 30 min at 4°C in a MSE 18 anglehead centrifuge, washed three times in BSS and finally resuspended in sterile d.w. to a 200-fold concentration of the original volume. After homogenisation, the suspensions were disintegrated ultrasonically with a MSE-Mullard Ultrasonic Disintegrator 60 W by the method of Mackay (1969). Based on estimations for total protein by a semi-micro Kjeldahl method, the concentrations of the mycoplasma suspensions were adjusted to 2 mg of protein per ml. A portion of the mycoplasma suspension was emulsified in an equal volume of adjuvant, Bayol and Falba containing 1 mg per ml of BCG or, more recently, commercially-prepared complete Freund's adjuvant (Difco Laboratories). The remainder of the mycoplasma suspension was retained for intravenous use.

For serological tests

Antigen concentrates for use in the indirect haemagglutination and agar gel double diffusion tests were prepared in the same manner as vaccine antigen grown in OB or AB, but final suspension of the organism was in PBS. After ultrasonication and total protein estimations, the suspensions were stored at -20°C or -70°C.

For polyacrylamide gel electrophoresis (PAGE)

Mycoplasmas were grown for two days in 200 ml of AB, which, for glycolytic mycoplasmas, was adjusted to pH 7.6. The cultures were centrifuged as before, washed once in BSS and resuspended in PBS.

PREPARATION OF HYPERIMMUNE SERA

Rabbits were vaccinated by a modification of the method of Morton and Roberts (1967). Primary vaccination with antigen in adjuvant was by the inoculation of 0.1 ml into each hind footpad, 0.1 ml intradermally into eight sites on the flanks and 2 ml intramuscularly into four sites in the shoulders. Three weeks later, 1 ml of antigen in adjuvant was inoculated into each hind leg. Two supplementary i.v. injections, each of 0.5 ml of antigen in d.w., were given one week apart and 4 - 5 weeks after the second intramuscular vaccination. The rabbits were bled out 4 - 7 days later. The separated serum was filtered through 0.22 μm a.p.d. membrane filters and stored in aliquots at -20°C.

Specificity and effectiveness of the antisera produced were tested by the growth inhibition and agar gel double diffusion tests.

In both tests, the antiserum from each individual rabbit was tested against the homologous antigen and against one or two other species of mycoplasmas. The broth medium in which the mycoplasma had been grown was included as a control in the agar gel double diffusion test.

SEROLOGICAL TESTS

Growth inhibition (GI)

The method of Clyde (1964), modified by Dighero, Bradstreet and Andrews (1970) was used. Antiserum discs were prepared by lyophilization of sterile paper discs of 6.5 mm diameter (Mast Laboratories, Liverpool) impregnated with 10 µl of antiserum. Digitonin and sodium polyanethol sulfonate (SPS) discs were prepared by the method of Freundt, Andrews, Ernø, Kunze and Black (1973). Discs soaked with a 1.5% (w/v) ethanolic solution of digitonin (Sigma Chemical Co.) were dried overnight at 37°C and stored at 4°C. Discs soaked with 5% and 20% (w/v) aqueous solutions of SPS (Sigma Chemical Co.) were used wet. The GI test was performed by flooding dried OA plates with 24 or 48 h cultures and dilutions thereof. The excess fluid was removed and the plates dried under a hood for 15 min, after which discs were placed on each plate. The results were read 2 - 10 days later.

The GI test was employed in two capacities:

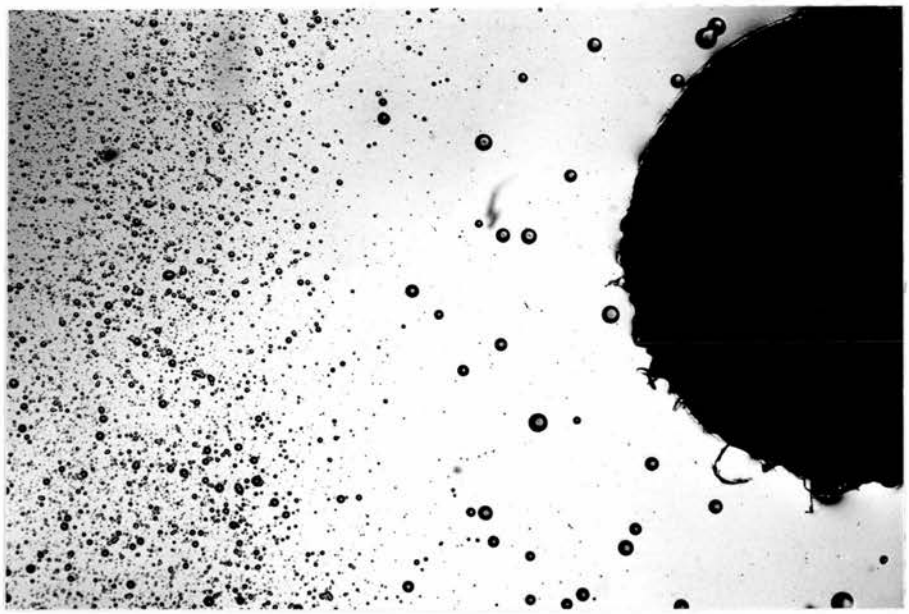
i. As the routine technique for identification of isolates. The test was performed using 50 mm diameter OA plates flooded with undiluted and 1 in 100 dilutions of broth cultures. In

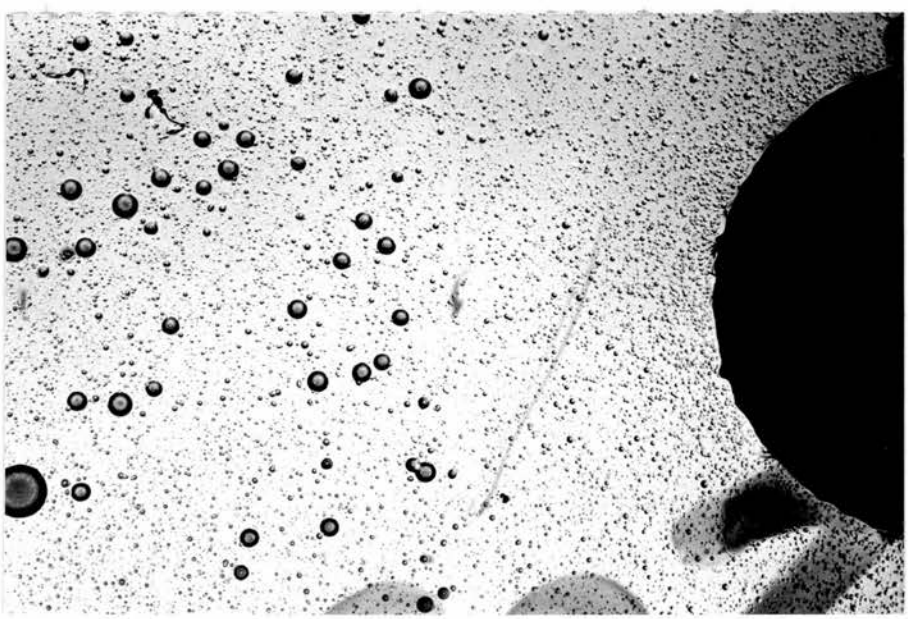
general, all isolates from the ovine respiratory tract were tested against discs containing digitonin and antisera to M. ovipneumoniae, (pooled antisera to strains 956/2, 697, 611 and 672/9; see Chapter 3), M. arginini (strain G230) and A. laidlawii (strain PG8): isolates from nasal swabs were also tested against M. conjunctivae (strain HRC581) antiserum discs. The use of this multi-disc technique frequently permitted the identification of mixed species of mycoplasmas present in the same broth culture, irrespective of the colour change shown (Figs. 2.3 and 2.4). This eliminated in many cases the necessity for cloning cultures before subjection to the GI test. The majority of the family Mycoplasmataceae have been shown to be susceptible to digitonin (and SPS), whereas members of the family Acholeplasmataceae are resistant to these reagents (Williams and Wittler, 1971; Freundt et al., 1973; Ernø and Stipkovits, 1973). Thus the inclusion of digitonin discs in the multidisc test assisted identification by adding confirmation to serological findings, by demonstration of mixed acholeplasma and mycoplasma species in the culture, or, where serological identification was not obtained, by indicating whether subsequent GI tests should employ antisera to acholeplasma or mycoplasma species.

Positive reactions in the GI test were indicated by zones of inhibition around the specific antiserum disc. Frequently, such zones were partially obscured by breakthrough colonies (Fig. 2.5) due to antigenic variation within the species, to excessive colony numbers, to poor antiserum

Figs. 2.3 (upper) and 2.4 (lower)

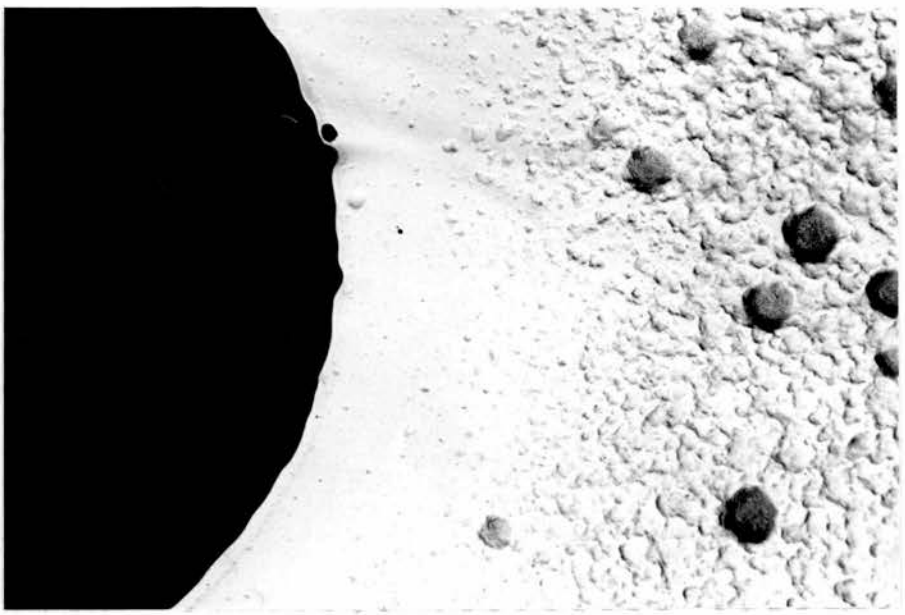
Identification of mixed mycoplasma species present in field material by the multidisc growth inhibition (GI) test. A broth culture obtained from a nasal swab and showing acid change was subjected to the GI test: discs containing antiserum to four mycoplasma species including A. laidlawii (Fig. 2.3) and M. conjunctivae (Fig. 2.4) were placed on the same plate. In each figure, two distinct populations of mycoplasmas are visible. In Fig. 2.3, the smaller but more numerous colonies of A. laidlawii show a zone of inhibition around the disc, whereas the larger colonies, indicated by the converse situation in Fig. 2.4 to be M. conjunctivae, are impervious to the A. laidlawii antiserum.





Figs. 2.5 (upper) and 2.6 (lower)

Identification of field isolates of M. ovipneumoniae by the growth inhibition test. Breakthrough (Fig. 2.5) or bizarre-shaped (Fig. 2.6) colonies are apparent in the zone of inhibition around the disc containing antiserum to M. ovipneumoniae.



quality or to combinations of all three factors. The least difficulty was experienced with M. arginini and M. conjunctivae. Adequate inhibitory zones could always be demonstrated with isolates of these two species by the use of antiserum directed solely against the type strain, provided colony numbers were not excessive. Over-abundant growth was the usual cause for poor results in the GI test for A. laidlawii. Cultures suspected of being this organism were diluted 1 in 10 and 1 in 100 for the GI test. The greatest difficulties were encountered with isolates of M. ovipneumoniae, which as a species demonstrates a broad antigenic spectrum (see Chapter 3): thus antiserum directed against one strain was found to identify only a proportion of field isolates by the GI test. This was overcome by the use of pooled antisera against four strains of M. ovipneumoniae, a technique which has been used in the identification of Mycoplasma hominis (Lin, Alpert and Radnay, 1975). In addition, newly isolated strains of M. ovipneumoniae often provided insufficient colony growth for the demonstration of inhibition zones. Thus fresh isolates were passaged at least twice and generally 3 - 4 times in broth before identification was attempted. Growth inhibition of M. ovipneumoniae was often manifested not only by reduced numbers and size or elimination of colonies around the specific antiserum disc, but also by the presence of bizarre colonies in this area (Fig. 2.6), and a

metabolism inhibition effect in which acidic change of the agar occurred except in the vicinity of the M. ovipneumoniae antiserum and digitonin discs.

ii. In the characterisation of species and for the demonstration of intraspecific antigenic variations. OA plates of 90 mm diameter were flooded with several decimal dilutions of each strain. The antiserum discs were tested in duplicate at each dilution. The results were taken from that dilution which allowed optimal development of inhibition zones commensurate with ease of reading.

Metabolism inhibition (MI)

The method of Taylor-Robinson, Purcell, Wong and Chanock (1966) was used for M. ovipneumoniae, the broth being OB.

Aliquots of cultures stored at -70°C diluted to provide $10^5$ - $10^6$ ccu/0.2 ml were used as antigens. Fresh guinea-pig serum was included at a final concentration of 5.7%. For M. arginini, the method of Purcell, Taylor-Robinson, Wong and Chanock, (1966) and AB broth were used. Tests were run with both stored (-70°C) and 24 h broth cultures, diluted to provide $10^4$ - $10^5$ ccu/0.2 ml. The volumes of guinea-pig serum were also varied; the final concentrations tested were 6.25% and 3.12%. Both M. ovipneumoniae and M. arginini MI tests were performed in U-bottomed microtitration plates (Cookes Engineering Co., Alexandria, Virginia, USA), which were sealed with individual plate sealers.

Difficulties were experienced in the use of the MI test,

particularly in the assay of serum antibodies to M. ovipneumoniae in experimentally-infected animals. Colour changes in the medium, on which the test depends, were often variable within a plate and between plates, and titres obtained inconsistent. Attempts to overcome this by the use of 24 h broth cultures, and by pre-washing the microtitration plates in a NaOH-HCl-deionized water system or with ethanol and deionized water were generally unsuccessful. The test was therefore later discarded in favour of the tetrazolium reduction inhibition test.

Tetrazolium reduction inhibition (TRI)

The technique employed was similar to that of the MI test, except that TRI broth was used and the test was read when the control wells showed the presence of red forazan crystals. Fresh guinea-pig serum was included at a final concentration of 6.25%. Eighteen to 24 h broth cultures of strain 956/2 were used as antigen and were diluted to provide $10^5$ - $10^6$ ccu/0.2 ml.

Indirect haemagglutination (IHA)

The method used was essentially that of Herbert (1967). The test was performed with fresh sheep RBC collected in Alsever's solution. The same source of RBC was used throughout. The cells were washed twice in PBS and stored at $4^{o}$C in Alsever's solution, or latterly stored as whole blood in the same solution. Before use 2 - 10 days later, the cells were washed and finally resuspended in PBS to a concentration of 6%. This suspension was

treated with a 1 in 20,000 solution of tannic acid (BDH Chemicals, Ltd.) in PBS (final concentration), incubated at 37°C for 15 min, then washed once with PBS and sensitized with antigen suspension. The concentrated suspensions of M. ovipneumoniae were found to agglutinate sheep and guinea-pig RBC, and were therefore absorbed for 1 h at room temperature with one quarter of their volume of washed, packed, sheep RBC. The optimal concentration of M. ovipneumoniae antigen was found by chequerboard titrations to be 0.5 mg of protein per ml. The suspensions of M. arginini did not agglutinate sheep RBC and pre-absorption was therefore unnecessary: these were also adjusted to a concentration of 0.5 mg of protein per ml. Equal volumes of the antigen and 6% tanned RBC suspensions were incubated at 37°C for 30 min, during which time the mixture was shaken several times. The cells were then washed three times with 1% "serum saline" and finally resuspended in the same diluent to a concentration of 1% or 1.5%. The "serum saline" used was PBS supplemented with 1% inactivated serum from a hysterectomy-produced, colostrum-deprived lamb that had never been exposed to mycoplasmal infection. The tests were performed in U- or V-bottomed microtitration plates, and were read after 18 h at room temperature. All test samples were inactivated at 56°C for 30 min before titration. The controls comprised known positive and negative sera, the titration of four dilutions of each sample against control cells, and the inclusion of sensitized and control cells with serum-saline only. When the positive control serum did not achieve titres within two dilutions of expected levels, the results were discarded.

Agar gel double diffusion (AGDD)

The method of Ouchterlony (1948) was used. Cleaned slides were flooded with 2% purified agar (Oxoid, Ltd.) to a depth of 5 mm. Wells were cut with a metal punch: the centre well was 10 mm in diameter and 10 mm distant from the peripheral wells, which were 7 mm in diameter. The antigen suspensions contained 1.0 - 1.4 mg protein per ml. The gels were kept in a moist atmosphere at room temperature; photography was performed after five days.

TECHNIQUES EMPLOYED BY MAIN COLLABORATORS

Bacteriology

Media

7% sheep blood agar (Ba) and nutrient broth number 2 (NB2) (Oxoid Ltd.) were employed.

Isolation and culture

Tissue samples of approximately 1 g were homogenized in 10 ml of peptone water, pH 7.0, using a Colworth stomacher 80 (A.J. Seward, London). Viable counts from these suspensions or from swabs shaken in NB2 were performed on Ba. Cultures were incubated aerobically at 37°C overnight.

Identification of P. haemolytica strains

Serotyping was performed by the IHA test described by Shreeve, Biberstein and Thompson (1972).

Serology

The IHA test as described by Biberstein, Gills and Knight (1960) was used.

Virology

Homogenates prepared from lung samples as 6 - 10% (w/v) suspensions in PBS were clarified by centrifugation and filtration through membrane filters of 0.45 μm a.p.d. Primary sheep thyroid or secondary foetal lamb kidney cultures were inoculated with 0.2 ml of the filtrate, adsorbed for 1 h at 37°C, after which maintenance medium was added. The cultures were examined for 7-14 days: if no cytopathic effect was observed, 0.5% guinea-pig erythrocytes were added to test for haemadsorption.

Serology

Assays for antibody to PI3 virus were performed by the haemagglutination inhibition (HAI) test (Smith, 1975).

Histopathology

Unless otherwise stated, blocks were prepared from formol-fixed tissues, post-fixed in a saturated solution of mercuric chloride, dehydrated in alcohols and embedded in paraffin wax. Sections at 6 μm were prepared and stained with haematoxylin and eosin.

The terminologies employed for the pathological changes observed in the lungs of sheep are defined below. This system of nomenclature is of necessity arbitrary, and intended solely for the

provision of descriptions of histopathological syndromes without assumptions as to their aetiology. It may be that several of the syndromes, in particular those facets of atypical pneumonia described by Stamp and Nisbet (1963), do represent different stages or degrees of reaction to a single aetiology, but until their causal factor(s) are known, each must be considered separately.

Lymphoid hyperplasia

Lymphoid hyperplasia (Fig. 2.7) is defined as the formation around air-passages and blood vessels of lymphoid nodules with germinal centres. This hyperplasia frequently causes constriction of bronchiolar lumina, and is often associated with collapse of surrounding lung tissue. Lymphocytes, macrophages and plasma cells may be observed in the bronchial and bronchiolar mucosa. Macrophages, lymphocytes and occasionally neutrophils are seen in the alveoli. A mild perivascular fibrosis occurs. These changes correspond closely with the description of "peribronchitis nodosa" by Pavlov (1969).

Interstitial thickening

Interstitial thickening (Figs. 2.7, 2.8) is defined as mild increase in cellularity of alveolar septa without resultant occlusion of alveolar lumina.

Interstitial pneumonia

Interstitial pneumonia (Fig. 2. 9) is defined as consolidation of lung parenchyma due to marked interstitial thickening,

accompanied by variable degrees of peribronchial, peribronchiolar and perivascular lymphoid cell cuffing. Only occasional small amounts of cellular exudate are found in the alveoli and bronchioles.

Proliferative exudative (P.E.) pneumonia

P.E. pneumonia (Figs. 2.10, 2.11) is defined as consolidation of lung parenchyma due to the presence of marked lymphoid hyperplasia; interstitial thickening; collapse of alveoli; exudation of neutrophils and macrophages into alveoli, bronchioles and bronchi; degenerative or hyperplastic changes in bronchiolar epithelium; and Type II (great alveolar) cell proliferation. Epithelialization, thought to be due to complete replacement of normal alveolar epithelium with Type II cells (Alley and Manktelow, 1971) may be observed. Hyaline scars are also frequently present. P.E. pneumonia corresponds with the interstitial form of atypical pneumonia of Stamp and Nisbet (1963), and is closely similar to the "chronic enzootic pneumonia" of Alley and Manktelow (1971), to the "lobar pneumonia" of Sullivan, St. George and Horsfall, (1973a and b) and to the "pneumonitis lymphocytaria" of Pavlov (1969).

Congestive pneumonia

Congestive pneumonia (Fig. 2.12) is defined as an acute condition often affecting the whole lungs, which have a dark pink or purple appearance. Microscopically, there is alveolar oedema and congestion, some haemorrhage and slightly increased numbers of macrophages within the alveoli. Neutrophil exudation

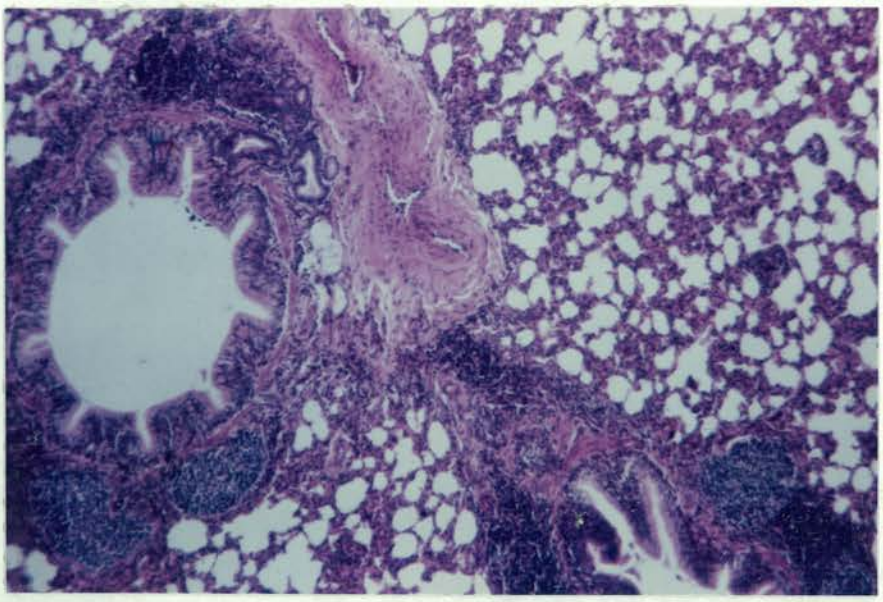
is not observed. Affected and normal areas of lung merge indistinctly.

Purulent bronchopneumonia

Purulent bronchopneumonia (Fig. 2.13) is defined as necrotizing bronchiolitis and alveolitis, with inflammatory cell exudate, serum leakage and haemorrhage.

Fig. 2.7 Lung section showing lymphoid hyperplasia with interstitial thickening.
Haematoxylin and eosin X 165

Fig. 2.8 Lung section showing mononuclear cell infiltration leading to interstitial thickening but not alveolar collapse.
Haematoxylin and eosin X 96



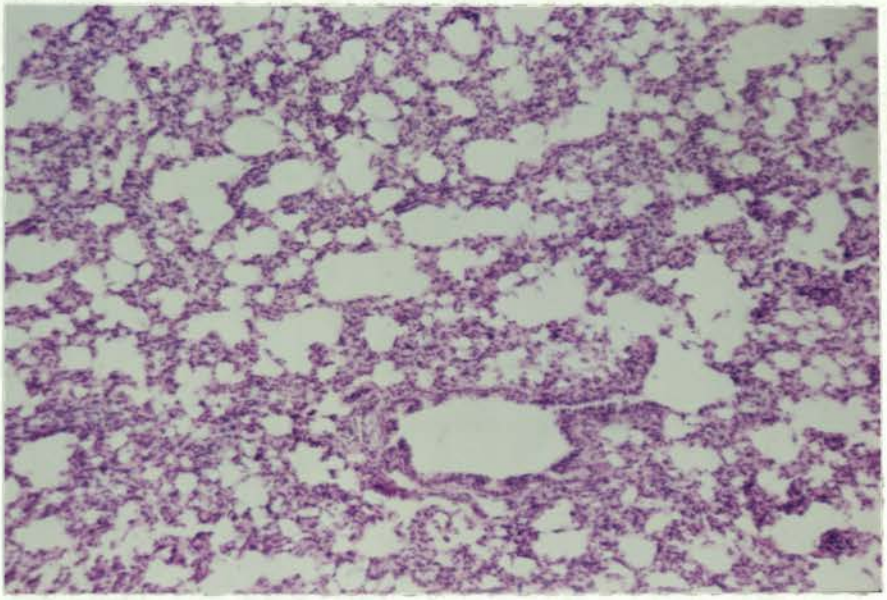
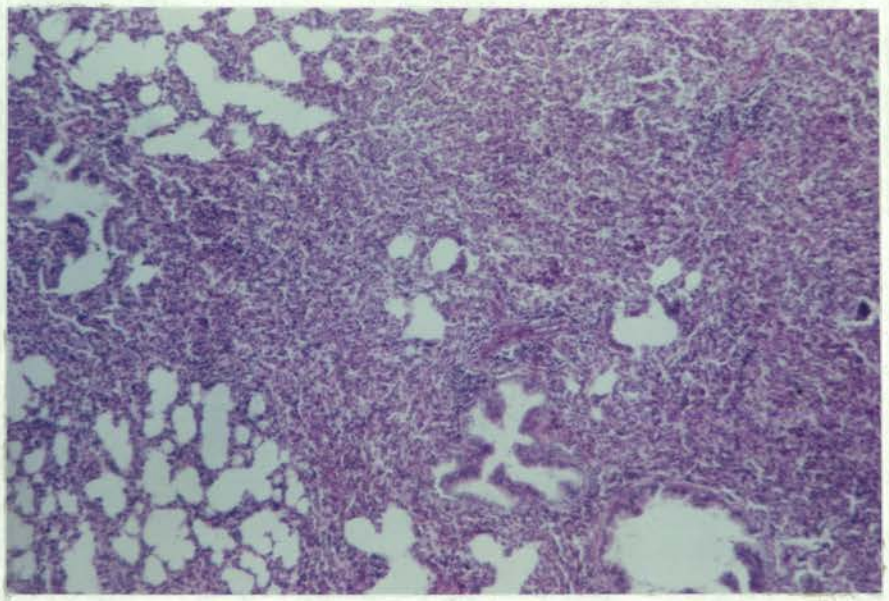


Fig. 2.9 Lung section showing heavy mononuclear cell infiltration of interalveolar septa leading to interstitial pneumonia and collapsed areas. No cellular exudate is present.
Haematoxylin and eosin X 165

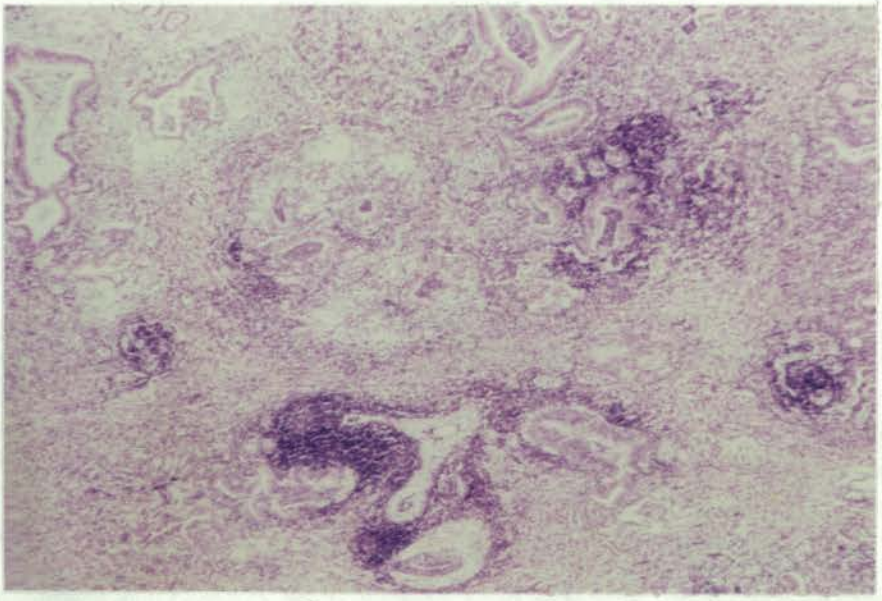


Figs. 2.10 (upper) and 2.11 (lower)

Lung sections showing P.E. pneumonia. Bronchiolar epithelial hyperplasia, peribronchiolar and perivascular lymphoid cell cuffing, alveolar collapse, interstitial thickening and hyaline scar formation (in Fig. 2.10 only) are apparent.

Haematoxylin and eosin

Fig. 2.10 X 43
Fig. 2.11 X 400



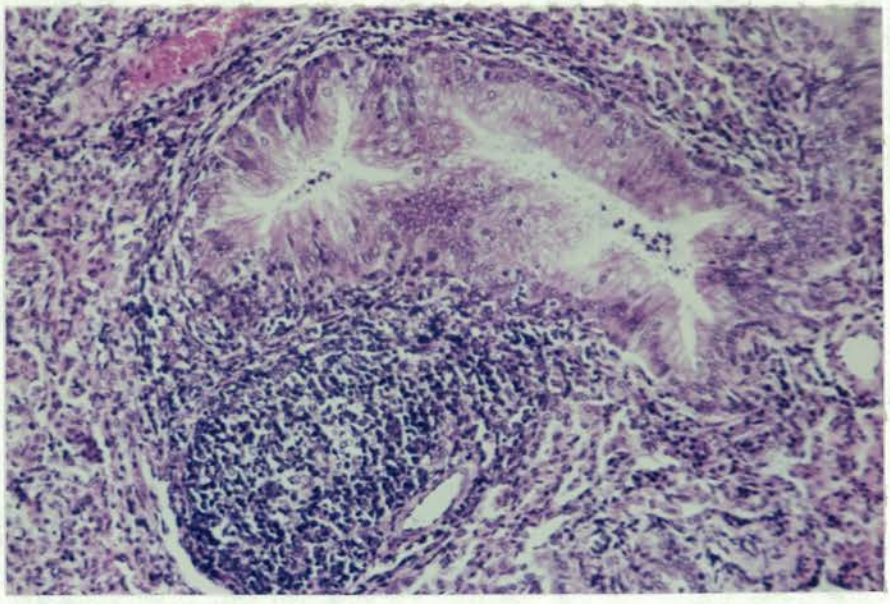
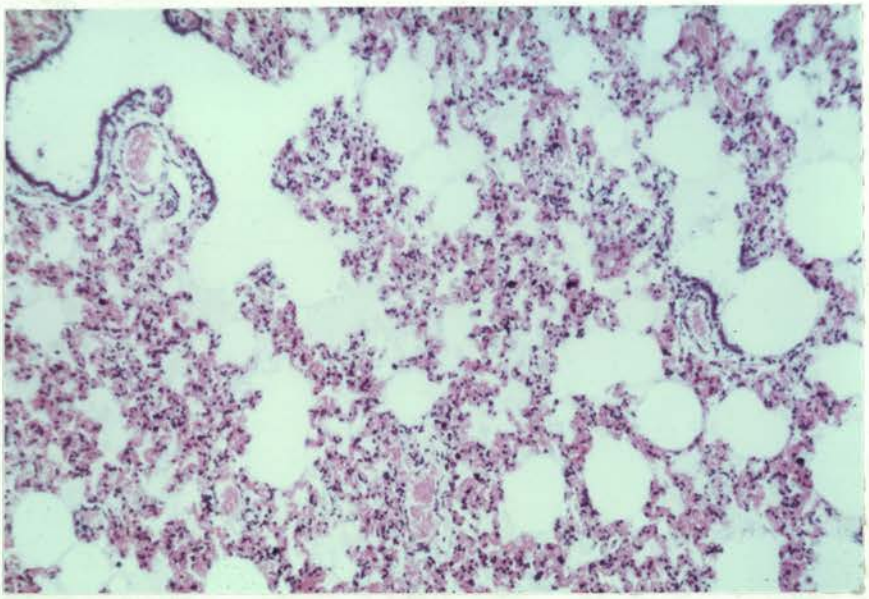


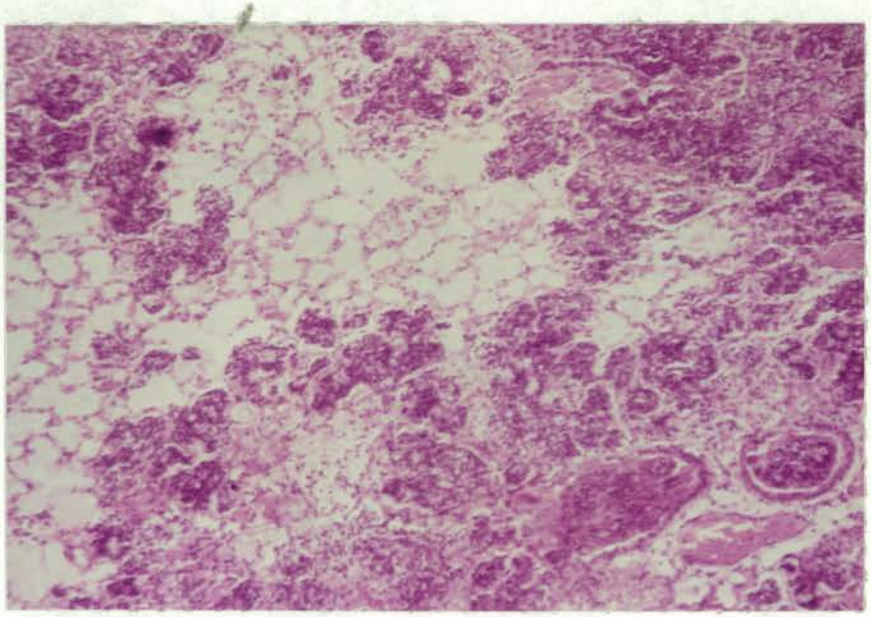
Fig. 2.12 Lung section showing congestive pneumonia. Alveolar oedema, congestion, haemorrhage and increased numbers of macrophages are present.

Haematoxylin and eosin X 96

Fig. 2.13 Lung section showing purulent bronchopneumonia. Heavy inflammatory cell exudate, necrotizing alveolitis, serum exudate and bronchiolitis are present.

Haematoxylin and eosin X 96





# CHAPTER 3

## THE COMPARISON OF A REPRESENTATIVE STRAIN OF M. OVIPNEUMONIAE WITH SIMILAR ORGANISMS ISOLATED FROM SHEEP IN SCOTLAND

### INTRODUCTION

The change in mycoplasma media employed, from Mackay's Medium No. 2 to OA and OB (Chapter 2), resulted in markedly improved efficiency of isolation and propagation of mycoplasmas with biological characteristics similar to M. ovipneumoniae. Conclusive identification of such isolates necessitated comparison with a representative strain of M. ovipneumoniae: though no type strain for the species had been formally designated by Carmichael et al. (1972), strain Y98 had been used by these workers as representative of their isolates. Complete characterisation of the biochemical properties of M. ovipneumoniae, or serological comparison with all known species of mycoplasmas as required by the Subcommittee of the Taxonomy of Mycoplasmatales (1972) had not been performed. Thus the several aims of the comparison of nine Scottish mycoplasma strains with strain Y98 were to establish the identity of the Scottish strains, to investigate possible variations among isolates and to extend the biochemical and serological characterisation of M. ovipneumoniae.

### MATERIALS AND METHODS

#### Strains examined

These are listed in Table 3.1. All strains isolated at this laboratory were cloned at least four times. The possibility

Table 3.1 Strains of ovine glycolytic mycoplasmas examined

| Strain number | Origin of strains | |
|---|---|---|
| | Material | Animal |
| 7Z97 | Lung | Naturally-occurring case of SPA |
| 8Z56 | Lung | Naturally-occurring case of SPA |
| 611 | Lung | Naturally-occurring case of SPA |
| 652 | Lung | Naturally-occurring case of SPA+ |
| 658 | Lung | Experimental SPA case produced by the intra-tracheal inoculation of SPA lung material |
| 650/21 | Nasal swab | Apparently healthy 6-month-old lamb |
| 672/9 | Nasal swab | One of a flock of housed adult sheep showing pneumonic symptoms |
| 956/2 | Nasal swab | One of a flock of ewes at grass showing pneumonic symptoms |
| 697 | Lung | Housed lamb with pneumonia |
| Y98* | Lung | Sheep |

* The Queensland strain of M. ovipneumoniae obtained from Dr. T.D. St. George.

\+ Isolated initially in primary sheep-lung cultures.

that the strains were L-forms was eliminated by five consecutive subcultures on OA from which bacterial inhibitors had been omitted. No reversion to bacterial forms occurred.

Isolation media

Isolation and propagation of strains were performed with OA and OB.

Biochemical test media

The basic media, EA and EB, were based on OA and OB respectively but with MEM substituted for Medium 199 and phenol red omitted unless specifically required. All strains were subcultured twice in EB before they were tested. Paired broth cultures were incubated aerobically and anaerobically, overlaid with sterile paraffin. Paired agar plates were incubated, one in a candle-jar and the other in an atmosphere of 95% $N_2$ and 5% $CO_2$, as described by Aluotto, Wittler, Williams and Faber (1970). For all biochemical tests, controls included test media inoculated with sterile broth or agar blocks.

Biochemical tests

The oxidation-fermentation, phosphatase production, optochin sensitivity and tetrazolium, tellurite and methylene blue reduction tests were performed according to the methods of Aluotto et al. (1970) but with EA or EB as the basic medium. The complete methods and media of Aluotto et al. (1970) were used for the hydrolysis of gelatin and for the casein and serum digestion tests. The inhibitory effect of 0.02% methylene blue on 24 h broth cultures was

investigated by the method of Kraybill and Crawford (1964) but EB was used as the culture medium. The test for the hydrolysis of aesculin was performed by the method of Williams and Wittler (1971), with EA as the basic medium. Ability to form "films and spots" was examined on (a) EA containing 10% (v/v) of concentrated egg yolk emulsion (Difco Laboratories) and 5% (v/v) of inactivated swine serum (Fabricant and Freundt, 1967), and (b) EA incorporating 20% (v/v) of inactivated swine serum and 50% of MEM. Hydrogen peroxide production was detected by two methods, with EA as the basic medium: (a) the technique of Lind (1970), in which human group O cells and methylene blue solution are added to mycoplasma colonies. Hydrogen peroxide production is indicated by blue staining of the RBC in contact with the colonies. (b) the technique of Cole, Ward and Martin (1968), in which cultures were inoculated onto EA supplemented with 4% (v/v) sheep blood and 0.01% (w/v) benzidine hydrochloride. Hydrogen peroxide production is indicated by brownish colouration of the colonies and underlying agar.

The determination of specific substrate requirement was performed as follows. The medium used (TSM) comprised (v/v) 60% MEM, 18% BSS, 10% brain-heart infusion, 1% serum fraction (Difco Laboratories), 1% fresh yeast extract and 10% test substrate solution, and was supplemented with ampicillin, thallous acetate and phenol red at the same concentrations as in OB. Before addition of the test substrates, the medium was exhaustively dialysed against several changes of BSS, filtered through Millipore filters of 0.22 μm a.p.d., and adjusted to pH 7.2. The test substrates

contained 10% (w/v) of either glucose, arginine or urea in d.w. The control substrate was BSS. Suspensions of organisms were prepared according to the method of Edward (1971): 1-2 d.o. cultures were centrifuged at 3600 g (5500 r.p.m.) at 4°C for 45 min in an MSE 18 angle-head centrifuge. The deposits were washed three times with BSS and resuspended in medium (PM), which was BSS containing 1% (v/v) serum fraction and the same concentrations of antibacterial supplements as in OB. The test method consisted of pipetting 0.2 ml of the organism suspension or sterile PM into 1.8 ml of test and control TSM, and measuring the pH of the broth after aerobic incubation for 1 - 2 days.

Sensitivity to antibacterial substances

Sensitivities of the mycoplasmas examined to digitonin, SPS, optochin (Oxoid, Ltd.) 0.025% (w/v) and antibiotics were determined with impregnated discs by the same flooded plate technique used in the GI test. All antibiotic discs were obtained from Mast Laboratories Ltd., Liverpool, except those containing tylosin which were from Elanco Products Ltd., London.

Colony and organism morphology

Colonies were photographed through transmitted light. Smears prepared from 18 h broth cultures which had been centrifuged at 3600 g for 45 mins and resuspended in PBS were stained with MacNeal's stain.

Electron microscopy

Material for thin-sections was prepared by prefixing 18-24 h broth cultures of strain 956/2 by the addition of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The final concentration of fixative was adjusted to 0.5% and calcium chloride was added to a final concentration of 0.05%. After prefixation for 2 h at 4°C, the suspensions were centrifuged at 3600 g for 15 mins. The pellets were fixed for 2 h at 4°C in buffered 2.5% glutaraldehyde, washed briefly with buffer and fixed again for 1 h in aqueous 1% osmium tetroxide. The pellets were exposed to 0.5% uranyl acetate in Michaelis buffer pH 5.0 for 2 h at 20°C. They were then dehydrated in graded alcohols and embedded in araldite. Thin sections were stained with lead citrate and examined with a Siemens Elmiskop 1 electron microscope. For capsule staining, ruthenium red was added at a concentration of 700 p.p.m. during both prefixation and fixation steps (Luft, 1971).

Direct examination of mycoplasmas was performed by prefixation and pelleting of 18 - 24 h broth cultures as before. Pellets were washed in d.w., and then resuspended in 1% phosphotungstic acid pH 5 - 8, 1% ammonium molybdate pH 5 - 8, and 2% methylamine tungstate pH 6.4. A drop was placed on copper grids, coated with carbon-Formvar, the surplus removed and the grid allowed to dry.

Filtration studies

Filtration experiments were performed with 24 h broth cultures by means of Millipore 25 mm filter holders and filters of 0.8 μm,

0.45 μm and 0.22 μm a.p.d. The filtrations were performed sequentially on one sample of each test strain. The numbers of organisms (c.f.u./ml) were titrated before and after each filtration.

Serological studies

Antisera were produced against strain Y98 and four Scottish strains (956/2, 697, 658 and 611). Because of import regulations it was necessary to incorporate formaldehyde in the vaccine antigen of Y98 to a final concentration of 1 in 2000. The cross-reactions of these five strains were compared by the IHA and AGDD tests, and the five antisera were titrated against all 10 mycoplasma strains studied by the MI and GI tests. In addition, four strains (956/2, Y98, 658 and 611) were titrated against 24 antisera by the IHA test, and strains 658 and Y98 were tested against 40 antisera by the MI test.

Statistical analysis of results

Antibody-titre ratios between the five strains tested in cross-serological reactions were calculated from the MI and GI tests by the method of Archetti and Horsfall (1950) as applied by Gois, Kuksa, Franz and Taylor-Robinson (1974). The geometric mean of the ratios is given by the formula $r = r_1 \times r_2$ where

$$r_1 = \frac{\text{heterologous titre (mycoplasma 2)}}{\text{homologous titre (mycoplasma 1)}}$$

$$r_2 = \frac{\text{heterologous titre (mycoplasma 1)}}{\text{homologous titre (mycoplasma 2)}}$$

The rank correlation coefficient of the two tests for each antiserum was calculated by the method of Spearman (1904), from the formula

$$r_s = 1 - \frac{6\ d^2}{n(n^2 - 1)}$$

where $r_s$ is the rank correlation coefficient, $d^2$ is the sum of the squares of the values obtained by subtraction of ranked pair values and n is the number of strains compared.

PAGE

Phenol-acetic acid-water (2:1:0.5 v/v/v) extraction of the sedimented organisms and PAGE of the extracted proteins was essentially as described by Forshaw (1972). The gels, which contained 35% (v/v) acetic acid, 5 M urea and 7.5% (w/v) acrylamide, were stained with amido black. Background stain was removed electrolytically. The gels, in tubes, were photographed through similar tubes containing water, as described by Fiske (1974).

RESULTS

Biochemical reactions

The biochemical reactions of the 10 strains examined are summarised in Table 3.2. In general, the reactions of all strains were very similar and differed only in degree. The tests in which the greatest variations were observed were methylene-blue reduction (anaerobic conditions) and hydrogen peroxide production as determined by the method of Lind (1970).

Table 3.2 Summary of the biochemical and haemolytic reactions of the 10 ovine mycoplasma strains examined.

| Biochemical tests | Reaction* |
|---|---|
| Glycolysis | + |
| Oxidation-fermentation | Fermentation |
| Phosphatase production | - |
| Tetrazolium reduction | |
| (i) aerobic | ± to + |
| (ii) anaerobic | + |
| Tellurite reduction aerobic and anaerobic | Complete or partial growth inhibition (no reduction) |
| Methylene-blue reduction (0.003%) | |
| (i) aerobic | ± to + |
| (ii) anaerobic | ± to ++ |
| Methylene-blue inhibition (0.02%) | Inhibition |
| Hydrogen-peroxide production | |
| (i) Lind (1970) | ± to ++ |
| (ii) Cole et al. (1968) | + |
| Casein digestion | - |
| Serum digestion | - |
| Gelatin hydrolysis | - |
| Aesculin hydrolysis | - |
| "Films and spots" production | |
| (i) 20% swine serum | - |
| (ii) 10% egg yolk emulsion plus 5% serum | - |
| Haemolysis of erythrocytes | |
| (i) sheep | ∝pr or Br |
| (ii) guinea-pig | ∝p or B |
| (iii) horse | ∝p or B |
| (iv) human group 0 | ∝ or ∝p |

* - = Negative, ± = weak, + = moderate, and ++ = strong reaction; ∝ = green zone around colonies; ∝p = clear area, with some unhaemolysed erythrocytes remaining in the zone around colony; B = clear, cell-free zone around colonies; r = green ring around indicated zone type.

Sensitivity to antibacterial substances

The sensitivities of the strains to optochin, digitonin, SPS, and 11 antibiotics are given in Table 3.3. All strains were sensitive to digitonin and SPS, although sensitivity to SPS (5%) was low, possibly because of the use of undiluted broth cultures as inocula. All strains were also sensitive to kanamycin, chlortetracycline, oxytetracycline, gentamycin and tylosin, and resistant to erythromycin, streptomycin, oleandomycin, polymixin B and nystatin. Two strains only (Nos. 956/2 and 650/21) were sensitive to neomycin.

Colony and organism morphology

Colonies are generally centreless, with a granular, radially-striated or reticular appearance (Figs. 3.1, 3.2), although fresh field isolates or cultures grown on solid media with lowered concentrations of agarose occasionally show the presence of an ill-defined centre (Fig. 3.3). Confluence of colonies readily occurs where inoculum titres are high (Fig. 3.4).

Smears of broth cultures stained by MacNeal's method generally show the organismsas ring or coccoid forms with uni-, bi- or tri-polar accumulations of material (Fig. 3.5). These bodies are approximately 1100 x 750 nm in size, although large ring forms of up to 2500 x 2000 nm in size, and small densely-staining bodies are also seen.

By the electron microscope, the organisms appear in thin sections as round, coccoid, oval or kidney-shaped bodies with a mean size of 975 nm x 600 nm (Fig. 3.6). Filament formation was

Figs. 3.1, 3.2, 3.3 and 3.4

Different colony morphologies of M. ovipneumoniae. Fig. 3.1 (upper left, X 15) shows centreless, granular, radially-striated colonies. The colonies in Fig. 3.2 (upper right, X 15) are less granular, and some show slight centres, a feature more clearly apparent in Fig. 3.3 (lower left, X 15). Confluence of colonies readily occurs where inoculum titres are high (Fig. 3.4, lower right, X 5).

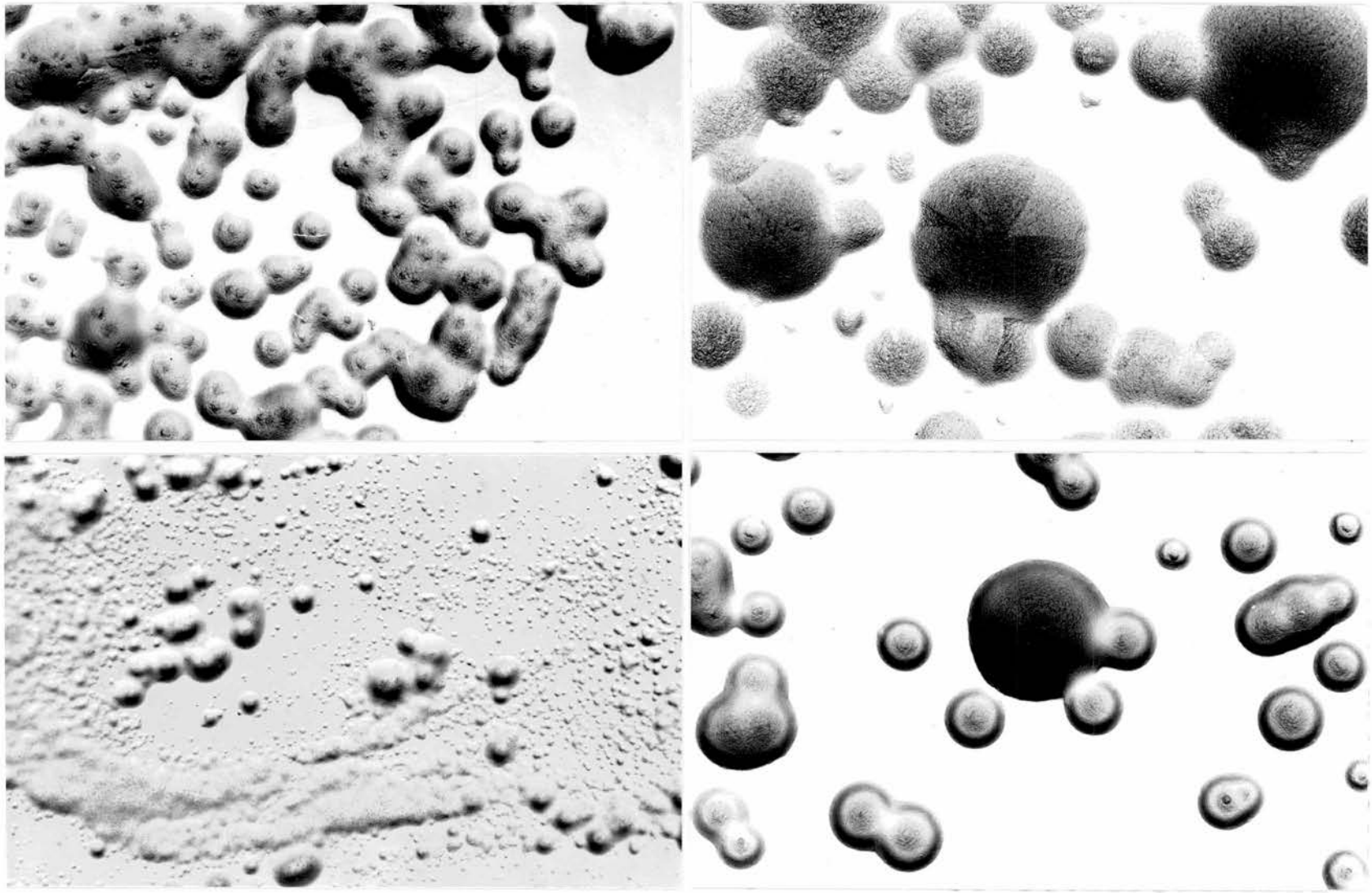
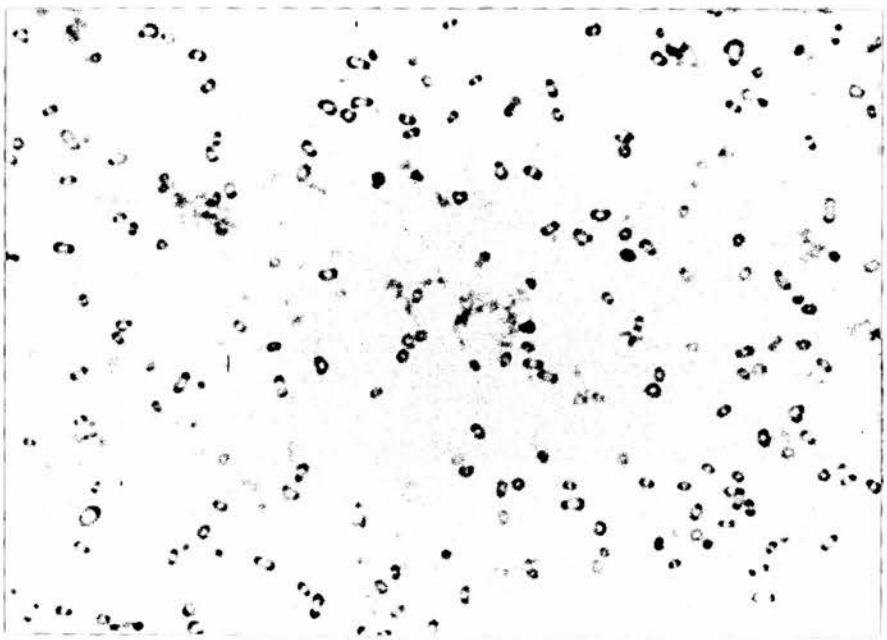
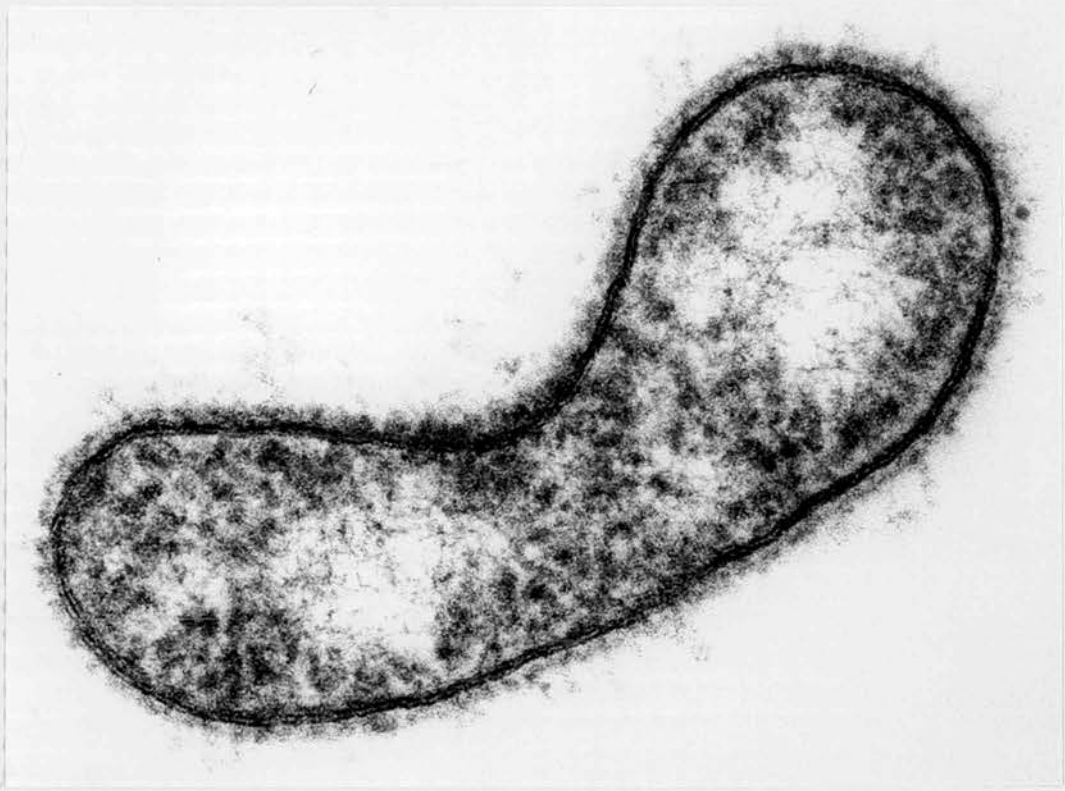


Fig. 3.5 Smear of broth culture of M. ovipneumoniae (strain 956/2) stained by MacNeal's method. The organisms display pleomorphism, but generally appear as round or coccal ring forms, often with uni, bi- or tri- polar accumulations of material. X 1800



Figs. 3.6 (upper) and 3.7 (lower)

Electron micrographs of thin sections of *M. ovipneumoniae* (strain 956/2) stained in uranyl acetate, lead citrate and ruthenium red. The cells contain amorphous ribosomes and strands of DNA, which in Fig. 3.6 (X 180,000) are at each end of the cell, suggesting binary fission. The cells lack capsule, but the trilaminar membrane is coated with extramembraneous material which frequently has a segmented appearance (Fig. 3.7, X 240,000).



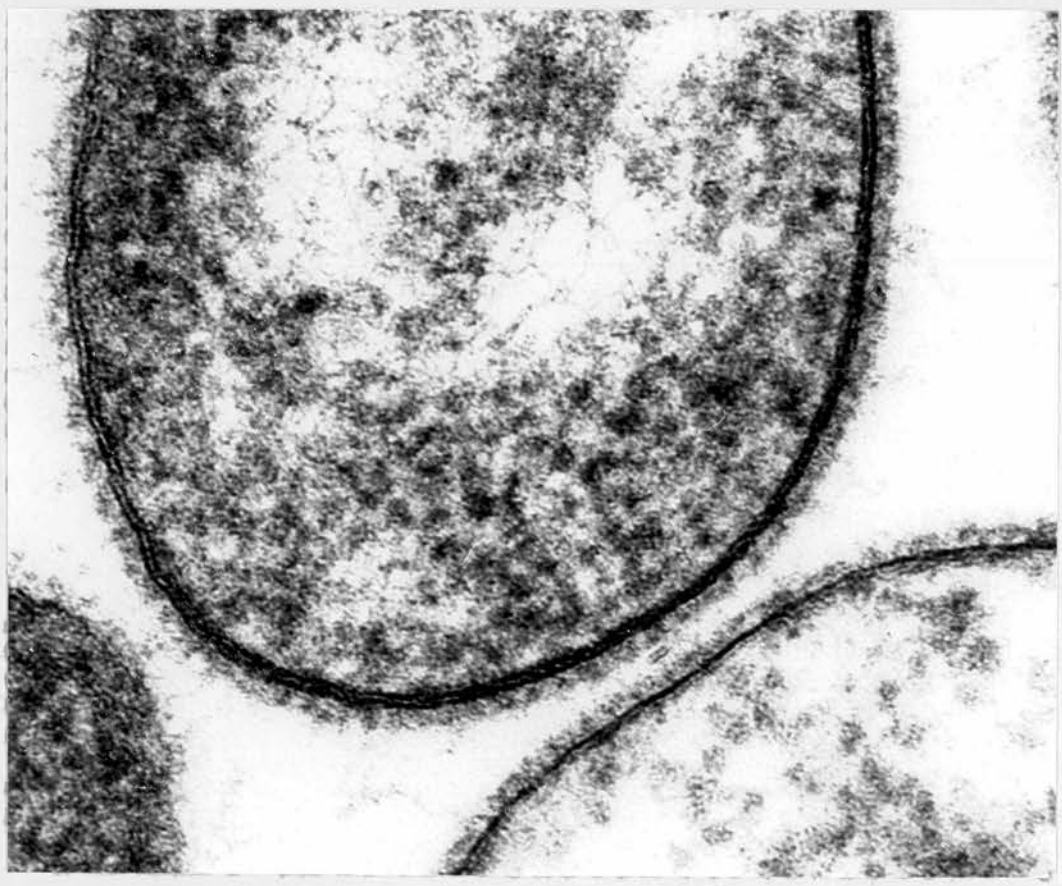


Fig. 3.8 Electron micrographs of whole cell preparations of M. ovipneumoniae (strain 956/2) stained by ammonium molybdate (upper), phosphotungstic acid (centre) and methylamine tungstate (lower).
X 60,000

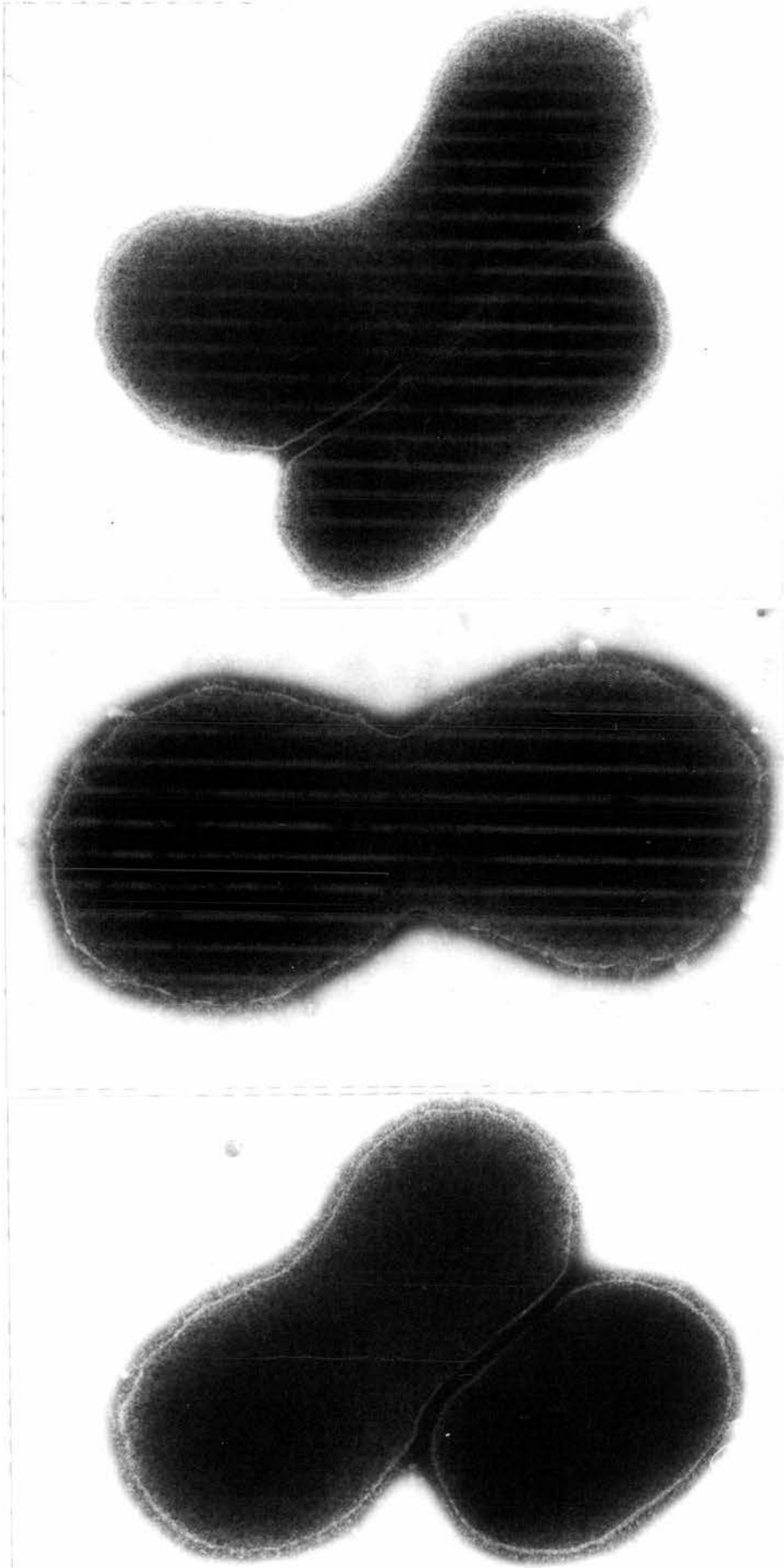


Table 3.3 Sensitivity of ovine mycoplasma strains to optochin, digitonin, SPS, antibiotic and fungicide discs

| Strain Number | Size of zone of inhibition (mm) of growth of the stated strain produced by | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OP | DIG | SPS 5% | SPS 20% | K | E | NE | CT | S | OL | NY | OT | GM | PB | TY |
| 956/2 | <1 | 2 | 3 | 5 | 10 | <1 | 5 | 15 | 0 | 0 | 0 | >20 | 13 | 0 | 13 |
| 650/21 | <1 | 2.5 | 0.5 | 3 | 8 | 1 | 6 | 9 | 0 | 0 | 0 | 10 | 10 | 0 | 13 |
| Y98 | <1 | 1.5 | 2.5 | 6 | 10 | 0 | 0 | 15 | 0 | 0 | 0 | 6 | 6 | 0 | 5 |
| 697 | 1 | 1 | 1.5 | 3 | 15 | 0 | 1 | 11 | 0 | 0 | 0 | 10 | 5-10 | 0 | 15 |
| 652 | 1 | 2.5 | 1 | 1.5 | 5-10 | 0 | <1 | 13 | 0 | 0 | 0 | 15 | 10 | 0 | 15 |
| 658 | <1 | 3 | 1.5 | 3 | 5 | 0 | 1 | 15 | 0 | 0 | 0 | 15 | 8 | 0 | 10 |
| 7Z97 | <1 | 3 | 2.5 | 4 | 10 | 0 | 0 | 10 | 0 | 0 | 0 | 15 | 5 | 0 | 15 |
| 8Z56 | <1 | 4 | 2 | 5 | 13 | 0 | 0 | 15 | 0 | 0 | 0 | 15 | 5-10 | 0 | 15 |
| 611 | <1 | 1.5 | 1 | 6 | 5 | 0 | 0 | 12 | 1 | 0 | 0 | 10-15 | 4 | 0 | 10 |
| 672/9 | <1 | 2.5 | 2 | 3 | 10 | 0 | 1 | 16 | 0 | 0 | 0 | 15 | 10 | 0 | 10 |

OP = Optochin; DIG = digitonin; SPS = sodium polyanethol sulphonate; K = kanamycin (30 μg); E = erythromycin (5 μg); OL = oleandomycin ( 5 μg); NY = nystatin (100 units); OT = Oxytetracycline (25 μg); GM = gentamicin (10 μg); PB = polymixin B (100 units); TY = tylosin (30 μg).

not observed, but large numbers of apparently dividing forms were generally present. The limiting membrane of cells consists of a three-layered structure 7.5 nm thick, comprising two electron-dense bands separated by an electron lucent layer (Fig. 3.7). An extramembraneous coat approximately 13 nm thick surrounds the membrane. This coat is of varying electron density and may appear segmented. Material processed with and without ruthenium red showed no differences in appearance. Bodies demonstrate a variable amount of cellular content, but within each cell strands and amorphous material are present which correspond respectively with the descriptions for DNA material and ribosomes in other mycoplasma species (e.g. Anderson, 1969).

The three stains used in preparations of whole cells showed no major differences in the morphology of bodies, but best definition was achieved by the use of methylamine tungstate (Fig. 3.8). Such preparations showed good correlation with the morphology of sectioned organisms, but the mean size of bodies, 1350 nm (S.E. $\pm$ 35 nm) x 645 nm (S.E. $\pm$ 16 nm), was larger than observed in thin-section preparations. Cellular contents are electron dense, and the limiting membrane appears as an electron lucent band surrounded by an extramembraneous coat of approximately 25 nm in thickness.

Filtration studies

The results for two strains are presented in Table 3.4. Both strains failed to pass through filters of 0.22 $\mu$m a.p.d., and showed considerable reduction in titre following passage through filters of

Table 3.4 Filtration studies with two ovine mycoplasma strains

| Filtration details | Titre (c.f.u. per ml) of strains | |
|---|---|---|
| | 658 | Y98 |
| Unfiltered | $1.0 \times 10^9$ | $5.5 \times 10^8$ |
| Filter 0.80 μm a.p.d. | $1.1 \times 10^7$ | $6.8 \times 10^6$ |
| Filter 0.45 μm a.p.d. | $6.6 \times 10^3$ | $1.1 \times 10^3$ |
| Filter 0.22 μm a.p.d. | 0 | 0 |

a.p.d. = Average pore diameter.

c.f.u. = Colony forming units.

The filtrations were performed sequentially on one sample of each test strain.

0.45 μm. This might have been due to growth in clumps.

AGDD tests

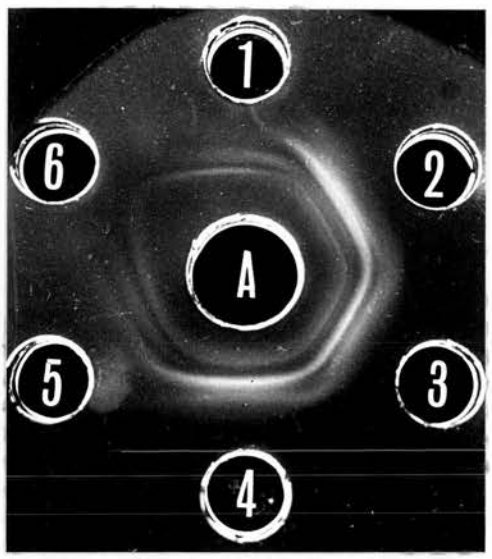
The antigen suspensions of the five selected strains showed three to seven precipitin lines against each of the five prepared antisera (Figs. 3.9, 3.10). The number of lines produced by each mycoplasma suspension varied little with different antisera. Fewest lines (three to four) were given by strains 956/2 and Y98. Strain 697 produced four to five lines against each antiserum, No. 658 produced four to six lines and No. 611 produced four to seven lines.

IHA tests

The results of IHA cross-titration tests between the five strains to which antisera were raised are given in Table 3.5. No significant differences could be demonstrated between any of the strains. In the same tests four of the strains were titrated against antisera to 24 other mycoplasma species (Table 3.6). These titrations revealed moderate reactivity against four antisera (M. gallinarum, M. gallisepticum, M. neurolyticum and M. spumans) and high reactivity to a further four antisera (M. anatis, M. hyorhinis (strains 7 and GDL), M. maculosum and M. pulmonis). However, of the strongly reacting antisera only the antiserum to M. hyorhinis (strain GDL) showed reactivity by the AGDD test, a single faint precipitin line developing between this antiserum and all five antigen wells.

Figs. 3.9 and 3.10

Agar gel double diffusion, with antisera to strains 658 (well A) and 611 (well B) in the centre wells, and concentrated mycoplasma suspensions in wells 1 - 5 (1 = No. 956/2, 2 = No. 611, 3 = No. 658, 4 = No. 697, 5 = No. Y98). Well 6 contains normal rabbit antiserum.


1
6
2
A
5
3
4

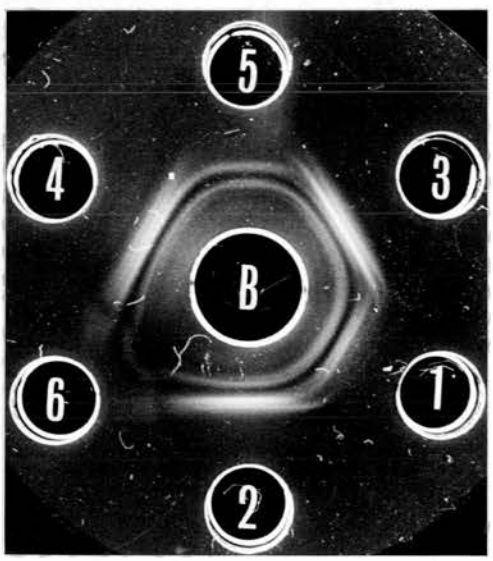

5
4
3
B
6
1
2

Table 3.5 Titres obtained in cross-titration of five ovine mycoplasma strains by the indirect haemagglutination test

| Strain number | Titres for the stated strain of rabbit antisera prepared against strains | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | 81,920 | 163,840 | 40,960 | 327,840 | 81,920 |
| Y98 | 20,480 | 20,480 | 10,240 | 5,120 | 5,120 |
| 697 | 40,960 | 10,240 | 40,960 | 20,480 | 20,480 |
| 658 | 5,120 | 640 | 2,560 | 2,560 | 2,560 |
| 611 | 10,240 | 320 | 2,560 | 1,280 | 2,560 |

Homologous titres are underlined.

Table 3.6 Titres obtained in indirect haemagglutination tests with ovine mycoplasma strains against reference antisera to other mycoplasmas

| Antiserum against strain | Titres of the stated antiserum as demonstrated by ovine mycoplasma strain | | | |
|---|---|---|---|---|
| | 956/2 | Y98 | 658 | 611 |
| M. anatis (1340)* | 640-1280 | 80 | 320-640 | 640 |
| M. arginini (G230)+ | ND | 10 | 20 | 10-20 |
| M. arthritidis (PG6) | ND | 10 | 20 | 20 |
| M. canis (PG14) | 40 | 20 | 40-80 | 40 |
| M. capri (PG3) | 10 | <10 | <10 | <10 |
| M. fermentans (PG18) | 10 | <10 | 10 | <10 |
| M. gallinarum (PG16) | 160-320 | 80 | 80 | 80 |
| M. gallisepticum (PG31) | 80 | 160-320 | 160 | 80-160 |
| M. granularum (BTS39) | 10 | <10 | 10 | <10 |
| M. hyorhinis (7) | 320-640 | 160-320 | 320 | 320 |
| M. hyorhinis (GDL) | >1280 | 1280 | 640-1280 | 1280 |
| M. iners (PG30) | 80 | 40 | 40 | 40 |
| M. maculosum (PG15) | 640 | >1280 | >1280 | >1280 |
| M. meleagridis (17529) | 40 | 10 | 20 | 40 |
| M. mycoides (PG1) | <10 | <10 | <10 | <10 |
| M. neurolyticum (TypeA) | ND | 80 | 320 | 320 |
| M. orale 1 (CH-19299) | ND | 20 | 20 | 40 |
| M. orale 2 (CH-20247) | 40 | ND | 40 | 20-40 |
| M. orale 3 (DC-333) | 40 | <10 | 20 | 20 |
| M. pneumoniae (FH-Liu) | 10 | 10 | 10 | 10 |
| M. pulmonis (Ash) | 1280 | 640-1280 | 320-640 | 1280 |
| M. salivarium (PG20) | <10 | <10 | <10 | <10 |
| M. spumans (PG13) | 320 | ND | 40 | 40 |
| Acholeplasma laidlawii (PG8) | 40 | 40 | 80 | 80 |

All sera were titrated in duplicate.
ND = not done.
* = Strain designations are given in parenthesis.
\+ = Prepared by the author by the method described in the text. All other mycoplasma reference antisera were obtained from the National Institutes of Health, Bethesda, USA.

MI and GI tests

Tables 3.7 (MI tests) and 3.8 (GI tests) show the results of the titration of all 10 ovine mycoplasma strains against the antisera produced against five of the strains. All strains cross-reacted by these tests, confirming that they belong to the same species. The MI and GI tests, however, showed antigenic differences among the strains tested, and a comparison for relatedness was made by the method of Gois et al. (1974). The antibody-titre ratios shown in Table 3.9 were calculated from the results of the MI and GI serological tests only, as no intraspecific distinctions were made by the IHA tests. Strains 956/2 and Y98 were most closely related to each other and most dissimilar to 611, which, like strain 658, was most closely related to strain 697. The rank correlation coefficient, a measure of the agreement between tests in the ranking of relatedness, was 1.0 for strains 956/2, Y98 and 658 (significant at the 5% level), 0.925 for strain 697 (significant at the 10% level) and 0.675 for strain 611 (not significant). Consideration of the relation, calculated or apparent, of all 10 strains emphasises the apparent polarisation of SPA and non-SPA strains at opposite ends of the antigenic spectrum, strains 658, 7Z97, 8Z56 and 611 appearing at one end and strains 956/2, 650/21 and Y98 at the other. However, strains 652 and 672/9 did not fit this observation, although the animal from which strain 672/9 was isolated belonged to a flock in which SPA occurred.

Strains 658 and Y98 were also tested by the MI test against 40 hyperimmune sera, including the 24 antisera listed in Table 3.6,

Table 3.7 Comparison of 10 ovine mycoplasma strains by the metabolic-inhibition test.

| Strain number | Titres of inhibition of the indicated strain by antisera prepared against strain . | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | 5120 | 320 | 320 | 80 | 40 |
| 650/21 | 320 | 10 | 40 | 10 | 10 |
| Y98 | 640 | 640 | 640 | 640 | 160 |
| 697 | 320 | 20 | 2560 | 160 | 320 |
| 652 | 160 | 20 | 320 | 40 | 20 |
| 658 | 160 | 10 | 320 | 2560 | 160 |
| 7Z97 | 80 | 40 | 2560 | 5120 | 160 |
| 8Z56 | 80 | 40 | 640 | 1280 | 40 |
| 611 | 20 | 10 | 40 | 40 | 1280 |
| 672/9 | 80 | 10 | 40 | 40 | 160 |

Homologous titres are underlined.

Table 3.8 Comparison of 10 ovine mycoplasma strains by the growth-inhibition test.

| Strain number | Size (mm) of the zone of inhibition* of the stated strain produced by rabbit antiserum to strain | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | 11.0-11.5 | 2.5 | 3.5 | 3.5-4.5 | 3.0-3.5 |
| 650/21 | 4.0-5.5 | 1.0-2.0 | 4.5 | 2.5-3.0 | 1.0-1.5 |
| Y98 | 9.5 | 7.5-9.5 | 7.5-10.0 | 8.0-10.5 | 2.0-3.5 |
| 697 | 2.5-4.0 | 1.0 | 5.5 | 3.5-4.0 | 3.5 |
| 652 | 6.0 | 1.5-2.0 | 9.0-10.0 | 3.0-3.5 | 3.5 |
| 658 | 3.0 | 1.5-2.0 | 4.5-5.0 | 10.5-11.5 | 1.5 |
| 7Z97 | 3.5-4.0 | 0.5 | 5.0-6.0 | 13.0-14.0 | 3.0 |
| 8Z56 | 4.0-5.0 | 2.5-3.0 | 6.5-7.0 | 14.0 | 2.0-3.0 |
| 611 | 3.0 | 1.0 | 3.0 | 3.4-4.0 | 8.0 |
| 672/9 | 5.5 | 2.0 | 3.5-4.0 | 4.0 | 5.0-5.5 |

Homologous reactions are underlined.

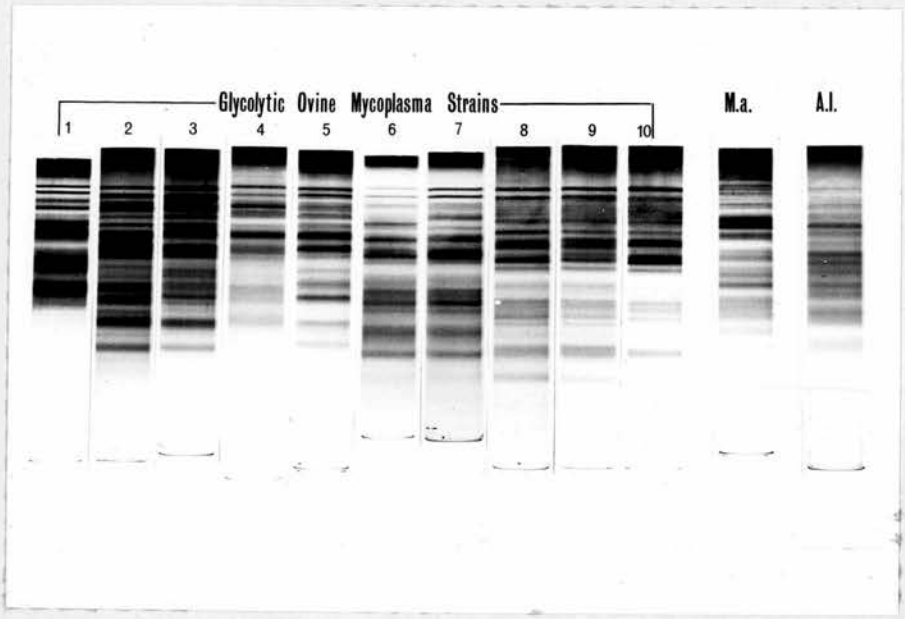
*The results indicate the range of zone widths from duplicate tests of each antiserum, taken from the concentration of inoculum that provided maximal zone development commensurate with ease of reading.

Table 3.9 Antigenic relatedness among five ovine mycoplasma strains calculated from the results of metabolic-inhibition and growth-inhibition tests.

| Strain Number | Homologous/heterologous ratios obtained in the stated test with antiserum against strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 956/2 | | Y98 | | 697 | | 658 | | 611 | |
| | MI | GI | MI | GI | MI | GI | MI | GI | MI | GI |
| 956/2 | 1/1 | 1/1 | - | - | - | - | - | - | - | - |
| Y98 | 1/4 | 1/2 | 1/1 | 1/1 | - | - | - | - | - | - |
| 697 | 1/11.3 | 1/2.3 | 1/11.3 | 1/2.3 | 1/1 | 1/1 | - | - | - | - |
| 658 | 1/32 | 1/3.2 | 1/16 | 1/2.4 | 1/11.3 | 1/2.0 | 1/1 | 1/1 | - | - |
| 611 | 1/90.5 | 1/3.3 | 1/22.6 | 1/4.7 | 1/16 | 1/2.5 | 1/22.6 | 1/2.8 | 1/1 | 1/1 |

The homologous ratio is defined as 1/1, and the degree of antigenic relatedness is indicated by the closeness of the fractions to unity.

Fig. 3.11 Polyacrylamide gel electrophoresis with sheep mycoplasmas. 1 = No. 7Z97, 2 = No. 956/2, 3 = No. 652, 4 = No. 650/21, 5 = No. 611, 6 = No. Y98, 7 = No. 697, 8 = No. 658, 9 = No. 8Z56, 10 = No. 672/9, M.a. = *M. arginini* and A.l. = *A. laidlawii*.


Glycolytic Ovine Mycoplasma Strains
1
2
3
4
5
6
7
8
9
10
M.a.
A.I.

and the following 15 antisera obtained from the FAO/WHO International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark: M. agalactiae subsp. agalactiae (PG2), M. agalactiae subsp. bovis (Donetta), M. bovigenitalium (PG11), M. bovirhinis (PG43), M. bovoculi (M165/69), M. capricolum (Calif. Kid), M. dispar (462/2), M. gateae (CS), M. putrefaciens (KS-1), bovine serogroups L(B144 P), 7 (PG50), and 8 (PG51), and ovine serogroups 5(Goat 189) and 11 (2-D). Antiserum to M. verecundum (107), obtained from Drs. Gourlay and Howard of IRAD, Compton, nr. Newbury, Berks, was also included in the tests. No cross-reactions were noted between strain 658 or strain Y98 and any of the antisera tested.

PAGE

The protein-banding patterns obtained by PAGE (Fig. 3.11) demonstrated a very close resemblance between all 10 strains. The patterns obtained with M. arginini and A. laidlawii showed no similarities to those of the 10 strains under examination.

DISCUSSION

Nine strains of glycolytic mycoplasmas isolated from the respiratory tract of sheep in Scotland and the Australian strain Y98 were almost completely homogeneous in their reactions in 14 biochemical tests, and in their sensitivities to optochin, digitonin, SPS and 11 antibiotics. Where the same tests were performed, the results obtained agree with those of Carmichael et al. (1972). Since the inception of this work, four further reports on the

biochemical properties of mycoplasma isolates which cross-reacted serologically with strain Y98 have appeared. Furlong and Cottew (1973) compared six Queensland strains, six of Cottew's "Type 2" strains isolated from sheep in Victoria and M. dispar by tests for glycolysis, arginine and urea breakdown, methylene blue and tetrazolium reduction (aerobic and anaerobic), phosphatase production, "films and spots" formation, gelatin hydrolysis, casein and inspissated serum digestion, haemolysis of sheep RBC, and sensitivity to optochin. Leach, Cottew, Andrews and Powell (1976) compared strain Y98 with two Victorian strains and a British isolate, M333/68, by tests for glycolysis, arginine hydrolysis, tetrazolium reduction, "films and spots" formation, sensitivity to SPS and digitonin, and growth on medium without serum. Clarke, Brown and Alley (1974) compared a "standard strain" of M. ovipneumoniae from Queensland with six mycoplasma strains isolated from sheep in New Zealand by tests for glycolysis and haemolysis. St. George and Carmichael (1975) compared strain Y98 with two strains isolated from sheep in the USA by tests for glycolysis, arginine hydrolysis, tetrazolium reduction and haemolysis of sheep RBC. In each communication, the results of all tests on ovine mycoplasmas concur with those reported here. The failure of the strains to hydrolyse aesculin and their sensitivity to SPS and digitonin confirm that M. ovipneumoniae is a member of the family Mycoplasmatales, the only previous evidence for this being lack of growth at 22°C and in media without serum (Carmichael et al., 1972). Further evidence that M. ovipneumoniae is a typical member of the family Mycoplasmatales is given by the DNA base ratio values, found for Leach's strain

M333/68 and Mackay's "Type A" strain to be within the range 27 - 29% G + C (Hill, Leach and Andrews, 1970).

The in vitro sensitivity of strains of M. ovipneumoniae to antibiotics has also been investigated by Quinlan, Alley and Clarke (1975), with similar findings to those reported here. The relative insensitivity of all strains to erythromycin, found both in this study and by Quinlan et al., is of interest, only two of 17 species of mycoplasma tested by Ernø and Stipkovits (1973) showing similar resistance.

The morphology of colonies and of stained organisms from broth cultures correspond with the descriptions given for these by Carmichael et al. (1972) and Furlong and Cottew (1973). The ultrastructural appearance of M. ovipneumoniae, not previously described, is typical of the "coccoid" form of mycoplasma (Anderson, 1969; Maniloff, 1972). Diffusely scattered nuclear material and ribosomes are contained within a trilaminar membrane, and specialized structures such as have been described for M. pneumoniae (Biberfeld and Biberfeld, 1970) and M. gallisepticum (Maniloff, Morowitz and Barrnett, 1965) were not observed. Tangential sectioning probably accounts for the smaller measurements obtained for the length of M. ovipneumoniae in thin section compared with whole cell preparations, particularly as the mean width of cells was similar in both techniques. The more accurate measurements should therefore be reflected from unsectioned material, and these are closely similar to the sizes of organisms observed by light microscopy. Conversely, the differences in the thickness of extramembranous

material as measured by the two techniques may be due to the accumulation of stain around the extramembranous coat in whole cell preparations: the figures obtained from thin section material may thus be more accurate in this case.

The possession of extramembranous material has been described for several members of the genus Mycoplasma; however, such material appears floccular in nature (Domermuth, Nielsen, Freundt and Birch-Andersen, 1964), or as relatively large spiky projections (Hummeler, Tomassini and Hayflick, 1965; Chu and Horne, 1967). The layer of uniform thickness seen in M. ovipneumoniae more closely resembles the extramembranous coat observed in ureaplasmas (Black, Birch-Andersen and Freundt, 1972; Black and Vinther, 1977), the composition of which has been found to include glucosyl-like residues (Robertson and Smook, 1976). The morphology of mycoplasmas is, however, considerably affected by the methods of preparation, fixation and staining employed (Freundt, 1969), and little can be inferred concerning the extramembranous coat of M. ovipneumoniae by comparison with observations made in other species of mycoplasma. Staining by ruthenium red has been used to demonstrate the presence of a capsule in M. meleagridis (Green and Hanson, 1973) and in M. mycoides subsp. mycoides and M. dispar (Howard and Gourlay, 1974). The capsules demonstrated by this technique displayed no obvious structure and extended for 17 - 24 nm beyond the trilaminar membrane in the case of M. dispar, and up to 30 nm in the other two mycoplasmas. In preparations of M. dispar not treated with ruthenium red only a small amount of material was evident outside the membrane

(Howard and Gourlay, 1974). Ruthenium red reacts most strongly with substances such as acidic mucopolysaccharides, which have a high negative charge and high molecular weight (Luft, 1971). Since preparations of M. ovipneumoniae stained with ruthenium red did not demonstrate more intense staining or greater depth of the extramembranous coat than preparations stained without its use, the composition of this coat presumably does not include mucopolysaccharides. The possible rôles of extramembranous material in mycoplasmas, summarised by Green and Hanson (1973), may be to lend strength to the trilaminar membrane, to afford environmental protection for saprophytes, to act as an anti-phagocytic virulence factor for pathogens, to allow attachment of organisms to epithelial surfaces, to assist proliferation within the connective tissue of the trachea, and to permit gliding motility: haemagglutination has also been suggested to be a property of extramembranous material in M. gallisepticum and M. mycoides subsp. mycoides (Chu and Horne, 1967).

The absence of filament or bud formation in the preparations studied, coupled with the large number of apparently dividing forms observed, suggests that M. ovipneumoniae replicates by binary fission. Robertson, Gomersall and Gill (1975) found that under "minimally distorting" conditions of culture, M. hominis showed relatively uniform exponential growth, and appeared to be dividing by binary fission: pleomorphic forms only appeared upon further incubation of cultures. These studies with M. ovipneumoniae do not exclude the possibility that the organism may, under certain

conditions, form filaments and replicate by filament fragmentation or budding. However, binary fission is now considered to be the most common form of replication, at least in coccoid forms of mycoplasma (Maniloff, 1972). Baskerville, Wright, Meneely and Curran (1972) observed both binary fission and budding in broth cultures of M. hyorhinis, but binary fission only in the infected lungs of pigs - from which they concluded that budding in M. hyorhinis occurs only in vitro.

The diameters of the various mycoplasma species range from 0.33 μm to 1.0 μm (Maniloff, 1972). The results of light and electron microscopy measurements indicate that M. ovipneumoniae is a comparatively large mycoplasm, in which respect it resembles M. dispar (Gourlay and Leach, 1970). The filtration experiments performed in these studies and by Leach et al. (1976) bear out the morphological observations. Considerable reductions in titre followed the passage of cultures through filters of 0.8 μm and 0.45 μm a.p.d., with complete exclusion of organisms by filters of 0.22 μm a.p.d., despite the natural plasticity of mycoplasmas.

The protein-banding patterns obtained with PAGE substantiated the apparent homogeneity of the strains as indicated by the biochemical tests. In the IHA test, high levels of sensitivity were obtained with fresh sheep RBC, but no significant differences between strains were revealed, and in fact, considerable cross-reactions with antisera to other mycoplasma species occurred. A similar lack of specificity with a high degree of cross-reaction between species was also obtained with fresh sensitised cells by Freundt,

Ernø, Black, Krogsgaard-Jensen and Rosendal (1973). Serological intraspecific differences have been shown to be due to membrane antigens: where non-membrane antigens participate in a serological test, as in the complement-fixation test, the ability to make intraspecific distinctions becomes lost (Hollingdale and Lemcke, 1970). Despite the finding that in the IHA test for M. hominis only membrane antigens participate (Hollingdale and Lemcke, 1969), it is likely that non-membrane antigens are also involved in IHA tests with M. ovipneumoniae. This would account not only for the inability to differentiate between strains with this test, but also for the heterologous reactions that occurred with antisera to other species of mycoplasma.

The AGDD tests were also more useful in demonstrating similarities than differences between strains, because there was little difference in the number of precipitin lines produced by each antigen suspension irrespective of the opposing antiserum. For M. pulmonis, Deeb and Kenny (1967) found that heated antigens were required to obtain sub-type specificity with this test, although this finding was not corroborated by Forshaw and Fallon (1972). Pollack, Somerson and Senterfit (1969) showed that M. pneumoniae harvested from culture media that had become acid lost its reactivity in immunodiffusion tests. This effect was considered to be due to activation of previously released mycoplasmal enzymes rather than to a direct hydrogen-ion effect. The bulk culture media used to produce AGDD antigens all achieved final pH levels of 6.2 - 6.4. It is possible that the poor antigenicity of strains

956/2 and Y98 in these tests compared with that of the other strains examined may have been due to their greater production of, or sensitivity to, such enzymes.

This phenomenon may also be partly responsible, in conjunction with the obligatory treatment of the hyperimmunising antigen with formaldehyde, for the poor performance of Y98 antiserum in the MI and GI tests.

The MI and GI tests have been found to be extremely specific (Purcell, Chanock and Taylor-Robinson, 1969; Freundt et al., 1973), and therefore suitable for the demonstration of intraspecific differences (Hollingdale and Lemcke, 1970; Forshaw and Fallon, 1972; Haller, Boiarski and Somerson, 1973; Gois et al., 1974). Both tests revealed considerable antigenic heterogeneity among the 10 strains of M. ovipneumoniae, with possible polarisation of the SPA strains towards one end of the spectrum and non-SPA strains towards the other. The practical implications in the application of the GI test have been pointed out by Gois et al. (1974), namely that, for routine identification of fresh isolates, antisera to several antigenically different strains would be required. Similar implications in the use of the MI test in sero-epidemiological studies have been observed by Hollingdale and Lemcke (1970), namely that full coverage would be achieved only by testing each serum against several strains of the mycoplasma under consideration. For this reason, the highly sensitive but moderately specific IHA test is probably more suitable for such studies.

In the characterisation of the Y98 biotype (Carmichael et al., 1972), serological comparison was made with only 12 other ovine and caprine stains. Furlong and Cottew (1973) compared their Victoria strains with the Queensland Y98 biotype and with M. dispar. Thus at the time the studies reported herein were performed, the serological relation of strain Y98 to other members of the Mycoplasmatales had been insufficiently investigated. The negative results obtained in the MI tests of strains 658 and Y98 against 40 hyperimmune sera to 33 named mycoplasma species or subspecies and six serogroups of bovine or ovine origin, though not fully conclusive, indicates that these strains should be considered as a distinct species with the name M. ovipneumoniae (FAO/WHO Committee on Comparative Virology, 1974). The status of M. ovipneumoniae as a distinct species has been corroborated by Leach et al. (1976), who found that strain Y98, the British strain M333/68 and two Victorian strains RXP and GIP, were serologically indistinguishable from each other by the GI and MI tests, but gave negative results by the same tests with antisera to 25 other glycolytic mycoplasmas and M. arginini. Included in the tests by Leach et al. (1976) but not in the studies above were antisera to M. felis, M. synoviae, M. suipneumoniae, and M. conjunctivae.

The findings of Leach et al. (1976) and Furlong and Cottew (1973) confirm that the "Type 2" strains of Cottew (1971) were M. ovipneumoniae, and indicate by inference that the "Type A" strains of Mackay et al. (1963), the "Serotype 1" of Nayil (1971) and the "Serogroup 2" of Krauss and Wandera (1970) were also this species.

# CHAPTER 4

## A LONGITUDINAL SURVEY OF RESPIRATORY MYCOPLASMA INFECTIONS IN HOUSED LAMBS AND EWES

### INTRODUCTION

That a mycoplasma-associated pneumonia similar to that described in Australia (St. George *et al.*, 1971; Carmichael *et al.*, 1972) may be present in Scotland was suggested by the recovery, at Moredun Institute, of *M. ovipneumoniae* and *M. arginini* from sheep. (Foggie and Angus, 1972; Chapter 3, this thesis). A survey was therefore undertaken in an intensive indoor lambing and fat-lamb rearing system in which chronic respiratory disease occurred annually in the lambs. The aim of this survey was to provide a basis for future pathogenicity studies by investigation of the species, extent of involvement and serology of the mycoplasmas present, these factors being related to the involvement of other respiratory micro-organisms and the age and pulmonary pathology of the lambs.

### MATERIALS AND METHODS

#### Survey flock

This commercial flock consisted of Finnish Landrace X Dorset Horn sheep. The ewes were housed in early December, approximately 10 days before lambing, and were returned to pasture two and a half months later. The lambs were housed during the whole of the survey. At peak stocking periods some 600 ewes and

lambs were maintained in one house and shared the same air-space, but groups of ewes and lambs were separated into straw-covered pens within that house. Temperature and relative humidity (RH) conditions within the house were monitored by a thermohygrograph for the first three and a half months of the survey.

Sampling

Three pens containing 39 ewes and their 70 lambs were involved. The lambs were born over a 15 day period from mid-December onwards. Each lamb was bled and clinically examined at 1 - 5 d.o., and swabs taken from the nasopharynx. Thereafter, the lambs in the pens were sampled in the same manner in rotation, each pen being sampled at three week intervals on three occasions, such that the lambs were examined at 6 - 14, 25 - 35 and 45 - 55 d.o. A final examination of all lambs was made at 99 - 114 d.o., by which time culling had reduced the lamb numbers to 64. The ewes were sampled on a single occasion shortly before removal from the house.

Serology

Lamb and ewe sera were examined for antibodies to M. ovipneumoniae and M. arginini by the IHA test only. It was necessary to pre-adsorb lamb sera with packed, washed RBC, due to non-specific agglutination possibly caused by chylaemia. Culling and difficulties experienced in the taking of blood samples produced incomplete serum series for most lambs: the results presented have been limited to the 60 lambs from which at least four serum

samples were obtained over the 180 day survey period, including one sample from the 0 - 5 d.o. age group. For calculation purposes, titres of less than one in 10 (the minimum dilution tested) have been assumed to be positive at the undiluted level. The serum series from the same 60 lambs were also assayed for antibodies to PI3 virus by the HAI test.

Colostrum samples obtained at varying times post partum from 25 ewes were treated with rennin and, after absorption with packed RBC, the resultant wheys were assayed for antibodies to M. ovipneumoniae by the IHA test.

Post mortem examination

Thirty-four lambs were examined in five groups, the details of which are presented in Table 4.1. The heads and lungs of 31 culled ewes of the same flock, 22 of which were dams of the lambs involved in the survey, were also removed at the abattoir for examination.

Microbiology

Samples from lambs examined for mycoplasmas consisted of tonsillar tissue, trachea, bronchial swab and 2 - 6 pieces of lung, comprising pneumonic areas and apparently normal areas of lung. Ewe samples examined for mycoplasmas comprised tonsillar tissue and bronchial swabs from all animals, and lung samples from the 16 ewes that showed macroscopic pneumonia. The usual isolation procedures were employed and the presence and morphology of any

Table 4.1 Lamb groups examined post mortem.

| Group | Age of lambs in days | No. of lambs | Condition |
|---|---|---|---|
| 1 | 71-80 | 7* | Culled due to poor growth |
| 2 | 121-132 | 5 | Lambs at slaughter |
| 3 | 136-144 | 10 | Lambs at slaughter |
| 4 | 150-155 | 4+ | Culled due to poor growth |
| 5 | 165-180 | 8 | Lambs at slaughter |

* One lamb outwith the pre-necropsy survey sample.

\+ All lambs outwith the pre-necropsy survey sample.

growth on solid media was recorded, but serological identification was deferred by storage at -70°C of broths showing colour change. All harvested broth cultures removed from storage for identification by the GI test were initially inoculated into OB, OA, AB and TB2, irrespective of the originating broth type: this procedure allowed easier recognition of the presence of mixed mycoplasma species in positive broth cultures. Lung samples from the 34 lambs and 16 ewes were also examined for P. haemolytica. Virus isolation, using primary sheep thyroid tissue cultures, was attempted on 26 lamb lung samples.

Histopathology

Sections stained by haematoxylin and eosin were prepared from 1 - 5 representative sites from the lungs of lambs. Examination of ewe lung material was confined to eight animals which had macroscopic pneumonic lesions.

RESULTS

Ambient conditions in the sheep house

The mean daily maximum RH during the period of the survey was 88% (SD ±3.1%), and the minimum mean was 73% (SD ± 10%). The full range was 43-93%, with diurnal variations of 1 - 47%. The mean daily maximum temperature was 6.6°C (SD ± 2.0°C) and the mean daily minimum value was 2.7°C (SD ± 1.9°C). The full range was -1°C to 18°C, with diurnal variations of 1 - 14°C. The extremes of range and diurnal variation in both RH and temperature occurred in March.

## Nasal swabbings

### Lambs

The principal findings are shown in Fig. 4.1. The recovery rates of M. ovipneumoniae from the nasal cavity of lambs increased rapidly from less than 3% in 0 - 5 d.o. lambs to over 92% in 99 - 114 d.o. lambs. M. arginini was recovered at low, fluctuating levels from 6 - 14 d.o. until the final sampling, which yielded 28% positive recoveries. Isolations of M. conjunctivae from the nasal cavity showed a peak of 40% in 25 - 35 d.o. lambs, and rapidly declining levels thereafter. Two further mycoplasma species were recovered: A. laidlawii was isolated from seven animals including one 2 d.o. lamb, and ureaplasmas were isolated from two lambs of 55 and 99 d.o.

### Ewes

The only mycoplasmas isolated were M. ovipneumoniae from 25 of the 39 ewes (64%) and M. arginini from two ewes (5%).

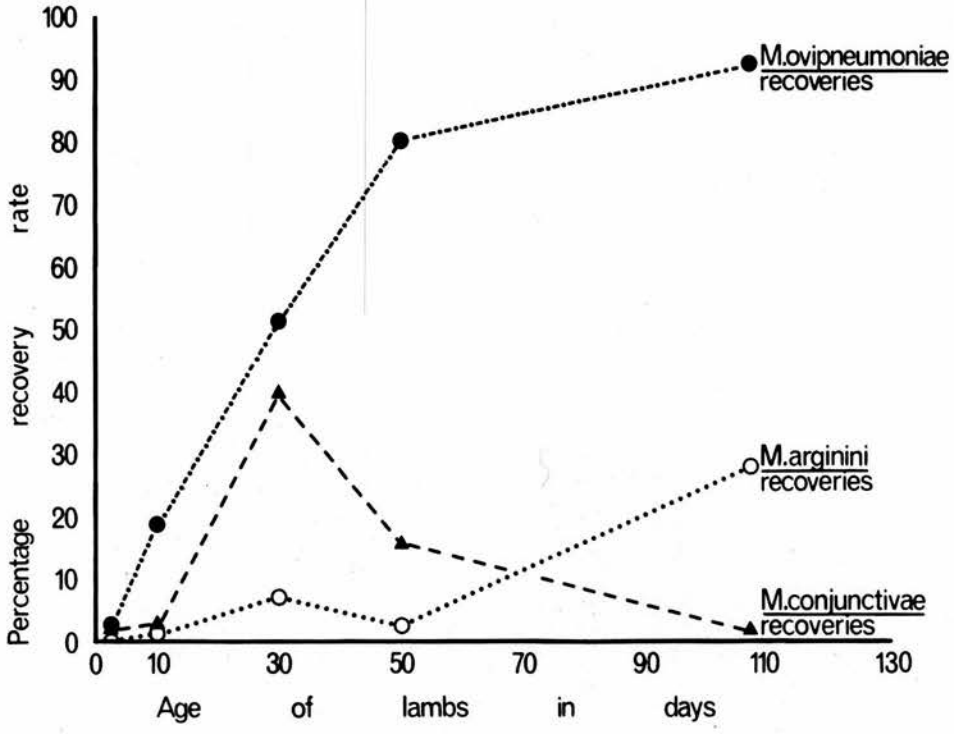
## Clinical examinations

### Lambs

Evidence of mild respiratory disease, indicated by abnormal auscultatory sounds, nasal discharge and occasionally coughing, was first detected in some lambs at 6 - 14 d.o., and from 25 to 55 d.o. between 10 and 25% of animals were mildly affected. However, in 99 - 114 d.o. lambs, moderate to severe clinical signs of respiratory disease were detected in 54% of animals.

Fig. 4.1

Recovery rates of three mycoplasma species from nasal swabs of survey lambs at the different ages of sampling



Ewes

Specific clinical examination of the dams was not performed, but at no time were severe respiratory symptoms observed, although nasal discharge and coughing were present in some animals.

Serology

Lambs

The geometric means of serum IHA antibody titres to M. ovipneumoniae and M. arginini at the different ages of sampling are shown in Fig. 4.2. The M. ovipneumoniae antibody titre profile clearly indicates a declining antibody status in the lambs from the earliest age group examined until the 45 - 55 d.o. age group, after which titres rise again to a plateau at 140 d.o. Seven animals only (11.7%) yielded titres of less than 1/10 from the first serum sample. In contrast, although the M. arginini antibody titre profile shows an initial fall, there appears to be a gradual rise from the 6 - 14 d.o. group onwards. That this is a distortion due to the high number of lambs without measur able antibody in their first serum sample (18 animals (30%) with titres of $<1/10$) or with low levels only (28 animals (46.7%) with titres of 1/10) is shown by Fig. 4.3. This compares the M. arginini antibody profiles in lambs with titres at dilutions of 1/20 or greater in their first serum sample, with those giving no measurable titre at the same age. The profile of the "positive" group shows a similar pattern to that obtained for M. ovipneumoniae, the decline in antibody titres reaching a trough at 45 - 55 d.o. It is also

Fig.4.2

Geometric mean titres of lambs' sera to M.ovipneumoniae and M.arginini, measured by the I.H.A. test, at the different ages of sampling

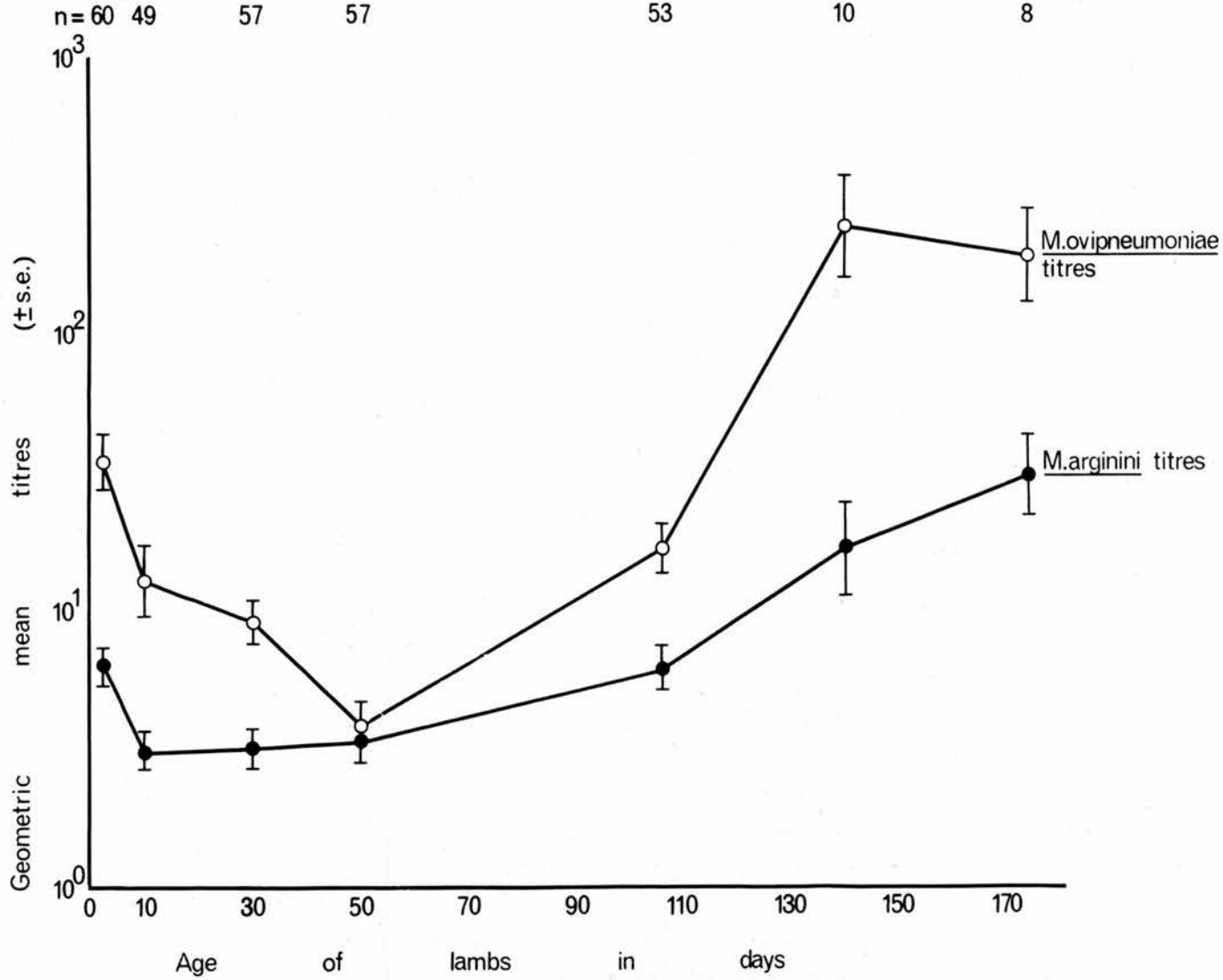
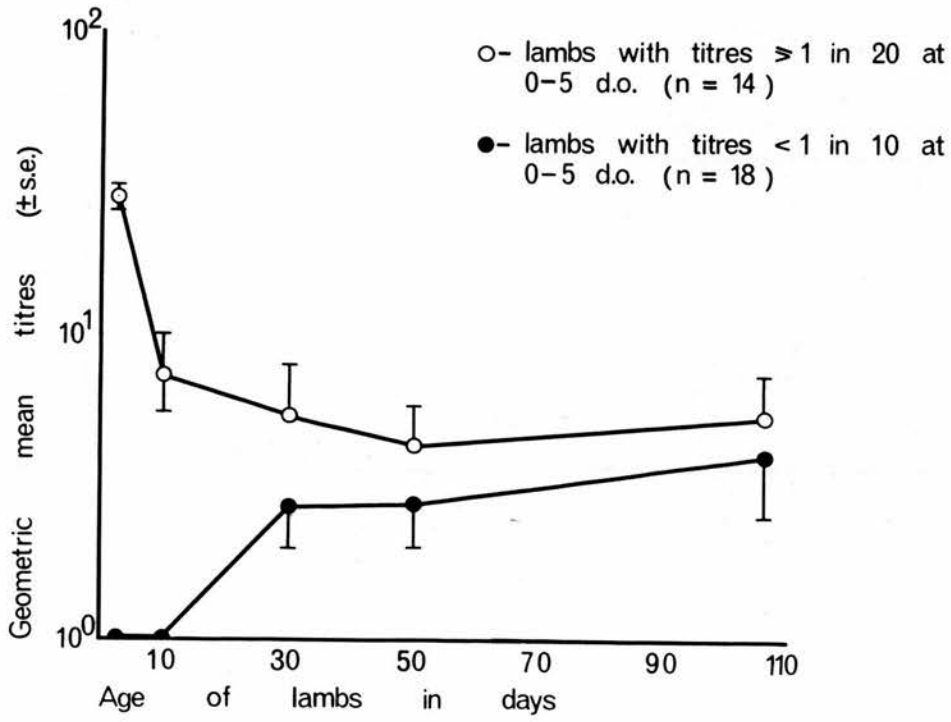


Fig.4.3

I.H.A. antibody titres to M.arginini at the different ages of sampling; comparison of the antibody titre profile of lambs showing titres of 1 in 20 or greater at 0 to 5 d.o. with that of lambs showing titres of less than 1 in 10 at the same age.



apparent that in the "negative" group, several lambs demonstrate rising antibody levels as early as 25 - 35 d.o.

Thirty-seven of the 60 lambs (61.7%) had titres of 1/10 or greater to PI3 virus in the first serum sample. In the 45 - 55 d.o. group, titres of $\geq$1/10 were present in only 13 of 53 samples (24.5%), but in the 99 - 114 d.o. group, 48 of these (90.6%) had titres of 1/10 to 1/20.

Ewes

M. ovipneumoniae antibody titres in the sera of the 39 ewes ranged from 1/10 - 1/320; M. arginini titres ranged from $<$ 1/10 - 1/40; colostrum whey titres to M. ovipneumoniae ranged from 1/10 - 1/320.

Microbiology

The recoveries of M. ovipneumoniae, M. arginini and P. haemolytica from necropsy specimens are presented in Table 4.2. Three of seven lambs in Group 1 yielded M. ovipneumoniae from the lower respiratory tract (LRT) (lungs and bronchial swab or trachea), a significantly lower proportion ($P < 0.001$ Chi-square test) than in Groups 2 - 5, where all 27 animals were positive for the organism from these sites. Similarly, the proportion of lung lobes examined from Group 1 which were positive for M. ovipneumoniae (13%) were significantly lower ($P < 0.001$) than the proportion of positive recoveries from Groups 2 - 5 (84% mean). No significant differences were found between Group 1 and Groups 2 - 5 in the number of animals positive for M. arginini from the lungs, but the proportion

Table 4.2 Recoveries of M. ovipneumoniae, M. arginini and P. haemolytica from the respiratory tract of lambs and ewes.

| Group | No. of animals | No. of animals yielding M.ovipneumoniae | | | M. arginini | | | P. haemolytica | Percentage of lung lobes positive for M. ovipneu. | | M. arginini |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Lung | Bs/Tra | Ton | Lung | Bs/Tra | Ton | Lung | | | |
| 1 (Lambs) | 7 | 2 | 3 | 6 | 0 | 1 | 4 | 1 | 13 | (31*) | 0 |
| 2 " | 5 | 5 | 5 | 2 | 3 | 0 | 4 | 1 | 82 | (17) | 18 |
| 3 " | 10 | 10 | 10 | 7 | 4 | 1 | 9 | 4 | 76 | (45) | 15 |
| 4 " | 4 | 4 | 4 | 3 | 3 | 3 | 4 | 3 | 100 | (12) | 50 |
| 5 " | 8 | 8 | 8 | 8 | 3 | 2 | 8 | 7 | 88 | (50) | 16 |
| Ewes | 31 | 13** | 26+ | 16 | 6** | 5+ | 6 | 9** | | | |

Bs = Bronchial swab

Tra= trachea

Ton= tonsillar tissue

* = Figures in parentheses indicate total number of lung lobes examined in group.

** = 16 samples only examined

+ = Trachea not examined

of lung lobes yielding the organism was significantly lower ($P = 0.02$) in Group 1 (0%) than in Groups 2 - 5 (19% mean). Thus it would appear that a significant increase occurred in the colonisation of lungs with mycoplasmas between 71 - 80 d.o. and 121 - 132 d.o.

In contrast, the appearance of P. haemolytica biotype A (serotypes A2, A5 and A1) in the LRT of lambs occurred later, with a progressive increase in incidence with age. In Groups 1 + 2, Group 3 and Groups 4 + 5, two of 12, four of 10 and 10 of 12 lambs respectively yielded P. haemolytica. The differences between Groups 1 + 2 and Group 3 are not significant, and between Group 3 and Groups 4 + 5 of marginal significance ($P = 0.048$). However, Groups 4 + 5 are significantly higher than Groups 1 + 2 ($P = 0.002$) and Groups 1 + 2 + 3 ($P = 0.0024$) in terms of the number of animals yielding P. haemolytica.

No differences were found in the incidence of M. ovipneumoniae and M. arginini in tonsillar tissue of lambs, nor were any age variations detected in the recovery of mycoplasmas from this site. Of the total 34 lambs, 26 (76%) yielded M. ovipneumoniae and 29 (85%) M. arginini from tonsillar tissue.

Other mycoplasmas recovered from lamb necropsy specimens were ureaplasmas from one lung lobe and four tonsillar tissue samples, and A. laidlawii from the lungs of two lambs.

No viruses were recovered from the lungs of any lamb.

The only mycoplasmas recovered from ewes were M. ovipneumoniae, recovered from 84% of bronchial swabs, 52% of tonsillar tissue samples and 81% of lung samples, and M. arginini, the figures for

which were 16%, 19% and 37% respectively. The differences in recoveries between M. ovipneumoniae and M. arginini from all sites are significant ($P < 0.015, > 0.001$). P. haemolytica strains, recovered from nine of 16 (56%) lung samples, comprised serotype A2 (two specimens), biotype T (T4 and T10) (two specimens) and non-typable strains (five specimens).

## Histopathology

### Lambs

The histopathological findings in the lungs of lambs are presented in Table 4.3. Seven of the 12 lambs in Groups 1 and 2, and one lamb in Group 4 showed small lymphoid nodules and occasional minor bands of collapse in otherwise normal lung parenchyma; these were not considered to represent pathological change. Otherwise, pathological changes found in the lungs of lambs consisted of lymphoid hyperplasia, accompanied in later groups by interstitial thickening, and P.E. pneumonia. Consolidated lesions of interstitial pneumonia were not observed. There was an increasing incidence of P.E. pneumonia with age. Groups 4 + 5 had significantly more P.E. pneumonia than Groups 1 + 2 ($P = 0.006$) and Groups 1 + 2 + 3 ($P = 0.0098$).

The correlation of microbial lung recoveries with histopathological findings is shown in Table 4.4. M. ovipneumoniae was recovered from all cases of P.E. pneumonia, from all but one of the lambs with lymphoid hyperplasia and from half the animals with no significant lesions. The highest recovery rate in terms

Table 4.3 Histopathological findings in lungs of lambs.

| Group | No. of lambs | No. of lambs with lungs showing | | |
|---|---|---|---|---|
| | | Proliferative, exudative pneumonia | Lymphoid hyperplasia with or without interstitial thickening | No abnormalities* |
| 1 | 7 | 1 | 2 | 4 |
| 2 | 5 | 1 | 1 | 3 |
| 3 | 10 | 4 | 6 | 0 |
| 4 | 4 | 3 | 0 | 1 |
| 5 | 8 | 6 | 2 | 0 |
| Totals | 34 | 15 (44%) | 11 (32%) | 8 (24%) |

* Small lymphoid follicles and/or bands of alveolar collapse were assumed to be insignificant.

Table 4.4 Correlation of microbial recoveries with histopathological findings in lungs of lambs.

| Condition | No. of lambs showing condition | Percentage yielding lung recoveries of *M. ovipneumoniae* | *M. arginini* | *P. haemolytica* |
|---|---|---|---|---|
| P.E. pneumonia | 15 | 100 (86%*) | 40 (19%) | 100 |
| Lymphoid hyperplasia ± interstitial thickening | 11 | 91 (65%) | 42 (13%) | 9 |
| No abnormalities | 8 | 50 (39%) | 25 (11%) | 0 |

* Percentages in parentheses indicate proportion of lung lobes in each category which yielded the mycoplasm.

of that proportion of the total lung lobes examined from all animals in any category which were positive for M. ovipneumoniae was from the lambs which had lesions of P.E. pneumonia. For M. arginini, little difference was discernible among the categories either in the number of animals or the proportion of lung lobes yielding the organism. All recoveries of P. haemolytica except one were from cases of P.E. pneumonia. This exception and two of the P.E. pneumonia cases yielded single colonies only of the organism.

Ewes

Five of the eight animals examined histopathologically showed lesions of SPA, two purulent bronchopneumonia and one eosinophilic bronchopneumonia in which larvae of Muellerius capillaris were observed. All but one animal, a case of SPA, yielded M. ovipneumoniae from lung samples. M. arginini was not isolated from any of the eight ewes, and the only isolations of P. haemolytica (serotype A2) were from the two cases of purulent bronchopneumonia.

## DISCUSSION

The paramount factors affecting morbidity, rapidity of spread and pathogenicity of respiratory infections are population density, ambient conditions, the susceptibility of the exposed animals, the "weight" of infective challenge and the infectivity and pathogenicity of the organism involved, both singly and in

combination. The system of management under which the survey flock was maintained provided favourable conditions for the spread and development of respiratory disease. All 600 animals within the house shared a common airspace and no controlled system of ventilation or temperature regulation was operated. Conditions of high humidity and low temperature, which prevailed within the house for most of the survey period, are known to increase the susceptibility of ruminants to respiratory disease (Parker, 1968). Accumulations of noxious gases and infective droplets and particles have been suggested as contributory causes to the high prevalence of enzootic pneumonia of pigs noted where dense populations are maintained in poorly ventilated buildings (Whittlestone, 1976). The lack of adequate ventilation and the use of deep-litter in the sheep house would permit the build-up of dust and ammonia. Ammonia has been shown to increase significantly the severity of respiratory disease caused by M. pulmonis in mice (Broderson, Lindsey and Crawford, 1976). Turkeys exposed to high concentrations of dust and ammonia show loss of tracheal epithelial cilia, an increase in mucus secreting cells and areas of consolidation and inflammation in the lungs (Anderson, Wolfe, Chermus and Roper, 1968).

Spread of M. suipneumoniae, the primary agent of enzootic pneumonia of pigs, is considered to be almost exclusively by the airborne route (Whittlestone, 1976). The available evidence indicates that infection with M. ovipneumoniae and M. arginini occurs in the same manner. Apart from the small number of isolations from eyes (Carmichael et al., 1972; Jones, Foggie, Sutherland and

Harker, 1976a), and a single isolation from a vaginal swab (Leach et al., 1976), M. ovipneumoniae has been recovered only from the respiratory tract of sheep. Similarly, recoveries of M. arginini have been from the respiratory tract and eyes only, with the exception of a single isolation from brain (see Chapter 1). The initial "donors" of respiratory micro-organisms, the ewes, remained in the house until the lambs were approximately 2 m.o. All samples from ewes indicated high infection rates with M. ovipneumoniae and low with M. arginini, and this may have been a major factor in the different recovery rates of the two organisms from lambs. The spread of respiratory mycoplasmas may also be influenced by the physical factors that affect their survival in the airborne state. The viability of aerosols of M. pneumoniae has been shown to be a function of both temperature and RH, with provision of optimal conditions by low temperatures and the extremes of RH (Wright, Bailey and Goldberg, 1969). These conditions pertained in the survey sheep house.

The susceptibility of individual animals to infectious agents is influenced mainly by age, genetic factors, which include reaction to ambient conditions and "stress", and possession of immunity, either active, passive or hypersensitive, to the agents involved. The antibody titre profiles suggest that colostral transfer of maternal antibody to mycoplasmas can occur, and titres of maternally derived antibody decline to a minimum at about 50 d.o. In a survey of this nature, the effectiveness of colostrally-acquired immunity in resisting mycoplasmal infection is difficult

to assess. Suggestive of a temporary protective function of passively-acquired immunoglobulin is the low mycoplasma recovery rate from the lungs of Group 1 (71-80 d.o.) animals compared with other groups (120-180 d.o.), despite the high proportions of animals yielding M. ovipneumoniae from tonsillar tissue in Group 1 (6/7) and from nasal swabs at 45 - 55 d.o. (80%). Conversely, there appears to have been no suppressive effect on the infection rates of the nasal cavity with M. ovipneumoniae, although colostral immunoglobulin has been shown to appear on the surface of the nasal mucosa of lambs (Smith, Wells, Burrells and Dawson, 1976). The waning of colostral immunoglobulin levels may be the reason for the increase in morbidity and severity of clinical signs in the survey lambs as a whole between the samplings at 45 - 55 d.o. and 99 - 114 d.o. This increase cannot however be ascribed to mycoplasmal infection alone, as might be inferred from the necropsy results from Group 2 lambs, since serology indicates that PI3 virus infection also occurred in the lambs at this time. Possible alternatives to colostrally-acquired immunity as reasons for the low recovery rates of mycoplasmas from Group 1 animals are age, implying increasing susceptibility with maturation or exposure to the organisms, or the weight of challenge, which would escalate as more animals became infected with, and therefore excretors of, respiratory organisms. The latter possibility may apply particularly to M. arginini, where both numbers of initial donor animals and lung recoveries of the organism from Group 1 lambs were low.

The post mortem examinations revealed no correlation between

the presence of M. arginini and any of the histopathological changes observed in the lungs of lambs or ewes. The effects ascribable to M. ovipneumoniae are ambiguous. Lung recoveries of the organism alone were obtained both from animals which had insignificant pulmonary changes and from animals with lymphoid hyperplasia, with or without interstitial thickening. M. ovipneumoniae was implicated in every case of P.E. pneumonia, but the recovery of only single colonies of P. haemolytica from two cases lends support to the observation of Stamp and Nisbet (1963) that isolations of this bacterium from lesions of P.E. pneumonia are inconstant. Thus P.E. pneumonia may be due entirely to the effects of M. ovipneumoniae, of PI3 virus or of other organisms not examined for, in particular chlamydiae; in this context, P. haemolytica may be merely a non-pathogenic invader. A second possibility is that P.E. pneumonia results from the superinfection of P. haemolytica on the milder lesions created by a primary pathogen, suggested by this survey to be M. ovipneumoniae, but that in some cases the bacterium is rapidly cleared from the lungs thereafter. Finally, the disease might be due to P. haemolytica per se, the nature of the lesion and the inconstant presence of the organism being due to the immune status of the animal. The later appearance of P. haemolytica in the lungs of lambs compared with M. ovipneumoniae favours the first two possibilities.

The recoveries of M. conjunctivae from nasal swabs of lambs was consistent with the observation of keratoconjunctivitis associated with this organism in the same flock (Jones et al., 1976a),

although the age of peak recovery rate from nasal swabs (25 - 35 d.o.) preceded slightly that from eye swabs (45 - 55 d.o.). Recovery of A. laidlawii from nasal swabs has been reported by other workers (Leach et al., 1976), but presence of the organism in the lungs of sheep has not previously been recorded. Furthermore, although ureaplasmas have been isolated from the urogenital tract of sheep (Livingston and Gauer, 1975; Doig and Ruhnke, 1977), the seven isolations of ureaplasmas from nasal swabs, tonsillar tissue and lungs reported herein appear to be the first recorded recoveries of these organisms from the ovine respiratory tract. Neither A. laidlawii nor ureaplasma lung recoveries were associated with any specific pathological changes or with the presence of P.E. pneumonia. Thus the rôle of these mycoplasmas in ovine pneumonia would seem to be negligible, unlike the situation in cattle where ureaplasmas appear to be of some importance in pneumonic conditions (Gourlay and Thomas, 1970; Shimizu, Nosaka and Nakamura, 1975).

The association of mycoplasmas and bacteria, notably P. haemolytica, with outbreaks of respiratory disease in sheep has been described by several workers. Mohn and Utklev (1974) investigated outbreaks of tracheobronchitis and fibrinous pneumonia in 11 flocks in Norway in which both lambs and adults were involved. Lambs first become affected at about 2 w.o., and during the initial acute phase a mortality rate of 1 - 5% was observed. Surviving lambs developed chronic infections and showed reduced growth rates. P. haemolytica biotype A was recovered from the lungs of most cases;

biotype T was also recovered from those animals that died of fibrinous pneumonia. Mycoplasmas were recovered from the LRT of affected sheep in eight of the 11 flocks examined. In all cases where mycoplasmas were isolated, P. haemolytica strains were also present. P. haemolytica was considered by the authors to be the main causative agent in the diseases described, but that predisposing factors were involved in the pathogenesis. It was observed that colostrum did not appear to provide adequate protection against the pneumonias encountered. The mycoplasmas isolated were not identified, but Giemsa-stained preparations of a broth culture show a marked resemblance to the light microscopy appearance of M. ovipneumoniae as described in Chapter 3. Preliminary experiments conducted with some of the isolated mycoplasma strains produced lesions of interstitial pneumonia in colostrum deprived lambs.

Subsequent to the implication of M. ovipneumoniae in pneumonia of sheep in Queensland (St. George et al., 1971; Carmichael et al., 1972), the natural history of the disease was studied in 100 ewes and their 107 lambs from one of the pneumonic flocks (Sullivan et al., 1973a). Pneumonia in the lambs was observed to progress through three distinct clinical stages. Minimal evidence of pneumonia was seen until the lambs were 35 - 70 d.o., when a sudden onset of severe clinical pneumonia affecting the whole flock occurred; this reached its peak 14 days after onset, when 30% of lambs were severely affected. Four weeks after onset, when the lambs were 63 - 98 d.o., the outbreak

had begun to subside, and by 6 m.o. the lambs appeared virtually normal. Eight lambs of 2 - 50 d.o., necropsied when the flock was clinically normal, had lobular atelectasis, but only one animal yielded mycoplasmas of a centreless colony morphology. Though not identified, these were similar to those recovered by St. George et al. (1971), and are presumed to be M. ovipneumoniae. A further nine lambs of 57 - 180 d.o. were necropsied when overt pneumonia was present in the flock. M. ovipneumoniae-like organisms were recovered from the lungs of seven lambs. The "primary" lung lesion in these animals was a proliferative interstitial pneumonia resulting from proliferation of alveolar septal cells; perivascular and peribronchiolar lymphoid cell cuffing was also observed. The histology of these lambs was closely similar to that termed lymphoid hyperplasia with interstitial thickening in the survey described in this chapter. From the results presented, the precise relationship between mycoplasmal presence and lung histopathological changes is not clear. Five animals showed lobar pneumonia which, in every case except one, was associated with the presence of various species of bacteria. Histologically, this pneumonia conformed in virtually all aspects with the definition proposed for P.E. pneumonia in this work.

The similarities between the Australian and Scottish findings are striking. Sullivan et al. (1973a, b) considered that their inability to recover mycoplasmas from younger animals may have been due to mishandling of tissue specimens, but results presented herein indicate that maternally-derived antibody may

also have been responsible. Other correlative findings are the association of mycoplasmas with interstitial pneumonia (lymphoid hyperplasia with interstitial thickening) and a general though not invariable relationship between mycoplasmal and bacterial presence and lobar (P.E.) pneumonia. Sullivan et al. did not report recovery of mycoplasmas of morphology similar to M. arginini. The major differences between the two surveys, the ages at which clinical symptoms, microorganism recoveries from the lungs and pulmonary lesions occurred in the lambs supports a previous suggestion that this may relate to the weight of challenge. In the Australian survey, the whole flock was involved in the outbreak of respiratory disease that occurred when the lambs were 35 - 70 d.o., implying the excretion of high numbers of respiratory microorganisms by all animals; no precipitating cause for the involvement of ewes as well as lambs in this outbreak was suggested. In contrast, in the Scottish survey pneumonia occurred principally in the lambs, with no major involvement of the ewes.

The flock observed by Sullivan et al. was maintained outdoors, but two other surveys of mycoplasma prevalence have concerned housed sheep. The English flock of 1800 sheep investigated by Leach et al. (1976) was kept in controlled environment houses, and the health of the flock was generally good. Over a nine month period, respiratory and urinogenital tract samples were obtained from 266 sheep from this or a similar unit. Mycoplasmas identified on serological or biological grounds as M. ovipneumoniae were recovered from 30% of nasal swabs, 38% of lungs

and one of 167 vaginal swabs. Six of the 32 lungs examined had macroscopic pneumonic lesions, and all six yielded M. ovipneumoniae. No other mycoplasmas were recovered from lungs, but A. laidlawii was isolated from 22 specimens (nasal and vaginal swabs and semen), M. arginini from 12 nasal swabs and unidentified strains from 24 specimens. Ureaplasmas were not isolated.

St. George and Carmichael (1975) in the USA investigated a chronic debilitating pneumonia which occurred in a flock of over 1000 ewes and their lambs. Nasal swabs from 32 lambs yielded 18 isolates of M. ovipneumoniae and 24 of M. arginini. Histological examination of consolidated areas in typical cases showed pathological changes similar to the chronic proliferative pneumonia described by Sullivan et al.(1973a, b,), accompanied by a suppurative bronchiolitis. Nineteen lambs examined microbiologically at slaughter yielded M. ovipneumoniae only from the LRT: 15 tracheal samples, 16 bronchial swabs but only three triturated lung suspensions yielded the organism. Most lungs also contained E. coli, Pasteurella spp. and occasionally Klebsiella species. No histopathological examinations were performed on the lungs of these animals.

The controlled environment conditions under which the animals investigated by Leach et al. (1976) were maintained may account for their low mycoplasmal infection rates and good health status compared with the surveys reported herein and by St. George and Carmichael (1975). In the American survey, conditions within the barns in which the sheep were kept were stated to be good, but

no details were given of ambient conditions or husbandry methods.

An initial report on the occurrence of M. ovipneumoniae and M. arginini in New Zealand (Clarke et al., 1974) was followed by an investigation into the prevalence of mycoplasmas in 6 - 9 m.o. sheep sampled at slaughter (Alley, Quinlan and Clarke, 1975) The animals were classified as "normal" or "pneumonic": histopathological details were not given, but the pneumonia observed was described as subacute or chronic. Nasal swabs from 27 normal and 27 pneumonic sheep yielded 48% and 70% recoveries of M. ovipneumoniae and 33% and 52% recoveries of M. arginini respectively. Lung material was examined initially only for the presence or absence of mycoplasmas: 63% of 27 normal lungs yielded M. ovipneumoniae and 19% yielded M. arginini. In contrast, 79% of 63 pneumonic lungs yielded M. ovipneumoniae and 32% M. arginini. Due to the possibilities of contamination, particularly during meat inspection, a further 60 pneumonic and 40 normal lungs were assayed for numbers of M. ovipneumoniae. All pneumonic lungs yielded $10^3$ - $10^9$ ccu of M. ovipneumoniae per 0.3 ml, with a geometric mean of $10^{6.6}$ ccu per 0.3 ml. Ten of the normal lungs yielded up to $10^7$ ccu of M. ovipneumoniae per 0.3 ml, but generally $10^3$ ccu per 0.3 ml or less.

The lung recoveries of M. arginini by the New Zealand workers were considerably higher than those obtained in the survey reported herein. However, both surveys indicate a greater degree of involvement of the organism in sheep pneumonia than appears from the work of Sullivan et al. (1973), St. George and Carmichael (1975) and

Leach et al. (1976). More significantly, the New Zealand findings corroborate those of this survey and of Leach et al. (1976) that M. ovipneumoniae may be recovered from the nasal cavity and lungs of a high proportion of healthy sheep, although "normal" lung histology was not defined by Alley et al. (1975) or by Leach et al. (1976). Although the assertion by St. George and Carmichael (1975) that "... the main significance of this mycoplasm (M. ovipneumoniae) is that it allows localization and multiplication of secondary bacterial invaders" may be correct, nevertheless this viewpoint must, at present, be regarded with reservation. To quote Alley et al. (1975), "... M. ovipneumoniae may play a rôle in the pathogenesis of chronic pneumonia. Whether this rôle is that of a primary pathogen or represents efficient colonisation by an ubiquitous organism of lung damaged by other agents requires further investigation".

# CHAPTER 5

## INVESTIGATIONS INTO THE PATHOGENICITY OF *M. OVIPNEUMONIAE* IN SPECIFIC PATHOGEN FREE LAMBS

### GENERAL METHODS AND MATERIALS

#### Specific pathogen free (SPF) lambs

The hysterectomy-produced, colostrum-deprived lambs were maintained under SPF conditions at an ambient temperature of 18°C and 40% RH (Hart, Mackay, MacVittie and Mellor, 1971; Brotherston, 1968). The lambs, which were generally Suffolk, Blackface or Dorset Horn cross-breeds, were housed in numbers of up to eight per pen.

#### Inoculation technique

In all experiments, inoculation was by the e.b. route. The animals were anaesthetized with i.v. or i.m. "Immobilon" (Reckitt and Colman Pharmaceutical Division, Hull). Lambs of 10 d.o. or less were premedicated with i.m. atropine to prevent vagal stimulation on insertion of the endotracheal tube. Each animal was laid on its left side, an endotracheal tube inserted and through this a 600 mm length of sterilized nylon tubing (1.5 mm internal diameter) passed as far as possible. At this site, presumed to be within the left bronchus, half the inoculum was deposited. The tube was partly withdrawn, the animal turned onto its right side and the procedure repeated. Anaesthesia was terminated with i.v. "Revivon" (Reckitt and Colman).

# EXPERIMENTS 1 AND 2

## INTRODUCTION

The possible importance of *M. ovipneumoniae* in chronic respiratory disease of sheep in Scotland had been confirmed by the survey reported in Chapter 4. The experimental infection of sheep with a strain of *M. ovipneumoniae* isolated and subcultured in tissue culture by St. George *et al.* (1971) had reproduced a proliferative interstitial pneumonia. The same group reproduced similar pulmonary lesions in sheep by the use of lung lesion suspensions or broth cultures containing *M. ovipneumoniae*: macroscopic lesions occurred in just over 50% of all experimental animals (Sullivan *et al.*, 1973a, b). However, mycoplasmas were not recovered from experimental sheep in these subsequent experiments, nor were hysterectomy-produced, colostrum-deprived animals used. The aims of Experiments 1 and 2 were to investigate the effects and transmissibility of two Scottish strains of *M. ovipneumoniae* in SPF lambs.

## MATERIALS AND METHODS

### Inocula

Both strains of *M. ovipneumoniae* used were isolated from nasal swabs, strain 956/2 (Experiment 1) from a ewe, and strain 956/3 (Experiment 2) from a lamb. The animals belonged to a conventionally-managed flock in which respiratory signs had been present for three months before sampling.

To avoid possible attenuation by passage through artificial media, the two isolates were treated as follows. After primary isolation in OB, each was subcultured in 40 ml of OB, and following incubation for two days, aliquots were stored at -70°C. No mycoplasma species other than M. ovipneumoniae, identified by the GI test, were detected in this culture, and bacteriological and virological examination gave negative results. Before use aliquots of the strains were thawed and diluted in OB; the final titre was $10^7$ ccu per 0.2 ml. The volume of inoculum for each animal was 2 ml. The inoculum for control lambs was sterile OB.

Experimental animals

In Experiment 1, the SPF lambs were inoculated when 5 d.o. The experimental lambs (1 to 6) were housed in one pen and the three uninfected control lambs (7 to 9) in a separate pen. In Experiment 2, lambs A and B were inoculated when 5 d.o. and one day later six lambs (C, D, E, F, G and H) were placed in the same pen.

Sampling and clinical examination

Rectal temperatures were recorded daily, and the lambs auscultated daily for the first 12 days and at intervals of one to three days thereafter. In Experiment 1, the respiration rates were also recorded at the same intervals as for auscultation. Nasal swabs for examination for mycoplasmas were taken every second day for the first 12 days, and every fourth day thereafter: serum samples were taken every four days. In Experiment 2, nasal swabs

for examination for mycoplasmas were taken at intervals of two to four days until 34 days post exposure (d.p.e.). The remaining three lambs were finally sampled at 41 d.p.e. Serum samples were taken at weekly intervals. Nasal swabs for bacteriological and virological examination were taken every week in both experiments.

## Necropsy

### Experiment 1

The animals were killed with i.v. sodium pentobarbitone at predetermined times, lambs 1, 2 and 7 at 7 days, lambs 3, 4 and 8 at 14 days and lambs 5, 6 and 9 at 28 d.p.i.

The trachea was exposed and clamped off 50 - 100 mm below the larynx to prevent collapse of the lungs. The thorax was opened with aseptic precautions and the lungs and trachea removed to a sterile tray. The left bronchus was exposed, clamped off and the left lung taken for microbiological examination. The right lung was infused via the trachea with 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 from a reservoir 300 mm above the bench. The trachea was then clamped below the infusion point and the lung allowed to fix for 30 mins in a bath of the fixative.

The following tissues were removed for examination for mycoplasma: nasal mucosa, tonsillar tissue, retropharyngeal lymph node, trachea, left bronchial lymph node, left bronchial swab, portions of the apical, cardiac and diaphragmatic lobes of the left lung and liver, spleen, kidney and cerebrum.

Samples from the three lobes of the left lung, nasal mucosa and spleen were also taken for bacteriological and virological examination.

Experiment 2

The animals were killed by the same method as in Experiment 1, lambs A and C being killed at 16 d.p.i. and 15 d.p.e. respectively, lambs D, E and F at 35 d.p.e. and lambs B, G and H at 43 d.p.i. and 42 d.p.e. respectively.

No attempt was made to prevent collapse of the lungs at necropsy. Samples collected for examination for mycoplasma were similar to those collected in Experiment 1, with the omission of liver, kidney, spleen and cerebrum samples, and the addition of samples from the three lobes of the right lung. Samples of the lungs and trachea were also taken for bacteriological and virological examination.

Portions from both sides of the lung were fixed by immersion in 10% formol saline.

Microbiology

The techniques and media employed were as described in Chapter 2, except that for mycoplasmology treatment of tissues prior to seeding of growth media was by placement of approximately 0.5 g of chopped material in 4.5 ml of OB, followed by incubation at 37°C for 1 h. Examinations for viruses were by three passages in primary sheep thyroid cell cultures.

Serology

The sera of lambs were examined for antibodies to M. ovi-pneumoniae by the IHA and MI tests.

Histopathology

In Experiment 1, blocks of 1 $mm^3$ were excised from all lesions in the right lung and from equivalent areas in all animals not having lesions. These were processed through to araldite and 1 µm thick sections were stained with Giemsa at 60°C for light microscopy. Thin sections were stained with lead citrate and examined in a Siemens Elmiskop 51. A comparable series of larger blocks were post fixed in 10% formol saline, trimmed and processed through to paraffin wax. Samples from the left lung, trachea, tonsillar tissue, retropharyngeal lymph nodes and nasal turbinates from Experiment 1, and all tissues examined from Experiment 2 were fixed in 10% formol saline, trimmed and processed through to paraffin wax. Sections were cut at 6 µm and stained with haematoxylin and eosin. Selected sections were also stained by periodic acid Schiff (PAS), Hale's colloidal iron, Southgate's mucicarmine method for mucin and Best's carmine method for glycogen (Carleton, 1967).

RESULTS

Experiment 1

Clinical examinations

No rectal temperatures exceeding 40.6°C (105°F) were recorded. Respiration rates varied considerably. Lamb 2 exhibited dyspnoea

alternating with periods of polypnoea from day 2 p.i. onwards.

Abnormal auscultatory sounds were generally of a harsh, dry nature and only heard in lambs 2, 3, 4 and 6. Lamb 2 had harsh sounds on auscultation prior to infection; in this animal, the sounds increased in intensity after infection until crepitations and sonorous rhonchi were also audible. Mild transient harshness was also detected in control lambs 7 and 9 at day 5 p.i.

Apart from lamb 2, no obvious respiratory symptoms were noted in the other animals except in lamb 6, which developed a serous nasal discharge from day 20 p.i. onwards.

Microbiology

No mycoplasmas were recovered at any time from the three control lambs Nos. 7, 8 and 9. Table 5.1 shows the incidence of mycoplasma infection in the nasal cavities. All the experimental animals were infected at this site except lamb No. 4. Table 5.2 shows that five lambs also yielded mycoplasmas from the lungs. All six infected lambs were positive for mycoplasmas from tracheal and tonsillar tissue, and mycoplasma were also recovered from the retropharyngeal lymph nodes of four animals and the bronchial lymph node of one animal. No mycoplasmas were recovered from the spleen, kidney, liver or cerebrum of any of the animals.

Nasal swabs also yielded Escherichia coli, Enterobacter cloacae, Citrobacter spp. and Streptococcus mitis; coagulase positive Staphylococcus pyogenes was isolated from one nasal swab.

No bacteria were isolated from the lungs or trachea of any of the animals except lamb 2, from which heavy growths of E. coli

Table 5.1 Experiment 1. Recovery of M. ovipneumoniae from nasal swabs

| Lambs | Days post inoculation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 |
| 1 | + | + | +K | | | | | | | | | | | |
| 2 | - | + | +K | | | | | | | | | | | |
| 7 | - | - | -K | | | | | | | | | | | |
| 3 | - | - | - | + | + | C | K | | | | | | | |
| 4 | - | - | - | - | - | - | K | | | | | | | |
| 8 | - | - | - | - | - | - | K | | | | | | | |
| 5 | + | + | + | - | + | - | | + | | - | | - | | -K |
| 6 | + | + | + | + | + | + | | + | | C | | + | | +K |
| 9 | - | - | - | - | - | - | | - | | - | | - | | -K |

Lambs 7, 8 and 9 were control animals given OB broth only.

K = Killed

C = Contaminated

Table 5.2 Experiment 1. Recovery of M. ovipneumoniae at necropsy

| | 7 d.p.i. | | | 14 d.p.i. | | | 28 d.p.i. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Infected lambs | | Control lamb | Infected lambs | | Control lamb | Infected lambs | | Control lamb |
| Sample | 1 | 2 | 7 | 3 | 4 | 8 | 5 | 6 | 9 |
| Nasal mucosa | + | + | - | + | + | - | - | + | - |
| Tonsillar tissue | 1 | 6 | - | 4 | 1 | - | 2 | 6 | - |
| Retropharyngeal l.n. | - | + | - | + | + | - | + | - | - |
| Tracheal mucosa | 5 | 7 | - | 4 | 4 | - | 2 | 6 | - |
| Bronchial l.n. | - | - | - | + | - | - | - | - | - |
| Bronchial swab | 0 | 7 | - | 6 | 5 | - | 5 | 4 | - |
| L. apical | 0 | 8 | - | 8 | 6 | - | 0 | 5 | - |
| L. cardiac | 0 | 8 | - | 7 | 6 | - | 0 | 7 | - |
| L. diaphragmatic | 0 | 7 | - | 6 | 6 | - | 4 | 5 | - |
| Liver | - | - | - | - | - | - | - | - | - |
| Spleen | - | - | - | - | - | - | - | - | - |
| Kidney | - | - | - | - | - | - | - | - | - |
| Cerebrum | - | - | - | - | - | - | - | - | - |

Figures represent titres (ccu per 0.2 ml) expressed as $\log_{10}$.
+ and - indicate presence or absence in culture (no titrations performed)
l.n. = lymph node apical, cardiac, diaphragmatic = lung lobes
L = left.

were obtained from the apical and diaphragmatic lobes of the left lung. No viruses were isolated from any of the specimens.

Pathology

The lungs of infected lambs 1, 4 and 5 and control lambs 7, 8 and 9 appeared normal both on macroscopic and microscopic examination. Macroscopically, lambs 2, 3 and 6 showed clearly demarcated focal areas of collapse and consolidation with a lobular distribution generally confined to the apical and cardiac lobes (Fig. 5.1).

Microscopically, the areas of collapse in lamb 2 consisted of alveoli packed with neutrophils and alveolar macrophages. An exudate with the staining characteristics of acid mucopolysaccharide was seen to fill groups of alveoli. This exudate gave a strong positive reaction with Hale's colloidal iron, but only weak reactions by the PAS method and Southgate's method for mucin, and a negative reaction by Best's method for glycogen (Fig. 5.2).

In lamb 3 the collapsed foci were firmer. The affected tissue contained more neutrophils than alveolar macrophages. The neutrophils tended to be clumped in end bronchioles whereas the macrophages were spread more evenly throughout the alveoli (Fig. 5.3). Peribronchiolar cuffing with lymphoid cells was seen, as was an increase in Type II alveolar cells (Fig. 5.4).

These last two changes were marked in lamb 6. The peribronchiolar cuffs were often many cells thick and on occasions lymphoid cells could be seen infiltrating the bronchiolar epithelial lining: in such areas cilia were absent. An increase in numbers

Fig. 5.1 Left apical and cardiac lung lobes of lamb 3, Experiment 1, showing brown/grey focal areas of consolidation with a lobular distribution.

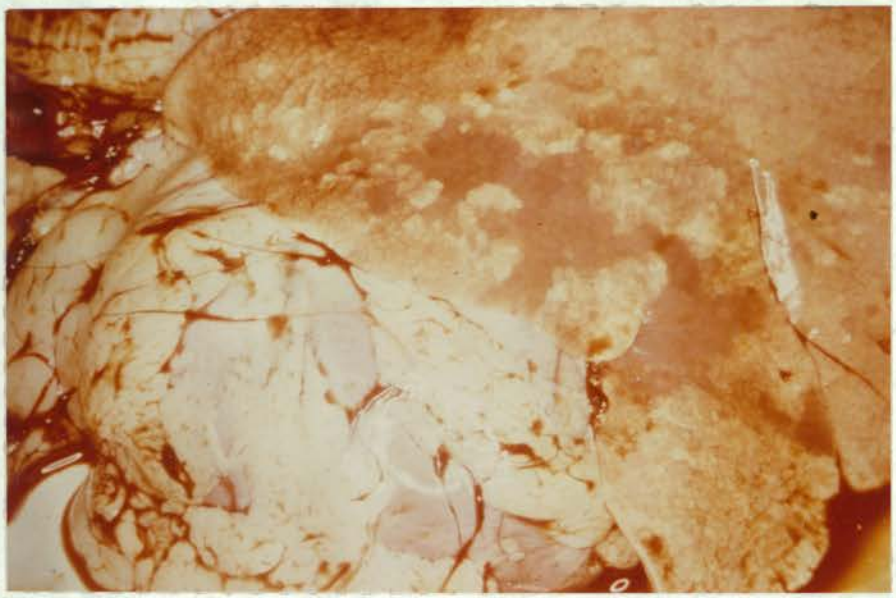
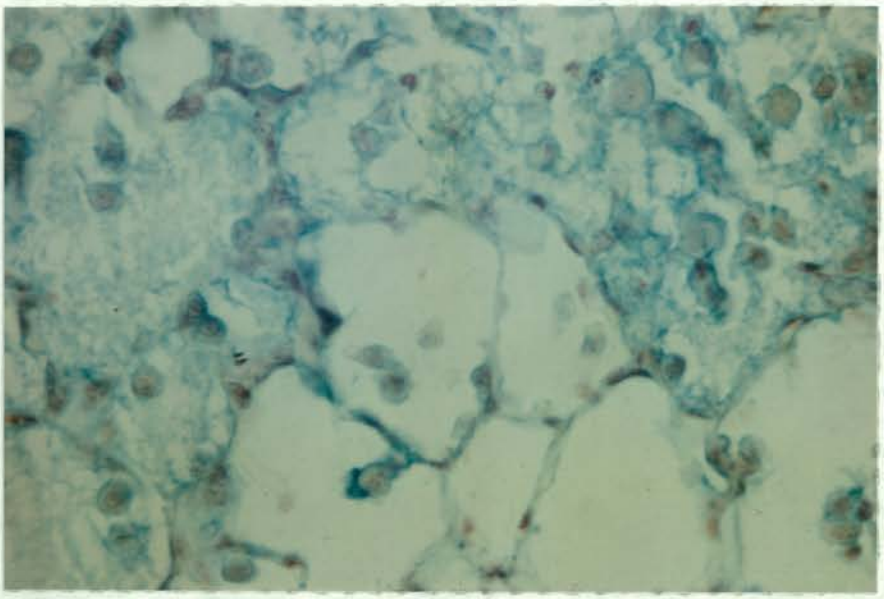


Fig. 5.2 Section of lung from lamb 2, Experiment 1 (killed 7 d.p.i), stained by Hale's colloidal iron to show exudate present in the alveoli.

X 1000

Fig. 5.3 Section of lung from lamb 3, Experiment 1 (killed 14 d.p.i.) showing mild peribronchial lymphoid hyperplasia, clumping of neutrophils in airways, and intra-alveolar macrophages and Hale-positive material.

Hale's colloidal iron X 104



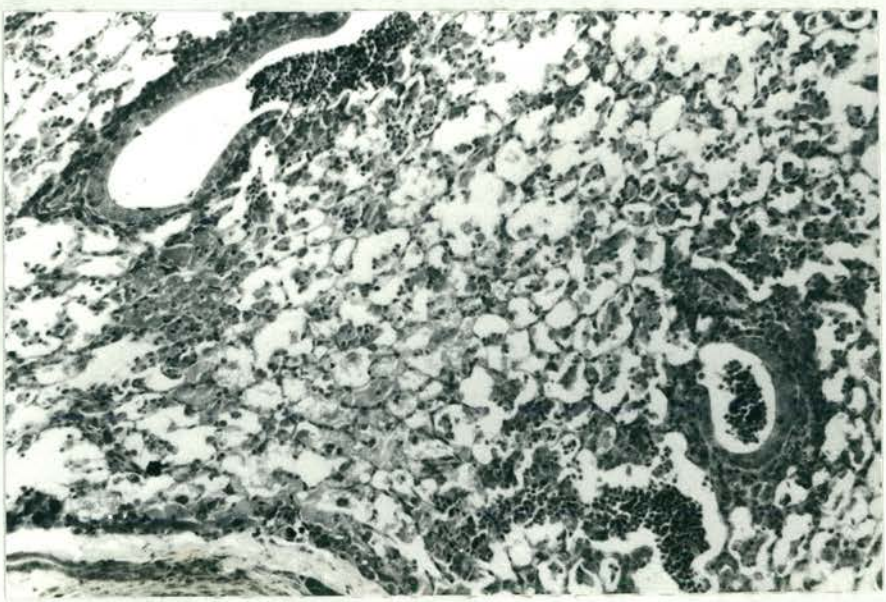
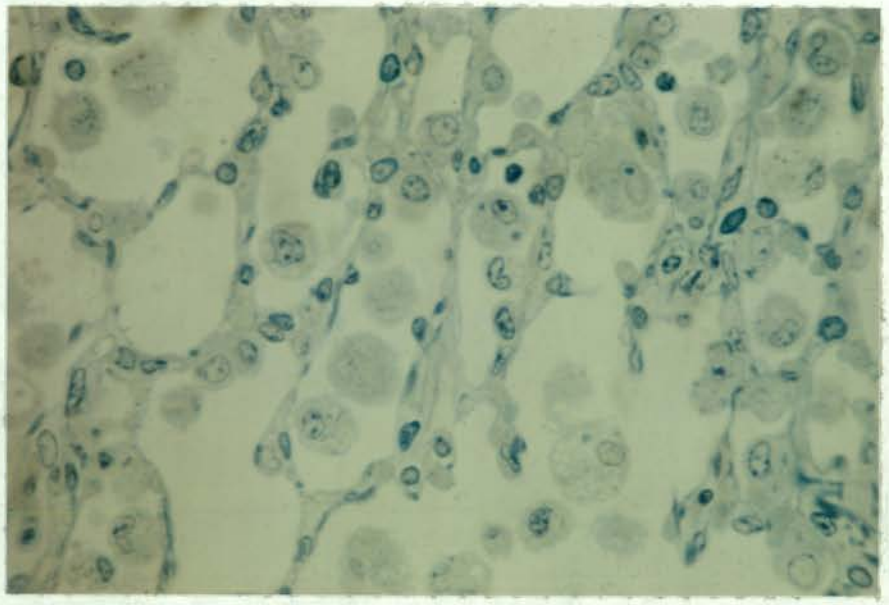


Fig. 5.4 Section of lung from lamb 3, Experiment 1 (killed 14 d.p.i.) showing increased numbers of Type II alveolar cells and intra-alveolar macrophages.
Giemsa X 1000

Fig. 5.5 Section of lung from lamb 6, Experiment 1 (killed 28 d.p.i.) showing a clearly demarcated area of collapse, in which marked peribronchiolar and perivascular lymphoid cell hyperplasia and intra-alveolar inflammatory cells are present.
Giemsa X 10$^4$



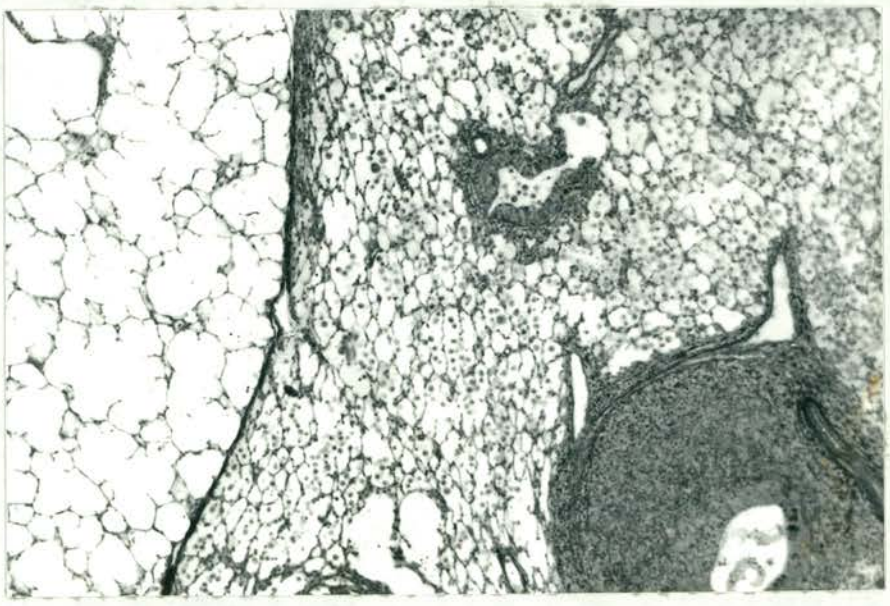
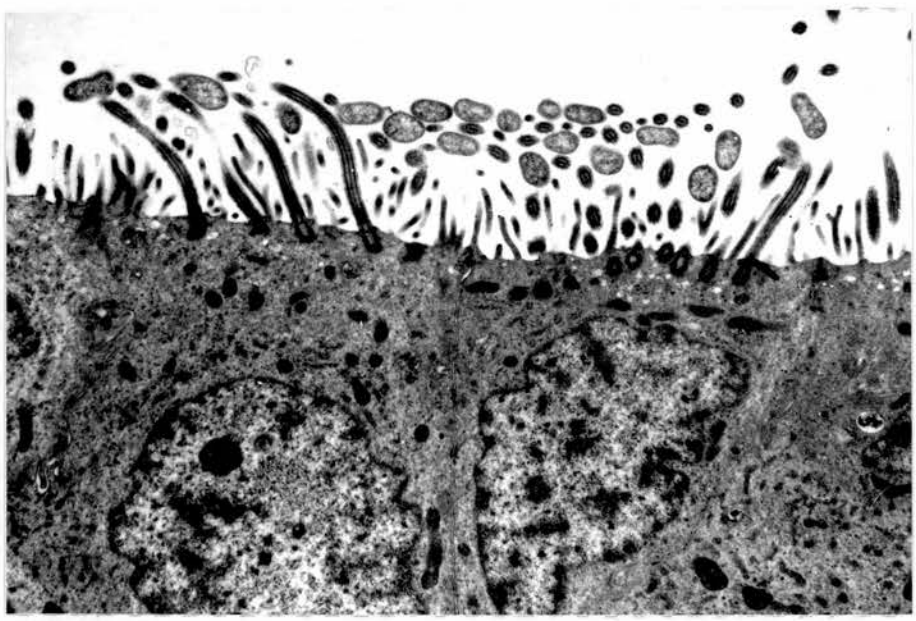


Fig. 5.6 Electron micrograph showing mycoplasmas in association with bronchiolar cilia in lamb 2, Experiment 1 (killed 7 d.p.i.).

X 8600



of Type II cells occurred only in areas of collapse, where they could be seen in twos and threes in these alveoli. The inflammatory cells present were predominantly macrophages, few neutrophils being present. Little excess Hale positive material was seen (Fig. 5.5).

The retropharyngeal lymph nodes of lambs 3 and 6 were enlarged and their medullary cords mildly hypercellular. No significant changes were observed in tonsillar tissue, trachea or nasal turbinates.

By electron microscopy, mycoplasmas were only seen in lamb 2, where they were in association with bronchiolar cilia (Fig. 5.6), and sometimes adjacent to cells displaying ciliary loss. Generally, however, ciliary loss was minimal in all infected lambs. Neutrophils often occurred in the submucosa or lying between epithelial cells in the vicinity of these mycoplasmas. Structures which were less dense but of a similar size to mycoplasma were seen in alveoli, both free and in the process of being phagocytosed by macrophages and neutrophils, but no positive identification could be made.

## Experiment 2

### Clinical examinations

No rectal temperatures exceeding 40.6°C were recorded. Mild, abnormal sounds were heard on auscultation in lambs A and H.

Lambs B, F and G developed a mild serous nasal discharge at various times during the experiment.

### Microbiology

*M. ovipneumoniae* was recovered in nasal swabs from all eight lambs (Table 5.3), but only from the lungs of the donor lambs (Table 5.4). In the recipient lambs, mycoplasmas were recovered from the

Table 5.3 Experiment 2. Recovery of M. ovipneumoniae from nasal swabs.

| Lambs | Days post inoculation of lambs A + B (lambs C-H placed in contact 1 day later) | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | 42 | 44 |
| A | - | + | + | + | + | | + | + | + | K | | | | | | | | | | | | |
| B | - | - | + | + | + | | + | + | + | | + | + | | + | + | | + | + | | | | CK |
| C | - | | - | + | + | | + | + | C | K | | | | | | | | | | | | |
| D | - | | - | + | + | | + | + | + | | + | + | | + | + | | - | CK | | | | |
| E | - | | - | C | C | | C | C | C | | + | C | | + | C | | + | +K | | | | |
| F | - | | - | - | - | | + | + | + | | + | + | | + | + | | C | CK | | | | |
| G | - | | - | - | - | | - | + | + | | + | + | | + | + | | + | + | | | | +K |
| H | - | | - | - | - | | + | + | + | | + | + | | + | + | | + | + | | | | -K |

K = Killed

C = Contaminated

Table 5.4 Experiment 2. Recovery of M. ovipneumoniae at necropsy.

| | 16 d.p.i. | 15 d.p.e. | | 35 d.p.e. | | 43 d.p.i. | 42 d.p.e. | |
|---|---|---|---|---|---|---|---|---|
| Sample | A | C | D | E | F | B | G | H |
| Nasal mucosa | + | - | - | + | + | - | + | - |
| Tonsillar tissue | + | - | + | + | + | + | + | + |
| Retropharyngeal l.n. | + | - | - | - | - | - | - | - |
| Tracheal mucosa | + | - | - | - | - | + | + | - |
| Bronchial l.n. | - | - | - | - | - | + | - | - |
| Bronchial swab | + | - | - | - | - | - | - | - |
| L. apical | - | - | - | - | - | + | - | - |
| L. cardiac | + | - | - | - | - | + | - | - |
| L. diaphragmatic | - | - | - | - | - | + | - | - |
| R. apical | ND | - | - | - | - | - | - | - |
| R. cardiac | ND | - | - | - | - | - | - | - |
| R. diaphragmatic | ND | - | - | - | - | - | - | - |

R = right

ND = not done

Otherwise, symbols and abbreviations as for Table 5.2.

nasal mucosa of three animals, from tonsillar tissue of five animals and from the trachea of one animal.

All animals yielded coagulase positive Staphylococcus pyogenes from nasal swabs at various stages during the experiment. E. cloacae, E. coli and Streptococcus epidermidis were also isolated from some nasal swabs.

No bacteria or viruses were isolated from any of the tissues examined at necropsy.

Pathology

None of the lambs showed any macroscopic lesions in the lungs. Microscopically, only lamb A had pathological changes in the lungs, which consisted of mild interlobular oedema and small bands of collapse. No peribronchiolar cuffing or Hale positive exudate was seen, and intra-alveolar inflammatory cells in collapsed areas were sparse.

Serology

No antibodies due to the mycoplasmal infection could be demonstrated in any lambs from either experiment by means of the IHA and MI tests.

## DISCUSSION

In Experiment 1, the endobronchial inoculation of a broth culture containing $10^7$ ccu per 0.2 ml of a low passage strain of M. ovipneumoniae produced mild macroscopic and microscopic lesions in three of six SPF lambs. Mycoplasmas were recovered from the lungs of five of the lambs, in four of them at high titres. The

recovery of mycoplasmas from retropharyngeal and bronchial lymph nodes indicates that local spread of the infection into the lymphatic system occurred.

Except in lamb 4, in which the lungs appeared to be normal, abnormal respiratory sounds were heard only in those lambs with macroscopic lesions. Lamb 2, in which respiratory symptoms were particularly marked, yielded a heavy growth of E. coli from the lungs. Furthermore, during endotracheal intubation of this lamb froth was produced from the tube. The significance of the clinical symptoms and pathology of this animal with regard to the mycoplasma infection is therefore doubtful.

In Experiment 2, the endobronchial inoculation of M. ovipneumoniae into two donor lambs produced a pulmonary infection but no macroscopic lesions. Only minimal microscopic lesions were seen in the lungs of one of the donor lambs. Although all six contact lambs became infected with M. ovipneumoniae, no mycoplasmas were recovered from the lungs and no pathological changes were noted in any of these animals.

No seroconversion due to the mycoplasmal infection could be demonstrated in any of the animals by either the IHA or MI tests. Other workers (Goodwin, Hodgson, Whittlestone and Woodhams, 1969; Cole, Cahill, Wiley and Ward, 1969) have also been unable to demonstrate IHA or MI activity early after the experimental infection of animals with mycoplasmas, although Goodwin et al. (1969) found that high titres to the IHA test had developed by 16 w.p.i. Fernald (1969) was similarly unable to demonstrate IHA activity following the experimental infection of hamsters with M. pneumoniae,

although high levels of GI antibodies were detected from two weeks onwards.

As only three lambs had lung lesions, and since in-contact transmission caused no colonisation of the lungs, no definite conclusions can be reached concerning the pathogenicity for SPF lambs of the strains of M. ovipneumoniae used. Interpretation of these results is further complicated by the recovery of E. coli from the lungs of one of the lambs with lesions. However, the pathology of the lungs is similar, though milder, to that described for the experimental infection of pigs with M. hyorhinis (Baskerville, 1972) and M. hyopneumoniae (Livingston, Stair, Underdahl and Mebus, 1972), of mice with M. pulmonis (Lindsey and Cassell, 1973) and of hamsters with M. pneumoniae (Dajani, Clyde and Denny, 1965). The pathogenesis of respiratory mycoplasmal disease has been considered to be due in part to their close attachment to respiratory epithelium (Collier, 1972). M. ovipneumoniae was observed in these studies to be above or enmeshed within the tips of the bronchiolar cilia at a relatively distant site from the epithelial surface compared with virulent strains of other mycoplasmas (e.g. Hu, Collier and Baseman, 1976; Abu-Zahr and Butler, 1976), and this factor may partly explain the mild nature of the lesions induced with M. ovipneumoniae.

The pathology of affected lambs in Experiments 1 and 2 fits with the observation by Fernald (1969) "that the changes observed are not part of an invasive or destructive process, but rather the marshalling of a local immune defence system in response to the

superficial infection within the bronchial tree". The total lack of pathological change in the lungs of lambs 4, 5 and B and the minimal changes in lamb A indicate that activation of the immune system in these animals did not occur despite the presence of mycoplasmas in the lungs, suggesting that M. ovipneumoniae was poorly or non-antigenic in these animals.

No attempt was made to clone or dilute the isolates used beyond the second passage because of the possibility of concomitant attenuation (Couch, Cate and Chanock, 1964; Dajani et al., 1965). Despite this, the pathogenicity of these strains would seem to be considerably less than that of the strains isolated by St. George et al. (1971) and Sullivan et al. (1973a, b). It is therefore necessary to compare the results of Scottish and Australian pathogenicity experiments with M. ovipneumoniae in detail. Initial experiments by Sullivan et al. (1973a) involved the i.t. inoculation of homogenized lung from a naturally infected 2 d.o. survey lamb (see Chapter 4) into two 23 d.o. and two 8 m.o. lambs. Macroscopic lesions of "greyness" and atelectasis were observed in three lambs and microscopic lesions of interstitial pneumonia in all four lambs at necropsy. Broth cultures administered to 8 m.o. lambs by i.t. inoculation and aerosol induced microscopic lesions of interstitial pneumonia in all four lambs, but macroscopic lesions only in the two lambs inoculated by the i.t. route. Subsequently, conventionally-born lambs which had been fed colostrum were exposed at 1 d.o. to infection with M. ovipneumoniae by aerosol (two lambs), i.v. inoculation (two lambs) or contact with the aerosolized lambs

(22 lambs). The lambs were killed 1 - 56 d.p.i. or d.p.e. Evidence of pneumonia was detected on auscultation in lambs exposed to infection by all three routes, and from as soon as 1 d.p.e. At necropsy, greyish discolouration of the lungs was observed in 21 lambs, lobar consolidation and pleurisy in three lambs and lobular atelectasis in 10 lambs. All animals had microscopic evidence of interstitial pneumonia with marked thickening of alveolar septa. In early cases there was an increase in free alveolar cells resembling macrophages, and a few neutrophils were present, but otherwise exudative changes were minimal. Alveolar collapse was a prominent feature. Peribronchiolar and perivascular cuffing with lymphoid cells was seen in 11 lambs. Bronchiolar epithelium, especially of terminal and respiratory bronchioles, showed hyperplastic and degenerative changes. Occasionally, marked goblet cell hyperplasia was observed in the large bronchioles. Considerable variation was noted in the severity of changes found in lambs killed at similar intervals after exposure: well developed interstitial pneumonia was present in two lambs at 2 d.p.e. No mycoplasmas or viruses were recovered from the lungs of any of the lambs in the experiments conducted by Sullivan *et al.* (1973a, b). Two of the 26 lambs in the latter experiments yielded *E. coli* from the lungs, but the pulmonary histology of these animals was not indicated.

Further discussion of the Australian work must be based on the assumption that, despite their failure to fulfil Koch's postulates by recovery of the organism from experimental animals, the effects observed by Sullivan *et al.* were due to exposure of lambs to *M. ovipneumoniae* and to this factor alone. These workers

considered that their inability to recover mycoplasmas from lung tissue might have been due to the release of mycoplasmacidal substances from ground tissue (Kaklamanis et al., 1969), or to the storage of such tissue at -100°C. Similar poor recoveries of mycoplasmas from lung tissue by Carmichael et al. (1972) and St. George and Carmichael (1975) were ascribed to the same reason. Assessment of the work of Sullivan et al. is further confounded by the absence of control animals in any of the experiments performed. The sera of lambs were not tested for antibody activity against mycoplasmas: however, it would appear that little or no protection to challenge with M. ovipneumoniae was afforded by the prior feeding of lambs with colostrum. Transmission of M. ovipneumoniae by contact, achieved with apparently total success using two donor animals and a contact period of 48 h, caused the rapid subsequent development of clinical symptoms and pathological pulmonary changes. These findings do not fit with either the Scottish or Australian survey observations nor with the results obtained in Experiment 2. The histopathological changes in the lungs of lambs also show differences from those observed in Experiment 1, in particular regarding the thickening of alveolar septa, hyperplasia and degenerative changes of bronchiolar epithelium and hyperplasia of goblet cells.

The reproduction of pneumonia in lambs by i.v. inoculation of a broth culture of M. ovipneumoniae is particularly surprising. With virulent strains of M. mycoides subsp. mycoides, it has not proved possible to induce CBPP by i.v. inoculation without the

simultaneous injection of agar emboli, blood clots or antiserum (Lloyd and Trethewie, 1970).

The experimental pathogenicity of M. ovipneumoniae for lambs has also been investigated by Hungarian workers (Tury, Belak, Palfi and Stipkovits, 1975). Four colostrum-deprived lambs of 3 d.o. were inoculated by the i.t. route with a cloned culture of M. ovipneumoniae. One lamb necropsied at 9 d.p.i. demonstrated interstitial thickening, bronchial epithelial hyperplasia, proliferation of alveolar macrophages and the presence of a few neutrophils. Two lambs killed at 59 and 79 d.p.i. had intralobular interstitial pneumonia with desquamation and proliferation of bronchial epithelial cells, peribronchiolar and perivascular infiltration with histiocytes, lymphocytes and eosinophils, and hyperplasia of peribronchial lymphoid follicles. No effects were apparently observed in the fourth animal killed at 27 d.p.i., nor were recoveries of mycoplasmas or other micro-organisms from experimental animals reported.

Survey and experimental studies have demonstrated that alveolar septal thickening and lymphoid hyperplasia constitute the two major forms of lung reaction to the presence of M. ovipneumoniae, and one or other or both lesions may be present in the lungs of infected animals. Whether this variation is dependent on the strain of M. ovipneumoniae, the duration of exposure or external factors is uncertain at present.

# EXPERIMENTS 3 AND 4

## INTRODUCTION

The inoculation of M. ovipneumoniae into SPF lambs in Experiment 1 had reproduced lymphoid hyperplasia accompanied by inflammatory cell exudate, one of the lesion forms observed in uncomplicated cases of naturally-occurring mycoplasmosis. Interstitial proliferative pneumonia, possibly an alternative form of lesion associated with M. ovipneumoniae, had been reproduced by the Queensland workers. The question was, did these effects represent the limit of pathogenicity of M. ovipneumoniae in lambs, or could more severe lesions, in particular P.E. pneumonia, be induced by the organism per se? It has been observed that the pathological changes elicited in the lungs of three lambs in Experiment 1 appeared to be due to host response rather than to toxicity of the organism. It was therefore postulated that more severe effects might stem from an exaggerated host response provoked by challenge of a hypersensitized animal. This hypothesis has been advanced by other workers. Brunner, James, Horswood and Chanock (1972) considered that, from the ecology of M. pneumoniae infections, pre-sensitization with the organism might be an important factor in pathogenesis of human atypical pneumonia. Evidence that delayed hypersensitivity occurs following mycoplasmal infection has been demonstrated by macrophage migration inhibition, lymphocyte transformation and intradermal skin tests in several animal species (e.g. Arai, Hinuma, Matsumoto and Nakamura, 1971; Biberfeld, 1973; Roberts, 1973;

Biberfeld, Biberfeld and Sterner, 1974; Biberfeld, 1974). A different form of hypersensitivity, the Arthus phenomenon, has been suggested as being involved in the pathogenesis of CBPP (Provost, 1969).

To investigate the pathogenic importance of prior exposure of lambs to M. ovipneumoniae, sensitization was attempted by both e.b. inoculation of a low dose of the organism and by i.n. inoculation. Three different preparations of the same strain of M. ovipneumoniae were used for secondary challenge, to investigate additionally whether the method of preparation had any bearing on pathogenicity.

## MATERIALS AND METHODS

### Inocula

The strain used (5759) was isolated from the lung of a survey lamb (Chapter 4) with P.E. pneumonia. Strain 5759 in its second passage was inoculated into a SPF lamb which was killed at 10 d.p.i. The primary isolation culture in OB from the lungs of this lamb was incubated for 40 h, then harvested and stored in aliquots at -70°C. This formed the seed culture (SC) for the inocula used.

#### Experiment 3

Two cultural methods were employed to avoid the possibility of hypersensitivity reactions to medium components. The sensitizing inocula were produced in primary sheep thyroid tissue cultures, the initial maintenance medium of which had been replaced with a

medium (TCM) of the following composition: MEM, 90%; sheep serum (inactivated), 10%; and penicillin G, 1000 units per ml (final concentration). The second passage of SC through tissue cultures was incubated for eight days, then the supernatant (ITC) was harvested and stored at -70°C. Control tissue cultures (CTC), inoculated in the first passage with sterile OB, were treated in the same manner. Immediately before inoculation of lambs, ITC and CTC were removed from storage and diluted 1:5 with TCM. CTC was heated at 56°C for 30 min to eliminate possible mycoplasma contaminants. The final titre of ITC was $10^5$ ccu per 0.2 ml, and the inoculum volume of ITC and CTC was 2 ml.

The challenge inocula were undiluted sterile OB and an unstored and undiluted 40 h culture of SC in OB (Ch 3), the titre of which was $10^9$ ccu per 0.2 ml. The inoculum volume was 5 ml.

Experiment 4

The sensitizing inoculum was an undiluted 24 h culture of SC (Sen 4) which had been stored at -70°C. The titre was $10^8$ ccu per 0.2 ml, and the volume administered on each occasion was 0.5 ml.

Two challenge inocula were employed: lambs A and B were inoculated with 5 ml of an undiluted 24 h broth culture of SC (Ch 4), titre $10^8$ ccu per 0.2 ml. Lambs C and D were inoculated with a lung homogenate (LH) prepared from a lamb in Group 2, Experiment 3 in the following manner. The lung was coarsely chopped and suspended in BSS supplemented with ampicillin and thallous acetate (1 mg per ml and 1 in 4000 final concentration

respectively). The suspension was shaken well, incubated at 37°C for 1 h, clarified through muslin and stored in aliquots at -70°C. The titre of LH was $10^6$ ccu per 0.2 ml, and the inoculum volume 5 ml.

All inocula were monitored for the presence of bacteria and mycoplasmas, but the only organism detected was M. ovipneumoniae, the identity of which was confirmed by the GI test.

Experimental animals and design

SPF lambs were used.

Experiment 3

The design of Experiment 3 is indicated in Table 5.5. The groups were housed in separate pens. The lambs were killed seven weeks after the primary inoculation, i.e. one week after secondary challenge.

Experiment 4

Four lambs were inoculated intranasally with 0.5 ml of Sen 4 at weekly intervals, starting when the lambs were 2 d.o. Two weeks after the sixth inoculation, lambs A and B were challenged with Ch 4 by the e.b. route, and killed one and two weeks later. Lambs C and D were challenged with LH by the e.b. route three weeks after the seventh i.n. inoculation, and killed one week later.

Sampling and clinical examination

Rectal temperatures were taken daily, otherwise clinical examination including auscultation was confined to the week following

Table 5.5 Design of Experiment 3.

| Group | No. of lambs | Treatments 1st e.b. inoculation* | 2nd e.b. inoculation** |
|---|---|---|---|
| 1 | 2 | CTC+ | Sterile OB |
| 2 | 6 | CTC | Ch 3 |
| 3 | 6 | ITC | Sterile OB |
| 4 | 6 | ITC | Ch 3 |

* Lambs 2 - 4 d.o.

** Lambs 6 w.o.

\+ For details of inocula, see text.

primary inoculation and the period between secondary challenge and necropsy. Serum samples and nasal swabs were collected before inoculation and at necropsy.

Necropsy and microbiological and histopathological examination

The samples taken and the procedures followed were the same as in Experiment 2.

Serology

The sera of all animals were examined for antibodies to M. ovipneumoniae by the IHA test.

RESULTS

Clinical examination

In Experiment 3, slight depression of appetite in Groups 2 and 4, and transient coughing in one animal in Group 4 were observed, both following secondary challenge. No elevated rectal temperatures were recorded other than in one animal in Group 2 and one in Group 3, which had temperatures of 40.8°C at 5 - 8 d.o. In Experiment 4, no clinical signs or elevated rectal temperatures were observed in any animal throughout the experiment.

Microbiology

No mycoplasmas were isolated from pre-inoculation nasal swabs. The mycoplasma recoveries at necropsy from both experiments are shown in Table 5.6. In Experiment 3, no mycoplasmas were recovered

Table 5.6 Experiments 3 and 4. Recovery of M. ovipneumoniae at necropsy.

| Sample | Number of animals yielding M. ovipneumoniae Experiment 3, groups* 1 | 2 | 3 | 4 | Experiment 4, lambs A + B | C + D |
|---|---|---|---|---|---|---|
| Nasal swab | 0 | 5 | 2 | 2 | 1 | 2 |
| Tonsillar tissue | 0 | 2 | 4 | 1 | 2 | 1 |
| Retropharyngeal l.n. | 0 | 1 | 0 | 0 | 1 | 1 |
| Tracheal mucosa | 0 | 4 | 3 | 4 | 2 | 1 |
| Bronchial l.n. | 0 | 0 | 0 | 0 | 0 | 1 |
| Bronchial swab | 0 | 6 (4.0+) | 2 (4.5) | 6 (3.0) | 2 (4.5) | 1 (1.0) |
| L. apical | 0 | 5 (3.0) | 1 (2.0) | 3 (3.3) | 1 (1.0) | 0 |
| L. cardiac | 0 | 6 (3.3) | 0 | 2 (2.5) | 1 (1.0) | 0 |
| L. diaphragmatic | 0 | 6 (3.7) | 2 (2.5) | 5 (2.4) | 1 (3.0) | 0 |
| R. apical | 0 | 5 | 1 | 2 | 0 | 0 |
| R. cardiac | 0 | 3 | 0 | 1 | 1 | 0 |
| R. diaphragmatic | 0 | 3 | 1 | 1 | 0 | 1 |

* 6 animals per group except Group 1 (2 animals).

\+ Figures in parentheses indicate geometric mean titres (ccu per 0.2 ml) of positive recoveries only, given as $\log_{10}$.

For abbreviations, see footnotes of Tables 5.2 and 5.4.

from either control animal in Group 1. Of the infected groups in this experiment, the least number of isolations from all sites except tonsillar tissue were from Group 3. Groups 2 and 4 showed no significant differences, although more isolations and slightly higher recovery titres were obtained from animals in Group 3 (secondary challenge only infective) than in Group 4 (both inoculations infective).

In Experiment 4, the major differences between lambs A and B and lambs C and D were in the recoveries of M. ovipneumoniae from the LRT. More positive samples and higher titres were obtained from lambs A and B, but in both experiments the recovery titres throughout were low, the highest obtained being $10^6$ ccu per 0.2 ml.

Bacteriological examination indicated the majority of animals harboured Staphylococcus aureus, Staphylococcus albus, Streptococcus mitis, and E. coli in the nasal cavity before inoculation. At necropsy, Staphylococcus spp., Proteus sp., and E. coli were recovered from nasal swabs and tonsillar tissue. No bacteria were isolated from the LRT.

Pathology

The results are shown in Table 5.7. In Experiment 3, macroscopic abnormalities of the lungs consisting of emphysema, oedema and diffuse areas of consolidation were observed in two animals in Group 2. Microscopically, both these animals had interstitial pneumonia. Four lambs from Groups 2, 3 and 4 had lymphoid hyperplasia. The observation of mild interstitial

Table 5.7 Experiments 3 and 4. Histopathological findings in lungs of lambs.

| Experiment | Group | No. of lambs | Numbers of lambs with lungs showing | | | | |
|---|---|---|---|---|---|---|---|
| | | | Interstitial pneumonia | Lymphoid hyperplasia | Interstitial thickening | Alveolar collapse | No abnormalities |
| 3 | 1 | 2 | 0 | 0 | 2 | 0 | 0 |
| | 2 | 6 | 2 | 1 | 0 | 0 | 3 |
| | 3 | 6 | 0 | 1 | 0 | 3 | 2 |
| | 4 | 6 | 0 | 2+ | 1 | 0 | 3 |
| 4 | A + B | 2 | 0 | 0 | 1 | 1 | 0 |
| | C + D | 2 | 0 | 0 | 0 | 0 | 2 |

+ Interstitial thickening also present in one lamb.

thickening in the two control lambs suggests this change to be a nonspecific reaction to the inoculum; its presence in lambs of infected groups cannot therefore be considered significant. That M. ovipneumoniae infection caused the mild alveolar collapse seen in three animals in Group 3 is also dubious. No abnormalities were detected in eight lambs.

No significant histopathological changes were detected in any of the lambs in Experiment 4.

Serology

The pre-inoculation serum samples of all animals were negative for antibodies to M. ovipneumoniae by the IHA test. Positive titres in necropsy serum samples were shown by two animals in Group 2, one animal in Group 3 and one animal in Group 4.

DISCUSSION

The finding of lymphoid hyperplasia in four lambs and an absence of lesions in the lungs of 10 lambs confirms the observations of Experiments 1 and 2. Furthermore, the presence of interstitial pneumonia in two lambs supports the findings by Sullivan et al. (1973b) and Tury et al. (1975) that this form of lesion may be induced by the experimental inoculation of M. ovipneumoniae into sheep. The age at which lambs are exposed to the organism is presumably irrelevant to the development of interstitial pneumonia, as both Australian and Hungarian workers used lambs of 1 - 3 d.o. compared with the 6 w.o. lambs used in Group 2 of Experiment 3. Thus it would seem, as suggested in Experiment 1, that the type of histopathological change

induced in the proportion of lambs that respond, whether lymphoid hyperplasia, interstitial pneumonia or both, could be dependent on the strain of M. ovipneumoniae used.

The poor lung recoveries of M. ovipneumoniae in Group 2 animals may be attributable to the age of lambs at first exposure to the organism: the fewer positive animals and even lower recovery titres from Group 4 animals suggest that immunity induced by prior exposure may also be a factor. It is surprising, therefore, that seroconversion could be demonstrated in only one animal in this group.

Experiments 3 and 4 failed in their objective of inducing a magnified response to challenge with M. ovipneumoniae by pre-exposure of lambs. If hypersensitivity is a factor of importance in the pathogenicity of M. ovipneumoniae, failure to demonstrate this may have been due to the methods of sensitization employed, or to the time interval between sensitization and secondary challenge. Adegboye (1975) has shown that delayed-type hypersensitivity following exposure of pigs to M. suipneumoniae occurs during late recovery stages, but not during early pneumonic stages.

The different preparatory methods employed for the inocula in Experiments 3 and 4 caused only minor and probably insignificant differences in effect. The minimal effects observed in Group 3, inoculated with infective tissue culture, and the lack of response in lambs C and D, inoculated with lung homogenate, may have been due to the relatively low titres of M. ovipneumoniae in these inocula. Insufficient numbers of animals were used to compare the effectiveness

of stored and unstored broth cultures of M. ovipneumoniae, but the poor results obtained in lambs A and B compared with Groups 2 and 4 suggest that fresh broth cultures are the method of choice for the preparation of inocula.

# CHAPTER 6

# INVESTIGATION INTO THE PATHOGENICITY OF M. ARGININI FOR SPF LAMBS

## EXPERIMENT 5

### INTRODUCTION

The literature review and the survey reported in Chapter 4 indicated that M. arginini may frequently be recovered from the ovine respiratory tract, but that the organism does not appear to possess virulence on its own, or enhance the pathogenic effects of other respiratory microorganisms in sheep. Experimental proof of the non-pathogenicity of M. arginini for the ovine respiratory tract was, however, confined to the inoculation of two SPF lambs with a single strain of the organism by Foggie and Angus (1972). The following experiment was therefore undertaken to obtain more substantial proof of the pathogenicity of M. arginini for sheep.

### MATERIALS AND METHODS

#### Inocula

The strain of M. arginini employed (5882) was isolated from the lung of a survey lamb (Chapter 4) in which there was lymphoid hyperplasia in the lungs. The uncloned isolate was stored at its second passage at $-70^{\circ}C$; immediately before use, the strain was removed, thawed and used undiluted at a titre of $10^{7}$ ccu per 0.2 ml. Examination of this culture for bacteria and mycoplasmas revealed the

presence of no organisms other than M. arginini, the identity of which was confirmed by the GI test. Control animals were inoculated with sterile AB. Two ml of inoculum were administered to all animals by the e.b. route.

Experimental animals

The SPF lambs employed were not inoculated until 4 w.o., due to an outbreak of enteritis in the lambs in the first week of life. Six lambs were inoculated with strain 5882 and three lambs with AB. The two groups were housed in separate pens.

Sampling and clinical examination

Clinical examinations including the taking of rectal temperatures were performed daily. Nasal swabs and serum samples were taken before inoculation and at necropsy.

Necropsy, microbiology and histopathology

Two infected and one control animal were killed at 7, 14 and 28 d.p.i. The samples removed at necropsy and the techniques of their subsequent microbiological and histopathological examinations were the same as in Experiment 2.

Serology

The sera of lambs were examined for antibodies to M. arginini by the IHA test.

## RESULTS

### Clinical examinations

No rectal temperatures exceeding 40.6°C were recorded nor signs of respiratory disease detected in any of the lambs.

### Microbiology

No mycoplasmas were recovered from pre-inoculation nasal swabs. The single isolation of *M. arginini* from nasal swabs taken at necropsy was from an infected lamb at 28 d.p.i. *Streptococcus* spp., *Citrobacter* spp., other coliforms and *Staphylococcus albus* were recovered from both pre-inoculation and necropsy nasal swabs of several animals.

Necropsy samples yielded *M. arginini* from tonsillar tissue of all six infected animals and retropharygeal lymph nodes of five animals. Mycoplasmas were not recovered from any other site in infected animals or from any of the control lambs. Coliforms, isolated from tonsillar tissue of all animals, were the only bacteria recovered from necropsy samples.

### Histopathology

No differences were observed between control and infected animals. Mild interlobular oedema, with or without small areas of alveolar collapse, were present in five animals, including two control lambs.

### Serology

Four infected animals showed four-fold or greater increases in IHA antibody titres to *M. arginini*. No significant rises were

detected in two infected lambs or any of the control lambs.

## DISCUSSION

The results of this experiment confirm the earlier findings of Foggie and Angus (1972) in that pulmonary lesions ascribable to infection with M. arginini were not produced in any of six lambs inoculated by the e.b. route with the organism. Furthermore, lung infection was not established, although all animals yielded M. arginini from tonsillar tissue at necropsy, and four animals demonstrated seroconversion to the mycoplasm. These findings suggest that M. arginini is not a primary pathogen in sheep pneumonias; its frequent recovery from diseased lung tissue (Chapter 4) is presumably due to secondary invasion from the upper respiratory tract, in particular tonsillar tissue.

# CHAPTER 7

## INVESTIGATIONS INTO THE RÔLES OF MYCOPLASMAS AND *P. HAEMOLYTICA* IN P.E. PNEUMONIA BY THE INOCULATION OF LUNG LESION SUSPENSIONS OR THEIR MICROBIOLOGICAL CONSTITUENTS INTO SHEEP

## EXPERIMENT 6

### INTRODUCTION

It has been suggested that the major importance of mycoplasmas in ovine pneumonias lies in their ability to predispose lungs to secondary bacterial invasion (Sullivan *et al.*, 1973; St. George and Carmichael, 1975). The survey reported in Chapter 4 supported this hypothesis; *M. ovipneumoniae* appeared to precede *P. haemolytica* in its establishment in the lungs of lambs, and their combined presence coincided in all but one case with the observation of P.E. pneumonia. Experiments were thus undertaken to investigate the aetiology of P.E. pneumonia, and the involvement and interrelationships of *M. ovipneumoniae* and *P. haemolytica* in this disease. As an initial step, it was considered necessary to assess the reproducibility of P.E. pneumonia using as inoculum a lung lesion suspension prepared from natural cases and administered by the e.b. route.

### MATERIALS AND METHODS

#### Inoculum

Three sheep lungs with consolidated lesions were obtained from the abattoir. Histological examination showed the lesions to consist of P.E. pneumonia. Consolidated areas were excised, coarsely chopped, suspended in BSS and shaken well. The suspension (LS1) was clarified

through muslin and stored in aliquots at -70°C. Before use, aliquots were removed from storage, thawed and diluted 1:1 with BSS. LS1 was found to contain $10^7$ ccu per 0.2 ml of M. ovipneumoniae, $10^{5.2}$ orgs per ml of P. haemolytica and $10^3$ orgs per ml of E. coli. Small numbers of Streptococci, Staphylococci and Pseudomonas were also present, but chlamydiae or viruses were not detected. The P. haemolytica strains comprised a mixture of serotypes, in the proportions A1, 34%; A2, 8%; A6, 54%; and an untypable strain, 4%.

LS1 was administered in 4 ml volumes by the e.b. route.

Experimental animals and experiment design

Ten Cheviot sheep of 6-7 m.o. were inoculated with LS1, and killed in pairs at 18 - 22 d.p.i.

Sampling and clinical examination

Nasal swabs were taken before inoculation only. Serum samples were collected pre-inoculation and at necropsy. Rectal temperatures and examinations for evidence of respiratory disease, indicated by anorexia, depression, tachypnoea or dyspnoea and coughing, were performed before inoculation and daily thereafter.

Necropsy

Animals were stunned electrically or by captive bolt pistol, and bled out from the jugular vein. The lungs were removed, and the proportion of the surface area showing consolidation was estimated from a diagrammatic sketch of the lungs using a point-grid

system. The average of dorsal and ventral measurements was taken as the final consolidated lung lesion score (CLS).

Samples examined for mycoplasmas comprised specimens from the apical, cardiac and diaphragmatic lobes of both right and left lungs, trachea, tonsillar tissue and a bronchial swab. Samples examined for bacteria comprised right and left lung pools prepared from the three major lobes of that lung, and tonsillar tissue. Consolidated lung lesions were also examined for viruses using secondary foetal lamb kidney tissue cultures.

Microbiology and histopathology

The techniques employed were as previously described. Lung tissue samples for microbiological examination were approximately 0.5 c.c. in volume: consolidated lung specimens of these dimensions weighed approximately 1.5 g, and normal lung specimens approximately 0.5 g.

Serology

Serum samples were assayed for antibodies to M. ovipneumoniae, M. arginini and P. haemolytica (serotypes A1, A2 and A6) by the IHA test, and to PI3 virus by the HAI test.

Chlamydiology

The inocula only were screened for the presence of chlamydiae, using 6 d.o. fertilized eggs (Stevenson and Robinson, 1970).

## RESULTS

### Clinical examinations and nasal swabbing

No symptoms of respiratory disease were detected in any animal before inoculation, but a non-typable strain of *P. haemolytica* was recovered from the nose of one sheep. Following inoculation, all animals developed fever (rectal temperatures above 41.0°C), and showed anorexia, depression and tachyphoea or dyspnoea. Seven animals showed coughing. Onset of fever was generally from 1 - 3 d.p.i., with a mean duration of 8.1 (SE $\pm$ 1.6) days occurring as 1 - 4 peaks.

### Microbiology

*M. ovipneumoniae* was recovered from all lung samples, tracheas and bronchial swabs, and from the tonsillar tissue of nine animals. All specimens titrated yielded $10^6$ ccu per 0.2 ml or greater. *P. haemolytica* A serotypes were recovered from the lungs of all animals and from the tonsillar tissue of nine. Multiple serotype infections of lungs occurred in three sheep, with serotype A1 recovered from seven, A2 from four and A6 from three animals. One sheep yielded serotype A7 from both lungs and tonsillar tissue. Titres of *P. haemolytica* reached $10^6$ - $10^7$ orgs per ml in eight animals. No other bacteria or mycoplasmas were recovered from lungs, with the exception of *Listeria monocytogenes* from a solitary lung abscess. No viruses were isolated.

Pathology

All 10 animals had well-defined consolidated lesions. The CLS ranged from 17 - 73%, with a mean of 42.2% (SE $\pm$ 4.5%). Histologically, P.E. pneumonia indistinguishable from that seen in the lesions used as inoculum was present. The non-consolidated areas showed mild to moderate interstitial thickening. In six animals there were areas of pleurisy, the presence of which could not be correlated with any particular serotype of P. haemolytica.

Serology

Pre-inoculation antibody titres to M. ovipneumoniae were 1/16 or less. Two animals showed 3-fold and all others 4-fold or greater increases in titre in necropsy serum samples. Pre-inoculation titres to P. haemolytica were 1/8 or less. Significant seroconversion in necropsy serum samples was observed in only one animal, in which a 4-fold rise to A1 serotype and a 3-fold rise to A6 serotype was demonstrated. No seroconversions to PI3 virus occurred.

DISCUSSION

The results obtained from the inoculation of LS1 into 10 sheep were remarkably consistent in almost all respects. P.E. pneumonia indistinguishable from the natural lesions which constituted the inoculum were produced, and M. ovipneumoniae and P. haemolytica were recovered from the lungs of all animals, generally in large numbers. E. coli and the other bacterial spp. present in small numbers in LS1

were not isolated from the lungs of any animal, which suggests their presence in the inoculum was irrelevant to the effects produced.

The P. haemolytica component of LS1 comprised four serotypes, and although serotype A1 was the most commonly recovered, no significant differences were demonstrable in the distribution of serotypes and no dissimilarities observed in the histology of the lesions. The presence of serotype A7 in the lungs of one animal might indicate pre-existing lung infection with this serotype, or its invasion of diseased lungs from tonsillar tissue.

The observation of severe symptoms of respiratory disease in all animals contrasts sharply with the asymptomatic natural outbreak of chronic apical pneumonia reported by Gilmour and Brotherston (1963), and was probably due to the rigorous nature of the challenge. The extensive lung damage inflicted may also have been responsible for the much higher serological response to M. ovipneumoniae than had previously been obtained by inoculation of this organism alone into SPF lambs, although low titres detected in some pre-inoculation sera suggest that the response may have been anamnestic. The failure to demonstrate seroconversion to P. haemolytica in more than one animal is not understood.

The results confirm the findings of Boidin et al. (1958) that pneumonia may be reproduced in sheep by the i.t. or e.b. inoculation of lung lesion suspensions containing mycoplasmas and pasteurellae. The effectiveness and severity of this form of challenge may owe much

to an enhancing effect of pneumonic lung tissue and inflammatory exudate, as has been shown for challenge systems involving M. mycoides subsp. mycoides (Lloyd and Trethewie, 1970).

# EXPERIMENT 7

## INTRODUCTION

P.E. pneumonia had been reproduced by e.b. inoculation of a lung lesion suspension of the disease. It was therefore decided to test for repeatability of this form of challenge, and to attempt reproduction of P.E. pneumonia with pure cultures of the organisms recoverable from this suspension. The possible enhancement of effects by lung tissue components has been pointed out: to allow for this possibility, sterilized lung suspension was included in two of the inocula as controls.

## MATERIALS AND METHODS

### Inocula

Lung suspension (LS2) was prepared in the same manner and using similar source material as LS1, but constituted pooled lesions from seven pneumonic lungs. "Sterilized" LS, from which no organisms could be recovered, was prepared by exposure of LS2 to 2.5 Mrads gamma irradiation.

Microorganism suspension (MS) was prepared from the organisms recoverable from LS2. The strains of *M. ovipneumoniae* (P4/1) and *M. arginini* (P4/2) were passaged through nine and 11 subcultures respectively, including three clonings. Both strains were incorporated in the inocula as 24 h cultures, the final three passages of each being in OB or AB from which bacterial inhibitors had been omitted (OB(-) and AB(-)). *P. haemolytica* serotype A2 was stored

as a second passage culture at -70°C. For use, an 18 h broth culture prepared from a stored aliquot was subcultured for 6 h in NB2, then diluted 1 in 1000 in NB2 and mixed with an equal volume of premixed mycoplasma cultures.

LS2 was diluted 1:1 with BSS, and MS was diluted 1:1 with sterilized LS or NB2 before inoculation. The titres of the organisms present in the inocula are given in Table 7.1: no other microorganisms were recovered.

The inocula were administered in 4 ml volumes by the e.b. route.

Experimental animals and design

A preliminary test of the efficacy of LS2 was performed in two 6 m.o. Blackface hoggs derived from the Institute flock (Flock A).

At the time of the main experiment mild respiratory disease, manifested by coughing and serous nasal discharge, was present in Flock A, and as many sheep as possible were therefore obtained from an outside source (Flock B). These animals, which were randomly placed in Groups 2 - 5, were judged free of respiratory microorganisms by nasal swabbing and of respiratory disease by clinical examination at the premises of origin. Group 1 comprised animals from Flock A. All sheep were 6 - 7 m.o. Cheviots.

The five groups of seven sheep each were inoculated as follows: Group 1 - LS2 and BSS: Group 2 - Sterilized LS2 and MS: Group 3 - BSS and MS: Group 4 - Sterilized LS2 and BSS: Group 5 - No treatment. Survivors were killed at 14 - 21 d.p.i.

Table 7.1 Microbiological constituents of inocula.

| Inoculum[+] | Group(s) | Microorganism titres* | | |
|---|---|---|---|---|
| | | M.ovipneu. | M.arginini | P.haem.A2 |
| LS2 | Pilot expt. and Group 1 | 7 | 2 | 2.8** |
| MS | Groups 2 and 3 | 7 | 7 | 5.7 |

[+] Inoculum volume 4 ml.

* Titres of mycoplasmas as $\log_{10}$ ccu/0.2 ml and bacteria as $\log_{10}$ orgs/ml.

** Titre of P. haemolytica in LS2 inoculated into the 2 pilot experiment lambs was $10^{3.6}$ orgs/ml: on storage, this titre fell to $10^{2.8}$ orgs/ml.

Sampling, clinical examination, necropsy procedures, microbiology and histopathology

The techniques were the same as in Experiment 6, except that the samples assayed for M. ovipneumoniae in the animals killed according to schedule were bronchial swabs and "lesion" and "non-lesion" pools taken from macroscopic lesions and apparently normal areas of lung respectively: samples removed from those animals that died or were killed in extremis were the same as in Experiment 6. Bronchial swabs in the main experiment were also assayed for numbers of M. arginini. Examinations for bacteria were performed on the six major lung lobes from each animal, and viable counts of P. haemolytica were made of lesion and non-lesion pools.

Serology

Antibodies to M. ovipneumoniae were assayed by the IHA and TRI tests, to M. arginini by the IHA and MI tests, to P. haemolytica by the IHA test and to PI3 virus by the HAI test.

RESULTS

Preliminary experiment

Both animals appeared normal, and neither yielded mycoplasmas nor P. haemolytica from nasal swabs before inoculation.

Following inoculation both animals had rectal temperatures in excess of 41.0°C from 1 d.p.i. until death, and showed anorexia, depression, coughing and respiratory distress. One animal was killed in extremis at 5 d.p.i., and the other was found dead at 13 d.p.i.

At necropsy the lungs of both lambs were grossly discoloured. The CLS of the lamb killed *in extremis* was 20% and of the lamb found dead 43%: the latter also had extensive pleuritic adhesions of the right lung. Histologically, these lesions consisted of P.E. pneumonia. *M. ovipneumoniae*, *M. arginini* and *P. haemolytica* A2 were recovered from all lung and tonsillar tissue samples examined. Viable counts, performed only in the animal killed *in extremis*, yielded $10^6$ ccu per 0.2 ml or greater of *M. ovipneumoniae* from all lung samples, and $10^{5.8}$ orgs per ml of *P. haemolytica* from a lung pool. Serological examinations were not performed.

Main experiment

Clinical examinations

Before inoculation some animals in Group 1 were observed to be coughing, and nasal swabs from all sheep in this group yielded *M. ovipneumoniae*. One animal also yielded *M. arginini* and four *P. haemolytica* (various serotypes). Groups 2 - 5 appeared normal and no mycoplasmas or pasteurellae were recovered except from two sheep in Group 4, which yielded *P. haemolytica* serotype A6.

The post-inoculation clinical findings are summarised in Table 7.2. Respiratory symptoms were observed in most animals in Groups 1, 2 and 3. All eight animals which died or were killed *in extremis* 2 - 13 d.p.i. had severe pleurisy at necropsy. The control animals in Groups 4 and 5 appeared normal throughout, although one animal showed an elevated rectal temperature on three occasions. The differences between Groups 2 and 3 and Groups 4 and 5 were very

Table 7.2 Main Experiment 7. Summary of clinical findings.

| Group | Treatment | Number of sheep dying or killed in extremis+ | with rectal temperature ≥41.0°C | with clinical response* |
|---|---|---|---|---|
| 1 | LS2 | 1 | 4 | 6 |
| 2 | Sterile LS2 + MS | 3 | 6 | 5 |
| 3 | BSS + MS | 4 | 4 | 5 |
| 4 | Sterile LS2 | 0 | 0 | 0 |
| 5 | No treatment | 0 | 1 | 0 |

7 animals per group

\+ 2 - 13 d.p.i.

* Observation of respiratory distress and/or depression and/or coughing.

slight for all parameters measured, so for statistical purposes the groups were combined. Significant differences ($P < 0.01$) occur between Groups 2 + 3 and Groups 4 + 5 for all three clinical parameters in Table 7.2. Group 1 had significantly higher numbers of animals showing clinical response ($P = 0.01$) and elevated rectal temperatures ($P = 0.05$) compared with Groups 4 + 5. There were no significant differences in any respect between Group 1 and Groups 2 + 3.

Microbiology

The results are summarised in Table 7.3. M. ovipneumoniae was recovered from the upper respiratory tract and virtually all parts of the lungs of every animal in Groups 1, 2 and 3. Numbers of organisms recovered were higher in lung lesion pools than in other samples. M. ovipneumoniae was also recovered from the upper respiratory tract of five animals in Groups 4 and 5, and three animals in Group 5 yielded the organism from, on average, 78% of lung lobes examined: recovery titres of $10^7$ ccu per 0.2 ml were obtained from the lesion pools in two of these sheep. M. arginini, recovered from the upper respiratory tract of 16 animals and the lungs of 14 in Groups 1, 2 and 3, was not as widely distributed through the lungs as M. ovipneumoniae. Significant numbers were demonstrated in the bronchial swabs of only 12 of these sheep: these 12 also had pleurisy. Lung samples from only one pleuritic animal were negative for M. arginini, although the organism was recovered from the trachea in this case. M. arginini was also recovered from the lung samples

Table 7.3 Main Experiment 7. Summary of microbiological findings.

| Group | No. of animals with upper respiratory tract +ve for | | No. of animals with lungs +ve for | | | % of lung[1] lobes +ve for | | | Geometric mean titres of positive lung samples[2] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | M. ovipneumoniae | | | | M. arg. | P. haem. | |
| | M.o. | M.a. | M.o. | M.a. | P.h. | M.o. | M.a. | P.h. | Lt.L. | B.S. | Le | NLe | B.S. | Le | NLe |
| 1 | 7 | 4 | 7 | 4 | 2 | 88 | 24 | 7 | 4.7 (1/1)[3] | 6.1 (7/7) | 6.3 (6/6) | 4.0 (5/6) | 5.2 (3/7) | 5.1 (2/7) | 0.0 (0/6) |
| 2 | 7 | 6 | 7 | 4 | 7 | 93 | 43 | 71 | 4.8 (3/3) | 6.3 (7/7) | 7.2 (4/4) | 3.5 (4/4) | 4.7 (4/7) | 6.4 (7/7) | 0.0 (0/4) |
| 3 | 7 | 6 | 7 | 6 | 7 | 90 | 74 | 81 | 5.0 (4/4) | 5.3 (7/7) | 7.0 (3/3) | 3.3 (3/3) | 5.8 (5/7) | 7.5 (7/7) | 4.2 (2/3) |
| 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ND (-) | 0 (0/7) | 0 (0/5) | 0 (0/7) | 0 (0/7) | 0 (0/5) | 0 (0/7) |
| 5 | 4 | 0 | 3 | 2 | 0 | 33 | 5 | 0 | ND (-) | 4.0 (4/7) | 5.3 (3/6) | 4.0 (3/7) | 0 (0/7) | 0 (0/6) | 0 (0/7) |

7 animals per group

1 - 6 lung lobes examined in every animal

2 - Titres expressed as $\log_{10}$

3 - Figures in parentheses indicate number positive/number examined.

upper respiratory tract - trachea and/or tonsillar tissue

M.o. - M. ovipneumoniae M.a. - M. arginini

P.h. - P. haemolytica A2 B.S. - Bronchial swab

Lt.L. - Left lung: mean of log values of recovery titres from left apical, cardiac and diaphragmatic lobes

Le - Lesion pool NLe - Non-lesion pool

ND - Not done

of two animals in Group 5, but no growth was obtained from their bronchial swabs. P. haemolytica serotype A2 was recovered from the lungs of all sheep in Groups 2 and 3, most lung lobes being infected. Only two animals in Group 1 yielded the organism, from a total of three lung lobes. The numbers of P. haemolytica recovered were very much higher in lesion than in non-lesion pools, and ranged from $10^{3.0}$ - $10^{9.6}$ orgs per ml. P. haemolytica was not isolated from the lungs of any sheep in Groups 4 and 5. No viruses were recovered from any animal.

Pathology

The findings are summarised in Table 7.4. All animals in Groups 2 + 3, and five in Group 1 had large individual areas of grey/red consolidation (Figs. 7.1, 7.2), whereas only linear lesions or small foci of consolidation were present in Groups 4 + 5. One animal in Group 5, in which the consolidated foci were widespread, was estimated to have a CLS of 11%.

As in Experiment 6, the predominant lesion was P.E. pneumonia, which was present in 16 of 19 animals examined in Groups 1, 2 and 3, and one of 14 in Groups 4 and 5 (only five of the seven animals in Group 3 were examined histologically as the samples from two sheep were mislaid). Bronchiolar hyperplasia and metaplasia, lymphoid hyperplasia and accumulations of macrophages, giant cells and polymorphs were present regularly, and hyaline scars and perivascular fibrosis were seen frequently. Pleurisy was present in 13 animals from Groups 1, 2 and 3 (Fig. 7.3). Sheep in all groups were infected with both Dictyocaulus filaria and Muellerius capillaris.

Table 7.4 Main Experiment 7. Summary of pathological findings.

| Group | Mean CLS (%) (± SE) | Number of sheep showing pleurisy | acute broncho-pneumonia | proliferative/exudative pneumonia | focal or linear collapse lesions only |
|---|---|---|---|---|---|
| 1 | 23.3 ± 7.94 | 4 | 0 | 6 | 1 |
| 2 ) | | 4+ | 2 | 5 | 0 |
| ) | 48.9 ± 9.53 | | | | |
| 3 ) | | 5 | 0* | 5* | 0* |
| 4 ) | | 0 | 0 | 1 | 4 |
| ) | 2.4 ± 0.72 | | | | |
| 5 ) | | 0 | 0 | 0 | 6 |

7 animals per group.

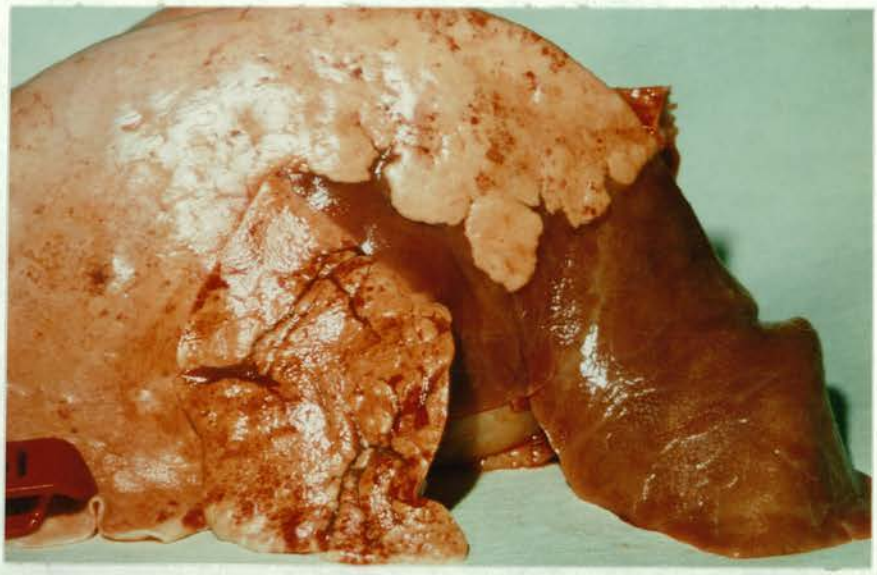
CLS - Consolidated lung lesion score.

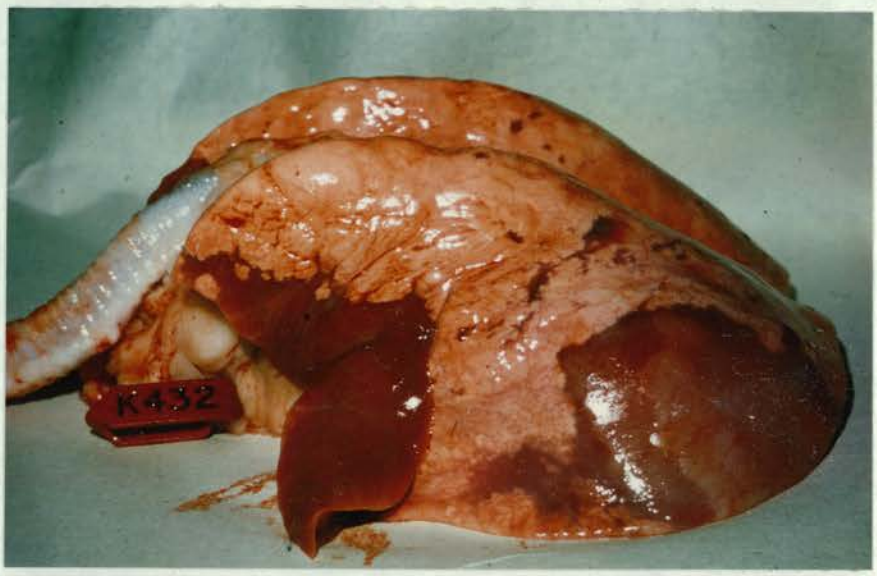
* - Only 5 animals examined histologically.

\+ - Pleurisy in one animal observed microscopically only.

Figs. 7.1 (upper), 7.2 (centre) and 7.3 (lower)

Lungs of sheep from Groups 2 and 3, Experiment 7. These were inoculated endobronchially with mixed cloned cultures of M. ovipneumoniae, M. arginini and P. haemolytica serotype A2, combined either with sterile lung homogenate or nutrient broth. Fig. 7.1 shows red/brown consolidation of the right apical lobe of an animal killed 17 d.p.i. Linear collapse lesions are present in the cardiac lobe. Fig. 7.2 shows consolidated lesions present in all three lobes of the left lung of an animal killed 15 d.p.i. The area of consolidation in the diaphragmatic lobe probably represents the deposition site of the inoculum. Fig. 7.3 shows the opened thorax of an animal killed in extremis 3 d.p.i. Fibrinous pleurisy is evident, and areas of consolidation are present in all three lobes of the right lung.

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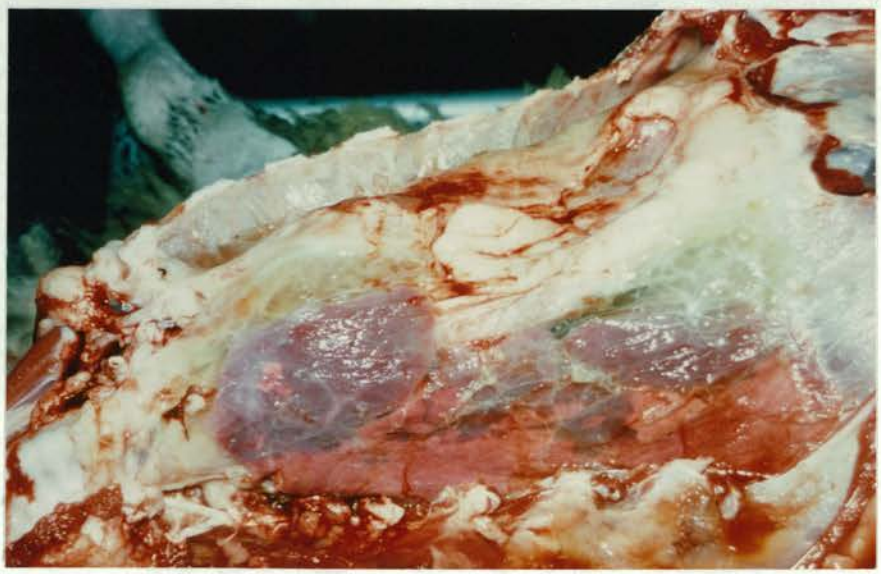


Two animals in Group 2, both of which died at 3 d.p.i., had acute bronchopneumonia and pleurisy. There was necrosis of the bronchiolar epithelial cells, capillary congestion, serous alveolar exudation and an inflammatory cell infiltration of the parenchyma.

Groups 2 + 3 and Group 1 had significantly higher ($P = <0.01$) numbers of animals with pleurisy and with proliferative pneumonia than Groups 4 + 5.

Serology

The results are summarised in Table 7.5.

M. ovipneumoniae: By the IHA test, pre-inoculation titres of 1/2 or greater were detected only in animals of Group 1. Four-fold or greater rises in necropsy serum samples were found in the nine animals in Groups 2 and 3 which survived longer than 6 d.p.i., and in three animals in Group 1. By the TRI test, six animals in Group 1 and one in Group 3 were found to have positive pre-inoculation titres, but in contrast to the IHA tests, seroconversion in necropsy serum samples was detected only in the three animals in Group 1 which also seroconverted by the IHA test. The single animal from Group 3 died at 3 d.p.i.

M. arginini: Low antibody titres to M. arginini were detected by the IHA test in all animals, and five animals from Groups 1, 2 and 3 showed subsequent seroconversion. One sheep from Group 2, which died at 3 d.p.i., had a high pre-inoculation titre. Despite repeated attempts with the MI test, neither positive pre-inoculation titres nor seroconversion could be demonstrated in any animal, though some sheep showed necropsy serum titres of 1/2 to 1/4.

Table 7.5 Main Experiment 7. Summary of serological findings.

| Organism | Test | Pre-inoculation titres | No. showing seroconversion* in necropsy serum samples. Gp 1 | Gp 2 | Gp 3 |
|---|---|---|---|---|---|
| *M. ovipneumoniae* | IHA | 1/2 - 1/4 for all animals except Gp 1 (1/4 - 1/128) | 3 | 5 | 4 |
| | TRI | 1/2 for all animals except 6 in Gp 1 (1/8 - 1/256) and 1 in Gp 3 (1/256) | 3 | 0 | 0 |
| *M. arginini* | IHA | 1/4 - 1/32 for all animals except 1 in Gp 2 (1/128) | 2 | 1 | 2 |
| | MI | <1/2 | 0 | 0 | 0 |
| *P. haemolytica* A2 | IHA | <1/2 - 1/8 | 1 | 4 | 3 |
| PI3 virus | HAI | <1/10 - 1/80 | 6 | 0 | 0 |

* - A four-fold or greater rise in titre.
No animals from Groups 4 and 5 showed seroconversion to any of the organisms tested.

Antiserum prepared in rabbits to M. arginini gave titres of 1/640 to 1/1280 in the same tests.

P. haemolytica: Little or no antibody to P. haemolytica was detected by the IHA test in pre-inoculation samples. Seroconversion in necropsy serum samples was found in one animal in Group 1, and seven of the nine animals in Groups 2 and 3 which survived longer than 6 d.p.i.

Pl3 virus: Eleven animals representing all groups except Group 1 showed pre-inoculation antibody titres of 1/20 to 1/80, but seroconversion was found only in animals of Group 1.

## DISCUSSION

The combined results of Experiments 6 and 7 demonstrate that two different preparations from naturally-occurring lesions of P.E. pneumonia produced very similar effects when inoculated endobronchially into a total of 19 sheep, with the development in 18 animals of respiratory symptoms and P.E. pneumonia. LS2 produced, in the Group 1 animals of Experiment 7, milder clinical symptoms, a smaller mean CLS and fewer lung recoveries of P. haemolytica than the LS1 used in Experiment 6. This may have been due to a lower content of P. haemolytica in LS2: however, the presence of mild respiratory disease, M. ovipneumoniae and P. haemolytica in the Group 1 (Experiment 7) animals before inoculation may also be relevant to the differences. This possibility is supported by the marked response of both lambs in the preliminary experiment to challenge with fresh LS2, the P. haemolytica content of which was little greater than in the stored LS2 used in the main experiment.

Rising antibody titres also indicate that a P13 virus infection occurred within Group 1 during the experiment. As P13 virus was not recovered from LS2, the sheep were probably infected with virus before inoculation. How infection with P13 virus, mycoplasmas and P. haemolytica may have affected results is uncertain: no histopathological differences were noted between Group 1 and other infected animals in Experiments 6 and 7, but at necropsy, P. haemolytica was recovered from the lungs of only two animals in Group 1.

The combined cloned cultures of mycoplasmas and P. haemolytica given in Groups 2 and 3 simulated the effects of LS. No differences were found between these two groups, indicating that sterile lung material included in the inoculum given to Group 2 had no exacerbative effects.

The 14 control animals in Groups 4 and 5 showed no signs of respiratory disease. Respiratory microorganisms were not isolated from the mild and localized P.E. pneumonia observed in one control animal. Three sheep from Group 5 yielded M. ovipneumoniae from the lungs, and in two of these the highest recovery titres were obtained from areas of linear collapse, but as observed earlier, this does not necessarily imply a causal relationship.

The death of several animals from the infected groups in Experiment 7 is a major difference between this experiment and the preceding one. Pleurisy, present in all animals that died or were killed in extremis, was also seen in six sheep in Experiment 6. However, M. arginini was recovered from the LRT of each animal which died in Experiment 7, though this organism was neither injected nor

recovered from any animal in Experiment 6. Thus these results suggest a relationship only between the presence of M. arginini and mortality.

The anomalies apparent in the serological results relating to mycoplasmas are investigated further in Chapter 8; discussion of these results is therefore deferred to that chapter. The results obtained by the IHA test for P. haemolytica show a strong resemblance to those found for M. ovipneumoniae by the same test, with the exception that Group 1 animals had only low titres to P. haemolytica in pre-inoculation serum samples.

# EXPERIMENT 8

## INTRODUCTION

The results of Experiment 7 confirmed the hypothesis that endobronchial challenge of sheep with combined cultures of mycoplasmas and P. haemolytica allows establishment in the lungs of all organisms contained in the inoculum and results in the reproduction of P.E. pneumonia, but did not demonstrate that this effect was due to additive or synergistic interactions among the organisms concerned. Despite the results obtained with pure cultures of M. ovipneumoniae and M. arginini in SPF lambs, it was possible that the severe symptoms and lesions seen in Experiment 7 were caused solely by either one or both mycoplasmas, due to the particular strains of these mycoplasmas employed or the use of conventional sheep rather than SPF lambs as experimental animals. Similarly, the findings by other authors (see Chapter 1) relating to the challenge of conventionally-reared sheep with P. haemolytica are not directly applicable to the serotype, strain, passage level and cultural treatment of the isolate used in Experiment 7, and the observed effects may have been attributable to this organism alone. Experiment 8 thus attempts to establish whether both M. ovipneumoniae and P. haemolytica are involved in the aetiology of P.E. pneumonia. Since previous work has suggested that M. arginini is more probably a secondary invader than a primary agent in sheep pneumonia, this organism was omitted from the experiment. A preliminary experiment was performed in 10 sheep to reaffirm the effectiveness of challenge with the strains of M. ovipneumoniae and P. haemolytica used.

## MATERIALS AND METHODS

### Inocula

The P4/1 strain of *M. ovipneumoniae* was used at its thirteenth passage: the final four passages were in OB(-). The strain was incorporated in the inocula as a 24 h broth culture. The strain and manner of preparation of the *P. haemolytica* serotype A2 were the same as in Experiment 7. The constituents of the inocula were mixed in equal proportions according to the scheme shown in Table 7.6. The inocula were administered in 4 ml volumes by the e.b. route.

### Experimental animals and design

The preliminary experiment was performed in 4 m.o. Suffolk X lambs derived from Flock A. Sterile medium was administered to three sheep (Group A) and combined cultures of *M. ovipneumoniae* and *P. haemolytica* A2 to seven sheep (Group B). The survivors were killed at 7 d.p.i.

The main experiment employed 7-7½ m.o. Cheviot lambs derived from Flock B. The four groups of seven sheep each were inoculated according to the design shown in Table 7.6. The animals were killed at 14-17 d.p.i.

### Sampling, clinical examination, necropsy procedures, microbiology, serology and histopathology

Techniques were the same as in Experiments 6 and 7, except that examinations for chlamydiae or antibodies to *M. arginini* were not performed. Necropsy samples examined for *M. ovipneumoniae*

Table 7.6 Preliminary and main Experiment 8. Constitution of inocula and experiment design.

| Inoculum* constituents | Experiment | Group | Microorganism numbers+ M.ovipneu. | P.haem A2 |
|---|---|---|---|---|
| OB(-) + NB2 | Preliminary<br>Main | A<br>1 | - | - |
| P4/1 + P. haem.A2 | Preliminary | B | 8.0 | 6.0 |
| P4/1 + NB2 | Main | 2 | 7.0 | - |
| P. haem.A2 + OB(-) | Main | 3 | - | 5.4 |
| P4/1 + P.haem.A2 | Main | 4 | 7.0 | 5.4 |

* Constituents were mixed in equal proportions immediately before administration. The inoculum volume was 4 ml.

\+ Titres of M. ovipneumoniae as $\log_{10}$ ccu/0.2 ml, and P. haemolytica as $\log_{10}$ orgs/ml.

comprised lesion (where present) and non-lesion pools, bronchial swab, trachea and tonsillar tissue, of which all but tonsillar tissue were titrated in OB. Examinations for bacteria were performed on lesion and non-lesion pools and tonsillar tissue, of which the lung pools were assayed for the number of P. haemolytica present. Examinations for viruses were performed on lesion pools where available, otherwise on non-lesion pools.

## RESULTS

### Preliminary experiment

One sheep in Group A yielded P. haemolytica serotype A2 and one M. ovipneumoniae from pre-inoculation nasal swabs: the same sampling of Group B yielded P. haemolytica serotype T3 from two sheep and A. laidlawii from one animal. One sheep in Group B was observed to be coughing prior to inoculation.

Post-inoculation and necropsy findings in the two groups are summarised in Table 7.7. All animals within Group B (experimental group) showed symptoms of coughing, hyperpnoea or both, and appeared dull and depressed. Five animals developed elevated rectal temperatures, and one animal died at 2 d.p.i. In Group A (control group), the only abnormality observed was the presence of hyperpnoea and marked râles sounds in one animal. At necropsy, macroscopic areas of consolidation were present in the lungs of all animals, but the mean CLS of Group B sheep was 29.3% compared with 9.7% for Group A. Three animals in Group B had pleurisy. Histopathologically, the lung lesions in the two groups were indistinguishable. One animal from each group had lesions of interstitial thickening with severe

Table 7.7 Pilot Experiment 8. Summary of clinical, pathological and microbiological findings.

| Condition | No. of animals showing condition | |
|---|---|---|
| | Group A (3 control sheep) | Group B (7 infected sheep) |
| Temperature $\geqslant$ 41.0°C | 0 | 5 |
| Clinical signs | 1 | 7 |
| Death | 0 | 1 |
| Macroscopic lesions | 3 (9.7% SE ± 2.6%)* | 7 (29.3% SE ± 7.3%)* |
| Pleurisy | 0 | 3 |
| P.E. pneumonia | 2 | 5 |
| Interstitial thickening with lymphoid cell hyperplasia | 1 | 1 |
| Purulent bronchopneumonia | 0 | 1 |
| Lung presence of *M. ovipneumoniae* | 3 (5.33)+ | 7 (7.28)+ |
| Lung presence of *P. haemolytica* A2 | 0 | 7 (6.37)+ |

* Figures in parentheses indicate mean CLS, with standard errors. CLS for Group B excludes findings in single animal which died at 2 d.p.i.

\+ Figures in parentheses indicate geometric mean for group of highest recoveries from each animal (*M. ovipneumoniae* as ccu/0.2 ml, *P. haemolytica* as orgs/ml) expressed as $\log_{10}$.

lymphoid hyperplasia, and the animal from Group B which died had purulent bronchopneumonia: all other animals had lesions of P.E. pneumonia.

All animals from both groups yielded M. ovipneumoniae from the lungs. Lowest recoveries were obtained from the three sheep without lesions of P.E. pneumonia, in particular sheep 622 of Group A, which yielded only $10^2$ ccu per 0.2 ml from a bronchial swab, and no M. ovipneumoniae from lung tissue. M. ovipneumoniae was also recovered from the trachea of all sheep and from the tonsillar tissue of all but two animals in Group A. P. haemolytica serotype A2 was recovered from the lungs and tonsillar tissue of Group B animals only. Recovery numbers from lungs, which ranged from $10^{3.8}$ - $10^{9.2}$ orgs per ml, were highest in the two animals which did not have lesions of P.E. pneumonia. P. haemolytica T3 or T4 serotypes were recovered from the tonsillar tissue of two sheep in Group A and one in Group B. No viruses or other organisms were recovered from the respiratory tract of any animal. However, a significant rise in antibody titre to P13 virus occurred in sheep 622 (Group A).

The IHA test showed all animals to have low or undetectable antibody titres to M. ovipneumoniae in pre-inoculation serum samples. By the TRI test, only sheep No. 622 (Group A) had a positive pre-inoculation titre (1/16) to M. ovipneumoniae. No significant rises in titre were found in necropsy serum samples by either test. Pre-inoculation titres to P. haemolytica A2 ranged from 1/4 - 1/32; a significant rise in titre was found in the necropsy serum sample from one animal from Group B.

Main experiment

Clinical and pathological findings

No signs of respiratory disease were noted in any animal before inoculation, nor were mycoplasmas or P. haemolytica isolated from nasal swabs.

The post-inoculation clinical and pathological findings are summarised in Table 7.8. Clinical signs, where present, were mild; coughing was seen in one sheep in Group 3 shortly after inoculation, and in some animals in Groups 2 and 4. In addition, hyperpnoea and/or dyspnoea and elevated rectal temperatures were seen in a proportion of Group 4 sheep.

Macroscopic lung lesions were seen in animals from all four groups at necropsy, with the highest mean CLS shown by Group 4. The lesions present in the animals of Groups 1 and 3 consisted of bands or foci of collapse and consolidation; one sheep in Group 1 had an estimated CLS of 15%. Areas of consolidation histologically confirmed as P.E. pneumonia were found only in eight animals in Groups 2 and 4. In this respect, Group 4 is significantly different from Groups 1 and 3 ($P = 0.01$). Group 2, though not significantly different from the individual Groups 1 and 3, is significantly different by the one-tailed Chi square test from combined Groups 1 + 3 ($P = 0.026$). Interstitial thickening accompanied by lymphoid cell cuffing was present in all other animals except one sheep from Group 1.

Microbiology

The findings are summarised in Table 7.9. M. ovipneumoniae was recovered from the upper respiratory tract and lungs of all animals

Table 7.8 Main Experiment 8. Summary of clinical and pathological findings.

| Condition | No. of sheep showing condition in Group | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Temperature ≥ 41.0°C | 0 | 0 | 0 | 2 |
| Clinical signs | 0 | 3 | 1 | 4 |
| Macroscopic lesions | 6 (5.0% SE ± 1.9%)* | 5 (5.8% SE ± 2.2%) | 3 (0.3% SE ± 0.1%) | 6 (11.9% SE ± 4.0%) |
| Pleurisy | 0 | 0 | 0 | 1 |
| P.E. pneumonia | 0 | 3 | 0 | 5 |
| Interstitial thickening with lymphoid cell hyperplasia | 6 | 4 | 7 | 2 |
| Bands/foci of collapse and consolidation | 6 | 2 | 3 | 1 |
| No abnormalities | 1 | 0 | 0 | 0 |

7 sheep per group

* Figures in parentheses indicate mean CLS, with standard errors.

Table 7.9 Main Experiment 8. Summary of microbiological findings.

| Organism | Sample* | No. of sheep positive from Group | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| *M. ovipneumoniae* | Ton | 2 | 7 | 1 | 6 |
| | Tra | 1 | 7 | 0 | 7 |
| | BS | 2 (3.5)+ | 7 (5.7) | 0 | 7 (6.3) |
| | Le | 0/2++ | 4/4 (6.5) | ND | 6/6 (7.0) |
| | Nle | 1 (5.0) | 7 (4.3) | 1 (2.0) | 7 (5.4) |
| *P. haemolytica* A2 | Ton | 0 | 0 | 1 | 3 |
| | Le | 0/2 | 0/4 | ND | 5/6 (5.2) |
| | Nle | 0 | 0 | 1 (3.4) | 2 (4.0) |
| *P. haemolytica* biotype T serotypes | Ton | 7 | 7 | 4 | 5 |
| | Le or Nle | 0 | 2 | 1 | 0 |

7 sheep per group.

* Ton = tonsillar tissue. Tra = tracheal mucosa. Otherwise abbreviations as for Table 7.3.

+ Figures in parentheses indicate geometric mean of positive recoveries only, expressed as $\log_{10}$.

++ The denominator indicates the number of sheep from which lesion pools were removed for examination.

in Groups 2 and 4: the numbers of organisms recovered were marginally higher in Group 4 than in Group 2, and in lesion pools and bronchial swabs compared with non-lesion pools. M. ovipneumoniae was also recovered in low titres from the lungs or bronchial swabs of two animals in Group 1 and one in Group 3. P. haemolytica A2 was isolated in low numbers from one sheep in Group 3, and from the five sheep in Group 4 which displayed P.E. pneumonia. P. haemolytica serotypes T3, T4 or T10 were recovered from tonsillar tissue of the majority of animals in all groups, and, at $10^3$ - $10^4$ orgs per ml, from the lung tissue of three animals in Groups 2 and 3, including one sheep in which P.E. pneumonia was present.

PI3 virus was recovered from the lungs of two sheep in Group 4, both of which had lesions of P.E. pneumonia. Demonstration of seroconversion in six animals confirmed that the whole of Group 4 had suffered concurrent infection with PI3 virus during the period of the experiment. No other microorganisms were recovered from the lungs of any animal.

Serology

Titres to M. ovipneumoniae in pre-inoculation serum samples were found by both IHA and TRI tests to be low or undetectable. No significant rises in necropsy serum samples were found. Pre-inoculation titres to P. haemolytica A2 were 1/8 or less in 17 sheep: 11 sheep, which had titres between 1/16 and 1/512, were randomly distributed through the four groups. Seroconversion in necropsy serum samples was found in four sheep from Group 3 and five from Group 4.

DISCUSSION

Lesions of P.E. pneumonia were again observed in the majority of animals challenged with combined cultures of P. haemolytica and M. ovipneumoniae. However, the presence of P.E. pneumonia in two control sheep in the preliminary experiment, and of natural infections with various respiratory microorganisms in both preliminary and main experiments, confound to some degree interpretation of the results obtained. PI3 virus infection was detected serologically or microbiologically in all animals of Group 4, but the relevance of this infection to the subsequent development of P.E. pneumonia is uncertain. Sharp, Gilmour, Thompson and Rushton (1978) have recently shown that when biotype A serotypes of P. haemolytica are administered by aerosol to SPF lambs 4 - 7 days after an endobronchial injection of PI3 virus, acute pneumonia develops in approximately 90% of lambs. Similar findings after the combined i.n. and i.t. challenge of conventionally reared lambs with PI3 virus and P. haemolytica have been reported by workers in New Zealand (Davies, Dungworth, Humphreys and Johnson, 1977). In Group 4, Experiment 8 it is possible that infection with PI3 virus produced the mild clinical response, pulmonary lesions and lung establishment of P. haemolytica in some animals. However, P. haemolytica became established in the lungs of sheep in Groups 2 and 3, Experiment 7, in which infection with PI3 virus did not occur during the period of the experiment. Furthermore, the lesions observed in these sheep were similar histologically to those seen in the animals of Group 4, Experiment 8. The pneumonia seen by Sharp et al. (1978) in the combined challenge system with PI3 virus and P. haemolytica was generally an acute, necrotizing

bronchopneumonia: the pathology of lesions was not described by Davies et al. (1977).

Concurrent lung infections with biotype T serotypes of P. haemolytica were not associated with any specific histopathological change, and were seen in only three animals, despite the high number of sheep yielding these organisms from tonsillar tissue. These findings suggest that there is little or no synergism between mycoplasmas and P. haemolytica biotype T serotypes.

Natural lung infections with M. ovipneumoniae were found in six sheep in the preliminary and main experiments, of which four displayed interstitial thickening with lymphoid hyperplasia, and two had P.E. pneumonia from which P. haemolytica could not be recovered. The isolation of M. ovipneumoniae only from lungs showing both forms of histopathological change has been noted before, as has the possibility that P. haemolytica may be involved in the early phase of the development of P.E. pneumonia, but be eliminated rapidly by immune mechanisms thereafter. The low but detectable antibody titres to P. haemolytica present in the pre-inoculation sera of all sheep involved in the preliminary experiment lend some support to this possibility. However, in the main experiment, P.E. pneumonia of moderate extent was observed in three sheep in Group 2, which were inoculated with, and yielded lung recoveries of, M. ovipneumoniae only. The absence of P.E. pneumonia in Groups 1 and 3 of this experiment suggests that the findings in Group 2 were associated with the inoculum. The implication that M. ovipneumoniae per se may cause P.E. pneumonia

is in direct contrast to the findings from the inoculation of M. ovipneumoniae into SPF lambs; this difference may be due to the strain of M. ovipneumoniae employed, or to the use of conventional rather than SPF lambs as experimental animals.

The conclusion of other workers (see Chapter 1) that P. haemolytica by itself does not, at "realistic" challenge doses, cause pneumonia or become established in the lungs of conventionally-reared sheep is corroborated by the findings in Group 3, main experiment. No lung lesions were noted in these sheep, and the organism was recovered, in low numbers, from the lungs of only one animal: in contrast, five sheep in Group 4 showed pneumonia and yielded P. haemolytica from the lungs. If, as the results suggest, M. ovipneumoniae alone may cause P.E. pneumonia, then the role of P. haemolytica in the disease is merely that of secondary invader, the presence of which does not radically alter the pathological picture observed. However, a comparison of the animals showing P.E. pneumonia in Group A with those in Group B (preliminary experiment) and of those in Group 2 with those in Group 4 (main experiment) shows the mean CLS of these categories to be 12% and 30%, and 11% and 16% respectively. This suggests that the secondary invasion of P. haemolytica may have the effect of increasing the volume of lung affected. Compared with Experiments 6 and 7, the clinical signs and lesions seen in animals of Group 4, Experiment 8, were unexpectedly mild. It is unlikely that this was due to the omission of M. arginini from the inoculum, since the animals of Experiment 6, which were not inoculated with this organism, all showed severe clinical signs of disease and had extensive lesions of P.E. pneumonia at

necropsy. The higher passage level of P4/1 used compared with that employed in Experiment 7 may be relevant, but alternatively, the mildness of reaction of Group 4, Experiment 6 animals may relate to a suppressed P. haemolytica involvement due to the prior possession of immunity to P. haemolytica. The relatively high antibody titres to P. haemolytica in the pre-inoculation sera of some sheep, and low recovery numbers of the organism from the lung tissue of these animals supports this latter suggestion.

The failure of the serological tests applied to show significant rises in antibody titre to M. ovipneumoniae following inoculation of animals may have been due, in the preliminary experiment, to the killing of animals at 7 d.p.i.: the sheep in Experiment 7 which died within 6 days of inoculation similarly failed to show seroconversion. In the main experiment, the moderate extent of the lesions may account for the lack of seroconversion in sheep of Groups 2 and 4. It is interesting to note that sheep 622 of Group A, preliminary experiment, had a positive pre-inoculation titre by the TRI test, and at necropsy yielded very much lower lung recoveries of M. ovipneumoniae than the other two control animals in the same group. That only one sheep in the preliminary experiment showed seroconversion to P. haemolytica may be due to the same reason suggested for M. ovipneumoniae serology. The significant rise in antibody titres to P. haemolytica shown by four animals in Group 3, main experiment, occurred despite failure to recover the organism from the lungs of three of these sheep, and the absence of pneumonia in

all four animals. This finding suggests that the response may have been anamnestic, and again implies that the animals used in Experiment 8 possessed some degree of immunity to P. haemolytica before inoculation.

# CHAPTER 8

## SOME INVESTIGATIONS INTO THE ASSAY OF ANTIBODIES TO *M. OVIPNEUMONIAE* BY THE IHA AND TRI TESTS

### INTRODUCTION

Both the IHA and MI tests are considered to be sensitive, but the IHA test to lack specificity, in the assay of mycoplasma antibodies (Purcell, Chanock and Taylor-Robinson, 1969). These observations are corroborated in these studies by application of the two tests to the assay of rabbit hyperimmune sera (Chapter 3). That more major discrepancies may exist between the two tests when applied to experimentally infected animals became apparent from the anomalous results obtained in Experiment 7. The following investigations were therefore performed to identify, if possible, the causes of these differences.

### MATERIALS AND METHODS

#### Serum fractionation studies

##### Gel filtration and fraction preparation

Serum samples of 1-2 ml were filtered through an upward flowing column (700 x 25 mm) of Biogel A 1.5M, eluting with PBS at 15 ml per h. Five ml fractions were collected and dialysed three times against BSS. Pairs of fractions were then pooled and concentrated ten-fold by dialysis in polyethylene glycol (20,000 M.W.).

Immunoglobulin (Ig) assay

The concentrated fractions were assayed for the presence of IgG, IgM and IgA by single radial diffusion (Fahey and McKelvey, 1965). A description of the preparation and purity of these ovine immunoglobulins and their monospecific antisera is given by Smith, Dawson, Wells and Burrells (1975).

Fractionated samples

i) Sera from a lamb (No. 5827) in the survey described in Chapter 4, taken when the lamb was 2 d.o. and 6 m.o. This animal had lesions of P.E. pneumonia at necropsy.

ii) Pre-inoculation and necropsy sera taken from sheep 744 (Group 1) and 354 (Group 3) of Experiment 7. Both IHA and TRI tests indicated that sheep 744 had antibodies to M. ovipneumoniae in the pre-inoculation sample: there were significant rises in antibody titres in the necropsy sample. The pre-inoculation sample from sheep 354 was negative for antibodies to M. ovipneumoniae by both tests; the necropsy serum sample was positive by the IHA test only.

Antigenicity studies

Vaccine preparation

A 24 h broth culture of strain 956/2 of M. ovipneumoniae in OB was centrifuged at 10,000 g for 30 min at 4°C, washed three times in BSS and finally resuspended in d.w. to give a 400-fold concentration of the original culture. This contained 6.6 mg protein of M. ovipneumoniae per ml. The concentration of

thixotropic $\beta$ type aluminium hydroxide gel ("Alhydrogel", Miles Laboratories Ltd., Slough, Bucks.) which gave optimal absorption with this mycoplasma suspension, i.e. greatest flocculation and clearest supernatant, was determined by titration of varying concentrations of Alhydrogel in d.w. against standard aliquots of antigen. This titration revealed that the optimal proportions of mycoplasma suspension, d.w. and Alhydrogel (10% w/v in d.w.) were 10:9:3 respectively. A suspension containing the three constituents in these proportions was emulsified with an equal volume of Bayol and Arlacel to provide a final concentration of M. ovipneumoniae in the vaccine of 1.5 mg protein per ml.

Experimental animals and treatment

Six 7 m.o. ewe hoggs from Flock B were injected by the s.c. route with 2 ml of vaccine on weeks 0 and 2. Clinical examination and nasal swabbing showed the animals to be free of respiratory signs and nasal infection with mycoplasmas at week 0. The animals were bled at weekly intervals between weeks 0 and 5, and at 2-3 week intervals thereafter.

## RESULTS

### Serum fractionation studies

Immunoglobulins of the IgA class were not detected in any of the five serum samples examined.

i) Lamb 5827 (Fig. 8.1). TRI-positive fractions could not be detected in either serum sample. Fractions containing IgG only were positive by the IHA test in both serum samples, with higher titres present in the sample collected at 6 m.o.

ii) Sheep 744 (Fig. 8.2). Low titres in fractions containing IgG only were detectable by both TRI and IHA tests in the pre-inoculation sample. Increases in titre in the post-inoculation sample were also evident by both tests, and included fraction samples containing IgM. The sensitivities of both tests were remarkably similar, and showed little discrepancy except for a "tail" present in the TRI test results in the last fractions of both samples.

iii) Sheep 354 (Fig. 8.3). All fractions of the pre-inoculation serum sample were negative by both tests. In the post-inoculation sample, antibodies at low titres were detectable only by the IHA test, where they occurred in fractions containing both IgM and IgG.

Antigenicity studies

The geometric mean antibody titres by the IHA test of the six ewe hoggs following vaccination are shown in Fig. 8.4. Titres rose rapidly to a plateau of greater than 1/1000 four weeks after primary vaccination. No response could be detected by the TRI test in any animal up to the final sampling at 12 weeks post primary vaccination, despite the use of unheated fowl or SPF lamb serum as alternative sources of complement. Rabbit hyperimmune serum to M. ovipneumoniae gave titres of 1/3200 - 1/4000 in the same tests.

Fig. 8.1
Characterisation of indirect haemagglutinating (I.H.A) antibodies to M.ovipneumoniae in serum samples taken at 2d.o. and 6m.o. from sheep 5827. The sera were fractionated by filtration through Biogel A 1.5M. Tetrazolium reduction inhibition antibodies were not detectable in any fraction.

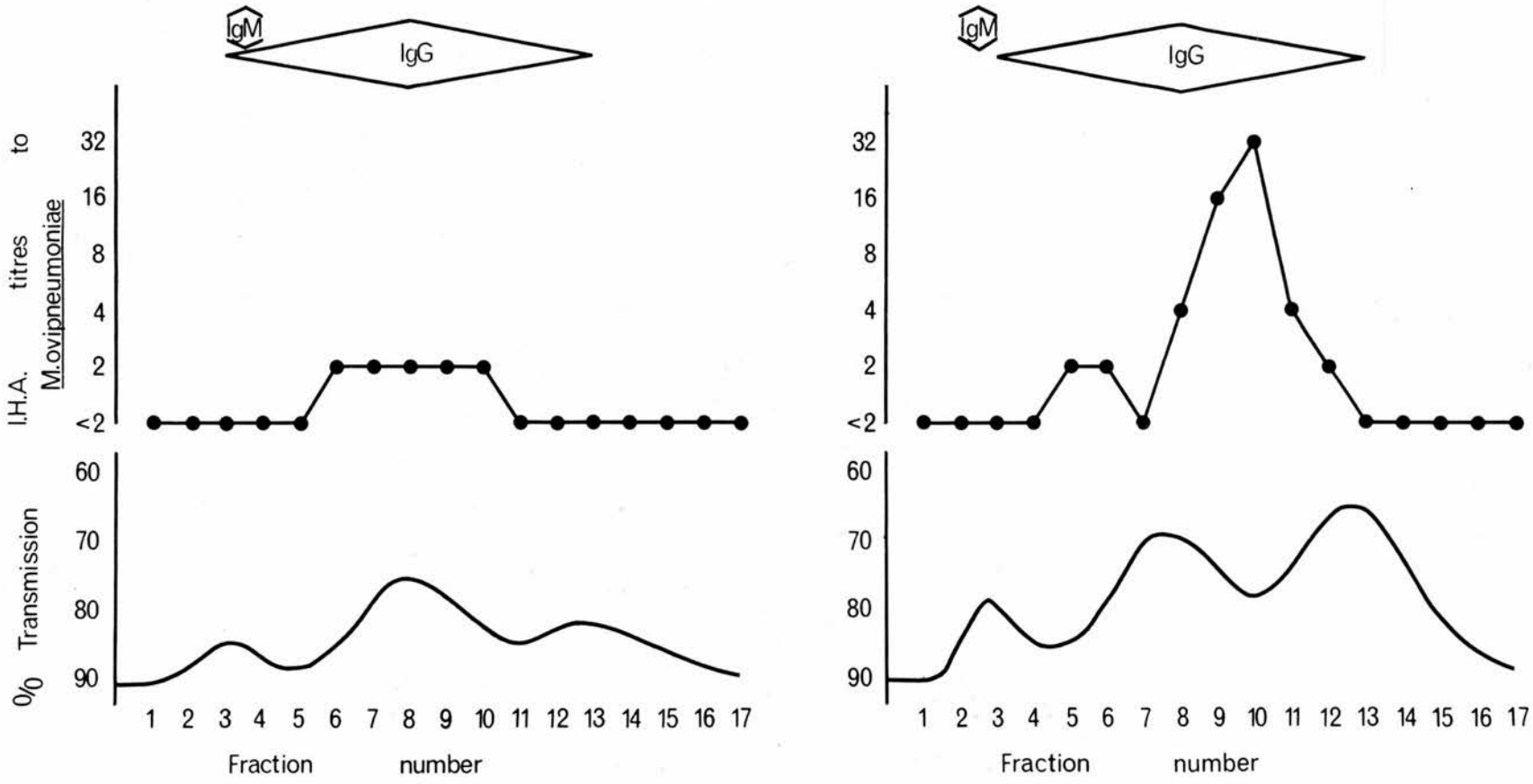


Fig.8.2
Characterisation of indirect haemagglutinating (I.H.A.) and tetrazolium reduction inhibition (T.R.I.) antibodies to M.ovipneumoniae in pre- and post-inoculation serum samples from sheep 744. The samples were fractionated by filtration through Biogel A 1.5M.

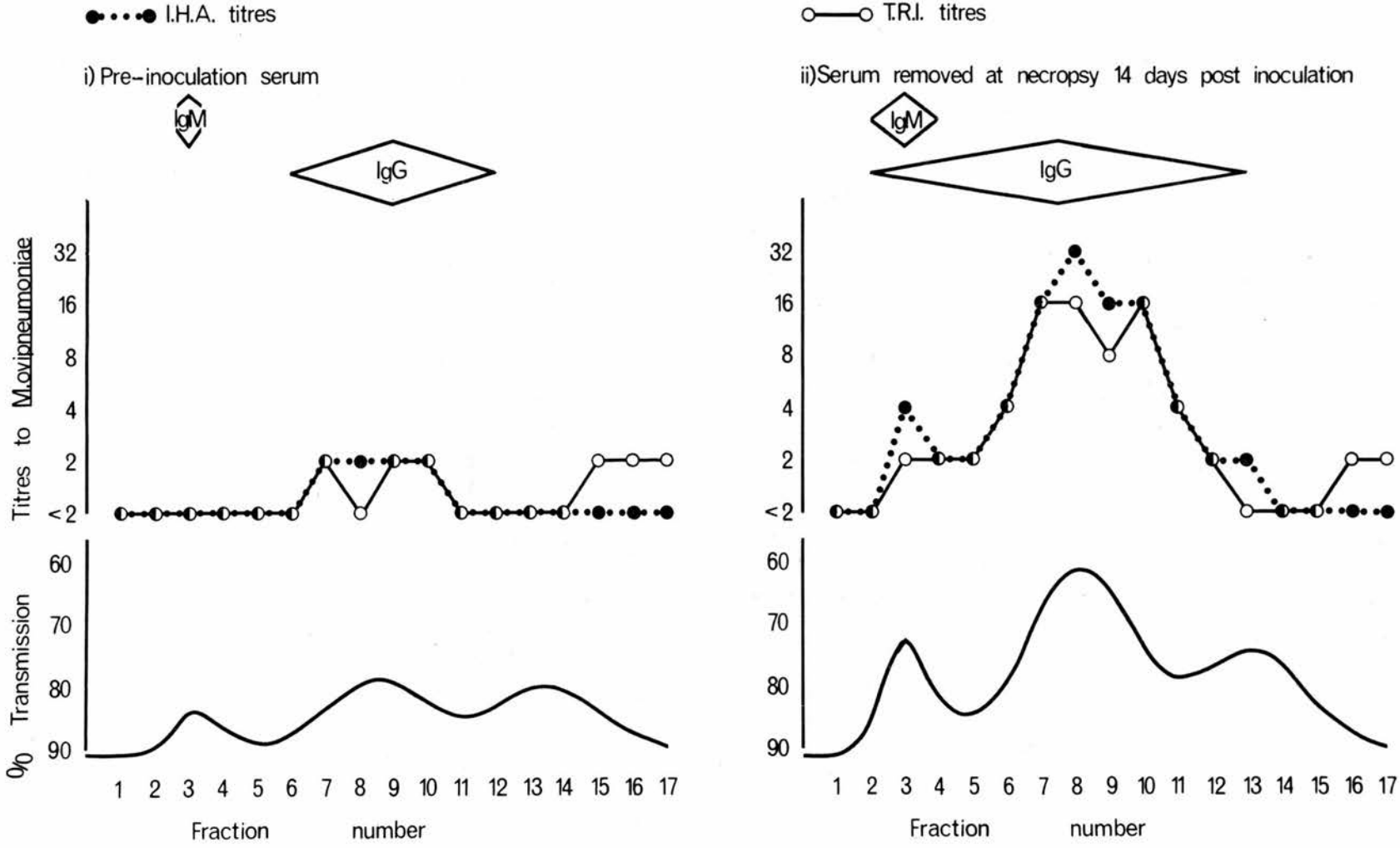


Fig.8.3
Characterisation of indirect haemagglutinating (I.H.A.) antibodies in the serum of sheep 354 removed at 15 days post inoculation. The serum was fractionated by filtration through Biogel A 1.5M. Tetrazolium reduction inhibition antibodies to M.ovipneumoniae were not detectable in any fraction.

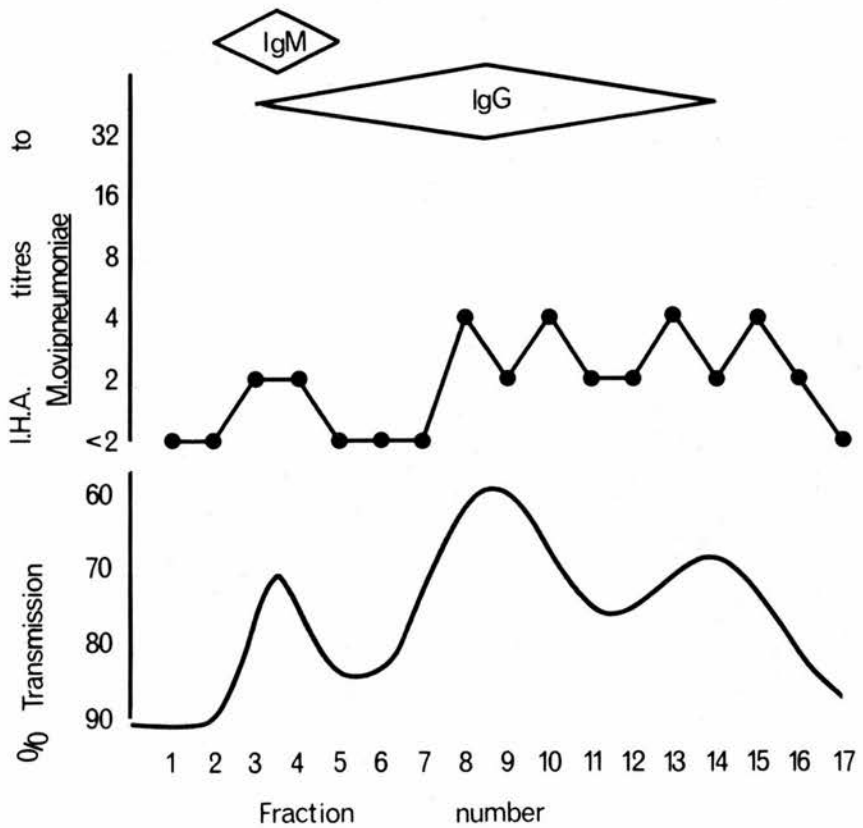
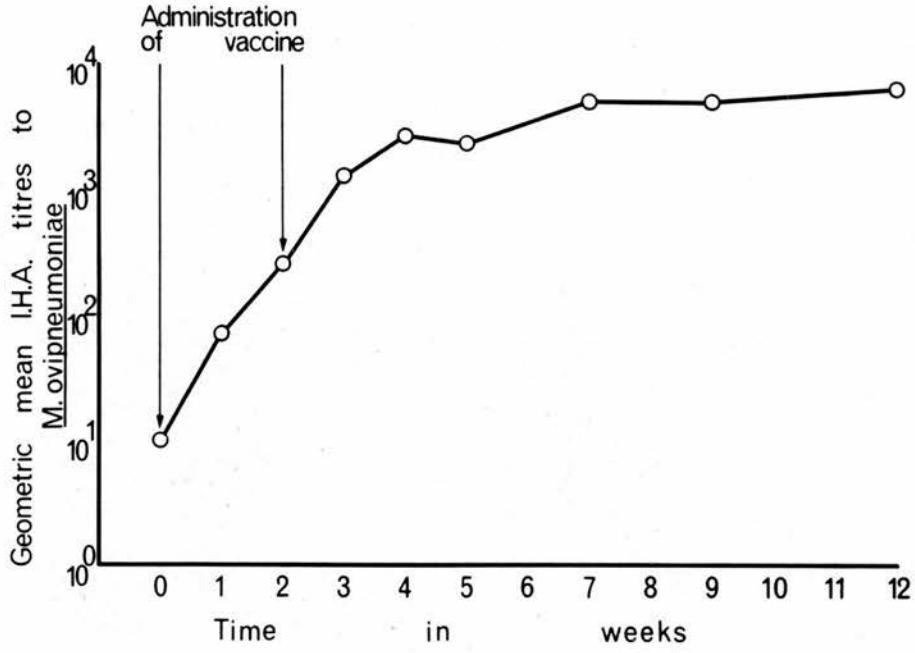


Fig.8.4
Geometric mean antibody titres of six sheep to M.ovipneumoniae, measured by the indirect haemagglutination (I.H.A.)test, following two subcutaneous injections of strain 956/2 in adjuvant administered two weeks apart. Tetrazolium reduction inhibition antibodies were not detectable in the sera of animals at any stage.



Assessment of the sensitivities of the IHA and TRI tests to IgM

Treatment of sera at 62.5°C for 30 min has been shown to denature IgM but not IgG (Jonas, 1969). Pools were made of the serum samples collected from all six vaccinated ewe hoggs at weeks 0, 1, 2 and 3, and heated at 62.5°C for 30 min in a waterbath. The results of the assay of heated and unheated serum pools by the IHA test are shown in Table 8.1.

Table 8.1

| Week of sampling | IHA antibody titres of serum pools (reciprocals) | |
|---|---|---|
| | Unheated pool | Heated pool |
| 0 | 4 | 4 |
| 1 | 256 | 32 |
| 2 | 256 | 256 |
| 3 | 1024 | 512 |

Significantly reduced titres were produced by the heat treatment only in the serum pool from the sampling at one week after primary vaccination.

Insufficient amounts of IgM containing fractions from the post-inoculation serum sample of sheep 744 were available for further assessment of the IgM sensitivities of the IHA and TRI tests. The unfractionated post-inoculation serum sample of a sheep (No. 746) from the same experiment and group as sheep 744 was therefore used for this purpose. Like sheep 744, both IHA and TRI tests detected antibodies to M. ovipneumoniae in the

pre-inoculation serum sample of sheep 746, and a significant rise in titre in the post-inoculation sample. Heated and unheated portions of this serum sample were assayed by the two tests. The results are shown in Table 8.2.

Table 8.2

| Test | Titres in serum of sheep 746 (reciprocals) Unheated | Heated |
|---|---|---|
| IHA | 4096 | 512 |
| TRI | 16 | 2-4 |

In both tests, heat treatment was found to reduce the titre of the sample, although the titres obtained by the TRI test were considerably lower than those obtained by the IHA test.

DISCUSSION

These studies confirm the indication from Experiment 7 that results obtained from the IHA and TRI tests may show discordance. These tests will therefore be considered independently then comparatively in relation to the serological findings in the foregoing chapters.

In all five sera tested, fractions containing IgG as the only form of immunoglobulin present were positive by the IHA test. That the test also measures immunoglobulin of the IgM class is

indicated by the reduction in titre, following heating, of both pooled sera taken from vaccinated sheep at one week after primary vaccination, and of the post-inoculation serum sample from sheep 746. The IHA test has previously been shown to detect both IgM and IgG classes (Biberfeld, 1968), but is considered to be more sensitive to IgM antibodies (Greenbury, Moore and Nunn, 1963; Fernald, Clyde and Denny, 1967), and to be particularly efficient in detecting anti-mycoplasmal activity in the acute phase of disease (Taylor-Robinson, Sobeslavsky, Jensen, Senterfit and Chanock, 1966b; Senterfit and Jensen, 1966), when IgM levels are at their highest (e.g. Fernald and Clyde, 1976). The results of these studies indicate that the IHA test for M. ovipneumoniae, in our hands, is efficient in detecting antibodies of both immunoglobulin classes. Fractions containing IgG only produced, in fact, higher titres than those containing IgM, although this may be related to the quantity of each specific immunoglobulin present. In addition, heat treatment of pooled sera from vaccinated sheep indicated that IgM activity against M. ovipneumoniae was negligible by the second week after primary vaccination, yet antibody titres in the group as a whole continued to rise to a plateau at four weeks post primary vaccination.

The time after infection at which IHA antibodies appear varies according to the mycoplasma species and host involved. In humans infected with M. pneumoniae, peak IHA titres are reported to occur at 3-4 weeks after initial infection (Fernald et al., 1967). Infections of humans with M. hominis resulted in high IHA antibody

titres within 10 days of inoculation (Taylor-Robinson, Ludwig, Purcell, Mufson and Chanock, 1965). In pigs infected with M. suipneumoniae, no IHA antibodies could be detected at 22 d.p.i., but high titres were found at 16-60 w.p.i. (Goodwin, Hodgson, Whittlestone and Woodhams, 1969). A similar situation described by Lam and Switzer (1972) showed IHA titres to M. suipneumoniae in infected pigs to be detectable at 2-3 w.p.i., with maximal titres apparent at 8-11 w.p.i. In Experiment 7, seroconversion was not detectable in animals which died up to 6 d.p.i., but was present in all animals challenged with M. ovipneumoniae at 13 or more d.p.i. In vaccinated animals, increases in titre were present one week after primary vaccination. The failure to detect IHA (and MI or TRI) antibody titres in almost all SPF lambs challenged with M. ovipneumoniae may relate to the mildness of lung reaction in these animals - a possibility borne out by a similar failure to detect seroconversion in the conventionally-reared animals of Groups 2 and 4, Experiment 8, in which lung lesions were also mild. This suggests that a certain degree of lung damage is necessary for adequate presentation of antigen to the reticulo-endothelial system, and implies that previous or current lung infection with M. ovipneumoniae need not necessarily be detectable by the IHA test if the consequences of infection are minor. The development of local humoral or cellular immunity may, however, confer resistance to the organism in the host, as the presence or absence of circulating antibodies need not necessarily correlate with resistance to challenge (Whittlestone, 1976a;

Fernald and Clyde, 1976).

The TRI test detected antibodies in fractionated serum samples of sheep 744 only. Fractions containing IgG as the only immunoglobulin class present were positive by the TRI test, and heat treatment of post-inoculation serum from sheep 746 showed a reduction in titre, indicating that the test also detects antibodies of the IgM class. This agrees with the findings of Biberfeld (1968).

Failure to detect TRI antibodies to M. ovipneumoniae in more than three of the 21 animals in Experiment 7 which were inoculated with infective material may be related to the timescale of their development in sheep: the surviving animals in this experiment were killed between 14 and 21 d.p.i. Taylor-Robinson et al. (1966b) found that at 2 w.p.i. with M. pneumoniae, only nine of 17 volunteers had four-fold or greater rises in antibody titre. However, this explanation would seem to be inadequate. Lamb 5827, which was exposed to infection with M. ovipneumoniae from birth, had no detectable TRI antibody at 6 m.o., despite lung infection with the organism and the presence of P.E. pneumonia in the animal at this time. Furthermore, TRI antibodies to M. ovipneumoniae were not detectable in the sera of vaccinated sheep up to the final sampling at 12 weeks after primary vaccination. In contrast, rabbits immunized with M. pneumoniae have been found to develop maximum TRI antibody titres 30 days after vaccination (Fernald et al., 1967). The hyperimmune rabbit serum to M. ovipneumoniae used during these studies was obtained 9-10 weeks after primary vaccination, and gave titres of 1/3200-1/4000 in the same tests.

An alternative explanation for the largely negative results obtained with the TRI test is provided by the concept of biological mimicry of hosts by their specific mycoplasma pathogens, advanced by workers in Utah (Cole, Cahill, Wiley and Ward, 1969; Cole, Golightly-Rowland, Ward and Wiley, 1970). The inoculation of M. arthritidis, M. pulmonis, M. neurolyticum and M. felis into rats, mice and guinea pigs demonstrated a positive correlation between the ability of a mycoplasm to produce disease and lack of MI antibody formation by its natural host (Cole et al., 1970). Further work by Cole and Ward (1973) showed that mycoplasmacidal antibody to M. arthritidis may be detected in the serum of infected rats and mice by use of a modified mycoplasmacidal test. The test showed peak mycoplasmacidal antibody titres to occur at seven days after i.v. inoculation with M. arthritidis. Since mice and rats were found to be capable of producing MI antibody against a number of non-murine mycoplasmas, Cole and Ward (1973) postulated that the mycoplasmacidal antibody produced to M. arthritidis is defective, and may be directed towards only minor components of the mycoplasma membrane, due to similarity of the major membrane antigen to host antigens.

Correlation between the TRI and IHA tests in M. pneumoniae infection of man has been found to be closest in the convalescent phase of illness (Taylor-Robinson, Shirai, Sobeslavsky and Chanock, 1966a). Marked disparity between the two tests was noted in the acute phase of illness and in persons without illness. Taylor-Robinson et al. (1966a) considered this to be due to the measurement

of different antibodies by the two tests, and pointed out that the IHA test probably involves both internal as well as surface envelope mycoplasma antigens, in contrast to the TRI test, which probably involves only mycoplasma membrane antigens. This explanation alone however is not sufficient to explain why sheep vaccinated with a whole cell mycoplasma suspension developed high IHA antibody titres but no detectable TRI titres.

Although the MI (or TRI) test has been claimed to correlate with immunity (e.g. Taylor-Robinson et al., 1966a), in general neither the TRI nor the IHA test is a good indicator of the resistance or susceptibility of hosts to their mycoplasma pathogens (Whittlestone, 1976a). Thus the failure to detect TRI antibodies in the serum of lamb 5827 at 2 d.o. does not infer that the colostrally-acquired immunoglobulin in this animal was ineffective in suppressing infection with M. ovipneumoniae. Conversely, the presence of both IHA and TRI antibodies in the sera of three animals in Group 1, Experiment 7 did not appear to afford any protection against challenge with lung homogenate, since all three sheep had M. ovipneumoniae in high titres ($\geqslant 10^6$ ccu per 0.2 ml), P.E. pneumonia and pleurisy in the lungs at necropsy. The CLS of these animals was 4%, 25% and 50%. However, the findings in these sheep may have been due to natural disease prior to experimental inoculation. The efficacy of the vaccine and vaccination schedule used in these studies was not assessed by challenge of the vaccinated sheep with M. ovipneumoniae, so the significance of IHA antibody presence and TRI antibody absence cannot be evaluated. The varied and conflicting experience of other workers concerning the efficacy of different mycoplasma vaccines in adjuvants (Whittlestone, 1976a) prohibits

useful comparison with their results.

# CHAPTER 9

## GENERAL DISCUSSION

The results of these studies, and the literature relating to ovine mycoplasmas reviewed in Chapter 1 or referred to in subsequent chapters, indicates that the two most commonly occurring mycoplasmas of the ovine respiratory tract are M. ovipneumoniae and M. arginini. Since 1972, only two serotypes (EHM and QEW), have been added to the list of mycoplasmas known to occur in the respiratory tract of sheep (Cottew, 1974). It is nevertheless possible that other ovine mycoplasma species exist which are as common or more important in disease terms than M. ovipneumoniae or M. arginini, but which have remained undetected due to the use of inadequate media or isolation techniques.

### M. OVIPNEUMONIAE

#### Distribution and epidemiology

M. ovipneumoniae has been isolated from sheep only and has been recovered in Australia (Carmichael et al., 1972), New Zealand (Clarke et al., 1974), the USA (St. George and Carmichael, 1975), Hungary (Stipkovits, Belak, Palfi and Tury, 1975), the UK (Jones, Foggie, Mould and Livitt, 1976; Leach et al., 1976), and Iceland (Friis, Palsson, and Petursson, 1976). There have been no studies to date on the prevalence of M. ovipneumoniae on a flock-to-flock basis. Within flocks, infection rates may reach very high levels, particularly in lambs, but spread of the organism

may largely depend on management factors (see results and discussion, Chapter 4). The relatively high recovery rates from ewes implicates dams as the probable initial source of infection, as has been found, for example, in mycoplasmoses of swine (e.g. Durisic and Vrbanac, 1969; Ross and Spear, 1973). It is also probable that infection is by the respiratory route: survey findings in Chapter 4 showed the isolation rates of M. ovipneumoniae from the nasal cavities of lambs to increase with age. Experiment 2 showed that nasal colonisation of lambs could be established by in-contact transmission from infected lambs; and M. ovipneumoniae has rarely been recovered from sites other than the respiratory tract and its associated lymph nodes.

Pathogenicity

The experiments in SPF lambs demonstrated that a proportion of animals show no discernible response to challenge with M. ovipneumoniae; lymphoid cell cuffing and phagocytic cell and fluid exudation or interstitial pneumonia may be observed in other animals. In addition, the results of Experiment 8, performed in conventionally-reared sheep, suggest that M. ovipneumoniae alone may produce P.E. pneumonia. The findings from these experimental studies are reflected in the results of the survey into naturally-occurring respiratory mycoplasmosis of lambs (Chapter 4). M. ovipneumoniae was recovered from the respiratory tract of 33, and from the lungs of 29 of 34 lambs. Almost 24% of these animals showed no significant pulmonary changes, 32% had lymphoid hyperplasia with or without interstitial thickening, and 44% had P.E. pneumonia. It is

apparent, therefore, that a considerable spectrum of lung changes is associated with both natural and experimentally-produced infection with M. ovipneumoniae, and that the response by individual animals to this infection varies enormously. No attempt has been made in these studies to investigate the pathogenic mechanisms of M. ovipneumoniae: however, the similarity of the pathological changes induced in sheep by this organism to those produced by respiratory mycoplasma pathogens of other hosts (Freundt, 1974a; Experiment 1, Chapter 5) permits speculation of the possible pathogenicity mechanisms of M. ovipneumoniae by consideration of those factors thought to operate in other mycoplasma species. These factors have been reviewed recently by Whittlestone (1972a), Clyde (1975), Bredt (1976) and Brunner (1976).

i) Possible pathogenicity factors involving M. ovipneumoniae per se.

M. neurolyticum is the only mycoplasm known to produce a soluble exotoxin. Toxic effects have also been observed with M. gallisepticum, M. pulmonis and M. arthritidis, but i.v. inoculation of these organisms is necessary to elicit toxicity (Whittlestone, 1972a). M. mycoides subsp. mycoides may also produce an exotoxin (Lloyd and Trethewie, 1970), and certainly does produce a galactan which causes acute respiratory, circulatory and other effects when injected intravenously into calves (Buttery, Lloyd and Titchen, 1976). It is extremely unlikely that M. ovipneumoniae produces comparable substances. No signs of toxaemia or neurological damage were noted in any of the animals which yielded M. ovipneumoniae

only from the lungs, either in the survey or in the experimental studies. It is also unlikely that M. ovipneumoniae possesses membranes with toxic properties: electron microscopy studies in Experiment 1 could find little evidence of ciliary loss or damage to bronchiolar epithelium - effects elicited by membrane preparations of M. pneumoniae in hamster tracheal organ cultures (Gabridge, Johnson and Cameron, 1974).

Many mycoplasmas are known to release hydrogen peroxide, other haemolysins and enzymes (e.g. Whittlestone, 1972a). Peroxide elaboration alone is known not to be a determinant of virulence, as saprophytic mycoplasmas and avirulent strains of pathogens are also capable of this function (Cole et al., 1968). In M. pneumoniae, cytadsorption and morphology but not peroxide release, growth, glycolysis or PAGE patterns have been found to distinguish virulent from avirulent strains (Lipman and Clyde, 1969; Lipman, Clyde and Denny, 1969). The importance of cytadsorption capacity to the pathogenicity of M. pneumoniae supports a suggestion made initially by Sobeslavsky, Prescott and Chanock (1968), and subsequently by other workers (e.g. Collier, 1972; Fernald and Clyde, 1976) that the toxic effects of peroxide release are mediated in M. pneumoniae by an intimate association between the mycoplasm and host cells. M. pneumoniae has been shown to possess a specialized tip (Biberfeld and Biberfeld, 1970), by which adherence to host cell surfaces (Collier and Clyde, 1971) employing neuraminic acid receptor sites (Manchee and Taylor-Robinson, 1969) is accomplished. It has been suggested that the close adherence of M.

pneumoniae to epithelial cell membranes may also interfere with ciliary beating, result in membrane damage due to disturbance of surface charges needed for synchrony of ciliary motility, cause nutritional deprivation (Collier, Clyde and Denny, 1971), and permit alterations in macromolecular biosynthesis and metabolic activity of the host respiratory epithelial cells by the release of mycoplasma products (Hu, Collier and Baseman, 1975). In pathogenic mycoplasmas of other species, the roles of peroxide release and cell attachment as pathogenic mechanisms may be totally different from these functions in M. pneumoniae. Peroxide production but not cytadsorption has been shown to be important in the pathogenesis of M. mycoides subsp. capri infection of chicken tracheal organ cultures, but neither function was found to be significant in the pathogenesis of M. gallisepticum in the same in vitro system (Cherry and Taylor-Robinson, 1973). M. suipneumoniae neither secretes peroxide (Pijoan, 1974) nor causes pathological effects in porcine tracheal organ cultures (Pijoan, Roberts and Harding, 1972), but is recognised to be the primary agent of enzootic pneumonia of pigs (Whittlestone, 1972b), in which it causes loss of cilia from bronchiolar epithelium, and a reduction in adherence between individual epithelial cells (Whittlestone, 1972a). It is apparent therefore that the capacity of M. ovipneumoniae for hydrogen peroxide production (Chapter 3) may be irrelevant to its mode of pathogenesis, particularly since the organism possesses no specialized structures for attachment (Chapter 3), and where visualised by the electron microscope in lung section of an infected lung (Experiment 1) was found to be enmeshed in the tips of cilia,

at some distance from the epithelial cell surface. These observations relate, however, to the bronchial and bronchiolar levels of infection. There remains the possibility that the release of soluble products by M. ovipneumoniae may be of importance at the alveolar level. Lindsey and Cassell (1973) found that the inoculation of high doses of M. pulmonis ($10^5$ cfu or greater) into pathogen free mice produced two effects, an acute disease occurring at 1 - 10 d.p.i., or a chronic disease which was manifested from 11 d.p.i. onwards. The acute disease involved pneumonia which principally affected the alveoli. Immunofluorescence techniques demonstrated the location of mycoplasmas in the acute disease to be mainly within the alveoli at 3 - 5 d.p.i.; from 7 - 10 d.p.i., organisms were observed as a continuous line on the surface of respiratory epithelium, and from 10 d.p.i. onwards the majority of organisms were in the major airways. Lindsey and Cassell (1973) considered that the oedema, neutrophilic infiltration, congestion and haemorrhage seen in the alveoli of acutely affected mice might be wholly or partly attributable to some product of the mycoplasm. In Experiment 1 (Chapter 1), intra-alveolar exudation of Hale positive material was observed in the lungs of infected lambs. The transient and early nature of this reaction suggests that it occurred during the presence of M. ovipneumoniae in alveolar lumina in the initial phases of infection, and may have been due to the release of products by the mycoplasm at this site.

Prolonged passage of mycoplasmas in artificial media is known to affect the virulence of strains (Couch et al. 1964; Smith, Chanock, Friedewald and Alford, 1967). Four different

strains of M. ovipneumoniae were used during the course of these experimental studies. The three strains inoculated into SPF lambs were used at passage levels ranging from 2 - 6 -levels at which marked attenuation of the organism is unlikely to occur. The single strain inoculated into conventional animals in Experiments 7 and 8 was used in its ninth and 13th passages respectively, and it is perhaps possible that passage levels of this order decreased the virulence of the strain. Smith et al. (1967) challenged groups of volunteers with the PI-1428 strain of M. pneumoniae at different passage levels. All volunteers inoculated with a second and a 25th passage level culture became infected (as judged by antibody rise), but whereas 17 of the 22 men challenged with the second passage culture became ill, only seven of 13 men showed a similar response to the 25th passage culture.

ii) Possible pathogenicity factors involving interactions between host and organism

The hypothesis that delayed hypersensitivity may be an important factor in the pathogenesis of respiratory mycoplasmosis has been discussed in Chapter 5. Several observations support this hypothesis. Smith et al. (1967) found that vaccination with killed M. pneumoniae vaccine stimulated antibody response in a proportion of volunteers, and that these were resistant to subsequent challenge with virulent M. pneumoniae. However, challenge provoked a more serious illness, compared with non-immunized controls, in those vaccinees that did not develop detectable antibodies. In guinea pigs inoculated by the i.n. route with M.

pneumoniae, the first histopathological changes are noted one week after infection; the lesions increase in extent and severity, then resolve and disappear by about 16 w.p.i. (Brunner, 1976). If the animals are reinfected several months after excretion of M. pneumoniae has ceased and the lesions have disappeared, cellular infiltration of the lungs occurs within a few days and is of greater extent compared with the first infection. A similar protocol will produce the same effects in hamsters (Clyde, 1971). The failure of Experiments 3 and 4 to demonstrate enhanced lung reaction following secondary challenge with M. ovipneumoniae may therefore be due, as indicated earlier, to an insufficient time interval between primary and secondary challenges.

The antigenicity of mycoplasmas for their hosts may be a particularly important facet of their pathogenicity and capacity for survival for lengthy periods in their specific predilection sites. The importance of the immune system to the development of "typical" mycoplasmal-induced lung lesions, i.e. peribronchial and perivascular cuffing with lymphoid cells, has been demonstrated in mice immuno-suppressed by thymectomy and X-irradiation and challenged with M. pulmonis (Denny, Taylor-Robinson and Allison, 1972; Taylor-Robinson, Denny, Allison, Thompson and Taylor, 1972), or hamsters treated with antithymocyte serum and infected with M. pneumoniae (Taylor, Taylor-Robinson and Fernald, 1974). Immuno-suppressed animals developed much less severe lung lesions than immunologically-normal control animals due to reduced lymphoid cell and macrophage exudation. The pneumonic lesions in those animals

that respond to infection with M. ovipneumoniae appear to be due almost entirely to host reaction involving lymphoid cell, macrophage, neutrophil and fluid exudation rather than overt tissue damage. That this reaction is immunological in origin and does not involve tissue damage due to the mycoplasm or to inflammatory by-products released by the interactions of mycoplasmas with serum constituents (Bredt and Bitter-Suermann, 1975), is indicated by the total lack of reaction to challenge seen in three lambs in Experiment 1, in two of which M. ovipneumoniae became established in the lungs. The absence of response to M. ovipneumoniae, seen in a proportion of experimental and survey lambs, may perhaps be explained by the concept of biological mimicry discussed in Chapter 8. This hypothesis, developed by workers in Utah from studies in laboratory animals, is supported by evidence from other mycoplasma-host relationships. Shared antigens have been demonstrated between bovine pneumogalactan and M. mycoides subsp. mycoides (Shifrine and Gourlay, 1965). In man, infection with M. pneumoniae is followed by the development of cold agglutinins in approximately 50% of patients (Couch, 1969). It has been suggested that this autoimmune reaction is due to the sharing of a galactose-containing carbohydrate moiety between human erythrocytes and the membrane of M. pneumoniae (Razin, Kahane and Kovartovsky, 1972). Shared antigens between M. pneumoniae, brain and other human tissues have also been demonstrated (Biberfeld, 1971). It may therefore be that sheep which show little or no reaction to the presence of M. ovipneumoniae in the lungs are those which share antigens in common

with the mycoplasm. However, the demonstration that strains of M. ovipneumoniae exhibit a broad antigenic spectrum (Chapter 3) might mean that the failure of some sheep to respond applies only to certain strains of the organism, and that other strains are capable of provoking pneumonia in these sheep. This postulated balance between the strain of M. ovipneumoniae involved and the individual animal must depend on M. ovipneumoniae being of low antigenicity for sheep, for which circumstantial evidence is provided by the failure to demonstrate TRI antibodies to M. ovipneumoniae in vaccinated sheep (Chapter 8), or in more than four animals in Experiments 7 and 8. Low antigenicity may also account for the chronic colonisation of lungs with M. ovipneumoniae suggested by recovery of the organism from 6 m.o. lambs and adult ewes (Chapter 4), although antiphagocytic surface factors (Bredt, 1976) may be more important in this respect.

The biology, distribution and pathogenicity of M. ovipneumoniae invite comparison with one other mycoplasma in particular, namely M. dispar. Both species are glycolytic, form centreless colonies on solid media, do not possess any specialised organelles, and are found in the respiratory tract of ruminants (Freundt, 1974). M. dispar may commonly be recovered from the respiratory tract, including lungs, of both pneumonic and normal calves (Gourlay, Mackenzie and Cooper, 1970; Thomas and Smith, 1972), although recovery rates are higher from pneumonic calves (Pirie and Allan, 1975). Naturally-infected calves show a range of lesions, including mild interstitial reaction, proliferative interstitial pneumonia associated with bronchiolitis, and lymphoid hyperplasia

and cuffing pneumonia (Gourlay et al., 1970; St. George, Horsfall and Sullivan, 1973; Pirie and Allan, 1975). The experimental infection of gnotobiotic calves has shown that M. dispar produces a subclinical pneumonia comprising peribronchiolar cuffing lesions and catarrhal bronchiolitis (Howard, Gourlay, Thomas and Stott, 1976). All animals were killed at 3 w.p.i. The caesarean-derived or colostrum-deprived calves inoculated by St. George et al. (1973) with cultures or an homogenate of affected lung containing M. dispar developed, in contrast, a proliferative interstitial pneumonia. These animals, however, died or were killed within eight days of inoculation - possibly too early for the manifestation of cuffing lesions (Pirie and Allan, 1975).

The numerous similarities between M. dispar and M. ovipneumoniae suggest that both mycoplasmas may exert their pathogenic effects in the same manner. Thomas and Howard (1974) found that large numbers of M. dispar produced a rapid cytopathic effect in organ cultures of bovine foetal trachea which involved degeneration and sloughing of the respiratory epithelium. Electron micrographs showed the organism to be closely attached to cil ia and not epithelial cell surfaces, in a manner similar to that observed for M. ovipneumoniae in Experiment 1. No evidence of penetration of the intercellular spaces was found, a feature described for M. pneumoniae (Collier et al., 1971). Washed, live suspensions of M. dispar did not produce a cytopathic effect, which suggested to the authors that toxic membrane components were not responsible. Further work in the same in vitro system (Howard and Thomas, 1974)

showed that M. dispar inhibited the ciliary activity of the organ cultures, and that this inhibition was dependent on the presence of serum in the maintenance medium. The inhibitory activity was unaffected by the addition of catalase to infected cultures, suggesting therefore that peroxide production was not responsible. Furthermore, no toxic substance could be demonstrated in the supernatant fluids of infected organ cultures. The authors pointed out, however, that the presence of serum may have stimulated the attachment of M. dispar to the cilia, which, as previously mentioned, is probably a necessary step for manifestation of the toxic effects of mycoplasma products.

It has already been suggested that the form of histopathological lesion produced experimentally by e.b. inoculation of M. ovipneumoniae into lambs may be dependent of the strain of M. ovipneumoniae employed (Chapter 5). The foregoing discussion offers possible explanations as to how these differences might occur, and why in the natural situation a range of pathological changes are found in the lungs of animals. Lymphoid cell hyperplasia and phagocytic cell exudation presumably occur where the mycoplasm is recognised by the individual host to be antigenic. Absence of this reaction would therefore be due to low antigenicity of the strain of mycoplasm involved. On the other hand, interstitial pneumonia may be a reaction, at the alveolar level, to the irritant effects of mycoplasma products, including peroxide. Absence of this reaction might thus be due to the inability of the mycoplasma strain involved to persist within the alveoli for any

length of time, or to produce only low levels of toxic substances. Pirie and Allan (1975) have suggested that interstitial pneumonia may be the earliest reaction in naturally-occurring mycoplasmosis of calves, and that lymphoid cell cuffing follows at a later stage. The absence of interstitial reaction and the observation of cuffing lesions as early as 14 d.p.i. in animals in Experiment 1 indicate that this may not be the case in M. ovipneumoniae infections of lambs. Combinations and varying degrees of severity of these two effects would thus depend on the properties of the strain of M. ovipneumoniae involved and its interrelationship with each animal. Whether or not P.E pneumonia may be produced by M. ovipneumoniae alone requires further investigation, but the disease, which essentially comprises both severe lymphoid and inflammatory cell exudation and interstitial thickening combined with bronchiolar hyperplasia, may represent the extreme of a range of effects produced by the mycoplasm. Additional host factors, including hypersensitivity, may however, be necessary for manifestation of the disease. The epidemiological factors which provoked the hypothesis that hypersensitivity is concerned in the pathogenesis of atypical pneumonia of man have their parallels in atypical pneumonia of sheep. In man, the disease occurs mainly in the age group 5 - 15 y.o. (Brunner, 1976), although infections of infants and children with M. pneumoniae are considerably higher than might therefore be expected: however, 74% of such infections have been found to be asymptomatic or associated with a mild, non-specific coryza and cough (Fernald, Collier and Clyde, 1975). In sheep, lambs of 3 - 10 m.o. are affected (Harris and Alley, 1977), although

lambs may be exposed from birth to the organisms commonly associated with atypical pneumonia (Chapter 4). High infection rates with M. ovipneumoniae were found to develop rapidly with increasing age, but mild clinical signs only were observed in a low proportion of the survey population until the animals were 3 m.o. or more. The corollary of these observations is that high proportions of children under 5 y.o. have low amounts of antibody to M. pneumoniae; these are detectable by sensitive techniques such as the mycoplasmacidal and radioimmunoprecipitation tests (Bunner, Prescott, Greenberg, James, Horswood and Chanock, 1977). In sheep, passively-acquired immunity may act to suppress but not eliminate infection with M. ovipneumoniae, such that animals become sensitized rather than immunized by contact with the organism. Fernald et al. (1975) have suggested that in man, "recurrent, unsuspected M. pneumoniae infections occur during infancy and early childhood, and that pneumonic disease, common above age 10 years, is an expression of increasing host response to the organism".

## M. ARGININI

### Distribution and epidemiology

M. arginini has a wide host range, including cattle, sheep, chamois, goats, cats and pigs (Barile et al., 1968; Leach, 1970; Heyward, Sabry and Dowdle, 1969; Tully, Del Giudice and Barile, 1972; Galli and Leach, 1976). The results of surveys (Carmichael et al., 1972; Alley et al., 1975; Chapter 4, this thesis) indicate that M. arginini may commonly be recovered from the respiratory tract of both normal and pneumonic sheep. The main site of

localization appears to be tonsillar tissue. The e.b. inoculation of SPF lambs with a culture of M. arginini resulted in colonisation of tonsillar tissue and the retropharyngeal lymph node (Chapter 6), which supports a suggestion made previously (Chapter 4) that infection probably occurs principally by the respiratory route: the frequent recovery of M. arginini from eyes suggests that the conjunctival route of infection may also be important (Leach, 1970).

Pathogenicity

Little evidence of pathogenicity has been found for M. arginini in these studies. No correlation was observed in Chapter 4 between lung presence of the organism and histopathology of the colonised lung. In Chapter 6, neither lung lesions nor lung colonisation resulted from the e.b. challenge of SPF lambs with a culture of M. arginini. Blackmore and Hill (1973) experimentally inoculated five SPF kittens by the oral, ocular and nasal routes with a strain of M. arginini isolated from a cheetah. Persistence of infection with the organism for 21 days occurred in only one animal, and no evidence of pathogenicity was seen. However, an indication that M. arginini may play an exacerbative rôle in respiratory disease of sheep was apparent in Experiment 7. This, the only experiment into the aetiology of P.E. pneumonia which included M. arginini in the infective inocula, was also the only experiment in which deaths occurred. Lung recoveries of M. arginini in significant titres were confined to the eight animals which died, and four

others which had pleurisy. Consideration of the possible pathogenicity factors of M. arginini will therefore be restricted to those which might facilitate or exacerbate infection and invasion by other microorganisms, notably P. haemolytica.

The Type N strains studied by Cottew et al. (1968) were reported to produce weak or no lysis of erythrocytes. In studies parallel to those reported in Chapter 3, a similar finding was obtained with the type strain of M. arginini. Furthermore, negative results for the organism were obtained by both tests used in these studies for demonstration of hydrogen peroxide release. It would seem therefore that haemolysin release by M. arginini is negligible or non-existent.

Cell growth in tissue cultures has been found to be impaired by arginine utilizing mycoplasmas, due to depletion of arginine in the medium (Kenny and Pollock, 1963). Similarly, possession of the enzyme arginine deiminase and consequent deprivation of cells of arginine have been considered to be the reason for inhibition of lymphocyte stimulation by mitogens following addition of mycoplasma organisms (Barile and Leventhal, 1968) or extracts (Simberkoff, Thorbecke and Thomas, 1969). These effects were observed with arginine utilizing species only, and no inhibition of lymphocyte stimulation was apparent with glucose utilizing mycoplasmas. The same effects have been observed with M. arginini (Callewaert, Kaplan, Peterson and Lightbody, 1975). The whole organism, or cell free media obtained after growth of M. arginini, were found to inhibit activation of human peripheral lymphocytes and mouse spleen

lymphocytes by mitogens or allogeneic cells. This would seem to be a plausible mechanism by which the invasiveness of P. haemolytica might be facilitated by M. arginini, but whether these effects elicited in vitro are significant in vivo remain to be proven. Virulent and avirulent strains of the arginine utilizing species M. meleagridis have been found not to differ in their metabolism of arginine, and no significant differences were found in plasma arginine concentrations between M. meleagridis-infected and uninfected turkey poults (Ibrahim and Yamamoto, 1977). Since loss of virulence of M. meleagridis correlates with loss of antigenicity and invasiveness (Ghazikhanian and Yamamoto, 1974), Ibrahim and Yamamoto (1977) concluded that the pathogenicity of M. meleagridis infection is not based on its competition with the host for arginine. On the other hand, it may be that arginine deiminase is effective in suppressing the immune response of the host at a local level, particularly when other adverse factors are operating. Foggie and Angus (1972) noted that N type mycoplasmas were not uncommonly recovered in Turkey from the lung, spleen, kidney and lymph nodes of goats dying of selenium deficiency or other debilitating disease.

## P.E. PNEUMONIA

### The rôle of mycoplasmas in the disease

In the course of these studies, lung homogenate preparations or mixed cultures containing principally M. ovipneumoniae and P. haemolytica biotype A serotypes were inoculated into a total of

47 conventionally-reared sheep. At necropsy, P.E. pneumonia was found in 38 (81%), acute bronchopneumonia in three, interstitial thickening with lymphoid hyperplasia in three, and focal or linear collapse lesions in one sheep. The lungs of two animals were not examined histologically, although macroscopic lesions identical to those of naturally-occurring P.E. pneumonia were present in these sheep. The objections to the use of conventionally-reared animals for experiments into respiratory disease were clearly apparent in these studies. Natural infections with various respiratory microorganisms including those under investigation, antibodies to these organisms and lung lesions were all encountered in some experimental animals before inoculation or in control animals at necropsy. These findings must therefore be regarded with caution, and require confirmation in SPF or gnotobiotic lambs. However, the constant association of M. ovipneumoniae with P.E. pneumonia noted in 15 survey lambs was substantiated by these experimental findings. Whether the additional presence of P. haemolytica is obligatory to development of the disease remains more doubtful at present. In preliminary and main Experiment 8, lesions of P.E. pneumonia from which only M. ovipneumoniae was recovered were present in two control sheep, and in three animals inoculated with a culture of M. ovipneumoniae. It may be, though, that P. haemolytica exacerbates the extent of P.E. pneumonia (Experiment 8), and negative evidence from the experimental challenge of SPF lambs with M. ovipneumoniae or M. arginini suggests that the febrile response, severe clinical symptoms and pleurisy

seen in many sheep challenged with a combination of P. haemolytica and mycoplasmas is due to the bacterial and not the mycoplasmal content of the inoculum.

The results of Experiment 8 confirm that M. ovipneumoniae can potentiate the establishment of P. haemolytica in the lungs of sheep. It is possible that this facilitation is due to the stimulation of cellular exudate. Lindsey and Cassell (1973) considered that the massive outpouring of neutrophils into the lungs of mice challenged with high doses of M. pulmonis was an extremely important and central feature of the pneumonia induced, and appeared to be linked directly with more dramatic changes such as bronchiectasis, destruction of bronchial epithelium, pulmonary abscesses and pleurisy. These authors suggested that "much of the actual tissue injury in these lesions might result from pressure effects of severe suppuration and/or the release of large quantities of lysosomal enzymes from neutrophils". An early neutrophil response was similarly noted in lambs in Experiment 1. Alternatively, the elicitation of Hale positive exudate may be of greater importance to the lung establishment of P. haemolytica. It is perhaps surprising, therefore, that the experimental production of P.E. pneumonia in these studies was achieved by the simultaneous inoculation of M. ovipneumoniae and P. haemolytica. Liu, Jayanetra, Voth, Muangmanee and Cho (1972) found that M. pneumoniae-mediated potentiation of Diplococcus pneumoniae infection in hamsters was successful only if active multiplication of the mycoplasm in the lungs preceded challenge with the pneumococcus. The time interval

between mycoplasmal and bacterial inoculations used by these workers was four days. Potentiation of pasteurellosis of sheep by PI3 virus has been found to be optimal if infection with the virus precedes that of the bacterium by 4 - 7 days (Sharp et al., 1978). This might suggest that the predisposing effect of M. ovipneumoniae is achieved very rapidly - within the normal clearance times of particle removal from the lungs. In calves, it has been shown that 90% of P. haemolytica administered by aerosol is removed from the lungs in 4 h (Lillie and Thomson, 1972). Alternatively, it may be that P. haemolytica reinvades the lung from a site of establishment in the upper respiratory tract. Anatomical considerations and the observation in a proportion of sheep of circumscribed lesions in the diaphragmatic lobes indicates that the endobronchial method of challenge deposits the inoculum in this site. However, the lesions of P.E pneumonia produced were almost invariably in the apical and cardiac lobes, and involvement of the diaphragmatic lobes was generally minor in extent. An explanation might therefore be that following inoculation, a localised focus of infection with M. ovipneumoniae in the diaphragmatic lobes is followed by the colonisation of those areas of lung which, due to anatomical or physiological reasons, are more susceptible to microbial invasion. Subsequent to this establishment, superinfection with P. haemolytica occurs from the upper respiratory tract.

Further investigations into the association of mycoplasmas and bacteria with "enzootic" (P.E.) pneumonia of sheep have recently been performed by two groups of workers in New Zealand. Alley and

Clarke (1977) studied the degree to which histopathological lesions found in the lungs of 60 6 - 9 m.o. lambs slaughtered at an abattoir correlated with the numbers of M. ovipneumoniae and bacteria recoverable from these lungs. The bacteria present were identified as P. haemolytica, Neisseria catarrhalis or Staphylococcus aureus. Large numbers ($>10^7$ orgs per g) of M. ovipneumoniae were found to be associated with chronic proliferative changes such as peribronchiolar fibrosis and alveolar interstitial thickening. Large numbers of M. ovipneumoniae and bacteria ($>10^5$ orgs per g) together were found to be associated with neutrophilic exudation and epithelial hyperplasia. Lymphoid hyperplasia and excess mucus production were observed where bacterial numbers were low. The significance of these findings is doubtful. The underlying assumption, that microbial numbers and species in infected lungs are a direct reflection of the pathological changes present at any moment in time, implies that pathological and immune processes occur synchronously, that all organisms present in the lungs contribute to the lesions observed, and that immunity, when acquired, is equally effective against bacteria and mycoplasmas. It is possible that bacteria may exert their effects and be eliminated from the lungs over a much shorter period than is required for resolution of the lesions induced. Conversely, mycoplasmas may be recoverable in high titres from lungs in the absence of pneumonic lesions, or after their resolution (Experiment 1), due to their capacity for prolonged survival in the lower respiratory tract (Whittlestone, 1972a). The nature of the material examined by Alley and Clarke (1977) precluded knowledge of either the duration of infection with M. ovipneumoniae and bacteria or the stage of disease process, and no

examinations for circulating or local antibodies were performed. Workers at Wallaceville (Thurley, Boyes, Davies, Wilkins, O'Connell and Humphreys, 1977) studied the incidence of subclinical pneumonia in lambs over a two year period. A total of 85 lambs were slaughtered between November and March or June; 54 of these had "enzootic" pneumonia, 15 yielded P. haemolytica, 14 unspecified mycoplasmas and two S. aureus from the lungs. In both years, the incidence of enzootic pneumonia and the numbers of isolations of P. haemolytica and mycoplasmas increased towards the later part of the sampling period. The low recovery rates of microorganisms, in particular mycoplasmas, is surprising in view of previous findings (Alley et al., 1975; Chapter 4, this thesis), and suggests that the techniques employed precluded the isolation of M. ovipneumoniae, despite use of the mycoplasma media devised by Carmichael et al. (1972).

The potentiating effect of mycoplasmal infection for subsequent bacterial invasion has been described for a number of different hosts and microorganisms. The i.n. inoculations of hamsters with M. pneumoniae and subsequently Diplococcus pneumoniae does not lead to an increased incidence of pneumococcal bacteraemia compared with hamsters infected with the bacterium alone, but does result in a more fulminating septicaemia, with higher recovery titres of pneumococci and more animals dying (Liu et al., 1972). Immunisation of the hamsters with live M. pneumoniae conferred a considerable protective effect on animals, and the use of a temperature sensitive mutant of the mycoplasm did not potentiate development

of the severe disease. Studies involving Pasteurella pneumotropica and M. pulmonis in conventional, SPF and gnotobiotic mice (Brennan, Fritz and Flynn, 1969) showed that the two organisms had an additive effect, and that both probably contribute to typical murine pneumonia. The combination of Bordetella bronchiseptica and M. hyorhinis produces more severe disease in piglets than either agent alone (Gois, Kuksa and Sisak, 1977). In chickens, M. synoviae (Springer, Luskus and Pourciau, 1974) and M. gallisepticum (Fabricant, 1969) have been shown to potentiate infection with infectious bronchitis virus and E. coli. Gourlay, Howard, Thomas and Stott (1976) have produced experimentally exudative bronchopneumonia in conventionally-reared calves by the endobronchial inoculation of pneumonic lung homogenates (LH). The average percentage of lung surface area with gross pneumonia (CLS) in 15 calves treated with two pools of LH was 39%. Ten calves treated with irradiated LH had a mean CLS of 3.5%; treatment of LH and the calves with tylosin tartrate produced in 10 calves a mean CLS of 0.5%. Ampicillin treatment of LH and the calves gave a mean CLS of 9% in 10 calves, the histological pattern of which was more cuffing and less exudative in nature. These results suggested to the authors that the total pneumonia was due to organisms susceptible to tylosin tartrate, and that the residual pneumonia after ampicillin treatment was due to organisms susceptible to tylosin tartrate but not ampicillin. Of the organisms isolated from the lungs, the ones in the latter category most likely to be responsible were M. dispar, isolated from 93%,

and ureaplasmas, isolated from 65% of the 58 pneumonic lungs from all groups of experimental calves. The bacteria present in the LH and recovered from the lungs of inoculated calves comprised a variety of species, without any one species predominating. The authors concluded that "the rôle of the many different species of bacteria isolated from the lungs appears to be that of opportune invaders which are nevertheless frequently of great importance in determining the severity of the disease". The situation in sheep would appear to be very similar, except that in the U.K. one bacterium predominates, namely P. haemolytica. Alley (1975) has remarked "that bacteria from the nasal cavity may contribute actively to the severity of the lesions in chronic pneumonia, but it is unlikely that they are responsible for initiating the disease process".

The importance of P.E pneumonia in sheep production

Quantitative assessment of the prevalence of P.E. pneumonia and its effect on weight gains and growth rates of lambs has only recently been attempted. Kirton, O'Hara, Shortridge and Cordes (1976) in New Zealand examined the lungs of 3243 conventionally-reared lambs at slaughter over a five year period. The flock from which the lambs derived had no history of clinical pneumonia. The lungs were graded 1 - 6 according to absence or presence of pneumonia of various types. There was an overall prevalence of pneumonia of 60%: 39.5% had no discernible lesions in the lungs (Grade 1); 20% had mild pneumonia consisting of small discrete areas of consolidation (Grade 2); 6% had moderate pneumonia,

with less than one third of the lung involved (Grade 3); 0.5% had severe pneumonia, with more than one third of the lung showing consolidation (Grade 4); 24% had atelectasis without evidence of active pneumonia; and 10% had verminous pneumonia due to Dictyocaulus sp. The pneumonia seen in Grades 2 - 4 was characterised histologically by some or all of the features - bronchial epithelial hyperplasia, peribronchial lymphoid hyperplasia and purulent bronchopneumonia. Mild pneumonia (Grade 2), atelectasis and verminous pneumonia were not associated with depressed carcass weights, but lambs with Grades 3 and 4 pneumonia had weights which were lower, on average over the five seasons, by just over 0.45 Kg per carcass. Harris and Alley (1977) have questioned the conclusions drawn by Kirton et al. (1976) from this data. They contended that the "unaffected" (Grade 1) lambs in the survey may have suffered pneumonia at an earlier stage - possibly pre-weaning - but resolution of the lesions would disguise this fact at slaughter. This factor would depress the growth rate of the control lambs and obscure differences in growth rates between the control lambs and the different pneumonia categories. Kirton, O'Hara, Shortridge and Cordes (1977) defended this issue by pointing out that the incidence and severity of enzootic pneumonia increased as the season progressed, and that the disease was of greatest prevalence in the late summer, so that a low prevalence might be expected in pre-weaning lambs. This point is borne out by the findings of Thurley et al. (1977) and in Chapter 4, this thesis. Harris and Alley (1977) also contended that the organisms

involved in enzootic pneumonia are likely to have systemic effects on metabolism in addition to reducing functional lung area. This is not supported by the data presented by Kirton et al. (1976), as only the group with moderate to severe pneumonia showed significantly reduced growth rates.

The effects of pneumonia on growth rates and feed conversion efficiencies in other domestic animals has been recognised for some time, particularly in pigs, although estimates of the economic effects of enzootic of pigs vary considerably (Huhn, 1970a). Whether the extent of pneumonic lesions in pigs correlates with the reduction in growth rates (Huhn, 1970b), or whether M. suipneumoniae also has some metabolic effect in addition to causation of pneumonia is uncertain (Goodwin, 1971). In beef cattle, respiratory disorders have been found to be the biggest single cause of production loss (Thomas, Wood and Longland, 1978). More importantly, it has been stressed that depression of the efficiency of food utilization, the marked variability in growth rates and production of a general debilitating effect by pneumonia can largely be overcome by good management (Huhn, 1970b; Goodwin, 1971; Thomas et al., 1978). The importance of "stress" factors in increasing the severity of chronic, low-grade mycoplasma infections is well recognised (Adler, 1965). The sheep flocks surveyed by Leach et al. (1976) and Kirton et al. (1976) were both maintained under good systems of husbandry, and the prevalence of pneumonia in these flocks was low. In contrast, the conditions of housing in the flock surveyed during these studies were poor, and a high

proportion of lambs at slaughter had lesions of P.E. pneumonia. It would seem therefore, that the effectiveness of M.ovipneumoniae and P. haemolytica as pathogens in the natural situation is largely dependent on management factors affecting the well-being of the animals at risk.

In conclusion, it is apparent that M. ovipneumoniae and M. arginini are common inhabitants of the respiratory tract of sheep. The pathogenic effects of M. ovipneumoniae per se are mild, and may be negligible in the natural situation if conditions of animal management are good. The combination of M. ovipneumoniae and bacteria, notably P. haemolytica, is generally found in P.E. pneumonia, but further investigations in SPF or gnotobiotic lambs are necessary to elucidate the rôles of both organisms in the disease, and the mechanisms of potentiation by the mycoplasm of the bacterium. M. arginini on its own does not appear to be pathogenic, and the frequent recovery of the organism from pneumonic lungs would therefore appear to be due to its secondary invasion from a site in the upper respiratory tract, probably tonsillar tissue. However, the possibility that M. arginini may enhance the invasiveness of P. haemolytica requires further study. The importance and prevalence of P.E. pneumonia are unknown quantities at present, but comparison with similar diseases of other domestic animals suggests that chronic pneumonia of sheep may be an important cause of economic loss, particularly where intensive or semi-intensive systems of management are practised.

## REFERENCES


AARSLEFF, B., BIBERSTEIN, E.L., SHREEVE, B.J. and THOMPSON, D.A. (1970) A study of untypable strains of Pasteurella haemolytica. Journal of Comparative Pathology 80 493-498

ABU-ZAHR, M.N. and BUTLER, M. (1976) Growth, cytopathogenicity and morphology of Mycoplasma gallisepticum and M. gallinarum in tracheal explants. Journal of Comparative Pathology 86 455-463

ADEGBOYE, D.S. (1975) Immune mechanisms in Mycoplasma suipneumoniae infection of pigs with special reference to cell-mediated immunity. Ph.D. thesis, University of Cambridge.

ADLER, H.E. (1965) Mycoplasmosis in animals. Advances in Veterinary Science 10 205-244 Eds. C.A. Brandly and C. Cornelius. New York Academic Press, New York

AKTAN, M. (1956) /Ïsolation of microorganisms of the pleuro-pneumonia group from lungs of lambs/ Türk Ijiyen ve Tecrübi Biyoloji Dergisi 16 170-178

AKTAN, M., GÜLEY, M. AND DOGUER, M. (1955) /Äbortion in sheep following immunization with contaminated sheep pox vaccine/ Türk Veteriner Hekimleri Dernegi Dergisi 25 2463-2477 Veterinary Bulletin (1956) 26 2598

AL-AUBAIDI, J.M., DARDIRI, A.H. and FABRICANT, J. (1972) Biochemical characterization and antigenic relationship of Mycoplasma mycoides subsp. mycoides Freundt and Mycoplasma mycoides subsp. capri (Edward) Freundt. International Journal of Systematic Bacteriology 22 155-164

AL-AUBAIDI, J.M., TAYLOR, W.D., BUBASH, G.R. and DARDIRI, A.H. (1972) Identification and characterization of Mycoplasma arginini from Bighorn sheep (Ovis canadensis) and goats. American Journal of Veterinary Research 33 87-90

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

ALLEY, M.R. and CLARKE, J.K. (1977) The influence of micro-organisms on the severity of lesions in chronic ovine pneumonia. New Zealand Veterinary Journal 25 200-202

ALLEY, M.R. and MANKTELOW, B.W. (1971) Alveolar epithelialisation in ovine pneumonia. Journal of Pathology 103 219-224

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

ALONSO, A.S. (1964) Estudio serologico del genero mycoplasma Revista del Patronato de Biologia Animal 4 33-91

ALUOTTO, B.B., WITTLER, R.G., WILLIAMS, C.O. and FABER, J.E. (1970) Standardized bacteriologic techniques for the characterization of Mycoplasma species. International Journal of Systematic Bacteriology. 20 35-58

ANDERSON, D.P., WOLFE, R.R., CHERMS, F.L. and ROPER, W.E. (1968) Influence of dust and ammonia on the development of air sac lesions in turkeys. American Journal of Veterinary Research 29 1049-1058

ANDERSON, D.R. (1969) Ultrastructural studies of mycoplasmas and the L-phase of bacteria. In The Mycoplasmatales and the L-phase of Bacteria, pp. 365-402 Ed. L. Hayflick. Appleton-Century-Crofts, New York

ANDREWS, B.E., LEACH, R.H., GOURLAY, R.N. and HOWARD, C.J. (1973) Enhanced isolation of Mycoplasma dispar by substitution of ampicillin for benzylpenicillin in growth media. Veterinary Record 93 603

ARAI, S., HINUMA, Y., MATSUMOTO, K. and NAKAMURA, T. (1971) Delayed type hypersensitivity in hamsters infected with Mycoplasma pneumoniae as revealed by macrophage migration inhibition test. Japanese Journal of Microbiology 15 509-514

ARCHETTI, I. and HORSFALL, F.L., Jr. (1950) Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. Journal of Experimental Medicine 92 441-462

ARISOY, F., ERDAG, O., COTTEW, G.S. and WATSON, W.A. (1967) Investigations into contagious agalactia of sheep and goats in Turkey. Türk Veteriner Hekimleri Dernegi Dergisi 37 11-17

BARB, K., FARKAS, E., ROMVARY, J. and TAKATSY, G. (1962) Comparative study of influenza virus strains isolated from domestic animals in Hungary. Acta Virologica, Prague 6 207-213
Veterinary Bulletin (1962) 32 3401

BARBER, T.L. and FABRICANT, J. (1962) Primary isolation of Mycoplasma organisms (PPLO) from mammalian sources. Journal of Bacteriology 83 1268-1273

BARILE, M.F., DEL GIUDICE, R.A., CARSKI, T.R., GIBBS, C.J. and MORRIS, J.A. (1968) Isolation and characterization of Mycoplasma arginini: spec. nov. Proceedings of the Society for Experimental Biology and Medicine 129 489-494

BARILE, M.F., DEL GIUDICE, R.A. and TULLY, J.G. (1972) Isolation and characterization of Mycoplasma conjunctivae sp. n. from sheep and goats with keratoconjunctivitis. Infection and Immunity 5 70-76

BARILE, M.F. and KERN, J. (1971) Isolation of Mycoplasma arginini from commercial bovine sera and its implication in contaminated cell cultures. Proceedings of the Society for Experimental Biology and Medicine 138 432-437

BARILE, M.F. and LEVENTHAL, B.G. (1968) Possible mechanism for mycoplasma inhibition of lymphocyte transformation induced by phytohaemagglutinin. Nature, London 219 751-752

BASKERVILLE, A. (1972) Development of the early lesions in experimental enzootic pneumonia of pigs: an ultrastructural and histological study. Research in Veterinary Science 13 570-578

BASKERVILLE, A., WRIGHT, C.L., MENEELY, J.D. and CURRAN, W.L. (1972) The ultrastructure of Mycoplasma hyorhinis in broth culture. Research in Veterinary Science 13 497-499

BELAK, S. and PALFI, V. (1974a) Characterization of an ovine strain of parainfluenza - 3 virus isolated from sheep showing respiratory disease. Acta Veterinaria Academiae Scientiarum Hungaricae 24 281-285

BELAK, S. and PALFI, V. (1974b) Pneumoenteritis of lambs caused by adenovirus. Acta Veterinaria Academiae Scientiarum Hungaricae 24 327-328

BELAK, S. and PALFI, V. (1974c) Isolation of reovirus type 1 from lambs showing respiratory and intestinal symptoms. Archiv für die Gesamte Virus-forschung 44 177-183

BELAK, S. and PALFI, V. (1974d) Experimental infection of lambs with reovirus type 1 isolated from sheep. Acta Veterinaria Academiae Scientiarum Hungaricae 24 241-247

BERTHIAUME, L., JONCAS, J., BOULAY, G. and PAVILANIS, V. (1973) Serological evidence of respiratory syncytial virus infection in sheep. Veterinary Record 93 337-338

BIBERFELD, G. (1968) Distribution of antibodies within 19S and 7S immunoglobulins following infection with Mycoplasma pneumoniae. Journal of Immunology 100 338-347

BIBERFELD, G. (1971) Antibodies to brain and other tissues in cases of Mycoplasma pneumoniae infection. Clinical and Experimental Immunology 8 319-333

BIBERFELD, G. (1973) Macrophage migration inhibition in response to experimental Mycoplasma pneumoniae infection in the hamster. Journal of Immunology 110 1146-1150

BIBERFELD, G. (1974) Cell-mediated immune response following Mycoplasma pneumoniae infection in man. II Leucocyte migration inhibition. Clinical and Experimental Immunology 17 43-49

BIBERFELD, G. and BIBERFELD, P. (1970) Ultrastructural features of Mycoplasma pneumoniae. Journal of Bacteriology 102 855-861

BIBERFELD, G., BIBERFELD, P. and STERNER, G. (1974) Cell-mediated immune response following Mycoplasma pneumoniae infection in man. I Lymphocyte stimulation. Clinical and Experimental Immunology 17 29-41

BIBERSTEIN, E.L. and GILLS, M. (1962) The relation of the antigenic types to the A and T types of Pasteurella haemolytica. Journal of Comparative Pathology and Therapeutics 72 316-320

BIBERSTEIN, E.L., GILLS, M. and KNIGHT, H. (1960) Serological types of Pasteurella haemolytica. Cornell Veterinarian 50 283-300

BIBERSTEIN, E.L., NISBET, D.I. and THOMPSON, D.A. (1967) Experimental pneumonia in sheep. Journal of Comparative Pathology 77 181-192

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

BIBERSTEIN, E.L., SHREEVE, B.J. and THOMPSON, D.A. (1970) Variation in carrier rates of Pasteurella haemolytica in sheep flocks. I Normal flocks. Journal of Comparative Pathology 80 499-507

BIBERSTEIN, E.L. and THOMPSON, D.A. (1966) Epidemiological studies on Pasteurella haemolytica in sheep. Journal of Comparative Pathology 76 83-94

BLACK, F.T., BIRCH-ANDERSEN, A. and FREUNDT, E.A. (1972) Morphology and ultrastructure of human T-mycoplasmas. Journal of Bacteriology 111 254-259

BLACK, F.T. and VINTHER, O. (1977) Morphology and ultrastructure of Ureaplasma urealyticum in agar growth. Acta Pathologica et Microbiologica Scandinavica; Sect. B 85 281-285

BLACKMORE, D.K. and HILL, A. (1973) The experimental transmission of various mycoplasmas of feline origin to domestic cats (Felis catus). Journal of Small Animal Practice 14 7-13

BOIDIN, A.G., CORDY, D.R. and ADLER, H.E. (1958) A pleuropneumonialike organism and a virus in ovine pneumonia in California. Cornell Veterinarian 48 410-430

BREDT, W. (1976) Pathogenicity factors of mycoplasmas. Infection 4 Suppl. 1 S9-S12

BREDT, W. and BITTER-SUERMAN, D. (1975) Interactions between Mycoplasma pneumoniae and guinea pig complement. Infection and Immunity 11 497-504

BRENNAN, P.C., FRITZ, T.E. and FLYNN, R.J. (1969) Role of Pasteurella pneumotropica and Mycoplasma pulmonis in murine pneumonia. Journal of Bacteriology. 97 337-349

BRODERSON, J.R., LINDSEY, J.R. and CRAWFORD, J.E. (1976) The role of environmental ammonia in respiratory mycoplasmosis of rats. American Journal of Pathology 85 115-130

BROTHERSTON, J.G. (1968) Report of the Animal Diseases Research Association, Edinburgh. 1967-68. p. 26

BRUNNER, H. (1976) Pathogenitätsmechanismen von Mycoplasmen Zentralblatt für Bakteriologie, Parasitenkunde Infektionskrankheiten und Hygiene, 1. Abt. Orig. A 235 122-133

BRUNNER, H., JAMES, W.D., HORSWOOD, R.L. and CHANOCK, R.M. (1972) Measurement of Mycoplasma pneumoniae mycoplasmacidal antibody in human serum. Journal of Immunology 108 1491-1498

BRUNNER, H., PRESCOTT, B., GREENBERG, H., JAMES, W.D., HORSWOOD, R.L. and CHANOCK, R.M. (1977) Unexpectedly high frequency of antibody to Mycoplasma pneumoniae in human sera as measured by sensitive techniques. Journal of Infectious Diseases 135 524-530

BUTTERY, S.H., LLOYD, L.C. and TITCHEN, D.A. (1976) Acute respiratory, circulatory and pathological changes in the calf after intravenous injections of the galactan from Mycoplasma mycoides subsp. mycoides. Journal of Medical Microbiology 9 379-391

CALLEWAERT, D.M., KAPLAN, J., PETERSON, W.D., Jr. and LIGHTBODY, J.J. (1975) Suppression of lymphocyte activation by a factor produced by Mycoplasma arginini Journal of Immunology 115 1662-1664

CARLETON'S HISTOLOGICAL TECHNIQUE (1967) 4th ed. R.A.B. Drury and E.A Wallington, Oxford Medical Publications.

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

CARTER, M.E. and HUNTER, R. (1970) Isolation of parainfluenza type 3 virus from sheep in New Zealand. New Zealand Veterinary Journal 18 226-227

CELLO, R.M. (1967) Ocular infections in animals with PLT (Bedsonia) group agents. American Journal of Ophthalmology 63 1270-1273

CHERRY, J.D. and TAYLOR-ROBINSON, D. (1973) Mycoplasma pathogenicity studies in organ cultures. Annals of the New York Academy of Sciences 225 290-303

CHU, H.P. and HORNE, R.W. (1967) Electron microscopy of Mycoplasma gallisepticum and Mycoplasma mycoides using the negative staining technique and their comparison with myxovirus. Annals of the New York Academy of Sciences 143 190-203

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

CLYDE, W.A., Jr. (1964) Mycoplasma species identification based upon growth inhibition by specific antisera. Journal of Immunology 92 958-965

CLYDE, W.A., Jr. (1971) Immunopathology of experimental Mycoplasma pneumoniae disease. Infection and Immunity 4 757-763

CLYDE, W.A., Jr. (1975) Pathogenic mechanisms in mycoplasma diseases. In Microbiology - 1975 pp. 143-146 Ed. D. Schlessinger. American Society for Microbiology, Washington, D.C.

COLE, B.C., CAHILL, J.F., WILEY, B.B. and WARD, J.R. (1969) Immunological responses of the rat to Mycoplasma arthritidis. Journal of Bacteriology 98 930-937

COLE, B.C., GOLIGHTLY-ROWLAND, L., WARD, J.R. and WILEY, B.B. (1970) Immunological response of rodents to murine mycoplasmas. Infection and Immunity 2 419-425

COLE, B.C. and WARD, J.R. (1973) Detection and characterization of defective mycoplasmacidal antibody produced by rodents against Mycoplasma arthritidis Infection and Immunity 8 199-207

COLE, B.C., WARD, J.R. and MARTIN, C.H. (1968) Haemolysin and peroxide activity of Mycoplasma species. Journal of Bacteriology 95 2022-2030

COLEMAN, L.H. and LYNN, R.J. (1972) Immune inhibition of Mycoplasma pneumoniae. Proceedings of the Society for Experimental Biology and Medicine 140 383-386

COLLIER, A.M. (1972) Pathogenesis of Mycoplasma pneumoniae infection as studied in the human foetal trachea in organ culture. In Pathogenic Mycoplasmas pp. 307-320. Associated Scientific Publishers, Amsterdam.

COLLIER, A.M. and CLYDE, W.A., Jr. (1971) Relationships between Mycoplasma pneumoniae and human respiratory epithelium. Infection and Immunity 3 694-701

COLLIER, A.M., CLYDE, W.A., Jr. and DENNY, F.W. (1971) Mycoplasma pneumoniae in hamster tracheal organ culture: immunofluorescent and electron microscopic studies. Proceedings of the Society for Experimental Biology and Medicine 136 569-573

COLUSI, A.D., GARBINI, J., SEQUEIRA, A. and DELPIETRO, H. (1964) Nueva enfermedad infecciosa en ovinos, neumonia enzootica, debida un organismo del genero Mycoplasma: primera comprobacion en la Rep. Argentina. G aceta Veterinaria 26 205-227

CONTINI, A. (1960) Morphological and cultural characters of pleuropneumonia-like organisms from sheep. Veterinaria Italiana 11 280-292

CORDY, D.R., ADLER, H.E. and YAMAMOTO, R. (1955) A pathogenic pleuropneumonia-like organism from goats. Cornell Veterinarian 45 50-68

COTTEW, G.S. (1971a) Characterisation of mycoplasmas isolated from sheep with pneumonia. Australian Veterinary Journal 47 591-596

COTTEW, G.S. (1971b) Mycoplasmas of sheep. Victorian Veterinary Proceedings 29 45

COTTEW, G.S. (1974) The mycoplasmas of sheep and goats. INSERM 33 357-362

COTTEW, G.S. and LEACH, R.H. (1969) Mycoplasmas of cattle, sheep and goats. In the Mycoplasmatales and the L-phase of bacteria. pp. 527-570 Ed. L. Hayflick. Appleton-Century-Crofts, New York

COTTEW, G.S. and LLOYD, L.C. (1965) An outbreak of pleurisy and pneumonia in goats in Australia attributed to a Mycoplasma species. Journal of Comparative Pathology 75 363-374

COTTEW, G.S., WATSON, W.A., ARISOY, F., ERDAG, O. and BUCKLEY, L.S. (1968) Differentiation of Mycoplasma agalactiae from other mycoplasmas of sheep and goats. Journal of Comparative Pathology 78 275-282

COUCH, R.B. (1969) Diagnosis and treatment of M. pneumoniae disease in man. In The Mycoplasmatales and the L-phase of Bacteria. pp. 683-695. Ed. L. Hayflick. Appleton-Century-Crofts, New York

COUCH, R.B., CATE, T.R. and CHANOCK, R.M. (1964) Infection with artificially propagated Eaton agent (Mycoplasma pneumoniae). Implications for development of attenuated vaccine for cold agglutinin-positive pneumonia. Journal of the American Medical Association 187 442-447

DAJANI, A.S., CLYDE, W.A., Jr. and DENNY, F.W. (1965) Experimental infection with Mycoplasma pneumoniae (Eaton's agent). Journal of Experimental Medicine 121 1071-1086

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DAVIES, G. and WANDERA, J.G. (1967) Unpublished observations cited by Wandera, J.G. (1968) Veterinary Record 83 478-482

DEEB, B.J. and KENNY, G.E. (1967) Characterization of Mycoplasma pulmonis variants isolated from rabbits. II Basis for differentiation of antigenic subtypes. Journal of Bacteriology 93 1425-1429

DEIANA, S. and CERETTO, F. (1967) Reperti istoimmunologici nella adenomatosi polmonare enzootica degli ovini in Sardegna. Atti della Societa Italiana delle Scienze Veterinarie 21 612-615

DENNY, F.W., TAYLOR-ROBINSON, D. and ALLISON, A.C. (1972) The rôle of thymus-dependent immunity in Mycoplasma pulmonis infections of mice. Journal of Medical Microbiology 5 327-336

DIGHERO, M.W., BRADSTREET, C.M.P. and ANDREWS, B.E. (1970) Dried paper discs for serological identification of human mycoplasmas. Journal of Applied Bacteriology 33 750-757

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

DOIG, P.A. and RUHNKE, H.L. (1977) Isolation of ureaplasma from sheep with granular vulvitis. Veterinary Record 100 179-180

DOMERMUTH, C.H., NIELSEN, M.H., FREUNDT, E.A. and BIRCH-ANDERSEN, A. (1964) Ultrastructure of Mycoplasma species. Journal of Bacteriology 88 727-744

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

DUNGWORTH, D.L. (1963) Enzootic ovine pneumonia (a situation report) Proceedings of the 67th Annual Meeting of the U.S. Livestock Sanitary Association, New Mexico, pp. 301-304

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

DURISIC, S. and VRBANAC, I. (1969) /Mycoplasma infection in pigs. V. Role of carrier sows in transmission of infection to their litters/ Veterinarski Glasnik 23 439-444. Veterinary Bulletin (1970) 40 494

DURUSAN, R. and DOGUER, M. (1955) /Cultivation of pleuropneumonia organisms from lung lesions in lambs./ Türk Veteriner Hekimleri Dernegi Dergisi 25 2217-2230

EDWARD, D.G.ff (1971) Determination of sterol requirement for Mycoplasmatales. Journal of General Microbiology 69 205-210

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

EDWARD, D.G.ff and FREUNDT, E.A. (1970) Amended nomenclature for strains related to Mycoplasma laidlawii Journal of General Microbiology 62 1 - 2

ERDAG, O. (1972) /The significance of Mycoplasmas in lamb pneumonia/ Pendik Veteriner Kontrol ve Arastirma Enstitüsü Dergisi 5 15-20. Veterinary Bulletin (1973) 43 3309

ERNØ, H., FREUNDT, E.A., KROGSGAARD-JENSEN, A. and ROSENDAL, S. (1972) The identification of an organism isolated from maned sheep as Mycoplasma mycoides subsp. mycoides. Acta Veterinaria Scandinavica 13 263-265

ERNØ, H. and STIPKOVITS, L. (1973) Bovine mycoplasmas: cultural and biochemical studies. I. Acta Veterinaria Scandinavica 14 436-449

ESTOLA, T. and SALMELA, E. (1970) /Occurrence of Chlamydia infections in Finnish sheep. II. Antibodies in 18 flocks/. Finsk Veterinärtidskrift 76 431-436. Veterinary Bulletin (1971) 41 1681

FABRICANT, J. (1969) Avian mycoplasmas. In The Mycoplasmatales and L-phase of Bacteria, pp. 621-641. Ed. L. Hayflick. Appleton-Century-Crofts, New York

FABRICANT, J. and FREUNDT, E.A. (1967) Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasmas. Annals of the New York Academy of Sciences 143 50-58

FAHEY, J.L. and MCKELVEY, E.M. (1965) Quantitative determination of serum immunoglobulins in antibody-agar plates Journal of Immunology 94 84-90

FAO/WHO PROGRAMME ON COMPARATIVE VIROLOGY (1974) FAO/WHO programme on comparative mycoplasmology. Veterinary Record 95 457-461

FARZALIEV, M.M. KHALIMBEKOV, M.M. DANDAMAEV, S.G. and ALIEV, A.I. (1962) /Pneumonia in lambs: the rôle of PPLO/. Veterinariya 39 27-30. Veterinary Bulletin (1962) 32 2580

FERNALD, G.W. (1969) Immunologic aspects of experimental Mycoplasma pneumoniae infection. Journal of Infectious Diseases 119 255-266

FERNALD, G.W. and CLYDE, W.A., Jr. (1976) Pulmonary immune mechanisms in Mycoplasma pneumoniae disease. In Immunologic and Infectious Reactions in the Lung. pp. 101-130. Eds. C.H. Kirkpatrick and H.Y. Reynolds. Marcel Dekker, Inc., New York

FERNALD, G.W., CLYDE, W.A., Jr. and DENNY, F.W. (1967) Nature of the immune response to Mycoplasma pneumoniae. Journal of Immunology 98 1028-1038

FERNALD, G.W., COLLIER, A.M. and CLYDE, W.A., Jr. (1975) Respiratory infections due to Mycoplasma pneumoniae in infants and children. Pediatrics 55 327-335

FISKE, P.J. (1974) Auxiliary cylindrical lenses for recording bands in narrow tubes. Medical and Biological Illustration 24 160-161

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

FORSHAW, K.A. (1972)Electrophoretic patterns of strains of Mycoplasma pulmonis. Journal of General Microbiology 72 493-499

FORSHAW, K.A. and FALLON, R.J. (1972) Serological heterogeneity of Mycoplasma pulmonis. Journal of General Microbiology 72 501-510

FRASER, C.E.O. and BERMAN, D.T. (1965) Type-specific antigens in the psittacosis-lymphogranuloma venereum group of organisms. Journal of Bacteriology 89 943-948

FREUNDT, E.A. (1955)The classification of the pleuropneumonia group of organisms (Borrelomycetales) International Bulletin of Bacteriological Nomenclature and Taxonomy 5 67-78

FREUNDT, E.A. (1969)Cellular morphology and mode of replication of the mycoplasmas. In The Mycoplasmatales and the L-phase of Bacteria. pp. 281-315. Ed. L. Hayflick. Appleton-Century-Crofts, New York

FREUNDT, E.A. (1974)The Mycoplasmatales pp. 929-955. In Bergey's Manual of Determinative Bacteriology 8th Edition. Eds. R.F. Buchanan and N.E. Gibbons. The Williams and Wilkins Co., Baltimore

FREUNDT, E.A. (1974a) Present status of the medical importance of mycoplasmas. Pathologia et Microbiologia 40 155-187

FREUNDT, E.A., ANDREWS, B.E., ERNØ, H., KUNZE, M. and BLACK, F.T. (1973) The sensitivity of Mycoplasmatales to sodium-polyanethol-sulfonate and digitonin. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene: I Abt. Orig. A 225 104-112

FREUNDT, E.A., ERNØ, H., BLACK, F.T., KROGSGAARD-JENSEN, A. and ROSENDAL, S. (1973) Evaluation of reference reagents for mycoplasmas. Annals of the New York Academy of Sciences 225 161-171

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

FURLONG, S.L. and COTTEW, G.S. (1973) Identity of mycoplasmas from sheep pneumonia in Queensland and Victoria. Australian Veterinary Journal 49 215-216

FURNESS, G. (1973) T-mycoplasmas: some factors affecting their growth, colonial morphology, and assay on agar Journal of Infectious Diseases 128 703-709

GABRIDGE, M.G., JOHNSON, C.K. and CAMERON, A.M. (1974) Cytotoxicity of Mycoplasma pneumoniae membranes. Infection and Immunity 10 1127-1134

GALLI, G. and LEACH, R.H. (1976) /Ī/solation and identification of Mycoplasma arginini from swine with lung lesions. Preliminary studies/7 Clinica Veterinaria 99 597-598. Veterinary Bulletin (1977) 47 3040

GHAZIKHANIAN, G. and YAMAMOTO, R. (1974) Characterization of pathogenic and nonpathogenic strains of Mycoplasma meleagridis: in ovo and in vitro studies. American Journal of Veterinary Research 35 425-430

GIAUFFRET, A. and RUSSO, P. (1972) /Ī/solation of a virus in a respiratory infection of lambs/7. Bulletin de l'Academie Veterinaire de France 45 207-213. Veterinary Bulletin (1973) 43 1097

GIAUFFRET, A. and RUSSO, P. (1974) /S/tudy of a virus isolated from a pneumonic lesion in a lamb/7. Recueil de Medecine Veterinaire 150 801-806. Veterinary Bulletin (1975) 45 2413

GILMOUR, N.J.L. and BROTHERSTON, J.G. (1963) Clinical and pathological findings in an outbreak of pneumonia in sheep. Journal of Comparative Pathology and Therapeutics 73 329-335

GILMOUR, N.J.L., THOMPSON, D.A. and FRASER, J. (1974) The recovery of Pasteurella haemolytica from the tonsils of adult sheep. Research in Veterinary Science 17 413-414

GILMOUR, N.J.L., THOMPSON, D.A., SMITH, W.D. and ANGUS, K.W. (1975) Experimental infection of lambs with an aerosol of Pasteurella haemolytica. Research in Veterinary Science 18 340-341

GOIS, M., KUKSA, F., FRANZ, J. and TAYLOR-ROBINSON, D. (1974) The antigenic differentiation of seven strains of Mycoplasma hyorhinis by growth-inhibition, metabolism-inhibition, latex-agglutination, and polyacrylamide-gel-electrophoresis tests. Journal of Medical Microbiology 7 105-115

GOIS, M., KUKSA, F. and SISAK, F. (1977) Experimental infection of gnotobiotic piglets with Mycoplasma hyorhinis and Bordetella bronchiseptica. Zentralblatt für Veterinärmedizin B 24 89-96

GOODWIN, R.F.W. (1971) The economics of enzootic pneumonia. Veterinary Record 89 77-81

GOODWIN, R.F.W., HODGSON, R.G., WHITTLESTONE, P. and WOODHAMS, R.L. (1969) Immunity in experimentally induced enzootic pneumonia of pigs. Journal of Hygiene, Cambridge. 67 193-208

GOODWIN, R.F.W. and HURRELL, J.M.W. (1970) Further observations on the problem of isolating Mycoplasma suipneumoniae from field cases of enzootic pneumonia in pigs. Journal of Hygiene, Cambridge 68 313-325

GOURLAY, R.N., BROWNLIE, J. and HOWARD, C.J. (1973) Isolation of T-mycoplasmas from goats, and the production of subclinical mastitis in goats by the intramammary inoculation of human T-mycoplasmas. Journal of General Microbiology 76 251-254

GOURLAY, R.N., HOWARD, C.J., THOMAS, L.H. and STOTT, E.J. (1976) Experimentally produced calf pneumonia. Research in Veterinary Science 20 167-173

GOURLAY, R.N. and LEACH, R.H. (1970) A new mycoplasma species isolated from pneumonic lungs of calves (Mycoplasma dispar sp. nov.) Journal of Medical Microbiology 3 111-123

GOURLAY, R.N., MACKENZIE, A. and COOPER, J.E. (1970) Studies on the microbiology and pathology of pneumonic lungs of calves. Journal of Comparative Pathology 80 575-584

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

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

GREENBURY, C.L., MOORE, D.H. and NUNN, L.A.C. (1963) Reaction of 7S and 19S components of immune rabbit antisera with human group A and AB red cells. Immunology 6 421-433

GREIG, A.S. (1955) The isolation of pleuropneumonia-like organisms from the respiratory tracts of sheep. Canadian Journal of Comparative Medicine 19 265-271

HALLER, G.J., BOIARSKI, K.W. and SOMERSON, N.L. (1973) Comparative serology of Mycoplasma pulmonis. Journal of Infectious Diseases 127 Suppl. S38-S42.

HAMDY, A.H. (1958) Observations on respiratory disease agents in lambs. Ph.D. dissertation, Ohio State University, Columbus. Dissertation Abstracts (1958) 19 210

HAMDY, A.H. and POUNDEN, W.D. (1959) Experimental production of pneumonia in lambs. American Journal of Veterinary Research 20 78-83

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

HANKO, E. and OTTERLIN, S.-E. (1955) On an outbreak of PPLO infection in goats and sheep in Sweden. Nordisk Veterinaermedicin 7 609-624

HARRIS, G. (1974) An outbreak of pasteurellosis in lambs. Veterinary Record 94 84-85

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

HART, R., MACKAY, J.M.K., McVITTIE, C.R. and MELLOR, D.J. (1971) A technique for the derivation of lambs by hysterectomy. British Veterinary Journal 127 419-424

HEIKKILA, I. (1956) Pleuropneumonie infectieuse des moutons. Bulletin de l'Office International des Epizooties 46 585-590

HERBERT, W.J. (1967) Passive haemagglutination. In Handbook of Experimental Immunology pp. 720-744. 1st Edition. Ed. D.M.Weir. Blackwell Scientific Publications. Oxford and Edinburgh.

HEYWARD, J.T., SABRY, M.Z. and DOWDLE, W.R. (1969) Characterization of Mycoplasma species of feline origin. American Journal of Veterinary Research 30 615-622

HILL, L.R., LEACH, R.H. and ANDREWS, B.E. (1970) Reports from the Conference on the Taxonomy of Bacteria (Brno, 1969) Publications of the Faculty of Sciences, J.E. Purkyne University, Brno (Series K) 47 79

HOLLINGDALE, M.R. and LEMCKE, R.M. (1969) The antigens of Mycoplasma hominis. Journal of Hygiene, Cambridge 67 585-602

HOLLINGDALE, M.R. and LEMCKE, R.M. (1970) Antigenic differences within the species Mycoplasma hominis. Journal of Hygiene, Cambridge 68 469-477

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

HORE, D.E. (1968) Aspects of the pathogenesis of pneumonia in sheep. Ph.D. thesis, University of Edinburgh.

HORE, D.E. (1969) A survey of sheep sera for antibodies to an ovine strain of parainfluenza 3 virus. British Veterinary Journal 125 311-316

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

HORE, D.E. and STEVENSON, R.G. (1967) Experimental virus pneumonia in lambs. Veterinary Record 80 26-27

HORE, D.E. and STEVENSON, R.G. (1969) Respiratory infection of lambs with an ovine strain of parainfluenza virus type 3. Research in Veterinary Science 10 342-350

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

HOWARD, C.J., GOURLAY, R.N., THOMAS, L.H. and STOTT, E.J. (1976) Induction of pneumonia in gnotobiotic calves following inoculation of Mycoplasma dispar and ureaplasmas (T-mycoplasmas) Research in Veterinary Science 21 227-231

HOWARD, C.J. and THOMAS, L.H. (1974) Inhibition by Mycoplasma dispar of ciliary activity in tracheal organ cultures. Infection and Immunity 10 405-408

HU, P.C., COLLIER, A.M. and BASEMAN, J.B. (1975) Alterations in the metabolism of hamster tracheas in organ culture after infection by virulent Mycoplasma pneumoniae. Infection and Immunity 11 704-710

HU, P.C., COLLIER, A.M. and BASEMAN, J.B. (1976) Interaction of virulent Mycoplasma pneumoniae with hamster tracheal organ cultures. Infection and Immunity 14 217-224

HUDSON, J.R. (1959)Pasteurellosis. In Infectious Diseases of Animals. Diseases Due to Bacteria. pp. 413-436. Eds. A W. Stableforth and I.A. Galloway. Butterworths Scientific Publications, London.

HUDSON, J.R., COTTEW, G.S. and ADLER, H.E. (1967) Diseases of goats caused by mycoplasma: a review of the subject with some new findings. Annals of the New York Academy of Sciences 143 287-297

HUHN, R.G. (1970a) Enzootic pneumonia of pigs: a review of the literature. Veterinary Bulletin 40 249-257

HUHN, R.G. (1970b) Swine enzootic pneumonia: incidence and effect on rate of body weight gain. American Journal of Veterinary Research 31 1097-1108

HUMMELER, K., TOMASSINI, N. and HAYFLICK, L. (1965) Ultrastructure of a mycoplasma (Negroni) isolated from human leukemia. Journal of Bacteriology 90 517-523

IBRAHIM, A.A. and YAMAMOTO, R. (1977) Arginine catabolism by Mycoplasma meleagridis and its role in pathogenesis. Infection and Immunity 18 226-229

JENSEN, R. (1974) Diseases of the respiratory system. In Diseases of Sheep, pp. 171-181. Lea and Febiger, Philadelphia.

JONAS, W.E. (1969) The distribution and properties of IgM in some body fluids of sheep. Research in Veterinary Science 10 83-92

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

JONES, G.E., FOGGIE, A., SUTHERLAND, A. and HARKER, D.B. (1976a) Mycoplasmas and ovine keratoconjunctivitis. Veterinary Record 99 137-141

KAKLAMANIS, E., THOMAS, L., STAVROPOULOS, K., BORMAN, I. and BOSHWITZ, C. (1969) Mycoplasmacidal action of normal tissue extracts. Nature, London 221 860-862

KENNY, G.E. and POLLOCK, M.E. (1963) Mammalian cell cultures contaminated with pleuropneumonia-like organisms. I Effect of pleuropneumonia-like organisms on growth of established cell strains. Journal of Infectious Diseases 112 7-16

KHAN, A.Q. (1960) A note on the isolation of pleuropneumonia like organism from sheep in the Sudan. Sudan Journal of Veterinary Science and Animal Husbandry 1 72-73

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

KIRTON, A.H., O'HARA, P.J., SHORTRIDGE, E.H. and CORDES, D.O. (1977) Pneumonia in sheep: does it affect weight gain? New Zealand Veterinary Journal 25 195-196

KRAUSS, H. and WANDERA, J.G. (1970) Isolation and properties of mycoplasma from the respiratory tract of sheep with jaagsiekte in Kenya. Journal of Comparative Pathology 80 389-397

KRAUSS, H., WANDERA, J.G. and LAUERMAN, L.H., Jr. (1971) Isolation and identification of Chlamydia in Kenya sheep and serologic survey. American Journal of Veterinary Research 32 1433-1437

KRAYBILL, W.H. and CRAWFORD, Y.E. (1964) Tetrazolium and methylene blue for isolation and recognition of Mycoplasma pneumoniae. Bacteriological Proceedings, p. 70.

LAM, K.M. and SWITZER, W.P. (1972) Mycoplasmal pneumonia of swine: serologic response in pigs. American Journal of Veterinary Research 33 1329-1332

LANGFORD, E.V. (1971) Mycoplasma and associated bacteria isolated from ovine pink-eye. Canadian Journal of Comparative Medicine 35 18-21

LEACH, R.H. (1970) The occurrence of Mycoplasma arginini in several animal hosts. Veterinary Record 87 319-320

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

LILLIE, L.E. and THOMSON, R.G. (1972) The pulmonary clearance of bacteria by calves and mice. Canadian Journal of Comparative Medicine 36 129-137

LIN, J.-S.L., ALPERT, S. and RADNAY, K.M. (1975) Combined type-specific antisera in the identification of Mycoplasma hominis. Journal of Infectious Diseases 131 727-730

LIND. K. (1970) A simple test for peroxide secretion by mycoplasma. Acta Pathologica et Microbiologica Scandinavica Sect. B. 78 256-257

LINDLEY, E.P. and ABDULLA, A.E.D. (1969) Some notes on the host specificity of the etiological agents of contagious caprine pleuropneumonia and contagious bovine pleuropneumonia. Bulletin of Epizootic Diseases of Africa. 17 153-158

LINDSEY, J.R. and CASSELL, G.H. (1973) Experimental Mycoplasma pulmonis infection in pathogen-free mice: Models for studying mycoplasmosis of the respiratory tract. American Journal of Pathology 72 63-90

LIPMAN, R.P. and CLYDE, W.A., Jr. (1969) The interrelationship of virulence, cytadsorption and peroxide formation in Mycoplasma pneumoniae. Proceedings of the Society for Experimental Biology and Medicine 131 1163-1167

LIPMAN, R.P., CLYDE, W.A., Jr. and DENNY, F.W. (1969) Characteristics of virulent, attenuated and avirulent Mycoplasma pneumoniae strains. Journal of Bacteriology 100 1037-1043

LIU, C., JAYANETRA, P., VOTH, D.W., MUANGMANEE, L. and CHO, C.T. (1972) Potentiating effect of Mycoplasma pneumoniae infection on the development of pneumococcal septicaemia in hamsters. Journal of Infectious Diseases 125 603-612

LIVINGSTON, C.W., Jr. and GAUER, B.B. (1975) Isolation of T-strain mycoplasma from sheep and goats in Texas. American Journal of Veterinary Research 36 313-314

LIVINGSTON, C.W., Jr., STAIR, E.L., UNDERDAHL, N.R. and MEBUS, C.A. (1972) Pathogenesis of mycoplasmal pneumonia in swine. American Journal of Veterinary Research 33 2249-2258

LLOYD, L.C. and TRETHEWIE, E.R. (1970) Contagious bovine pleuropneumonia. In The Role of Mycoplasmas and L-forms of Bacteria in Disease. Ed. J.T. Sharp. C.C Thomas, Springfield, Illinois

LUFT, J.H. (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anatomical Record 171 347-368

MCFERRAN, J.B., BASKERVILLE, A. and NELSON, R. (1974) Experimental infection of sheep with a type 3 reovirus of ovine origin. Research in Veterinary Science 17 356-359

MCFERRAN, J.B., NELSON, R. and CLARKE, J.K. (1973) Isolation and characterisation of reoviruses isolated from sheep. Archiv für die Gesante Virusforschung 40 72-81

MCFERRAN, J.B., NELSON, R. and KNOX, E.R. (1971) Isolation and characterisation of sheep adenoviruses. Archiv für die Gesamte Virusforschung 35 232-241

MCFERRAN, J.B., NELSON, R., MCCRACKEN, J.M. and ROSS, J.G. (1969) Viruses isolated from sheep. Nature, London 221 194-195

MCILWAIN, P.K. and BOLIN, F.M. (1967) A mycoplasma associated with ovine orchitis. American Journal of Veterinary Research 28 885-887

MACKAY, J.M.K. (1965) Association of pleuropneumonia-like organisms (PPLO) with pulmonary adenomatosis in sheep - cultural and serological findings. In Lung Tumours in Animals: Proceedings of the 3rd Quadrennial International Conference on Cancer, University of Perugia, pp. 403-412

MACKAY, J.M.K. (1969) Tissue culture studies of sheep pulmonary adenomatosis (jaagsiekte). II Transmission of cytopathic effects to normal cultures. Journal of Comparative Pathology 79 147-154

MACKAY, J.M.K. and NISBET, D.I. (1966) Jaagsiekte - a hazard of intensified sheep husbandry. Veterinary Record 78 18-20

MACKAY, J.M.K., NISBET, D.I. and FOGGIE, A. (1963) Isolation of pleuropneumonia-like organisms (genus Mycoplasma) from cases of sheep pulmonary adenomatosis (S.P.A.) Veterinary Record. 75 550-551

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

MANCHEE, R.J. and TAYLOR-ROBINSON, D.H. (1969) Utilization of neuraminic acid receptors by mycoplasmas. Journal of Bacteriology 98 914-919

MANCHEE, R.J. and TAYLOR-ROBINSON, D.H. (1969) Enhanced growth of T-strain mycoplasmas with N-2-hydroxy-ethylpiperazine-N'-2 ethane-sulfonic acid buffer. Journal of Bacteriology 100 78-85

MANILOFF, J. (1972) Cytology of the mycoplasmas. In Pathogenic Mycoplasmas. pp. 67-87. Associated Scientific Publishers, Amsterdam

MANILOFF, J., MOROWITZ, H.J. and BARRNETT, R.J. (1965) Ultrastructure and ribosomes of Mycoplasma gallisepticum. Journal of Bacteriology. 90 193-204

MANKTELOW, B.W. (1970) The problem of pneumonia in sheep. Sheepfarming Annual, pp. 115-119

MARMION, B.P. (1967) The mycoplasmas: new information on their properties and their pathogenicity for man. In Recent Advances in Medical Microbiology. p. 170-253. Ed. A.P. Waterson, Churchill, London.

MASALSKI, N. (1971) /Respiratory disease of sheep associated with Mycoplasma/ Veterinarna Sbirka, Sofia 68 9-10. Veterinary Bulletin (1971) 41 4573

MEYER, K.F. (1967) The host spectrum of psittacosis-lymphogranuloma venereum (PL) agents. American Journal of Ophthalmology 63 1225-1246

MOHN, S.F. and UTKLEV, H.E. (1974) Pasteurella hemolytica and enzootic respiratory infections in sheep. Nordisk Veterinaer Medicin 26 658-669

MONTGOMERIE, R.F., BOSWORTH, T.J. and GLOVER, R.E. (1938) Enzootic pneumonia in sheep. Journal of Comparative Pathology and Therapeutics 51 87-107

MORTON, H.E. and ROBERTS, R.J. (1967) Production of anti-mycoplasma (PPLO) antibodies in rabbits. Proceedings of the Society for Experimental Biology and Medicine 125 538-543

NAYIL, A.A. (1971) Mycoplasmas isolated from the pneumonic lungs of sheep. Ph.D. thesis, University of Cambridge.

NEUVONEN, E. and ESTOLA, T. (1974) Occurrence of antibodies to group specific Chlamydia antigen in Finnish sheep, cattle and horse sera. Acta Veterinaria Scandinavica 15 256-263

NOBEL, T.A. (1958) Pulmonary adenomatosis (jaagsiekte) in sheep with special reference to its occurrence in Israel. Refuah Veterinarith 15 101-98

O'GAIRBHIDHE, C. (1965) An outbreak of pneumonia in a flock of sheep. Irish Veterinary Journal 19 26-28

OMORI, T., ISHII, S., HARADA, K., ISCHIKAWA, O., MURASE, N., KATADA, M. and ARAUMI, W. (1953) Study on an infectious pneumonia of goats caused by a virus. I. Isolation of the causative agent and characteristics. Experimental Report of the Government Experimental Station of Animal Hygiene, Tokyo 27 101-119

OTTE, E. (1960) Clinical studies on "Abu nini" in the Sudan: a contagious disease of goats and sheep, possibly caused by pleuropneumonia-like organisms. Veterinary Record 72 140-145

OUCHTERLONY, Ö. (1948) In vitro method for testing the toxic-producing capacity of diphtheria bacteria. Acta Pathologica et Microbiologica, Scandinavica 25 186-191

PAGE, L.A. (1966) Revision of the family Chlamydiaceae Rake (rickettsiales): Unification of the psitticosis-lymphogranuloma-venereum-trachoma group of organisms in the genus Chlamydia Jones Rake and Stearns, 1945. International Journal of Systematic Bacteriology 16 223-252

PAGE,L.A. (1968) Proposal for the recognition of two species in the genus Chlamydia Jones, Rake and Stearns, 1945. International Journal of Systematic Bacteriology 18 51-66

PAGE, L.A. (1974) Chlamydiales. pp. 914-918. In Bergey's Manual of Determinative Bacteriology 8th Edition. Eds. R.F. Buchanan and N.E. Gibbons. The Williams and Wilkins Co., Baltimore

PAGE, L.A. and ERICKSON, K. (1969) Serological evidence of natural and experimental transfers of Chlamydia psittaci between wild and domestic animals. Bulletin of the Wildlife Diseases Association 5 284-290

PARKER, W.H. (1968) Housing of ruminants. II. Requirements of good housing and effects of bad housing. Veterinary Record 83 364-369

PAUL, J. (1960) Cell and Tissue Culture. 2nd Edition. E. and S. Livingstone Ltd., Edinburgh and London

PAVLOV, N. (1969) Histopathology of the interstitial and proliferative forms of pneumonia in calves, pigs and sheep. Zentralblatt für Veterinärmedizin 16B 164-172

PEGREFFI, G., MURA, D. and CONTINI, A. (1957) /Ovine contagious pleuropneumonia caused by pleuropneumonia-like organisms/. Atti della Societa Italiana delle Scienze Veterinarie 11 829-833. Veterinary Bulletin (1958) 28 3905

PERK, K. and HOD, I. (1971) Demonstration of virus particles in ovine pulmonary adenomata. Experientia 27 437-438

PERK, K., HOD, I. and NOBEL, T.A. (1971) Pulmonary adenomatosis of sheep (jaagsiekte). I. Ultrastructure of the tumour. Journal of the National Cancer Institute 46 525-537

PIERCE, K.R., MOORE, R.W., CARROLL, L.H. and BRIDGES, C.H. (1963) Experimental ornithosis in ewes. American Journal of Veterinary Research 24 1176-1188

PIJOAN, C. (1974) Secretion of hydrogen peroxide by some common pig mycoplasmas. Veterinary Record 95 216-217

PIJOAN, C., ROBERTS, D.H. and HARDING, J.D.J. (1972) The effect of porcine mycoplasmas on pig tracheal organ cultures. Journal of Applied Bacteriology 35 361-365

PIRIE, H.M. and ALLAN, E.M. (1975) Mycoplasmas and cuffing pneumonia in a group of calves. Veterinary Record 97 345-349

POLLACK, J.D., SOMERSON, N.L. and SENTERFIT, L.B. (1969) Effect of pH on the immunogenicity of Mycoplasma pneumoniae. Journal of Bacteriology 97 612-619

POYNTER, D. and SELWAY, S. (1966) Diseases caused by lungworms Veterinary Bulletin 36 539-554

PROVOST, A. (1960) Clinical studies on "Abu nini". Veterinary Record. 72 353

PROVOST, A. (1969) Immunological studies on pleuropneumonia XII Immuno-pathogenic conception of the disease. Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux. 22 319-334

PURCELL, R.H., CHANOCK, R.M. and TAYLOR-ROBINSON, D. (1969) Serology of mycoplasmas in man. In The Mycoplasmatales and the L-phase of Bacteria. pp. 221-264. Ed. L. Hayflick. Appleton-Century-Crofts, New York

PURCELL, R.H., TAYLOR-ROBINSON, D. WONG, D.C. and CHANOCK, R.M. (1966) A colour test for the measurement of antibody to the non-acid forming human Mycoplasma species. American Journal of Epidemiology 84 51-66

QUINLAN, J.R., ALLEY, M.R. and CLARKE, J.K. (1975) In vitro sensitivity of New Zealand strains of Mycoplasma ovipneumoniae to some antibiotics. New Zealand Veterinary Journal 23 188-189

RAZIN, S., KAHANE, I. and KOVARTOVSKY, J. (1972) Immunochemistry of mycoplasma membranes. In Pathogenic Mycoplasmas, pp. 93-117. Associated Scientific Publishers, Amsterdam.

ROBERTS, D.H. (1973) Preliminary studies on the cell-mediated immune response in pigs to Mycoplasma hyopneumoniae British Veterinary Journal 129 427-438

ROBERTSON, J., GOMERSALL, M. and GILL, P. (1975) Mycoplasma hominis: growth, reproduction and isolation of small, viable cells. Journal of Bacteriology 124 1007-1018

ROBERTSON, J. and SMOOK, E. (1976) Cytochemical evidence of extramembranous carbohydrates on Ureaplasma urealyticum (T-stain mycoplasma) Journal of Bacteriology 128 658-660

ROMVARY, J. (1963) Catarrhal pneumonia of sheep caused by a virus of the psittacosis lymphogranuloma venereum (PLV) group. Acta Veterinaria Academiae Scientiarum Hungaricae 13 355-361

ROMVARY, J., TAKATSY, G., BARB, K. and FARKAS, E. (1962) Isolation of influenza virus strains from animals. Nature, London 193 907-908

ROSENDAL, S. (1976) Canine mycoplasmas. Doctorate Thesis; University of Aarhus

ROSS, R.F. and SPEAR, M.L. (1973) Role of the sow as a reservoir of infection for Mycoplasma hyosynoviae. American Journal of Veterinary Research 34 373-378

ST. GEORGE, T.D. (1969) II. The isolation of Myxovirus para-influenza type 3 from sheep in Australia. Australian Veterinary Journal. 45 321-325

ST. GEORGE, T.D. (1971) A survey of sheep throughout Australia for antibody to parainfluenza type 3 virus and to mucosal disease virus. Australian Veterinary Journal 47 370-374

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

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

ST. GEORGE, T.D., HORSFALL, N. and SULLIVAN, N.D. (1973) A subclinical pneumonia of calves associated with Mycoplasma dispar. Australian Veterinary Journal 49 580-586

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

ST. GEORGE, T.D. and SULLIVAN, N.D. (1973) Pneumonias of sheep in Australia. pp. 3-22. The University of Sydney, The Post-Graduate Foundation in Veterinary Science. No. 13

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

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

SCHACHTER, J., BANKS, J., SUGG, N., SUNG, M., STORZ, J. and MEYER, K.F. (1974) Serotyping of Chlamydia. I. Isolates of ovine origin. Infection and Immunity 9 92-94

SCHACHTER, J., STORZ, J., TARIZZO, M.L. and BÖGEL, K. (1973) Chlamydiae as agents of human and animal diseases. Bulletin of The World Health Organization 49 443-449

SENTERFIT, L.B. and JENSEN, K.E. (1966) Antimetabolic antibodies to Mycoplasma pneumoniae measured by tetrazolium reduction inhibition. Proceedings of The Society for Experimental Biology and Medicine 122 786-790

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

SHARP, J.M., MCFERRAN, J.B. and RAE, A. (1974) A new adenovirus from sheep. Research in Veterinary Science 17 268-269

SHEPARD, M.C. (1969) Fundamental biology of the T-strains. In The Mycoplasmatales and the L-phase of Bacteria, pp. 49-65. Ed. L. Hayflick. Appleton-Century-Crofts, New York

SHEPARD, M.C., LUNCEFORD, C.D., FORD, D.K., PURCELL, R.H. TAYLOR-ROBINSON, D., RAZIN, S. and BLACK, F.T. (1974) Ureaplasma urealyticum gen. nov., sp. nov: proposed nomenclature for the human T (T-strain) mycoplasmas. International Journal of Systematic Bacteriology 24 160-171

SHIFRINE, M. and GOURLAY, R.N. (1965) Serological relationship between galactans from normal bovine lung and from Mycoplasma mycoides. Nature, London 208 498-499

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

SHIRLAW, J.F. (1959) The pneumonias of the sheep with special reference to enzootic pneumonia and jaagsiekte. Veterinary Reviews and Annotations 5 37-57

SHREEVE, B.T., BIBERSTEIN, E.L. and THOMPSON, D.A. (1972) Variation in carrier rates of Pasteurella haemolytica in sheep. II Diseased flocks. Journal of Comparative Pathology 82 111-116

SIMBERKOFF, M.S., THORBECKE, G.J. and THOMAS, L. (1969) Studies of PPLO infection. V. Inhibition of lymphocyte mitosis and antibody formation by mycoplasmal extracts. Journal of Experimental Medicine 129 1163-1181

SMITH, C.B., CHANOCK, R.M. FRIEDEWALD, W.T. and ALFORD, R.H. (1967) Mycoplasma pneumoniae infections in volunteers. Annals of the New York Academy of Sciences 143 471-483

SMITH, G.R. (1959) Isolation of two types of Pasteurella haemolytica from sheep. Nature, London 183 1132-1133

SMITH, G.R. (1960a) The pathogenicity of Pasteurella haemolytica for young lambs. Journal of Comparative Pathology and Therapeutics 70 326-338

SMITH, G.R. (1960b) Virulence and toxicity of Pasteurella haemolytica in the experimental production of ovine septicaemia. Journal of Comparative Pathology and Therapeutics 70 429-436

SMITH, G.R. (1961) The characteristics of two types of Pasteurella haemolytica associated with different pathological conditions in sheep. Journal of Pathology and Bacteriology 81 431-440

SMITH, G.R. (1964) Production of pneumonia in adult sheep with cultures of Pasteurella haemolytica type A. Journal of Comparative Pathology and Therapeutics 74 241-249

SMITH, W.D. (1975) The nasal secretion and serum antibody response of lambs following vaccination and aerosol challenge with parainfluenza 3 virus. Research in Veterinary Science 19 56-62

SMITH, W.D., DAWSON, A.M., WELLS, P.W. and BURRELLS, C. (1975) Immunoglobulin concentrations in ovine body fluids. Research in Veterinary Science 19 189-194

SMITH, W.D., WELLS, P.W., BURRELLS, C. and DAWSON, A. McL. (1976) Maternal immunoglobulins and parainfluenza 3 virus inhibitors in the nasal and lachrymal secretions and serum of newborn lambs. Clinical and Experimental Immunology 23 544-553

SNOWDON, W.A. (1970) CSIRO, Division of Animal Health, Annual Report, p. 28

SOBESLAVSKY, O., PRESCOTT, B. and CHANOCK, R.M. (1968) Adsorption of Mycoplasma pneumoniae to neuraminic acid receptors of various cells and possible role in virulence. Journal of Bacteriology 96 695-705

SPEARMAN, C. (1904)Quoted by Snedecor, G.W. and Cochran, W.C. in Statistical Methods. 6th edition, 1967. p. 194. The Iowa State University Press, Ames, Iowa.

SPRADBROW, P.B. and MARLEY, J. (1971) Ovine kerato-conjunctivitis. Possible T-strain mycoplasmas in the conjunctival sac. Australian Veterinary Journal 47 116-118

SPRINGER, W.T., LUSKUS, C. and POURCIAU, S.S. (1974) Infectious bronchitis and mixed infections of Mycoplasma synoviae and Escherichia coli in gnotobiotic chickens. I. Synergistic role in the air-sacculitis syndrome. Infection and Immunity 10 578-589

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

STAMP, J.T., WATT, J.A.A. and THOMLINSON, J.R. (1955) Pasteurella haemolytica septicaemia of lambs. Journal of Comparative Pathology and Therapeutics 65 183-196

STEVENS, A. (1957) Respiratory diseases of sheep. Veterinary Record 69 1249-1254

STEVENSON, R.G. (1968) Studies in pneumonia of animals. Ph.D. Thesis, University of Edinburgh.

STEVENSON, R.G. (1969) Respiratory diseases of sheep. Veterinary Bulletin 39 747-759

STEVENSON, R.G. (1974) Streptococcus zooepidemicus infection in sheep. Canadian Journal of Comparative Medicine 38 243-250

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

STIPKOVITS, L., BELAK, S., PALFI, V. and TURY, E. (1975) Isolation of Mycoplasma ovipneumoniae from sheep with pneumonia. Acta Veterinaria Academiae Scientiarum Hungaricae 25 267-273

STORZ, J. (1966) Psittacosis-lymphogranuloma infection of sheep: Antigenic structures and interrelations of PL agents associated with polyarthritis, enzootic abortion, intrauterine and latent intestinal infections. Journal of Comparative Pathology 76 351-362

STORZ, J. (1971) Chlamydia and chlamydia-induced diseases. C.C. Thomas, Springfield, Illinois.

SUB-COMMITTEE ON THE TAXONOMY OF MYCOPLASMATALES (1972) International Committee on Systematic Bacteriology Proposal for minimal standards for descriptions of new species of the order Mycoplasmatales. International Journal of Systematic Bacteriology 22 184-188

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

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

SURMAN, P.G. (1968) Cytology of "pink-eye" of sheep, including a reference to trachoma of man, by employing acridine orange and iodine stains, and isolation of Mycoplasma agents from infected sheep eyes. Australian Journal of Biological Sciences 21 447-467

TAMARIN, R. (1969) Laboratory diagnosis of Bedsonia infections in sheep and cattle. Refuah Veterinarith 26 28-39

TAURASO, N.M. (1967) Effect of diethylaminoethyl dextran on the growth of mycoplasma in agar. Journal of Bacteriology 93 1559-1564

TAYLOR, G., TAYLOR-ROBINSON, D. and FERNALD, G.W. (1974) Reduction in the severity of Mycoplasma pneumoniae-induced pneumonia in hamsters by immunosuppressive treatment with anti-thymocyte sera. Journal of Medical Microbiology 7 343-348

TAYLOR-ROBINSON, D., DENNY, F.W., ALLISON, A.C., THOMPSON, G.W. and TAYLOR, G. (1972) Cell-mediated immunity and the response to mycoplasma infections. In Pathogenic Mycoplasmas, pp. 349-362 Associated Scientific Publishers, Amsterdam

TAYLOR-ROBINSON, D., LUDWIG, W.M., PURCELL, R.H., MUFSON, M.A. and CHANOCK, R.M. (1965) Significance of antibody to Mycoplasma hominis Type 1 as measured by indirect haemagglutination. Proceedings of The Society for Experimental Biology and Medicine 118 1073-1083

TAYLOR-ROBINSON, D., PURCELL, R.H., WONG, D.C. and CHANOCK, R.M. (1966) A color test for the measurement of antibody to certain Mycoplasma species based upon the inhibition of acid production. Journal of Hygiene, Cambridge 64 91-104

TAYLOR-ROBINSON, D., SHIRAI, A., SOBESLAVSKY, O. and CHANOCK, R.M. (1966a) Serological response to Mycoplasma pneumoniae infection. II Significance of antibody measured by different techniques. American Journal of Epidemiology 84 301-313

TAYLOR-ROBINSON, D., SOBESLAVSKY, O., JENSEN, K.E., SENTERFIT, L.B. and CHANOCK, R.M. (1966b) Serological response to Mycoplasma pneumoniae infection. I Evaluation of immunofluorescence, complement fixation, indirect hemagglutination and tetrazolium reduction inhibition tests for the diagnosis of infection. American Journal of Epidemiology 83 287-298

TAYLOR-ROBINSON, D., SOMERSON, N.L., TURNER, H.C. and CHANOCK, R.M. (1963) Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. Journal of Bacteriology 85 1261-1273

THOMAS, L.H. and HOWARD, C.J. (1974) Effect of Mycoplasma dispar, Mycoplasma bovirhinis, Acholeplasma laidlawii and T-mycoplasmas on explant cultures of bovine trachea. Journal of Comparative Pathology 84 193-201

THOMAS, L.H. and SMITH, G.S. (1972) Distribution of mycoplasmas in the non-pneumonic bovine respiratory tract. Journal of Comparative Pathology 82 1-4

THOMAS, L.H., WOOD, P.D.P. and LONGLAND, J.M. (1978) The influence of disease on the performance of beef cattle. British Veterinary Journal 134 152-161

THOMPSON, D.A. (1973) Experimental studies on Pasteurella haemolytica. M.Phil. Thesis, University of Edinburgh

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

TULL, A.H., BLAIR, E.B., FISHMAN, D.L. and HEATLEY, G.J. (1975) Significant enhancement of T-strain mycoplasmal growth on supplemented Shepard's A6 differential agar. Journal of Clinical Microbiology 1 234-236

TULLY, J.G., DEL GIUDICE, R.A. and BARILE, M.F. (1972) Synonymy of Mycoplasma arginini and Mycoplasma leonis. International Journal of Systematic Bacteriology 22 47-49

TURY, E., BELAK, S., PALFI, V. and STIPKOVITS, L. (1975) Mycoplasma infection of sheep with pneumonia. In Vitro V CSSR pp. 189-193. Proceedings of 3rd Conference on Taxonomy and Physiology of Animal Mycoplasmas, Brno. Eds. K. Jurmanova and Z. Dreslerova

UNGUREANU, C. (1969) Identification of some ovine mycoplasma strains by the growth inhibition test in the presence of specific serum. Archiva Veterinaria 6 75-82

UNGUREANU, C. and CIRSTET, I. (1969) Characters of some mycoplasma strains isolated from sheep with lung affections. Archiva Veterinaria 6 53-63

UNGUREANU, C., POPOVICI, V., HIASTRU, F., SIRBU, Z., FROMUNDA, V. and CIRSTET, I. (1972) /Pneumonic Conditions in sheep/ Lucrarile Institutului de Cercetari Veterinare si Biopreparate "Pasteur" 8 151-166. Veterinary Bulletin (1972) 42 6421

VOGELZANG, A.A. and BEUVERY, E.C. (1974) Isolation of mycoplasmas from bovine sera used for cell cultures. International Congress on Mycoplasmas of Man, Animals, Plants and Insects, Bordeaux

WANDERA, J.G. (1968) Experimental transmission of sheep pulmonary adenomatosis (jaagsiekte) Veterinary Record 83 478-482

WANDERA, J.G. (1971) Sheep pulmonary adenomatosis (jaagsiekte) Advances in Veterinary Science and Comparative Medicine 15 251-283

WATSON, W.A., COTTEW, G.S., ERDAG, O. and ARISOY, F. (1968) The pathogenicity of mycoplasma organisms isolated from sheep and goats in Turkey. Journal of Comparative Pathology 78 283-291

WHITTLESTONE, P. (1972a) Pathogenicity of mycoplasmas in animals. In Microbial Pathogenicity in Man and Animals. pp. 217-250. Eds. H. Smith and J.H. Pearce. Cambridge University Press

WHITTLESTONE, P. (1972b) The role of mycoplasmas in the production of pneumonia in the pig. In Pathogenic Mycoplasmas pp. 263-279. Associated Scientific Publishers, Amsterdam

WHITTLESTONE, P. (1976) Effect of climatic conditions on enzootic pneumonia of pigs. International Journal of Biometeorology 20 42-48

WHITTLESTONE, P. (1976a) Immunity to mycoplasmas causing respiratory diseases in man and animals. Advances in Veterinary Science and Comparative Medicine 20 277-307

WILLIAMS, C.O. and WITTLER, R.G. (1971) Hydrolysis of aesculin and phosphatase production by members of the order Mycoplasmatales which do not require sterol. International Journal of Systematic Bacteriology 21 73-77

WILSON, M.R. (1966)The differentiation of some psittacosis-lymphogranuloma-venereum group agents isolated from sheep and cattle. Journal of Infectious Diseases 116 319-323

WINDSOR, G.D., EDWARD, D.G. ff. and TRIGWELL, J.A. (1975) A solid medium for culture and identification of human T-mycoplasmas. Journal of Medical Microbiology 8 183-187

WITTLER, R.G. (1973) Heterogeneity of the members of the class Mollicutes. Journal of Infectious Diseases 127 Supp. S5-S7

WOODS, G.T., SIBINOVIC, K. and MARQUIS, G. (1965) Experimental exposure of calves, lambs and colostrum-deprived pigs to bovine Myxovirus parinfluenza 3. American Journal of Veterinary Research 26 52-56

WOOLF, A., KRADEL, D.C. and BUBASH, G.R. (1970) Mycoplasma isolates from pneumonia in captive Rocky Mountain Bighorn sheep. Journal of Wildlife Diseases 6 169-170

WRIGHT, D.N., BAILEY, G.D. and GOLDBERG, L.J. (1969) Effect of temperature on survival of airborne Mycoplasma pneumoniae. Journal of Bacteriology 99 491-495

## ERRATUM

Page 244; insert

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

## PUBLICATIONS

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

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

JONES, G.E., FOGGIE, A., SUTHERLAND, A. and HARKER, D.B. (1976) Mycoplasmas and ovine keratoconjunctivitis. Veterinary Record 99 137-141

JONES, G.E., GILMOUR, J.S. and RAE, A. (1976) Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasmas isolated from them. Journal of Comparative Pathology 88 85-96

# Papers and Articles

## Mycoplasmas and ovine keratoconjunctivitis

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**The clinical course of an outbreak of keratoconjuctivitis in housed lambs and their dams was followed. Signs were transient generally and became severe in only a small proportion of lambs. The outbreak became most obvious when the lambs were 46 to 55 days old, when 46·9 per cent were affected. *Mycoplasma conjunctivae* isolations, confirmed by comparison with the type strain by biochemical and serological reactions, increased to 62·1 per cent of all eyes swabbed, but no correlation could be demonstrated between presence of the organism and clinical status. The reasons for this are discussed. *Mycoplasma ovipneumoniae* was also recovered from the eyes of a small number of lambs. Instillation of a broth culture of *M conjunctivae* into the conjunctival sacs of four hoggs produced a transient keratoconjunctivitis similar to that observed in the field, but no effect was observed in animals inoculated intravenously. *M conjunctivae* may therefore be the aetiological agent of non-follicular infectious ovine keratoconjunctivitis, although further work in gnotobiotic or specific pathogen free lambs is required to establish the fact beyond doubt.**

THE aetiology of infectious ovine keratoconjunctivitis (OKC) remains obscure, despite the number of micro-organisms known to be associated with the disease. *Neisseria ovis* was initially described by Lindqvist (1960), who recovered the organism from about 40 cases of OKC, but subsequent findings on distribution (Spradbrow 1968, Kjolleberg 1971, Nicolet and others 1974) and pathogenicity (Spradbrow 1971, Fairlie 1966) have indicated that *N ovis* is probably not pathogenic, even when the cornea is damaged. The recovery of *Moraxella* from sheep with and without OKC has been reported (Baker and others 1965, Wood and others 1965), but experimental pathogenicity studies led Wood and his colleagues to conclude that the isolates were unrelated to OKC.

The recovery in Norway of *Listeria monocytogenes* type 04 from sheep with OKC has been described (Kummeneje and Mikkelsen 1975). The organism was considered to be a possible causal agent of the condition.

The implication of Chlamydia in OKC, first suggested by Dickinson and Cooper (1959), has been substantiated by subsequent findings regarding the distribution (Stephenson and others 1973, Hopkins and others 1973, Cello 1967, Storz and others 1967) and pathogenicity (Storz and others 1967, Cooper 1974) of these organisms.

A rickettsial aetiology for OKC, postulated by Coles (1931) on the basis of bodies observed in conjunctival epithelial cells, has received support from other workers (Beveridge 1942, Pavlov and others 1964, Leeflang and others 1969). However, the supposed organism, originally called *Rickettsia conjunctivae* (Coles 1931) and later *Colesiota conjunctivae* (Rake 1948), has never been cultivated.

Mycoplasmas have been recovered from cases of OKC in Switzerland (Leach 1970, Nicolet and others 1974), Canada (Langford 1971, Australia (Surman 1968, 1973, Spradbrow and Marley 1971, Carmichael and others 1972), and America (Barile and others 1972, Cello 1967, Barner 1952). Barile and others (1972) characterised and named their isolates as *Mycoplasma conjunctivae*, and retrospectively identified the strains of both Surman (1968) and Langford (1971) as consisting of *M conjunctivae* and *Mycoplasma arginini*. The isolates of Leach (1970) were also identified as *M arginini*, those of Nicolet and others (1974) as *M conjunctivae* and the strain recovered by Carmichael and others (1972) as *Mycoplasma ovipneumoniae*.

During a survey of housed sheep in Scotland, the occurrence of conjunctivitis and keratitis in housed lambs, and to a lesser extent in their dams, became apparent. This paper reports the recovery of mycoplasmas from the eyes of survey animals, the identification of isolates and the experimental inoculation of one isolate into sheep.

### Materials and methods

#### MEDIA

The media employed for the cultivation of glycolytic mycoplasmas (OB and OA) have been described previously (Jones and others 1976). Briefly, OB comprised 60 per cent medium 199 (Burroughs Wellcome) 20 per cent tryptose phosphate broth (Oxoid) or brain-heart infusion (Oxoid), 10 per cent inactivated swine serum, 10 per cent fresh yeast extract supplemented with ampicillin 1 mg per ml (Penbritin, Beecham), thallium acetate 0·025 per cent, and phenol red 0·006 per cent. For performance of the metabolic inhibition test the concentration of swine serum was increased to 20 per cent and the proportion of medium 199 decreased accordingly. OA differed from OB in containing 15 per cent inactivated swine serum, 55 per cent medium 199 and 0·75 per cent agarose (BDH). Both media were adjusted to pH 7·6 to 7·8.

The broth medium employed for the cultivation of arginine-hydrolising mycoplasmas (Arg B) was essentially OB but arginine was added to provide a final concentration of 1 per cent (w/v) and the medium was adjusted to pH 6·6 to 6·8. Similarly, media (TB and TA) for the cultivation of ureaplasmas (T-mycoplasmas) resembled OB and OA, but contained 20 per cent inactivated swine serum, 50 per cent medium 199 and 1 per cent (w/v) final concentration of urea, thallium acetate being omitted. TB and TA were adjusted to pH 6·0 to 6·3.

Transport medium (MTM) comprised 90 per cent Hartley's infusion broth (Oxoid) or brain-heart infusion broth, 5 per cent fresh yeast extract, 5 per cent inactivated swine serum and ampicillin 1 mg per ml, and was adjusted to pH 7·0 to 7·2.

#### COLLECTION OF SAMPLES

Conjunctival swabs were taken by sampling both eyes with a single cotton wool swab. Both nasal cavities of experimental animals were also sampled with a single swab. Swabs taken for mycoplasma isolation were placed in bijoux containing 3 ml MTM. Bacterial isolations were attempted only from

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conjunctival swabs taken during the experimental investigations.

CULTIVATION AND CLONING PROCEDURES

Primary mycoplasma isolations were performed by inoculating approximately 0·2 ml from the MTM in which the swabs had been placed into bijoux containing 1·8 ml OB, Arg B and TB. For isolations from experimental animals OA and TA plates were also inoculated from MTM. During the investigation of a field outbreak of OKC, apparently positive broth cultures as indicated by an acid change of medium were stored at –70°C to await identification at a later date, but identification of strains recovered from experimental animals was performed at the time of isolation. Mycoplasma broth cultures were incubated aerobically and solid medium cultures in candle jars.

Primary bacterial isolations were performed by smearing swabs on paired sheep blood agar (SBA) plates, then placing the swabs in 5 per cent bovine serum nutrient broth. One of each pair of plates was incubated aerobically, and the other in an atmosphere of 95 per cent $N_2$ 5 per cent $CO_2$. Broth cultures were streaked onto SBA plates after incubation for 24 hours. All cultures were incubated at 37°C.

A mycoplasma strain (5777) isolated during the field investigation from a severe case of keratoconjunctivitis in a 29-day-old lamb, was cloned four times by serial transfer of single colonies from agar to broth. The possibility that this strain was an L-form was eliminated by five consecutive passages on solid medium from which anti-bacterial inhibitors had been omitted. No reversion to bacterial form occurred.

BIOCHEMICAL TESTS

The methods and media employed have been described previously (Jones and others 1976). Strain 5777 and HRC 581, the type strain of *M conjunctivae* (kindly donated by Dr R. Lemcke, Lister Institute), were compared by the following tests: oxidation-fermentation reaction; tetrazolium reduction; "films and spots" and phosphatase production; and sensitivities to digitonin, sodium polyanethol sulphonate (SPS) (5 per cent and 20 per cent), optochin (Oxoid) and 11 antibiotics. The antibiotic discs were obtained from Mast Laboratories Ltd, Liverpool, except for those containing tylosin, which were obtained from Elanco Products Ltd, London.

PREPARATION OF ANTISERA

The modification of the method of Morton and Roberts (1967) described previously (Jones and others 1976) was used to produce antisera in rabbits against *M conjunctivae* (Strain HRC 581), *M arginini* (Strain G230, NCTC 10129) and *M ovipneumoniae* (Strain 956/2).

SEROLOGICAL METHODS

*Growth inhibition (GI test).*—The method of Dighero and others (1970) was used for the routine identification of isolates. Where mixed cultures occurred, colonies were cloned according to morphological type before being identified by the GI test.

*Growth precipitation (GP) test.*—The method of Jordan (1973) was used to compare strain 5777 with HRC 581, using antiserum to HRC 581.

*Metabolic inhibition (MI test).*—The method of Taylor-Robinson and others (1966) was employed. Fresh guinea-pig serum was included at a final concentration of 3·12 per cent.

EXPERIMENTAL INFECTIONS OF SHEEP

*Experimental animals.*—Eight Suffolk x Greyface hoggs, six months old, were randomly divided into two groups of four, the groups being housed in separate pens. The hoggs were examined twice before experimental inoculation for the presence of eye lesions and ocular mycoplasmas and bacteria. No lesions or mycoplasmas were detected.

*Inoculum.*—The strain used (5809) was isolated during the field investigation from a mild case of keratoconjunctivitis in a 35 day-old lamb. The strain was passaged five times in OB before being stored at –70°C. When required, the culture was thawed and used undiluted at a titre of $10^8$ colour changing units per 0·2 ml. No limiting dilutions or cloning procedures were performed in any of the passages, but no bacteria or other mycoplasmas were recovered from the cultures at any stage. The identity of the strain was confirmed as *M conjunctivae* by the GI test.

INOCULATION

*Group* 1.—Approximately 0·2 ml of a broth culture of strain 5809 was instilled into the conjunctival sacs of both eyes on three occasions, each at an interval of one hour.

*Group* 2.—2 ml of broth culture was injected intravenously (iv).

SAMPLING AND CLINICAL EXAMINATION

Clinical examination was performed daily for the first 12 days and on occasions thereafter. Conjunctival and nasal swabs for mycoplasma isolation were taken from Group 1 at 0, 4, 8, 11, 18, 25, 35, 44 and 53 days post-inoculation (dpi), and from Group 2 at 0, 2, 4, 7, 11 and 18 dpi. Conjunctival swabs for bacterial examination were taken from Group 1 only at 0, 4 and 8 dpi. Serum samples were obtained from all animals at 0, 35 and 53 dpi.

Corneal scrapings taken under anaesthesia from an animal infected 19 dpi by the intra-conjunctival route were stained by May—Grünwald—Giemsa.

## Results

INVESTIGATION OF FIELD OUTBREAK OF CONJUNCTIVITIS AND KERATITIS

The animals involved were housed during the whole of the investigation. At peak stocking periods some 600 ewes and their lambs were maintained in one house sharing the same air-space, but groups of ewes and lambs were separated by pens.

Initially three pens containing 39 ewes and their 71 lambs were involved in the investigation, but death and culling reduced the lamb numbers to 62 by the last time of sampling. The pens were sampled in rotation, each pen being sampled three times at three week intervals, with a final examination of all lambs when 94 to 114 days old. The occurrence of mild conjunctivitis first became apparent when the lambs were six to 10 days old, but mycoplasma isolations were not attempted until more severe symptoms appeared in some of the lambs. With the recovery of mycoplasmas from the eyes of affected animals, routine sampling of all survey animals was initiated. No bacterial isolations were attempted. The results of clinical and mycoplasmal examinations are presented in Table 1.

The symptoms exhibited by affected lambs ranged from a mild conjunctivitis involving hyperaemia of the palpebral conjunctivae, excessive lachrymation and varying degrees of blepharospasm and photophobia, to engorgement of blood vessels of the bulbar conjunctivae accompanied by opacity of the cornea and the development of a mucopurulent exudate. Ulceration of the cornea was observed in severe cases.

The highest incidence of clinical symptoms occurred at 46 to 55 days old, when 46·9 per cent of the lambs were affected. The isolation rate for *M conjunctivae* was also highest from this age range, with 62·1 per cent of the animals being positive. The differences in isolation rates of *M conjunctivae* between clinically affected and unaffected animals are comparable only for the two age groups 46 to 55 days and 94 to 114 days old, and were found to be not significant.

Of the total of 71 lambs, 29·6 per cent never appeared to show symptoms of OKC at any stage, but as 58·1 per cent were only observed to have eye lesions at one visit it is possible that transient symptoms occurred in some lambs between examinations. One lamb, in which symptoms were observed on only one occasion, yielded *M conjunctivae* at each of three samplings spanning 81 days.

*M ovipneumoniae* was recovered from the eyes of a small

TABLE 1: Occurrence of ocular lesions and mycoplasma infections in lambs

| Age (days) | No of lambs | Percentage with ocular lesions | | | Percentage of *M conjunctivae* recoveries | |
|---|---|---|---|---|---|---|
| | | Mild[1] | Moderate[2] | Severe[3] | With eye lesions | Without eye lesions |
| 6-10 | 19 | 10·5 | 0 | 0 | ND | ND |
| 23-35 | 71 | 28·2 | 2·8 | 4·2 | 100† (0)* | ND (ND) |
| 46-55 | 66 | 34·8 | 12·1 | 0 | 67·7 (6·4) | 57·1 (0) |
| 94-114 | 62 | 16·1 | 4·9 | 1·6 | 57·1 (0) | 52·1 (8·3) |

(1) Palpebral conjunctivitis with moderate/profuse lachrymation.
(2) Moderate keratoconjunctivitis—palpebral and bulbar conjunctivitis, often with pannus formation of the cornea.
(3) Severe keratoconjunctivitis—as for 2 but with increased involvement of the cornea, including purulent keratitis and occasionally corneal ulceration.
ND—Not done
†Only 13 animals sampled, all clinically positive.
*Figures in parentheses indicate recoveries of *M ovipneumoniae*.

number of lambs. There was little correlation between its presence and the severity of eye lesions. Neither *M arginini* which was recoverable from the respiratory tracts of some of the survey animals, nor ureaplasmas were isolated from eye swabs.

Conjunctival swabs were taken from the 39 ewes on one occasion, when 12·8 per cent yielded *M conjunctivae*. None of those sampled showed the presence of marked ocular lesions, although a small proportion of other ewes in the same house were severely affected with OKC.

COMPARISON OF STRAIN 5777 WITH *M conjunctivae* (STRAIN HRC 581)

*Biochemical reactions*

With the exception of the formation of "films and spots", in which strain 5777 was positive and HRC 581 negative, the biochemical reactions obtained from the two strains corresponded with each other and with the results obtained by Barile and others (1972). Other, uncloned strains isolated from the same outbreak and from other outbreaks of OKC in Scotland have also been found to produce "films and spots" on OA plates.

The sensitivities of strains 5777 and HRC 581 to digitonin, sodium polyanethol sulphonate (SPS), optochin and 11 antibiotics are given in Table 2. Both strains were sensitive to digitonin, SPS (5 per cent and 20 per cent), neomycin, chlortetracycline, oxytetracycline, gentamycin and tylosin, and were resistant to erythromycin, oleandomycin, nystatin and Polymixin B. Optochin produced minimal levels of inhibition and streptomycin low levels of inhibition in strains 5777 only.

Fig 1 shows the results of a GI/GP test, the running-drop culture was strain 5777 and the well contained antiserum to strain HRC 581. An inhibition zone of 4 to 6 mm around the well, and the formation of three precipitin lines each side are visible. By the MI test, antiserum to HRC 581 produced titres of 1/160 to 1/320 against strain 5777 and to its homologous strain titres of 1/320 to 1/640.

INTRACONJUNCTIVAL INFECTION OF SHEEP (GROUP 1)

*Clinical symptoms.*—Symptoms were first noted in animal 120 at three days post inoculation, with the rapid development of blepharospasm, copious lachrymation with epiphora, hyperaemia of the palpebral conjunctivae, congestion of the bulbar conjunctival vessels which gave a "bloodshot" appearance to the eyes and slight pannus formation at the limbus. At four dpi all four animals showed similar symptoms, with some variation occurring in the degree of pannus formation. At five dpi, the inflammatory reaction had lessened in three of the sheep, but had increased in one (122), particularly in the extent of opacity spreading from the limbus towards the centre of the cornea. The improvement in the three sheep continued gradually until by 18 dpi all appeared normal. except for slight residual hyperaemia of the palpebral conjunctivae. In sheep 122, vascularisation and blue-black pigmentation of the affected areas of the cornea followed the development of opacity. Resolution of these changes in sheep 122 was also complete by 18 dpi.

FIG 1: Identification of field isolate by the GI/GP test. The running-drop culture is of strain 5777, and the well contains antiserum to *M conjunctivae* (strain HRC 581)

*Mycoplasmas.*—*M conjunctivae* was recovered from all conjunctival swabs taken up to 35 dpi in one animal, 44 dpi in two animals and until the last sampling at 53 dpi in the fourth animal. Nasal swabs were positive for *M conjunctivae* up to 25 dpi in one animal and 44 dpi in three animals. The recovery titres of *M conjunctivae* were not determined, but the density of growth on primary isolation on OA plates indicated that infection levels were considerably higher in the eyes than the nasal cavities. No other mycoplasma species were recovered from any samples.

*Bacteria.*—The most commonly isolated bacteria were *Bacillus* spp. In addition, *Escherischia coli*, *Staphylococcus epidermidis*, *Staph pyogenes*, *Streptococcus* spp, *Neisseria* spp, diptheroids and fungi were recovered, though not consistently or in large numbers.

*Serology.*—All animals in both groups showed pre-inoculation titres of 1/2 to 1/4 by the MI test, and no significant seroconversions following inoculation were detected.

*Cytology.*—Figs 2 and 3 show cells scraped from the corneal surface of an animal 19 dpi with *M conjunctivae* by the intraconjunctival route, and stained by May-Grünwald-Giemsa. Numerous round, oval or triangular bodies of 0·25 to 1·10 μm are visible. Most of the organisms have a vacuolated centre with unipolar or bipolar accumulations of material, though a proportion stain homogeneously throughout. In some cells the bodies showed linear arrangements, giving a streaming effect (Fig 3) and a general but not invariable association with cells was noted. In about one-third of the cells examined

TABLE 2: Sensitivity of strains HRC 581 and 5777 to digitonin, SPS, optochin, antibiotic and fungicide discs

| Strain | Zones of inhibition (mm) produced by | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Dig | SPS 5 per cent | SPS 20 per cent | Op | K | E | NE | CT | S | OL | NY | OT | GM | PB | TY |
| M. conjunctivae (HRC 581) | 3·5 | 4 | 5·5 | 1·0 | ND | 0·0 | 9·0 | 18·0 | 0·0 | 0·0 | 0·0 | 24·0 | 27·0 | 0·0 | 15·0 |
| 5777 | 3·0 | 5·0 | 6·0 | 2·0 | 9·0 | 1·0 | 6·0 | 6·0 | 2·0 | 0·0 | 0·0 | 20·0 | 14·0 | 1·0 | 18·0 |

Dig = Digitonin SPS = Sodium polyanethol sulphate Op = Optochin K = Kanamycin (30 μg) E = Erythromycin (5 μg) NE = Neomycin (10 μg) CT = Chlortetracycline (25 μg) S = Streptomycin (10 μg) OL = Oleandomycin (5 μg) NY = Nystatin (100 units) OT = Oxytetracycline (25 μg) GM = Gentamycin (10 μg) PB = Polymixin B (100 units) TY = Tylosin (30 μg)

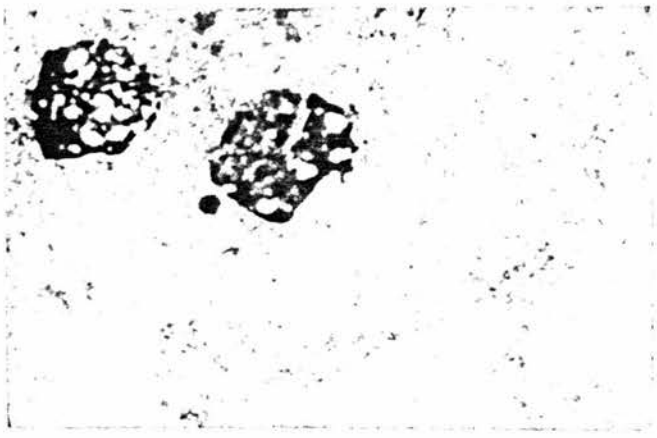

FIG 2: Corneal epithelial scraping taken from an animal 19 dpi with *M conjunctivae* by the intra conjunctival route and stained by May-Grünwald-Giemsa. 1350

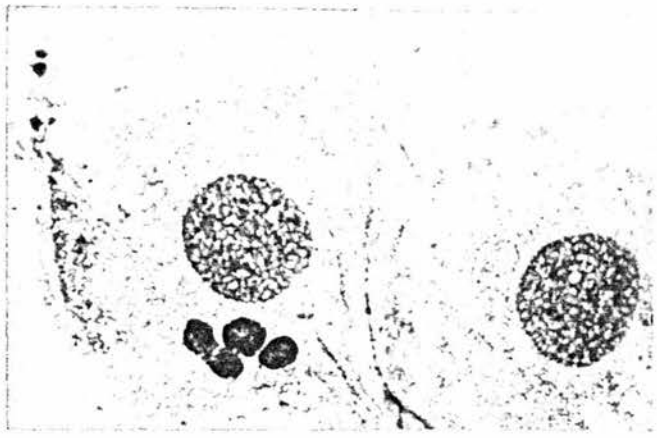

FIG 3: Similar preparation to Fig 2, illustrating distribution of mycoplasmas relative to the corneal epithelial cells. 985

homogenously-staining round or oval bodies of 1·35 to 2·70 $\mu$m were also visible. The presence of these larger bodies has been noted by other workers (eg, Barner and Riley 1951, Beveridge 1942). Their origin is unknown but they may correspond with the melanin granules described by Barner (1952) and Dickinson and Cooper (1959), who observed their presence in clinically normal as well as affected eyes. In stained smears of tears large numbers of neutrophils were present.

INTRAVENOUS INOCULATION OF SHEEP (GROUP 2)

No clinical symptoms were observed or mycoplasmas recovered from any of the animals.

## Discussion

No recognised definitions of different forms of infectious OKC exist at present, but at least two separate clinical forms have been described. In the first, so-called follicular conjunctivitis (FC) described by Storz and others (1967), Hopkins and others (1973) and Stephenson and others (1973), the characteristic feature is the development of numerous, often confluent enlarged lymphoid follicles on the lower and third eyelids. A second feature generally observed in cases of FC and infrequently in the nonfollicular form of OKC is the development of chemosis. An associated sign in flocks affected with FC is the presence of polyarthritis in a proportion of lambs. In all reports of FC, chlamydia have been implicated as the causal agents, but Dickinson and Cooper (1959) and Cooper (1974), in reporting cultivation, demonstration and pathogenicity studies with chlamydia-like organisms from cases of OKC do not describe the presence of conjunctival follicles, but consider involvement of the sclera as being pathognomonic of the disease.

The second form of OKC, in which follicle formation is negligible or non-existent, has been described by Johnson (1938), Beveridge (1942), Blakemore (1947), Barner and Riley (1951) and Surman (1968). The ocular signs observed by us in both survey and experimental animals closely resembled descriptions of this form of OKC and comprised a transient conjunctivitis/keratitis which became severe in only a small proportion of survey animals. Follicle formation was not observed. In addition, the organisms observed in stained conjunctival scrapings from experimentally infected sheep showed marked similarities to those described by other authors (Coles 1931, Beveridge 1942, Blakemore 1947, Surman 1968). It should be noted that similar bodies were also observed by Dickinson and Cooper (1959), but they were thought to represent cytoplasmic changes in the epithelial cells. It is likely, therefore, that the disease observed by us and reproduced experimentally by the instillation into the conjunctival sac of broth culture containing *M conjunctivae* is the same as that described by these authors. We are thus in agreement with the opinion expressed by Barner and Riley (1951), Barner (1952), Surman (1968) and Nicolet and others (1974) that the aetiology of this form of OKC is not the putative *Colesiota conjunctivae* but the mycoplasma *M conjunctivae*. However, we differ from those authors in that we consider there is no evidence for an intracellular site for these organisms, but that they are more likely to be extracellular and closely adherent to the cytoplasmic membrane of epithelial cells in a fashion similar to other mycoplasmas (Anderson 1969).

In the investigation of the field outbreak of OKC, the correlation between recovery of *M conjunctivae* and the presence of clinical symptoms was poor, but several reasons may account for this apparent discrepancy. First, approximately 25 per cent of apparently positive primary isolations, which were stored at –70°C for 10 to 12 months before removal, failed to grow on subculture, and it may be that storage adversely affected their viability. Second, both experimental and survey studies established that symptoms are usually transient and might therefore have been missed altogether in some animals due to the examination regime. Third, though symptoms are generally transient colonisation of the conjunctivae may persist for a considerable time, in one case 81 days. Beveridge (1942) found that infection, as judged by the presence of "rickettsial bodies" in conjunctival cells, persisted in 13 per cent of affected sheep for 200 days or longer. On microbiological grounds, therefore, the difference between clinically positive and clinically negative animals will become negligible after the disease has been present within a flock for a sufficient length of time. Finally, it is possible that some lambs infected with *M conjunctivae* may not develop recognisable signs.

The only reported isolation of *M ovipneumoniae* from eyes (Carmichael and others 1972) was from a severe case of OKC in a lamb which was concurrently affected with pneumonia, although a non-pathogenic, glycolytic mycoplasma was also recovered from cases of OKC by Spradbrow and Marley (1971). Our survey findings would suggest that *M ovipneumonia* probably has no role, primary or secondary, in the pathogenesis of OKC.

Outbreaks of OKC from which *M conjunctivae* was frequently isolated from affected animals have been described by Nicolet and his colleagues (1974), but those workers did not observe any pannus formation or ulceration in clinical cases of the disease. This may be attributable to the presence or absence of predisposing factors or pyogenic bacteria capable of establishing a secondary superinfection, or to variations in host resistance or virulence of *M conjunctivae*. Surman (1968, 1973) experimentally produced mild symptoms of OKC in a proportion of sheep and in two goats by the intra conjunctival inoculation of both conjunctival scrapings from affected sheep and isolates of mycoplasma from the conjunctiva. None of the inocula derived from conjunctival sources nor the strains recovered from experimentally inoculated animals were satisfactorily identified. Spradbrow and

Marley (1971) have also experimentally produced keratoconjunctivitis in two sheep by the intracorneal inoculation of broth cultures of ureaplasma-like organisms isolated from sheep with OKC.

In the experimental studies reported in this work, the inoculum employed consisted of a field isolate clearly identified as *M conjunctivae*. The intra conjunctival inoculation of a fifth-passage broth culture of this strain induced ocular lesions in all four experimental sheep, and mycoplasmas identified as *M conjunctivae* were recovered from the eyes and nasal cavities of each animal. No effects were observed following the intravenous inoculation of the same strain, adding confirmation to the findings of Beveridge (1942) that infection is established only by the ocular route. Examination for the presence of chlamydia in eye-swabs of experimental animals was not attempted, but no chlamydia-like bodies were observed in stained smears of corneal cells. To obviate the possibility of latent infections with other organisms, however, further studies on the pathogenicity of *M conjunctivae* should be performed in specific pathogen free or gnotobiotic lambs.

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REFERENCES

ANDERSON, D. R. (1969). In "The Mycoplasmatales and the L-phase of Bacteria." Ed. Hayflick, p. 382. New York, Appleton-Century-Crofts.
BAKER, J. R., FAULL, W. B., & WARD, W. R. (1965). *Vet. Rec.* 77. 402.
BARILE, M. F., DEL GIUDICE, R. A., & TULLY, J. G. (1972). *Inf. Imm.* 5. 70.
BARNER, R. D. (1952) .*Am. J. Vet. Res.* 13. 132.
BARNER, R. D., & RILEY, W. F. JR. (1951). *Mich. St. Coll. Vet.* 12. 12.
BEVERIDGE, W. I. B. (1942). *Aust. vet. J.* 18. 155.
BLAKEMORE, F. (1947). *J. comp. Path.* 57. 223.
CARMICHAEL, L. E., ST. GEORGE, T. D., SULLIVAN, N. D., & HORSFALL, N. (1972). *Cornell Vet.* 62. 654.
CELLO, R. M. (1967). *Am. J. Ophthal.* 63. 1270.
COLES, J. D. W. A. (1931). 17th Rep. Dir. Vet. Serv. and Anim. Indust. p. 175.
COOPER, B. S. (1974). *N.Z. vet. J.* 22. 181.
DICKINSON, L., & COOPER, B. S. (1959). *J. path. Bact.* 78. 257.
DIGHERO, M. W., BRADSTREET, C. M. P., & ANDREWS, B. E. (1970). *J. Appl. Bact.* 33. 750.
FAIRLIE, G. (1966). *Vet. Rec.* 78. 649.
HOPKINS, J. B., STEPHENSON, E. H., STORZ, J., & PIERSON, R. E. (1973). *J. Am. vet. med. Ass.* 163. 1157.
JOHNSON, L. V. (1938). *Proc. Soc. Exp. Biol. Med.* (*N.Y.*). 38. 42.
JONES, G. E., FOGGIE, A., MOULD, D. L. & LIVITT, S. (1976). *J. med. microbiol* 9. 39.
JORDAN, F. T. W. (1973). *Res. vet. Sci.* 14. 387.
KJOLLEBERG, K. (1971). *Norsk. Vet. Tidsskr.* 83. 233.
KUMMENEJE, K., & MIKKELSEN, T. (1975). *Nord. Vet.—Med.* 27. 144.
LANGFORD, E. V. (1971). *Can. J. Comp. Med.* 35. 18.
LEACH, R. H. (1970). *Vet. Rec.* 87. 319.
LEEFLANG, P., HOFLAND, G., & DE VRIES, T. J. (1969). *Neth. J. Vet. Sci.* 2. 181.
LINDQVIST, K. (1960). *J. Infect. Dis.* 106. 162.
MORTON, H. E., & ROBERTS, R. J. (1967). *Proc. Soc. Exp. Biol. Med.* 125. 538.
NICOLET, J., WANNER, M., STURZENEGGER, N., MESSERLI, J., & DE MEURON, P. A. (1974). *Schweiz. Arch. Tierheilk.* 116. 435.
PAVLOV, P., MILANOV, M., & TSCHULEW, D. (1964). *Zentbl. Bakt. I.* (*Orig.*) 194. 439.
RAKE, G. W. (1948). In Bergey's Manual of Determinative Bacteriology, 6th Ed. Baltimore, Md. Williams and Wilkins Co.
STEPHENSON, E. H., STORZ, J., & HOPKINS, J. B. (1973). *J. Am. vet. med. Ass.* 163. 1179.
SPRADBROW, P. B. (1968). *Aust. vet. J.* 44. 117.
SPRADBROW, P. B. (1971). *Vet. Rec.* 88. 615.
SPRADBROW, P. B., & MARLEY, J. (1971). *Aust. vet. J.* 47. 116.
STORZ, J., PIERSON, R. E., MARRIOT, M. E., & CHOW, T. L. (1967). *Proc. Soc. Exp. Biol. Med.* 125. 857.
SURMAN, P. G. (1968). *Aust. J. biol. Sci.* 21. 447.
SURMAN, P. G. (1973). *Aust. J. Exp. Biol. and Med. Sci.* 51. 589.
TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C., & CHANOCK, R. M. (1966). *J. Hyg. Camb.* 64. 91.
WOOD, D. R., WATSON, W. A., & HUNTER, D. (1965). *Vet. Rec.* 77. 551.

# Type II ostertagiasis in adult cattle

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**During the spring of 1975 outbreaks of ostertagiasis affecting adult cattle were recorded from several beef herds. Two of these outbreaks, one involving an autumn calving herd and the other spring calving, were investigated in detail. The clinical, biochemical, haematological, parasitological and pathological findings are described and were similar to those characteristic of type II ostertagiasis in immature cattle.**

IN Scotland, as elsewhere in the more temperate parts of the world, ostertagiasis remains a common and economically significant disease of immature cattle (Anderson and others 1965, Armour 1970, 1975). Under certain circumstances, however, the condition may also arise as a clinical entity in adult cattle (Bailey 1956, Becklund 1962, Smith and Jones 1962, Hotson 1967, Wedderburn 1970, Armour 1975) and during the past five years, particularly since admissions of beef cattle to the Department of Veterinary Medicine, Glasgow, have increased, we have become aware of a rise in the number of such cases. The majority of affected animals have been single-suckling heifers and cows although a higher proportion than expected has been beef bulls; in all cases, confirmation of diagnosis has been made by plasma pepsinogen estimations (Anderson and others 1965) and post mortem examination. In many of the instances where a reliable history had been obtained, it was inferred that the problem was confined to a single animal but further investigations have indicated that this was not necessarily correct. During the spring of 1975, two farms (A and B) were investigated on which ostertagiasis was found to be widespread among the adult cattle.

## Materials and methods

Farms A and B were situated in Berwickshire and Ayrshire respectively. Visits were carried out in late May and June in order to ascertain the background and history of the problems; three affected cattle were acquired for further study. On admission to the department of veterinary medicine, routine biochemical, haematological and faecal examinations were carried out. Plasma pepsinogen concentrations were measured in milli-international units (mu) tyrosine (Edwards and others 1960) and levels in excess of 3000 were considered to be significantly elevated. After a variable period, the animals were slaughtered and post mortem procedures were as described by Anderson and others (1965).

## Results

### FARM A

The beef herd on Farm A had been built up from approximately 50 animals to over 200 in 18 months, mostly by the purchase of locally-bred in-calf heifers. The bulk of the herd calved during the autumn of 1974, and the poor condition of many of the adults at that time was attributed to reduced herbage growth during a very dry summer.

In July 1974, a bull and two heifers were confirmed as suffering from type I ostertagiasis and by October, several more were observed to be diarrhoeic. The following spring, while the animals were still housed, a large number of adults showed weight loss, weakness and profuse diarrhoea, with these signs being most severe in the younger adults. Diarrhoea had been preceded by marked weight loss in the majority of instances. In all, 24 animals required repeated anthelmintic treatments, 10 died or were slaughtered and several were still thin and/or diarrhoeic six months later.

The grazing history was as follows: approximately 140 adults were moved to 540 acres of rough grazing after their calves were weaned in May 1974. They remained there until September when they were moved to a 70-acre permanent

# THE COMPARISON AND CHARACTERISATION OF GLYCOLYTIC MYCOPLASMAS ISOLATED FROM THE RESPIRATORY TRACT OF SHEEP

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PLATES III AND IV

THE original isolations of glycolytic mycoplasmas in Scotland were made by Mackay, Nisbet and Foggie (1963) from cases of sheep pulmonary adenomatosis (SPA). Identical (" Type A ") mycoplasmas were later isolated from pneumonic but not from normal lungs of sheep, and from the upper respiratory tract of apparently healthy sheep (Mackay, cited by Cottew and Leach, 1969). Krauss and Wandera (1970) compared mycoplasmas recovered from cases of SPA in Kenya with other mycoplasmas, including the Scottish MS strain originally isolated by Mackay from a case of SPA. The Scottish strain was placed in one of three serogroups.

Mycoplasmas recovered from lambs and sheep in Queensland, Australia, by St George *et al.* (1971) have been characterised by Carmichael *et al.* (1972), and the name *Mycoplasma ovipneumoniae* proposed. Glycolytic mycoplasmas isolated from the respiratory tract of sheep in Victoria, Australia (Cottew, 1971; Furlong and Cottew, 1973), and in New Zealand (Clarke, Brown and Alley, 1974) were identical to the Queensland strains of *M. ovipneumoniae*.

The purpose of this investigation was to compare the biochemical and serological reactions of glycolytic mycoplasmas isolated from SPA cases, pneumonic sheep and apparently healthy sheep in Scotland with a Queensland strain of *M. ovipneumoniae*.

## MATERIALS AND METHODS

*Strains examined.* These are listed in table I. All strains isolated in this laboratory were cloned at least four times by serial transfer of single colonies from agar plates to broth. The possibility that the strains were L-forms was eliminated by five consecutive subcultures on solid medium (OA) from which bacterial inhibitors had been omitted. No reversion to bacterial forms occurred.

*Isolation media.* Broth medium (OB) comprised (v/v) 20% of Brain-Heart Infusion (Oxoid Ltd., London), 60% of Medium 199 (Burroughs Wellcome, Beckenham, Kent), 10% of inactivated swine serum and 10% of fresh yeast extract (Marmion, 1967), supplemented with ampicillin, 1 mg per ml (" Penbritin ", Beecham Veterinary Products, Crawley, Sussex) thallium acetate 0·025% (w/v) and phenol red 0·006% (w/v). The medium was adjusted to *p*H 7·6-7·8 with M NaOH. Solid medium (OA) differed in containing 15% (v/v) of

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TABLE I

*Strains of ovine glycolytic mycoplasmas examined*

| Strain number | Origin of strains | |
|---|---|---|
| | Material | Animal |
| 7Z97 | Lung | Naturally-occurring case of SPA |
| 8Z56 | Lung | Naturally-occurring case of SPA |
| 611 | Lung | Naturally-occurring case of SPA |
| 652 | Lung | Naturally-occurring case of SPA† |
| 658 | Lung | Experimental SPA case produced by the intra-tracheal inoculation of SPA lung material |
| 650/21 | Nasal swab | Apparently healthy 6-month-old lamb |
| 672/9 | Nasal swab | One of a flock of housed adult sheep showing pneumonic symptoms |
| 956/2 | Nasal swab | One of a flock of ewes at grass showing pneumonic symptoms |
| 697 | Lung | Housed lamb with pneumonia |
| Y98* | ? | Sheep |

* The Queensland strain of *Mycoplasma ovipneumoniae* obtained from Dr T. D. St George.
† Isolated initially in primary sheep-lung cultures.
SPA = Sheep pulmonary adenomatosis.

inactivated swine serum, 55% (v/v) of Medium 199, and Agarose (B.D.H., Poole, Dorset) 0·75% (w/v). The Agarose was autoclaved with the Brain-Heart Infusion and the other constituents were added when the suspension had cooled to 56°C.

*Biochemical test media.* The basic media, EA and EB, were based on OA and OB respectively, but with Eagle's Minimal Essential Medium (MEM) substituted for Medium 199 and phenol red omitted unless specifically required. All strains were subcultured twice in EB before being tested. Paired broth cultures were incubated aerobically and, by being overlaid with sterile paraffin, anaerobically. Paired agar plates were incubated, one in a candle-jar and the other in an atmosphere of 95% $N_2$ and 5% $CO_2$, as described by Aluotto *et al.* (1970). For all biochemical tests, controls included test media inoculated with sterile broth or agar blocks.

*Rabbit broth* (RB). For the production of hyperimmune serum in rabbits strains were grown in RB, which was based on OB but with rabbit infusion broth (Taylor-Robinson *et al.*, 1963) and inactivated rabbit serum substituted for brain-heart infusion and swine serum respectively.

All cultures were incubated at 37°C. Routine broth cultures were incubated aerobically and cultures on solid medium in a candle-jar.

*Biochemical tests.* The oxidation-fermentation, phosphatase production, optochin sensitivity and tetrazolium, tellurite and methylene-blue reduction tests were performed according to the methods of Aluotto *et al.* (1970) but with EA or EB as the basic medium. In addition, the complete methods and media of Aluotto *et al.* (1970) were used for the hydrolysis of gelatin and for the casein and serum digestion tests.

The inhibitory effect of 0·02% methylene blue on 24-h broth cultures was investigated by the method of Kraybill and Crawford (1964) but employing EB as the culture medium. The test for the hydrolysis of aesculin was performed by the method of Williams and Wittler (1971), with EA as the basic medium.

The determination of specific substrate requirement was performed as follows. The medium used (TSM) comprised (v/v) 60% of MEM, 18% of Hanks' balanced salt solution (BSS), 10% of Brain-Heart Infusion, 1% of Serum Fraction (Difco Laboratories, West

GLYCOLYTIC MYCOPLASMAS FROM SHEEP

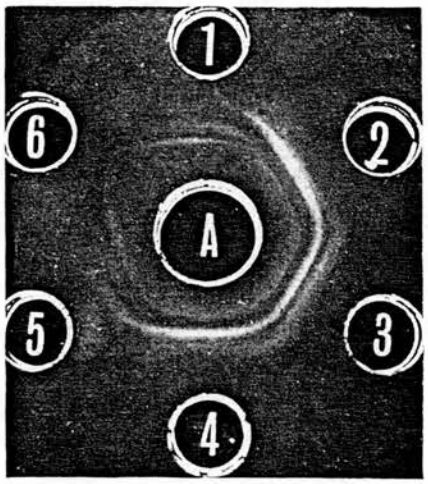


FIG. 1.—Agar-gel double diffusion with antiserum to strain 658 in the centre well (A), concentrated mycoplasma suspensions in wells 1–5 (1 = no. 956/2, 2 = no. 611, 3 = no. 658, 4 = no. 697, 5 = no. Y98), and normal rabbit serum in well 6.

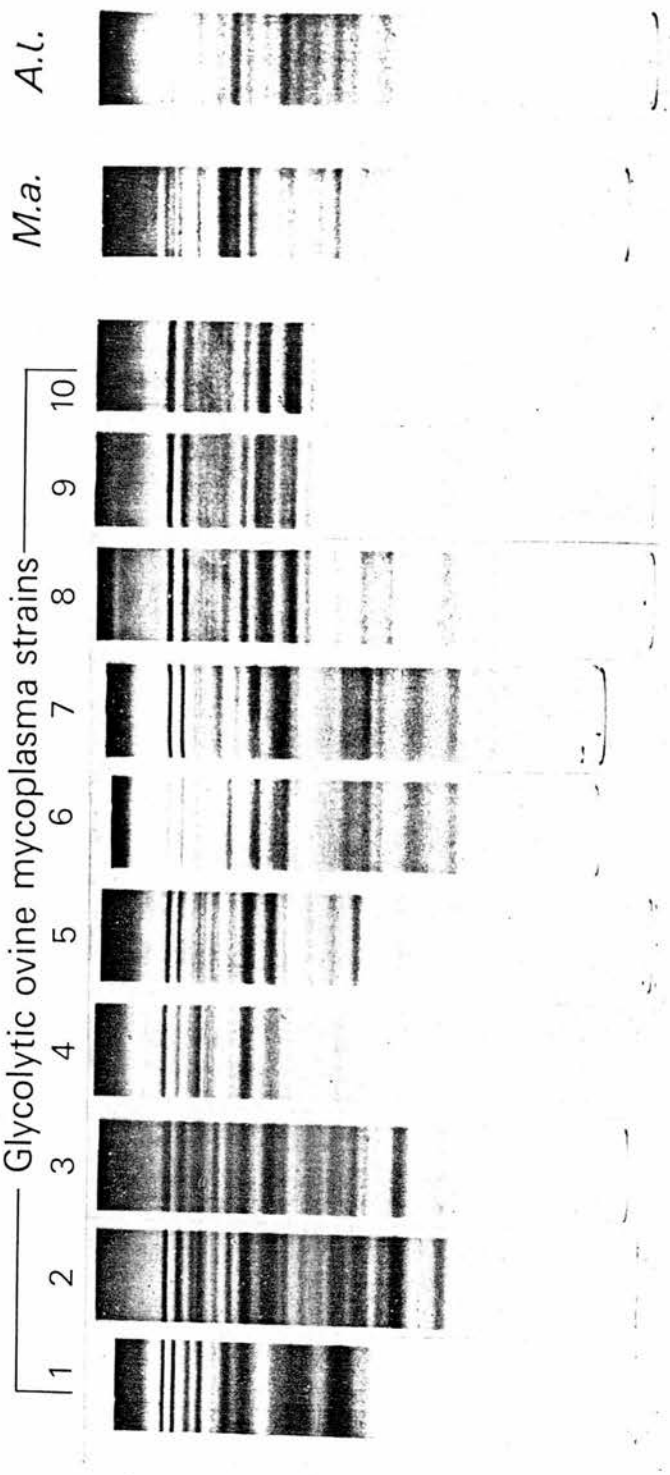


FIG. 2.—Polyacrylamide-gel electrophoresis with ovine mycoplasmas. 1 = no. 7Z97, 2 = no. 956/2, 3 = no. 652, 4 = no. 650/21, 5 = no. 611, 6 = no. Y98, 7 = no. 697, 8 = no. 658, 9 = no. 8Z56, 10 = no. 672/9, *M.a.* = *Mycoplasma arginini*, and *A.l.* = *Acholeplasma laidlawii*.

Molesey, Surrey), 1% of fresh yeast extract and 10% of test substrate solution, and was supplemented with ampicillin, thallium acetate and phenol red at the same concentrations as in OB. The BSS was prepared without glucose. Before the addition of the test substrates, the medium was exhaustively dialysed against several changes of BSS, filtered through Millipore filters of 0·22-$\mu$m average pore diameter and adjusted to *p*H 7·2. The test substrates contained 10% (w/v) of either glucose, arginine or urea in distilled water, the control substrate being BSS. Suspensions of organisms were prepared according to the method of Edward (1971), the washing and suspending medium (PM) being BSS with 1% (v/v) of Serum Fraction and the same concentrations of antibacterial supplements as in OB. The test method consisted of pipetting 0·2 ml of the organism suspension or sterile PM into 1·8 ml of test and control TSM, and measuring the *p*H of the broth after aerobic incubation for 1–2 days.

Ability to form " films and spots " was examined on (*a*) EA containing 10% (v/v) of concentrated egg yolk emulsion (Difco Laboratories) and 5% (v/v) of inactivated swine serum (Fabricant and Freundt, 1967), and (*b*) EA incorporating (v/v) 20% of inactivated swine serum and 50% of MEM.

Hydrogen peroxide production was detected by the methods of Lind (1970) and Cole, Ward and Martin (1968), with EA as the basic medium.

*Haemolysis.* Haemolysis of erythrocytes was examined by the method of Somerson, Taylor-Robinson and Chanock (1963). Guinea-pig, horse, sheep and human group O erythrocytes were used. The results were read 24 and 48 h after overlayering the agar-erythrocyte suspension on to the mycoplasma colonies and were classified according to the system of Aluotto *et al.* (1970).

*Sensitivity to antibacterial substances.* Sensitivities of the strains to digitonin (Sigma Chemical Co.) 1·5% (w/v), sodium polyanethol sulphonate (SPS) (Sigma Chemical Co.) 5% and 20% (w/v), optochin, (Oxoid Ltd) 0·025% (w/v) and 11 antibiotics were determined with impregnated disks by the flooded-plate technique. All antibiotic disks were obtained from Mast Laboratories Ltd, Liverpool, except those containing tylosin (Elanco Products Ltd, London). Filter-paper disks impregnated with digitonin and SPS were prepared by the method of Freundt *et al.* (1973*a*). Undiluted 24-h broth cultures were flooded on to dried OA plates 85 mm in diameter, the excess fluid was removed and the plates were dried for 15 min. before peripheral placement of the disks. The plates were incubated aerobically, and the results read 4–6 days later.

*Filtration studies.* Filtration experiments were performed on 24-h broth cultures by means of Millipore filters of 0·80 $\mu$m, 0·45 $\mu$m and 0·22 $\mu$m average pore diameter. Titrations of the number of organisms were performed before and after each filtration.

*Antigens for hyperimmune serum production.* Five strains (nos. 611, 658, 697, 956/2 and Y98) were selected for the production of antiserum. The strains were grown in RB for 2–4 days, centrifuged at 40 000*g* for 30 min. at 4°C in a MSE 18 angle-head centrifuge, washed three times in BSS and finally resuspended in sterile distilled water to a 200-fold concentration of the original volume. After homogenisation, the suspension was disintegrated ultrasonically with a MSE-Mullard Ultrasonic Disintegrator 60W by the method of Mackay (1969). After estimations for total protein, the concentrations of the mycoplasma suspensions were adjusted to 2 mg of protein per ml. Because of import regulations, it was necessary to incorporate formaldehyde in the diluent of strain Y98 to a final concentration of 1 in 2000. A portion of the mycoplasma suspensions was emulsified in an equal volume of a mixture of Bayol and Falba containing 1 mg per ml BCG. The remainder was retained as the intravenous (i.v.) vaccine.

*Antigens for serological tests.* The methods employed were the same as for antigen production for the preparation of hyperimmune sera except that the medium employed was OB, and that the organisms were finally resuspended in phosphate buffered saline (PBS) *p*H 7·2. After total protein estimations, the suspensions were stored in portions at −20°C.

*Antigens for polyacrylamide-gel electrophoresis* (PAGE). All strains in table I as well as *Mycoplasma arginini*, strain G230 (no. NCTC 10129) and *Acholeplasma laidlawii*, strain PG8 (no. NCTC 10116) were grown in 200 ml of OB supplemented with arginine 1% (w/v) for

2 days, then centrifuged at 40 000*g* for 30 min. The deposits were washed once in BSS and suspended in PBS.

*Antiserum production.* New Zealand rabbits were vaccinated by the method of Morton, and Roberts (1967), 3 ml of antigen in adjuvant being given by injection into the muscle, dermis and footpad, followed 3 weeks later by 2 ml intramuscularly. The growth-inhibiting titres obtained by this technique alone were unsatisfactory however, and two supplementary i.v. injections of 0·5 ml each were given 1 week apart and 4–5 weeks after the second intramuscular injection. The rabbits were bled 1 week later.

*Agar-gel double diffusion (AGDD) test.* The method of Ouchterlony (1948) was used. The centre well was 10 mm in diameter and 10 mm distant from the peripheral wells, which were 7 mm in diameter. The antigen suspensions contained 1·0–1·4 mg of protein per ml. The gels were kept in a moist atmosphere at room temperature for 5 days before being photographed.

*Indirect haemagglutination (IHA) test.* The method used was essentially that of Herbert (1967), the test being performed with fresh sheep RBC. The mycoplasma suspensions were found to agglutinate sheep and guinea-pig RBC, and were therefore absorbed for 1 h at room temperature with 1/4 of their volume of washed, packed sheep RBC. The optimal preabsorption concentration of antigen was found by chequer-board titrations to be 0·5 mg of protein per ml. The 1% " serum-saline " used for washing the sensitised and control cells and for finally suspending the RBC contained inactivated serum from a hysterectomy-produced, colostrum-deprived lamb that had never been exposed to mycoplasmal infection; otherwise the diluent and washing medium used throughout was PBS. The tests were performed in U-bottomed microtitration plates (Cookes Engineering Co., Alexandria, Virginia) with a 1·5% cell suspension, and were read after 18 h at 4°C. All sera were inactivated at 56°C for 30 min. before titration. The controls comprised known negative sera, the titration of four dilutions (1 in 10 to 1 in 80) of each test serum against control cells, and the provision of sensitised and control cells with serum-saline only.

*Growth-inhibition (GI) test.* The method of Dighero, Bradstreet and Andrews (1970) was used. The strains were found to vary in their ability to grow on solid medium; in addition, the titre of the broth culture used to inoculate the plates affected the size of the inhibition zones. Accordingly, OA plates, 85 mm in diameter were flooded with decimal dilutions of each strain as described above, the antiserum disks being tested in duplicate at each dilution. The results were read up to 10 days later to permit full growth of colonies and expression of antiserum effect, and were taken from that dilution which allowed optimal development of inhibition zones commensurate with ease of reading.

*Metabolic-inhibition (MI) test.* The method of Taylor-Robinson *et al.* (1966), was used, the broth being OB. Fresh guinea-pig serum was included at a final concentration of 5·7% (v/v).

*PAGE.* Phenol-acetic acid-water (2 : 1 : 0·5 v/v/v) extraction of the sedimented organisms and PAGE of the extracted proteins was essentially as described by Forshaw (1972). The gels, which contained 35% (v/v) acetic acid, 5M urea and 7·5% (w/v) acrylamide, were stained with 1% amido black. Background stain was removed electrolytically. The gels were photographed as described by Fiske (1974).

*Statistical analysis of results.* Antibody-titre ratios between the five strains tested in cross-serological reactions were calculated from the MI and GI tests by the method of Archetti and Horsfall (1950) as applied by Goiš *et al.* (1974). The rank correlation coefficient of the two tests for each antiserum was calculated by the method of Spearman (1904), the rankings of antibody-titre ratios being scored from 1 (homologous system) to 5 (greatest difference from homologous system).

## RESULTS

### *Biochemical reactions*

The biochemical reactions of the 10 strains examined are summarised in table II. In general, the reactions of all strains were very similar, differing

TABLE II

*Summary of the biochemical and haemolytic reactions of the 10 ovine mycoplasma strains examined*

| Biochemical tests | Reaction* |
|---|---|
| Glycolysis | + |
| Oxidation-fermentation | Fermentation |
| Phosphatase production | − |
| Tetrazolium reduction | |
| (i) aerobic | ± to + |
| (ii) anaerobic | + |
| Tellurite reduction aerobic and anaerobic | Complete or partial growth inhibition (no reduction) |
| Methylene-blue reduction (0·003%) | |
| (i) aerobic | ± to + |
| (ii) anaerobic | ± to ++ |
| Methylene-blue inhibition (0·02%) | Inhibition |
| Hydrogen-peroxide production | |
| (i) Lind (1970) | ± to ++ |
| (ii) Cole *et al.* (1968) | + |
| Casein digestion | − |
| Serum digestion | − |
| Gelatin hydrolysis | − |
| Aesculin hydrolysis | − |
| " Films and spots " production | |
| (i) 20% swine serum | − |
| (ii) 10% egg yolk emulsion plus 5% serum | − |
| Haemolysis of erythrocytes | |
| (i) sheep | αpr or Br |
| (ii) guinea-pig | αp or B |
| (iii) horse | αp or B |
| (iv) human group O | α or αp |

* − = Negative, ± = weak, + = moderate, and ++ = strong reaction; α = green zone around colonies; αp = clear area, with some unhaemolysed erythrocytes remaining in the zone around colony; B = clear, cell-free zone around colonies; r = green ring around indicated zone type.

only in degree. The tests in which the greatest variations were observed were methylene-blue reduction (anaerobic conditions) and hydrogen-peroxide production as determined by the method of Lind (1970).

### *Sensitivity to antibacterial substances*

The sensitivities of the strains to optochin, digitonin, SPS, and 11 antibiotics are given in table III. All strains were sensitive to digitonin and SPS, although sensitivity to SPS (5%) was low, possibly because of the use of undiluted broth cultures as inocula. All strains were also sensitive to kanamycin, chlortetracycline, oxytetracycline, gentamicin and tylosin, and resistant to erythromycin, streptomycin, oleandomycin, polymixin B and nystatin. Two strains only (nos. 956/2 and 650/21) were sensitive to neomycin.

TABLE III

*Sensitivity of ovine mycoplasma strains to optochin, digitonin, SPS, antibiotic and fungicide disks*

| Strain number | Size of zone of inhibition (mm) of growth of the stated strain produced by | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OP | DIG | SPS 5% | SPS 20% | K | E | NE | CT | S | OL | NY | OT | GM | PB | TY |
| 956/2 | <1 | 2 | 3 | 5 | 10 | <1 | 5 | 15 | 0 | 0 | 0 | >20 | 13 | 0 | 13 |
| 650/21 | <1 | 2·5 | 0·5 | 3 | 8 | 1 | 6 | 9 | 0 | 0 | 0 | 10 | 10 | 0 | 13 |
| Y98 | <1 | 1·5 | 2·5 | 6 | 10 | 0 | 0 | 15 | 0 | 0 | 0 | 6 | 6 | 0 | 5 |
| 697 | 1 | 1 | 1·5 | 3 | 15 | 0 | 1 | 11 | 0 | 0 | 0 | 10 | 5–10 | 0 | 15 |
| 652 | 1 | 2·5 | 1 | 1·5 | 5–10 | 0 | <1 | 13 | 0 | 0 | 0 | 15 | 10 | 0 | 15 |
| 658 | <1 | 3 | 1·5 | 3 | 5 | 0 | 1 | 15 | 0 | 0 | 0 | 15 | 8 | 0 | 10 |
| 7Z97 | <1 | 3 | 2·5 | 4 | 10 | 0 | 0 | 10 | 0 | 0 | 0 | 15 | 5 | 0 | 15 |
| 8Z56 | <1 | 4 | 2 | 5 | 13 | 0 | 0 | 15 | 0 | 0 | 0 | 15 | 5–10 | 0 | 15 |
| 611 | <1 | 1·5 | 1 | 6 | 5 | 0 | 0 | 12 | 1 | 0 | 0 | 10–15 | 4 | 0 | 10 |
| 672/9 | <1 | 2·5 | 2 | 3 | 10 | 0 | 1 | 16 | 0 | 0 | 0 | 15 | 10 | 0 | 10 |

OP = Optochin; DIG = digitonin; SPS = sodium polyanethol sulphonate; K = kanamycin (30 μg); E = erythromycin (5 μg); NE = Neomycin (10 μg); CT = chlortetracycline (25 μg); S = streptomycin (10 μg); OL = oleandomycin (5 μg); NY = nystatin (100 units); OT = Oxytetracycline (25 μg); GM = gentamicin (10 μg); PB = polymixin B (100 units); TY = tylosin (30 μg).

TABLE IV

*Filtration studies with two ovine mycoplasma strains*

| Filtration details | Titre (c.f.u. per ml) of strains | |
|---|---|---|
| | 658 | Y98 |
| Unfiltered | $1{\cdot}0\times10^{9}$ | $5{\cdot}5\times10^{8}$ |
| Filter 0·80 μm a.p.d. | $1{\cdot}1\times10^{7}$ | $6{\cdot}8\times10^{6}$ |
| Filter 0·45 μm a.p.d. | $6{\cdot}6\times10^{3}$ | $1{\cdot}1\times10^{3}$ |
| Filter 0·22 μm a.p.d. | 0 | 0 |

a.p.d. = Average pore diameter.
c.f.u. = Colony-forming units.
The filtrations were performed sequentially on one sample of each test strain.

### *Filtration studies*

The results for two strains are presented in table IV. The strains resembled *Mycoplasma dispar* (Gourlay and Leach, 1970) in their poor filtrability through Millipore filters. This might have been due to growth in clumps.

### *Agar-gel double diffusion tests*

The antigen suspensions of the five selected strains showed three to seven precipitin lines against each of the five prepared antisera. The number of lines

TABLE V

*Titres obtained in cross-titration of five ovine mycoplasma strains by the indirect haemagglutination test*

| Strain number | Titres for the stated strain of rabbit antisera prepared against strains | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | **81 920** | 163 840 | 40 960 | 327 840 | 81 920 |
| Y98 | 20 480 | **20 480** | 10 240 | 5120 | 5120 |
| 697 | 40 960 | 10 240 | **40 960** | 20 480 | 20 480 |
| 658 | 5120 | 640 | 2560 | **2560** | 2560 |
| 611 | 10 240 | 320 | 2560 | 1280 | **2560** |

Homologous titres are in bold type.

produced by each mycoplasma suspension varied little with different antisera. Fewest lines (three to four) were given by strains 956/2 and Y98. Strain 697 produced four to five lines against each antiserum, no. 658 produced four to six lines and no. 611 produced four to seven lines (see fig. 1).

### *Indirect haemagglutination tests*

The results of IHA cross-titration tests between the five strains to which antisera were raised are given in table V. No significant differences could be demonstrated between any of the strains. In the same tests four of the strains were titrated against antisera to 24 other mycoplasma species (table VI). These titrations revealed moderate reactivity against four antisera (*M. gallinarum*, *M. gallisepticum*, *M. neurolyticum* and *M. spumans*) and high reactivity to a further four antisera (*M. anatis*, *M. hyorhinis* (strains 7 and GDL), *M. maculosum* and *M. pulmonis*). However, of the strongly reacting antisera only the antiserum to *M. hyorhinis* (strain GDL) showed reactivity by the AGDD test, a single faint precipitin line developing between this antiserum and all five antigen wells.

### *Metabolic-inhibition and growth-inhibition tests*

Tables VII (MI tests) and VIII (GI tests) show the results of the titration of all 10 ovine mycoplasma strains against the antisera produced against five of the strains. All strains cross-reacted by these tests, confirming that they belonged to the same species. The MI and GI tests, however, showed antigenic differences among the strains tested, and a comparison for relatedness was made by the method of Goiš *et al.* (1974). The antibody-titre ratios shown in table IX were calculated from the results of the MI and GI serological tests only, as no intraspecific distinctions were made by the IHA tests. Strains 956/2 and Y98 were most closely related to each other and most dissimilar to 611, which like strain 658 was most closely related to strain 697. The rank correlation coefficient, a measure of the agreement between tests in the ranking of

TABLE VI

*Titres obtained in indirect haemagglutination tests with ovine mycoplasma strains against reference antisera to other mycoplasmas*

| Antiserum against strain | Titres of the stated antiserum as demonstrated by ovine mycoplasma strain | | | |
|---|---|---|---|---|
| | 956/2 | Y98 | 658 | 611 |
| *M. anatis* (1340)* | 640–1280 | 80 | 320–640 | 640 |
| *M. arginini* (G230)† | ND | 10 | 20 | 10–20 |
| *M. arthritidis* (PG6) | ND | 10 | 20 | 20 |
| *M. canis* (PG14) | 40 | 20 | 40–80 | 40 |
| *M. capri* (PG3) | 10 | <10 | <10 | <10 |
| *M. fermentans* (PG18) | 10 | <10 | 10 | <10 |
| *M. gallinarum* (PG16) | 160–320 | 80 | 80 | 80 |
| *M. gallisepticum* (PG31) | 80 | 160–320 | 160 | 80–160 |
| *M. granularum* (BTS 39) | 10 | <10 | 10 | <10 |
| *M. hyorhinis* (7) | 320–640 | 160–320 | 320 | 320 |
| *M. hyorhinis* (GDL) | >1280 | 1280 | 640–1280 | 1280 |
| *M. iners* (PG30) | 80 | 40 | 40 | 40 |
| *M. maculosum* (PG15) | 640 | >1280 | >1280 | >1280 |
| *M. meleagridis* (17529) | 40 | 10 | 20 | 40 |
| *M. mycoides* (PGl) | <10 | <10 | <10 | <10 |
| *M. neurolyticum* (Type A) | ND | 80 | 320 | 320 |
| *M. orale* 1 (CH-19299) | ND | 20 | 20 | 40 |
| *M. orale* 2 (CH-20247) | 40 | ND | 40 | 20–40 |
| *M. orale* 3 (DC-333) | 40 | <10 | 20 | 20 |
| *M. pneumoniae* (FH-Liu) | 10 | 10 | 10 | 10 |
| *M. pulmonis* (Ash) | 1280 | 640–1280 | 320–640 | 1280 |
| *M. salivarium* (PG20) | <10 | <10 | <10 | <10 |
| *M. spumans* (PG13) | 320 | ND | 40 | 40 |
| *Acholeplasma laidlawii* (PG8) | 40 | 40 | 80 | 80 |

All sera were titrated in duplicate.
ND = not done.
* = Strain designations are given in parenthesis.
† = Prepared by the authors by the method described in the text.
All other mycoplasma reference antisera were obtained from the National Institutes of Health, Bethesda, USA.

relatedness, was 1·0 for strains 956/2, Y98 and 658 (significant to 5% level), 0·925 for strain 697 (significant to the 10% level) and 0·675 for strain 611 (not significant). Consideration of the relation, calculated or apparent, of all 10 strains emphasises the apparent polarisation of SPA and non-SPA strains at opposite ends of the antigenic spectrum, strains 658, 7Z97, 8Z56 and 611 appearing at one end and strains 956/2, 650/21 and Y98 at the other. However, strains 652 and 672/9 did not fit this observation, although the animal from which strain 672/9 was isolated belonged to a flock in which SPA occurred.

Strains 658 and Y98 were also tested by the MI test against 40 hyperimmune sera, including the 24 antisera listed in table VI, and the following 15 antisera obtained from the FAO/WHO International Reference Centre for Animal Mycoplasmas, Aarhus, Denmark: *M. agalactiae* subsp. *agalactiae* (PG2), *M. agalactiae* subsp. *bovis* (Donetta), *M. bovigenitalium* (PG11), *M. bovirhinis* (PG43), *M. bovoculi* (M165/69), *M. capricolum* (Calif. Kid), *M. dispar* (462/2),

TABLE VII

*Comparison of 10 ovine mycoplasma strains by the metabolic-inhibition test*

| Strain number | Titres of inhibition of the indicated strain by antisera prepared against strain | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | **5120** | 320 | 320 | 80 | 40 |
| 650/21 | 320 | <10 | 40 | 10 | 10 |
| Y98 | 640 | **640** | 640 | 640 | 160 |
| 697 | 320 | 20 | **2560** | 160 | 320 |
| 652 | 160 | 20 | 320 | 40 | 20 |
| 658 | 160 | 10 | 320 | **2560** | 160 |
| 7Z97 | 80 | 40 | 2560 | 5120 | 160 |
| 8Z56 | 80 | 40 | 640 | 1280 | 40 |
| 611 | 20 | 10 | 40 | 40 | **1280** |
| 672/9 | 80 | 10 | 40 | 40 | 160 |

Homologous titres are in bold type.

TABLE VIII

*Comparison of 10 ovine mycoplasma strains by the growth-inhibition test*

| Strain number | Size (mm) of the zone of inhibition* of the stated strain produced by rabbit antiserum to strain | | | | |
|---|---|---|---|---|---|
| | 956/2 | Y98 | 697 | 658 | 611 |
| 956/2 | **11·0–11·5** | 2·5 | 3·5 | 3.5–4·5 | 3·0–3·5 |
| 650/21 | 4·0–5·5 | 1·0–2·0 | 4·5 | 2·5–3·0 | 1·0–1·5 |
| Y98 | 9·5 | **7·5–9·5** | 7·5–10·0 | 8·0–10·5 | 2·0–3·5 |
| 697 | 2·5–4·0 | 1·0 | **5·5** | 3·5–4·0 | 3·5 |
| 652 | 6·0 | 1·5–2·0 | 9·0–10·0 | 3·0–3·5 | 3·5 |
| 658 | 3·0 | 1·5–2·0 | 4·5–5·0 | **10·5–11·5** | 1·5 |
| 7Z97 | 3·5–4·0 | 0·5 | 5·0–6·0 | 13·0–14·0 | 3·0 |
| 8Z56 | 4·0–5·0 | 2·5–3·0 | 6·5–7·0 | 14·0 | 2·0–3·0 |
| 611 | 3·0 | 1·0 | 3·0 | 3·4–4·0 | **8·0** |
| 672/9 | 5·5 | 2·0 | 3·5–4·0 | 4·0 | 5·0–5·5 |

Homologous reactions are in bold type.

* The results indicate the range of zone widths from duplicate tests of each antiserum, taken from the concentration of inoculum that provided maximal zone development commensurate with ease of reading.

*M. gateae* (CS), *M. putrefaciens* (KS-1), bovine serogroups L (B144 P), 7 (PG50), and 8 (PG51), and ovine serogroups 5 (Goat 145), 6 (Goat 189) and 11 (2-D). Antiserum to *M. verecundum* (107), obtained from Drs Gourlay and Howard of IRAD, Compton, nr. Newbury, Berks, was also included in the tests. No cross-reactions were noted between strain 658 or strain Y98 and any of the antisera tested.

TABLE IX

*Antigenic relatedness among five ovine mycoplasma strains calculated from the results of metabolic-inhibition and growth-inhibition tests*

| Strain number | Homologous/heterologous ratios obtained in the stated test with antiserum against strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 956/2 | | Y98 | | 697 | | 658 | | 611 | |
| | MI | GI | MI | GI | MI | GI | MI | GI | MI | GI |
| 956/2 | 1/1 | 1/1 | — | — | — | — | — | — | — | — |
| Y98 | 1/4 | 1/2·0 | 1/1 | 1/1 | — | — | — | — | — | — |
| 697 | 1/11·3 | 1/2·3 | 1/11·3 | 1/2·3 | 1/1 | 1/1 | — | — | — | — |
| 658 | 1/32 | 1/3·2 | 1/16 | 1/2·4 | 1/11·3 | 1/2·0 | 1/1 | 1/1 | — | — |
| 611 | 1/90·5 | 1/3·3 | 1/22·6 | 1/4·7 | 1/16 | 1/2·5 | 1/22·6 | 1/3·8 | 1/1 | 1/1 |

The homologous ratio is defined as 1/1, and the degree of antigenic relatedness is indicated by the closeness of the fractions to unity.

### *Polyacrylamide-gel electrophoresis*

The protein-banding patterns obtained by PAGE (fig. 2) demonstrated a very close resemblance between all 10 strains. The patterns obtained with *M. arginini* and *A. laidlawii* showed no similarities to those of the 10 strains under examination.

## DISCUSSION

Nine strains of glycolytic mycoplasma isolated from the respiratory tract of sheep in Scotland, and an Australian strain (Y98) of *M. ovipneumoniae*, were almost completely homogeneous in their reactions in 14 biochemical tests, and their sensitivities to optochin, digitonin, SPS, and 11 antibiotics. Where the same tests were performed, the results agreed with those of Carmichael *et al.* (1972) and Furlong and Cottew (1973). Furthermore, the appearance of colonies of the Scottish strains and the appearance of organisms from broth cultures stained with MacNeal's stain corresponded with the descriptions given by these authors. Failure to hydrolyse aesculin and sensitivity to SPS and digitonin have been accepted as evidence of belonging to the family Mycoplasmataceae (Williams and Wittler, 1971; Freundt *et al.*, 1973*a*; Ernø and Stipkovits, 1973) and *M. ovipneumoniae* can therefore be regarded as a member. The resistance shown by all strains to erythromycin is of interest, because only two of 17 species of mycoplasma tested by Ernø and Stipkovits (1973) showed a similar resistance.

The protein-banding patterns obtained with PAGE substantiated the apparent homogeneity of the strains studied. In the IHA test, high levels of sensitivity were obtained with fresh sheep RBC, but no significant differences between strains were revealed, and in fact considerable cross-reactions with antisera to other mycoplasma species occurred. A similar lack of specificity with a high degree of cross-reaction between species was also obtained with

fresh sensitised cells by Freundt *et al.* (1973*b*). Serological intraspecific differences have been shown to be due to membrane antigens: where non-membrane antigens participate in a serological test, as in the complement-fixation test, the ability to make intraspecific distinctions becomes lost (Hollingdale and Lemcke, 1970). Despite the finding that in the IHA test for *M. hominis* (Hollingdale and Lemcke, 1969) only membrane antigens participate, it is likely that non-membrane antigens are also involved in IHA tests with *M. ovipneumoniae*. This would account not only for the inability to differentiate between strains with this test, but also for the heterologous reactions that occurred with antisera to other species of mycoplasma.

The AGDD tests were also more useful in demonstrating similarities than differences between strains, because there was little difference in the number of precipitin lines produced by each antigen suspension irrespective of the opposing antiserum. For *M. pulmonis*, Deeb and Kenny (1967) found that heated antigens were required to obtain sub-type specificity with this test, although this finding was not corroborated by Forshaw and Fallon (1972). Pollack, Somerson and Senterfit (1969) showed that *M. pneumoniae* harvested from culture media that had become acid lost its reactivity in immunodiffusion tests. This effect was considered to be due to activation of previously released mycoplasmal enzymes rather than to a direct hydrogen-ion effect. The bulk culture media used to produce AGDD antigens all achieved final *p*H levels of 6·2–6·4. It is possible that the poor antigenicity of strains 956/2 and Y98 in these tests compared with that of the other strains examined may have been due to their greater production of, or sensitivity to, such enzymes.

This phenomenon may also be partly responsible, in conjunction with the obligatory treatment of the hyperimmunising antigen with formaldehyde, for the poor performance of Y98 antiserum in the MI and GI tests.

The MI and GI tests have been found to be extremely specific (Purcell, Chanock and Taylor-Robinson, 1969; Freundt *et al.*, 1973*b*), and therefore suitable for the demonstration of intraspecific differences (Hollingdale and Lemcke, 1970; Forshaw and Fallon, 1972; Haller, Boiarski and Somerson, 1973; Goiš *et al.*, 1974). Both tests revealed considerable antigenic heterogeneity among the 10 strains of *M. ovipneumoniae*, with possible polarisation of the SPA strains towards one end of the spectrum and non-SPA strains towards the other. The practical implications in the application of the GI test have been pointed out by Goiš *et al.* (1974), namely that, for routine identification of fresh isolates, antisera to several antigenically different strains would be required. Similar implications in the use of the MI test in sero-epidemiological studies have been observed by Hollingdale and Lemcke (1970), namely that full coverage would be achieved only by testing each serum against several strains of the mycoplasma under consideration. For this reason, the highly sensitive but moderately specific IHA test is probably more suitable for such studies.

In the characterisation of the Y98 biotype (Carmichael *et al.*, 1972), serological comparison was made with only 12 other ovine and caprine strains. Furlong and Cottew (1973) compared their Victoria strains with the Queensland

Y98 biotype and with *Mycoplasma dispar*. Thus, the serological relation of the organism to other members of the Mycoplasmatales has been insufficiently investigated. The negative results obtained in the MI tests of strains 658 and Y98 against 40 hyperimmune sera to 33 named mycoplasma species or subspecies and 6 serogroups of bovine or ovine origin, though not fully conclusive, indicates that these strains should be considered as a distinct species with the name *M. ovipneumoniae* (WHO/FAO Committee on Comparative Virology, 1974).

## Summary

Nine strains of glycolytic mycoplasmas isolated from the respiratory tract of apparently healthy sheep, pneumonic sheep and sheep with pulmonary adenomatosis (SPA) were compared with a Queensland strain (Y98) of *Mycoplasma ovipneumoniae*. All strains were very similar in their reactions in 14 biochemical tests and in their sensitivities to optochin, digitonin, sodium polyanethol sulphonate, and 11 antibiotics. Polyacrylamide-gel electrophoresis and serological cross-reactions by the agar-gel double diffusion, metabolic-inhibition (MI) and growth-inhibition (GI) tests also showed that all strains could be classified as *M. ovipneumoniae*.

The MI and GI tests, however, showed considerable intraspecific differences among strains, with apparent polarisation of SPA strains and non-SPA strains at opposite ends of the antigenic spectrum.

Two representative strains were tested by the MI test against antisera to 39 mycoplasma species or serogroups, with negative results.

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## REFERENCES

Aluotto, B. B., Wittler, R. G., Williams, C. O. and Faber, J. E. 1970. Standardized bacteriologic techniques for the characterization of mycoplasma species. *Int. J. syst. Bact.*, **20**, 35.

Archetti, I. and Horsfall, F. L. jr 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in ovo* with heterologous immune serum. *J. exp. Med.*, **92**, 441.

Carmichael, L. E., St George, T. D., Sullivan, N. D. and Horsfall, N. 1972. Isolation, propagation and characterization studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. *Cornell Vet.*, **62**, 654.

Clarke, J. K., Brown, V. G. and Alley, M. R. 1974. Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. *N.Z. vet. J.*, **22**, 117.

Cole, B. C., Ward, J. R. and Martin, C. H. 1968. Haemolysin and peroxide activity of mycoplasma species. *J. Bact.*, **95**, 2022.

Cottew, G. S. 1971. Characterisation of mycoplasmas isolated from sheep with pneumonia. *Aust. vet. J.*, **47**, 591.

Cottew, G. S. and Leach, R. H. 1969. Mycoplasmas of cattle, sheep and goats. *In* The Mycoplasmatales and the L-phase of bacteria, edited by L. Hayflick, *New York*, p. 527.

Deeb, B. J. and Kenny, G. E. 1967. Characterization of *Mycoplasma pulmonis* variants isolated from rabbits. II. Basis for differentiation of antigenic subtypes. *J. Bact.*, **93**, 1425.

DIGHERO, M. W., BRADSTREET, C. M. P. AND ANDREWS, B. E. 1970. Dried paper discs for serological identification of human mycoplasmas. *J. appl. Bact.*, **33**, 750.

EDWARD, D. G. FF. 1971. Determination of sterol requirement for Mycoplasmatales. *J. gen. Microbiol.*, **69**, 205.

ERNØ, H. AND STIPKOVITS, L. 1973. Bovine mycoplasmas: cultural and biochemical studies II. *Acta vet. scand.*, **14**, 450.

FABRICANT, J. AND FREUNDT, E. A. 1967. Importance of extension and standardization of laboratory tests for the identification and classification of mycoplasma. *Ann. N.Y. Acad. Sci.*, **143**, 50.

FISKE, P. J. 1974. Auxiliary cylindrical lenses for recording bands in narrow tubes. *Med. biol. Illust.*, **24**, 160.

FORSHAW, K. A. 1972. Electrophoretic patterns of strains of *Mycoplasma pulmonis*. *J. gen. Microbiol.*, **72**, 493.

FORSHAW, K. A. AND FALLON, R. J. 1972. Serological heterogeneity of *Mycoplasma pulmonis*. *J. gen. Microbiol.*, **72**, 501.

FREUNDT, E. A., ANDREWS, B. E., ERNØ, H., KUNZE, M. AND BLACK, F. T. 1973*a*. The sensitivity of Mycoplasmatales to sodium polyanethol sulphonate and digitonin. *Zbl Bakt. ParasitKde*, 1 Abt. Orig., **225**, 104.

FREUNDT, E. A., ERNØ, H., BLACK, F. T., KROGSGAARD-JENSEN, A. AND ROSENDAL, S. 1973*b*. Evaluation of reference reagents for mycoplasmas. *Ann. N.Y. Acad. Sci.*, **225**, 161.

FURLONG, S. AND COTTEW, G. S. 1973. Identity of mycoplasmas from sheep pneumonia in Queensland and Victoria. *Aust. vet. J.*, **49**, 215.

GOIŠ, M., KUKSA, F., FRANZ, J. AND TAYLOR-ROBINSON, D. 1974. The antigenic differentiation of seven strains of *Mycoplasma hyorhinis* by growth-inhibition, metabolism-inhibition, latex-agglutination, and polyacrylamide-gel electrophoresis. *J. med. Microbiol.*, **7**, 105.

GOURLAY, R. N. AND LEACH, R. H. 1970. A new mycoplasma species isolated from pneumonic lungs of calves (*Mycoplasma dispar* sp. nov.). *J. med. Microbiol.*, **3**, 111.

HALLER, G. J., BOIARSKI, K. W. AND SOMERSON, N. L. 1973. Comparative serology of *Mycoplasma pulmonis*. *J. infect Dis.*, **127** Suppl. S38.

HERBERT, W. J. 1967. Passive haemagglutination. *In* Handbook of experimental immunology, edited by D. M. Weir, *Oxford* and *Edinburgh*, p. 720.

HOLLINGDALE, M. R. AND LEMCKE, R. M. 1969. The antigens of *Mycoplasma hominis*. *J. Hyg., Camb.*, **67**, 585.

HOLLINGDALE, M. R. AND LEMCKE, R. M. 1970. Antigenic differences within the species *Mycoplasma hominis*. *J. Hyg., Camb.*, **68**, 469.

KRAUSS, H. AND WANDERA, J. G. 1970. Isolation and properties of mycoplasma from the respiratory tract of sheep with jaagsiekte in Kenya. *J. comp. Path. Ther.*, **80**, 389.

KRAYBILL, W. H. AND CRAWFORD, Y. E. 1964. Tetrazolium and methylene blue for isolation and recognition of *Mycoplasma pneumoniae*. *Bact. Proc.*, p. 70.

LIND, K. 1970. A simple test for peroxide secretion by Mycoplasma. *Acta path. microbiol. scand.*, **B78**, 256.

MACKAY, J. M. K. 1969. Tissue culture studies of sheep pulmonary adenomatosis (jaagsiekte). II. Transmission of cytopathic effects to normal cultures. *J. comp. Path. Ther.*, **79**, 147.

MACKAY, J. M. K., NISBET, D. K. AND FOGGIE, A. 1963. Isolation of pleuropneumonia-like organisms (genus *Mycoplasma*) from cases of sheep pulmonary adenomatosis (SPA). *Vet. Rec.*, **75**, 550.

MARMION, B. P. 1967. The mycoplasmas. *In* Recent advances in medical microbiology, edited by A. P. Waterson, *London*, p. 170.

MORTON, H. E. AND ROBERTS, R. J. 1967. Production of anti-mycoplasma (PPLO) antibodies in rabbits. *Proc. Soc. exp. Biol. Med.*, **125**, 538.

OUCHTERLONY, O. 1948. In vitro method for testing the toxin-producing capacity of diphtheria bacteria. *Acta. path. microbiol. scand.*, **25**, 186.

POLLACK, J. D., SOMERSON, N. L. AND SENTERFIT, L. B. 1969. Effect of *p*H on the immunogenicity of *Mycoplasma pneumoniae*. *J. Bact.*, **97**, 612.

PURCELL, R. H., CHANOCK, R. M. AND TAYLOR-ROBINSON, D. 1969. Serology of mycoplasmas in man. *In* The Mycoplasmatales and the L-phase of bacteria, edited by L. Hayflick, *New York*, p. 221.

ST GEORGE, T. D., SULLIVAN, N. D., LOVE, J. A. AND HORSFALL, N. 1971. Experimental transmission of pneumonia in sheep with a mycoplasma isolated from pneumonic sheep lung. *Aust. vet. J.*, **47**, 282.

SOMERSON, N. L., TAYLOR-ROBINSON, D. AND CHANOCK, R. M. 1963. Haemolysin production as an aid in the identification and quantitation of Eaton agent (*Mycoplasma pneumoniae*). *Am. J. Hyg.*, **77**, 122.

SPEARMAN, C. 1904. Quoted by G. W. Snedecor and W. C. Cochran, *in* Statistical methods, 6th ed., 1967. p. 194.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. AND CHANOCK, R. M. 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg., Camb.*, **64**, 91.

TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. AND CHANOCK, R. M. 1963. Serological relationships among human mycoplasmas as shown by complement-fixation and gel-diffusion. *J. Bact.*, **85**, 1261.

WHO/FAO PROGRAMME ON COMPARATIVE VIROLOGY, 1974. The FAO/WHO programme on comparative mycoplasmology. *Vet. Rec.*, **95**, 457.

WILLIAMS, C. O. AND WITTLER, R. G. 1971. Hydrolysis of aesculin and phosphatase production by members of the order Mycoplasmatales which do not require sterol. *Int. J. syst. Bact.*, **21**, 73.

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# The experimental infection of specific pathogen free lambs with *Mycoplasma ovipneumoniae*

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**Six colostrum-deprived SPF lambs inoculated endobronchially with a second passage broth culture of a Scottish strain of *Mycoplasma ovipneumoniae*, were killed in batches of two at seven, 14 and 28 days post-inoculation. One lamb from each batch showed macroscopic and microscopic lung lesions similar to but milder than those described for respiratory mycoplasmoses in other species of animals and exhibited minor clinical symptoms. Mycoplasma were recovered from all infected but from no control animals: five infected lambs yielded mycoplasma from lung tissue.**

**Two lambs infected with *M ovipneumoniae* by endobronchial intubation were placed in contact with six other SPF lambs. *M ovipneumoniae* was recovered from the upper respiratory tract only of all six contact lambs, but no pathological changes were noted in their lungs. Both donor lambs yielded mycoplasma from lung tissue, but microscopic lesions were detected in only one of them, and these were minimal.**

**No seroconversion due to the infection could be demonstrated in any of the lambs by either the indirect haemagglutination or metabolic inhibition tests.**

*Mycoplasma ovipneumoniae* was originally isolated in Queensland, Australia by St George *et al* (1971), and later characterised by Carmichael *et al* (1972). The homogeneity of *M ovipneumoniae* with strains isolated from sheep in Victoria, Australia (Furlong and Cottew 1973) and in Scotland (Jones *et al* 1976) has been confirmed on the basis of biochemical and serological reactions.

In a series of experiments (St George *et al* 1971; Sullivan *et al* 1973a, b), lambs and ewes infected with *M ovipneumoniae* by various methods subsequently showed microscopic evidence of a proliferative interstitial pneumonia. Macroscopic lesions occurred in just over 50 per cent of all experimental animals. However, recovery of mycoplasma from experimental animals was achieved only in the preliminary experiments (St George *et al* 1971).

The purpose of this study was to investigate the pathogenicity for specific pathogen free (SPF) lambs of strains of *M ovipneumoniae* isolated from sheep in Scotland.

## Materials and methods

### *Media*

Three media were used, one of them being a broth medium and the other two agar formulations. Broth OB comprised 60 per cent medium 199 (Wellcome Reagents Ltd), 20 per cent tryptose phosphate broth (Oxoid), 10 per cent inactivated swine serum and 10 per cent fresh yeast extract, together with ampicillin (Penbritin, Beecham Veterinary Products) at a final concentration of 1 mg/ml, thallium acetate at 0·025 per cent and phenol red at 0·006 per cent.

OA differs from OB in containing 15 per cent inactivated swine serum, 55 per cent medium 199 and 0·75 per cent (w/v) agarose (British Drug Houses).

The other medium used was a selective agar (1A) prepared by the addition of 0·75 per cent (w/v) agarose to a broth containing 68 per cent Hartley digest broth (Oxoid), 20 per cent inactivated horse serum, 10 per cent fresh yeast extract and the same concentrations of ampicillin and thallium acetate as in OB.

### *Inocula*

The mycoplasma used were glycolytic organisms which grew as centreless colonies on solid medium and which were subsequently shown to be inhibited in the growth inhibition (GI) test by antisera prepared in rabbits to *M ovipneumoniae*, strain Y98, supplied by Dr St George.

They were isolated from nasal swabs strain 956/2 (experiment 1) from a ewe, and strain 956/3 (experiment 2) from a lmb. Both animals belonged to a conventionally managed flock in which signs of respiratory disease had been present for three months before sampling.

To avoid possible attenuation by subcultivation the two isolates were treated as follows. After primary isolation in OB, each was subcultured in 40 ml of OB, and following incubation for two days aliquots were stored at −80°C. Bacteriological and virological examination of samples gave negative results. Before use the necessary number of aliquots

were thawed and diluted in OB, the final titre being $10^7$ colour-changing units (ccu)/0·2 ml. The volume of inoculum for each animal was 2 ml. The inoculum for control lambs was sterile OB.

*Experimental animals*

The animals used were hysterectomy-produced, colostrum-deprived lambs which were maintained under SPF conditions at an ambient temperature of 18°C and a relative humidity of 40 per cent (Hart *et al* 1971; Brotherston 1968).

In experiment 1, Greyface × Suffolk lambs were inoculated when five days old, the experimental lambs (1 to 6) being housed in one pen and the three control lambs (7 to 9) in a separate pen.

In experiment 2, lambs A and B were inoculated when five days old, and one day later six contact lambs (C, D, E, F, G and H) were placed in the same pen. All lambs in this experiment were 3/4 Suffolk crosses, except lamb H which was a Dorset Horn.

*Inoculation*

The lambs were premedicated with intramuscular (im) atropine and anaesthetised with intravenous (iv) Immobilon*. Each animal was laid on its left side, an endotracheal tube inserted and through this a 60 cm length of sterilised nylon tubing (1·5 mm internal diameter) passed as far as possible. At a site, presumed to be within the left bronchus, 1·0 ml of inoculum was deposited. The tube was partly withdrawn, the animal turned onto its right side and the procedure repeated. Anaesthesia was terminated with iv Revivon*.

*Sampling and clinical examination*

Rectal temperatures were recorded daily, and the lambs auscultated daily for the first 12 days and at intervals of one to three days thereafter. In experiment 1, the respiration rates were also recorded at the same intervals as for auscultation. Nasal swabs for examination for mycoplasma were taken every second day for the first 12 days, and every fourth day thereafter: serum samples were taken every four days. In experiment 2, nasal swabs for examination for mycoplasma were taken at intervals of two to four days until 34 days post exposure (pe), the remaining three lambs being finally sampled at 41 days pe. Serum samples were taken at weekly intervals. Nasal swabs for bacteriological and virological examination were taken every week in both experiments.

* Reckitt and Colman

AUTOPSY

*Experiment* 1

The animals were killed with iv sodium pentobarbitone at predetermined times, lambs 1, 2 and 7 at seven days, lambs 3, 4 and 8 at 14 days and lambs 5, 6 and 9 at 28 days post inoculation (pi).

The trachea was exposed and clamped off 5–10 cm below the larynx to prevent collapse of the lungs. The thorax was opened with aseptic precautions and the lungs and trachea removed to a sterile tray. The left bronchus was exposed, clamped off and the left lung taken for microbiological examination and light-microscope histopathology. The right lung was infused via the trachea with 3 per cent glutaraldehyde in 0·1 M phosphate buffer at pH 7·4, from a reservoir 30 cm above the bench. The trachea was then clamped below the infusion point and the lung allowed to fix for 30 min in a bath of the fixative.

The following tissues were removed for examination for mycoplasma: nasal mucosa, tonsillar tissue, retropharyngeal lymph node, trachea, left bronchial lymph node, left bronchial swab, portions of the apical, cardiac and diaphragmatic lobes of the left lung and liver, spleen, kidney and cerebrum.

Samples from the three lobes of the left lung, nasal mucosa and spleen were also taken for bacteriological and virological examination.

*Experiment* 2

The animals were killed by the same method as in experiment 1, lambs A and C being killed at 16 dpi and 15 dpe respectively, lambs D, E and F at 35 dpe and lambs B, G and H at 43 dpi and 42 dpe respectively.

No attempt was made to prevent collapse of the lungs at autopsy. Samples collected for mycoplasma examination were similar to those collected in experiment 1, with the omission of liver, kidney, spleen and cerebrum samples, and the addition of samples from the three lobes of the right lung. Samples of the lungs and trachea were also taken for bacteriological and virological examination.

Portions from both sides of the lung were fixed by immersion in 10 per cent formol saline.

MICROBIOLOGICAL EXAMINATION

*Mycoplasma*

*Swabs.* Swabs were placed in bijoux containing 1·8 ml OB, shaken well and incubated for 60 min at 37°C. The swabs were then removed, OA and 1A plates seeded from the broth and incubation of the bijoux continued at 37°C. OA plates were incubated in a candlejar and 1A plates aerobically, both at 37°C.

*Tissues.* Approximately 0·5 g of coarsely-cut tissue was placed into a Universal bottle containing 4·5 ml OB. The bottle was shaken well, and after incubation for 60 min at 37°C 0·2 ml of the suspending medium was inoculated into bijoux containing 1·8 ml OB: OA and 1A plates were also sown. Where titrations were performed in experiment 1 (tonsillar tissue, trachea, bronchial swab and apical, cardiac and diaphragmatic lobes), the first bijou of the series was termed the $10^{-2}$ dilution. Where the titration series remained negative, but the plates or single isolation bijoux were positive, the titre was considered to be $10^{1}$ ccu/0·2 ml.

Positive broth cultures were stored at −80°C to await later identification by the growth inhibition test.

*Bacteria and viruses*

Examinations for bacteria were made on 7 per cent sheep blood agar cultured aerobically and anaerobically, and for viruses by three passages in primary sheep thyroid cell cultures.

SEROLOGY

The serological methods used were the growth inhibition (GI) test (Dighero *et al* 1970), the metabolic inhibition (MI) test (Taylor-Robinson *et al* 1966) and the indirect haemagglutination (IHA) test (Herbert 1967).

HISTOPATHOLOGY

In experiment 1, blocks of 1 cu mm were excised from all lesions in the right lung and from equivalent areas in all animals not showing lesions. These were processed through to araldite and 1 μm sections stained with Giemsa at 60°C for light microscopy. Thin sections were stained with lead citrate and examined in a Siemens Elmiskop 51. A comparable series of larger blocks were post fixed in 10 per cent formol saline, trimmed and processed through to paraffin wax. Samples from the left lung, trachea, tonsillar tissue, retropharyngeal lymph nodes and nasal turbinates from experiment 1, and all tissues examined from experiment 2, were fixed in 10 per cent formol saline, trimmed and processed through to paraffin wax. Sections were cut at 6 μm and stained with haematoxylin and eosin. Selected sections were also stained by periodic acid Schiff (PAS), Hale's colloidal iron, Southgate's mucicarmine method for mucin and Best's carmine method for glycogen (Carleton 1967).

## Results

*Experiment 1*

*Clinical examinations.* No rectal temperatures exceeding 105°F (40·6°C) were recorded. Respiration rates varied considerably, but in general increased rates coincided with the presence of more marked respiratory sounds. Lamb 2 exhibited dyspnoea alternating with periods of polypnoea from day 2 pi onwards.

Abnormal auscultatory sounds were generally of a harsh, dry nature and only heard in lambs 2, 3, 4 and 6. Lamb 2 was harsh on auscultation before infection; in this animal, the sounds increased in intensity after infection until crepitations and sonorous rhonchi were also audible. Mild transient harshness was also detected in control lambs 7 and 9 at day 5 pi.

Apart from lamb 2, no obvious respiratory symptoms were noted in the other animals except in lamb 6, which developed a serous nasal discharge from day 20 pi onwards.

*Microbiology.* No mycoplasma were recovered at any time from the three control lambs 7, 8 and 9. Table 1 shows the incidence of mycoplasma infection in the nasal cavities. All the experimental animals were infected at this site except lamb 4. Table 2 shows that five lambs also yielded mycoplasma from the lungs. All six infected lambs were positive for mycoplasma from tracheal and tonsillar tissue, and mycoplasma were also recovered from the retropharyngeal lymph nodes of four animals and the bronchial lymph node of one animal. No mycoplasma were recovered from the spleen, kidney, liver or cerebrum of any of the animals.

Nasal swabs also yielded *Escherichia coli, Enterobacter cloacae, Citrobacter* spp and *Streptococcus mitis;* coagulase positive *Staphylococcus pyogenes* was isolated from one nasal swab.

No bacteria were isolated from the lungs or trachea of any of the animals except lamb 2, from which heavy growths of *E coli* were obtained from the apical and diaphragmatic lobes of the left lung. No viruses were isolated from any of the specimens.

*Pathology.* The lungs of infected lambs 1, 4 and 5 and control lambs 7, 8 and 9 appeared normal both on macroscopic and microscopic examination. Macroscopically, lambs 2, 3 and 6 showed clearly demarcated focal areas of collapse and consolidation with a lobular distribution generally confined to the apical and cardiac lobes.

Microscopically, the areas of collapse in lamb 2 consisted of alveoli packed with neutrophils and alveolar macrophages. An exudate with the staining characteristics of acid mucopolysaccharide was seen to fill groups of alveoli. This exudate gave a strong positive reaction with Hale's colloidal iron, but only weak reactions by the PAS method and Southgate's method for mucin, and a negative reaction by Best's method for glycogen.

In lamb 3 the collapsed foci were firmer. The

TABLE 1: Experiment 1. Recovery of *M ovipneumoniae* from nasal swabs

| Lambs | Days post inoculation 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | + | + | +K | | | | | | | | | | | |
| 2 | − | + | +K | | | | | | | | | | | |
| 7 | − | − | −K | | | | | | | | | | | |
| 3 | − | − | − | + | + | C | K | | | | | | | |
| 4 | − | − | − | − | − | − | K | | | | | | | |
| 8 | − | − | − | − | − | − | K | | | | | | | |
| 5 | + | + | + | − | + | − | | + | | − | | − | | −K |
| 6 | + | + | + | + | + | + | | + | | C | | + | | +K |
| 9 | − | − | − | − | − | − | | − | | − | | − | | −K |

Lambs 7, 8 and 9 were control animals K—killed C—contaminated

TABLE 2: Experiment 1. Recovery of *M ovipneumoniae* at autopsy

| Sample | 7 dpi Infected 1 | 2 | Control 7 | 14 dpi Infected 3 | 4 | Control 8 | 28 dpi Infected 5 | 6 | Control 9 |
|---|---|---|---|---|---|---|---|---|---|
| Nasal mucosa | + | + | − | + | + | − | − | + | − |
| Tonsil | 1 | 6 | − | 4 | 1 | − | 2 | 6 | − |
| Retropharyngeal lymph node | − | + | − | + | + | − | + | − | − |
| Trachea | 5 | 7 | − | 4 | 4 | − | 2 | 6 | − |
| Bronchial lymph node | − | − | − | + | − | − | − | − | − |
| Bronchial swab | 0 | 7 | − | 6 | 5 | − | 5 | 4 | − |
| Left lung apical lobe | 0 | 8 | − | 8 | 6 | − | 0 | 5 | − |
| cardiac lobe | 0 | 8 | − | 7 | 6 | − | 0 | 7 | − |
| diaphragmatic lobe | 0 | 7 | − | 6 | 6 | − | 4 | 5 | − |
| Liver | − | − | − | − | − | − | − | − | − |
| Spleen | − | − | − | − | − | − | − | − | − |
| Kidney | − | − | − | − | − | − | − | − | − |
| Cerebrum | − | − | − | − | − | − | − | − | − |

Figures represent titres (ccu/0·2 ml) expressed as powers of $\log_{10}$
+ and − indicate positive or negative recoveries only (no titrations performed)

affected tissue contained more neutrophils than alveolar macrophages and these tended to be clumped in end-bronchioles whereas the macrophages were spread more evenly throughout the alveoli. As in lamb 2, Hale-positive exudate was also present in some alveoli (Fig 1). Peribronchiolar cuffing with lymphoid cells was seen, as was an increase in Type II alveolar cells (Fig 2).

These last two changes were marked in lamb 6. The peribronchiolar cuffs were often many cells thick and on occasions lymphoid cells could be seen infiltrating the bronchiolar epithelial lining: in such areas cilia were absent. An increase in numbers of Type II cells occurred only in areas of collapse, where they could be seen in twos and threes in these alveoli. The inflammatory cells present were predominantly macrophages, few neutrophils being present. Little excess Hale-positive material was seen (Fig 3).

Ciliary loss was minimal in all lambs.

The retropharyngeal lymph nodes of lambs 3 and 6 were enlarged and their medullary cords mildly hypercellular. No significant changes were observed in tonsillar tissue, trachea or nasal turbinates.

By electron microscopy, mycoplasma were only seen in lamb 2 in close association with bronchiolar cilia (Fig 4), where they were sometimes adjacent to cells displaying ciliary loss. Neutrophils often occurred in the submucosa or lying between epithelial cells in the vicinity of these mycoplasma. Structures which were less dense but of a similar size to mycoplasma were seen in alveoli, both free and in the process of being phagocytosed by macrophages and neutrophils, but no positive identification could be made.

*Experiment* 2

*Clinical examinations.* No rectal temperatures exceeding 105°F (40·6°C) were recorded. Abnormal auscultatory sounds of a mild, harsh nature were heard in lambs A and H.

Lambs B, F and G exhibited a mild serous nasal discharge at various times during the experiment.

*Microbiology. M ovipneumoniae* was recovered in nasal swabs from all eight lambs (Table 3), but, in the lungs, only from the donor lambs (Table 4). In the

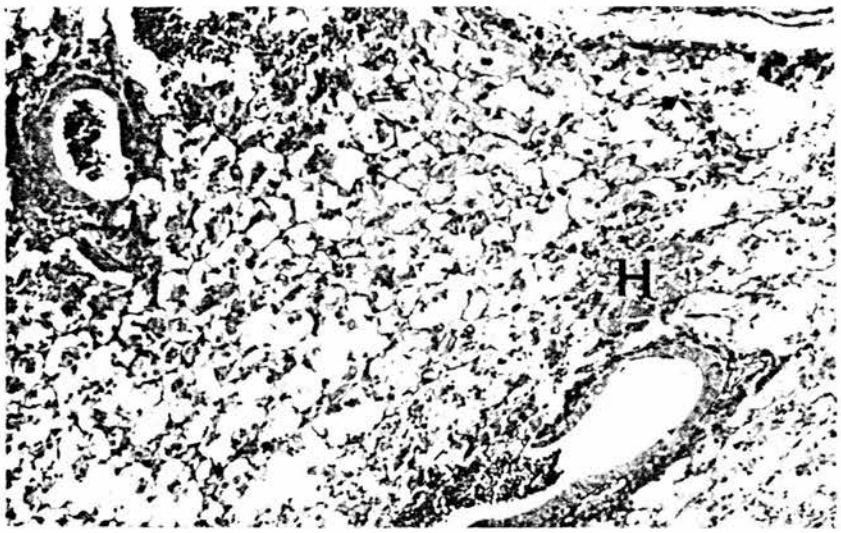


FIG 1: Mild peribronchial lymphoid hyperplasia with clumping of neutrophil polymorphs in airways and intra-alveolar Hale-positive exudate (H) in lamb 3, 14 days pi. Hale's colloidal iron × 25

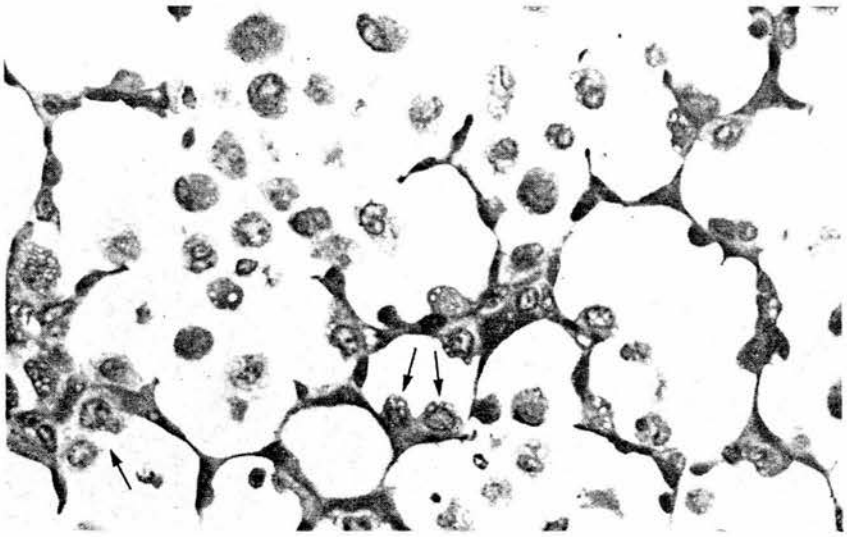

FIG 2: Increased numbers of Type II alveolar cells (arrows) and intra-alveolar macrophages in lamb 6, 28 days pi. Giemsa × 400

contact lambs, mycoplasma were recovered from the nasal mucosa of three animals, from tonsillar tissue of five animals and from the trachea of one animal.

All animals yielded coagulase positive *Staphylococcus pyogenes* from nasal swabs at various stages during the experiment. *E cloacae, E coli* and *Streptococcus epidermidis* were also isolated from some nasal swabs.

No bacteria or viruses were isolated from any of the tissues examined at autopsy.

*Pathology*. None of the lambs showed any macroscopic lesions in the lungs. Microscopically, only lamb A showed pathological change in the lungs, mild interlobular oedema and small bands of collapse being present. No peribronchiolar cuffing or

FIG 3: Clearly demarcated area of collapse with intra-alveolar inflammatory cells and marked peribronchial and perivascular lymphoid hyperplasia in lamb 6, 28 days pi. Giemsa × 25

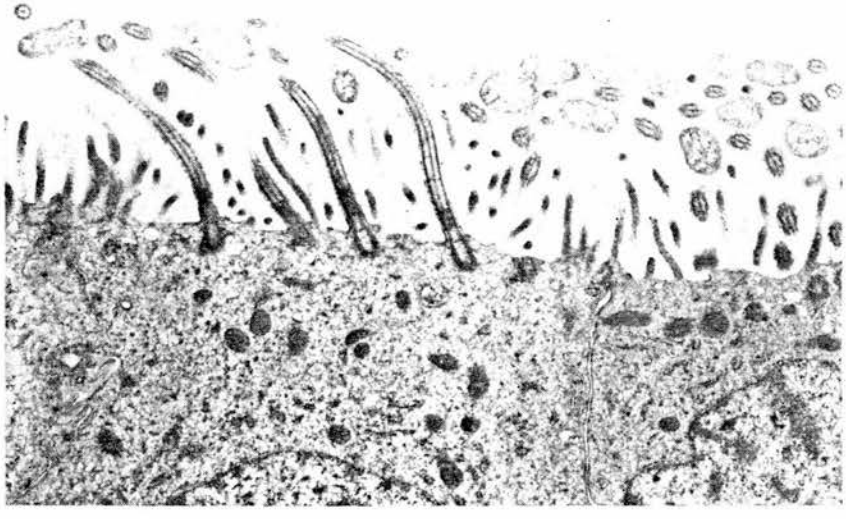

FIG 4: Mycoplasma in close association with bronchiolar cilia in lamb 2, seven days pi. EM × 12,500

Hale-positive exudate was seen, and intra-alveolar inflammatory cells in collapsed areas were sparse.

*Serology.* No specific antibody activity due to the mycoplasmal infection could be demonstrated in any lambs from either experiment by means of the IHA and MI tests.

## Discussion

In experiment 1, the endobronchial inoculation of a broth culture containing $10^7$ ccu/0·2 ml of a low-passage strain of *M ovipneumoniae* produced mild macroscopic and microscopic lesions in three out of six SPF lambs, although mycoplasma were recovered

TABLE 3: Experiment 2. Recovery of *M ovipneumoniae* from nasal swabs

| Lambs | Days post inoculation of lambs A + B (lambs C – H placed in contact 1 day later) | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 | 32 | 34 | 36 | 38 | 40 | 42 | 44 |
| A | – | + | + | + | + | | + | + | + | K | | | | | | | | | | | | |
| B | – | – | + | + | + | | + | + | + | | + | + | | + | + | | + | + | | | | CK |
| C | – | | – | + | + | | + | + | C | K | | | | | | | | | | | | |
| D | – | | – | + | + | | + | + | + | | + | + | | + | + | | – | CK | | | | |
| E | – | | – | C | C | | C | C | C | | + | C | | + | C | | + | + K | | | | |
| F | – | | – | – | – | | + | + | + | | + | + | | + | + | | C | CK | | | | |
| G | – | | – | – | – | | – | + | + | | + | + | | + | + | | + | + | | | | + K |
| H | – | | – | – | – | | + | + | + | | C | + | | + | + | | + | + | | | | – K |

K—killed C—contaminated

TABLE 4: Experiment 2. Recovery of *M ovipneumoniae* at autopsy

| Sample | 16 dpi | 15 dpe | | 35 dpe | | 43 dpi | 42 dpe | |
|---|---|---|---|---|---|---|---|---|
| | A | C | D | E | F | B | G | H |
| Nasal mucosa | + | – | – | + | + | – | + | – |
| Tonsil | + | – | + | + | + | + | + | + |
| Retropharnygeal lymph node | + | – | – | – | – | – | – | – |
| Trachea | + | – | – | – | – | + | + | – |
| Bronchial lymph node | – | – | – | – | – | + | – | – |
| Bronchial swab | + | – | – | – | – | – | – | – |
| Left lung—apical lobe | – | – | – | – | – | + | – | – |
| cardiac lobe | + | – | – | – | – | + | – | – |
| diaphragmatic lobe | – | – | – | – | – | + | – | – |
| Right lung—apical lobe | ND | – | – | – | – | – | – | – |
| cardiac lobe | ND | – | – | – | – | – | – | – |
| diaphragmatic lobe | ND | – | – | – | – | – | – | – |

ND—not done

from the lungs of five of the lambs, in four of them at high titres. The recovery of mycoplasma from retropharyngeal and bronchial lymph nodes indicates that local spread of the infection occurred into the lymphatic system.

Except in lamb 4 in which the lungs appeared to be normal, abnormal respiratory sounds were heard only in those lambs with macroscopic lesions. Lamb 2, in which respiratory symptoms were particularly marked, yielded heavy growths of *E coli* from the lungs. Furthermore, during endotracheal intubation of this lamb, froth was produced from the tube. The significance of the clinical symptoms and pathology of this animal with regard to the mycoplasma infection is therefore doubtful.

In experiment 2, the endobronchial inoculation of *M ovipneumoniae* in two donor lambs produced a pulmonary infection but no macroscopic lesions. Only minimal microscopic lesions were seen in the lungs of one of the donor lambs. Although all six contact lambs became infected with *M ovipneumoniae*, no mycoplasma were recovered from the lungs and no pathological changes were noted in any of these animals.

No specific seroconversion due to the mycoplasmal infection could be demonstrated in any of the animals by either the IHA or MI tests. Other workers (Goodwin *et al* 1969; Cole *et al* 1969) have also been unable to demonstrate IHA or MI activity early after the experimental infection of animals with mycoplasma although Goodwin *et al* (1969) found that high titres to the IHA test had developed by 16 weeks pi. Fernald (1969) was similarly unable to demonstrate IHA activity following the experimental infection of hamsters with *M pneumoniae*, although high levels of GI antibodies were detected from two weeks onwards.

As only three lambs showed the presence of lung lesions, and since in-contact transmission caused no colonisation of the lungs, no definite conclusions can be reached concerning the pathogenicity for SPF lambs of the strains of *M ovipneumoniae* used. Interpretation of these results is further complicated by the recovery of *E coli* from the lungs of one of the lambs showing lesions. However, the pathology of the lungs of affected lambs is similar, though milder, to that described for the experimental infection of pigs with *M hyorhinis* (Baskerville 1972) and *M hyopneumoniae* (Livingston *et al* 1972), of mice with *M pulmonis* (Lindsey and Cassell 1973) and of hamsters with *M pneumoniae* (Dajani *et al* 1965). Lindsey and Cassell (1973) have also noted

the essential similarities of the pathological findings from their mouse model with those found in naturally occurring respiratory mycoplasmoses of a wide variety of other species.

The pathology of affected lambs in these experiments fits with the observation by Fernald (1969) 'that the changes observed are not part of an invasive or destructive process, but rather the marshalling of a local immune defence system in response to the superficial infection within the bronchial tree'. The total lack of pathological change in the lungs of lambs 4, 5 and B and the minimal changes in lamb A in spite of the presence of mycoplasma infection would suggest that further work is required on the pathogenicity of *M ovipneumoniae* for lambs.

No attempt was made to clone or dilute the isolates used beyond the second passage because of the possibility of concommitant attenuation (Couch *et al* 1964; Dajani *et al* 1965). Despite this, the pathogenicity of these strains of *M ovipneumoniae* would seem to be considerably less than that of the strains isolated by St George *et al* (1971) and Sullivan *et al* (1973a, b). The pathology of the lesions produced by these strains in SPF lambs also differs from that described by the Queensland workers in that thickening of the alveolar septa due to proliferative changes in the alveolar walls, hyperplasia and degenerative changes in bronchiolar epithelium, or hyperplasia of goblet cells were not present in our cases. In their major experiments (Sullivan *et al* 1973a, b), no mycoplasma were recovered from any of the experimental animals, which they considered possibly due to the release of a mycoplasmacidal factor from ground tissue (Kaklamanis *et al* 1969). Our technique of minimal tissue destruction might therefore account for the good recovery rates of mycoplasma in experiment 1.

**Acknowledgments**

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## References

BASKERVILLE, A. (1972) *Research in Veterinary Science* **13,** 570
BROTHERSTON, J.G. (1968) *Report of the Animal Diseases Research Association,* Edinburgh, 1967–68, p 26
CARLETON'S HISTOLOGICAL TECHNIQUE (1947) 4th ed. R. A. B. Drury and E. A. Wallington, Oxford Medical Publications
CARMICHAEL, L.E., ST GEORGE, T.D., SULLIVAN, N.D. & HORSFALL, N. (1972) *Cornell Veterinarian* **62,** 654
COLE, B.C., CAHILL, J.F., WILEY, B.B. & WARD, J.R. (1969) *Journal of Bacteriology,* **98,** 930
COUCH, R.B., CATE, T.R. & CHANOCK, R.M. (1964) *Journal of the American Medical Association* **187,** 442
DAJANI, A.S., CLYDE, JNR, W.A. & DENNY, F.W. (1965) *Journal of Experimental Medicine* **121,** 1071
DIGHERO, M.W., BRADSTREET, C.M.B. & ANDREWS, B.E. (1970) *Journal of Applied Bacteriology* **33,** 750
FERNALD, G.W. (1969) *Journal of Infectious Diseases* **119,** 255
FURLONG, S.L. & COTTEW, G.S. (1973) *Australian Veterinary Journal* **49,** 215
GOODWIN, R.F.W., HODGSON, R.G., WHITTLESTONE, P. & WOODHAMS, R.L. (1969) *Journal of Hygiene,* Cambridge **67,** 193
HART, R., MACKAY, J.M.K., MCVITTIE, C.R. & MELLOR, D.J. (1971) *British Veterinary Journal* **127,** 419
HERBERT, W.J. (1967) *Handbook of Experimental Immunology,* pp 720–744. Ed. D. M. Weir. Blackwell, Oxford & Edinburgh
JONES, G.E., FOGGIE, A., MOULD, D.L. & LIVITT, S. (1976) *Journal of Medical Microbiology* **9,** 39
KAKLAMANIS, E., THOMAS, L., STRAVROPOULOS, K., BORMAN, I. & BOSHWITZ, C. (1969) *Nature* **221,** 860
LINDSEY, J.R. & CASSELL, G.H. (1973) *American Journal of Pathology* **72,** 63
LIVINGSTON, JNR, C.W., STAIR, E.L., UNDERDAHL, N.R. & MEBUS, C.A. (1972) *American Journal of Veterinary Research* **33,** 2249
ST GEORGE, T.D., SULLIVAN, N.D., LOVE, J.A. & HORSFALL, N. (1971) *Australian Veterinary Journal* **47,** 282
SULLIVAN, N.D., ST GEORGE, T.D. & HORSFALL, N. (1973a) *Australian Veterinary Journal* **49,** 57
SULLIVAN, N.D., ST GEORGE, T.D. & HORFSALL, N. (1973b) *Australian Veterinary Journal* **49,** 63
TAYLOR-ROBINSON, D., PURCELL, R.H., WONG, D.C. & CHANNOCK, R.M. (1966) *Journal of Hygiene,* Cambridge **64,** 91

# ENDOBRONCHIAL INOCULATION OF SHEEP WITH PNEUMONIC LUNG-TISSUE SUSPENSIONS AND WITH THE BACTERIA AND MYCOPLASMAS ISOLATED FROM THEM

By

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## INTRODUCTION

The 2 principal forms of ovine pneumonia occurring in the United Kingdom are enzootic pneumonia (syn. pasteurellosis, an acute necrotizing or exudative pneumonia) and atypical pneumonia (Stamp and Nisbet, 1963; Stevenson, 1969). *Pasteurella haemolytica* is associated with both forms (Smith, 1961; Gilmour and Brotherston, 1963), and is considered to be the causal agent of enzootic pneumonia (Smith, 1964), though experimental reproduction by the intra-tracheal or intra-nasal inoculation of cultures of the organism requires very large doses (Smith, 1960, 1964; Biberstein, Nisbet and Thompson, 1967). It has therefore been postulated that a predisposing agent is necessary (Biberstein *et al.*, 1967; Stevenson, 1969).

The aetiology of atypical pneumonia is similarly considered to be complex (Hore, 1970; Stevenson and Robinson, 1970), although lesions resembling those of atypical pneumonia have been reproduced with chlamydia (Dungworth and Cordy, 1962; Stevenson and Robinson, 1970).

The purpose of the studies reported here was to investigate the aetiology of atypical pneumonia by inoculating suspensions of lung lesions from naturally infected sheep.

## MATERIALS AND METHODS

*Inocula consisting of lung suspensions* (LS). Sheep lungs with consolidated lesions were obtained from the abattoir. Histological examination showed the lesions to consist of a proliferative exudative pneumonia, the principal features of which were lymphoid hyperplasia, bronchiolar hyperplasia, macrophage and polymorph exudation, giant cells, hyaline scar formation and areas of alveolar collapse. These lesions bore a close resemblance to the atypical pneumonia described by Stamp and Nisbet (1963). Consolidated areas of lung were excised, coarsely chopped, suspended in Hanks' Balanced Salt Solution (BSS) and shaken well. The suspensions were clarified through muslin and stored in aliquots at −70 °C. Lesions from 3 lungs were pooled for Experiment I (LS1), and lesions from 7 lungs for Experiment II (LS2). In the preliminary to Experiment II, the sheep were inoculated with freshly-prepared LS2. "Sterilized" LS, from which no organisms could be recovered, was prepared by exposure to 2·5 Mrads gamma irradiation.

LS1 and 2 were diluted 1:1 with BSS, and suspensions of micro-organisms (MS) were diluted 1:1 with sterilized LS2 or Oxoid Nutrient Broth Number 2 (NB2) before

inoculation. The main organisms present in the inocula are given in Table 1. In LS1, small numbers of streptococci, staphylococci and pseudomonads were additionally recovered, but chlamydiae or viruses were not detected in either LS. The titre of *P. haemolytica* in the fresh LS2 inoculum in the preliminary to Experiment II was $10^{3\cdot6}$ organisms (orgs) per ml, but after storage the titre was $10^{2\cdot8}$ orgs per ml.

TABLE 1

MICROBIOLOGICAL CONSTITUENTS OF INOCULA

| *Inoculum** | *Experiment* | *Inoculum administered to* | Titres† of | | | |
|---|---|---|---|---|---|---|
| | | | M. ovi-pneumoniae | P. haemolytica | E. coli | M. arginini |
| LS1 | I | all animals | $10^7$ | $10^{5\cdot2}$‡ | $10^{3\cdot0}$ | — |
| LS2 | II | sheep in pilot experiment and Group 1 | $10^7$ | $10^{2\cdot8}$§ | — | $10^2$ |
| MS | II | sheep in Groups 2 and 3 | $10^7$ | $10^{3\cdot7}$§ | — | $10^7$ |

* Inoculum volume 4 ml.

† Titres of mycoplasmas as ccu per 0·2 ml and bacteria as organisms per ml.

‡ Inoculum contained 4 types of *P. haemolytica*, in the following proportions: A1 (34 per cent); A2 (8 per cent); A6 (54 per cent); untypeable strain (4 per cent).

§ Serotype A2 only.

*Inocula consisting of suspensions of micro-organisms* (MS). All organisms used in MS were derived from LS2. The strains of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* were passaged through 9 and 11 subcultures respectively, including 3 clonings by transfer of single colonies from agar plates. Both strains were incorporated in the inocula as 24-h cultures, the final 3 passages of each being in media (OB or AB) from which bacterial inhibitors had been omitted. *P. haemolytica* serotype A2 was stored as a second passage culture at −70 °C. For use, an 18-h broth culture prepared from a stored aliquot was subcultured for 6 h in NB2, then diluted 1 in 1000 in NB2 and mixed with an equal volume of premixed mycoplasma cultures.

*Experimental animals.* Cheviot sheep 6 to 7 months old were used. Each experimental group was housed in a separate pen. The sheep used in Experiment I and in Group 1, Experiment II were derived from the Institute flock. However, at the time of Experiment II, mild respiratory disease of coughing and serous nasal discharge without anorexia, pyrexia or respiratory distress was present in this flock, and therefore as many sheep as possible were obtained from an outside source. These animals, which were randomly placed in Groups 2 to 5 of Experiment II, were judged free of respiratory disease by clinical examination at the premises of origin.

*Inoculation.* Four ml of inoculum were administered endobronchially to anaesthetized sheep (Foggie, Jones and Buxton, 1976).

*Clinical examination and necropsy.* Animals were examined daily for evidence of respiratory disease. At necropsy, the proportion of the lung surface area which showed consolidation was estimated from a diagrammatic sketch by means of a point-grid system, the average of dorsal and ventral measurements being taken as the final lung lesion score (LLS).

*Mycoplasma media.* The media used were glycolytic-mycoplasma broth and agar (OB and OA), arginine broth (AB), T-mycoplasma broth (TB) and transport medium (MTM) (Jones, Foggie, Sutherland and Harker, 1976), modified by

substitution of Brain Heart Infusion Broth (Oxoid Ltd) for tryptose phosphate broth, and the inclusion of 0·025 per cent thallium acetate in TB and MTM.

*Mycoplasma isolation and culture.* The techniques used were as described previously (Foggie *et al.*, 1976), with minor modifications. In brief, swabs or pieces of coarsely chopped tissue of approximately 1 g were placed in 3 ml of MTM: in Experiment II, where pools were made from "lesion" and "non-lesion" areas of lung, 2 to 3 g of chopped tissue were placed in 9 ml of MTM. The tissue or swab suspensions were shaken well and after incubation for 1 h at 37 °C, 0·2 ml of the suspension was inoculated into Bijoux bottles containing 1·8 ml of OB, AB or TB; OA plates were also sown. Cultures were retained for 3 weeks before being discarded as negative.

*Identification of mycoplasmas.* All strains were identified by the growth inhibition (GI) test (Dighero, Bradstreet and Andrews, 1970), with antiserum prepared by the method described by Jones, Foggie, Mould and Livitt (1976). For the identification of *M. ovipneumoniae*, equal volumes of antisera produced against 4 strains were pooled, but for *M. arginini* the single antiserum against the type strain G230 was found to be satisfactory.

*Samples from experimental animals* comprised pre-inoculation nasal swabs, and specimens taken with aseptic precautions from right and left apical, cardiac and diaphragmatic lobes; trachea; tonsillar tissue; and a bronchial swab. In Experiment I, *M. ovipneumoniae* in the 3 lobes of the left lung was assayed in OB [colour changing units (ccu) per 0·2 ml]. In Experiment II, the same regime was followed for those animals that died or were killed in extremis, but for those killed according to schedule the protocol was changed, the samples titrated in OB being "lesion pools" and "non-lesion pools" taken from macroscopic lesions and apparently normal areas of lung respectively. In this experiment, bronchial swabs were also titrated in AB to find the levels of *M. arginini* present.

*Isolation and identification of bacteria.* Viable counts from 10 per cent (w/v) tissue suspensions were made on 7 per cent sheep blood agar. *P. haemolytica* strains were serotyped by the indirect haemagglutination test (Shreeve, Biberstein and Thompson, 1972).

*Samples from experimental animals* comprised pre-inoculation nasal swabs and, in Experiment I, left and right lung pools and tonsillar tissues. In Experiment II, the 6 major lung lobes only from each animal were cultured for bacteria, and viable counts of *P. haemolytica* were made from "lesion" and "non-lesion" pools.

*Virology.* Prepared samples were inoculated on to secondary foetal-lamb kidney cultures. After 7 days, the cultures were tested for haemadsorption by the addition of a 0·5 per cent suspension of guinea-pig erythrocytes. In addition, sera from all animals were assayed for antibodies to PI3 virus by the HAI test (Smith, 1975).

*Chlamydiology.* The inocula only were screened for the presence of chlamydia, in 6-day-old fertilized eggs (Stevenson and Robinson, 1970).

*Histopathology.* Selected lung samples were fixed in a 10 per cent solution of formol saline, post-fixed in a saturated solution of mercuric chloride, dehydrated in alcohols and embedded in paraffin wax. Sections at 6 μm were prepared and stained with haematoxylin and eosin.

## RESULTS

### *Infection of Sheep with Lung Suspension LS1 (Experiment I)*

Ten sheep were inoculated with LS1 and killed daily in pairs at 18 to 22 days post-inoculation (d.p.i.).

Before inoculation, symptoms of respiratory disease were not detected in any animal, but a non-typeable strain of *P. haemolytica* was recovered from the nose of one sheep.

*Post-inoculation clinical examination.* All animals developed fever (rectal

temperatures above 41·5 °C), and showed anorexia, depression and tachypnoea or dyspnoea and 7 animals showed coughing. Fever generally began from 1 to 3 d.p.i., had a mean duration of 8·1 (s.e. ± 1·6) days and showed 1 to 4 peaks.

*Pathology*. All 10 animals had well-defined consolidated lesions. The LLS ranged from 17 to 73 per cent, with a mean of 42·2 per cent (s.e. ± 4·5 per cent). Histologically, a proliferative pneumonia very similar to that seen in the diseased tissue used as inoculum was present. The non-consolidated areas showed mild to moderate infiltration of the interalveolar septa with mononuclear cells. In 6 animals there were areas of pleurisy.

*Microbiology*. *M. ovipneumoniae* was recovered from all lung samples, tracheas and bronchial swabs, and from the tonsillar tissue of 9 animals. All specimens titrated yielded at least $10^6$ ccu per 0·2 ml. Various serotypes of *P. haemolytica* biotype A were recovered from the lungs of all animals and from the tonsillar tissue of 9. Multiple serotype infections of lungs occurred in 3 sheep, and the single serotypes A1, A2 and A6 were recovered from 7, 4 and 3 sheep respectively. One sheep yielded serotype A7—an organism not present in the inoculum—from both lungs and tonsillar tissue. No correlation was found between serotype and the presence of pleurisy. Titres of *P. haemolytica* reached $10^6$ to $10^7$ orgs per ml in 8 animals. No other bacteria were recovered from lungs, with the exception of *Listeria monocytogenes* from a solitary lung abscess. No viruses were isolated, nor was a rise in antibodies to PI3 virus detected in any animal.

### *Infection of Sheep with Lung Suspension LS2 or with Organisms Cultured from it (Experiment II)*

The results of Experiment I indicated that a proliferative pneumonia could readily be reproduced in sheep with LS1. An attempt was next made to repeat the experiment with a different inoculum (LS2) and to reproduce the disease with pure cultures of the organisms recovered from LS2. To take account of the possibility that lung tissue components in suspension may have had a contributory effect, sterilized LS2 was included in 2 control inocula.

A preliminary test of the efficacy of LS2 was performed in 2 sheep. After endobronchial inoculation, both animals exhibited severe respiratory symptoms: one was killed in extremis 5 d.p.i. and the other died 13 d.p.i. At necropsy extensive purple discoloration of the lungs was present in both animals. This, histologically, consisted of a proliferative and exudative pneumonia. *P. haemolytica*, *M. ovipneumoniae* and *M. arginini* were recovered from all samples examined.

In the main experiment, 5 groups of 7 sheep were inoculated endobronchially with the following materials: LS2 and BSS (Group 1); sterilized LS2 and MS (Group 2); BSS and MS (Group 3); sterilized LS2 and BSS (Group 4). Group 5 animals received no treatment. Survivors were killed at 14 to 21 d.p.i.

Before inoculation some animals in Group 1 were observed to be coughing, and nasal swabs from all sheep in this group yielded *M. ovipneumoniae*. One also yielded *M. arginini* and 4 animals *P. haemolytica* (various serotypes). Groups 2 to 5 appeared normal and no mycoplasmas or pasteurellae were recovered

from nasal swabs, except for 2 sheep in Group 4 that yielded *P. haemolytica* serotype A6.

*Post-inoculation clinical examination.* The results are summarized in Table 2. Respiratory symptoms were observed in most animals in Groups 1, 2 and 3. Eight animals died or were killed in extremis 2 to 13 d.p.i. and all had severe pleurisy at necropsy. The control animals in Groups 4 and 5 appeared normal throughout.

TABLE 2

SUMMARY OF CLINICAL FINDINGS IN EXPERIMENT II

| *Group* | *Treatment* | *Number of sheep* | | |
|---|---|---|---|---|
| | | *that died or were killed in extremis** | *with rectal temperature* $>41{\cdot}5\ ^{\circ}C$ | *showing clinical response†* |
| 1 | LS2 | 1 | 4 | 6 |
| 2 | Sterile LS2+MS | 3 | 6 | 5 |
| 3 | BSS+MS | 4 | 4 | 5 |
| 4 | Sterile LS2 | 0 | 0 | 0 |
| 5 | No treatment | 0 | 1 | 0 |

* 2 to 13 d.p.i.
† Clinical response consisted of one or more of the following abnormalities: respiratory distress; depression; coughing.
Each group contained 7 sheep.

The differences between Groups 2 and 3 and between Groups 4 and 5 were very slight for all parameters measured, so for statistical purposes the groups were combined. Significant differences ($P = < 0{\cdot}01$) occurred between Groups 2 + 3 and Groups 4 + 5 for all the clinical parameters in Table 2. Group 1 was significantly higher at the 99 per cent level than Groups 4 + 5 in the number of animals showing clinical response, and at the 95 per cent level in the number of animals showing elevated temperatures. There were no significant differences in any respect between Group 1 and Groups 2 + 3.

*Pathology.* The pathological findings are summarized in Table 3. All animals in Groups 2 + 3, and 5 in Group 1 displayed large individual areas of grey or red consolidation (Fig. 1), whereas only linear lesions or small foci of consolidation were present in Groups 4 + 5. One animal in Group 5 had widespread consolidated foci and was estimated to have a LLS of 11 per cent.

As in Experiment I, the predominant lesion was a proliferative and exudative pneumonia, which was present in 16 of 19 animals examined in Groups 1, 2 and 3, and in 1 of the 14 in Groups 4 and 5. Bronchiolar hyperplasia and metaplasia, lymphoid hyperplasia and accumulation of macrophages, giant cells and polymorphs were regularly present and hyaline scars and perivascular fibrosis were frequently seen (Figs 2 and 3). Pleurisy was present in 13 animals from Groups 1, 2 and 3. Sheep in all groups showed evidence of infestation with both *Dictyocaulus filaria* and *Muellerius capillaris*.

Two animals in Group 2, both of which died 3 d.p.i., had acute bronchopneumonia and pleurisy. There was necrosis of the bronchiolar epithelial cells, capillary congestion, serous alveolar exudation and an inflammatory cell infiltration of the parenchyma.

TABLE 3

SUMMARY OF PATHOLOGICAL FINDINGS IN EXPERIMENT II

| *Group* | *Mean LLS per cent* (± S.E.) | *Number of sheep with* *pleurisy* | *acute bronchopneumonia* | *proliferative and exudative pneumonia* | *focal or linear collapse lesions only* |
|---|---|---|---|---|---|
| 1 | 23·3 ± 7·94 | 4 | 0 | 6 | 1 |
| 2 | 48·9 ± 9·53 | 4* | 2 | 5 | 0 |
| 3 | | 5 | 0† | 5† | 0† |
| 4 | 2·4 ± 0·72 | 0 | 0 | 1 | 4 |
| 5 | | 0 | 0 | 0 | 6 |

* Pleurisy in one animal observed microscopically only.
† Only 5 animals examined histologically.
LLS, lung lesion score.
Each group contained 7 sheep.

Fig. 1. Right lung from sheep in Group 2, Experiment II (killed 17 d.p.i.), showing grey and red consolidation of the apical lobe and linear collapse lesions in the cardiac lobe.

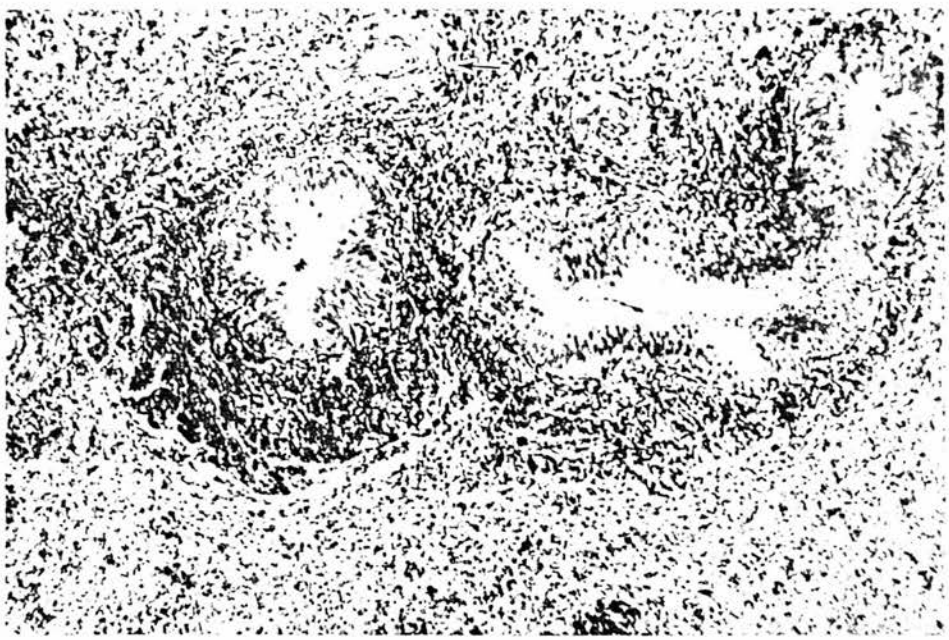

Fig. 2. Section from consolidated lesion in sheep from Group 2, Experiment II, killed 17 d.p.i. Bronchiolar and lymphoid hyperplasia and inflammatory cell infiltration of alveoli are present. Perivascular fibrosis (arrow) is also shown. HE. × 100.

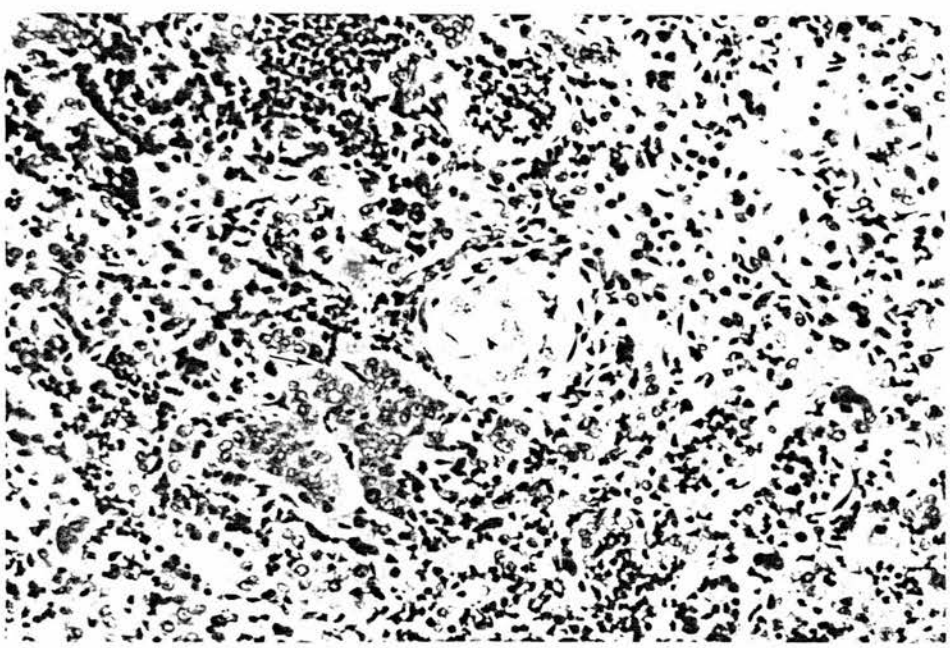

Fig. 3. Section from consolidated lesion in sheep from Group 2, Experiment II, killed 17 d.p.i. A hyaline scar is situated close to a hyperplastic bronchiolar wall (arrow). The surrounding alveoli are packed with macrophages and polymorphonuclear leucocytes. HE. × 256.

Groups 2 + 3 and Group 1 had significantly higher ($P = <0{\cdot}01$) numbers of animals with pleurisy and with proliferative pneumonia than Groups 4 + 5.

*Microbiology. M. ovipneumoniae* was recovered from the upper and lower respiratory tracts of every animal in Groups 1, 2 and 3. Titres of organisms recovered were higher in lung lesion pools than in other samples (Table 4). *M. ovipneumoniae* was also recovered from the upper respiratory tract (tonsil or trachea or both) of 5 animals and the lungs of 3 animals in Groups 4 and 5. Recovery titres of $10^7$ ccu per 0·2 ml were obtained from lesion pools in two

TABLE 4

SUMMARY OF MICROBIOLOGICAL FINDINGS IN EXPERIMENT II

| | *Geometric means of the titres ($log_{10}$) of the stated organisms in various lung samples** | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. ovipneumoniae (*ccu per 0·2 ml*) | | | | M. arginini (*ccu per 0·2 ml*) | P. haemolytica (*organisms per ml*) | |
| *Group* | *Lt.L* | *B.S.* | *DL* | *NL* | *B.S.* | *DL* | *NL* |
| 1 | 4·7 (1/1) | 6·1 (7/7) | 6·3 (6/6) | 4·0 (5/6) | 5·2 (3/7) | 5·1 (2/7) | 0·0 (0/6) |
| 2 | 4·8 (3/3) | 6·3 (7/7) | 7·2 (4/4) | 3·5 (4/4) | 4·7 (4/7) | 6·4 (7/7) | 0·0 (0/4) |
| 3 | 5·0 (4/4) | 5·3 (7/7) | 7·0 (3/3) | 3·3 (3/3) | 5·8 (5/7) | 7·5 (7/7) | 4·2 (2/3) |
| 4 | ND (—) | 0 (0/7) | 0 (0/5) | 0 (0/7) | 0 (0/7) | 0 (0/5) | 0 (0/7) |
| 5 | ND (—) | 4·0 (4/7) | 5·3 (3/6) | 4·0 (3/7) | 0 (0/7) | 0 (0/6) | 0 (0/7) |

* Negative samples excluded.
Lt.L., left lung: geometric mean of recovery titres from left apical, cardiac and diaphragmatic lobes.
B.S., bronchial swab.
DL, pool of diseased lung tissue.
NL, pool of normal lung tissue.
ND, not done.
Figures in parentheses indicate number of sheep positive/number examined.
Each group contained 7 sheep.

of these control sheep. *M. arginini* was recovered from the upper respiratory tract of 16 animals and the lungs of 14 in Groups 1, 2 and 3. Significant titres were demonstrated in the bronchial swabs of only 12 of these sheep: these 12 also had pleurisy. Lung samples from only one pleuritic animal were negative for *M. arginini*, although the organism was recovered from the trachea in this case. *M. arginini* was also recovered from the lung samples of 2 animals in Group 5, but no growth was obtained from their bronchial swabs. *P. haemolytica* serotype A2 was recovered from the lungs of all sheep in Groups 2 and 3 and of 2 animals in Group 1: the lungs of all animals in Groups 4 and 5 were negative for the organism. Titres of recovery in lesion pools were very much

higher than in non-lesion pools, and ranged from $10^{3\cdot0}$ to $10^{9\cdot6}$ orgs per ml. No viruses were detected, but a significant rise in serum antibody titres to PI3 virus was detected in 6 animals in Group 1.

## DISCUSSION

Two different preparations from atypical pneumonic lesions produced very similar effects when inoculated endobronchially into a total of 19 sheep, with the development in 18 animals of respiratory symptoms, and proliferative and exudative pneumonia. The combined cloned cultures of mycoplasmas and *P. haemolytica* given to Groups 2 and 3 in Experiment II simulated the effects of LS. No differences were found between these 2 groups, indicating that the sterile lung material included in the inoculum given to Group 2 had no exacerbative effects.

LS2 produced milder clinical symptoms, a smaller mean LLS and fewer *P. haemolytica* lung recoveries compared with LS1, which may have been due to a lower content of *P. haemolytica*, though the mild respiratory disease present before inoculation in the Group 1 (Experiment II) animals may also be relevant. Cultural findings from preinoculation nasal swabs suggest that mycoplasmas and *P. haemolytica* already established in the nose were involved in the disease; rising antibody titres also indicate that a PI3 virus infection occurred within Group 1 during the experiment. As PI3 virus was not recovered from LS2, the sheep were probably infected with virus before inoculation. How infection with these 3 organisms might have affected the results is uncertain, but no histopathological differences were noted between Group 1 and other infected animals in Experiments I and II.

The 14 control animals in Groups 4 and 5 (Experiment II) showed no symptoms of respiratory disease. Respiratory micro-organisms were not isolated from the mild and localized proliferative and exudative pneumonia observed in one control animal. Three sheep from Group 5 yielded *M. ovipneumoniae* from the lungs, and in two of these the highest recovery titres were obtained from areas of linear collapse, but *M. ovipneumoniae* in lung tissue is not always associated with pathological change: in an abattoir survey, 40 per cent of normal sheep lungs yielded *M. ovipneumoniae* (Alley, Quinlan and Clarke, 1975), and of 6 specific pathogen-free (SPF) lambs experimentally infected with *M. ovipneumoniae*, two that showed no histopathological change yielded $10^4$ to $10^6$ ccu per 0·2 ml from the lungs (Foggie *et al.*, 1976).

In Experiment II, pleurisy and death were closely associated with the presence of *M. arginini*. However, pleurisy was also present in 6 animals in Experiment I, though *M. arginini* was neither injected nor isolated, and the animals were not acutely ill. Thus there may be a relationship only between the presence of *M. arginini* and mortality.

The effects of the respiratory organisms contained in the infective inocula administered in these experiments have been studied individually by several workers. *P. haemolytica* biotype A strains when given intratracheally or endobronchially produced changes indistinguishable from natural cases of enzootic pneumonia, but doses in excess of $10^8$ organisms were necessary (Smith, 1960,

1964; Biberstein and Thompson, 1966; Biberstein *et al.*, 1967; Biberstein, Shreeve, Angus and Thompson, 1971). *M. ovipneumoniae* was found by Sullivan, St George and Horsfall (1973) to produce a proliferative interstitial pneumonia, but Foggie *et al.* (1976) found that endobronchial inoculation produced, in a proportion of SPF lambs, pulmonary cellular and fluid exudation which culminated in peribronchiolar and perivascular lymphoid cell cuffing. *M. arginini* produced virtually no pulmonary changes nor was lung infection established when the organism was administered by a variety of routes to SPF lambs (Foggie and Angus, 1972; Jones, Foggie and Buxton, unpublished work).

The results of experiments in which combinations of organisms were used are contradictory. The pathogenic effects of chlamydia were unaltered by the presence of mycoplasma spp. or *P. multocida* or both, or even by "stress" (Boidin, Cordy and Adler, 1958; Dungworth and Cordy, 1962), but Hamdy and Pounden (1959) consistently reproduced pneumonia when *P. multocida* and a mycoplasma sp., with and without chlamydia, were inoculated intratracheally and the animals subjected to "stress". Biberstein *et al.* (1967, 1971) studied changes produced by chlamydia or PI3 virus and *P. haemolytica* (biotypes A and T), alone and in combination, but were doubtful if synergism was demonstrated. In contrast, Sharp, Gilmour and Rushton (personal communication) claim to have shown synergism between PI3 virus and *P. haemolytica* in SPF lambs when the *P. haemolytica* was administered by aerosol.

Reproduction of enzootic pneumonia by the inoculation of lung lesion suspensions has been attempted by several workers (Montgomerie, Bosworth and Glover, 1938; Downey, 1957; Salisbury, 1957), but only Montgomerie *et al.* had some success in that simultaneous inoculation of material by several routes was followed by a severe clinical pneumonia, although histologically the lesions did not resemble those of the naturally occurring disease.

Our investigations show that proliferative and exudative lesions indistinguishable from atypical pneumonia may be produced by lesion suspensions of diseased lung and also by mixed cultures of *P. haemolytica*, *M. ovipneumoniae* and *M. arginini*. The atypical pneumonia produced by this combination was unlike the lesions reported in respect of infections with these agents given singly, but the contribution of the individual agents to the final effect requires further investigation, particularly as *P. haemolytica* and *M. arginini*, although injected, were not invariably recovered. Our findings also show that lung infections with *P. haemolytica* were established with inoculum doses as low as $10^{3\cdot4}$ to $10^{6\cdot3}$ organisms when combined with mycoplasmas. Abattoir and field surveys have demonstrated the ubiquity of *M. ovipneumoniae*, *M. arginini* and *P. haemolytica* in both normal and pneumonic sheep (Biberstein, Shreeve and Thompson, 1970; Shreeve *et al.*, 1972; Alley *et al.*, 1975) and an association between unidentified mycoplasmas and *P. haemolytica* has been observed in outbreaks of pneumonia (Mohn and Utklev, 1974). Thus it is suggested that the aetiology of atypical pneumonia may commonly involve the combination of mycoplasmas and *P. haemolytica*.

SUMMARY

A lung homogenate suspension prepared from lesions of naturally occurring atypical pneumonia was found to contain micro-organisms that included *P. haemolytica* (several serotypes), *M. ovipneumoniae* and *E. coli*. Ten sheep inoculated endobronchially with this lung suspension developed clinical symptoms of respiratory disease and pulmonary lesions indistinguishable from atypical pneumonia. Large numbers of *P. haemolytica* and *M. ovipneumoniae* were recovered from the lungs.

A second suspension prepared from atypical pneumonic lesions was found to contain *P. haemolytica* (serotype A2), *M. ovipneumoniae* and *M. arginini*. This lung suspension and suspensions containing combined cloned cultures of the 3 organisms isolated produced similar effects when injected endobronchially. Clinical symptoms of respiratory disease, with some deaths, and lesions of exudative and proliferative pneumonia indistinguishable from those found in field cases of atypical pneumonia were produced. *M. ovipneumoniae* was recovered from all animals inoculated with infective material, but the recoveries of *P. haemolytica* and *M. arginini* were less constant. However, these results may have been affected by the presence of all 3 respiratory micro-organisms in some sheep before inoculation, in particular those animals inoculated with lesion suspension. It is concluded that *P. haemolytica* and mycoplasmas may commonly be the aetiological agents involved in atypical pneumonia.

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REFERENCES

Alley, M. R., Quinlan, J. R., and Clarke, J. K. (1975). The prevalence of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in the respiratory tract of sheep. *New Zealand Veterinary Journal*, **23,** 137–141.

Biberstein, E. L., and Thompson, D. A. (1966). Epidemiological studies on *Pasteurella haemolytica* in sheep. *Journal of Comparative Pathology*, **76,** 83–94.

Biberstein, E. L., Nisbet, D. I., and Thompson, D. A. (1967). Experimental pneumonia in sheep. *Journal of Comparative Pathology*, **77,** 181–192.

Biberstein, E. L., Shreeve, B. J., Angus, K. W., and Thompson, D. A. (1971). Experimental pneumonia of sheep. Clinical, microbiological and pathological responses to infection with *Myxovirus parainfluenza*? 3 and *Pasteurella haemolytica*. *Journal of Comparative Pathology*, **81,** 339–351.

Biberstein, E. L., Shreeve, B. J., and Thompson, D. A. (1970). Variation in carrier rates of *Pasteurella haemolytica* in sheep flocks. I. Normal flocks. *Journal of Comparative Pathology*, **80,** 499–507.

Boidin, A. G., Cordy, D. R., and Adler, H. E. (1958). A pleuropneumonia-like organism and a virus in ovine pneumonia in California. *Cornell Veterinarian*, **48,** 410–430.

Dighero, M. W., Bradstreet, C. M. P., and Andrews, B. E. (1970). Dried paper discs for serological identification of human mycoplasmas. *Journal of Applied Bacteriology*, **33,** 750–757.

Downey, N. E. (1957). A preliminary investigation into the actiology of enzootic pneumonia of sheep ("Southland pneumonia"). *New Zealand Veterinary Journal*, **5,** 128–133.

Dungworth, D. L., and Cordy, D. R. (1962). The pathogenesis of ovine pneumonia. I. Isolation of a virus of the PLV group. *Journal of Comparative Pathology*, **72,** 49–70.

Foggie, A., and Angus, K. W. (1972). Observations on the distribution of *Mycoplasma arginini* as a respiratory tract infection in sheep and its pathogenicity for specific pathogen free lambs. *Veterinary Record*, **90,** 312–313.

Foggie, A., Jones, G. E., and Buxton, D. (1976). The experimental infection of specific pathogen free lambs with *Mycoplasma ovipneumoniae*. *Research in Veterinary Science*, **21,** 28–35.

Gilmour, N. J. L., and Brotherston, J. G. (1963). Clinical and pathological findings in an outbreak of pneumonia in sheep. *Journal of Comparative Pathology*, **73,** 329–335.

Hamdy, A. H., and Pounden, W. D. (1959). Experimental production of pneumonia in lambs. *American Journal of Veterinary Research*, **20,** 78–83.

Hore, D. E. (1970). Pneumonia in sheep. *Australian Veterinary Journal*, **46,** 169–172.

Jones, G. E., Foggie, A., Mould, D. L., and Livitt, S. (1976). The comparison and characterization of glycolytic mycoplasmas isolated from the respiratory tract of sheep. *Journal of Medical Microbiology*, **9,** 39–52.

Jones, G. E., Foggie, A., Sutherland, A., and Harker, D. B. (1976). Mycoplasmas and ovine keratoconjunctivitis. *Veterinary Record*, **99,** 137–141.

Mohn, S. F., and Utklev, H. E. (1974). *Pasteurella haemolytica* and enzootic respiratory infections in sheep. *Nordisk veterinaermedicin*, **26,** 658–669.

Montgomerie, R. F., Bosworth, T. J., and Glover, R. E. (1938). Enzootic pneumonia in sheep. *Journal of Comparative Pathology*, **51,** 87–107.

Salisbury, R. M. (1957). Enzootic pneumonia of sheep in New Zealand. *New Zealand Veterinary Journal*, **5,** 124–127.

Shreeve, B. J., Biberstein, E. L., and Thompson, D. A. (1972). Variation in carrier rates of *Pasteurella haemolytica* in sheep. II. Diseased flocks. *Journal of Comparative Pathology*, **82,** 111–116.

Smith, G. R. (1960). The pathogenicity of *Pasteurella haemolytica* for young lambs. *Journal of Comparative Pathology*, **70,** 326–338.

Smith, G. R. (1961). The characteristics of two types of *Pasteurella haemolytica* associated with different pathological conditions in sheep. *Journal of Pathology and Bacteriology*, **81,** 431–440.

Smith, G. R. (1964). Production of pneumonia in adult sheep with cultures of *Pasteurella haemolytica* Type-A. *Journal of Comparative Pathology*, **74,** 241–249.

Smith, W. D. (1975). The nasal secretion and serum antibody response of lambs following vaccination and aerosol challenge with parainfluenza 3 virus. *Research in Veterinary Science*, **19,** 56–62.

Stevenson, R. G. (1969). Respiratory disease of sheep. *Veterinary Bulletin*, **39,** 747–759.

Stevenson, R. G., and Robinson, G. (1970). The pathology of pneumonia in young lambs inoculated with Bedsonia. *Research in Veterinary Science*, **11,** 469–474.

Stamp, J. T., and Nisbet, D. I. (1963). Pneumonia of sheep. *Journal of Comparative Pathology*, **73,** 319–328.

Sullivan, N. D., St George, T. D., and Horsfall, N. (1973). A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 2. The experimental exposure of young lambs to infection. *Australian Veterinary Journal*, **49,** 63–68.

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