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CHAPTER FIVE

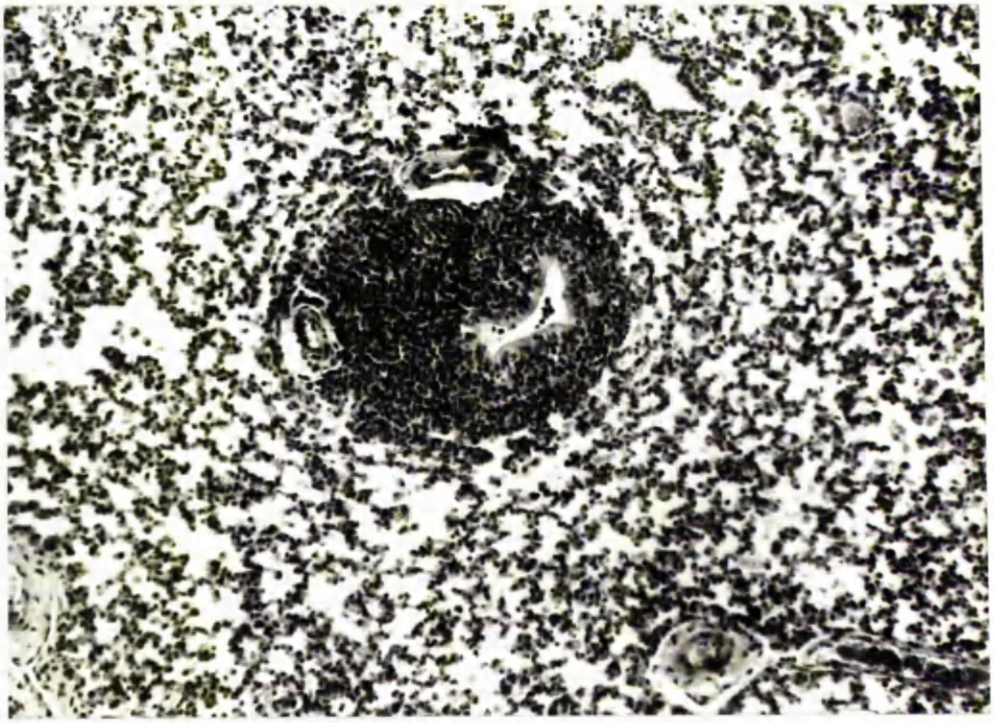
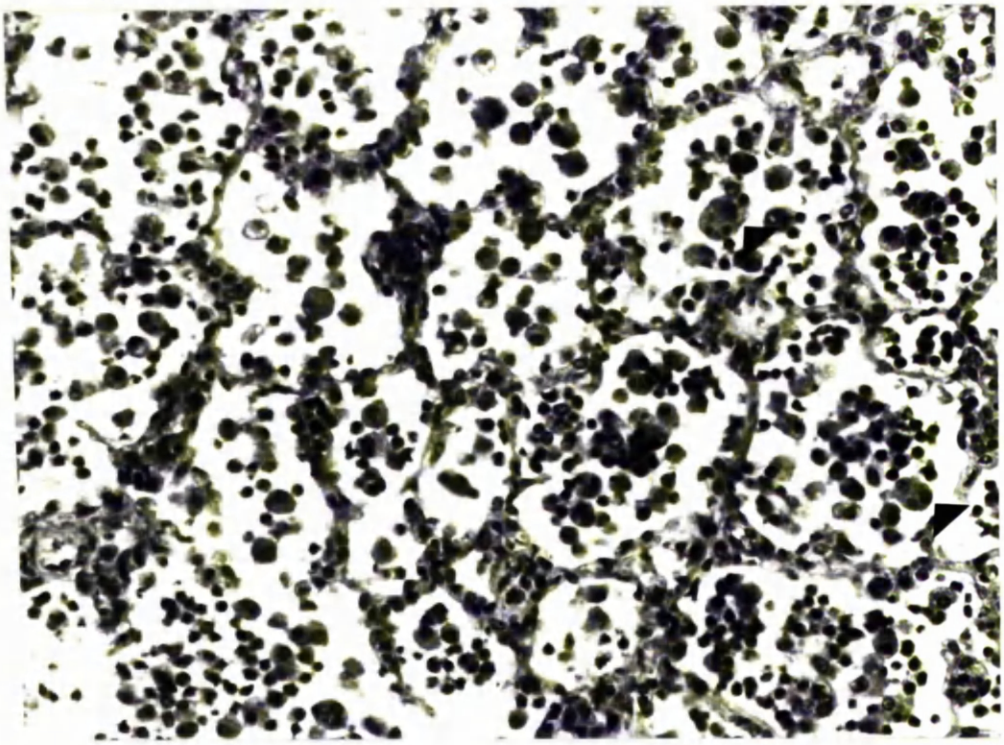


Fig 10 : One to two months old calf with a proliferative pneumonia. The lesion is a D(i) type characterised by an active alveolar reaction consisting of large numbers of macrophages. Multinucleated giant cells (arrows) are also seen. HE staining, x 250.

Fig. 11 : One to two months old calf. The alveolar reaction in a D(i) type pulmonary lesion often consists of large, foamy macrophages and neutrophils as seen here. HE staining, x 250.



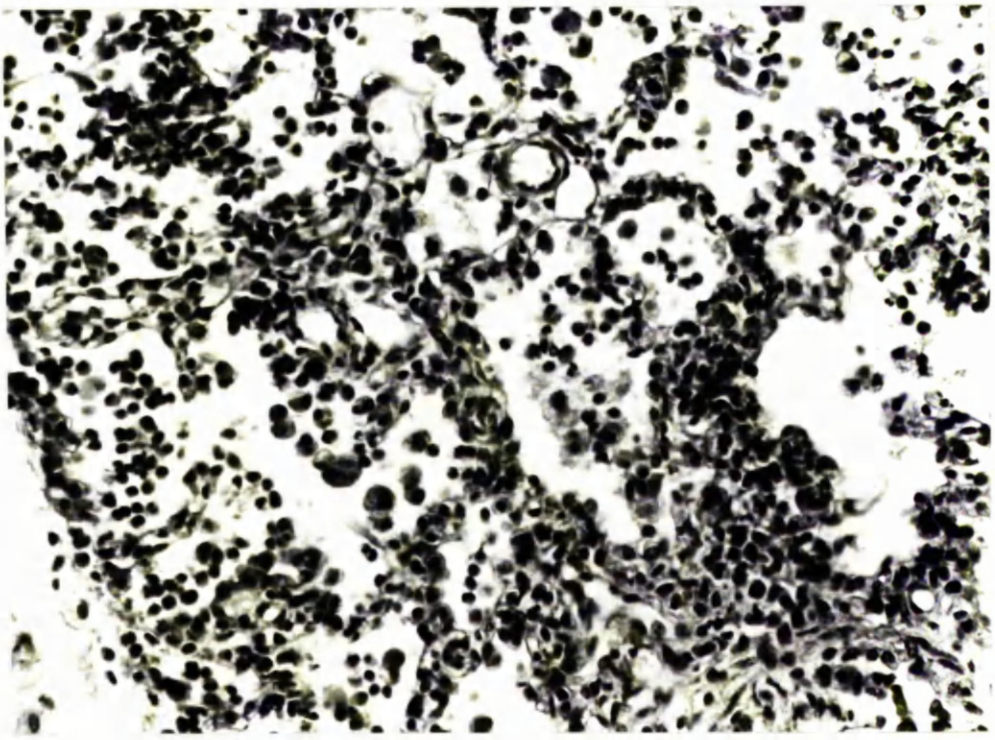


Fig. 12 : One to two months old calf. Bronchitis of a D type pulmonary lesion showing a heightened epithelial layer and cellular infiltration of the lamina propria. The bronchial submucosal glands are hypertrophied; the mucous tubules are slightly dilated and surrounded by plasma cells. HE staining, x 120.

Fig. 13 : One to two months old calf. Bronchitic epithelium of a large bronchus of D type lesion with a heightened epithelial layer and neutrophils infiltrating the cells (arrows). Numerous cells, particularly plasma cells, are seen in the lamina propria. The epithelial layer is intact and cilia are distinguishable. HE staining, x 250.

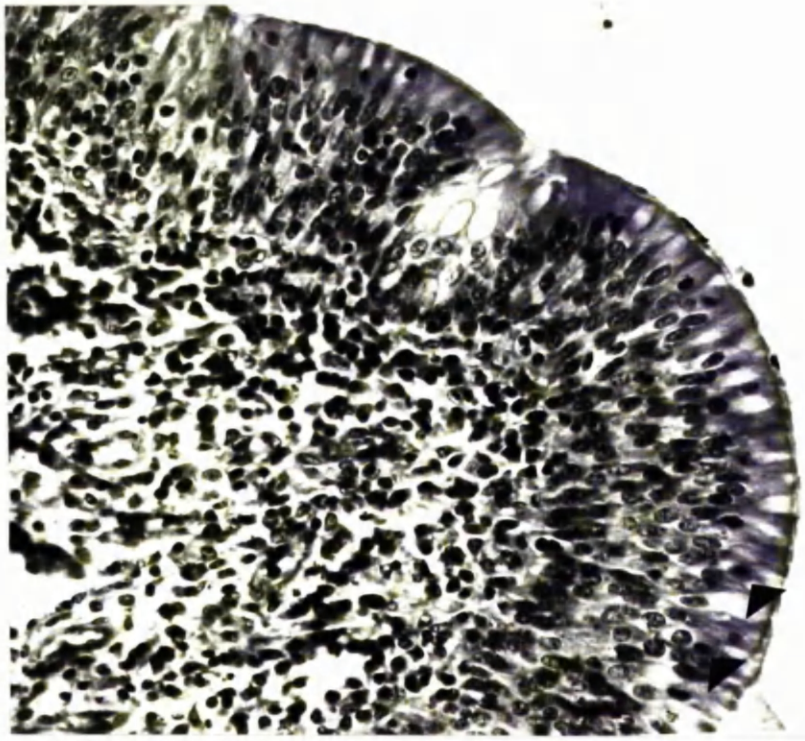
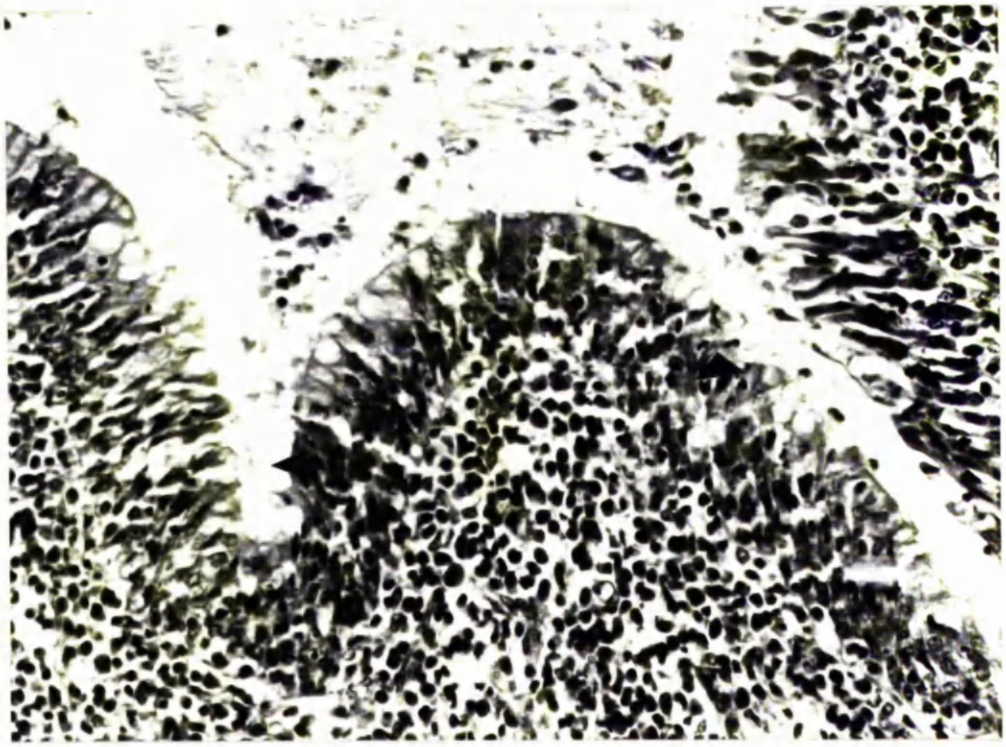
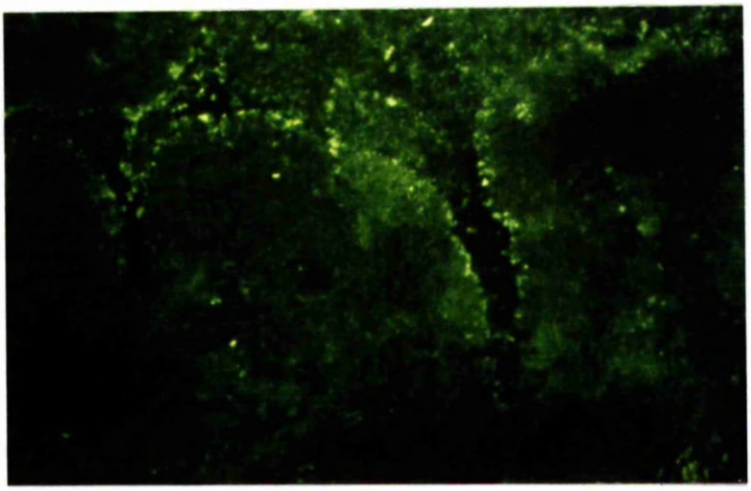


Fig. 14 : One to two months old calf. Bronchitic epithelium of a small bronchi with a D type lesion. Comparison with Fig. 13 illustrates the damaged epithelial cells in the narrower airways. Plasma cells are present in the lamina propria in large numbers and neutrophils infiltrate between the epithelial cells towards the lumen (arrows). A cellular exudate is seen in the lumen of the airway. HE staining, x 250.

Fig. 15 : A section of bronchial tissue fluorescing posidvely for the presence of M. dispar after staining by the indirect immuno-fluorescent technique. Bright yellow-green positive material is seen at the luminal surface of the epithelium, probably in the area of the cilia. Some fluorescing material is seen in the lumen of the airway. x 200 .





D. CALVES THREE TO FOUR MONTHS OLD

1. Introduction

The calves in this group were collected during an investigation into an outbreak of respiratory disease on a farm. The animals were three to four months of age and part of a group which had been reared together from birth. The history of the animals and the development of the respiratory disease will be given, followed by the details of the microbiological and pathological examination.

2. Materials and methods

a. Animals.

Twelve calves aged three to four months were examined from a dairy farm in Stirlingshire. On this farm, Hereford cross calves were born in July and August and reared in groups of two and three in adjacent pens which were small, open and well ventilated. All calves received colostrum and weresubsequently maintained on a milk substitute diet. During the summer of 1975 25 to 30 calves were being reared under such conditions. Many of the calves had a conjunctivitis at an early age; however, the animals thrived until they were about two months old when some of them developed a clinical respiratory disease characterised by a nasal discharge and much coughing. As these signs spread amongst the penned calves the animals were gathered into one large, well ventilated, warm, clean pen. The disease spread rapidly amongst the calves and developed into an acute condition five to six weeks after the signs were first observed. At the acute stage of the disease one calf died (M133); another calf (M167) received a single dose of terramycin but was destroyed a week later with severe respiratory symptoms. The ten most severely affected animals were purchased from the farmer a few days later and examined immediately. At that time 20 of the remaining calves were sampled for serological studies; a repeat sample was collected from them four weeks later.

b. Post mortem techniques.

The ten calves purchased from the farm were considered as one group (M183 - M192), while the other two animals (M133 and M167) were examined individually. The ten calves were collected from the farm in two groups of five during two consecutive days and slaughtered on the day of removal. Prior to slaughter the calves were examined clinically with measurements of respiratory rate, temperature and auscultatory findings being recorded. The examinations were performed by a clinician in the Department of Veterinary Medicine at the University of Glasgow Veterinary School. A sample of blood was taken from each calf prior to slaughter and examined haematologically for the packed cell volume (PCV), total red blood cell (RBC) and white-blood cell counts and a differential count.

The live animals were shot and exsanguinated. Two universals of blood were collected from each of the ten calves; the sera were later tested for antibody to M. dispar and certain bovine respiratory viruses. The lungs and trachea were removed into polythene bags, avoiding contact with foreign surfaces, and examined macroscopically. Specimens for all examinations were taken from the right cranial lobe; a similar site in each case was sampled to enable comparisons to be made. In addition, random pieces of tissue from other lobes were taken and examined by some of the techniques mentioned.

Samples of tissue were collected into GS, U3 and A broths and sterile PBS for subsequent examination for the presence of mycoplasmas and bacteria as described in chapter two, section C. Duplicate samples were collected for each microbiological examination and stored at -70^{o}C.

Six duplicate blocks of tissue from the right cranial and right diaghragmatic lobes of all animals were taken for histological examination. The main bronchus of the two lobes in each animal was sampled at three positions along its length; each sample included a piece of lung tissue. A specimen from each site was collected in corrosive formol, the duplicate block being fixed in ten per cent formol saline. Fixing, processing and staining were carried out as described in section E of chapter two; all blocks of tissue

Why just these ?

RS MD

Neo

Rhino

Chlamydia

were examined by HE staining.

The above examinations were performed on the 12 calves while immunofluorescent and serological studies were carried out on the ten calves purchased as a group.

Specimens of bronchus and lung from the right cranial lobe of all animals were collected and snap-frozen in acetone and dry ice. Samples from each animal were examined for the presence of M. dispar by the indirect immunofluorescent technique as described in chapter two, section G.

Serological studies were carried out on the serum collected from the calves at slaughter. Detection of antibody to M. dispar was carried out by the indirect immunofluorescent technique (chapter two, section H); the haemagglutination inhibition test for PI3 virus antibody, the gel diffusion test for precipitating antibody to adenovirus and the serum neutralising test for antibody to IBR virus were performed by the Department of Microbiology, Moredun Institute, Edinburgh. Subsequent to the respiratory outbreak in this group of calves, 20 of the remaining animals were tested for antibody to the above respiratory viruses. Two serum samples were tested, one taken at the acute stage of the disease and one during the convalescent period (four weeks later).

3. Results

a. Pre mortem examinations.

The results of the clinical and haematological findings are illustrated in Table 25. These examinations revealed that all animals were suffering from severe respiratory disease. Respiratory rates were above normal in all cases with one calf (M186) reaching 75 per minute; the average rate amongst these pneumonic animals was about 58 per minute.

The temperature of five calves was recorded and found to be significantly higher than normal. One calf (M188) had a pre mortem temperature of 105.1^{o}F; this calf also had an elevated respiratory rate (70 per minute).

An obvious cough was present in most calves; harsh respiratory sounds

with rhonchi were heard on auscultation. Fluid crackling was recognised in six of the animals with sibilant inspiratory/expiratory sounds on both sides in two of the more severe cases (M183 and M188).

Elevated packed cell volumes (PCV) and red blood cell counts (RBC) were characteristic of these animals. The total white blood cell (WBC) counts fell within the normal bovine range except for two cases (M184 and M186), with a massive increase in WBC counts to 23,900 per cmm in one case. The differential counts were within the normal range in most calves although an increase in lymphocytes was detected in two animals, M184 and M186.

b. Mycoplasmal and bacterial isolations.

The results of the microbiological examination of the 12 calves are shown in Table 26. Mycoplasmas, M. dispar, M. bovirhinis and A. laidlawii, were isolated from 11 of these calves; M. dispar was recovered from seven animals (58 per cent), while M. bovirhinis and A. laidlawii were cultured from the pneumonic lung tissue of one and four calves respectively. Ureaplasma spp. were not isolated from any lung tissue samples. Pulmonary tissue from one animal, M167, failed to yield any mycoplasmas on culture; this calf had received a dose of terramycin seven days before slaughter. Single isolations of mycoplasmas were made from the lungs of ten calves and A. laidlawii and M. dispar were cultured together from one calf (M183). The titres of mycoplasmas obtained varied; titres of 10^3 and 10^4 CCU of M. dispar per 0.2 ml of sample were found in most cases, while A. laidlawii was present at 10^4 and 10^6 CCU per 0.2 ml of sample. Mycoplasma bovirhinis was recovered in small numbers.

Bacteria were recovered from nine of the 12 calves. A few colonies of a Pasteurella sp. were isolated from one calf. An Acinetobacter sp. was recovered, in high numbers from two animals (M188 and M189), both calves yielding A. laidlawii too. Klebsiella aerogenes was isolated in high numbers from a calf (M185) together with M. dispar.

c. Pathology.

A summary of the findings from macroscopic and microscopic

pulmonary examination of the 12 calves is given in Table 27. Macroscopic lesions of pneumonia were seen in both lungs in 11 calves, being severe and involving all lobes to some degree in eight animals. In nine calves the right lung was more severely affected than the left with the most extensive lesions in the anterior lobes; the remaining three cases (M188, M133 and M167) showed vast areas of consolidation in both lungs. Figure 16 illustrates the anterior distribution of the lesions. This animal (M186) was one of the less severe cases although the anterior segment of the right cranial lobe was totally consolidated with a large area of pneumonia in the diaghragmatic lobe and scattered lesions in other lobes. These lesions were typical of nine of the calves being smooth, moist and deep plum-red as illustrated more clearly in Figure 17 which shows the middle lobe of case M183. This lesion occupied almost the entire lobe; at a higher magnification (Fig. 18) the plum-red lesions appeared smooth and the volume of the affected tissue was reduced. Clearly demarcated lesions was a typical feature of these cases as illustrated in Figures 17 and 18.

Lesions which differed slightly from those described above were found in three calves (M188, M189 and M167). Macroscopically, the pneumonia was more severe with extensive involvement of most lobes, as seen in case M167 illustrated in Figure 19. The lesions of the middle and diaghragmatic lobes were reddish-purple, moist and complicated by the presence of interstitial emphysema. The lesions of the cardiac lobes were less moist, smooth, deep purple with small greyish foci visible to the naked eye. The posterior lobes of case M188 (Fig. 20) had lesions similar to the anterior lesions of M167, being deep-purple with a moist, nodular surface; the nodules are seen clearly in Figure 21 which also illustrates the clear definition of the lesions. In contrast to the pneumonia of the posterior lobes, the lesions in the anterior lobes of this animal (M188) were smooth and plum-red, typical of the lesions in the other calves as shown in Figure17.

In the more severe cases mucus was present in the bronchi and bronchioles; the texture of the secretion varied from pale and frothy (M188) to stringy and yellow and it extended down the main bronchus of the

diaghragmatic lobe (M191).

The histological findings of these calves are illustrated in Table 27. The 12 animals had proliferative pneumonias with type D lesions; three of these animals with atypical type D histology (M188, M189 and M167), were designated with type D plasma cell lesions. A brief description of the type D lesion was given previously in this chapter (section B) indicating the characteristic presence of bronchitis and bronchiolitis. Peribronchiolar lymphoid accumulations was also a feature of type D lesions and were found in ten of these animals. Typically, these accumulations formed a diffuse layer of cells around the airway; a few follicular aggregates were present in most cases and in significant numbers in two animals (M191 and M192) (Fig. 22). In some areas these lymphoid aggregates, initially formed in the peribronchiolar region, were seen to infiltrate the lamina propria and destroy the muscularis at these points (Fig. 23). In one of the more severe cases (M192) the lymphocytes encircled a large proportion of the airway circumference, although not to the extent of forming a sheath or cuff of cells (Figs. 24 and 25). The cellular aggregates illustrated in Figure 24 have a germinal centre-like arrangement and have infiltrated the lamina propria; a large proportion of the muscle layer was destroyed. Alveolitis was present in the lung tissue adjacent to this affected bronchiole and consisted mostly of macrophages and a few plasma cells (Fig. 26). Figure 25 illustrates an active alveolitis with large numbers of neutrophils which have also plugged the bronchiolar lumen in this case. Although an alveolitis could be recognised in most cases (Table 27), alveolar collapse was a more significant finding in five of the nine calves; this was seen particularly in the lung tissue adjacent to severely affected airways.

The bronchitis of these cases was moderately severe characterised by hyperplasia of the epithelial cells which were ragged and torn in appearance (Fig. 27). Neutrophils were generally seen infiltrating through the lamina propria; large numbers of lymphocytes and plasma cells were found in the lamina propria.

Hypertrophy of the mucous glands was characteristic of most of these cases (Fig. 28). The glands were situated below the muscularis in clusters above and at the end of the cartilage plates. Increased numbers of tubules, containing cells actively secretory in appearance with large amounts of cytoplasm were seen (Fig. 29); the lumina of the tubules were slightly dilated with plasma cells in significant numbers around the tubules.

Pulmonary complications such as bronchiectasis, bronchial and bronchiolar polyps and abscesses were infrequently seen although plugging of the airways with a cellular exudate was a feature of some of the more severe cases (Fig. 24).

The three remaining calves were classified with plasma cell D type lesions and differed from the other nine animals by the presence of large numbers of plasma cells which had replaced many of the small lymphocytes typical of the lesions of the other nine cases. Figure 30 illustrates the cellular accumulations around the airways which consisted of plasma cells in large numbers occasionally infiltrating the epithelial cells of the bronchi (Fig. 31).

The epithelium of the large bronchi was hyperplastic in many cases and often a loss of differentiation to non-ciliated, cuboidal cells was seen. An acute alveolitis was present in all three cases and consisted of plugging of the alveolar spaces and bronchiolar lumina with neutrophils.

These three animals also differed, to some degree, in their macroscopic appearance from the other nine animals.

d. Immunofluorescence.

Employing the indirect immunofluorescent (IF) technique for the detection of M. dispar in the specimens of bronchial and alveolar tissue, ten calves purchased as a group (M183 - M192) were examined. Two cases (M187 and M191) fluoresced brightly at the luminal surface of the bronchial epithelium when stained specifically for M. dispar. Culturally, A. laidlawii and M. dispar were recovered from these two cases respectively. Of the eight calves which showed no specific IF for M. dispar, this organism was

cultured from the lungs of four of them.

e. Serology.

Serological examinations were carried out on the ten calves, purchased as a group (M183 - M192). No immunological response to M. dispar was detected by the indirect immunofluorescent technique. The examination for antibodies to P13 virus, adenovirus and IBR virus yielded insignificant results.

Samples collected during the acute and convalescent stages of the disease from 20 surviving calves were tested for the presence of antibody to some of the respiratory viruses; no increase in antibody titre to P13 virus was demonstrated and antibodies were not detected to adenovirus or IBR virus.

4. Discussion

Mycoplasma organisms were isolated from 11 of the 12 calves examined. Mycoplasma dispar, M. bovirhinis and A. laidlawii were the species isolated; Ureaplasma spp. were not recovered from any of the animals.

Mycoplasma dispar was the most frequently cultured organism, being recovered from seven of the animals at titres averaging 10^3 CCU per 0.2 ml of sample.

A variety of bacteria were isolated from the lung tissue of nine calves; an Acinetobacter sp. was cultured in large numbers from two animals which also harboured high titres of A. laidlawii in the lung tissue. These two animals, along with M167, differed histologically from the other calves by the presence of significant numbers of plasma cells in the lung tissue, particularly around the bronchi and bronchioles and also infiltrating through the epithelial cells into the airway lumen. An acute alveolitis was present in all three cases and consisted of a massive influx of neutrophils into the alveolar air spaces and bronchiolar lumina. These lesions were unusual and although Acinetobacter spp. were isolated in high numbers, no record of these organisms' pathogenesis is known.

Similar macroscopic and microscopic lesions were found in the other nine calves in this group, characterised by bronchitis, bronchiolitis and

accumulations of lymphocytes around the bronchiolar airways. These lesions have similarities to the second and third stage lesions found in experimental EPP (Whittlestone, 1972).

Gourlay and Thomas (1970) produced similar lesions of acute bronchiolitis associated with collapse and plugging of the bronchi and bronchioles with inflammatory exudate after the inoculation of nine calves with Ureaplasma spp. which were killed four weeks post-infection.

The results of the mycoplasmal isolations from these 12 cases examined suggests that M. dispar plays a significant role in the aetiology of the disease present amongst these animals.

The calves had been reared as a group since birth and although environmental conditions were reasonable, a respiratory infection quickly spread throughout the group. It is possible that M. dispar isolations were high among these calves because of the close contact between the animals, but no other microorganism was isolated at a significant rate. Additionally, serological studies eliminated the possibility of a viral causation for the disease, at least by PI3 virus, adenovirus and IBR virus. However, chlamydiae and other bovine viruses cannot be excluded; such organisms may have caused or may be connected with the outbreak of conjunctivitis seen within these animals at an earlier age.

Case No.	CLINICAL SIGNS			HAEMATOLOGICAL EXAMINATION				
	Respiratory rate min	Temperature °F	*Auscultatory results	PCV ml per 100 ml	RBC millions per cmm	Total WBC per cmm	Neutrophils per cent	Lymphocytes per cent
MI83	50	ND	++++	43	10.18	7,100	34	64
MI84	60	ND	+++	33	8.32	11,000	12	88
MI85	70	ND	+++	37	9.58	10,400	15	84
MI86	75	ND	++	36	8.59	23,900	40	60
MI87	45	ND	+++	42	10.47	8,100	31	69
MI88	70	105.1	++++	39	10.45	6,400	30	70
MI89	50	104	+++	45	9.17	8,600	26	74
MI90	60	103.2	++	34	9.14	7,400	32	68
MI91	50	103	++	42	10.23	10,300	29	71
MI92	50	102.2	++	36.5	9.63	9,000	29	69
MI33	60	ND	++	ND	ND	ND	ND	ND
MI67	** ND	ND	++	ND	ND	ND	ND	ND

* ++; harsh respiratory sounds with rhonchi : +++; very harsh respiratory sounds with fluid crackling on one side: ++++; very harsh respiratory sounds with fluid crackling on both sides. ** ND : not done.

Table 25 Results of the clinical and haematological examination of a group of 12 calves, aged from three to four months.

Case No.	Age (months)	*Mycoplasma isolations	*Bacterial isolations
MI83	3-4	M. dispar, 10^4; A. laidlawii, 10^4	-
MI84	3-4	M. dispar, 10^2	-
MI85	3-4	M. dispar, 10^3	Klebsiella aerogenes +++
MI86	3-4	M. dispar, 10^3	Corynebacterium pyogenes +
MI87	3-4	A. laidlawii, 10^4	Staph. sp. ; Coryn. sp.+
MI88	3-4	A. laidlawii, 10^6	Acinetobacter sp. +++
MI89	3-4	A. laidlawii, 10^4	Acinetobacter sp. +++
MI90	3-4	M. bovirhinis, 10^1	C. pyogenes +
MI91	3-4	M. dispar, 10^3	Micrococcus sp. +
MI92	3-4	M. dispar, 10^3	-
MI33	3-4	M. dispar, 10^4	Past. haemolytica var. haem. +
MI67	3-4	-	Strep. pneumoniae, Strep. bovis ++

* Lung tissue was examined for the presence of mycoplasmas and bacteria; the titre of mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++ 20-50 colonies; + 5-20 colonies.

Table 26. Frequency of isolations of mycoplasmas and bacteria from lung tissue of a group of three to four months old calves.

Case No.	*MACROSCOPIC LESIONS RCa	RCp	RM	RD	LCa	LCp	LD	Acc.	* MICROSCOPIC FINDINGS
MI83	+++	++	+++	-	+	+	+	+	Proliferative D(ii) lesion.
MI84	+++	++	+	+	+	++	+	+	Proliferative D(ii) lesion.
MI85	++	++	++	+	+	++	++	+	Proliferative D(i) lesion.
MI86	+++	+	+	++	+	+	+	+	Proliferative D(ii) lesion.
MI87	+	+	++	+	+	++	+	+	Proliferative D(ii) lesion.
MI88	+++	+++	+++	+	+++	+++	+	+++	Proliferative D(i) plasma cell type lesion.
MI89	+++	+++	+++	+	+	++	-	++	Proliferative D(i) plasma cell type lesion.
MI90	+	-	-	-	-	-	-	-	Proliferative D(i) lesion.
MI91	+++	+++	+	+	+	+	+	+	Proliferative D(i) lesion.
MI92	+++	++	-	+	-	+	++	+	Proliferative D(i) lesion.
MI33	+++	+++	++	++	+++	+++	++	++	Proliferative D(ii) lesion.
MI67	+++	+++	+++	+++	+++	+++	++	++	Proliferative D(i) plasma cell type lesion.

* Based on nomenclature and classification illustrated in Tables 16and 17 .

Table 27 . Pathological findings of pneumonic lungs of 12 calves aged between three and four months.

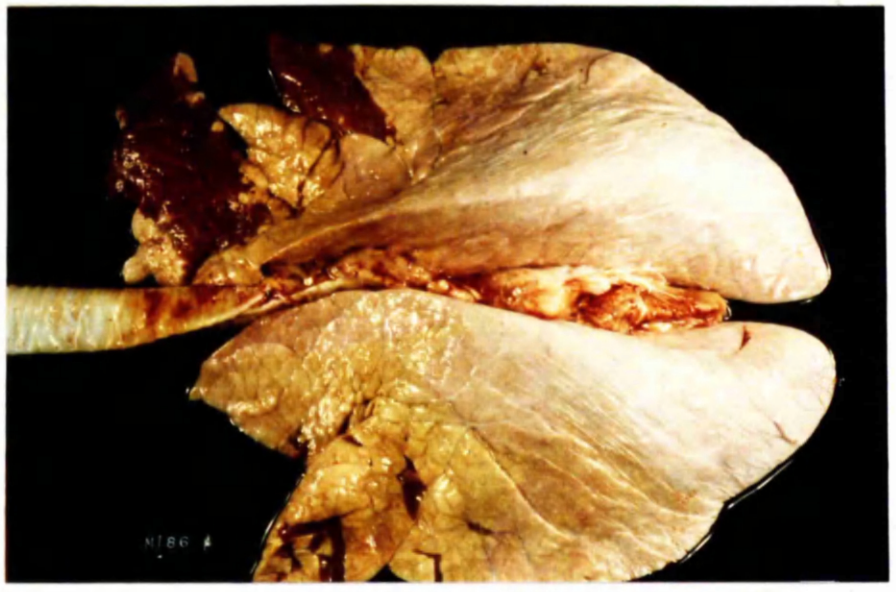
CHAPTER SIX

CHAPTER SEVEN

Fig. 16 : Three to four months old calf (M186). Smooth, moist lesions of consolidation are seen in the anterior lobes of the lungs particularly on the right side. The lesions are well-defined and deep plum-red.

Fig. 17 : The right middle lobe of case M183, a three to four months old calf, illustrating the smooth, moist appearance typical of the pulmonary lesions found in these calves. Almost the whole lobe is involved with only a few lobules of normal tissue seen.

Fig. 18 : A higher magnification of Fig. 17 illustrating the smooth, moist appearance of the well-demarcated lesions. The affected lobules are plum-red and of less than normal volume.




M 83 P

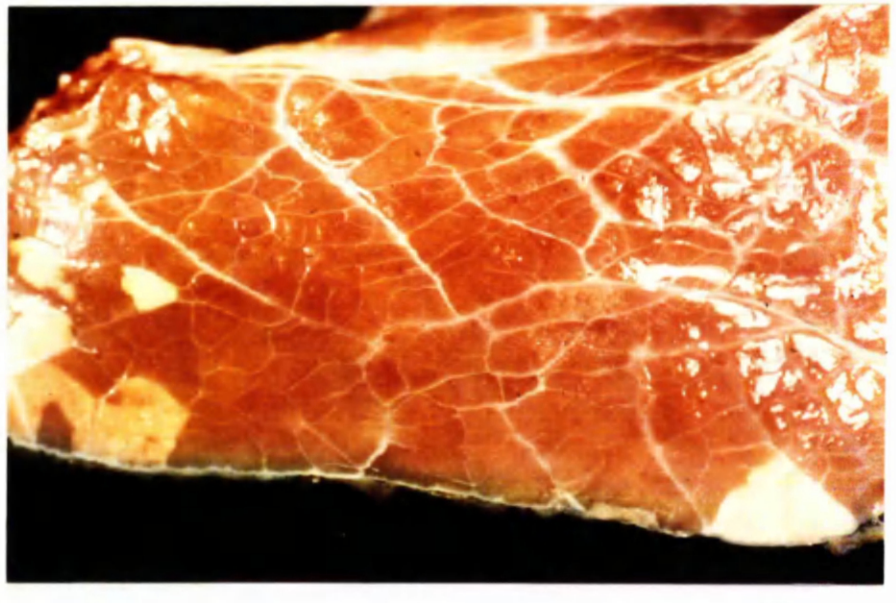
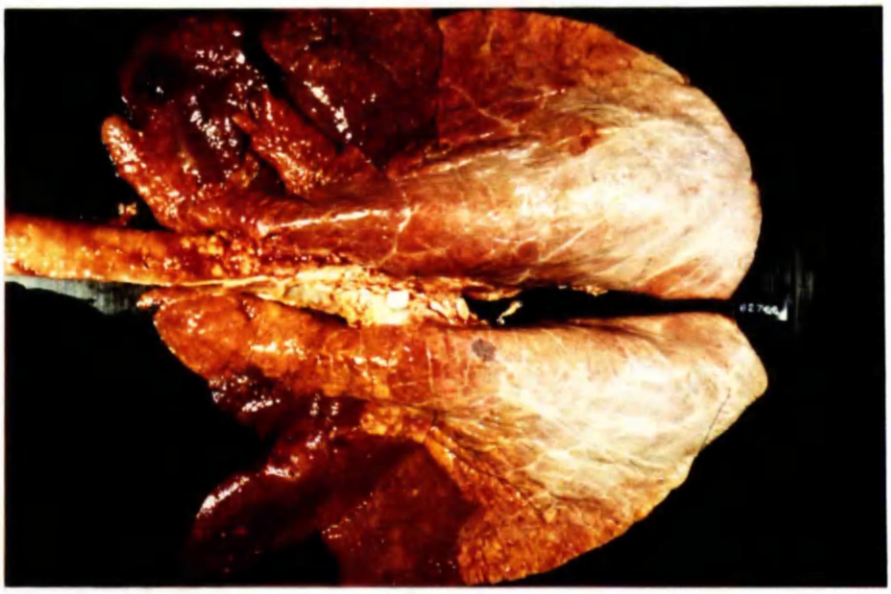


Fig. 19 : Case M167, a three to four months old calf, demonstrating extensive involvement of all lobes particularly the anterior lobes. The lesions of the middle and diaghragmatic lobes are reddish-purple and moist while the lesions of the cardiac lobes are smooth and deep-purple with small, greyish foci.

Fig. 20 : The left lung of case M188, a three to four months old calf. The cardiac lobe is completely consolidated with a moist nodular surface. The lesion of the diaghragmatic lobe, deep-purple with greyish nodules, is most common in the peripheral areas.

Fig. 21 : This is a close-up of the lesion illustrated in Fig. 20 showing the greyish nodular appearance of the pneumonic lobules. Areas of haemorrhage are present. The affected tissue is of less than normal volume and is clearly defined from the normal tissue.



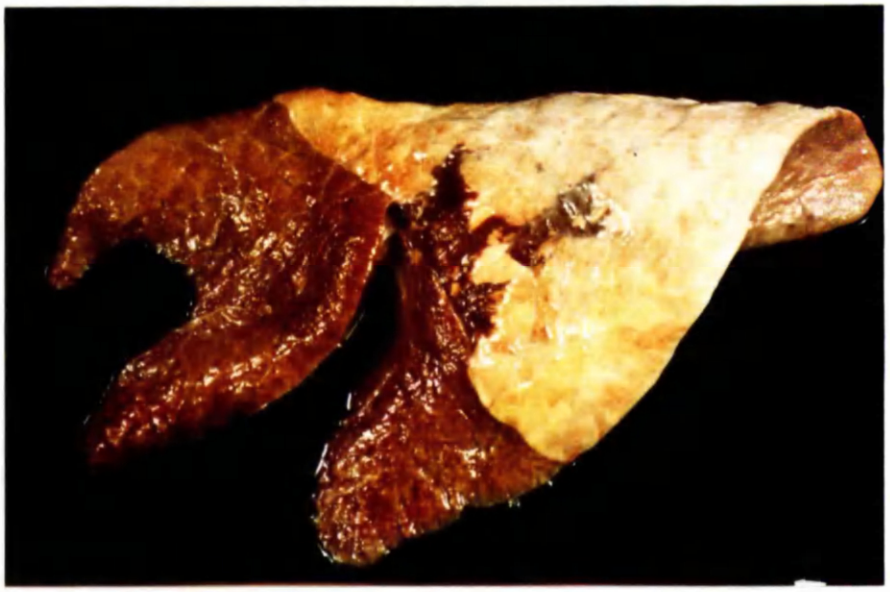
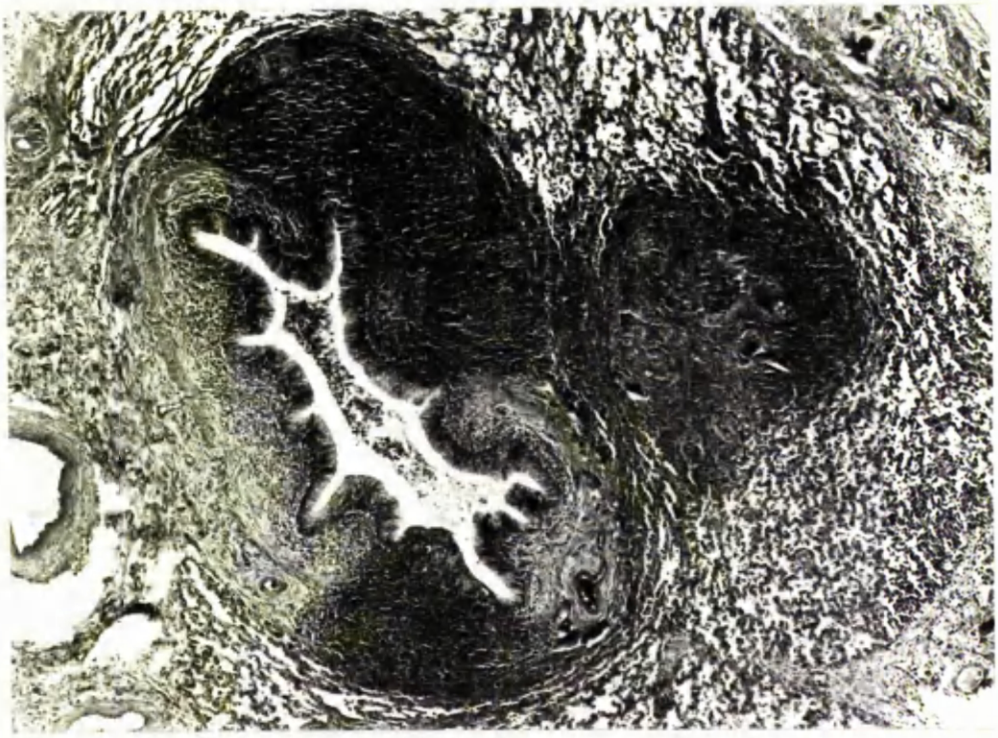


Fig. 22 : Three to four months old calf. D(i) type pulmonary lesion with accumulated lymphocytes in the perivascular and peribronchiolar regions. The lymphoid cells form a follicular arrangement around the airways in some areas, destroying the muscle where the aggregates penetrate the lamina propria. A cellular reaction in the alveoli and an exudate is present in the bronchiolar lumen. HE staining, x 40.

Fig. 23 : Three to four months old calf. D type pulmonary lesion with lymphocytic aggregates around the bronchiole forming a germinal centre-like organisation which destroys the muscle and infiltrates the lamina propria. An alveolitis is present in the adjacent lung tissue and a cellular exudate is seen in the airway lumen. HE staining, x 40.



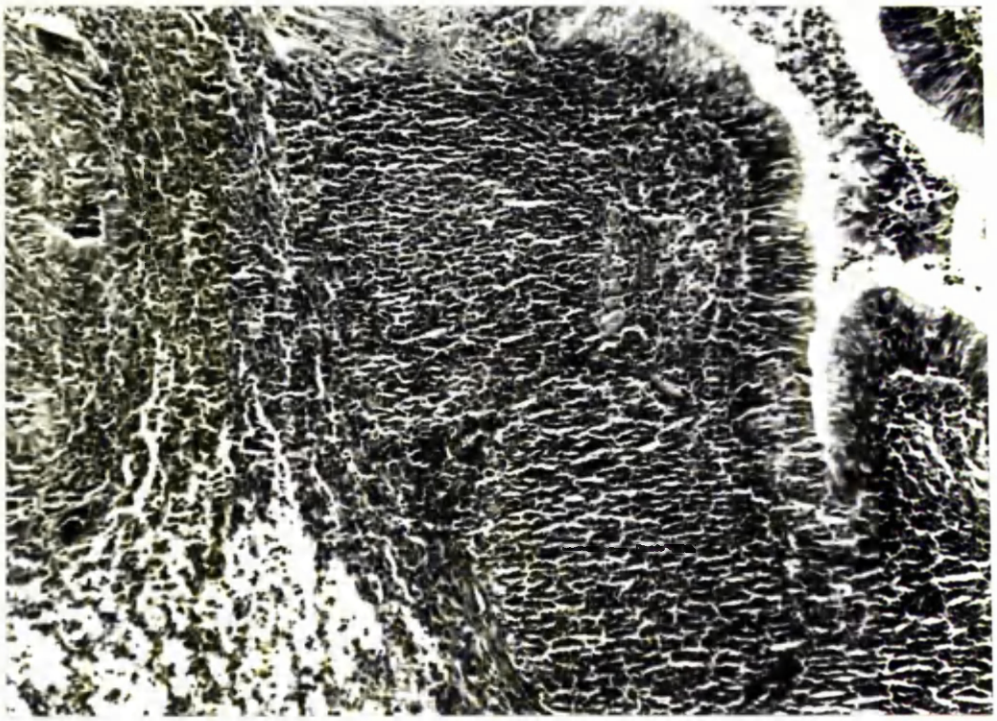
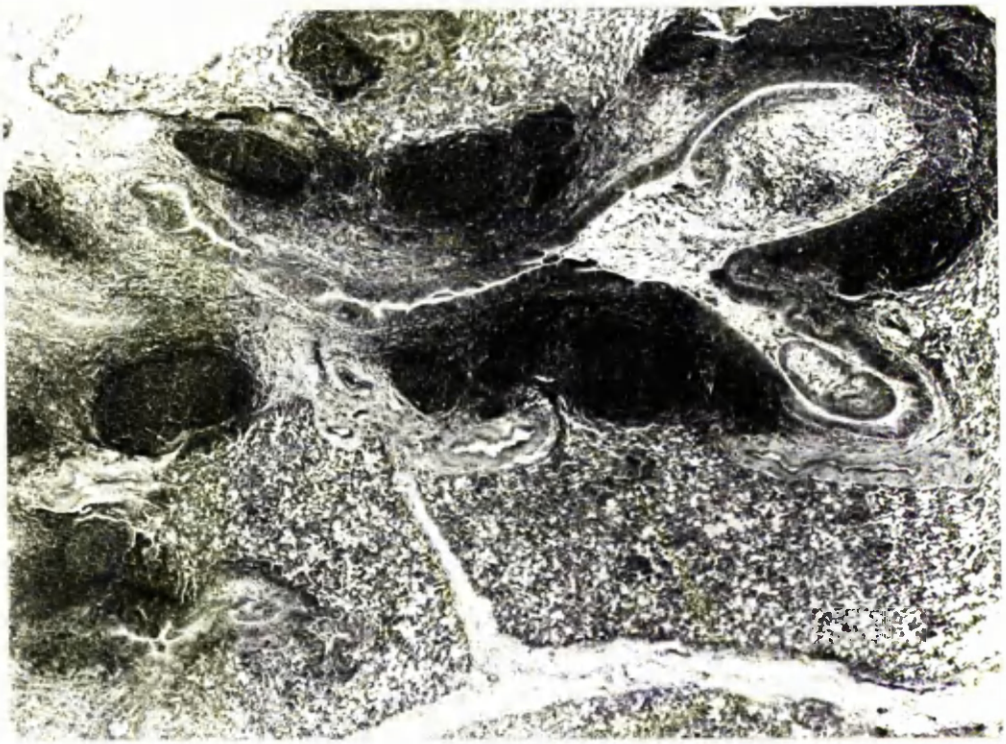


Fig. 24 : Three to four months old calf. D type pulmonary lesion with lymphocytic aggregates around the bronchiole forming a germinal centre-like organisation which destroys the muscle and infiltrates the lamina propria. An alveolitis is present in the adjacent lung tissue and a cellular exudate is seen in the airway lumen. HE staining, x 40.

Fig. 25 : Three to four months old calf. Lymphocytes in the peribronchiolar spaces surround most of the bronchiole. Neutrophils infiltrate the epithelium and form a plug in the airway lumen. An alveolitis consisting mostly of neutrophils is seen in the lung tissue adjacent to the bronchiole. HE staining, x 120.



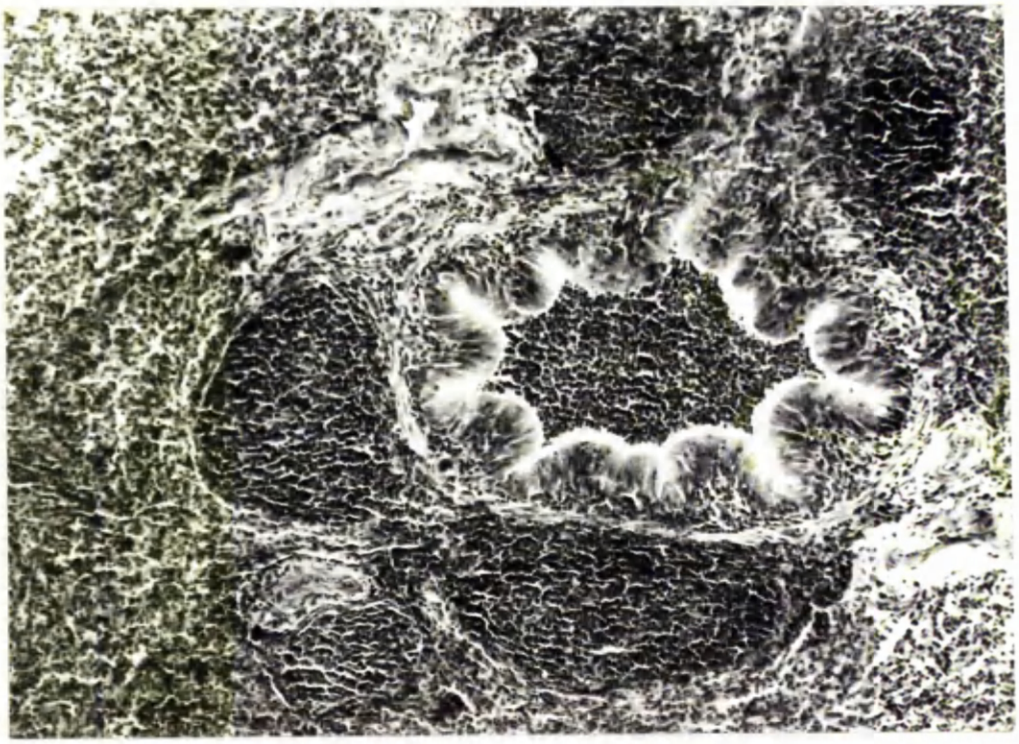
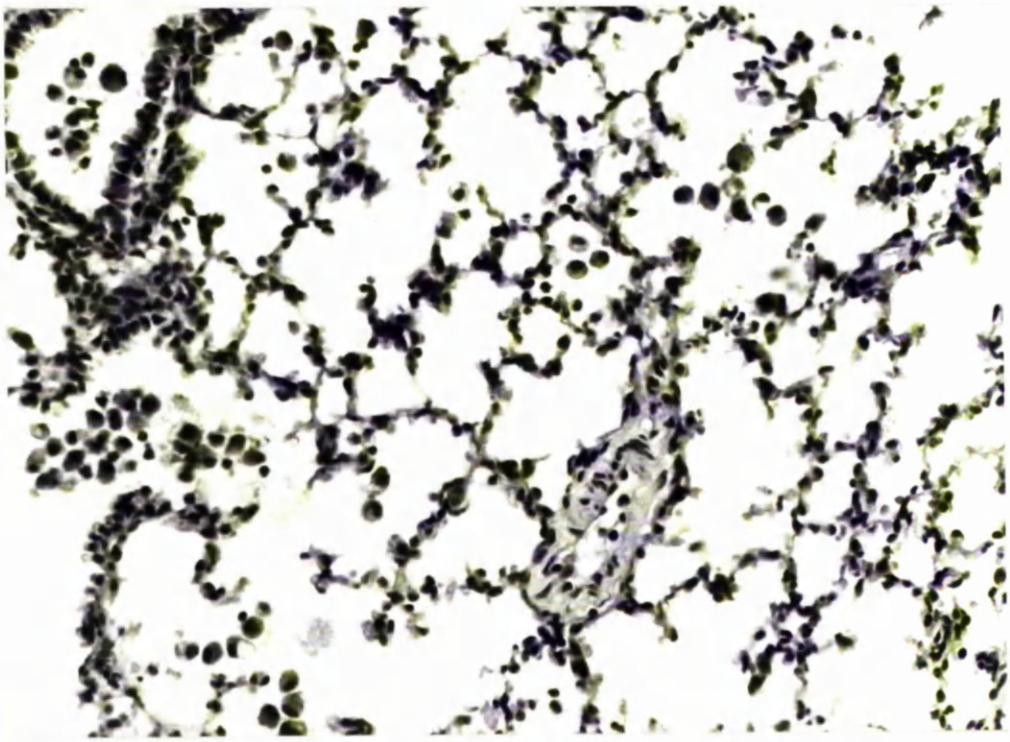
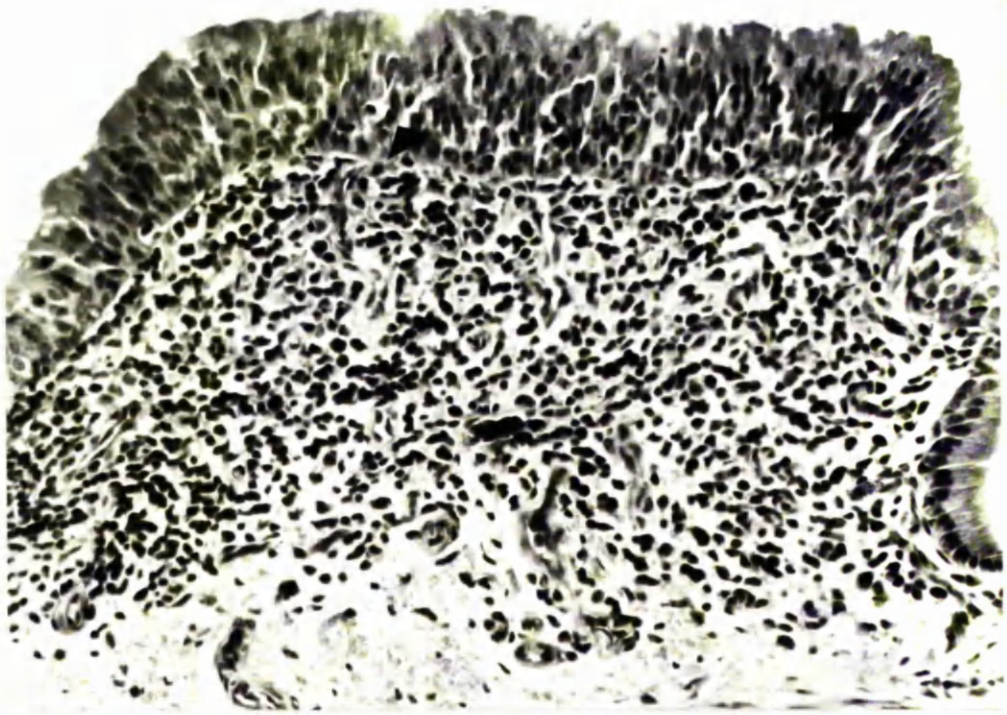


Fig. 26 : Three to four months old calf. Macrophages constitute the alveolitis seen in many D type pulmonary lesions. HE staining, x 250.

Fig. 27 : Three to four months old calf. Bronchitis is a common feature of D type pulmonary lesions and is characterised by epithelial hyperplasia and thickening of the lamina propria. Neutrophils penetrate the epithelial cells (arrows) and the cellular infiltrate of the lamina propria is mostly plasma cells. HE staining, x 250.





CHAPTER EIGHT

Fig. 28 : Three to four months old calf. Hypertrophy of the submucosal glands is a common feature in these animals. Hyperplasia of the epithelium and thickening of the lamina propria characteristic of the bronchitis in these cases, is seen here. HE staining, x 120.

Fig. 29 : Three to four months old calf. Hypertrophied submucosal gland; the cells of the tubules are columnar with basally situated nuclei and much cytoplasm. The tubules are slightly dilated with some mucus in the lumen. Plasma cells (arrows) can be seen surrounding the tubules. HE staining, x 250.

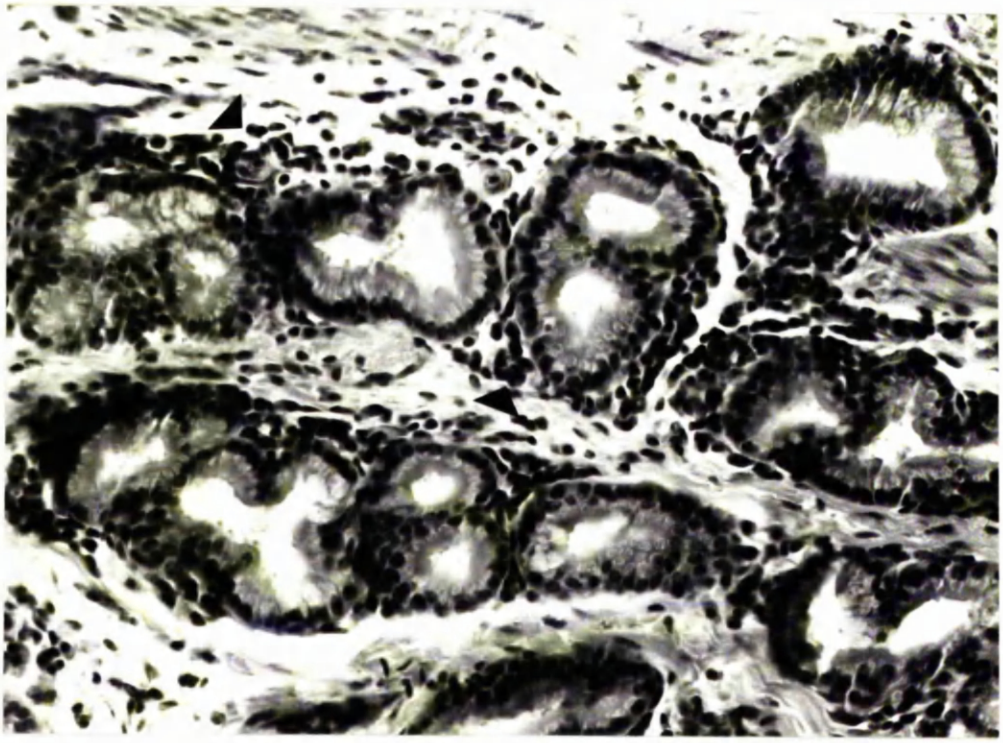
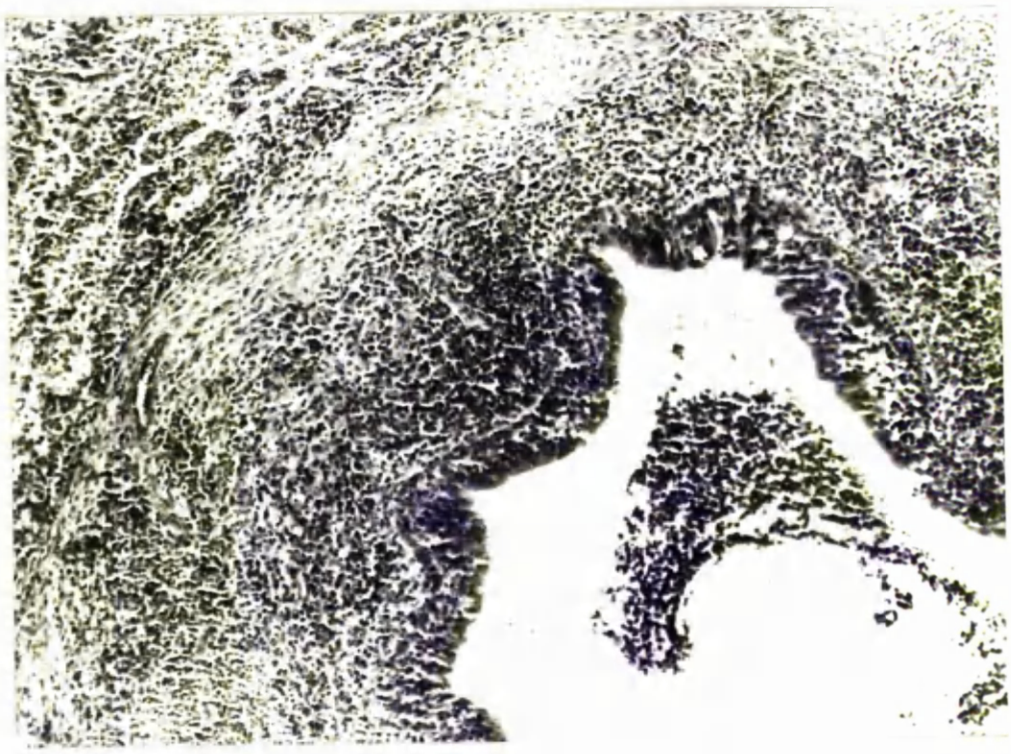
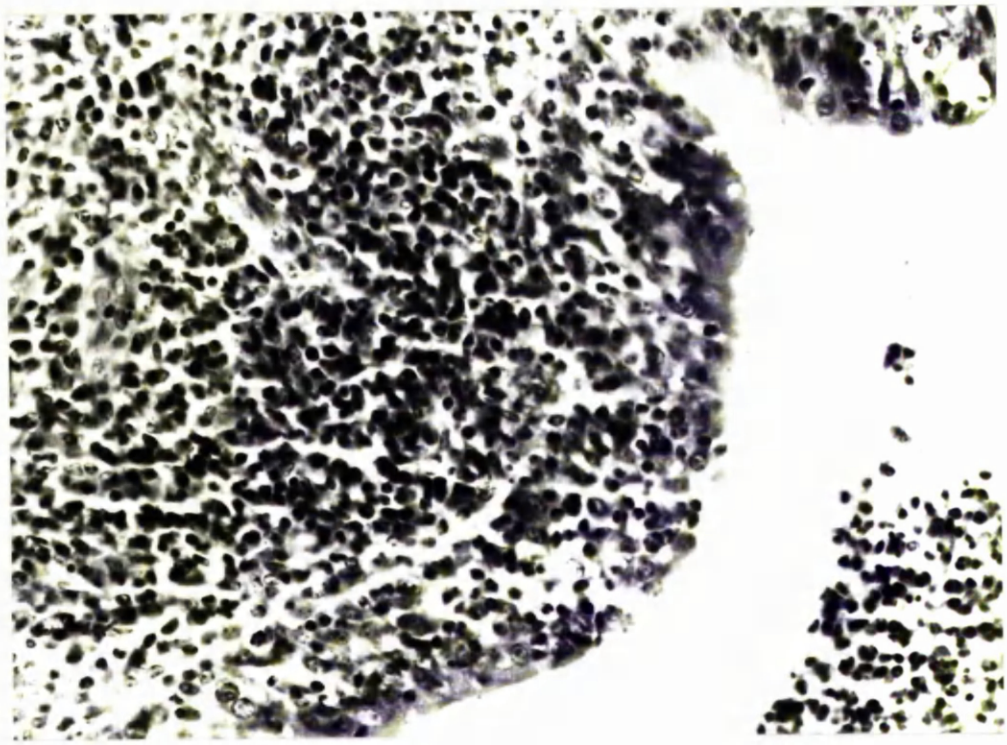


Fig. 30 : Three to four months old calf with a plasma cell D type lesion. Many features of this lesion are typical of D type reactions; however, large numbers of plasma cells can be seen in the lamina propria which is thickened. A cellular infiltrate is present in the lumen. HE staining, x 120.

Fig. 31 : This represents a higher magnification of Fig. 30 and demonstrates plasma cells in the lamina propria which can be seen infiltrating the epithelium and destroying it. HE staining, x 250.





E. CALVES SIX MONTHS OLD

1. Introduction

In many cattle rearing establishments calves may be kept indoors for periods as long as six months to one year. During this time they may develop clinical respiratory disease, varying in severity from a fatal condition to a mild infection indicated only by coughing. Many calves recover clinically from these infections but some may become carriers. Such animals during adverse environmental conditions, especially in the winter months, may initiate an outbreak of respiratory disease amongst newly introduced groups of calves.

Two groups of calves maintained together from birth were examined microbiologically and pathologically at the end of a six months period. Other examinations were carried out on some of the animals within these groups. In addition, six individual animals approximately six months of age were examined.

2. Materials and methods

a. Animals.

Two Groups of calves (D and E) were examined when six months of age. Group D contained 20 Friesian and Ayrshire cross calves and Group E contained nine similar animals. Each Group had been maintained as one unit from one week of age, the calves being reared in adjacent pens which held two or three animals. The pens were small and clean and the building was open and well ventilated. The calves were maintained on an adequate diet. Within both Groups, calves had suffered from an obvious outbreak of respiratory disease at some time during the first two to three months of their lives but had not been treated and none had died of pneumonia. The calves within Group D were still coughing and in poor condition when killed at the end of the six months period.

Six individual calves approximately six months of age were also studied; these animals had entered the University of Glasgow Veterinary

School for routine examination.

b. Post mortem techniques.

The calves in Groups D and E were killed randomly over periods of three and two days respectively. All animals were shot and exsanguinated; two universals of blood were collected from all calves in Groups Dand E for subsequent serological examination. The lungs and trachea from each calf were immediately removed into polythene bags avoiding contact with foreign surfaces. After macroscopic examination specimens were collected for all studies from the right cranial lobe of all animals. Tissue from other lobes was sampled in some cases.

Duplicate samples of lung tissue were collected into GS, U3 and A broths and sterile PBS for cultural examination for the presence of mycoplasmas and bacteria as described in chapter two, section C. In addition, a sample of bronchus from each animal in Group E was collected for microbiological examination.

Specimens of lung and lobar bronchus from the right cranial lobe were taken and fixed in corrosive formol and ten per cent formol saline. Fixing, processing and staining techniques were carried out as described in section E of chapter two; all sections were stained with HE for microscopical examination.

Immunofluorescent studies were carried out on some of the calves from Group E and some of the individual cases. Fresh tissue was collected from the right cranial lobe and examined as described in section G of chapter two (Materials and Methods).

Serological studies were carried out on the serum samples collected from calves in Groups D and E. Antibodies to M. dispar, M. bovirhinis and A. laidlawii were detected in the sera of Group D calves by the metabolic inhibition, latex agglutination and indirect immunofluorescent tests; the indirect immunofluorescent test was used to examine the sera of Group E calves for antibodies to M. dispar. The three serological tests were performed as described in chapter two, section H.

3. Results

a. Mycoplasmal and bacterial isolations.

The results of the isolations of mycoplasmas and bacteria from the calves in Group D are depicted in Tables 28 and 29. The results for Group E and for the six individual cases are illustrated in Table 30. In Group D (Tables 28 and 29) mycoplasmas were isolated from 17 of the calves, M. dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii being recovered from some of the calves. Mycoplasma dispar was isolated from six of the calves (30 per cent), recovered in multiple infections with other mycoplasmas; it was recovered at titres of 10^4 and 10^5 CCU per 0.2 ml of sample from four of the calves, while the other two isolates were present in low numbers. The lung tissue of nine of the 20 calves yielded Ureaplasma spp., with three single isolates being made. The titres were variable and ranged from 10^1 to 10^5 CCU per 0.2 ml of sample. Mycoplasma bovirhinis was recovered from four of the 20 calves, with no single isolates being made; the recovery titres were variable. Acholeplasma laidlawii was isolated from 14 of the 20 calves (70 per cent) including three single isolates; the recovery titres ranged from 10^1 to 10^6 CCU per 0.2 ml of sample. Multiple isolations of mycoplasmas were made from ten calves with all four species being isolated from two (M77 and M78).

Bacteria were recovered from the lungs of 16 of the 20 calves in Group D. The species recovered were Pasteurella haemolytica, Pasteurella multocida, Corynebacterium pyogenes, three species of streptococci and Neisseria catarrhalis; all organisms were recovered in small numbers. Pasteurella spp. were recovered from seven calves and C. pyogenes from three. The bacteria were recovered as single isolates from all calves except one (M73), whose lungs yielded Past. multocida and N. catarrhalis on culture.

Mycoplasmas were recovered from six of the nine calves in Group E; M. dispar and A. laidlawii were the two mycoplasmas isolated (Table 30). Mycoplasma dispar was isolated from the lung tissue of three of the calves and also from the bronchial sample in one of these animals (M104). The titres of the M. dispar isolates were small, 10^1 and 10^2 CCU per 0.2 ml of sample.

Acholeplasma laidlawii was recovered from four of the calves, all isolations being made from lung tissue; the titres were variable, ranging from 10^1 to 10^6 CCU per 0.2 ml of sample. Both organisms were cultured as single isolates except in one calf (M104) which yielded both species.

Four of the nine calves of Group E were positive bacteriologically. Four single isolates were cultured from the lung tissue, namely C. pyogenes, Strep. bovis, Aerococcus viridans and C. xerosis. Small numbers of colonies were isolated except for C. xerosis which was present in high numbers. This latter species and the C. pyogenes isolate were recovered from the lungs of calves which were negative for any mycoplasma isolation.

In Table 30 the microbiological findings of the lungs of six individual cases aged from five to seven months are shown. Mycoplasmas were recovered from four calves and included all four species. Two isolations at low titre of M. dispar were made; one isolate was recovered with A. laidlawii while the other was cultured on its own. A Ureaplasma sp. and M. bovirhinis were isolated in low numbers from one calf.

Bacteria were cultured from three calves; all three animals yielded single isolates in small numbers. The calf whose lungs cultured Past. haemolytica var. haemolytica harboured no other microorganism while the other two bacterial isolates were from calves whose lungs also yielded mycoplasmas.

The results of the microbiological examination on all the six months old calves are illustrated in Table 31. A total of 35 calves were examined; mycoplasmas were isolated from 27 (77 per cent). Mycoplasma dispar and Ureaplasma spp. were isolated from 11 and ten calves respectively; the distribution of M. dispar isolates was similar in all three groups while Ureaplasma spp. were not isolated from Group E and from only one of the individual calves (Misc.). Five isolations (14 per cent) of M. bovirhinis were were obtained, with none from any of the nine Group E calves. Acholeplasma laidlawii was isolated from 19 of the 35 calves (54 per cent) with a high proportion of the recoveries coming from the 20 Group D calves. Bacteria were recovered from many calves (66 per cent) but only nine isolates of

Pasteurella spp. were made.

b. Pathology.

A summary of the pathological findings from the calves in Groups D and E and individual cases are given in Tables 32, 33 and 34. Macroscopically no pulmonary lesions were found in eight of the 20 calves in Group D (Tables 32 and 33). The right lung was affected in the 12 pneumonic cases and five calves had lesions involving the left lung, present in the cranial lobe but not involving more than half of it. The lesions were confined to the anterior lobes in all cases except M60 which had extensive macroscopic pneumonia in the right middle lobe. The anterior distribution of the lesions is shown in Figure 32, illustrating almost total involvement of the cranial and middle lobes of the right side and extensive lesions in the left lung. The lesions were pink-grey, dry in texture and of less than normal volume (Fig. 33); the lesions were well defined with clearly demarcated edges. Two of the moderately severe cases (M62 and M72) had areas of oedema in the anterior segments of the lung. Mucus was present in the airways of the most severe cases, usually thin and grey and often extending into the trachea. In two calves (M74 and M79) the lesions were fawn-purple with circular greyish-white nodules, which on section, revealed dilated bronchi and bronchioles full of grey, thin mucus. Hyperplasia of the anterior dorsal right cranial lymph node was noted in most of the pneumonic cases; a weight of 11.9 g was recorded for one case (M74) which is significantly greater than from its non-pneumonic analogue (approximately 1-2 g).

In Group E nine calves were studied and macroscopic examinations showed lesions of pneumonia in six of them (Table 34). The lesions were small and patchy occupying less than half of any affected lobe, suggesting the presence of a mild pneumonic condition in these animals. As in Group D, the anterior lobes were more frequently affected than the posterior ones with lesions more common in the right lung than the left. The lesions were pink-purple and dry in nature, similar to those described in Group D. Nodular areas did not feature in any of the lesions; exudate or mucus was not

recorded in any of the airways of any of the animals.

The six six months old routine cases all had macroscopic lesions of pneumonia (Table 34). Both lungs were affected in four calves while only the right lung of M89 and M90 showed pneumonic lesions, present as patchy foci, occupying less than half the lobe. The anterior lobes of the lungs were the most severely affected; one animal (M80) had extensive pneumonic areas in both lungs. The lesions were similar to those described for the other six months old animals, generally pink-purple and dry in texture. A mucous exudate was present in the lungs of three of the calves and slight interstitial emphysema was recorded in one animal (M129). The lesion in the right cranial lobe of one calf (M84) was complicated; fawn-pink areas with large, yellowish spots which on section, revealed copious grey mucus extending throughout the bronchial tree.

Tables 32, 33 and 34 illustrate the findings of the histological examination of the lungs of these calves. According to the classification scheme described earlier (section B) all the calves except one had a pulmonary architecture defined as either type A, B or C. Calves with type A pulmonary architecture had no macroscopic pneumonia and there was no evidence of an active bronchiolitis when the lungs were examined microscopically. A few small peribronchiolar accumulations of cells of the lymphocyte series were seen in some sections of lung (Fig. 34). These were not considered to be significant.

The calves with type B pulmonary pathology had no macroscopic lesions of pneumonia but there was microscopic evidence of a mild bronchiolitis in some sections. There were peribronchiolar accumulations of lymphocytes which were sometimes suspiciously large but were not considered to be forming cuffs (Fig. 35). They were usually confined to the connective tissue in the peribronchiolar region and the muscularis was recognisable. In a few instances, however, there was a dense accumulation of cells thickening the lamina propria related to a peribronchiolar lymphoid accumulation and there was obliteration of the muscularis at this point (Fig. 36). Sometimes there were neutrophils in the wall and lumen of the

ACKNOWLEDGEMENTS.

bronchioles at these sites.

Calves with a proliferative pneumonia of type C had macroscopic lesions and, microscopically bronchitis and bronchiolitis were present in all cases. Changes characteristic of cuffing pneumonia were present; a high proportion of the bronchioles within the lobules had large peribronchiolar lymphocytic accumulations often extending completely around the circumference of the bronchiole and continuously along the wall of the bronchiole (Fig. 37). Germinal centres were seen in many of these accumulations with extensive infiltration of the walls of the affected bronchioles and complete obliteration of the muscularis and thickening of the lamina propria by masses of lymphocytes and plasma cells (Fig. 38). In a few cases the degree of infiltration by the lymphocytes into the lamina propria was extensive causing stenosis of the bronchiolar lumen (Fig. 39).

The C type proliferative pneumonias were sub-divided into categories (i), (ii) and (iii); the C(i) lesion was characterised by the presence of alveolitis (Fig. 37) which consisted of infiltration of the alveolar air spaces by neutrophils and macrophages in variable proportions (Fig. 40). Some animals had multi nucleated giant cells (Fig. 41) in the alveoli also. The distinguishing characteristic of type C(ii) lesions was alveolar collapse usually found in the lung tissue adjacent to affected airways. In a few cases the typical bronchiolar lesion with alveolitis or alveolar collapse was complicated by bronchiolar polyps, bronchiectasis or small abscesses (Fig. 42), and these lesions were designated as type C(iii).

The bronchitis in many of these cases was moderate to severe, characterised by elongation of the epithelial cells which became ragged, torn and vacuolated in appearance (Fig. 43). Plasma cells and lymphocytes were seen in the lamina propria of some of the pneumonic cases and neutrophils frequently infiltrated through the epithelium.

One calf (M30) had a proliferative pneumonia with D(i) type lesions considered extensive from macroscopic and microscopic examination. Lymphoid accumulations formed a diffuse layer around the bronchioles. Acute alveolitis was the most striking feature of this lesion and consisted of

macrophages and neutrophils in large numbers in the alveolar air spaces and the bronchiolar lumen.

An integration of the results of the pathological examination and the microbiological findings is illustrated in Table 35. Of the 35 six months old animals examined, 11 were non-pneumonic and 24 were pneumonic, most of which had classical lesions of cuffing pneumonia (type C).

Mycoplasma dispar was isolated from 11 of the pneumonic animals, being isolated from 50 per cent of the animals within the two Groups studied. This organism was not isolated from any of the non-pneumonic cases. Ureaplasma spp. were recovered from nine pneumonic animals; eight of the isolates were from the Group D calves while none of the six pneumonic Group E animals harboured this organism. A Ureaplasma sp. was isolated from one non-pneumonic animal which, although had no macroscopic lesions, had a microscopic bronchiolitis. Mycoplasma bovirhinis was cultured from four pneumonic calves and from one calf with no macroscopic lung lesions. The lungs of 13 pneumonic animals yielded A. laidlawii on culture, with a 75 per cent isolation rate in Group D. However, a similarly high number of isolations were made from the non-pneumonic animals.

Comparison of the results of the isolations from the pneumonic animals with the non-pneumonic animals in each Group (Table 35) indicates a considerable difference in the frequency of isolations of M. dispar and Ureaplasma spp. between the pneumonic and non-pneumonic animals. The difference in the isolations of M. dispar between the pneumonic and non-pneumonic calves was highly significant ($P < 0.01$) when tested statistically by the Chi-square test. Significantly ($P < 0.05$) different isolations of Ureaplasma spp. were also found between the pneumonic and non-pneumonic calves. On the other hand, the isolations of M. bovirhinis and A. laidlawii were not significantly different between the pneumonic and non-pneumonic animals.

Bacteria were isolated from pulmonary tissue of calves with and without macroscopic lung lesions in approximately equal frequency; Pasteurella spp. were the most commonly recovered.

c. Immunofluorescence.

Lung and bronchial tissue from seven of the nine calves in Group E and from five of the six individual cases were examined for the presence of M. dispar by the indirect immunofluorescent (IF) technique. The results are illustrated in Table 36 together with the mycoplasma isolates recorded from the animals examined. Positive fluorescence for M. dispar was detected in one of the seven calves examined in Group E (M102); this animal had normal pulmonary architecture (type A) and no mycoplasmas were cultured from the lung tissue. Mycoplasmas were isolated from five of the remaining six calves in Group E; M. dispar was recovered from two cases.

By IF studies on the bronchial and lung tissue one of the miscellaneous, routine calves was positive for the presence of M. dispar although the mycoplasma cultured from the lung tissue of this case was M. bovirhinis. Three of the remaining four calves were culturally positive for mycoplasmas; M. dispar was isolated from two of them.

In summary, pulmonary tissue from 12 six months old calves were examined by IF techniques for the presence of M. dispar. Mycoplasmas were cultured from nine of the calves, M. dispar from four of them; IF studies yielded only two cases positive for M. dispar, neither of which cultured the organism.

d. Serology.

Serological studies were carried out on the serum samples collected from calves in Groups D and E. The sera of Group D calves were examined for the presence of antibodies to M. dispar, M. bovirhinis and A. laidlawii using the metabolic inhibition test (MIT), indirect immunofluorescent technique (IF) and the latex agglutination test (LA); antibody to M. dispar was detected in the sera of Group E calves by the IF test.

Table 37 illustrates the serological results of Group D calves. Fifteen of the 20 calves showed a significant antibody titre to M. dispar by at least two of the techniques used; a reciprocal titre of at least 16 in two of the tests was found in seven calves, five of which were culturally positive for M. dispar. The titres obtained by the three techniques were variable

although the MIT and IF differed least for most cases and were most comparable. Low titres of antibody were obtained from most cases by the LA test.

Fourteen calves in Group D had significant titres to M. bovirhinis in at least two of the techniques employed, although a reciprocal titre of at least 16 was found in only six calves. Mycoplasma bovirhinis was isolated from four cases none of which demonstrated a high serum antibody response to this organism. Comparison of the results showed much variation amongst the three techniques.

Significant serum antibody titres to A. laidlawii were obtained in 17 of the 20 calves in Group D and a reciprocal titre of 16 or more in two techniques was found in three cases. Again the results were variable although the difference in titre by the MIT and IF tests appeared less than either of them with the LA test.

The sera collected from the nine calves in Group E were examined for the presence of antibodies to M. dispar by the IF technique. A reciprocal titre of four was detected in one calf (M107) which was culturally negative for this organism; the remaining eight animals were negative although M. dispar was isolated from three of them.

4. Discussion

This study has demonstrated that M. dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii can be recovered from the lungs of six months old calves. It has also shown that in the Groups studied the isolation frequency of M. dispar and Ureaplasma spp. was higher from the lungs of pneumonic animals than from those with no macroscopic pulmonary lesions. The presence of M. dispar in the pneumonic cases appeared to be more significant than the presence of Ureaplasma spp.; the latter organisms were found in the lungs of the pneumonic animals in only one Group of six months old calves, while no isolations were made from the pneumonic tissue in the second Group of calves (E) despite the fact that the histological lesions were similar in both. In addition, a Ureaplasma sp. was recovered from one calf with no macroscopic

lesions but with microscopic bronchiolitis, while M. dispar was only recovered from calves with visible lung lesions.

Gourlay et al. (1970) have studied the association between mycoplasmas and pneumonia in calves and the isolations of M. dispar and Ureaplasma spp. amongst one of their groups of pneumonic calves were similar to the rates found in these six months old animals.

Isolations of A. laidlawii in high numbers have been reported from the upper respiratory tract of calves by other workers (Davies, 1967; Thomas and Smith, 1972) but infrequently from the lungs. The high incidence of isolations from this age group of calves was not considered to be due to contamination as previous and subsequent examination of other calf lungs using identical techniques did not yield such numerous isolations. Acholeplasma laidlawii and M. bovirhinis were isolated from calves with and without macroscopic pneumonia and the distribution within the two groups was not significantly different.

The difference in the isolation rates of M. dispar and Ureaplasma spp. between the pneumonic and non-pneumonic six months old calves cannot be explained in terms of housing since 29 of the calves examined had been reared as two groups, each within the same air space. The pens used to maintain these animals did not have solid sides and the pneumonic calves were distributed amongst the non-pneumonic ones. This would suggest that Ureaplasma spp. or, more particularly, M. dispar may be important in the development of cuffing pneumonia either as primary pathogens or as secondary invaders. No data are available on these calves of viral infections early in the six months period, which could have allowed the establishment of mycoplasmas.

The pneumonia found in the animals with macroscopic pulmonary lesions was considered typical of cuffing pneumonia, with the peribronchiolar lymphocytic accumulations involving most of the circumference of the bronchiole and extending along it to form a sheath; the lymphocytic accumulation was frequently follicular with germinal centres and there was obliteration of the muscularis of the bronchioles by the cells.

The lesions in the pneumonic lungs of the six months old calves had probably been present for some time since experimental work with mice infected with M. pulmonis (Lindsey and Cassell, 1973) and with pigs infected with M. suipneumoniae (Whittlestone, 1972) would suggest that cuffing lesions, particularly those with a follicular pattern and germinal centres, only develop after an infection has been established for several weeks.

No relationship between the presence of mycoplasmas in the lung tissue of one Group of six months old calves and the detection of antibody to the organisms could be found using the MIT, IF and LA techniques. In this context it is interesting that Whittlestone (1972) could not detect an association between serum antibody to M. suipneumoniae in pigs and resistance to challenge with enzootic pneumonia.

Case No.	Age (months)	*Mycoplasma isolations.	*Bacterial isolations	
M60	6	M. dispar, 10^2; A. laidlawii, 10^2	Streptococcus bovis,	+
M61	6	-	-	
M62	6	M. dispar, 10^1; A. laidlawii, 10^3; Ureaplasma sp., 10^2	Corynebacterium pyogenes,	+
M63	6	M. dispar, 10^5; A. laidlawii, 10^3	Past. haemolytica var. haem.,	+
M64	6	-	-	
M65	6	A. laidlawii, 10^4; Ureaplasma sp., 10^1	-	
M66	6	A. laidlawii, 10^5	Strep. bovis,	+
M67	6	A. laidlawii, 10^6	C. pyogenes,	+
M68	6	A. laidlawii, 10^2	Past. haemolytica var. haem.,	+
M69	6	A. laidlawii, 10^6; M. bovirhinis, 10^1	Strep. mitis,	+

* The titre of the mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++ 20-50 colonies; +, 5-20 colonies.

Table 28. Frequency of isolations of mycoplasmas and bacteria from the lung tissue from Group D of the six months old calves.

Case No.	Age (months)	*Mycoplasma isolations	* Bacterial isolations	
M70	6	M. dispar, 10^5; A. laidlawii, 10^5 M. bovirhinis, 10^5	Streptococcus pneumoniae,	+
M71	6	-	Corynebacterium pyogenes,	+
M72	6	A.laidlawii, 10^3; Ureaplasma sp., 10^1	Pasteurella multocida,	+
M73	6	A.laidlawii, 10^1	Past. multocida,	+
			Neisseria catarrhalis,	+
M74	6	Ureaplasma sp., 10^4	-	
M75	6	Ureaplasma sp., 10^2	Strep. mitis,	+
M76	6	Ureaplasma sp., 10^5	Past. haemolytica var. haem.,	+
M77	6	M. dispar, 10^4; A.laidlawii, 10^2; M. bovirhinis, 10^2; Ureaplasma sp., 10^4	Past. multocida,	+
M78	6	M. dispar, 10^4; A.laidlawii, 10^4; M. bovirhinis, 10^4; Ureaplasma sp., 10^1	Strep. pneumoniae,	+
M79	6	A.laidlawii, 10^6; Ureaplasma sp., 10^1	Past. haemolytica var. haem.,	+

* The titre of the mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++, 20-50 colonies; +, 5-20 colonies.

Table 29. Frequency of isolations of mycoplasmas and bacteria from the lung tissue from Group D of the six months old calves.

Case No.	Age (months)	* Mycoplasma isolations	* Bacterial isolations
M102	6	-	Corynebacterium xerosis -L, +++
M103	6	A. laidlawii -L, 10^1	Aerococcus viridans -L, +
M104	6	M. dispar -L, B, 10^1 A. laidlawii -L, 10^1	-
M105	6	M. dispar -L, 10^1	-
M106	6	-	-
M107	6	A. laidlawii -L, 10^6	Streptococcus bovis -L, +
M108	6	-	C. pyogenes -L, +
M109	6	M. dispar -L, 10^2	-
M110	6	A. laidlawii -L, 10^1	-
M 80	5	Ureaplasma sp., 10^2	Staphylococcus aureus, +
M 84	5	-	Past. haemolytica var. haem., +
M 89	6	-	-
M 90	6	M. dispar, 10^2; A. laidlawii, 10^4	Pasteurella sp., +
M 91	6	M. dispar, 10^1	-
M129	6-7	M. bovirhinis, 10^1	-

* Bronchial (B) and lung (L) tissue were examined for the presence of mycoplasmas and bacteria. The titre of mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++, 20-50 colonies; +, 5-20 colonies.

Table 30. Frequency of isolations of mycoplasmas and bacteria from bronchial and/or lung tissue from nine calves in Group E and six individual cases.

Group	Number calves in Group	Number calves with mycoplasmas	Number of calves with				Number calves with bacteria	Number calves with Pasteurella spp.
			M. dispar	Ureaplasma spp.	M. bovirhinis	A. laidlawii		
D	20	17	6	9	4	14	16	7
E	9	6	3	0	0	4	4	0
Misc.	6	4	2	1	1	1	3	2
Total	35	27	11	10	5	19	23	9

Table 31 . This table illustrates the frequency of isolations of mycoplasmas and bacteria from Groups D and E and from the six individual cases (Misc.); all calves examined were approximately six months of age.

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Case No.	*MACROSCOPIC LESIONS								* MICROSCOPIC FINDINGS
	RCa	RCp	RM	RD	LCa	LCp	LD	Acc	
M60	+++	+++	+++	-	++	++	-	-	Proliferative C(ii) lesion with macrophage alveolitis.
M61	-	-	-	-	-	-	-	-	Type B pulmonary pathology.
M62	+++	+++	-	-	-	-	-	-	Proliferative C(i) lesion.
M63	++	++	-	-	-	-	-	-	Proliferative C(i) lesion with macrophage alveolitis.
M64	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M65	+	+	-	-	+	+	-	-	Proliferative C(ii) lesion.
M66	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M67	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M68	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M69	-	-	-	-	-	-	-	-	Type A pulmonary pathology.

* Based on nomenclature and classification illustrated in Tables 16 and 17 .

Table 32. Pulmonary pathological findings of ten calves of Group D which were six months old.

Case No.	* MACROSCOPIC LESIONS RCa	RCp	RM	RD	LCa	LCp	LD	Acc	* MICROSCOPIC FINDINGS.
M70	+	+	-	-	-	-	-	-	Proliferative C(i) lesion with neutrophil alveolitis.
M71	+++	+++	-	-	-	-	-	-	Proliferative C(i) lesion with neutrophil alveolitis.
M72	++	++	-	-	+	++	-	-	Proliferative C(i) lesion with neutrophil alveolitis.
M73	-	-	-	-	-	-	-	-	Type B pulmonary pathology.
M74	+++	++	-	-	+	-	-	-	Proliferative C(iii) lesion with macrophage alveolitis.
M75	+	-	-	-	+	-	-	-	Proliferative C(ii) lesion.
M76	-	-	-	-	-	-	-	-	Type B pulmonary pathology.
M77	+	-	-	-	-	-	-	-	Proliferative C(ii) lesion.
M78	+	-	-	-	-	-	-	-	Proliferative C(ii) lesion.
M79	+++	+++	-	-	-	-	-	-	Proliferative C(i) lesion with macrophage alveolitis.

* Based on nomenclature and classification illustrated in Tables 16 and 17.

Table 33. Pulmonary pathological findings of ten calves of Group D which were six months old.

Case No.	*MACROSCOPIC LESIONS RCa	RCp	RM	RD	LCa	LCp	LD	Acc	* MICROSCOPIC FINDINGS
M102	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M103	-	-	-	-	-	-	-	-	Type A pulmonary pathology.
M104	+	-	-	-	-	-	-	-	Proliferative C(ii) lesion.
M105	+	+	-	-	+	-	-	-	Proliferative C(ii) lesion with macrophage alveolitis.
M106	-	+	-	-	+	-	-	-	Proliferative C(ii) lesion.
M107	+	+	+	+	+	-	-	-	Proliferative C(ii) lesion with macrophage alveolitis.
M108	-	-	-	-	-	-	-	-	Type B pulmonary pathology.
M109	+	-	-	-	+	-	-	-	Proliferative C(ii) lesion.
M110	+	+	+	-	-	-	-	-	Proliferative C(ii) lesion.
M 80	+++	+++	+++	++	+++	+++	++	++	Proliferative D(i) lesion with neutrophil alveolitis.
M 84	++	++	-	-	+++	-	-	-	Proliferative C(iii) lesion with macrophage alveolitis.
M 89	+	-	-	-	-	-	-	-	Proliferative C(ii) lesion.
M 90	+	+	+	-	-	-	-	-	Proliferative C(i) lesion with macrophage alveolitis.
M 91	+++	++	+	+	+	+	+	+	Proliferative C(j) lesion with macrophage alveolitis.
M129	++	++	++	-	++	++	-	-	Proliferative C(iii) lesion with macrophage alveolitis.

* Based on nomenclature and classification illustrated in Tables 16 and 17 .

Table 34. Pulmonary pathological findings of nine calves in Group E and six individual cases.

Group	Total number calves in Group	Mycoplasma isolations from pneumonic calves.					Mycoplasma isolations from non-pneumonic calves.				
		Number of calves	M. dispar	Ureaplasma spp.	M. bovirhinis	A. laidlawii	Number of calves	M. dispar	Ureaplasma spp.	M. bovirhinis	A. laidlawii.
D	20	12	6	8	3	9	8	0	1	1	5
E	9	6	3	0	0	3	3	0	0	0	1
Misc.	6	6	2	1	1	1	-	-	-	-	-
Total	35	24	11	9	4	13	11	0	1	1	6

Table 35 . Number of isolates of four mycoplasma species from the lungs of pneumonic and non-pneumonic calves aged six months.

Group	Number calves in Group	Number calves examined IF	Case No.	Mycoplasma isolations	IF +ve or -ve *M. dispar*
E	9	7	M102	-	+
			M103	*A. laidlawii*	-
			M104	*M. dispar*; *A. laidlawii*	-
			M105	*M. dispar*	-
			M106	-	-
			M107	*A. laidlawii*	-
			M110	*A. laidlawii*	-
Misc.	6	5	M 80	*Ureaplasma* sp.	-
			M 84	-	-
			M 90	*M. dispar*; *A. laidlawii*	-
			M 91	*M. dispar*	-
			M129	*M. bovirhinis*	+
Total	15	12	Positive *M. dispar*	4 (culture)	2 (IF)

Table 36 . The results of the screening of pulmonary tissue from 12 calves, aged approximately six months, for *M. dispar* by the indirect immunofluorescent (IF) technique. The mycoplasmal isolations, by cultural methods, are also given.

Case No.	*Serum antibody to M. dispar			Serum antibody to M. bovirhinis			Serum antibody to A. laidlawii		
	MIT	IF	LA	MIT	IF	LA	MIT	IF	LA
M60	16	16	4	0	0	0	16	32	16
M61	2	2	0	16	32	4	16	32	4
M62	16	16	0	16	32	4	64	0	0
M63	16	32	4	0	4	4	4	8	0
M64	32	64	32	0	16	2	8	0	0
M65	0	2	0	8	16	16	8	0	0
M66	8	4	2	4	4	2	4	16	2
M67	0	8	2	4	8	4	8	32	4
M68	0	8	4	4	4	2	8	8	4
M69	0	0	0	4	4	4	4	4	0
M70	0	0	0	0	0	0	2	16	4
M71	0	8	0	0	2	0	4	32	0
M72	0	0	0	4	4	4	0	2	4
M73	16	0	0	0	0	0	2	4	4
M74	8	8	8	2	8	2	0	2	4
M75	16	4	4	16	16	8	4	8	2
M76	8	8	8	16	32	16	4	8	0
M77	32	16	8	8	8	4	4	16	4
M78	16	64	8	8	16	0	16	8	8
M79	32	16	16	16	32	16	32	32	16

*Reciprocal antibody titre to specific mycoplasma by metabolic inhibition test (MIT), indirect immunofluorescent test (IF) and latex agglutination (LA).

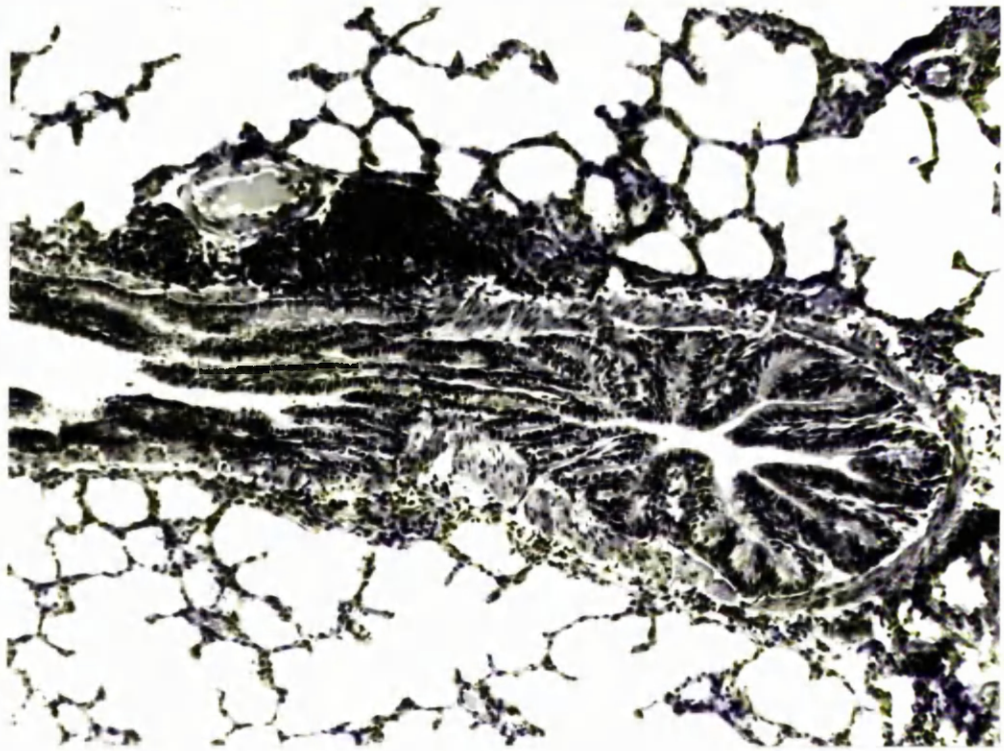
Table 37. Serum antibody titres to M. dispar, M. bovirhinis and A. laidlawii using the metabolic inhibition test (MIT), indirect immunofluorescent test (IF) and latex agglutination (LA) from Group D calves.

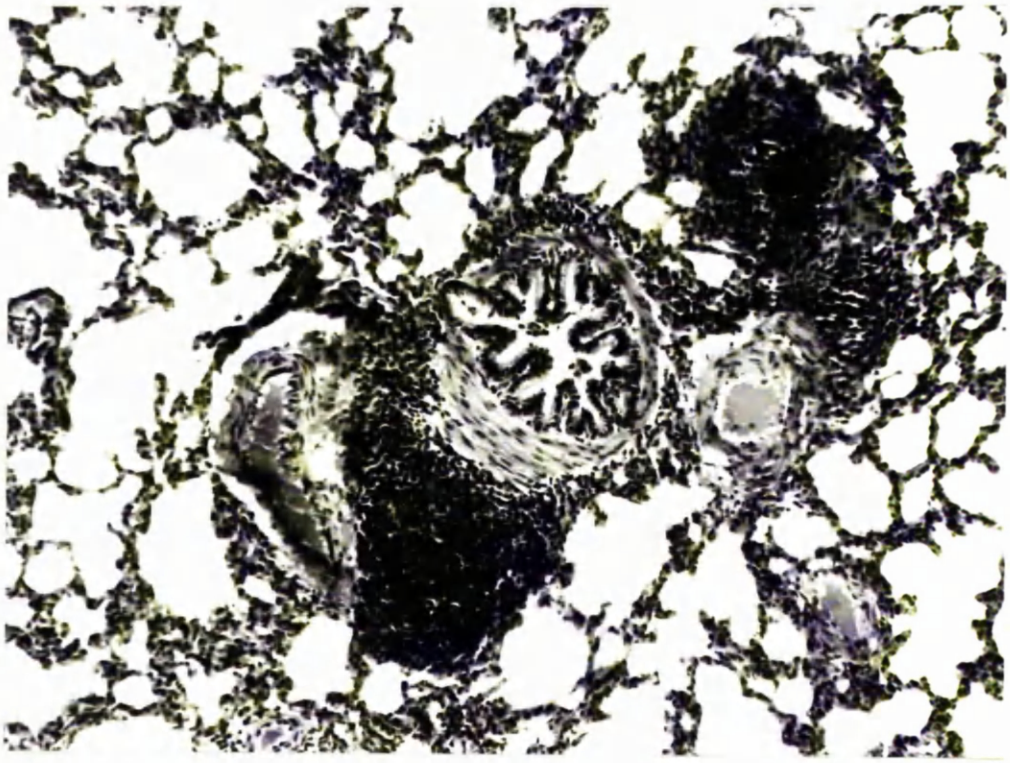
Fig. 32 : Six months old calf. Pneumonic lesions are present in both lungs with the anterior lobes most severely affected. The lesions are pink-grey, dryish and of less than normal volume.

Fig. 33 : A higher magnification of the pneumonic lesion typical of these six months old calves. The anterior lobes are most severely affected with well-defined pink-red areas of consolidation.

Fig. 34 : Six months old calf. Type A pulmonary architecture with no bronchiolitis but a small accumulation of lymphoid cells can be seen in the peribronchiolar region. HE staining, x 120.

Fig. 35 : Six months old calf. Type B pulmonary architecture. Although no macroscopic lesions are visible, a mild bronchiolitis is often evident and accumulations of lymphocytes in the peribronchiolar regions are seen. HE staining, x 120.





SUMMARY

Fig. 36 : Six months old calf. Accumulations of lymphocytes around the bronchiole can penetrate the lamina propria and destroy the layer of muscle in a few lobules of pulmonary tissue of type B. The surrounding alveolar tissue is normal. HE staining, x 45.

Fig. 37 : Six months old calf. Type C pulmonary lesion characteristic of cuffing pneumonia. The peribronchiolar lymphocytic accumulations have formed a sheath obliterating the muscularis and containing germinal centres. An alveolitis is also present. HE staining, x 40.

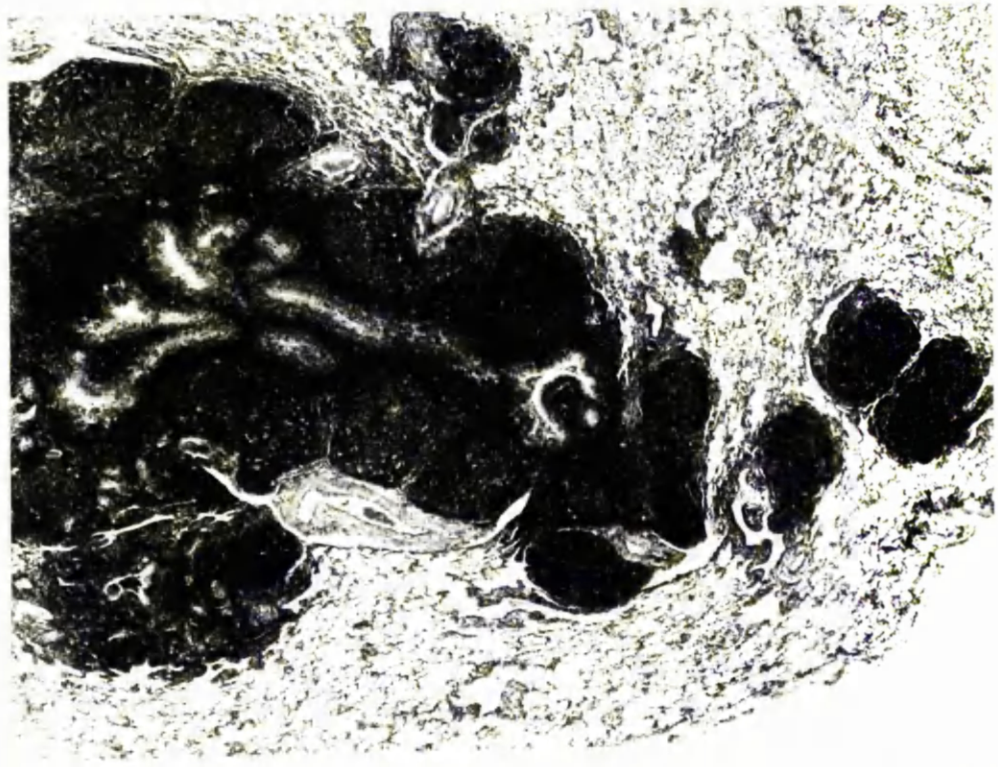


Fig. 38 : Six months old calf. Type C lesion showing germinal centres within the lymphoid cuff. The lymphocytes have displaced the muscle layer, forming a thickened lamina propria. HE staining, x 120.

Fig. 39 : Six months old calf. Extensive infiltration of the lamina propria causing thickening of this region and loss of epithelial differentiation (arrows) is seen in this type C pulmonary lesion. HE staining, x 120.

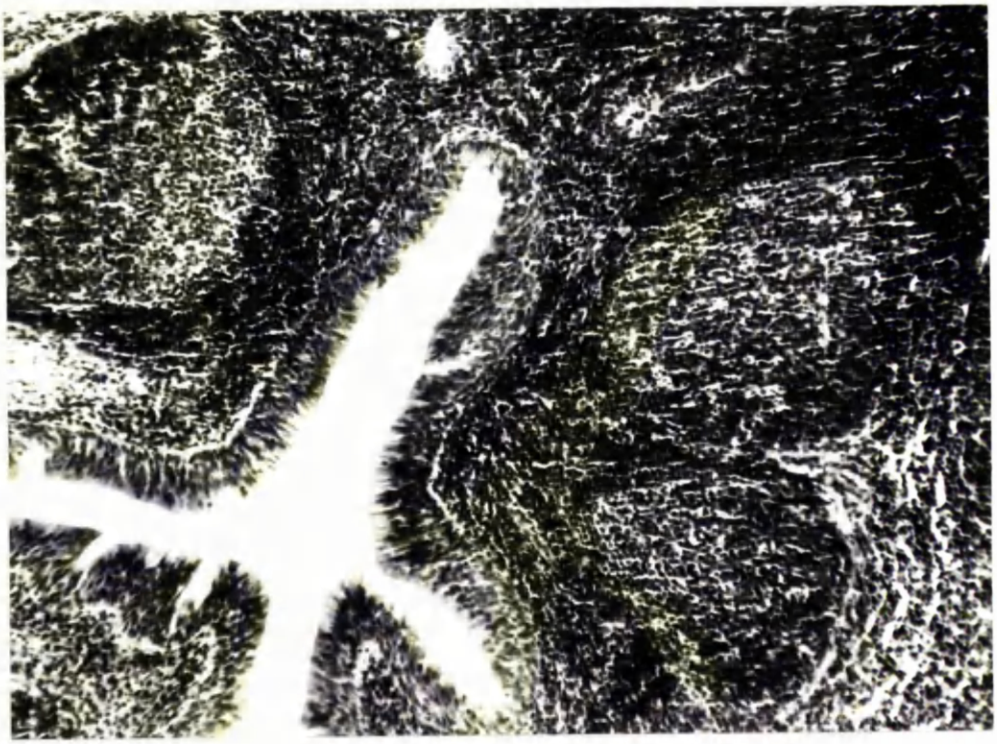
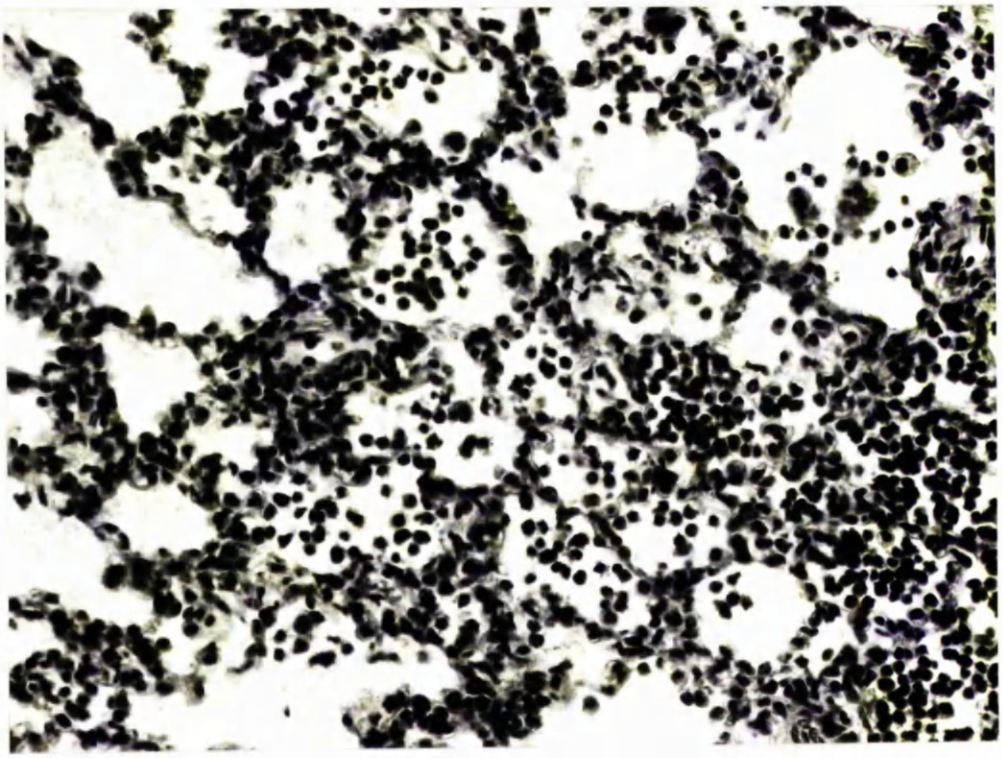


Fig. 40 : Six months old calf. Alveolitis, characteristic of C(i) pulmonary lesions is illustrated and consists of neutrophils and macrophages in the alveolar air spaces. HE staining, x 250.

Fig. 41 : Six months old calf. Multinucleated giant cells (arrow) are frequently seen in the alveolar spaces in C(i) type pulmonary lesions. Neutrophils and macrophages are also present in the air spaces. HE staining, x 250.



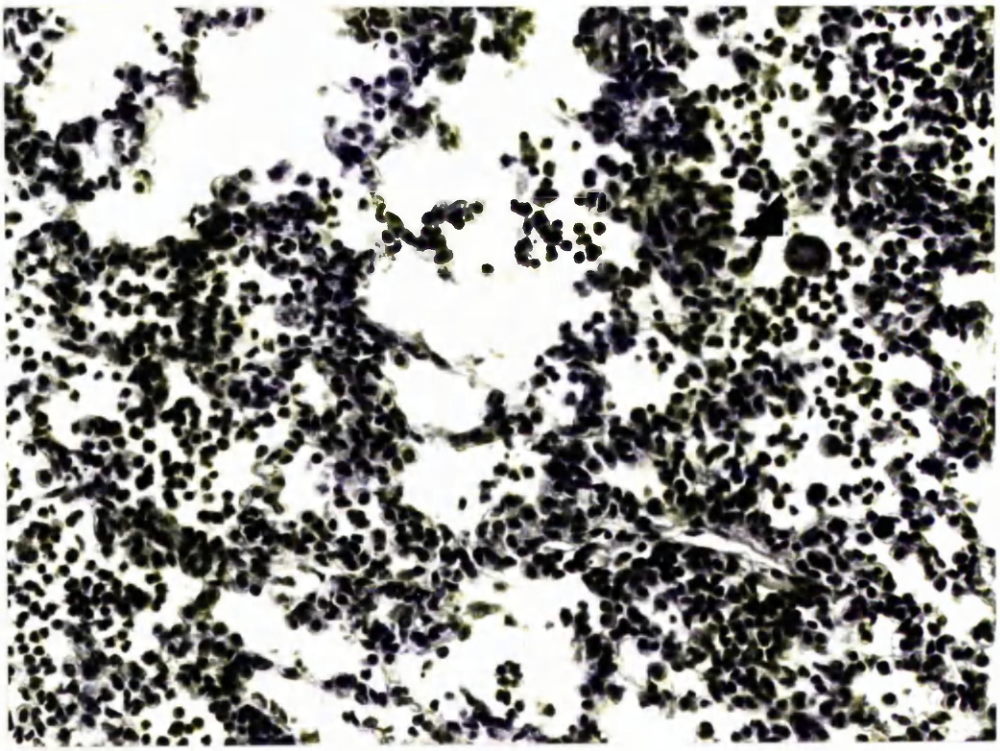
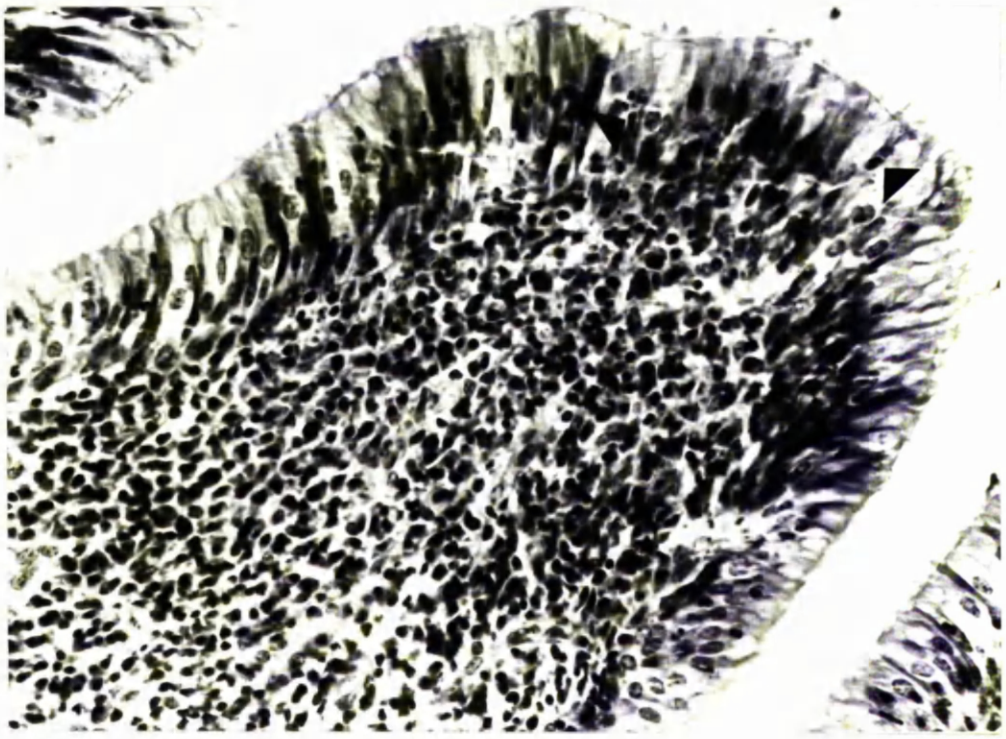


Fig. 42 : Six months old calf. The type C(iii) lesion is characterised by pulmonary complications such as bronchiolar polyps as illustrated in this figure. An alveolitis is also present. HE staining, x 40.

Fig. 43 : Six months old calf. The bronchitis of the C type lesion is characterised, in part, by hyperplasia of the epithelium and thickening of the lamina propria. Neutrophils (arrows) infiltrate the epithelium which has a ragged appearance due to the increased number of goblet cells. Plasma cells are seen in large numbers in the lamina propria. HE staining, x 250.



F. DISCUSSION

The findings of the mycoplasma isolations from all the pneumonic and non-pneumonic calves examined in this study are illustrated in Table 38 which contains the results of the recoveries made from each age group. The lungs of 67 calves were examined and 51 of them had moderate to severe macroscopic lung lesions.

Mycoplasma dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii were recovered from the lungs of the calves. Mycoplasma dispar was isolated from 21 of the 51 calves with pneumonia, but none were from the very young animals. The isolation frequency of M. dispar was similar from the two Groups of six months old calves and the Group of three months old animals (approximately 50 per cent). Although the calves within these Groups had been reared together from birth, isolations of M. dispar were not recorded in any of the 11 non-pneumonic animals. Mycoplasma dispar was recovered from three of the four pneumonic two months old calves. No isolations of M. dispar were made from the remaining 16 two months old and younger calves despite 11 of them having pneumonia.

Ureaplasma spp. were isolated from eight of the 12 pneumonic six months old calves in Group D, but no recoveries were made from the other six months old Group. One non-pneumonic six months old calf in Group D yielded a Ureaplasma sp. on culture. The pneumonic tissue from three of five calves in Group B (one to two months old) harboured ureaplasmas.

Mycoplasma bovirhinis was recovered from nine of the 51 pneumonic and four of the 16 non-pneumonic calves; the isolates were distributed throughout most of the Groups. Similarly, A. laidlawii was isolated from animals in most Groups from both pneumonic and non-pneumonic tissue; particularly high isolation rates were recorded from the six months old animals in Group D.

The present results suggest that mycoplasmas are found in the lung tissue in only a small number of non-pneumonic calves up to the age of six months. Mycoplasma bovirhinis was recovered from three of five one

month old calves and A. laidlawii from five of eight six months old animals. This is in contrast to the findings of Thomas and Smith (1972) who studied the mycoplasma distribution in the respiratory tract of non-pneumonic calves of various ages. Few mycoplasmas were isolated from the lung tissue except from the three to four months old calves from which 12 of 22 yielded M. dispar on culture. Seven of the eight Groups examined in the present studies formed bunches of calves which had been housed together from birth. Within many of these Groups one species of mycoplasma was commonly isolated from the lung tissue of the calves. For example, in the one to two months old calves examined, M. bovirhinis, Ureaplasma spp. and M. dispar were each recovered most frequently from the three Groups of animals.

Isolations of M. dispar and Ureaplasma spp. were most frequent from pneumonic cases, particularly in the three months old and six months old Groups; in fact, the difference in isolation rate between the pneumonic and non-pneumonic six months old calves for Ureaplasma spp. was significant and highly significant, statistically, for M. dispar. From these studies the latter organism appears to be the more important since it was cultured from about 50 per cent of the pneumonic calves in both Groups of six months old animals and the three months old Group. It was not isolated from any macroscopically normal lungs. The isolations of Ureaplasma spp., on the other hand, were confined to one of the six months old Group; one isolate was obtained from the non-pneumonic calves.

Gourlay et al. (1970) studied the association between mycoplasmas and pneumonia in calves. One of their groups of animals consisted of 45 three months old calves with pneumonia. These calves had been collected randomly from an abattoir over a period of 18 months and 50 per cent had cuffing pneumonia. Mycoplasma dispar was isolated from 60 per cent of the group and Ureaplasma spp. from 55 per cent of the calves; this is similar to the recovery rate of M. dispar from the three months old and six months old pneumonic calves described here. A similar recovery rate for Ureaplasma spp. was recorded above for one Group of six months old calves but isolations were not made from the calves in the other Group.

The aims of this work were to study the species of mycoplasmas present in the lungs of calves and in particular to see if any association could be made with these organisms and pneumonia in calves.

A literature review indicated the uniqueness of mycoplasmas as living cells and emphasised their general fragility and complex nutritional requirements. A study was carried out to find a suitable method of cultivating these organisms from bovine pulmonary tissue. Several techniques to release the organisms were examined, including enzymatic destruction of possible attachment sites and homogenisation of the tissue. The most satisfactory and reliable method devised was lightly chopping of a small piece of lung tissue followed by incubation in appropriate mycoplasma broth medium for 30 minutes at 37^{o}C and final serial ten-fold dilutions in broth media for subsequent cultivation.

Groups of calves ranging in age from less than one month to six months that had been housed together from birth were examined microbiologically and pathologically. Four species of mycoplasmas were recovered from the lungs of some of these calves, namely Mycoplasma dispar, Ureaplasma spp., M. bovirhinis and Acholeplasma laidlawii. A small number of mycoplasmal isolations were made from macroscopically and microscopically normal lungs but M. dispar was never recovered. On the other hand, mycoplasmas were frequently isolated from the lungs of calves with clinical or subclinical pneumonia. Mycoplasmas were not associated with the pneumonia in the young calves; histopathologically this pneumonia was usually an exudative type, generally considered to have a bacterial aetiology. However, in the older animals cuffing pneumonia and the lesions preceding it were the most commonly found pulmonary conditions. The four species of mycoplasmas were isolated from the lung tissue of such animals, M. dispar being the species most consistently recovered from the calves with lesions of cuffing pneumonia. Serological techniques excluded the presence of several bovine respiratory viruses from one group of calves studied and in other groups no pathognomic lesions of these agents were found. The results of the examination of these natural outbreaks of respiratory disease suggest that M. dispar is the most probable aetiological

Mycoplasma bovirhinis and A. laidlawii are considered non-pathogenic in the bovine respiratory tract (Gourlay, 1973). In these studies both species were isolated from calves with and without macroscopic pneumonia and their distribution within the two groups was not significantly different. Isolations of A. laidlawii in high numbers have been reported from the upper respiratory tract of calves by other workers (Davies, 1967; Thomas and Smith, 1972) but infrequently from the lungs. The high incidence of isolations from one Group of six months old calves was not considered to be due to contamination as previous and subsequent examination of other calf lungs using identical techniques did not yield such numerous isolations.

Multiple isolations of mycoplasmas from individual animals have been reported (Gourlay et al., 1970) and were frequent amongst the animals studied here with only four of the 21 calves culturally positive for M. dispar yielding a single isolate.

Bacteria of many genera were isolated, usually in small numbers from pneumonic and non-pneumonic calves within all Groups. In general, in the three months old calves M. dispar was multiply isolated with bacteria whereas other mycoplasmas and bacteria were recovered along with M. dispar from the older calves. The data presented here indicated no association between any bacteria with either a mycoplasmal infection or a specific microscopic lesion.

Examination for chlamydial infections in the lung tissue was not carried out for any of the animals studied. Data based on serological studies on the three to four months old calves, indicated that neither PI3 virus, adenovirus, or IBR virus played any role in the outbreak of respiratory disease amongst these animals. Unfortunately, the presence of viral infection amongst the calves in any of the other Groups is not available; however, current reports on the importance of viruses associated with respiratory outbreaks in calves suggests that their role lies in upper respiratory tract infections (Thomas, 1973; Thomas and Collins, 1974). This may, however, initiate a mycoplasmal respiratory infection since some viruses can damage bronchial epithelium thus enabling the establishment of mycoplasmas and their

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subsequent colonisation of the tissue; such a synergistic reaction has been noted in in vitro studies with organ cultures (Reed, 1972).

The pneumonia present in the calves within the Groups examined was variable although the lesions were similar within each age range of animals studied particularly within each Group of calves which had been reared together.

In the young calves up to one month of age a suppurative exudative pneumonia was generally present, usually complicated by abscess formation together with adhesive pleurisy. From the cultural examination no organism, neither bacterium nor mycoplasma, could be associated with these lesions but histologically this condition is considered to be due to bacterial infection (Jarrett, 1956; Omar, 1966). Calves of this age are particularly susceptible to infection, many suffering enteric disorders which lower their resistance and enable bacterial invasion of the lung tissue; chemotaxis by bacteria can cause massive outpouring of neutrophils into the alveolar air spaces and the bronchial and bronchiolar lumina, a feature seen in these cases. If the bacteria are present in large numbers the neutrophils can be overcome, producing areas of necrosis due to the release of tissue-damaging enzymes. Vaso-active substances can also be released, increasing the vascular permeability and allowing leakage of plasma substances, including complement, into the alveolar spaces, which can enhance the phagocytic activity of neutrophils and macrophages.

The remainder of the calves aged six months, three months and a few at two months of age, had a proliferative pneumonia with either type C or type D pulmonary lesions. The type C lesion was mainly found in the six months old calves and the type D lesion in the younger animals.

These two types of histological lesion were similar in many respects since bronchitis, bronchiolitis and either an alveolitis and/or alveolar collapse were present in both. Accumulations of lymphocytes were evident in the peribronchiolar and peribronchial regions in both types. However, in the type D pulmonary lesion the lymphocyte accumulations were represented as a diffuse layer of cells around the airway, while the lymphoid cells in the type C lesion were organised in a follicular manner around at least two-thirds

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of the airway circumference forming a cuff. Although lymphocytes were found around the airways in the D type lesion the lack of follicularly organised lymphoid tissue did not enable it to be classified as typical cuffing pneumonia. This is contrary to the pulmonary nomenclature employed by others (Taylor-Robinson et al., 1972; Lindsey and Cassell, 1973) who considered the presence of lymphocytes around the airway, not necessarily in an organised pattern, an adequate characteristic of cuffing pneumonia. In the bovine animal cuffing pneumonia was first described as a lesion with an expanding peribronchial lymphoid hyperplasia which appeared to the naked eye and microscopically as a peribronchial cuff (Jarrett et al., 1953). Jarrett (1954 and 1956) described the histology in more detail, defining the cuff as the appearance in the peribronchiolar area of excess lymphoid tissue which contained the structural elements of lymph nodes. In these present studies two criteria, based on a histological examination, had to be met before a lesion could be described as representing cuffing pneumonia. Firstly, germinal centres must be present within the lymphoid tissue forming a follicular pattern and secondly, the lymphoid sheath must surround at least two-thirds of the airway circumference.

The classification of peribronchiolar lymphocytic accumulations can present difficulties in relation to cuffing pneumonia. Accumulations of lymphocytic cells were seen, within the pulmonary lobules of the calves with type A and type B pulmonary architecture, in the peribronchiolar connective tissue and some of the accumulations, particularly in type B histology, even formed isolated follicles with germinal centres. However, apart from the absence of an alveolitis in the type A and type B appearance, the model appearance of the bronchiolar reactions differed in several respects from that in type C lesions. The peribronchiolar lymphocytic accumulations in type C involved most of the circumference of the bronchiole and extended along it to form a sheath, the lymphocytic accumulation was follicular with germinal centres and there was obliteration of the muscularis of the bronchioles by the cells. Many lymphocytes and plasma cells were seen in the adjacent lamina propria and finally most of the bronchioles in an affected lobule were involved. There was a clear cut difference between the bronchiolar reactions in type A

and type C but the distinction between some animals with type B and type C pulmonary architecture could be difficult if assessed on the bronchiolitis only.

Small peribronchiolar lymphocytic accumulations have been described in germ-free rats (Giddens, Whitehair and Carter, 1971) and some of the cells in type A could have represented this population. On the other hand, it is possible that they were residual accumulations from either a resolved cuffing pneumonia or a resolved bronchiolitis.

The type D and type C pulmonary lesions described in these Groups of calves have similarities to mycoplasmal infections in other animals. The cellular infiltrates, particularly chronic suppuration in airways and marked lymphoid infiltration, in diseases caused by mycoplasma species suggests a common pathogenetic mechanism. The obvious differences in clinical manifestations, duration of illness, relative importance of bronchial versus alveolar disease and the liklihood of complications such as bronchiectasis, abscess formation and pleuritis may be a matter of degree rather than separate mechanisms.

In the initial paper describing cuffing pneumonia in calves (Jarrett et al., 1953), attention was drawn to the histopathological similarity between cuffing pneumonia and porcine and murine pneumonias, both of which have now been shown to be caused by mycoplasmas (Goodwin et al., 1965; Lutsky and Organick, 1966).

Experimental infections in mice and pigs with M. pulmonis and M. suipneumoniae respectively, have been reported (Lutsky and Organick, 1966; Hodges et al., 1969; Livingston et al., 1972; Whittlestone, 1972; Lindsey and Cassell, 1973) in which the development of the histological lesion was followed during the course of the disease. The lesions seen in the acute stage of the murine pneumonia and early in the porcine infections are similar to the D type pneumonia described in these calves, characterised by the organisation of lymphocytes into the peribronchiolar areas and the packing of alveolar air spaces with neutrophils and varying numbers of macrophages. Descriptions of experimental EPP and CRD in mice suggested that cuffing lesions, particularly those with a follicular pattern and germinal centres

(analogous with type C lesions in calves), only develop after an infection has been established for several weeks. This means that, assuming these bovine mycoplasmas are pathogens, the peribronchiolar cuffing will only be present in chronic cases and not in those suffering the acute phase of the disease.

Gourlay and Thomas (1970) produced a pneumonia in calves after endobronchial inoculation with ureaplasmas. The animals used were three and four weeks of age and were killed four weeks after infection; the histopathological picture of the pneumonia produced was of an acute bronchiolitis associated with collapse and plugging of the bronchi and bronchioles by inflammatory exudate. Peribronchiolar lymphocytic cuffing was absent but was considered so due to the relatively short duration of the experimental disease.

The present investigation has demonstrated that M. dispar is the mycoplasma most significantly associated with the presence of cuffing pneumonia in calves, although in one Group Ureaplasma spp. were frequently isolated from the lungs of the pneumonic calves.

The study of the various age groups of calves reared together in individual groups, on their mycoplasmal respiratory population and pulmonary pathology suggests that younger calves are more susceptible to bacterial infections with pulmonary lesions of an exudative type while older calves are more likely to have mycoplasma colonisation of the respiratory tract and pulmonary lesions of a proliferative type. Whether or not the appearance of mycoplasmas in the respiratory tract is due to prior bacterial infection or viral infection lowering the resistance of the calves is not clear. Lowered resistance may not be necessary for some mycoplasmas to colonise the respiratory tract since bovine respiratory Ureaplasma spp. and M. dispar have been shown to be pathogenic experimentally in calves (Gourlay and Thomas, 1969 and 1970; St. George et al., 1973; Gourlay et al., 1976). In addition, M. dispar has been shown to be pathogenic for bovine foetal tracheal explant cultures (Thomas and Howard, 1974) and it appears to have a capsule as seen by electron microscopy, a feature associated with the pathogen M. mycoides var. mycoides (Howard and Gourlay, 1974).

Hypotheses explaining the pathogenesis of mycoplasmal respiratory disease in different animals have been published, mostly based on in vitro and a few in vivo experiments. Consideration of some of these theories has suggested several mechanisms of pathogenesis which may be applicable to the bovine infection. Respiratory mycoplasmas have a predilection for respiratory epithelium, attaching avidly and thus preventing their clearance from the major airways of infected animals. A close association forms between the mycoplasmas and the plasma membrane of epithelial cells and long cytoplasmic processes and microvilli often envelop them (Kohn, 1971; Cassell, Lindsey and Baker, 1974), possibly protecting them from the action of specific antisera and phagocytosis.

Close contact with respiratory epithelial cells by pathogenic mycoplasmas causes disorganisation and loss of ciliary activity (Collier and Clyde, 1971; Collier, 1972) which may affect the normal clearance mechanism of the bronchi. The production of hydrogen peroxide by some pathogens e.g. M. pneumoniae, produces tissue damage (Lipman and Clyde, 1969), but hydrogen peroxide liberation by the organisms does not fully explain the tissue damage which occurs. Recent work by Hu, Collier and Baseman (1975) on M. pneumoniae infection in hamster trachea organ culture demonstrated that initial infection increased the respiratory metabolism of the epithelial cells followed by an inhibition of host RNA and protein synthesis. Mycoplasma dispar infection in bovine tracheal explant cultures required large numbers of cells to cause degeneration and sloughing of the respiratory epithelium. Washing of the mycoplasmas, inactivation by heat and Millipore filtration removed the ability of the inoculum to cause tissue damage (Thomas and Howard, 1974).

Irritation of the bronchial epithelial surface by the invading mycoplasmas appears to have increased the number of goblet cells and, with the increased activity of the bronchial submucosal glands, excess mucus was produced in the infected animals. This mucus increase and loss of ciliary activity may have reduced the pulmonary clearance activity, enabling the mycoplasmas to infect the lower respiratory tract and invade the alveolar

tissue. It has been suggested that some mycoplasmas are strongly chemotactic for neutrophils (Lindsey and Cassell, 1973), a mechanism supported by the lesions, particularly in the alveoli, present in many of these cases. Unlike the organisms associated with the bronchial and bronchiolar epithelium which lie between the cilia, the alveolar mycoplasmas are probably exposed and more readily available for phagocytosis by neutrophils and macrophages (Lindsey and Cassell, 1973; Parkinson and Carter, 1975). Macrophages generally infiltrate the alveolar air spaces following the neutrophil infiltration.

Many mycoplasma species appear to adhere readily to the surface of alveolar macrophages but are phagocytosed only in the presence of homologous antisera (Jones and Hirsch, 1971; Bredt, 1975; Powell and Clyde, 1975). Recent work by Bredt and Bitter-Suermann (1975) indicated that the complement sequence is activated by M. pneumoniae even in the absence of antibody. In addition to killing the cell, this reaction resulted in a positive immune adherence suggesting the presence of C3b receptors on the mycoplasma surface. If this reaction occurs in vivo C3a would be expected to be present in the tissue; the chemotactic property of this compound would thus explain the outpouring of neutrophils into the alveolar air spaces in mycoplasma respiratory disease.

Much tissue damage results from the outpouring of the phagocytic cells which may account for the distension of airways, early destruction of the epithelial lining, and later, chronic bronchitis with such complications as bronchiectasis and abscess formation (Lindsey and Cassell, 1973).

Macrophages are considered to play a role in immunity; the ingestion of mycoplasmas by alveolar macrophages may produce degradation products which apparently stimulate the accumulation of lymphoid cells. The lymphocytes around the blood vessels may have resulted from their response to macrophages which had ingested mycoplasmas entering the alveoli (Livingston et al., 1972).

The presence of masses of lymphocytes in the lung suggests that a cell-mediated immune response might play a specific role in this disease although their possible involvement with B cells cannot be excluded. From

studies on immune mechanisms involved in mice with M. pulmonis infections, Taylor and Taylor-Robinson (1974) concluded that humoral immune mechanisms may be more important than cell-mediated ones in resistance to some mycoplasma infections, although T-cells did appear to play a role in the pathogenesis of disease and recovery from it.

T-cells may operate directly by contributing to the lung lesions and stimulating macrophage killing of mycoplasmas or indirectly, assuming antibody is important and its production is T-cell dependent, by stimulating the development of antibody-producing cells in the lung (Cassell et al., 1974). Depletion of T-cells in mice infected intranasally with M. pulmonis resulted in a reduction in the severity of the peribronchial and perivascular lymphoid cuffing. This suppression was accompanied by slightly greater numbers of organisms in the respiratory tract and greater extra-pulmonary dissemination when compared with immunologically normal mice (Denny, Taylor-Robinson and Allison, 1972). However, cell transfer gave no immunity to mice while transfer of immune sera resulted in immunity although mycoplasmas were readily recovered (Taylor and Taylor-Robinson, 1974).

Antibody, of the secretory IgA type, has been shown to be produced locally in the lung tissue in man infected with M. pneumoniae (Brunner et al., 1973), pigs infected with M. suipneumoniae (Holmgren, 1974) and mice infected with M. pulmonis (Cassell et al., 1974).

The influence of both humoral and cellular immunological responses to mycoplasmal respiratory disease may conform with the hypothesis proposed by Fernald and Clyde (1974) who studied the pathogenic mechanism of M. pneumoniae infection in man. A T-cell mediated accumulation of plasma cells in the lamina propria may serve as a source of antibody and lymphocytes which govern the neutrophilic and mononuclear phagocytic response to the invading organism. Systemic antibody and cells in the circulation may serve as indicators of these local reactions but do not directly influence the infection under normal circumstances.

As yet, the importance of the immune response in the bovine mycoplasma respiratory infection is not known. These studies indicate that

agent in cuffing pneumonia, particularly in calves housed and grouped together, up to the age of six months.

Experimental infections of rabbits and hamsters with M. dispar broth cultures and M. dispar-infected lung homogenates were largely unsuccessful.

Histologically, cuffing pneumonia is characterised by bronchitis and bronchiolitis with alveolar collapse and/or alveolitis. The main feature of this lesion is the accumulation of organised lymphoid tissue in the peri-bronchiolar and perivascular regions which extends to form a sheath or cuff around the airways. In other animal hosts this feature is found in pneumonic conditions that have been shown to have a mycoplasmal aetiology. The associated bronchitis involved a massive increase in the number of bronchial epithelial goblet cells which extended peripherally down the airways to the bronchioles, where they are not normally found. In addition, hypertrophy of the submucosal mucous glands often resulting in dilation of the tubules was a common feature. The types of mucosubstances present in the respiratory secretions of the non-pneumonic bovine animal were similar to those found in other animals. In the calves with cuffing pneumonia the mucosubstance composition of the secretions was quantitatively different from the non-pneumonic state; the alterations were found to be consistent among the calves with M. dispar infections.

Ultrastructural detection of mycoplasmas in naturally infected bovine pneumonic tissue has not been described previously. Organisms were found on the bronchial epithelium, forming close contact with the cilia of the cells. Intracellular organisms were never seen and their presence in alveolar tissue was detected in only two animals. Ultrastructural demonstration of mycoplasmas was closely related to the recovery of M. dispar from the lungs of the same animal; in contrast, isolation of A. laidlawii, even in high numbers, did not result in detection of mycoplasmas ultrastructurally. This suggested that M. dispar formed a closer association with bronchial epithelium than other mycoplasma species. Additionally, in the pneumonic cases from which M. dispar had been recovered there was ultrastructural damage to the cells of the bronchial epithelium.

M. dispar is the organism most likely to be responsible for respiratory disease in calves, characterised in its later stages by cuffing pneumonia, a lesion similar to those caused by mycoplasmas in other hosts.

Age Group of Calves	Total No. of calves in Group	Mycoplasma isolations from pneumonic calves					Mycoplasma isolations from non-pneumonic calves				
		No. of calves	M. dispar	Ureaplasma spp.	M. bovirhinis	A. laidlawii	No. of calves	M. dispar	Ureaplasma spp.	M. bovirhinis	A. laidlawii
Less than one month	5	0	-	-	-	-	5	0	0	3	0
1-2 months											
A	6	6	0	0	2	1	0	-	-	-	-
B	5	5	0	3	1	0	0	-	-	-	-
C	4	4	3	1	1	2	0	-	-	-	-
3-4 months	12	12	7	0	1	4	0	-	-	-	-
6 months											
D	20	12	6	8	3	9	8	0	1	1	5
E	9	6	3	0	0	3	3	0	0	0	1
Misc.	6	6	2	1	1	1	0	-	-	-	-
Total	67	51	21	13	9	20	16	0	1	4	6

Table 38 . Frequency of isolation of mycoplasma from eight Groups of calves. The ages of the Groups of calves ranged from less than one month to six months and some calves, in each Group, were affected with macroscopic pneumonia.

CHAPTER SIX

EXPERIMENTAL INFECTIONS IN LABORATORY ANIMALS

A. INTRODUCTION

B. RABBIT EXPERIMENT

1. Materials and methods

a. Animals.

b. Inocula.

c. Post mortem techniques.

2. Results

C. HAMSTER EXPERIMENT

1. Materials and methods

a. Animals.

b. Inocula.

c. Post mortem techniques.

2. Results

D. DISCUSSION

A. INTRODUCTION

Experimental infections with microorganisms in laboratory animals have the disadvantage of altering the factor of host-parasite specificity, but afford the opportunity to examine details of the infectious process which are sometimes not readily available in the natural host because of the problems of experimental animal numbers and expense. Pathogenesis and organism localisation can often be investigated in experimental infections.

The pathogenic mycoplasmas have generally been found to be either species specific or to have a very limited host range. Excluding the natural pathogenic mycoplasmas of small rodents, only a few mycoplasmas have been shown to induce recognisable lesions or disease in laboratory animals (Whittlestone, 1973).

Mycoplasma pneumoniae, a pulmonary pathogen of man, has been studied for many years and Eaton, Meiklejohn and van Herick (1944) successfully infected cotton rats and hamsters with the organism intranasally. Most subsequent studies continued to use hamsters as the experimental model (Dajani, Clyde and Denny, 1965), since these rodents have not, as yet, been found to harbour any natural respiratory tract mycoplasmas. More recently, a successful M. pneumoniae infection was established in guinea-pigs, in which an immune response was produced (Brunner et al., 1973). This was slightly different from that found in hamsters in that there was a more marked cellular response.

Infection of mice with respiratory tract organisms was troublesome because of the endemic occurrence of M. pulmonis in the murine respiratory tract. Lutsky and Organick (1966) attempted to infect gnotobiotic mice with M. pneumoniae but this was unsuccessful. However, a later trial in which multiple doses of M. pneumoniae were inoculated intranasally into gnotobiotic mice produced pneumonia, while single inoculations failed (Organick and Lutsky, 1968).

Goodwin et al. (1965) confirmed that M. suipneumoniae was the causal organism of EPP by inoculating a broth culture of the organism into

SPF piglets. However, several previous attempts had been made to infect laboratory animals with the infectious agent of EPP. Betts (1953) attempted, unsuccessfully, to induce lesions in Syrian hamsters and mice by intratracheal, intranasal and intraperitoneal inoculation of suspensions of pneumonic lung containing M. suipneumoniae. His attempts to produce lesions in the ferret were far more promising, in that transmissible pneumonia occurred on two occasions following primary inoculation. This work was not continued because of the problem of latent infection in the ferret and the introduction of pneumonia-free pigs into veterinary research. In addition to this work, Whittlestone (1958) inoculated mice of one to five days of age with the agent of EPP by intraperitoneal and intracerebral routes without any pathogenic effects.

Most experimental infections with pathogenic mycoplasmas have been confined to infection of the natural host. For example, M. suipneumoniae was considered highly pathogenic in piglets, M. neurolyticum in mice, young rats and field voles (Tully, 1969), M. gallisepticum in turkeys (Clyde and Thomas, 1973) and M. pulmonis in mice and rats (Lutsky and Organick, 1966; Kohn and Kirk, 1969; Lindsey and Cassell, 1973).

Experimental M. pulmonis infection in mice was studied in detail and it was found that the pathogenic results varied with the strain of organism and the breed of mouse used (Taylor-Robinson, 1975).

The bovine respiratory tract mycoplasmas have not been studied to any degree by experimental infections of laboratory animals, as far as one can judge from the literature. Test infections in conventionally reared calves have been carried out with A. laidlawii (Trapp et al., 1966) and M. bovirhinis (Hamdy et al., 1958; Langer and Carmichael, 1963; Dawson et al., 1966; Gourlay and Thomas, 1970) but only Gourlay and Thomas (1970) were successful; they produced tiny pneumonic lesions in colostrum-deprived calves infected endobronchially.

Colostrum-deprived and colostrum-fed calves inoculated endobronchially with bovine Ureaplasma spp. developed pneumonia (Gourlay and Thomas, 1969 and 1970). Similar results were obtained with M. dispar infection in eight calves (Gourlay and Thomas, 1969). Recently, pneumonia and polyarthritis

were produced in calves by endobronchial inoculation of pneumonic lung tissue infected with M. agalactiae var. bovis (Thomas et al., 1975).

Mycoplasma mycoides var. mycoides has been studied in great detail, largely with infection of the natural host. However, Smith (1968) infected mice intraperitoneally with this organism, producing an asymptomatic condition with the organism persisting in the circulation. This infection, although unnatural, has enabled some immunity studies to be followed.

The literature did not promise a successful laboratory animal infection with the bovine mycoplasma, M. dispar. However, in view of the fact that laboratory animals have been successfully infected with some mycoplasmas, experimental infections were undertaken in rabbits and hamsters since both animals are considered relatively free of natural respiratory tract mycoplasma infections. A lung homogenate infected with M. dispar was used as the inoculum. In addition, some hamsters were also infected with a pure broth culture of M. dispar.

In the first experiment rabbits were used as the experimental animals and in the second hamsters. The details of these two experiments are described separately but the results are discussed together.

B. RABBIT EXPERIMENT

1. Materials and methods

a. Animals.

Twelve six to eight weeks old New Zealand White and Dutch cross rabbits weighing approximately two kilograms each were used. The animals were divided into four groups according to inoculum and duration of infection, as illustrated in Table 39 . They were anaesthetised with 60 mg of sodium pentobarbitone (Euthatal, May and Baker, Dagenham, Essex) by intravenous injection of the marginal ear vein. Rabbits in Groups 1 and 3 were injected intratracheally with 0.5 ml of calf lung homogenate, while one rabbit from

each of Groups 2 and 4 received 0.5 ml of sterile PBS (pH 7.3). The two remaining rabbits acted as uninoculated controls. All rabbits were caged separately and managed as described in section A of chapter two.

b. Inocula.

A 50 per cent homogenate of pneumonic calf lung was prepared. The tissue was taken from a six months old calf in which M. dispar was the only microorganism cultured from the respiratory tract. Histologically the pneumonia was of the cuffing type. The tissue was homogenised in a Stomacher 80 for 30 seconds in an equal volume of sterile PBS (pH 7.3) containing one mg per ml ampicillin. The homogenate was spun lightly on an MSE bench centrifuge at speed three for three minutes. The supernatant was removed and used as inoculum. Pre-inoculation serum samples were collected from the marginal ear vein and again prior to necropsy.

c. Post mortem techniques.

The rabbits were killed as described in section B of chapter two, with Groups 1 and 2 killed at two weeks post-infection and Groups 3 and 4 at four weeks. The trachea and lungs were aseptically removed en bloc and examined for macroscopic lesions. Tissue from the anterior region of the lungs was examined for the presence of M. dispar, Ureaplasma spp. and bacteria employing the techniques of cultivation and identification described in section C of chapter two. Blocks of tissue for histological examination were taken from similar sites and from other areas as necessary. All sections were stained with haematoxylin and eosin. In addition, tissue for electron microscopy and for immunofluorescence was taken from some rabbit lungs, sampling from the regions used for the previous examinations. The techniques employed were described in chapter two, the frozen tissue being screened for the presence of M. dispar by the indirect immunofluorescent technique.

Paired serum samples were examined for the presence of antibodies to M. dispar using latex agglutination and indirect immunofluorescent techniques; these techniques were described in section H of chapter two.

2. Results

Mycoplasma dispar was reisolated from two of the eight infected rabbits; both belonging to Goup 1 as illustrated in Table 40. No other glucose-fermenting mycoplasma was isolated and Ureaplasma spp. were not found in any of the lungs. Both rabbits which harboured M. dispar were killed two weeks post-infection.

Alcaligenes bronchisepticus was isolated from three rabbits, one of which was a control animal. As may be seen in Table 40, A. bronchisepticus was not isolated from either rabbit harbouring M. dispar. This bacterium was isolated in small numbers and was the only organism found in rabbit lungs.

The results of the pathological examination are shown in Table 41. Macroscopic lung lesions were seen in only four of the eight infected rabbits, all belonging to Group 1. The lesions were small (less than 0.5 cm^2), purple-red, smooth, and restricted to the anterior lobes of the lungs. Histology revealed only slight differences between Groups 1 and 3 and the control rabbits. Lymphocytes were seen in small accumulations in the peribronchial and peribronchiolar areas in all rabbits except two (M120R and M125R). In Group 1, a follicular arrangement of the lymphocytes was present in two rabbits, with the cells infiltrating the lamina propria and destroying the muscularis to a small degree (Figs. 44 and 45). However, these accumulations were not large, did not form cuffs and were restricted to a small proportion of the airways. In three cases there were a small number of plasma cells in the bronchiolar lamina propria, although no plasma cells were seen in the peribronchiolar lymphoid accumulations. A mild macrophage alveolitis was seen in one of these cases (M119R). Rabbits in Group 2 had small peribronchiolar accumulations of lymphocytes which were diffuse and not follicular in appearance (Fig. 46).

Peribronchiolar lymphoid accumulations were present in two rabbits in Group 3; the accumulations were follicular in nature and often infiltrated the lamina propria and displaced the muscularis. As in Group 1, these cellular accumulations were small and were found only in a few airways in the lobes examined. Accumulations of lymphoid cells around the bronchioles were also

seen in one of the two other infected rabbits but the cells were arranged diffusely around the airways. The fourth member of Group 3 appeared to have histologically normal lungs except for the presence of a few macrophages in the alveoli. The inoculated control in Group 4 had a few small diffuse peribronchiolar accumulations of lymphocytes, while in the contact control the peribronchiolar lymphocytes had a follicular arrangement which caused slight destruction of the muscularis.

Tissue from two rabbits (M118R and M125R) was examined electron microscopically but no mycoplasmas were detected.

Serological examination for the presence of antibody to M. dispar was carried out using latex agglutination and indirect immunofluorescence; the results obtained with these two methods were similar but not identical. The reciprocal titres of each test have been tabulated in Table 40 : a slight antibody response to M. dispar was demonstrated. The response was most obvious in Group 1, in which an increase in titre was found in all four animals by both tests. One rabbit (M118R) had a low pre-infection antibody titre. The control animals in Group 2 had a very slight response by the latex agglutination test. At four weeks post-infection the response had dwindled and only three of the four Group 3 rabbits had any anti-M. dispar antibodies and these were at a low titre in both tests.

C. HAMSTER EXPERIMENT

1. Materials and methods

a. Animals.

Thirty five Golden Syrian hamsters, five to six weeks of age, weighing approximately 50 to 60 g were used. The animals were divided into five groups as illustrated in Table 42. They were anaesthetised by intraperitoneal injection of two mg of sodium pentobarbitone. The ten hamsters in Group 1 were inoculated intranasally with 0.2 ml of calf lung

homogenate while Group 2 received 0.2 ml of a broth culture of M. dispar containing approximately 10^5 CFU per ml. Control Group 3, consisting of five hamsters, received 0.2 ml of sterile calf lung homogenate. The five hamsters in control Group 4 were given 0.2 ml sterile GS broth, while the five remaining hamsters in control Group 5 were not inoculated and acted as contact controls. The hamsters were caged as five separate groups and managed as described in section A of chapter two.

b. Inocula.

The calf lung homogenate was prepared in the same manner as for the rabbit infections described in section B. A culture of M. dispar was grown in GS broth at 37^{o}C until the organisms were in a logarithmic growth phase, indicated by a slight drop in pH. The culture was titrated in suitable medium and was found to contain approximately 10^5 CFU per ml. The hamsters were killed, as described in section B of chapter two, three weeks post-infection.

c. Post mortem techniques.

The techniques used in the rabbit experiment (section B) were employed. Tissue was not cultured for the presence of Ureaplasma spp. and no specimens were collected for immunofluorescence or electron microscopy. Serology was not carried out.

2. Results

No glucose-fermenting mycoplasmas were isolated from the pulmonary tissue of any of the hamsters. Bacteriological examination of the lungs revealed the presence of a variety of organisms, which were found in all groups (Table 43). Bacteria were isolated from two hamsters in Group 1 and four in Group 2. The lung tissue from four of the control hamsters yielded bacteria, one isolate from Group 3, two isolates from Group 4 and one isolate from Group 5. All organisms were isolated in small numbers from the lung, and Staphylococcus aureus was the only organism recovered from more than one hamster.

Macroscopically, no lung lesions were visible and histological

CHAPTER THREE

MYCOPLASMAS OF THE BOVINE RESPIRATORY TRACT

A. REVIEW OF THE LITERATURE

B. CULTIVATION AND MORPHOLOGICAL CHARACTERISTICS OF FOUR BOVINE MYCOPLASMAS

examination of all cases demonstrated normal lungs, with no cell reaction being seen in any area.

C. DISCUSSION

Infection of eight rabbits with the supernate of a 50 per cent homogenate from a pneumonic calf lung known to be infected with M. dispar produced no clinical illness in any of the animals. Pulmonary pathological changes were minimal although a slight change could be detected when compared with the control animals. After two weeks infection, small macroscopic lesions were visible in all the lungs and these were seen histologically as small peribronchiolar lymphoid accumulations, with a few plasma cells present in the lamina propria. Mycoplasma dispar persisted in the lung during this period. At four weeks post-infection no macroscopic lesions were seen in any of the lungs, but the peribronchiolar lymphoid accumulations, when present, appeared more marked and were accompanied by a slight macrophage alveolitis. Mycoplasma dispar was not recovered.

The lung pathology of the rabbit infection is interesting, although a repeat experiment using either a large number of conventionally reared rabbits, SPF or gnotobiotic rabbits is necessary to eliminate any pulmonary reaction due to natural respiratory tract flora. This would ensure that the control rabbits could be used comparably. A higher dosage of organisms would probably increase the chances of establishing an infection. Microbiologically, however, the result was disappointing since only two of the eight infected rabbits yielded M. dispar on reisolation, both rabbits being killed two weeks post-infection. The persistence of this organism for only two weeks in only two rabbits may be the result of too small a dosage being used in the inoculum, although the isolation rate in pneumonic calves is comparable and so the low reisolation may be partly explained by the difficulty encountered in cultivation. Finally, other factors must be considered, such as breed of rabbit, strain and dosage of organism and incubation period, all of which have been found to

influence experimental mycoplasma infections.

The experiments described above have demonstrated hamsters to be totally refractile to infection with M. dispar in either broth culture or lung homogenate. This result was not surprising, in view of the previous studies of experimental infections in laboratory animals referred to in the introduction. In relation to M. dispar, hamsters appear to have an efficient pulmonary clearing system, since no mycoplasmas were reisolated from any of the infected animals. Interestingly, hamsters have not, as yet, been found to harbour any respiratory tract mycoplasmas although they have been found to be an ideal model system for M. pneumoniae infection experiments.

Successful experimental infections in laboratory animals are very useful as they enable pathogenic, pathologic and immunologic studies to be carried out at relatively low cost. However, in the studies carried out here, the results of the hamster experiment confirmed the host-parasite specificity generally found with pathogenic mycoplasmas. On the other hand, although the rabbit infection was not totally successful, there is a possibility that rabbits may be susceptible to a pulmonary M. dispar infection.

Group Number	Number of Rabbits	Inoculum	Duration of infection
1	4	0.5 ml lung homogenate	2 weeks
2	1	0.5 ml PBS	2 weeks
	1	uninoculated control	2 weeks
3	4	0.5 ml lung homogenate	4 weeks
4	1	0.5 ml PBS	4 weeks
	1	uninoculated control	4 weeks

Table 39. Rabbit grouping used in experimental infections.

Group Number	Rabbit No.	Organisms isolated from lung			Serology *			
		M. dispar	Ureaplasma spp.	Bacteria	Pre-infection		Post-infection	
					LA	IF	LA	IF
1	MI17R	-	-	-	-	-	2	4
1	MI18R	+	-	-	2	-	8	4
1	MI19R	+	-	-	-	-	2	4
1	MI20R	-	-	Alcaligenes bronchisepticus	-	-	16	8
2	MI21R	-	-	-	-	-	2	-
2	MI22R	-	-	A. bronchisepticus	-	-	2	-
3	MI23R	-	-	-	-	-	4	-
3	MI24R	-	-	-	-	-	-	2
3	MI25R	-	-	A. bronchisepticus	2	-	-	-
3	MI26R	-	-	-	-	-	-	2
4	MI27R	-	-	-	-	-	-	-
4	MI28R	-	-	-	-	-	-	-

* Reciprocal titre of antibody against M. dispar by latex agglutination (LA) and indirect immunofluorescent (IF) techniques.

Table 40 . Microbiological and serological results on rabbits infected with calf lung homogenate.

Group Number	Rabbit No.	Macroscopic lesions	Microscopic findings Peribronchiolar lymphoid accumulations	Alveolitis
1	M117R	Focal	Diffuse	-
1	M118R	Multifocal	Follicular	-
1	M119R	Multifocal	Follicular	+
1	M120R	Multifocal	-	-
2	M121R	-	Diffuse	-
2	M122R	-	Diffuse	-
3	M123R	-	Follicular	+
3	M124R	-	Diffuse	+
3	M125R	-	-	+
3	M126R	-	Follicular	-
4	M127R	-	Diffuse	-
4	M128R	-	Follicular	-

Table 41. Pathological results on rabbits infected with calf lung homogenate.

Group Number	Number of Hamsters	Inoculum	Duration of infection
1	10	0.2 ml calf lung homogenate	3 weeks
2	10	0.2 ml M. dispar broth culture	3 weeks
3	5	0.2 ml sterile calf lung homogenate	3 weeks
4	5	0.2 ml sterile GS broth	3 weeks
5	5	uninoculated control	3 weeks

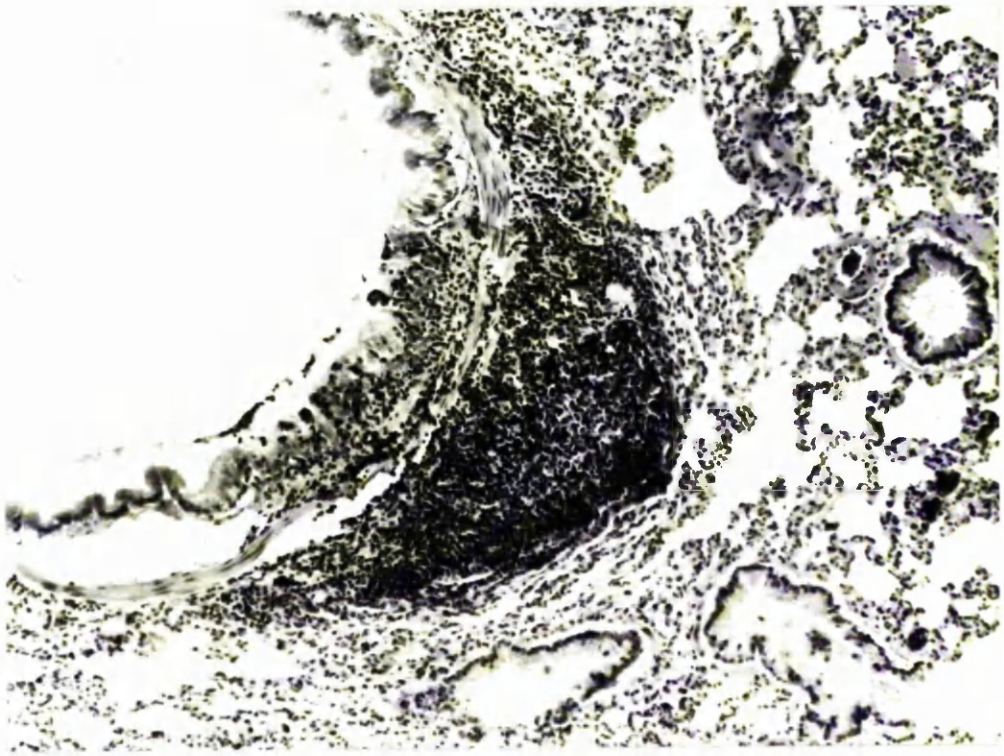
Table 42. Hamster groups used in experimental infections.

Group	Hamster No.	Type of infection	Bacterial species isolated
1	M136H)) M143H)	0.2 ml lung homogenate	Staphylococcus aureus Streptococcus pneumoniae
2	M148H)) M150H)) M151H) M153H)	0.2 ml M. dispar broth culture	Streptococcus sp. Enterobacter aerogenes Aerococcus viridans Streptococcus faecalis
3	M158H	0.2 ml sterile lung homogenate	Pasteurella haemolytica var. haemolytica
4	M160H)) M163H)	0.2 ml sterile broth	Staphylococcus aureus Pasteurella multocida
5	M168H	contact control	Micrococcus sp.

Table 43. Results of bacteriological examination on lungs of hamsters infected with calf lung homogenate or M. dispar broth culture.

Fig. 44 : Pulmonary histology of a Group 1 rabbit showing a small aggregate of lymphocytes in the peribronchiolar region. The lymphocytes are displacing the muscle tissue and infiltrating the lamina propria. HE staining, x 120.

Fig. 45 : Pulmonary histology of a Group 1 rabbit. The peribronchial lymphocytic accumulation has displaced the muscle layer and infiltrated the lamina propria. The epithelial layer has been lost in some areas (arrow) due to obliteration by the lymphoid cells. HE staining, x 120.



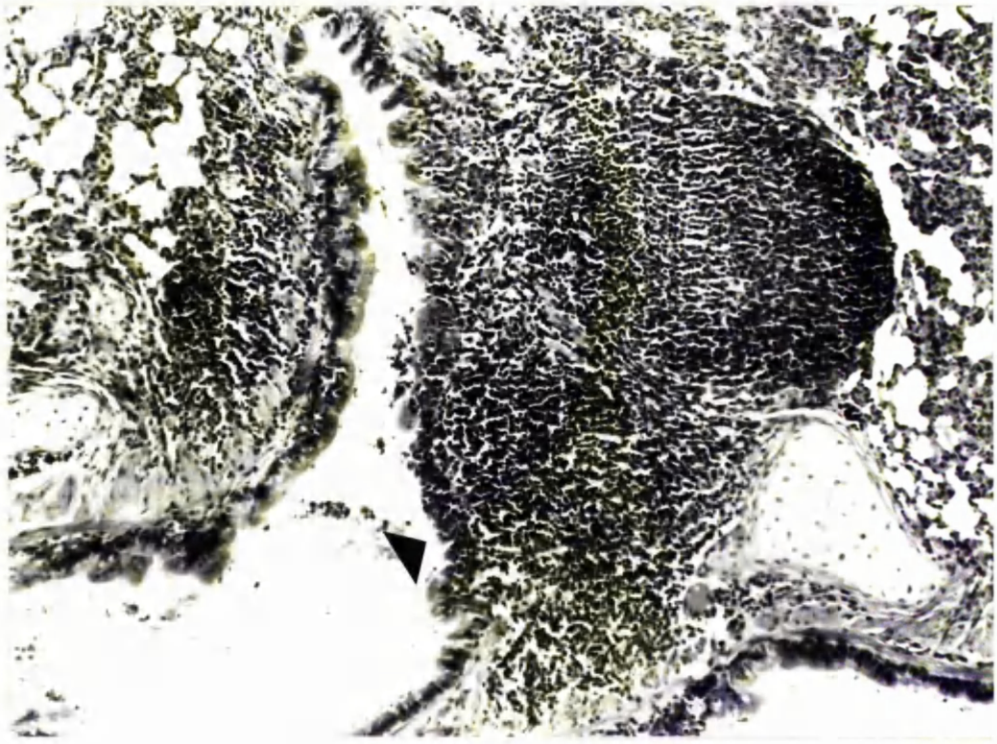
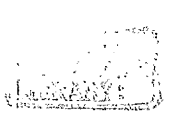


Fig. 46 : A Group 2 control rabbit. A small aggregate of lymphocytes is present in the peribronchial region. The muscle layer is still intact and the surrounding alveolar tissue appears normal. HE staining, x 120.



A. REVIEW OF THE LITERATURE

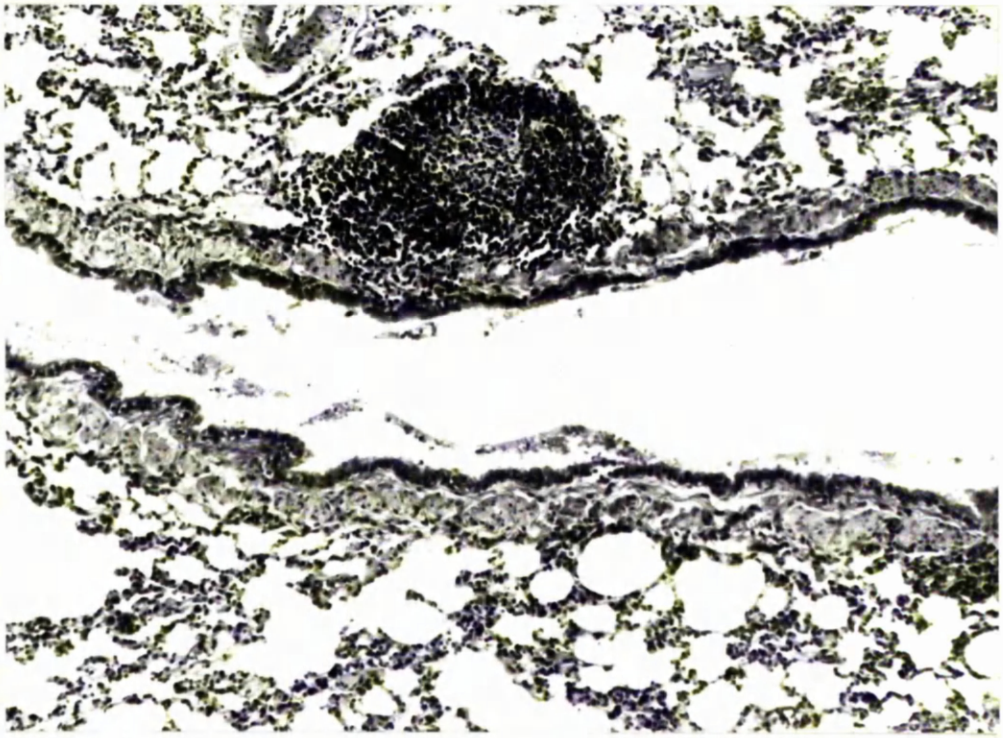
In chapter one some of the details of the distribution and possible significance of bovine respiratory tract mycoplasmas were described. Five species, A. laidlawii, Ureaplasma spp., M. bovirhinis, M. dispar and M. agalactiae var. bovis have been isolated from the bovine respiratory tract in Great Britain. Acholeplasma modicum, M. alkalescens, M. bovigenitalium and M. arginini have also been isolated from this site but not from animals in Great Britain.

This section will be limited to a more detailed review of the literature describing the isolations of four mycoplasmas, M. dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii, recovered from calves during this study. In section B their known biological characteristics will be reviewed.

1. Mycoplasma dispar

Gourlay (1969) modified the complex medium developed for the isolation of M. suipneumoniae, the causal agent of enzootic pneumonia of pigs (EPP), (Goodwin et al., 1965) and was then able to isolate M. dispar from the lungs of pneumonic calves. The modification consisted of the replacement of swine serum and yeast extract by foetal calf serum and calf thymus DNA (Gourlay and Leach, 1970). These latter workers noted that the colonial morphology of the organism was atypical, with a granular or lacy appearance, lacking well-defined centres. Mycoplasma dispar was isolated from 36 of 72 pneumonic calf lungs but no recoveries of this organism were made from the normal lungs of 20 calves and ten adult cows (Gourlay and Leach, 1970). Unfortunately, no histological examination was carried out on tissue from any of these animals either (i) to confirm the normality of the lung tissue of the 20 calves and ten cows or (ii) to give an indication of the type of lesion present in the pneumonic cases.

In a survey of the pathology and microbiology of pneumonic calf lungs, M. dispar was isolated from the lung tissue of 27 of 45 three months old calves which had been collected randomly from an abattoir over an 18 month period (Gourlay et al., 1970). The organisms were isolated in high numbers



CHAPTER SEVEN

A HISTOCHEMICAL STUDY OF MUCOSUBSTANCES IN THE BOVINE RESPIRATORY TRACT WITH SPECIAL REFERENCE TO CUFFING PNEUMONIA

A. INTRODUCTION AND REVIEW OF THE LITERATURE

B. MATERIALS AND METHODS

1. Animals
2. Tissue samples
3. Histochemical techniques
4. Quantification

    a. Goblet cells.

    b. Glands.

C. RESULTS

1. Non-pneumonic animals
2. Pneumonic animals

D. DISCUSSION

A. INTRODUCTION AND REVIEW OF THE LITERATURE

Mucus forms a continuous layer on the luminal surface of the tracheobronchial tree; it has several functions, but the prevention of close or prolonged contact between the epithelium and irritating or infective agents is its major role. The mucus is derived from epithelial goblet cells and submucosal glands. Goblet cells, scattered throughout the epithelium of large and small bronchi, discharge their cellular content directly onto the epithelial surface. The mucous glands are embedded in the submucosa of the airway wall and the mucosubstances produced are secreted into ducts which pass through the submucosa onto the epithelial surface.

The introduction of scanning and transmission electron microscopy and their application to cell morphology studies have enabled detailed descriptions to be made of cells associated with the secretory process. Comparative morphological features of the structures involved in respiratory tract secretions in animals are not adequately recorded; most studies have been carried out in man. However, cell structure and function in the mammalian tracheobronchial system was described in a recent review (Breeze, Wheeldon and Pirie, 1976) and both the light and electron microscopic details of ll epithelial cells and the cells of the submucosal glands were reviewed.

Goblet cells have been found in the respiratory epithelium with their number and distribution varying among species (McCarthy and Reid, 1964; Lamb, 1969; Baskerville, 1970; Wheeldon, 1974). Lamb (1969) described three morphologically distinct types of goblet cell in the human bronchial epithelium, similar to the description by Moe (1968) who studied the goblet cells of the intestinal tract and concluded that the shape of the cell indicated its secretory state; the distended cells found in the crypts of the villi were full of mucinogens. These actively discharged mucus as they ascended the villus to become wine-glass shaped cells, and eventually appeared as exhausted cells at the villus apex. Epithelial serous cells have been recently described in the trachea and extrapulmonary bronchi of SPF rats (Jeffery and Reid, 1975). The presence of these cells in the epithelium of other animals is uncertain and

they have not been attributed a specific function.

The tracheobronchial submucosal glands have been widely studied in man and it has been estimated that there are 6,000 in the tracheal wall (Thurlbeck, Benjamin and Reid, 1961). Earlier, Reid (1960) calculated that the submucosal glands of the human respiratory tract produced 40 times more mucosubstance than epithelial goblet cells and suggested that the gland secretions were thus more important than the epithelial secretions. Submucosal glands have been shown to be controlled by the parasympathetic nervous system (Lamb, 1969). The mucous cell secretions increased with cholinergic stimulation and decreased after atropine administration, although secretions never ceased but appeared to maintain a low threshold level (Lamb, 1969). The epithelial goblet cells have no nervous control but numbers can increase rapidly with irritation of the epithelium (Lamb, 1969; Jeffery and Reid, 1975).

Mucins are the major constituents of sputum and are mainly responsible for its physical properties, including its viscosity. Ideal characterisation of tracheobronchial secretion would be from biochemical procedures. However, such techniques are complicated by several factors including (i) the anatomical inaccessibility of the tracheobronchial mucosa, (ii) the small amount of secretion produced, (iii) contamination from saliva, blood and its transudate, and (iv) the inability to separate materials secreted from the two components (epithelial surface layer and the submucosal glands), or from individual cells which constitute these structures.

As the anatomical features of the secretory apparatus became known, it became clear that several cell types were associated with the respiratory tract secretions. During the past 20 years, the application of histochemistry has allowed the identification, localisation and quantification, of specific substances and reactive groups in these cells (Pearse, 1975). Many techniques are now available for the identification of mucosubstances and these include basic dye stains used singly or in sequence, enzymatic digestion, autoradiography, oxidation procedures, fluorescent antibody methods and chemical modification of tissue (Spicer and Hensen, 1967).

Until 1965, there was some confusion over nomenclature and

classification of the respiratory tract secretions. Then Spicer, Leppi and Stoward (1965) reviewed the current knowledge and proposed a more flexible terminology in an attempt to curb confusion and misinterpretations. These workers suggested the general term ' mucosubstance' be used to designate carbohydrate components of unknown nature; with such terms as 'mucopolysaccharide' and 'glycoprotein' being retained only to describe substances which had been analysed biochemically.

Spicer et al. (1965) recognised two broad groups of mucosubstances in tracheobronchial secretions - these were the neutral and acid mucosubstances. The acid group was subdivided into sialomucins (which may be further divided into neuraminidase-sensitive and neuraminidase-resistant) and sulphomucins. Neutral mucosubstances were considered to be those that stain red in the combined alcian blue/periodic acid-Schiff (AB/PAS) technique (this also stains glycogen but this can be eliminated by treating a section of tissue with diastase). The acid mucosubstances were recognised by their affinity for basic dyes, e.g. alcian blue (AB).

The alcianophilia of the acid mucosubstances was due to the presence of either sialic acid residues or sulphate ester residues attached to the basic glycoprotein molecule (Spicer et al., 1965). The presence of alcianophilic uronic acid residues was detected by treatment of a section of tissue with testicular hyaluronidase, which eliminated the uronic acid radicals (Spicer et al., 1971). However, to date uronic acid residues have not been detected in any respiratory tract secretions.

Sialomucins of the respiratory tract reacted in different ways to the specific enzyme neuraminidase (sialidase), which is usually derived from Vibrio cholerae. Sialomucins were highly susceptible, resistant or poorly susceptible to this enzyme. Lev and Spicer (1965) suggested several mechanisms of steric hindrance which would account for the resistance, but no proven biochemical or structural reason has been given (Lamb and Reid, 1969). However, from the work of Gibbons (1963) on whole sputum, Lamb and Reid (1969) developed an acid hydrolysis technique to eliminate all sialic acid residues by treating tissue sections with sulphuric acid. Jones and Reid (1973) detected a difference

in the staining reaction of the two sialomucins; the neuraminidase-sensitive sialomucins stained only between pH 2.6 and 1.7, while the neuraminidase-resistant sialomucins stained between pH 2.6 and 1.5.

Sulphomucins have been visualised by autoradiographic studies, using ^{35}S on tissue and organ cultures. However, this is a limited technique and several staining methods have been developed, these include aldehyde-fuchsin/alcian blue (Spicer and Meyer, 1960), alcian blue/aluminium sulphate (Heath, 1961), high-iron diamine (HID) and alcian blue (pH 1.0) (Spicer and Duvenci, 1964). Studies carried out by Lamb (1969) and Lamb and Reid (1969) on serial sections stained by the above techniques revealed that these stains were comparable with the acid hydrolysis method (which eliminated all sialic acid residues and left only sulphomucins) in staining most cells, but in comparison with ^{35}S uptake, all the staining techniques missed a few sulphomucin-containing cells.

The distribution of the mucus secreting apparatus' components varies considerably between species and the histochemical composition of the secretion is quite variable. For example, McCarthy and Reid (1964) noted that although the secretory structures in mice and rats had a similar histological appearance they differed histochemically. In mice the goblet cells and glands contained sialic acid and no sulphate, whereas sulphomucins predominated in the goblet cells of rats. Similar studies have been carried out in other animals and have also revealed variations between species (Korhonen, Holopainen and Paavolainen, 1969; Spicer et al., 1971; Wheeldon, 1974; Jones, Baskerville and Reid, 1975).

In several species studies of chronic respiratory diseases have detected both quantitative and qualitative alterations in the tracheobronchial mucosubstances when compared to the normal state. Most studies have been carried out on chronic bronchitis in man (de Haller and Reid, 1965; Reid, 1965; Lamb and Reid, 1969) in whom an increase in acid mucosubstance was detected; this mucus was resistant to neuraminidase and must have been either resistant sialomucin and/or sulphomucin. Lamb (1969) claimed that the increase in acid mucosubstance in chronic bronchitis in man was due to excess sulphomucin production.

Wheeldon (1974) compared the histochemical make-up of the respiratory tract mucosubstances in normal dogs and dogs with chronic bronchitis. He found that there was a reduction in epithelial sulphomucins together with an increase in sialomucins in chronic bronchitis; both neuraminidase-sensitive and neuraminidase-resistant sialomucins were detected. Spicer, Chakrin and Wardell (1972) exposed dogs to sulphur dioxide and found a resultant decrease in the sulphomucin content of the epithelial goblet cells and submucosal glands.

More recently, Jones et al. (1975) examined the tracheobronchial glands of normal pigs and compared them with those of pigs experimentally infected with M. hyorhinis. These workers found a shift from neutral mucosubstance to sulphomucin and neuraminidase-sensitive sialomucin in the mucous cells and in the serous cells there was an increase in sulphomucin and neuraminidase-resistant sialomucin.

Although studies of the secretory apparatus of the respiratory tract have been carried out in many animals, the bovine species does not appear to have been examined. Since very little is known about these structures in the bovine animal a special study was made of them; an additional reason for doing this was to interpret the changes which occur during bronchopulmonary diseases such as cuffing pneumonia. These studies involved histological and histochemical examinations of calves of varying ages. Non-pneumonic calves were investigated to establish the nature of the mucosubstances in the tracheobronchial secretion of the normal animal. Calves with cuffing pneumonia, some of which had pulmonary infections with M. dispar, were subsequently examined to see if any change could be found in the mucosubstances produced by bronchial epithelial goblet cells and submucosal glands.

B. MATERIALS AND METHODS

1. Animals

Details of the 15 calves examined are given in Table 44 . Four of the calves were considered to be normal, non-pneumonic animals and one was less than one month old. The 11 other calves had pneumonia. Six were six months of age and all had cuffing pneumonia (*M. dispar* was recovered from the lungs of three). Four were three months of age and were considered to have a pneumonia representing an earlier phase of the classical cuffing lesion (*M. dispar* was isolated from the lungs of three). One two months old animal, with pneumonia and an *M. dispar* infection, was also examined.

2. Tissue samples

Portions of bronchus were taken from the right cranial lobe of all animals examined. In many cases sections of the right cranial lobar bronchus were taken from three positions and from similar sites along the length of the lobar bronchus of the right caudal lobe. This enabled comparisons to be made between animals and also within individuals.

All material was taken immediately after death. The tissue was fixed in corrosive formol and/or formol saline and processed by the methods described in section E of chapter two. The section of bronchus used for quantification estimates was always from a tissue block which had been taken from the same lobe and in approximately the same site in each animal. The site sampled was the segmental bronchus of the cranial part of the cranial lobe of the right lung.

3. Histochemical techniques

The histochemical techniques utilised in this study are given in Appendix I, which details the methods and lists the reagents required. Stains selective for the different types of mucosubstance found in bronchial secretions were used. These included PAS and diastase digestion for neutral mucosubstances, neuraminidase digestion and acid hydrolysis prior to AB/PAS

staining for sialomucins, four staining techniques for sulphomucins and hyaluronidase digestion prior to AB staining for hyaluronic acid.

Four histochemical stains were used in the quantification studies and were carried out on serial transverse sections of bronchus in the order listed below.

Section 1. AB/PAS at pH 2.6; red staining indicative of neutral mucosubstance, blue of acid mucosubstance.

Section 2. Neuraminidase digestion followed by AB/PAS at pH 2.6; any increase in red staining compared to section 1 was due to the elimination of neuraminidase-sensitive sialomucins.

Section 3. Acid hydrolysis followed by AB/PAS at pH 2.6; any increase in red staining compared to section 2 was due to the elimination of neuraminidase- resistant sialomucins and the blue staining material was sulphomucins.

Section 4. High-iron diamine; to stain sulphomucins specifically brown-black. This was used as a check for the sulphomucin content of the cells and the results of the counts on these studies were not recorded.

4. Quantification

a. Goblet cells.

Goblet cell counts were done by examining the bronchial epithelium with a microscope fitted with a graticule in the x 10 eyepiece. The graticule was 1 cm square giving a unit length of epithelium of approximately 0.02 mm when viewed with a x 45 objective. The number of goblet cells was assessed by choosing randomly four areas of epithelium and counting the number of goblet cells within the unit length of the graticule. The folds of the epithelium were not examined as a high concentration of goblet cells was generally found in these regions. In addition, to avoid errors caused by plane of section, only goblet cells in the upper half of the epithelial layer were counted, avoiding goblet cell-like structures often seen in the lower regions of hyperplastic

epithelia in diseased animals. The three serial sections of bronchus were examined consecutively, counting the number of blue and red staining goblet cells within the graticule unit length in each of four standard fields. From these readings the total number of goblet cells in four graticule units for each section could be assessed, along with the percentage of blue and red staining cells. Finally, a mean value of the number of goblet cells in four unit lengths of epithelium was estimated from the three sections studied. This value was a measurement of the number of goblet cells in a standard length of bronchial epithelium(SLB) which was approximately 0.08 mm long.

b. Glands.

To evaluate the distribution and secretion of the submucosal glands, the total number of gland tubules in the submucosa of the section of bronchus was counted. Only tubules situated below the muscularis were counted and collecting ducts were omitted. Using the x 45 objective each cross section of gland tubule was assessed as being coloured predominantly blue or red. Although some tubules contained mixed secretions, the majority were seen to be prevalent in one or other of the colours. Occasionally a mixed tubule was encountered in which there was approximately equal proportions of blue and red cells in which case a point for each colour was recorded. Counts were carried out on the three serial sections described above and the percentage of tubules staining blue and red in each section was recorded.

Similarly an estimate was made of the mucosubstance in the gland lumen, although this was more difficult due to loss of material in some sections.

with an average titre of 10^4 CCU per 0.2 ml inoculum. The calves were described as being clinically healthy, although necropsy examinations revealed extensive macroscopic lesions, which were described, in most cases, as bronchiolitis accompanied by peribronchiolar lymphoid hyperplasia and frequently alveolitis. Cuffing lesions were recorded in 50 per cent of these pneumonic cases. A further group of 20 calves, aged from two weeks to nine months, which had also been collected randomly during 18 months from farms, were also examined microbiologically and pathologically after they had died or been killed in extremis. Mycoplasma dispar was isolated from only six animals in this group: three of these had pulmonary congestion, two had bronchiolitis and one had a cuffing lesion. It should be noted, however, that in this group of calves severe illness or death may not have been due to pneumonia in all instances.

In a survey carried out in the South of England, Thomas and Smith (1972) examined 70 animals, collected from two abattoirs, and stated that none had macroscopic pulmonary lesions. No histological examinations were carried out. The nasal cavity, trachea, small bronchus and lung tissue were examined microbiologically in three groups of calves aged one to two days, three to four months and ten months and older. The results indicated the presence of M. dispar in the respiratory tracts of a small number of 20 very young calves; four isolates were made from the upper respiratory tract while bronchial and lung tissue yielded one isolate each. Similar isolation rates were found in the ten months and older age group although the organism was confined to the nose and trachea. However, in the three to four months old calves, M. dispar was found to be the predominant mycoplasma of the lower respiratory tract and was isolated from 17 of 22 bronchial samples. These workers observed that the distribution of mycoplasmas in the bovine respiratory tract was age dependent and not due to a seasonal variation.

Mycoplasma dispar was isolated from 11 swabs of the lower respiratory tracts of 21 three to four weeks old calves, which were conventionally reared or colostrum-deprived and considered to be normal clinically (Gourlay and Thomas, 1970).

A similar age distribution of M. dispar colonisation of the respiratory

C. RESULTS

1. Non-pneumonic animals

In the normal, non-pneumonic calves, the epithelial lining of the large bronchi was of pseudo-stratified columnar ciliated cells; this became cdumnar ciliated in the smaller bronchi and bronchioles and cuboidal in the terminal bronchioles. A few plasma cells or small lymphocytes could occasionally be seen in the lamina propria. The muscularis was present as a continuous layer around all airways, the relative thickness varying with the diameter of the airway. The cartilage within all lobes was similar and was embedded in connective tissue below the muscularis. In the large airways, the cartilage plates formed a ring around the airway but became incomplete or absent as the airways became smaller. Most submucosal glands were situated in the connective tissue between the cartilage and the muscularis and were often seen between cartilage plates and at the ends of the plates.

Ciliated ducts left the epithelial surface and passed through the lamina propria. The ducts were composed of ciliated columnar epithelial cells with basal round or oval nuclei. The cytoplasm of these cells was opaque and eosinophilic and no mucus-staining material was seen, except for the contents of an occasional goblet cell. The ciliated ducts led into narrow collecting ducts which had an epithelium consisting of two cell types, low cuboidal and tall columnar; the former cell type (Fig. 47) lined the entrance to the gland tubule while the latter was present in the central segment. In both cases the nucleus was round and centrally placed. Nucleoli and chromatin were frequently seen at the nuclear membrane; mucosubstances were not seen within these cells, although mucus was frequently present within the lumen of the ducts.

The bronchial submucosal glands were generally seen as clusters of mucous tubules, in groups of four and five, in the connective tissue overlying the cartilage plates (Fig. 48) as well as at the ends of the plates (Fig. 49).

The glands were randomly distributed around the airways, becoming less frequent as the bronchi decreased in diameter and were absent before the cartilage had disappeared. The tubules were lined by a single layer of eosinophilic cells; histochemical staining revealed the presence of muco-substance, filling up to two-thirds of the cell. Plasma cells were seen in only small numbers around some gland tubules.

The goblet cells of the epithelium were difficult to distinguish by HE staining, but three types of cells were recognised histochemically (Fig.50). Most of the epithelial goblet cells had a goblet, wine-glass shape. The typical cell consisted of a 'foot', resting on the basement membrane, a narrow stalk containing the nucleus, and a distended apical theca filled with mucin droplets, which were often seen to be spilling into the airway lumen. The secreted mucus was found on top of the cilia (Fig. 50). This was the most common goblet cell type in the non-pneumonic epithelium and Figure 51 , stained with HID, illustrates the typical cell shape with the stalk tapering towards the basement membrane. The second type of goblet cell, generally situated at the apex of the bronchial epithelial folds (Fig . 50), was the exhausted cell, which had a slender, narrow theca, due to previous expulsion of mucus. Finally, in the crypts where the epithelium was low, the goblet cells were often cylindrical or barrel-shaped without a stalk. Large numbers of goblet cells were found in this area. The goblet cell type and distribution tended to be similar throughout the airways. In the normal animal the goblet cells did not extend far down the airway peripherally and were not found in the small bronchi without submucosal glands. Goblet cells and submucosal mucous glands were absent in the bronchioles (Fig. 52); AB/PAS staining detected no mucus-containing cells.

The results of histochemical examination of the bronchial epithelial goblet cells and submucosal glands of the four non-pneumonic calves are illustrated in Tables 45 and 46 . Case M134 was a two weeks old calf which differed slightly in its histochemical content from the six months old calves. Neutral mucosubstances and sialomucins were present in approximately 15 and

20 per cent of the goblet cells respectively; the remaining 65 per cent of goblet cells contained sulphomucins. Eighty gland tubules were counted in the transverse section of bronchus; the cells of the tubules contained mostly neutral mucosubstance and in approximately 30 per cent of the cells sulphomucin was detected after acid hydrolysis treatment of the section (Fig. 53). The luminal content of the gland tubules consisted of a mixture of neutral mucosubstance, sialomucins and sulphomucins in approximately equal amounts.

The number of epithelial goblet cells in the older non-pneumonic calves (M64, M67 and M102) was reduced in comparison to the youngest calf, averaging about 47 per SLB, (range 16 to 73). The contents of these cells were almost exclusively sulphomucin in nature, since the cells stained bright blue after acid hydrolysis (Fig. 50). The brown-black, HID-stained sulphomucins in the goblet cells are illustrated in Figure 51 . However, M67 had a few sialomucin-containing goblet cells. The number of gland tubules counted within the wall of the bronchus of these three calves was quite variable (121, 98 and 73 respectively). The quantities of mucosubstances found in the gland cells varied widely, although, in general, equal amounts of neutral mucins, sialomucins and sulphomucins were being produced by these cells. Comparison of the staining of the cells in Figure 54 (AB/PAS) and Figure 55 (neuraminidase digestion, AB/PAS) demonstrates that the sialomucins which were present were mostly neuraminidase-sensitive, since there was a loss of alcianophilia after digestion. Figure 56, stained with HID, illustrates the sulphomucins present in approximately one third of the gland tubules. The variation in mucosubstances found in the gland lumina was considerable; the contents of one animal (M102) consisted of approximately 15 per cent neutral mucosubstance, ten per cent sulphomucin and 75 per cent sialomucin, while M67 had 90 per cent neutral mucosubstance and ten per cent sialomucin.

In addition, although not tabulated, a nine months old non-pneumonic calf was examined and a similar histochemical result to that for the six months old animals was found.

2. Pneumonic animals

Eleven pneumonic animals were examined. Details of the histological characteristics of the pneumonic lesions present in these animals were presented in chapter five.

In all ages, three types of goblet cell were seen in the bronchial epithelium. In the six months old calves the bronchial epithelial layer was heightened, thus elongating the goblet cells and making them thin (Figs. 57, 58 and 59). There was a massive increase in the number of goblet cells in the epithelium of the large bronchi, forming an almost continuous layer of goblet cells. Exhausted goblet cells appeared greater in number than in the non-pneumonic calves. Similarly, in the three months old pneumonic calves the bronchial epithelium was hyperplastic resulting in only the theca of the goblet cells being clearly visible (Fig. 60). A large number of wine-glass shaped cells were present and were actively secreting mucus into the airway lumen. A common feature of these pneumonic cases was extension of goblet cells down the airways to the small bronchioles (Fig.61), although in many bronchioles goblet cells were absent where the epithelium had dedifferentiated or been displaced by lymphocytes.

The distribution of submucosal glands in the large bronchi of the pneumonic calves appeared to be similar to that in the normal and the mucous tubules were present in clusters mostly at the ends of the cartilage plates, and sometimes below the cartilage. The collecting ducts were dilated and mucus was often seen in the lumina. Large numbers of plasma cells were generally found around the ducts. The majority of the mucous tubules were dilated (Fig. 62), particularly in the six months old animals; the glands were slightly hypertrophied and surrounded by plasma cells in significant numbers. A few tubules were lined by columnar cells and appeared active in mucus secretion, unlike the dilated tubules which were lined by cuboidal cells with very little obvious cytoplasm (Fig. 62). Glands were not always seen in the smaller bronchi, possibly due to displacement by lymphocytic accumulations.

In turning to the histochemical examination, the number of goblet cells per SLB, the percentage of goblet cells staining blue or red, the number

of gland tubules around a bronchus and the percentage of gland tubule cells and luminal contents staining blue and red with three staining techniques are set out in Tables 47, 48, 49 and 50. To illustrate the changes found in these diseased calves, the findings in the three six months old non-pneumonic calves are compared with the pneumonic ones.

The six six months old calves examined all had a cuffing pneumonia, as previously described, and three of these animals (M60, M62 and M63) were culturally positive for M. dispar. The goblet cell number in these three calves was considerably greater than in their three non-pneumonic analogues; an average of 100 goblet cells per SLB was observed. Sulphomucin was still the predominant mucosubstance found in the goblet cells (Fig. 57), although comparison of Figure 58 (AB/PAS) with Figure 59 reveals a loss in alcianophilia due to the removal of sialic acid residues by the acid hydrolysis treatment, and indicates the presence of a significant amount of sialomucins in the goblet cells. Neuraminidase digestion indicated that sensitive and resistant sialomucins were present and were contained in 15 to 20 per cent of the cells. No goblet cells were found to contain neutral mucosubstances.

The remaining six months old pneumonic calves (M72, M74 and M79), which were negative by culture for M. dispar, also had an increase in the number of goblet cells compared to the normal six months old animals, but the increase was not as dramatic as that seen in the M. dispar-infected calves. The change in the mucosubstances produced by the goblet cells was similar to that in the M. dispar-infected calves, although, as with the goblet cell numbers, the change was not so intense and there was a less marked increase in the sialomucin production.

In comparison to the three non-pneumonic cases, the M. dispar-infected six months old calves appeared to have an increased number of gland tubules in the bronchial wall (Fig. 62). Neutral mucosubstances, sialomucins and sulphomucins were all demonstrated within the cells of these glands; sialomucins, neuraminidase-sensitive and resistant, were both detected. The mucosubstance contents of the gland tubule cells in two of the three cases (M60 and M62) were present in similar proportions, i.e. approximately ten to 15 per cent neutral

mucosubstance, 30 to 35 per cent sialomucins and 45 to 50 per cent sulphomucins. M63, on the other hand, had increased amounts of neutral mucosubstances and sulphomucins and less sialomucins. The luminal contents varied, but, in general, approximately equal proportions of all mucosubstances could be seen, although no neuraminidase-resistant sialomucins were detected.

The remaining three pneumonic six months old calves (M72, M74 and M79) had similar numbers of gland tubules in the wall of the bronchus as the non-pneumonic cases. The gland cell content had only altered slightly from the normal state. The mucosubstance content was uniform amongst the individual animals. There was an increase in the amount of neutral mucosubstances produced by these cells, which amounted to, in these calves, approximately 50 per cent (Fig.62); the quantity of sulphomucin was similar to the normal state but a reduction in the sialomucin content was noted in all cases.

Three of the three months old animals, M183, M185 and M191, had type D(ii) pneumonic lesions and were culturally positive for M. dispar. As noted with the older group of pneumonic calves the number of goblet cells per SLB varied between individual cases, and ranged from 51 to 130 in these animals. Despite this variation the average was approximately 100 goblet cells per SLB and indicated an increase in number from the normal. In two of the calves (M183 and M191) approximately 15 to 25 per cent of the goblet cells contained neutral mucosubstances, the remainder of the cells containing sulphomucins (Fig.60). M185, on the other hand, appeared to have fewer cells containing these two mucosubstances which were replaced by neuraminidase-resistant sialomucins in approximately 15 per cent of the goblet cells. The number of gland tubules in these three months old calves was extremely variable; as few as 37 were counted in M191. Despite this, the mucosubstance content of the cells in the gland tubules was similar in all three calves, and consisted of approximately 60 to 70 per cent neutral mucosubstances and 20 to 30 per cent acid mucins (Fig.63). The acid mucosubstances were predominantly sulphomucins as detected by neuraminidase and acid hydrolysis treatment of serial sections. The contents of the gland lumina were

extremely variable and all four types of mucosubstances were present.

A three months old calf, M188, that was culturally negative for M. dispar was also examined histochemically. In addition, this animal differed from the other three months old calves in the histological appearance of its lungs. A fairly severe pneumonia was evident; bronchitis and bronchiolitis were present with plasma cells in large numbers in the lamina propria and occasionally infiltrating the epithelial layer. An accompanying alveolitis consisted of neutrophils and a few plasma cells which packed the alveolar spaces and plugged many of the small airway lumina. The epithelium of the larger bronchi was hyperplastic and dedifferentiated in many areas (Fig. 64); this resulted in a decrease in goblet cell numbers (approximately 22 per SLB). The smaller bronchi and large bronchioles, despite some epithelial hyperplasia, consisted of normal epithelial cells with large numbers of goblet cells containing sulphomucins exclusively (Fig. 65).

There was a drop in gland tubule numbers in the bronchial wall, due, perhaps, to obliteration by infiltrating plasma cells. Neuraminidase-sensitive sialomucins were present in approximately 40 per cent of the gland tubule cells, the remainder containing sulphomucins with only an occasional cell staining for neutral mucosubstances. The luminal contents of the glands of M188 reflected the relatively large amounts of neuraminidase-sensitive sialomucins produced.

One two months old calf was examined. Similar pulmonary lesions to the three months old M. dispar-infected calves were present histologically and M. dispar was isolated. An estimate of 90 goblet cells per SLB, containing only sulphomucins, was made. A large number of gland tubules was present in the bronchial wall, the cells containing mostly neutral mucosubstances (approximately 60 per cent) with some sulphomucins (Fig. 66). Approximately equal amounts of neutral mucosubstances, sialomucins and sulphomucins were detected in the lumina of the gland tubules.

D. DISCUSSION

Quantitative and qualitative assessments of biological systems generally include problems of standardisation and some of these difficulties were recognised during the present studies. Individual animals could differ considerably within one group, despite being typical members of the group in some of their other characteristics.

Studies on the changes of the respiratory mucosubstances of dogs with chronic bronchitis were carried out by Wheeldon (1974). Statistical analyses of his results revealed that examination of one site in the respiratory tract was adequate for histological and histochemical statements that would be representative of the case in question. Consequently, this examination was carried out on one section of bronchus from approximately the same position in the right cranial lobe of each animal. This site was chosen since the lesions encountered were found in the anterior lobes of the lungs. A single specimen from each animal was commendable in this study as the work time involved was reduced, thus enabling comparisons with several animals to be made.

The techniques employed in the quantitative estimates in these studies were not ideal, but a review of the current literature did not provide a suitable alternative. A measure of the number of goblet cells present in a standard length of bronchial epithelium was useful and reasonably accurate after 16 fields had been examined in each case.

In the evaluation of the mucosubstance content of the submucosal glands, each gland tubule was counted and assessed, as one entity, as either predominantly blue or red. Although some tubules contained mixed secretions, the majority were seen to be either one or other of the colours. Occasionally, a tubule with approximately equal proportions of colours was encountered and a point for each colour was recorded. Most other workers in the field of histochemical quantitation have employed the point-count technique to assess the contents of individual cells and have categorised the stained mucosubstances into four colours, blue, blue-red, red-blue and red. Then

subsequent to the point-counting of the individual cells, the colours were grouped as blue or red in the final estimation of the mucosubstances present. It was difficult to decide which method merited more favour; superficially, the point-count appeared more accurate since each cell was examined individually, but the final staining reactions of the cells were presented as either blue or red. In addition, only a few glands were examined and these were not randomly chosen but selected such that the gland acinus/tubule filled the microscopic field (Jones et al., 1975). Thus the method devised and employed for the current examination was probably as accurate as any other method reviewed since all the gland tubules in a complete transverse section of a bronchus were examined.

The histochemical composition of the normal bovine bronchial secretions appears to vary with the age of the animal. The very young calf examined in this study (M134) had quite a large number of goblet cells present in the bronchial epithelium. Approximately 30 per cent of the goblet cells contained neutral mucosubstances and sialomucins, the remainder sulphomucins. In the six months old calves, two of the three non-pneumonic animals studied had sulphomucin-containing goblet cells only, while one calf (M67) had a small number of sialomucin-containing cells. These six months old non-pneumonic animals had been housed along with the pneumonic calves and may have experienced a respiratory disease at an earlier age. The presence of a small amount of sialomucin-containing goblet cells may reflect a state of recovery in this apparently healthy calf. The contents of the glands also differed with age.

The development of the secretory components of the human bronchial tree from their pre-natal conception to maturation post-natally has been followed (de Haller, 1969; Lamb, 1969) and an alteration in the histochemical appearance of the mucosubstance was observed during this period. Initially the goblet cells produced sulphomucin, but as the subject matured this decreased and the sulphomucin was gradually replaced by sialomucin and a little neutral mucosubstance. Similarly, as the glands matured the predominant sulphomucin was replaced by sialomucin and neutral mucosubstance,

this change occurring to a greater degree than that in the goblet cells.

Although the types of mucosubstance present in the bovine species differ from those in man, the present observations suggest that the secretory apparatus of the calf alters with age. However, it would appear that sulphomucin-containing goblet cells accompanied by mucous glands producing sulphomucins, sialomucins and neutral mucosubstances can be considered as the normal composition of bovine respiratory secretions.

In the pneumonic calves, particularly the six months old calves, with cuffing pneumonia, there was a significant increase in the number of goblet cells which extended even into the bronchioles, where they are not normally found. Sulphomucin was still the predominant mucosubstance produced by the goblet cells of these pneumonic cases, but unlike the non-pneumonic animals, some sialomucins were also produced. Neutral mucosubstances, sialomucins and sulphomucins were produced by the bronchial glands of the non-pneumonic and pneumonic animals; but, in general, there was an increase in sialomucin and sulphomucin production by the glands of the pneumonic calves, compared to the non-pneumonic state. This was particularly evident in the gland secretions of calves whose lungs were positive on culture for M. dispar. In contrast, in the pneumonic calves from which M. dispar was not recovered, mucins of the submucosal glands were more similar to those of the normal, non-pneumonic animals, with more neutral mucosubstances and sulphomucins being secreted at the expense of sialomucin production. These cases may represent animals recovering from the pneumonia, since M. dispar, the possible aetiological agent, could not be isolated from the lung material and the mucosubstances produced by the respiratory secretory apparatus were similar to those of the non-pneumonic calves.

In all these pneumonic cases the goblet cells increased in number in the epithelium of the large bronchi and extended peripherally down the airways to the small bronchi and bronchioles. Goblet cells are not considered to be under nervous control but proliferate and increase their rate of secretion following irritation of the bronchial epithelium (Thurlbeck et al., 1961; Jones,

tract was described in Australia from studies carried out on 52 conventionally reared calves (St. George et al., 1973). Seven to 16 weeks old calves were the most commonly affected; M. dispar was isolated from eight of 16 calves with macroscopic lung lesions in this age group and all these 16 had microscopic lesions of bronchiolitis and/or peribronchiolar lymphoid hyperplasia. The organism was also isolated from three of the 18 one to six weeks old calves, 11 of which were pathologically pneumonic, but no recoveries were made from the 16 animals older than 16 weeks due to loss in viability of the tracheal ring organ cultures used for mycoplasma isolations.

In Canada the isolation of this organism from the pneumonic lung of a calf was reported, although no details of the pathology were described (Ose and Meunster, 1975).

Experimental infections with this organism were briefly reported by Gourlay and Thomas (1969), who stated that macroscopic lung lesions were produced in seven of eight three weeks old conventionally reared calves inoculated endobronchially with a broth culture of M. dispar; no macroscopic lung lesions were produced in three control calves inoculated with sterile broth.

In Australia, St. George et al. (1973) inoculated three caesarian-derived and two colostrum-deprived calves (one to two days old) intratracheally with a broth culture of M. dispar or the supernate of a lung homogenate prepared from a calf naturally infected with this organism. Clinical signs of pneumonia, the result of a "proliferative interstitial pneumonia", were observed three to eight days after infection. However, M. dispar was reisolated from only two of the five experimentally infected calves and the control calves were found to have microscopic lung lesions, although they were apparently healthy clinically.

Thomas and Howard (1974) studied the growth of several mycoplasmas in foetal bovine tracheal explant cultures. Mycoplasma dispar grew to 10^6 CCU per ml in this tissue culture and caused progressive sloughing of the ciliated epithelial cells with inhibition of ciliary activity and patchy flattening of the epithelial layer during the first six days. The maximum titre of

Bolduc and Reid, 1973). Extension of goblet cells into the small bronchi and bronchioles is another feature of epithelial irritation (Reid, 1958; de Haller and Reid, 1965), and the massive goblet cell proliferation seen in the calves suggests the presence of a severe irritant in cuffing pneumonia.

The mucous gland tubules of many pneumonic cases were dilated and surrounded by large numbers of plasma cells. The numbers of tubules within the clusters had increased with their dilatation particularly marked in the older calves; the lumina of many of these tubules were empty. In addition, a proportion of the cells of these tubules were apparently lacking in mucosubstance since only occasional granules stained. This appearance, in comparison with the younger pneumonic calves and the non-pneumonic animals, suggested that with infection the glands became hypersecretory and the gland tubules and collecting ducts dilated due to the presence of excess mucus. In the later stages of the disease, the cells of the tubules lost their histochemical staining, either because they were exhausted and no longer able to produce mucosubstances or perhaps because they were replaced by a different or immature cell type. The large numbers of plasma cells surrounding the tubules may play an important role in the production of secretory IgA, which reaches the epithelial surface via the respiratory tract secretions.

The changes in the composition of the mucus in the pneumonic calves studied were not considered specific for cuffing pneumonia and M. dispar infection.

The continuously moving mucus layer of the bronchial tree prevents irritating or infective agents from being in close and prolonged contact with the epithelium. Phagocytosis by macrophages may also play a role in the host's defence mechanism, particularly in the peripheral part of the lung. The increased sialomucin content of the mucus in the pneumonic cases may act in the host's defence mechanism by limiting the attachment of the infective microorganisms to epithelial cells. Many organisms have been shown to attach to epithelial cells via a sialic acid residue, e.g. M. gallisepticum and M. pneumoniae (Manchee and Taylor-Robinson, 1969b), although the receptor

site for M. dispar on erythrocytes of several animal species does not appear to contain sialic acid (Howard et al., 1974). However, the increased sialomucin production will probably increase the viscosity of the respiratory mucus in pneumonic cases, since it has been shown by Gibbons (1959) that increased amounts of sialic acid residues in cervical mucus can increase viscosity. This increased viscosity together with an increased quantity of mucus will protect the epithelium from infective agents, but may slow the movement of the mucus blanket and thus inhibit the clearance mechanism of the respiratory tract and reduce the ability of the phagocytic cells to reach the infective agents.

Age Group	Number Calves Examined	Non-pneumonic Calves		Pneumonic Calves				
		No.	Case No.	No.	M. dispar +ve	Case No.	M. dispar -ve	Case No.
Six months	9	3	M 64 M 67 M102	6	3	M 60 M 62 M 63	3	M 72 M 74 M 79
Three months	4	-	-	4	3	M183 M185 M191	1	M188
Two months	1	-	-	1	1	M 95	-	-
Less than one month	1	1	M134	-	-	-	-	-

Table 44. Number and age of calves examined for the histochemical nature of their bronchial mucosubstances.

Non-pneumonic and pneumonic animals were studied; their infectivity with M. dispar is also indicated.

Case No.	Total Number Goblet Cells in Four Standard Fields				% Goblet Cells Staining Blue (B) or Red(R) in Section 1		Section 2		Section 3	
	Section 1	Section 2	Section 3	Mean No.	B	R	B	R	B	R
M 134	161	111	116	130	77	23	72	28	65	35
M 64	19	17	12	16	100	0	100	0	100	0
M 67	52	52	48	51	100	0	87	13	78	22
M 102	77	72	72	73	100	0	100	0	100	0

Table 45 . The total number of epithelial goblet cells in four standard fields in three serial sections of bronchus were recorded for four non-pneumonic calves. The three serial sections were treated and stained as follows: section 1. AB/PAS, section 2. neuraminidase AB/PAS and section 3. acid hydrolysis AB/PAS. The percentage of cells staining blue or red in each was noted.

Case No.	Total Number Gland Tubules	% Tubules Staining Blue (B) or Red (R)						% Gland Luminal Content Staining Blue (B) or Red(R)					
		Section 1		Section 2		Section 3		Section 1		Section 2		Section 3	
		B	R	B	R	B	R	B	R	B	R	B	R
M 134	80	25	75	30	70	30	70	58	42	70	30	30	70
M 64	121	75	25	42	58	45	55	82	18	40	60	40	60
M 67	98	47	53	20	80	20	80	10	90	0	100	0	100
M 102	73	80	20	22	78	20	80	85	15	10	90	10	90

Table 46 . The total number of gland tubules counted in the submucosa of a section of bronchus from four non-pneumonic calves together with the percentage of gland tubules and luminal contents staining blue or red in three serial sections: section 1. AB/PAS, section 2. neuraminidase AB/PAS, and section 3. acid hydrolysis AB/PAS.

Case No.	Total Number of Goblet Cells in Four Standard Fields				% Goblet Cells Staining Blue (B) or Red (R) in					
					Section 1		Section 2		Section 3	
	Section 1	Section 2	Section 3	Mean No.	B	R	B	R	B	R
M 64	19	17	12	16	100	0	100	0	100	0
M 67	52	52	48	51	100	0	87	13	78	22
M102	77	72	72	73	100	0	100	0	100	0
M 60	102	111	114	109	100	0	84	16	82	18
M 62	92	112	105	103	100	0	98	2	84	16
M 63	94	87	85	87	100	0	72	28	85	15
M 72	90	104	106	100	100	0	96	4	89	11
M 74	80	76	71	76	100	0	100	0	92	8
M 79	76	79	66	74	100	0	82	18	79	21

Table47. The total number of epithelial goblet cells in four standard fields in three serial sections of bronchus were recorded for nine six months old calves. The three sections were treated and stained as follows: section 1. AB/PAS, section 2. neuraminidase AB/PAS and section 3. acid hydrolysis AB/PAS, and the percentage of cells which stained blue or red in each section was noted. Calves M64, M67 and M102 were non-pneumonic while M60, M62 and M63 had a pneumonia from which M. dispar was isolated. M72, M74 and M79 had a similar pneumonia but M. dispar was not isolated.

Case No.	Total Number Gland Tubules	% Tubules Staining Blue (B) or Red (R).						% Gland Luminal Content Staining Blue (B) or Red (R)					
		Section 1		Section 2		Section 3		Section 1		Section 2		Section 3	
		B	R	B	R	B	R	B	R	B	R	B	R
M 64	121	75	25	42	58	45	55	82	18	40	60	40	60
M 67	98	47	53	20	80	20	80	10	90	0	100	0	100
M 102	73	80	20	22	78	20	80	85	15	10	90	10	90
M 60	125	90	10	75	25	50	50	60	40	27	73	30	70
M 62	104	85	15	60	40	44	56	70	30	20	80	20	80
M 63	191	61	39	46	54	57	43	43	57	12	88	47	53
M 72	92	44	56	42	58	40	60	38	62	20	80	24	76
M 74	90	45	55	34	66	30	70	10	90	15	85	10	90
M 79	75	54	46	22	78	30	70	80	20	50	50	50	50

Table 48. The total number of gland tubules seen in a section of bronchus from nine six months old calves together with the percentage of gland tubules and luminal contents staining blue or red in three serial sections: section 1. AB/PAS, section 2. neuraminidase AB/PAS, and section 3. acid hydrolysis AB/PAS were noted. Calves M64, M67 and M102 were non-pneumonic while M60, M62 and M63 had pneumonia from which M. dispar was isolated. M72, M74 and M79 had a similar pneumonia but M. dispar was not isolated.

Case No.	Total Number of Goblet Cells in Four Standard Fields				% Goblet Cells Staining Blue (B) or Red (R) in					
					Section 1		Section 2		Section 3	
	Section 1	Section 2	Section 3	Mean No.	B	R	B	R	B	R
MI83	142	118	130	130	85	15	87	13	82	18
MI85	44	66	42	51	93	7	94	6	70	30
MI91	133	122	101	119	77	23	75	25	78	22
MI88	25	23	18	22	100	0	100	0	100	0
M 95	93	95	82	90	100	0	100	0	100	0

Table 49. The total number of epithelial goblet cells in four standard fields in three serial sections of bronchus from five pneumonic calves. The three sections were treated and stained as follows: section 1. AB/PAS, section 2. neuraminidase AB/PAS and section 3. acid hydrolysis AB/PAS. The percentage of cells staining blue or red in each section was noted. M95 was two months old, while the remaining four animals were three months of age. M. dispar was cultured from all animals except MI88.

Case No.	Total Number Gland Tubules	% Tubules Staining Blue (B) or Red (R)						% Gland Luminal Content Staining Blue (B) or Red (R)					
		Section 1		Section 2		Section 3		Section 1		Section 2		Section 3	
		B	R	B	R	B	R	B	R	B	R	B	R
M183	175	39	61	32	68	24	76	38	62	40	60	20	80
M185	81	35	65	30	70	33	66	63	37	35	65	10	90
M191	37	23	77	20	80	20	80	66	33	66	33	66	33
M188	27	84	16	45	55	45	55	50	50	16	84	16	84
M 95	141	36	64	50	50	40	60	72	28	40	60	27	63

Table 30. The total number of gland tubules seen in a section of bronchus from five pneumonic calves and the percentage of gland tubules and luminal contents staining blue or red in three serial sections: section 1. AB/PAS, section 2. neuraminidase AB/PAS, and section 3. acid hydrolysis AB/PAS. M95 was two months old and the remaining four animals were three months of age. All animals except M188 were positive culturally for M. dispar.

Fig. 47 : This figure illustrates the submucosal gland of a non-pneumonic calf. The gland is embedded in the submucosa of the bronchial wall, situated near a cartilage plate. A duct can be seen leading into the gland tubule. No staining mucosubstances are found in the collecting duct which is composed of low cuboidal epithelial cells. The cells of the mucous tubule contain mucosubstance occupying up to two-thirds of the cell cytoplasm. A little mucus is present in the lumen of the gland tubule. Note the absence of plasma cells in the connective tissue surrounding the tubule. PAS staining, x 250.

Fig. 48 : Clusters of mucous tubules are embedded in the connective tissue of the bronchial submucosa of the non-pneumonic animal. The tubules are in groups of four and five and consist of mucus-containing cells. AB/PAS staining, x 120.

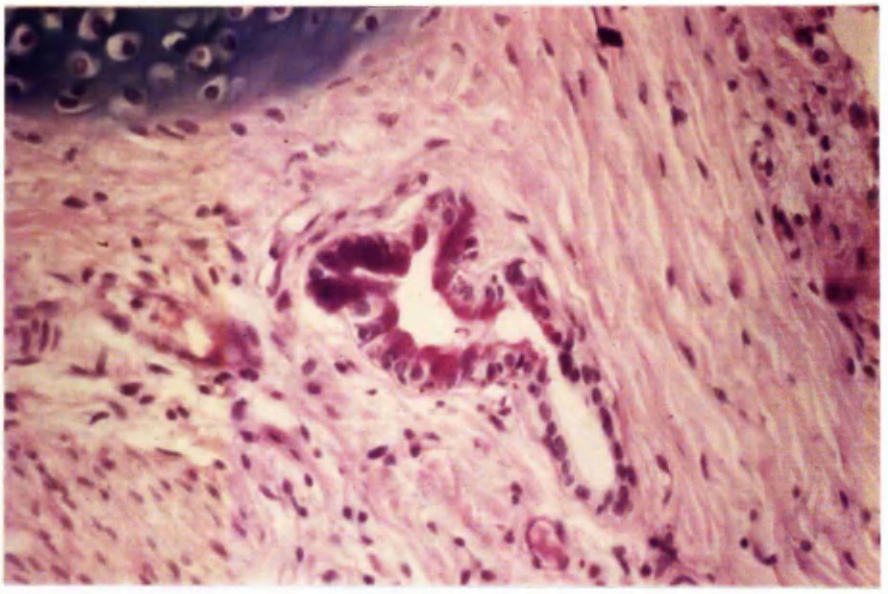
Fig. 49 : The size of mucous glands and their distribution in the bronchial wall of a non-pneumonic calf are illustrated in this figure. The glands consist of small numbers of mucus-secreting tubules, situated at the edge or overlying the cartilage plates. AB/PAS staining, x 35.

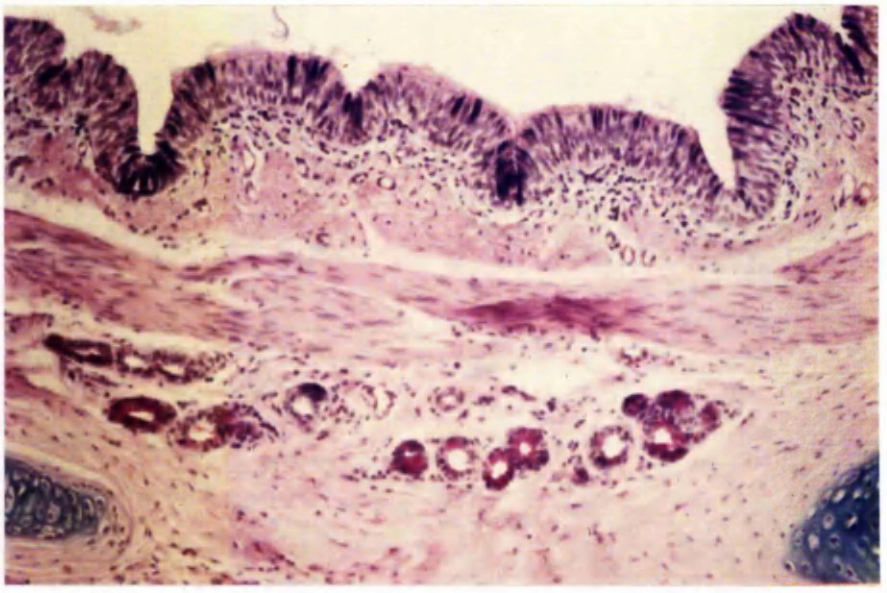
organisms was required to produce this effect, which did not develop if the mycoplasmas were washed, heat-inactivated or filtered through a Millipore membrane. Electron microscopical examination of these cultures demonstrated large mycoplasma cells up to 1 μm in diameter in close association with the cilia. In a subsequent study, the inhibition of ciliary activity by M. dispar was found to be dependent on the presence of serum in the maintenance medium (Howard and Thomas, 1974). The inhibitory activity was unaffected by catalase and no toxic substance was demonstrated in the organ culture supernatant fluid.

2. Ureaplasma spp.

Ureaplasma spp., initially known as T-strain mycoplasmas, were first isolated by Shepard in 1954 from the human urogenital tract. Since then they have been isolated from animals and were discovered in the bovine respiratory tract by Gourlay (1968). He used the liquid medium employed by Taylor-Robinson et al. (1967), in which growth was indicated by a rise in pH due to the breakdown of urea. Very small (tiny) colonies were produced after subculture onto solid medium. Gourlay (1968) isolated T-strains from nine of the 16 calf lungs examined. Seven of the isolates were from calves between six and 12 months of age while the remaining two were from young calves, 14 to 30 days old. Macroscopic lesions of pneumonia, generally involving more than one lobe, were found in all lungs, but no histological description of the lesions was given. Ureaplasma spp. were subsequently isolated from 25 of 45 clinically healthy three months old calves and from 13 of 20 calves, of varying ages, which had been collected from an abattoir after they had died or been killed in extremis (Gourlay et al., 1970). Macroscopic lesions of pneumonia were seen in all of the 45 clinically healthy calves at necropsy and microscopically most of these lesions consisted of bronchiolitis with peribronchiolar lymphoid hyperplasia. Approximately half of the pulmonary disorders in this group were classified as cuffing pneumonia.

Ureaplasma spp. were not believed to be present in healthy bovine lungs (Thomas and Smith, 1972). They considered this to be the case after an examination of the respiratory tracts, in three sites in three age groups, of





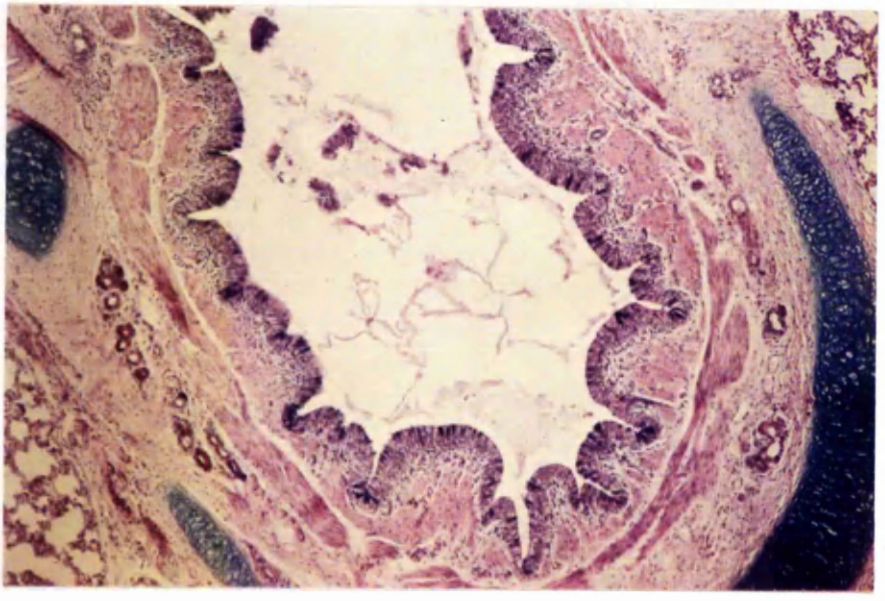
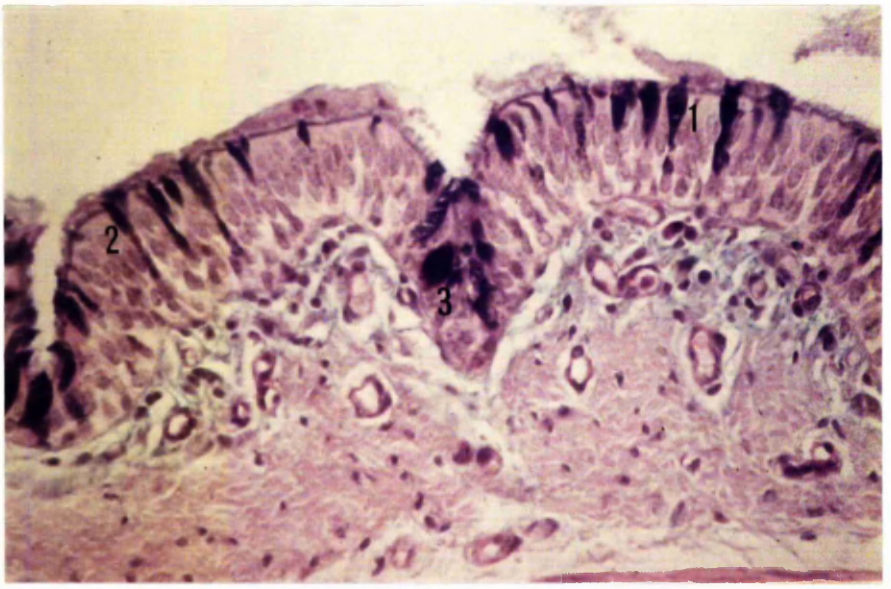


Fig. 50 : In the non-pneumonic calf goblet cells are scattered along the length of the bronchial epithelium. Three morphological types of goblet cells are described : 1. flask-shaped with a narrow stalk from the basement membrane widening into a mucus-filled theca which discharges mucins onto the surface of the epithelium, 2. the exhausted cell with a narrow theca due to recent discharge of mucosubstance and 3. barrel-shaped cells situated in the folds of the bronchial epithelium. This specimen was from a six months old calf and the section of tissue was acid hydrolyzed prior to AB/PAS staining. The goblet cells stain blue due to the presence of sulpho-mucin. Acid hydrolysis, AB/PAS staining, x 400.

Fig. 51 : The goblet cells in the bronchial epithelium of a six months old non-pneumonic calf illustrating the flask-shaped appearance of the cells which are stained brown-purple due to the presence of sulphomucins. HID staining, x 400.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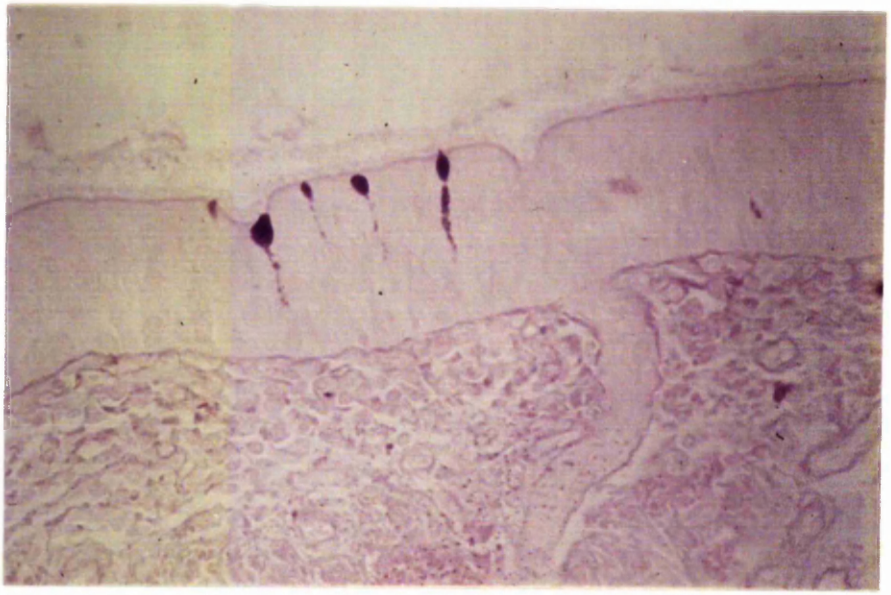
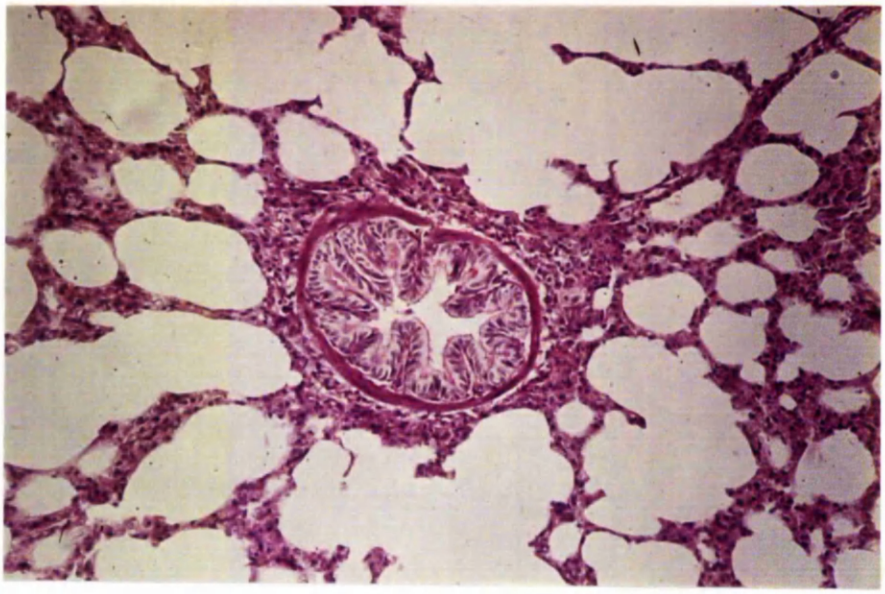


Fig. 52 : The bronchiole of a non-pneumonic six months old calf. Goblet cells and glands are absent from the epithelium and submucosa respectively, indicated by the absence of stained mucosubstance. AB/PAS staining, x 120.

Fig. 53 : The mucous glands are embedded in the submucosa of the bronchial wall in the young calf. The cells of the gland tubules produce neutral and acid mucosubstances; the latter consists of some sulphomucins present as the blue staining in this section which has been acid hydrolyzed prior to AB/PAS staining. x 400.



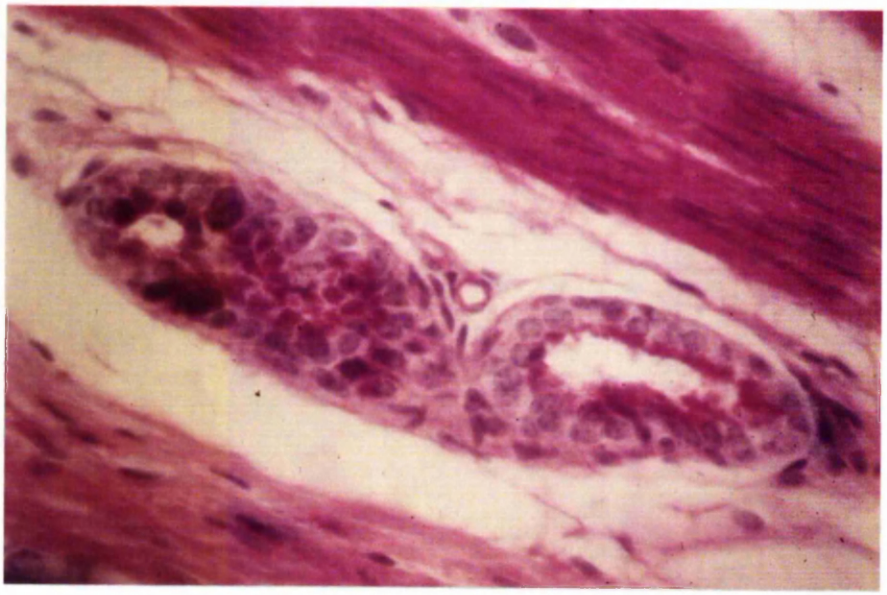
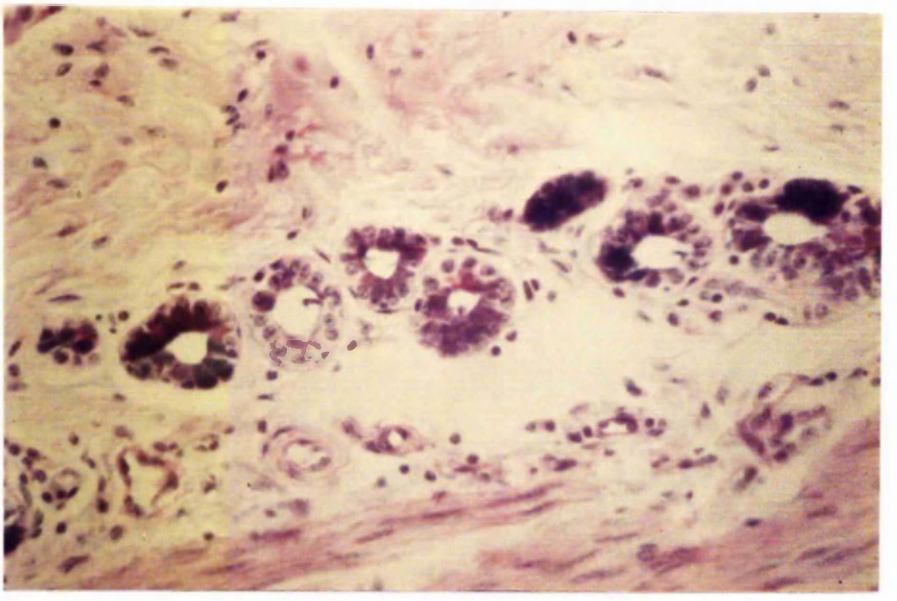
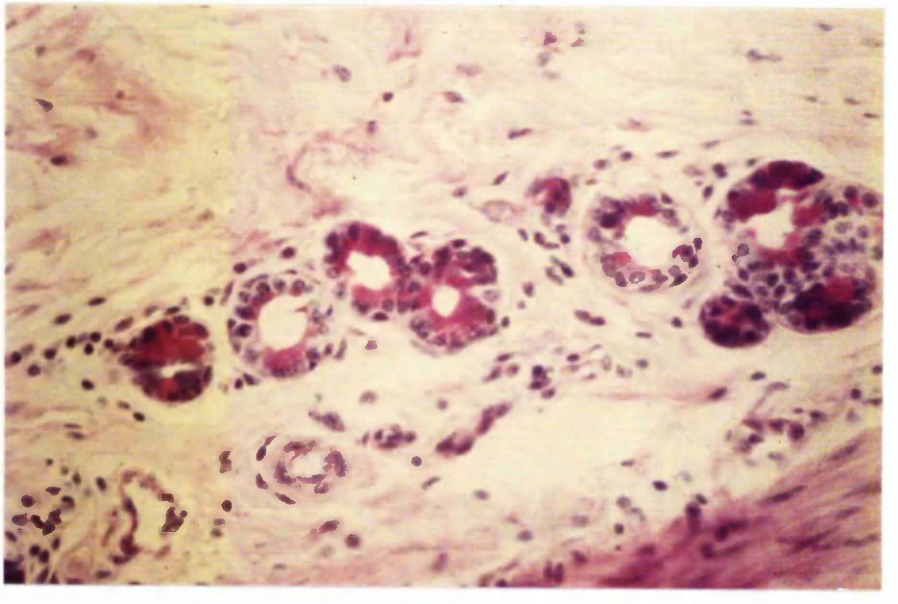


Fig. 54 : This figure illustrates neutral mucins staining red in the cells of the mucous gland tubules of a six months old non-pneumonic calf . AB/PAS staining, x 250.

Fig. 55 : This is a serial section of the sample of bronchus illustrated in Fig. 54. This section was treated with neuraminidase prior to AB/PAS staining and a decrease in alcianophilia is detected when compared with Fig. 54. This loss in blue staining is due to the removal, by neuraminidase, of sialic acid residues. Neuraminidase digestion, AB/PAS staining, x 250.

Fig. 56 : The distribution of sulphomucins in the bronchial epithelium and submucosal glands in a six months old non-pneumonic calf. Most goblet cells in the bronchial epithelium appear to stain brown-purple with HID while only a few gland tubules are stained. HID staining, x 120.





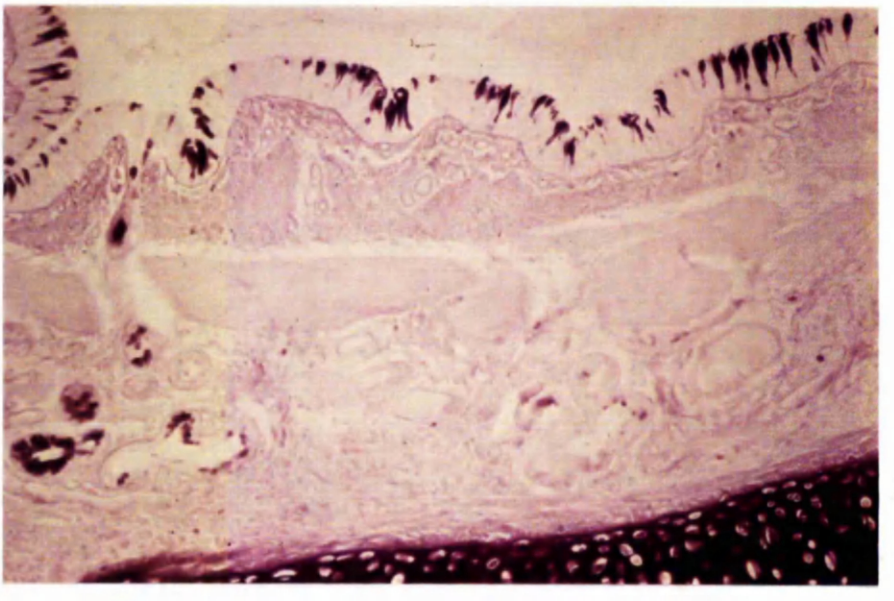
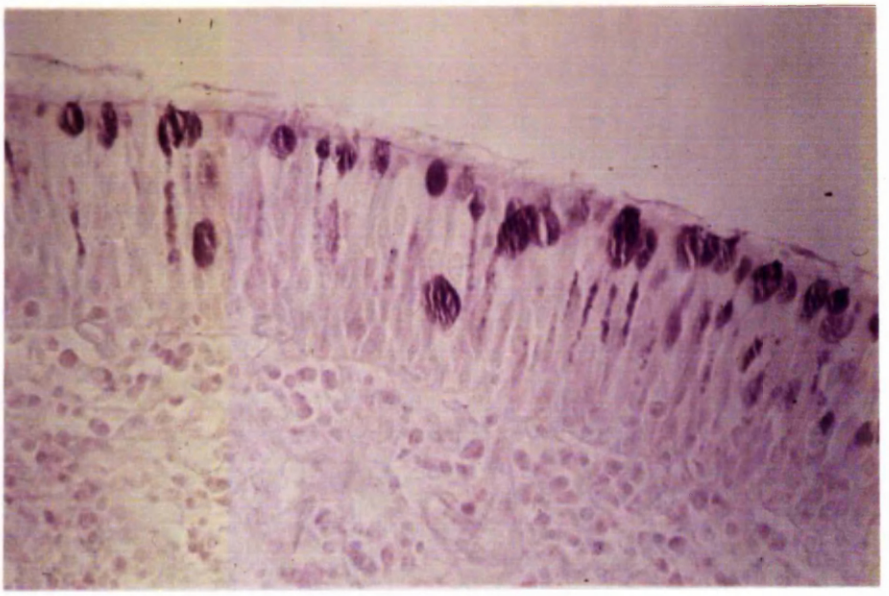
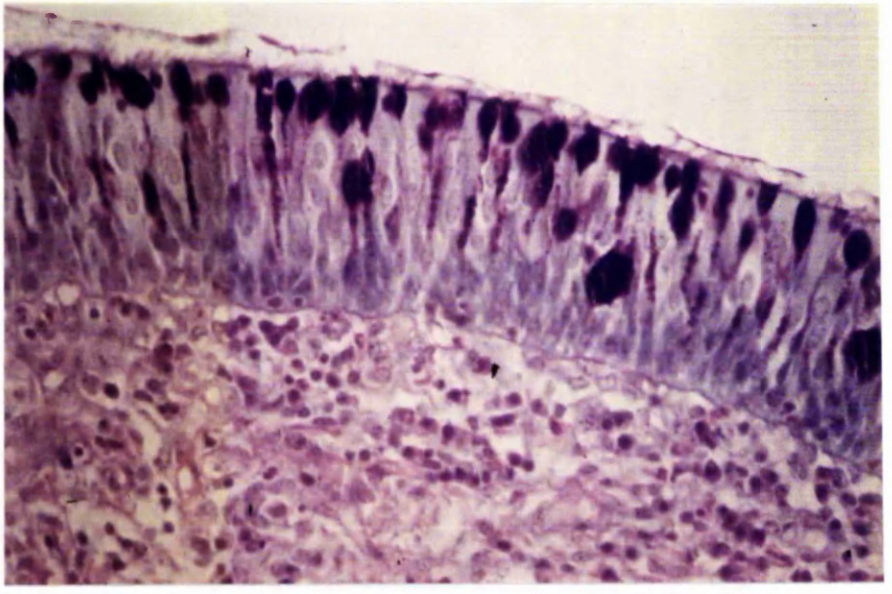


Fig. 57 : The heightened bronchial epithelium of a pneumonic six months old calf has goblet cells tightly packed along the surface. The goblet cells stain brown-purple due to the presence of sulphomucins. HID staining, x 400.

Fig. 58 : The goblet cells in the bronchial epithelium of six months old pneumonic calves are abundant along the surface; they are narrower than in the non-pneumonic calf (compare with Figs. 50 and 51). This section was stained with AB/PAS and has stained all the goblet cells blue. AB/PAS staining, x 400.

Fig. 59 : This is a serial section of the bronchial specimen of Fig. 58, hydrolyzed prior to AB/PAS staining. Comparison with Fig. 58 indicates a loss in alcianophilia due to loss of sialic acid residues. Acid hydrolysis, AB/PAS staining, x 400.





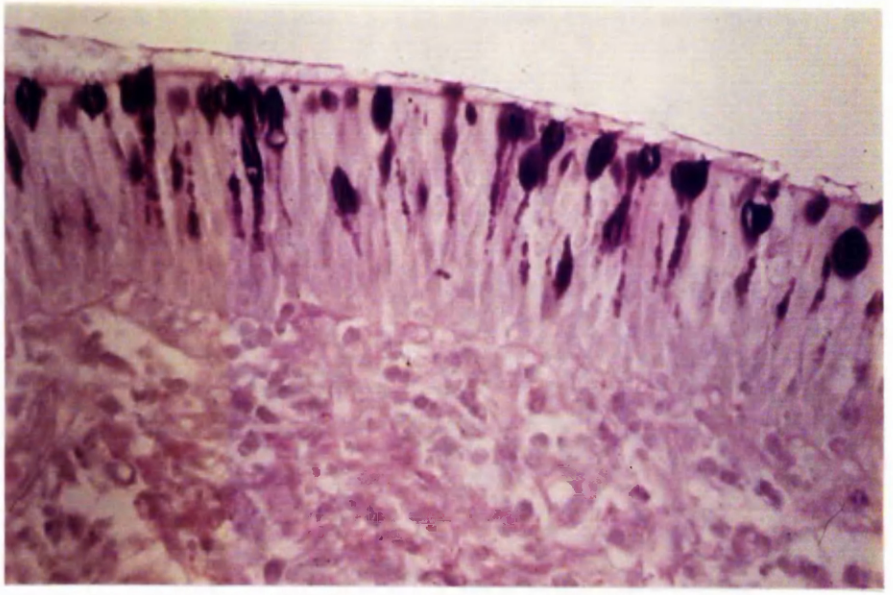
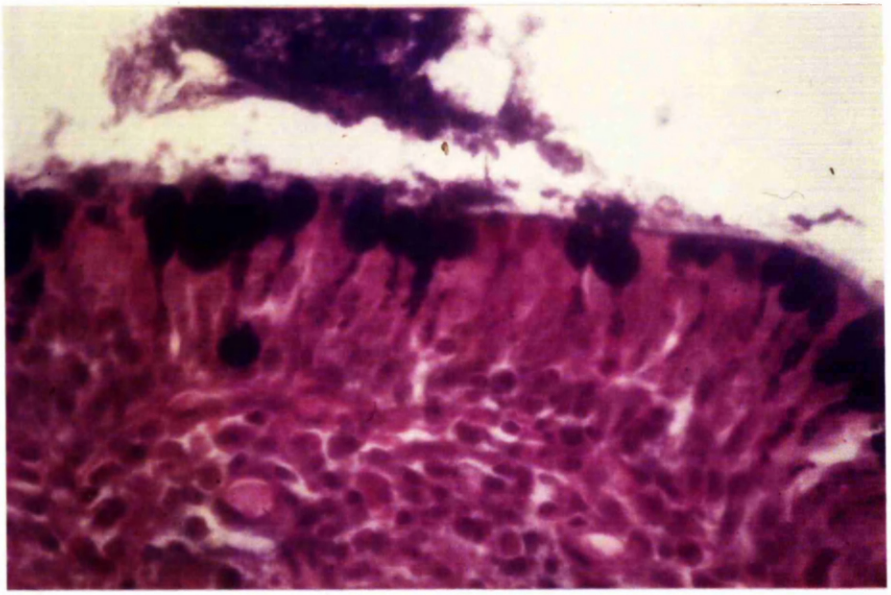


Fig. 60 : This illustrates the bronchial epithelium of a pneumonic three months old calf. The epithelium is heightened and an increase in the number of goblet cells compared to the non-pneumonic case (Fig. 50) is detected. Most goblet cells are flask-shaped secreting mucus onto the luminal surface. Goblet cells containing neutral, red mucins are present amongst the larger blue-staining acid mucosubstance-containing cells. AB/PAS staining, x 400.

Fig. 61 : In the pneumonic six months old calf the goblet cells extend peripherally to many bronchioles. The goblet cells are packed tightly in the epithelium and stain blue due to the reaction of acid mucosubstances with alcian blue. AB/PAS, x 120.

70 non-pneumonic calves. However, lower respiratory tract swabs of 21 three to four weeks old colostrum-deprived or colostrum-fed calves revealed the presence of ureaplasmas in three of these animals (Gourlay and Thomas, 1970). Reports from other countries have included ureaplasma isolations from the pneumonic lung of an aborted foetus in Italy (Romano et al., 1971), from five of seven pneumonic lung specimens in Texas (Livingston, 1972) and one of eight pneumonic lungs from two months old calves in Canada, with the positive isolation coming from lung tissue in which there was bronchitis, alveolar collapse and a marked fibrino-cellular-exudate in the alveoli (Ruhnke and van Dreumel, 1972). More recently, Shimizu et al. (1975) isolated ureaplasmas from 15 of 22 pneumonic calf lungs, although in nine of the cases the recovery site was the trachea.

Bovine Ureaplasma spp., isolated from pneumonic calf lungs, were inoculated endobronchially into ten colostrum-deprived and six colostrum-fed calves and resulted in the production of macroscopic pneumonia in 14 animals, while only a mild pneumonia developed in two of the nine control calves inoculated with sterile medium (Gourlay and Thomas, 1969 and 1970). Some of the test calves harboured ureaplasmas and other mycoplasmas in their respiratory tracts before inoculation so the exact role that the inoculated Ureaplasma spp. played in the aetiology of the pneumonia found at necropsy was not clear. Serum samples obtained before inoculation and at slaughter were examined for antibodies to the Ureaplasma spp. by the metabolic inhibition test and a four-fold or higher rise in titre was observed in the paired sera of all calves inoculated except two, while no significant antibody response developed in the control animals. There is no doubt that the inoculation of the Ureaplasma spp. resulted in pneumonia whereas the inoculation of sterile medium did not. The experimental calves were killed four weeks post-inoculation and an acute bronchiolitis associated with alveolar collapse and plugging of the bronchi and bronchioles by inflammatory exudate was found. These workers postulated that this reaction could initiate events leading to later peribronchiolar lymphocytic cuffing, which they considered had not developed because of the relatively short duration of the experimental disease.



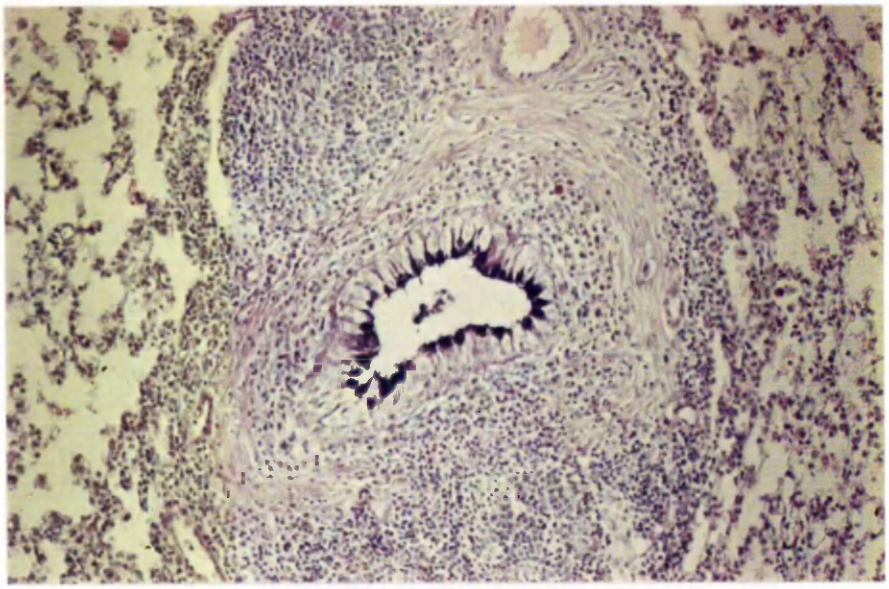
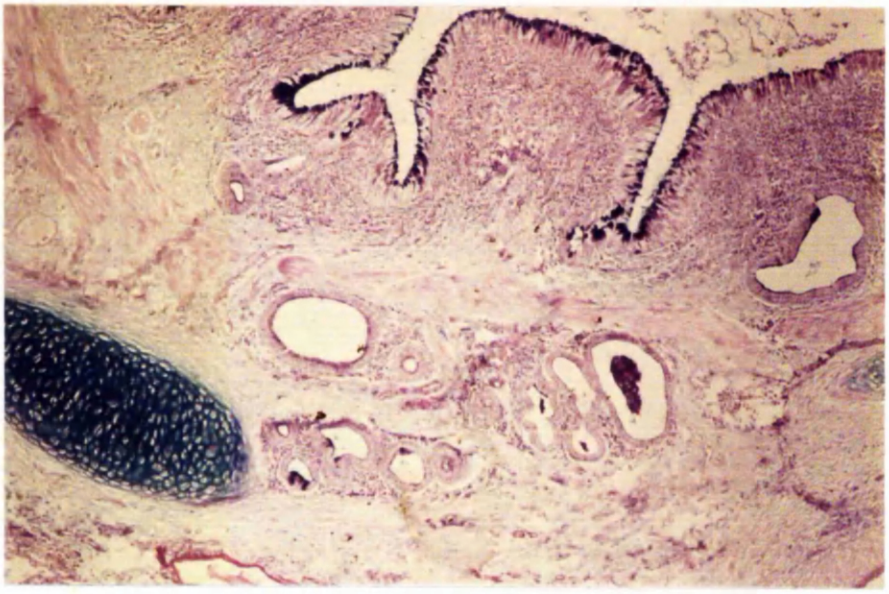


Fig. 62 : In the pneumonic six months old calves the bronchial mucous glands are embedded in the submucosa at the edge or overlying the cartilage plates. The glands are hypertrophied with extensive dilation of the tubules in many areas. Plasma cells surround most of the tubules. The cells of the tubules are cuboidal and many are devoid of mucosubstances in the cytoplasm. Some mucus is present in the lumina of these tubules. AB/PAS staining, x 35.

Fig. 63 : Neutral and acid mucosubstances are detectable in the mucous glands of the three months old pneumonic calves. The majority of the mucosubstances are PAS positive and neutral. AB/PAS staining, x 250.



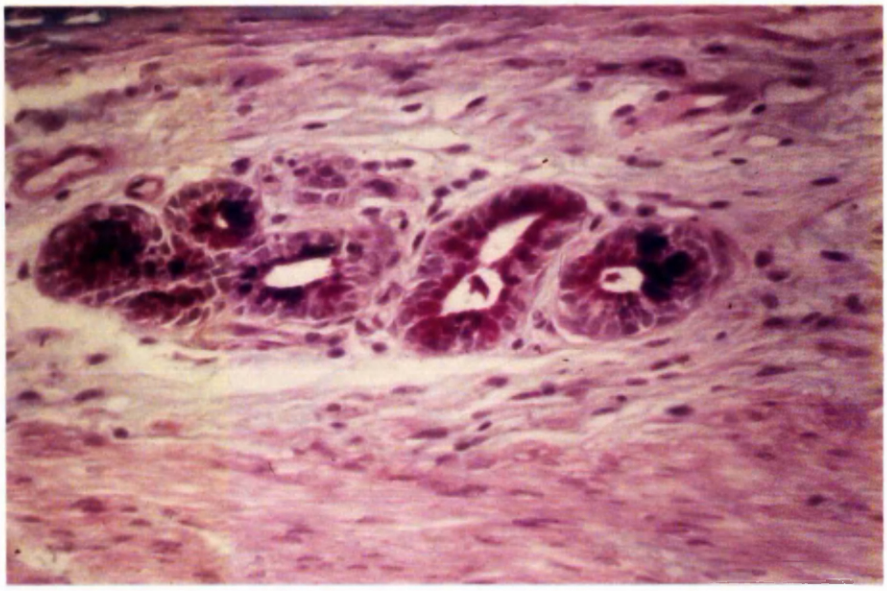
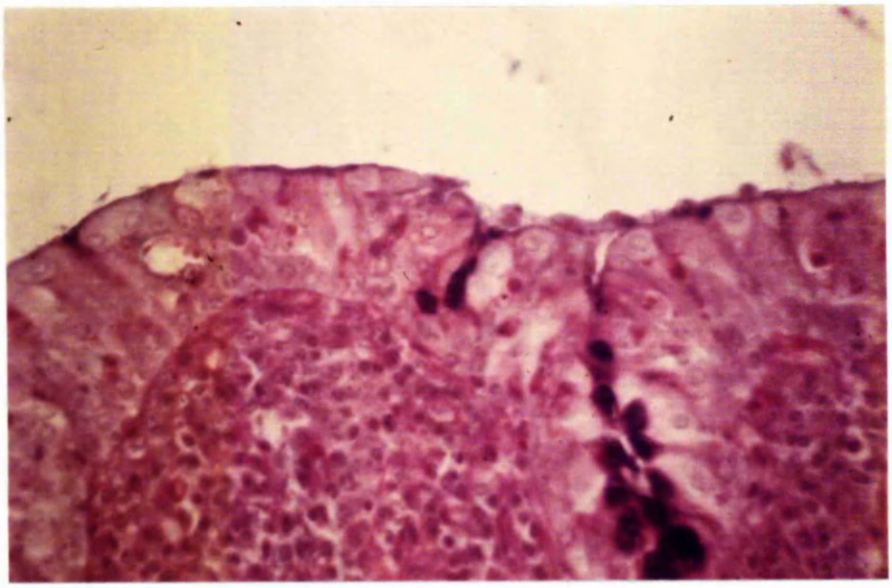
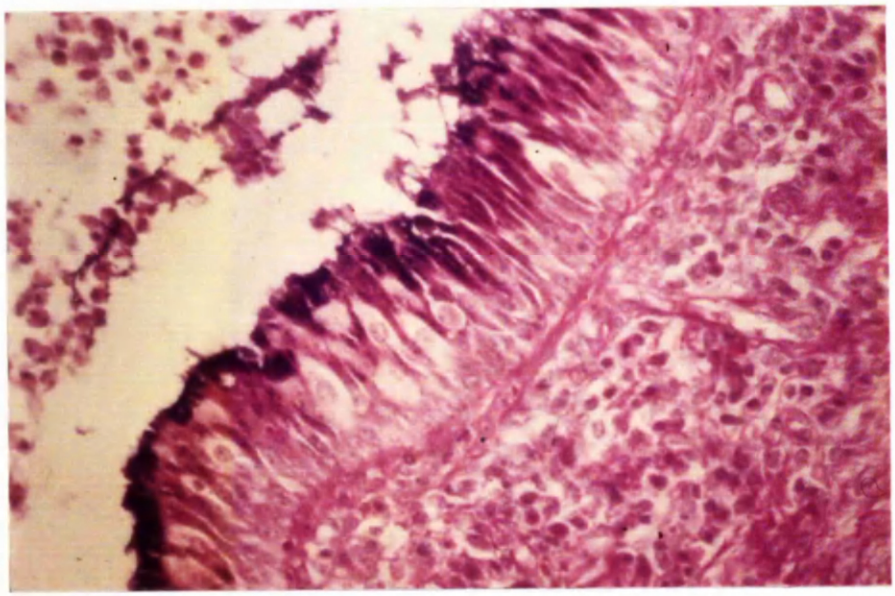


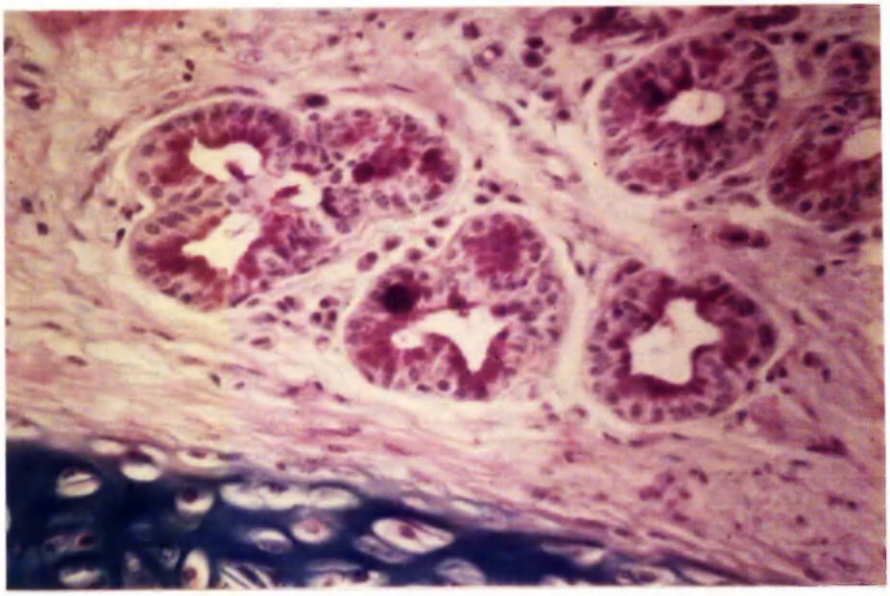
Fig. 64 : The epithelium of a large bronchus of a three months old calf, M188, has a plasma cell type D(ii) pneumonic lesion. Very few goblet cells are present due to hyperplasia and dedifferentiation of the epithelium. AB/PAS staining, x 400.

Fig. 65 : In comparison with Fig. 64, goblet cells are numerous in the epithelium of small bronchi and large bronchioles of this same case (M188). Note the ragged and vacuolated appearance of the epithelium and the cellular exudate in the airway lumen. AB/PAS staining, x 400.

Fig. 66 : The mucous gland of a pneumonic one to two months old calf. The tubules are slightly dilated and the cells contain muco-substance, mostly neutral, in their cytoplasm. AB/PAS staining, x 250.







CHAPTER EIGHT

ELECTRON MICROSCOPICAL EXAMINATION OF BRONCHIAL AND LUNG TISSUE FROM PNEUMONIC CALVES

A. INTRODUCTION

B. MATERIALS AND METHODS

1. Animals

2. Electron microscopical techniques

C. RESULTS

1. Mycoplasma detection by electron microscopy

2. Ultrastructural findings in the pneumonic tissue

D. DISCUSSION

A. INTRODUCTION

Descriptions at the ultrastructural level of mycoplasmas in the bovine respiratory tract do not appear to have been recorded in the literature.

Reports of ultrastructural studies on respiratory diseases in other species caused by mycoplasmas are limited; *in vivo* descriptions have been mostly confined to experimental infections of mice and rats by *M. pulmonis* (Organick, Siegesmund and Lutsky, 1966; Kohn,1971). In both these studies *M. pulmonis* caused a fatal respiratory disease. Ultrastructurally, the organisms were found on the surface of the bronchial epithelium in close contact with the cilia, often lying several layers deep on top of the cilia and penetrating between them. Organisms were seen in the alveoli only early in the experimental infective process, generally in association with phagocytic cells (neutrophils). A brief electron microscopical examination was carried out on hysterectomy-derived piglets infected with *M. suipneumoniae* (Livingston *et al.*, 1972). Mycoplasmas were seen adjacent to cilia and plasma membranes of epithelial cells lining the bronchioles and bronchi; the organisms were confined to surface structures and were not seen within epithelial cells. Similar findings were described in four weeks old pigs experimentally infected with *M. hyorhinis* (Baskerville, 1972), the organisms being present in the lumina of the bronchioles and in small numbers in the alveolar air spaces.

The respiratory tract mycoplasmas in general have a strong affinity for ciliated epithelium; this is evident from the many studies carried out on tracheal explant organ cultures. Detailed electron microscopical studies of the human species, *M. pneumoniae*, in both human foetal and hamster tracheal explant cultures (Collier and Clyde, 1971; Collier and Baseman, 1973; Hu *et al.*, 1975; Muse, Powell and Collier, 1976) have been carried out. Avian, caprine and bovine respiratory tract mycoplasmas have also been examined; all species grew in chicken and bovine tracheal explant cultures but only the pathogenic strains of mycoplasmas caused any cytopathological changes (Cherry and Taylor-Robinson, 1970; Thomas and Howard, 1974).

The purpose of this study was to examine bovine respiratory tissue by electron microscopy (i) for the presence of mycoplasma organisms in bronchial and alveolar tissue and (ii) to detect the changes in the bronchial epithelium in the pneumonic cases. A total of 30 calves was examined and these ranged in age from one to six months; all were members of the groups of animals studied microbiologically and pathologically and described in chapter five.

B. MATERIALS AND METHODS

1. Animals

Pulmonary tissue from 30 calves aged between one and six months was examined. The animals studied were members of the groups described microbiologically and pathologically in chapter five. The cases examined have been detailed in Tables 51, 52 and 53 ; 12 one to two months old calves, ten three to four months old calves and eight six months old animals were studied. The classification of the pulmonary lesions in these animals has also been indicated in these Tables; pneumonia was evident in all cases examined except M102, which was a six months old animal. Pulmonary tissue from this animal, together with samples from three non-pneumonic cases, previously studied but not belonging to any of the present groups of calves, acted as control material for the ultrastructural changes found in the diseased calves.

2. Electron microscopical techniques

Samples of fresh tissue from the right cranial lobe of all calves were fixed as soon as possible after slaughter in 1.3 per cent paraformaldehyde/ 1.6 per cent glutaraldehyde or directly in one per cent osmium tetroxide. Lung tissue and sections of bronchus were sampled in all cases. In each animal examined, four blocks of lung tissue and four blocks of bronchial epithelium were screened for the presence of mycoplasma organisms. The

electron microscopical techniques have been detailed in the general Materials and Methods chapter. The bronchial samples were mostly embedded employing the flat-embedding technique.

C. RESULTS

1. Mycoplasma detection by electron microscopy

Tissue from 12 calves aged between one and two months was examined and mycoplasmas were detected electron microscopically in five cases (Table 51). Mycoplasmas had been isolated by cultural techniques from eight calves; all four species were recovered. Four of these eight animals were also positive for mycoplasmas by electron microscopy. One case (M158) positive for mycoplasmas by electron microscopy did not yield any mycoplasmas by cultural methods. Mycoplasma dispar and A. laidlawii were isolated from two of the cases positive by electron microscopy, while one of the other two calves was infected with M. bovirhinis and the remaining animal, positive electron microscopically, yielded a Ureaplasma sp. on culture.

Ten calves aged three to four months, previously examined as a group, were studied for the presence of mycoplasmas by electron microscopical techniques. Mycoplasma organisms were detected in the respiratory tissue of five of the ten calves examined. These results and the mycoplasma isolations by cultural examination have been recorded in Table 52 . Mycoplasma dispar had been isolated from the lung tissue of four of the five calves that were positive for the presence of mycoplasmas by electron microscopy. Mycoplasma bovirhinis was recovered in very small numbers from the lungs of M190 and mycoplasma organisms were detected ultrastructurally. On the other hand, mycoplasmas could not be detected in either the bronchial or lung tissue of five cases by electron microscopy, although two calves were culturally positive for M. dispar and three for A. laidlawii; both organisms were recovered at relatively high titres, particularly the A. laidlawii isolate of case M188, which attained a titre of 10^6 CCU per 0.2 ml

of sample (see chapter five, section D).

A similar examination was carried out on the pulmonary tissue from eight six months old calves (Table 53); five of these animals were positive by electron microscopy. Mycoplasmas were detected culturally in all five cases and M. dispar was recovered from the pulmonary tissue of three of them. Two of the three cases negative electron microscopically were culturally positive for A. laidlawii, which was isolated at a titre of 10^6 CCU per 0.2 ml of sample (see chapter five, section E) in one instance. Mycoplasma dispar was isolated in small numbers in calves M104 and M105, but organisms were detected in these cases electron microscopically.

Mycoplasmas could be found by electron microscopy in 15 of the 30 cases examined, 25 of which were culturally positive for mycoplasmas. All four species of mycoplasmas were recovered from the lung tissue from these calves both singly and in double isolations as illustrated in Tables 51, 52 and 53; cultural recovery of two mycoplasma species was made in four of the electron microscopically positive cases. However, it appeared that M. dispar was the most frequently isolated mycoplasma in the cases in which mycoplasmas were detected. The results of the electron microscopical examination for mycoplasmas in pneumonic lung tissue from which M. dispar and other mycoplasmas had been cultured have been illustrated in Table 54. Electron microscopical examination of 13 pneumonic calves culturally positive for mycoplasmas other than M. dispar revealed five cases positive for mycoplasmas. Twelve pneumonic calves from which M. dispar was cultured either alone or with other mycoplasmas were examined by electron microscopy. Mycoplasmas were found in nine of these calves by this technique. Statistical examination of these results by the Chi-square test indicated that there was a significant difference ($P < 0.05$) between the frequency of detection of mycoplasmas by electron microscopy in pneumonic calves from which M. dispar had been cultivated and the detection rate in pneumonic lungs negative on culture for M. dispar. Only one of the four calves from which no mycoplasmas were cultured was positive by electron microscopic examination.

In the absence of suitable germ-free calves, bovine ureaplasmas were inoculated into the bovine mammary gland, which, while not germ-free, had a limited and readily-determined bacterial flora. Howard, Gourlay and Brownlie (1973) investigated this technique and found that mastitis developed after inoculation via the teat canal of four strains of ureaplasmas isolated from pneumonic calf lungs, one strain from the eye of an animal affected by infectious bovine keratoconjunctivitis and two of four strains isolated from apparently normal bovine urogenital tracts. The fact that some of the strains were avirulent indicated that not every ureaplasma was capable of producing mastitis in the bovine gland and that genuine virulent and avirulent strains (for the bovine mammary gland) existed and could be isolated from cattle. The production of mastitis also demonstrated that virulent strains were undoubtedly pathogenic for bovine tissues, producing disease in an apparently germ-free environment and that the disease produced was still of considerable significance even though it was not in the organ in which the natural disease occurred and from which the organisms were originally isolated (Gourlay, 1974). A bovine ureaplasma strain from pneumonic lung was also able to cause mastitis in the caprine mammary gland (Howard et al., 1973). This was of interest in that it indicated that this organism could cross the species barrier, at least experimentally.

The inoculation of foetal bovine tracheal organ cultures with 13 bovine Ureaplasma spp. isolated from pneumonic calf lungs, the eyes of animals affected by infectious bovine keratoconjunctivitis and from the urogenital tract revealed that all species became established in the cultures and multiplied, but did not produce any cytopathogenic effects (Thomas and Howard, 1974). There was no correlation between the ability to grow in tracheal culture and virulence as assayed in the bovine mammary gland. Similar results were also reported following the inoculation of bovine Ureaplasma spp. into human fallopian tube organ cultures (Taylor-Robinson and Carney, 1974).

A bovine Ureaplasma sp. isolated from the urogenital tract was inoculated into the uretha of a pathogen-free bull calf and was reported to have caused infection but not disease (Taylor-Robinson and Purcell, 1966).

2. Ultrastructural findings in the pneumonic tissue

In the 15 cases positive for mycoplasmas by electron microscopy the organisms were always detected in the bronchial epithelium. In two cases a few organisms were also seen in the alveolar tissue. Mycoplasmas in pneumonic lung tissue were generally seen as individual organisms in close association with inflammatory cells such as neutrophils and macrophages in the alveolar air spaces. Occasionally they were detected within intracytoplasmic vacuoles of these phagocytic cells but they were never seen in the proximity of the alveolar epithelial cells.

In the samples of bronchial epithelium the mycoplasmas were found on and between the cilia (Fig. 67), generally grouped as two to three layers of cells, having the appearance of a microcolony on the surface of the bronchial epithelium.

Most mycoplasma cells were oval to round in shape although cells penetrating between the cilia appeared elongated, as if they had squeezed between these structures (Fig. 67). The organisms were never seen within any of the epithelial cells. Very close contact with the cilia was usual (Fig. 68); the surface of the cilium was in contact with amorphous material on the outside of the mycoplasma cell in many cases (Fig. 68). Cellular connections were never seen, however, although what appeared to be engulfment by microvillous projections from the epithelium was sometimes evident (Fig. 69).

Mycoplasma cells were pleomorphic, although the majority were round or oval (Figs. 67, 68 and 69) and had cellular characteristics typical of the Mycoplasmatales. Figures 67, 68 and 69 illustrate the main features, (i) the triple-layered unit membrane enclosing the cell, with no evidence of a cell wall, (ii) the densely-stained ribosomes generally situated at the cell periphery, and (iii) the aggregates of nuclear material, nucleoids, present in the clear central area of the cell with electron dense strands stretching towards the periphery. In addition, there was an outer fringe of amorphous material that often had a regular beaded appearance (Fig. 68).

Ultrastructural changes were evident in the pulmonary tissue; most of the changes were in the bronchial epithelium. Loss of cilia was common (Fig. 70) leaving only the basal bodies at the apex of the ciliated cell. Perhaps the most obvious cytological feature in these cases was loss of the regular contour of the epithelial surface due to apical cytoplasmic protrusions into the bronchial lumen. Ciliated cells frequently bulged into the bronchial lumen often producing a 'sunburst' effect in cells in which the cilia were still intact. Epithelial cytoplasmic protrusions were often extensive in non-ciliated cells (Fig. 71); at the apex of such cells electron dense granules were sometimes present which extended into the bulging cytoplasm. Microvillous projections, but no cilia, were seen at the luminal surface. Some protrusions contained very little cytoplasmic material (Fig. 72) and consisted of granular material in which the rudiments of cilia and a few small electron-dense granules could be recognised; intracellular organelles were absent in most structures.

The mitochondria in many of the bronchial epithelial cells were distended (Fig. 73) with loss of cristae and total organelle disruption in some cells. Cytoplasmic vacuoles were a typical feature of the bronchial cells in the cases examined (Fig. 70, 73 and 74). Intercellular junctions of the bronchial epithelium were usually intact, although in several of the older animals distension of the intercellular spaces was noted (Fig. 75).

Neutrophils packed with lysosomes were frequently seen passing through the epithelium (Figs. 74 and 75) towards the bronchial lumen. An increase in the number of goblet cells was also a common feature in the pneumonic calves (Fig. 76). The goblet cells were tightly packed with mucinogen granules and these were seen secreting into the bronchial lumen.

The submucosal glands of most calves were hypertrophied and the tubules had slightly dilated lumina packed with mucins of different electron density (Fig. 77). The cell borders were very closely interdigitated and

tight junctions were clearly visible at the apex of the secretory cells. The mitochondria appeared large and slightly distended with apparent loss of some cristae, while the arrays of rough or smooth endoplasmic reticulum were distorted, possibly with exhaustion due to excess secretion since only a few secretory granules were visible at the cell apex. Active mucous tubules were seen (Fig. 78) and in these the apical cell cytoplasm was tightly packed with discrete granules, which were being discharged into the lumen. Lateral cytoplasmic interdigitations were tight due to the large number of granules in the cells: large, active mitochondria with many cristae were situated in the peripheral cytoplasm.

Inflammatory cells and cell debris were frequently seen in the bronchiolar lumina and alveolar air spaces of most animals (Fig. 79). Neutrophils and macrophages were generally found in large numbers; the macrophages appeared active and had long cytoplasmic projections.

D. DISCUSSION

Mycoplasmas were seen in the respiratory tracts of pneumonic calves using electron microscopical techniques and they were found in 15 of the 30 cases examined. Mycoplasmas were cultured from 25 of these animals. All four species of mycoplasmas were recovered from these calves both singly and in double isolations, with two species recovered culturally from four of the electron microscopically positive cases.

The electron microscopical technique for examining respiratory tissue for the presence of mycoplasma organisms is limited, in that the species of mycoplasma detected is not known. However, in these studies, M. dispar was the most frequently isolated mycoplasma from the cases in which mycoplasmas were detected electron microscopically. Twelve pneumonic calves from which M. dispar was cultured either alone or with other mycoplasmas were examined electron microscopically and organisms were detected in nine of them. In contrast, electron microscopical examinations

of 13 pneumonic calves culturally positive for mycoplasmas other than M. dispar revealed mycoplasmas in only five cases. Statistical examination of these results with the Chi-square test suggested that the presence of M. dispar in bovine pneumonic lung tissue significantly increases the probability of detecting mycoplasmas by electron microscopy. Mycoplasma dispar may form a better association with the cells of the respiratory tissue than other mycoplasma species and these may be lost during preparation of the sample for electron microscopy.

Studies on the bovine respiratory tract mycoplasmas in foetal bovine tracheal explant cultures by Thomas and Howard (1974) revealed that these organisms all became established and multiplied in the explant cultures, but only M. dispar produced cytopathic effects on the ciliated epithelial cells, causing progressive sloughing of cells and patchy flattening of the epithelial layer. The damage to the epithelium only occurred when large numbers of actively growing M. dispar organisms were inoculated and their presence in close association with the cilia caused degeneration and sloughing of the epithelium. Washing of the mycoplasmas, inactivation by heat and Millipore filtration all removed the ability of the inoculum to cause damage. The failure of viable washed M. dispar to induce a cytopathic effect excludes the possible existence of a toxic membrane component, a feature associated with M. fermentans (Gabridge and Murphy, 1971).

The mechanism by which M. dispar causes damage to the epithelial surface is unknown; cytadsorption to the epithelial cells is an established prerequisite for M. pneumoniae infections in human and hamster tracheal organ cultures (Collier, 1972). An extracellular capsule has been detected on M. dispar in cultures stained with ruthenium red and examined electron microscopically (Howard and Gourlay, 1974) and is similar to the capsular material surrounding M. mycoides var. mycoides, a known bovine pathogen; this structure may possibly act as the attachment site of M. dispar to the respiratory epithelium. In the present studies, amorphous material was seen on the outside of some of the mycoplasma cells and this was similar to the capsular material seen in pure cultures of M. dispar with no ruthenium red

staining. However, the presence of antigen-antibody complexes, mucus and precipitated oedema fluid in the bovine pulmonary tissue may be responsible for the amorphous material seen around the cells.

The position of the mycoplasmas seen in the above studies is very similar to that of mycoplasmas in other hosts, both in in vivo experimental infections (Organick et al., 1966; Kohn, 1971; Baskerville, 1972) and in tracheal explant organ culture studies (Collier and Clyde, 1971; Collier and Baseman, 1973; Hu et al., 1975; Muse et al., 1976). The mycoplasmas were seen in close contact with the ciliated epithelium, situated on and between the cilia. Intracellular organisms were never seen, although Organick et al. (1966) and Kohn (1971) described M. pulmonis in the intercellular spaces and within intracytoplasmic vacuoles in mouse and rat bronchial epithelium respectively. Mycoplasmas were seldom present in the alveolar tissue and then only as single organisms. The organisms may be removed quickly when they reach the respiratory tissue by neutrophils and macrophages of the inflammatory process; in experimental infections with M. pulmonis, organisms were infrequently encountered in the alveolar tissue after one week due to rapid phagocytosis and destruction by neutrophils and macrophages (Organick et al., 1966). Neutrophils are known to contain mycoplasmacidal factors (e.g. myeloperoxidase) and there may also be enhanced macrophage phagocytosis as a result of the action of complement or antibody. Ciliated epithelium appears to be a predilection site for respiratory tract mycoplasmas possibly due to specific secretions from these cells producing a suitable growth environment. In addition, and possibly more importantly, the position of the mycoplasmas below the mucus layer and between the cilia may protect them from many of the host's natural and acquired defence mechanisms.

Ultrastructural changes were evident in the bronchial epithelium of cases in which mycoplasmas were detected. Sloughing of cilia and apical cytoplasmic protrusions of both ciliated and non-ciliated cells were seen. In addition, intracytoplasmic alterations including distension and disruption of the mitochondria were present along with fairly extensive cytoplasmic vacuole formation. Similar ultrastructural changes have been recorded in experimental

mycoplasma infections in other species and also in mycoplasma-infected tracheal organ cultures. Organick et al. (1966) and Kohn (1971), in experimental infections with M. pulmonis in mice and rats, reported distension of mitochondria and extensive vacuolation of the cytoplasm in the bronchial epithelium. These features were also recorded in human tracheal organ cultures infected with M. pneumoniae (Collier, 1972). Distension of the intercellular spaces was a common feature of these in vivo and in vitro studies. Collier and Baseman (1973) noted that M. pneumoniae could be found in the distended intercellular spaces of hamster epithelium but not between the human epithelial cells.

Apical cytoplasmic protrusions into the bronchial lumen have been noted in previous mycoplasma infections (Organick et al., 1966; Kohn, 1971; Baskerville, 1972; Collier, 1972), the ciliated cell often having a sunburst appearance (Collier, 1972); this configuration was considered to represent a cell sloughing off the epithelial surface (Muse et al., 1976). Pierce and Hirsch (1958), studying sputum samples of adults with acute respiratory disease, found ciliocytophthoria in nearly all sputum samples from patients with influenza pneumonia, cold agglutinin-positive atypical pneumonia and viral pneumonia of undetermined aetiology. Many of these ultrastructural changes are typical of mycoplasma infections, but similar cytological changes have been reported from diseased respiratory tissue of non-mycoplasmal aetiology, so the lesions are not pathognomonic. For example, ultrastructural changes in the canine respiratory tract were described by Frasca et al. (1968 a and b), who exposed dogs for certain periods of time to cigarette smoke. Initially, an increase in the number of goblet cells was noted and there was a decrease in the number of cilia. Cytoplasmic processes on the apical border of the ciliated cells increased and there was protrusion of apical cytoplasm into the bronchial lumen.

Watson and Brinkman (1964), studying the ultrastructural changes in the human bronchus in chronic bronchitis, did not observe a loss of cilia and found that the alterations in the cells were a matter of degree only. They listed the main changes as: a decrease in the number of pellicular structures,

the alteration in mitochondria with a loss of cristae, the presence of numerous secretion droplets in the apical cytoplasm of ciliated cells and an increase in the number and size of the intercellular spaces.

Walsh et al. (1961) carried out tracheobronchial mucosal biopsies on six patients with type A Asian strain influenza uncomplicated by bacterial infection. A spectrum of changes was described, ranging from vacuole formation, oedema and loss of cilia in epithelial cells to desquamation of the mucosa down to the basement membrane; these findings were similar to the pathologic effects of M. pneumoniae in human tracheal organ cultures (Collier, Clyde and Denny, 1969).

The agent responsible for the disease in the calves described above produced a marked response in the secretory apparatus of the tracheobronchial system with an increase in the number of goblet cells together with hypertrophy and hypersecretion of the submucosal glands probably due to irritation of the epithelial surface by the causative agent. Neutrophils and macrophages were seen in large numbers in the alveolar tissue and neutrophils were seen infiltrating between the cells of the bronchial epithelium, migrating towards the airway lumen.

Many pathogenic, or at least potentially pathogenic, mycoplasmas cause a common basic cytopathic effect on their host's respiratory epithelium, i.e. inhibition of ciliary activity followed by loss of cilia. For many mycoplasma species this cytopathic effect has been recognised only after damage to the epithelial mucosa. In natural infections, this damage can readily be produced by respiratory viruses, which are considered either to damage the epithelial cells, releasing certain products into the extracellular spaces for the growth of mycoplasmas or to destroy some of the cilia thus making the epithelial cells more accessible for the attachment of mycoplasmas (Reed, 1972; Chu and Uppal, 1975). However, this cell damage is not a prerequisite for all mycoplasma infections since disease may be produced by some species in experimental animals with healthy respiratory tracts (Goodwin et al., 1965; Lutsky and Organick, 1966).

Kenny and Pollock (1963) suggested three possibilities by which

mycoplasmas could damage bronchial epithelium, thus allowing growth and establishment of an infection, (i) the mycoplasmas produce an extracellular toxin, (ii) the mycoplasmas parasitise and destroy the cells, or (iii) the mycoplasmas compete with the cells for nutritional materials. A fourth possibility was introduced by Pijoan, Roberts and Harding (1972), who noted that extensive damage to tracheal organ cultures was produced by hydrogen peroxide, a compound elaborated by several pathogenic mycoplasmas. However, recent studies by Hu et al. (1975), who exposed hamster tracheal rings in organ culture to virulent M. pneumoniae, revealed alterations in molecular biosynthesis and metabolic activity of the respiratory epithelial cells. An initial increase in the metabolic rate of the epithelial cells was noted which was followed within 24 hours by inhibition of the host cell ribonucleic acid and protein synthesis. These effects were not produced with infection of avirulent strains of M. pneumoniae into the hamster tracheal rings.

The electron microscopical examination of pneumonic calf tissue presented some interesting points. This was the first ultrastructural description of mycoplasmas in bovine respiratory tissue in vivo; their location appeared to conform with that found in mycoplasma infections of other species. A close association was found between the isolation of M. dispar from bovine tissue and the presence of mycoplasmas ultrastructurally. This feature, together with other characteristics of this organism, indicates the potential pathogenicity of M. dispar. Many ultrastructural changes in the respiratory tract of cases positive electron microscopically for mycoplasmas were similar to thosereported in mycoplasma infection in other species.

Case No.	Age (months)	* Histopathological Classification	Mycoplasma Isolations	EM +ve or -ve for Mycoplasmas
M 141	1	Exud. Pn.	-	-
M 153	1	Collapse	-	-
M 154	1	Supp. Exud. Pn.	M.bovirhinis	-
M 155	1	Supp. Exud. Pn.	M.bovirhinis, A.laidlawii	-
M 157	1	Supp. Exud. Pn.	-	-
M 158	1	Chr. Exud. Pn.	-	+
M 99	1	Supp. Exud. Pn.	Ureaplasma sp.	+
M 100	1	D(i)	Ureaplasma sp.	-
M 92	2	D(ii)	M.bovirhinis	+
M 93	2	C(i)	M.dispar, A.laidlawii	+
M 94	2	E	M.dispar, A.laidlawii	+
M 95	2	D(i)	M.dispar, Ureaplasma sp.	-

* Based on the classification illustrated in Table 16.

Table 51. The results of the electron microscopical examination (EM) for the detection of mycoplasmas in bronchial and lung tissue from calves aged one and two months. The pneumonic classification and the mycoplasmal isolations, by cultural techniques, are also given.

Case No.	Age (months)	* Histopathological Classification	Mycoplasma Isolations	EM + ve or - ve for Mycoplasmas
M 183	3 - 4	D (ii)	M.dispar, A.laidlawii	+
M 184	3 - 4	D (ii)	M.dispar	+
M 185	3 - 4	D (i)	M.dispar	+
M 186	3 - 4	D (ii)	M.dispar	-
M 187	3 - 4	D (ii)	A.laidlawii	-
M 188	3 - 4	D (i) pl	A.laidlawii	-
M 189	3 - 4	D (i) pl	A.laidlawii	-
M 190	3 - 4	D (i)	M.bovirhinis	+
M 191	3 - 4	D (i)	M.dispar	-
M 192	3 - 4	D (i)	M.dispar	+

* Based on the classification illustrated in Table 16.

Table 52. The results of the electron microscopical examination (EM) for the detection of mycoplasmas in bronchial and lung tissue from calves aged three to four months. The pneumonic classification and the mycoplasmal isolations, by cultural techniques, are also given.

3. Mycoplasma bovirhinis

This species appears to be common in the respiratory passages of cattle. The majority of the reported strains were isolated during investigations of respiratory diseases associated with the transport of cattle or with intensive methods of rearing calves. The first description of the group as a distinct species concerned strains isolated from farms in North-East England on which there was a history of respiratory disease (Harbourne et al., 1965). Mycoplasmas were isolated from both nasal swabs and lung specimens and most were later confirmed to be M.bovirhinis (Leach, 1967). This organism has a widespread geographical distribution (Cottew and Leach, 1969) and has been found in non-pneumonic calves, colonising the upper respiratory tract in most cases (Dawson et al., 1966; Davies, 1967; Hamdy and Trapp, 1967; Cottew, 1970; Thomas and Smith, 1972), although it has been found in the lower respiratory tract too (Davies, 1967; Gourlay and Leach, 1970; Gourlay and Thomas, 1970; Thomas and Smith, 1972). An age distribution of the respiratory tract colonisation has been demonstrated, three to four months old calves being most commonly infected (Thomas and Smith, 1972). Mycoplasma bovirhinis has been isolated most frequently from pneumonic animals (Hamdy et al., 1958; Langer and Carmichael, 1963; Davies, 1967; Gourlay et al., 1970; Nicolet and de Meuron, 1970a; Jurmanová and Krejčí, 1971; Shimizu et al., 1973) and the Japanese workers have associated it with an enzootic calf pneumonia and have isolated it from 63 of 117 calves examined. Isolations were also made from the trachea, spleen, kidneys and nose in these 63 cases; no other mycoplasmas were isolated although unspecified bacteria were present (Shimizu et al., 1973).

Intratracheal inoculation of pure cultures of M. bovirhinis into colostrum-deprived calves failed to induce clinical signs of respiratory disease although there was a serological response (Langer and Carmichael, 1963; Dawson et al., 1966). Calves affected by a natural respiratory disease also developed some degree of serological response to M. bovirhinis (Harbourne et al., 1965; Dawson et al., 1966). The serological response also coincided

Case No.	Age (months)	* Histopathological Classification	Mycoplasma Isolations	EM + ve or - ve for Mycoplasmas
M 102	6	A	-	-
M 104	6	C (ii)	M.dispar, A.laidlawii	+
M 105	6	C (ii)	M.dispar	+
M 107	6	C (ii)	A.laidlawii	-
M 110	6	C (ii)	A.laidlawii	-
M 80	6	D (i)	Ureaplasma sp.	+
M 91	6	C (i)	M.dispar	+
M 129	6	C (iii)	M.bovirhinis	+

* Based on the classification illustrated in Table 16 .

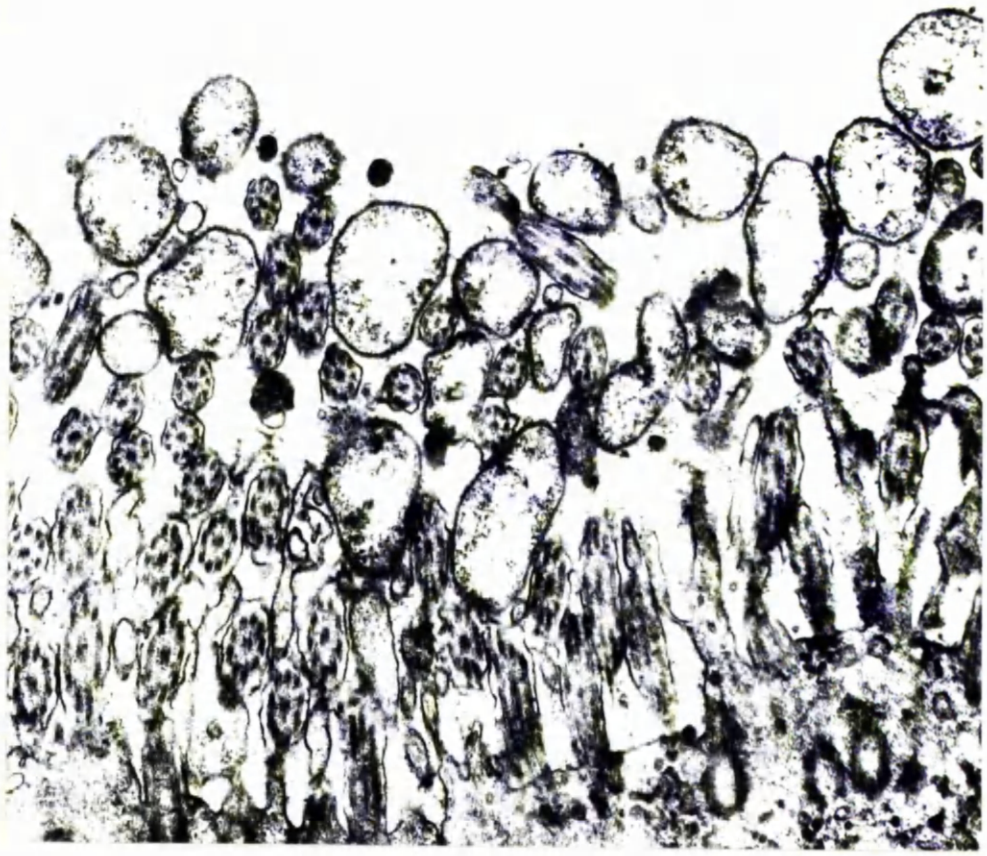
Table 53. The results of the electron microscopical examination (EM) for the detection of mycoplasmas in bronchial and lung tissue from calves aged six months. The pneumonic classification and the mycoplasmal isolations, by cultural techniques, are also given.

Cultural Examination for Mycoplasmas	Electron Microscopical Examination for Mycoplasmas	
	No. Positive	No. Negative
Pneumonic calves with mycoplasmas other than M. dispar	5	8
Pneumonic calves with M. dispar alone or with other mycoplasmas	9	3
Pneumonic calves with no mycoplasmas	1	3

Table 54. The results of electron microscopical examination of the lungs of calves with and without M. dispar or other mycoplasma infection.

Fig. 67 : Mycoplasmas situated on and between the cilia of bronchial epithelial cells of a pneumonic calf. The organisms are pleomorphic, usually round or oval, enclosed in the characteristic triple-layered membrane. Densely stained ribosomes occur at the cell periphery and aggregates of nuclear material occupy the centre of the cell. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 26,250.

Fig. 68 : Mycoplasmas in close contact with the cilia of the bronchial epithelium of a pneumonic case. The characteristic features of mycoplasma organisms, i.e. triple-layered membrane, peripherally situated ribosomes and central nuclear material are recognisable. Amorphous material is evident on the external surface of the membrane. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 60,000.



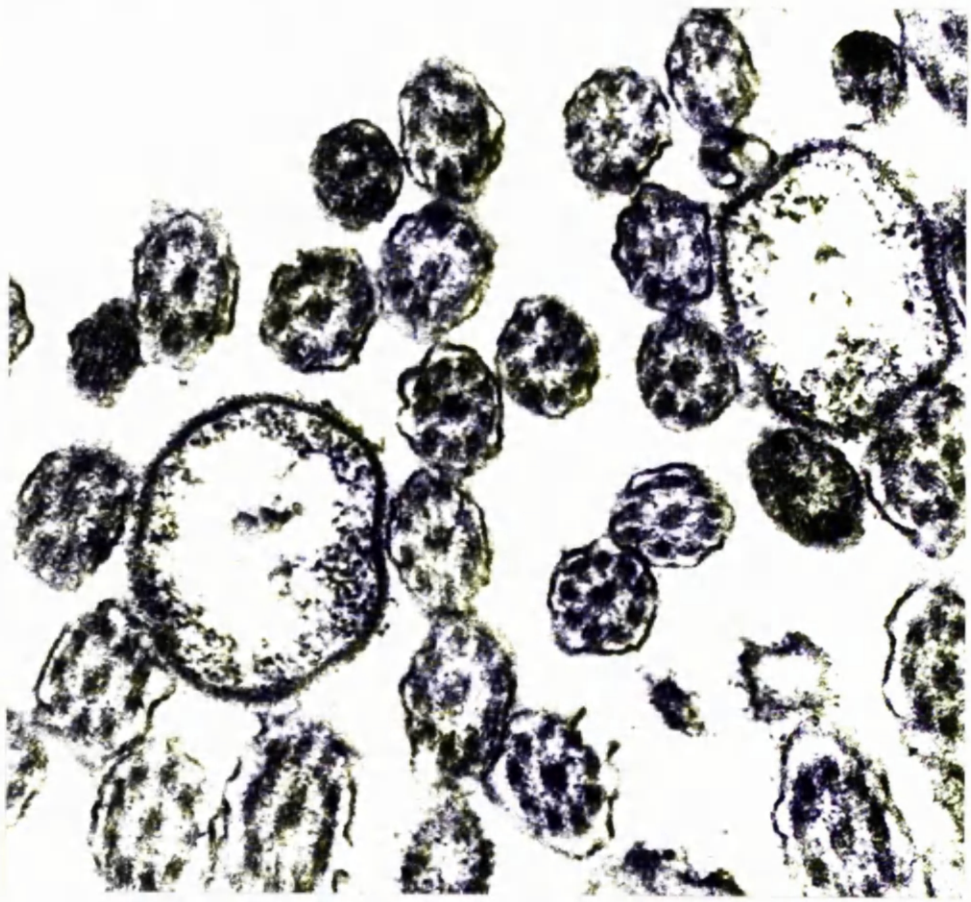
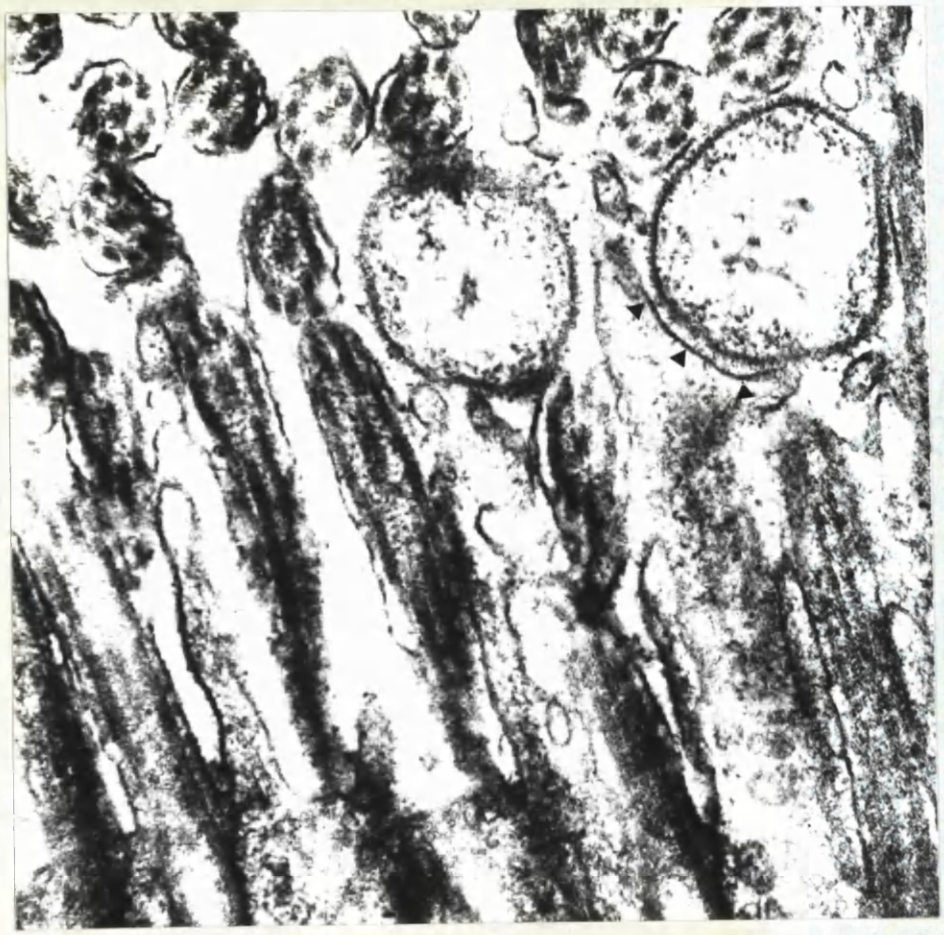


Fig. 69 : Mycoplasmas associated with the bronchial epithelium in pneumonic calves. The mycoplasmas are in close contact with the cilia and a microvillous projection has surrounded approximately one-third of an organism (arrows). The general features of mycoplasmas, as illustrated in Figs. 67 and 68, are evident in these cells. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 50,000.

Fig. 70 : Bronchial epithelium of a calf with pneumonia and a pulmonary mycoplasma infection. When compared with the epithelium from a healthy calf loss of cilia is evident, leaving the basal bodies at the cell apex (arrows). Intracellular cytoplasmic vacuolisation is a feature of many of the epithelial cells in these pneumonic cases. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 10,000.

p. 244 Fig 70

How can you tell that there
chia are lost.?



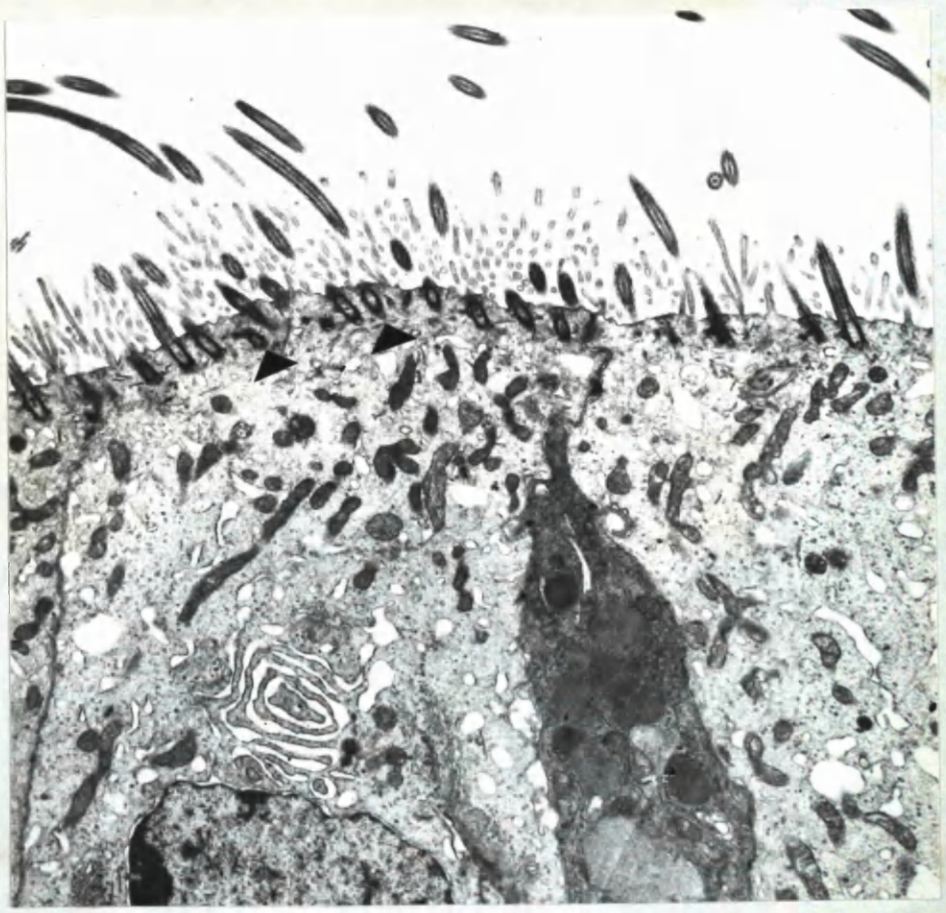
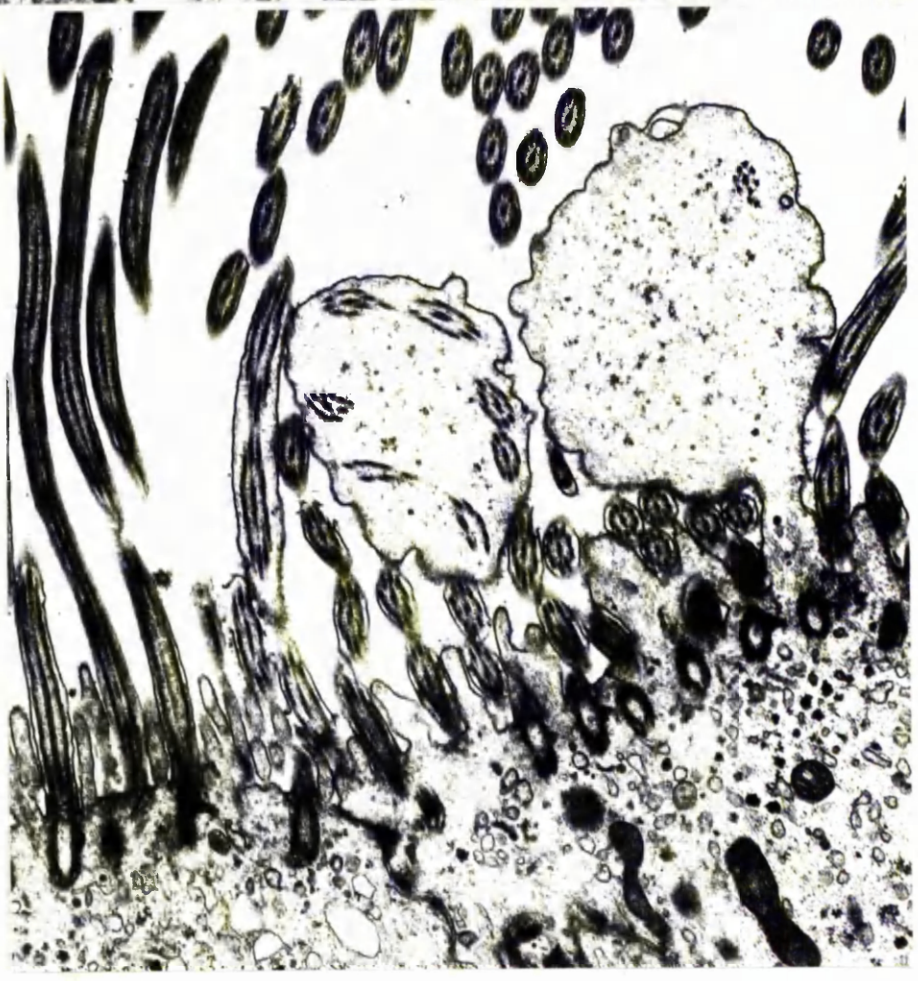


Fig. 71 : The bronchial epithelium of a pneumonic calf : apical cytoplasmic protrusions extend into the lumen of the airway. Electron dense granules at the apex of the protrusion and microvillous projections at the membrane surface are present. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 20,000.

Fig. 72 : Apical cytoplasmic protrusions frequently found in the bronchial epithelium of pneumonic calves. The rudiments of cilia and tiny electron dense granules are recognisable in the cytoplasm of the protrusions. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 20,000

P. 245 How do these cytoplasmic
protrusions develop?



with the spread of M. bovirhinis through the nasal passages of calves in one herd and was detectable even before respiratory disease, characterised by coughing and some deaths, became obvious (Dawson et al., 1966). However, reports concerning isolations of M. bovirhinis in association with calf respiratory disease have also indicated simultaneous infections with viruses, such as PI3 or with pathogenic bacteria including Pasteurella spp. (Harbourne et al., 1965; Dawson et al., 1966; Hamdy and Trapp, 1967). Thomas and Howard (1974) reported the ability of M. bovirhinis to multiply in foetal bovine tracheal explant cultures, but no cytopathogenic effects were recorded.

Gourlay and Thomas (1970) inoculated two calves endobronchially with a broth culture of M. bovirhinis: only tiny pneumonic lesions were found at post mortem examination four weeks later and the organism was not reisolated, although an eight-fold rise in the antibody titre to this organism was detected by the latex agglutination technique.

The frequent presence of this mycoplasma in pneumonic lungs may indicate a capacity to act as a secondary invader of the lower respiratory tract, even if not as a primary aetiologic agent (Cottew and Leach, 1969). This view was also expressed by Gourlay et al. (1970) who isolated it from five of 45 three months old clinically normal calves, all of which had macroscopic lung lesions and from ten of 20 calves which had died or been killed in extremis.

Some evidence of systemic infection by this organism was provided by its isolation from many organs in calves with pneumoenteritis or pneumonia (Langer and Carmichael, 1963; Shimizu et al., 1973).

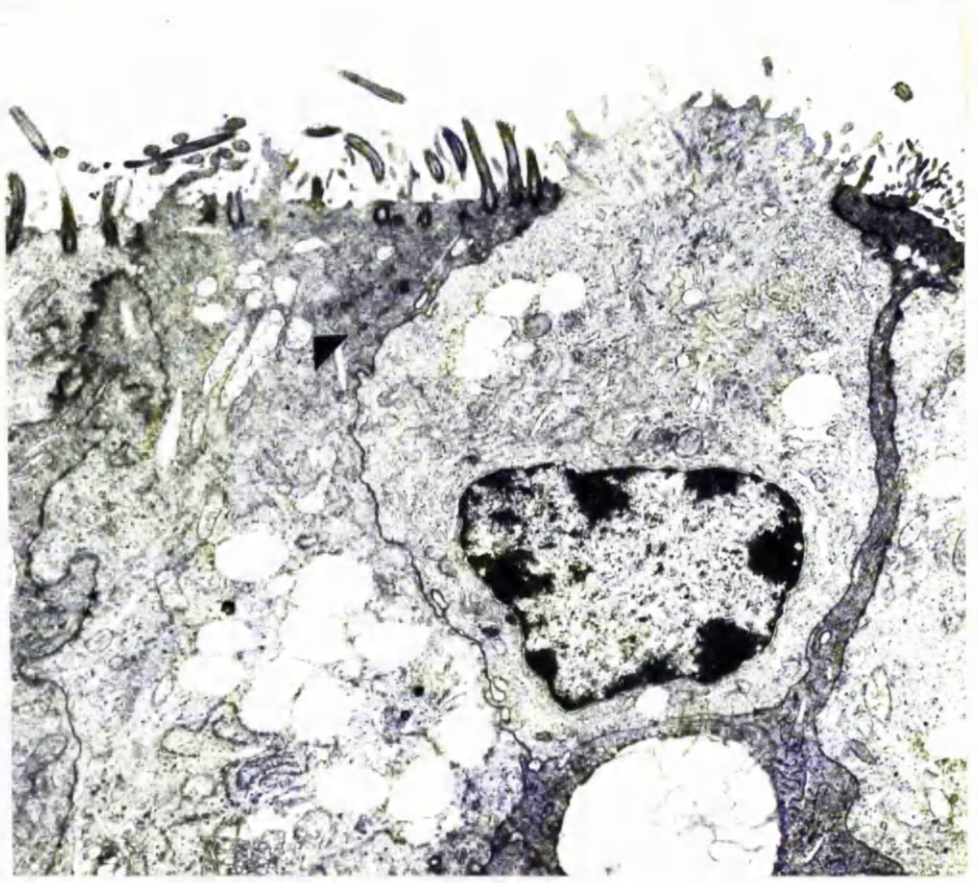
Some strains of M. bovirhinis are pathogenic for the bovine udder experimentally and there is one recorded natural case of mastitis due to this mycoplasma. The disease was reproduced by experimental inoculation with the mycoplasma isolated from the natural case and also with another strain of M. bovirhinis isolated from the respiratory tract (Langer and Carmichael, 1963).

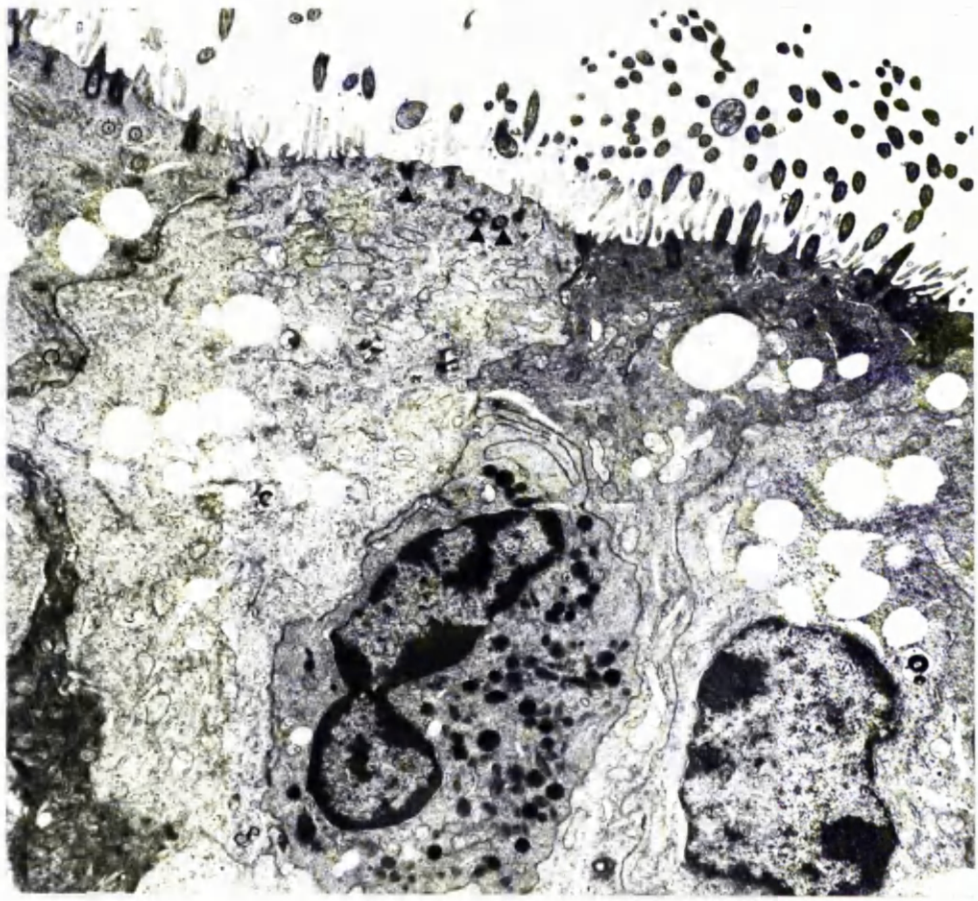
4. Acholeplasma laidlawii

This organism was first isolated from sewage (Laidlaw and Elford, 1936)

Fig. 73 : Bronchial epithelium of a pneumonic calf illustrating some of the intracellular changes. Many of the mitochondria are distended and show loss of cristae (arrow). Cytoplasmic vacuolisation is extensive. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 8,750.

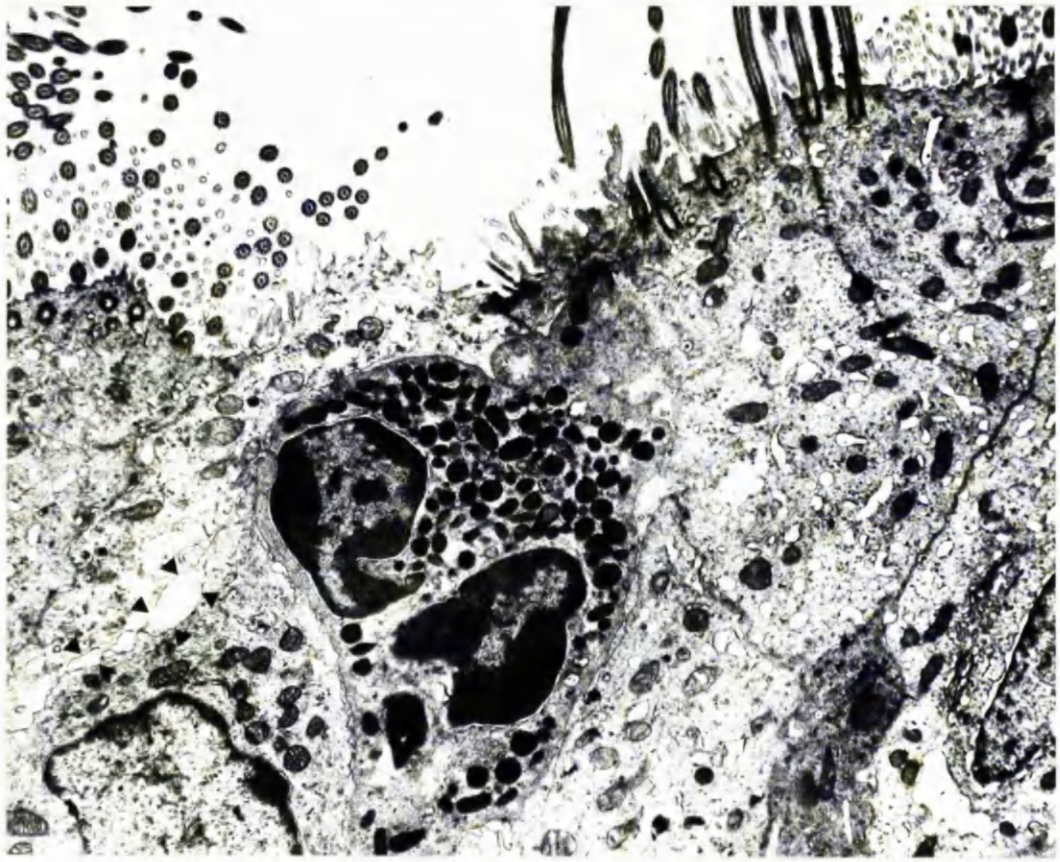
Fig. 74 : Vacuoles are common in the cytoplasm of the epithelial cells of this pneumonic tissue. A neutrophil is seen infiltrating between the epithelial cells. Loss of cilia is evident, leaving only basal bodies at the cell apex (arrows). Note the mycoplasmas on the luminal surface. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 8,750.

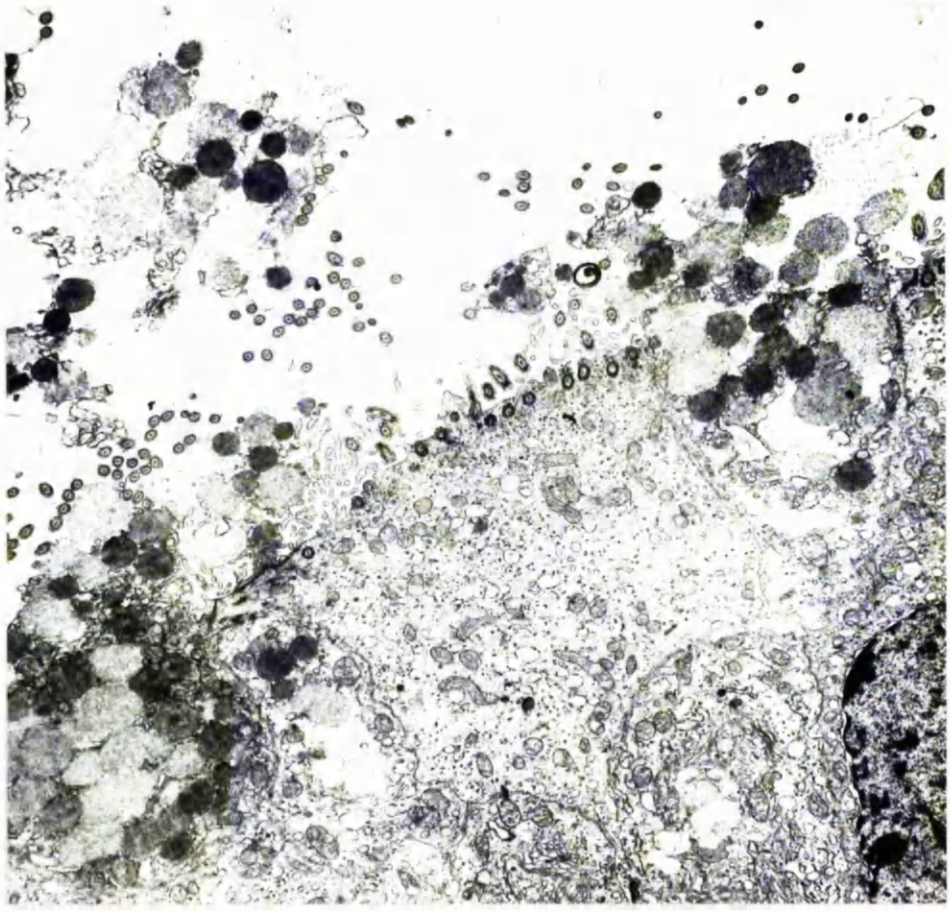




ig. 75 : The bronchial epithelium of a pneumonic case illustrating the infiltration of a neutrophil through the epithelial cells. The cytoplasm of the neutrophil is packed with electron dense granules. Distension of intercellular spaces is evident in this tissue (arrows). Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 10,000.

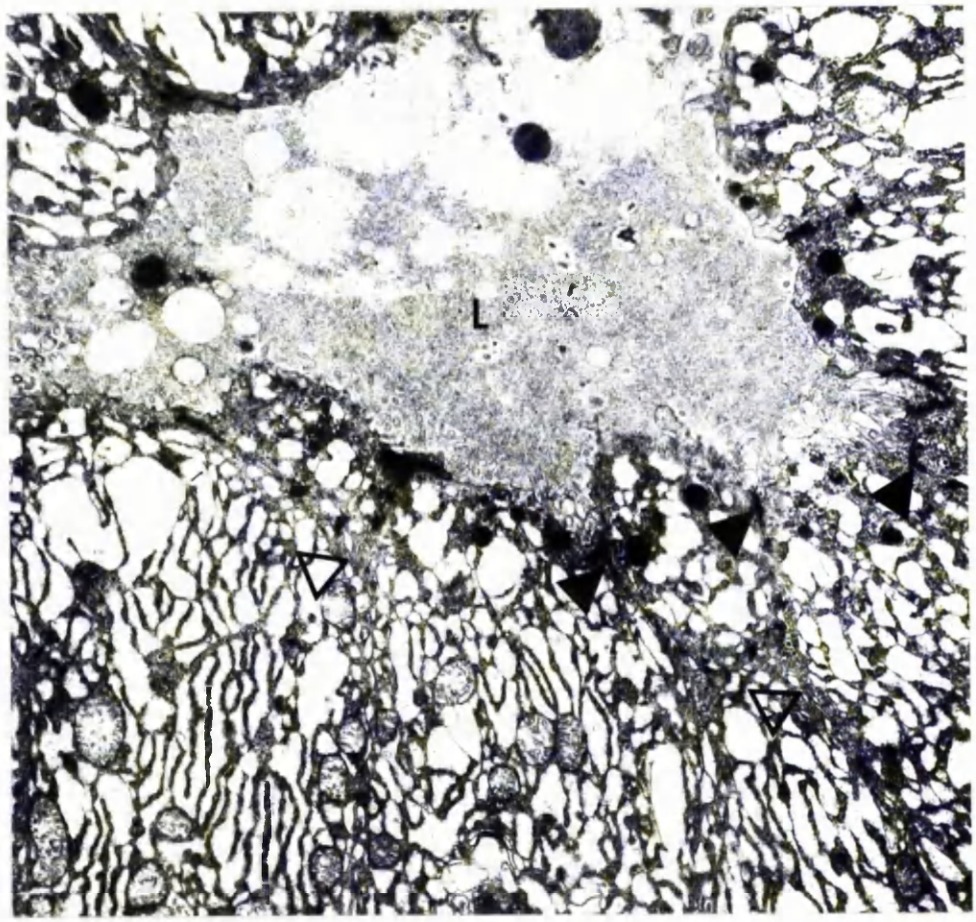
ig. 76 : An increase in the number of goblet cells in the bronchial epithelium was present in most pneumonic calves. The mucin granules, of different electron density, are tightly packed in the goblet cells seen in this Figure. The contents of the goblet cells spill onto the epithelial surface and into the lumen. Paraformaldehyde/glutarldehyde followed by osmium tetroxide fixation, x 6,000.





ig. 77 : A mucous tubule of a hypertrophied submucosal gland from a pneumonic calf. The lumen of the tubule (L) is slightly dilated and packed with mucins of different electron density. The cytoplasmic interdigitations appear tight and compact (△)with tight junctions at the apex (▲). The mitochondria are large and slightly distended with loss of cristae in some. The endoplasmic reticulum appears distorted possibly due to excess secretion. The cells of this tubule appear exhausted of secretion with only a few mucinogen granules at the cell apex. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 8,750.

ig. 78 : In contrast to the cells of the mucous tubule illustrated in Fig. 77 the cells of this tubule are actively producing mucus; electron dense granules are packed tightly within the cell and are being discharged into the gland lumen (L). Mitochondria are large and situated at the cell periphery. Paraformaldehyde/glutaraldehyde followed by osmium tetroxide fixation, x 10,000.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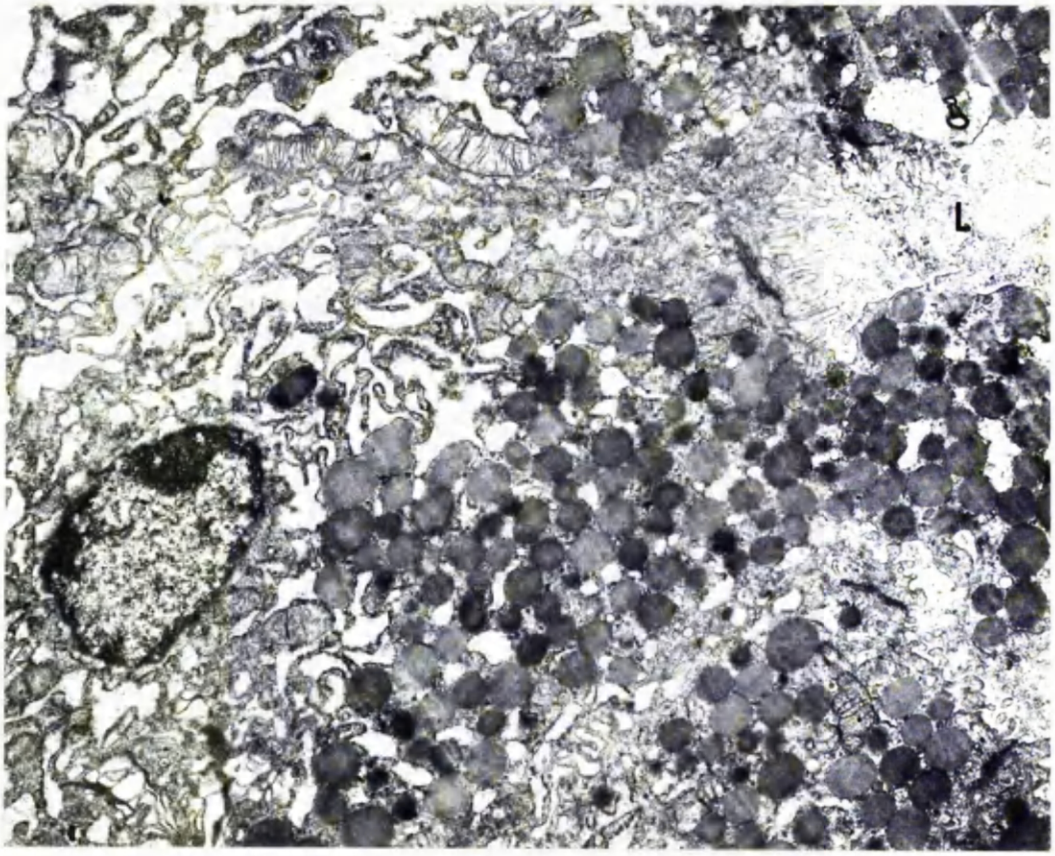
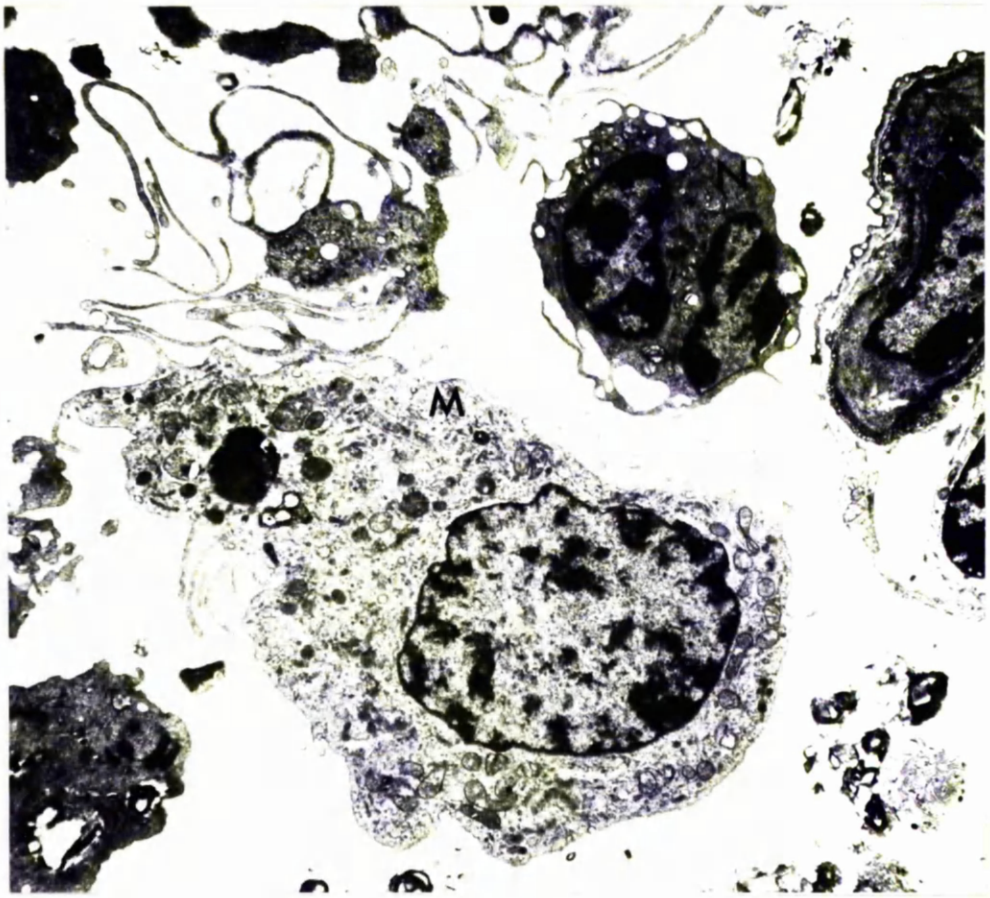

L

Fig. 79 : Alveolar tissue from a pneumonic calf. Debris is present in the alveolar air spaces; inflammatory cells including macrophages (M) and neutrophils (N) are seen. The macrophage appears active with many filamentous projections on its surface. Paraformaldehyde/ glutaraldehyde followed by osmium tetroxide fixation, x 6,000.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CONCLUSIONS

Mycoplasma dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii were isolated from the lungs of calves in the West of Scotland. These organisms were recovered most frequently from pneumonic lung tissue of calves over four weeks of age.

Mycoplasma dispar was the organism most consistently isolated from the lungs of pneumonic calves, especially from older animals with cuffing pneumonia and was believed to be associated with this pulmonary condition. Younger calves (two to four months old) with pulmonary lesions considered to be early cuffing pneumonia frequently yielded M. dispar from their lung tissue on culture, providing further support for the view that this organism is the aetiological agent of cuffing pneumonia. In addition, no isolations of M. dispar were made from the lungs of non-pneumonic calves.

Experimental infections of rabbits and hamsters with M. dispar broth culture and M. dispar-infected lung homogenates were largely unsuccessful.

Electron microscopical detection of mycoplasmas in pneumonic pulmonary tissue was closely related to the cultural recovery of M. dispar from the lung tissue; this suggested that M. dispar formed a closer association with bronchial epithelium than other mycoplasma species. Ultrastructurally, mycoplasmas were seen in close contact with the cilia of the bronchial epithelium. Intracellular organisms were never seen and their presence in alveolar tissue was recorded in only two cases. Ultrastructural lesions in the cells of the bronchial epithelium were seen in many pneumonic cases and the changes were similar to those described in respiratory mycoplasma infections in other animals.

Histochemical examination of the tracheobronchial mucosubstances of the normal calf indicated the presence of neutral mucins, sialomucins and sulphomucins in the secretory apparatus; the epithelial goblet cells of the non-pneumonic calf appeared to contain sulphomucins exclusively. In the calves with cuffing pneumonia the mucous-secreting apparatus was hypertrophied. The mucosubstances in the respiratory secretions in these cases were normal although their proportions in epithelial goblet cells and submucosal glands were altered; this change was consistent in the animals with M. dispar pulmonary infections.

and was regarded as exclusively saprophytic, rather than parasitic, because of its simple growth and temperature requirements (there is no requisition for sterols and it grows readily at 22°C). However, Edward (1950) reported its presence in the bovine genital tract and it has been isolated from the nasal passages of calves affected by bronchopneumonia (Harbourne et al., 1965).

In the survey of Thomas and Smith (1972) A. laidlawii was recovered most frequently from the nasal passages of very young calves, one to two days old, and of animals ten months and older, but no isolations were made from the three to four months old calves, unlike M. dispar and M. bovirhinis. All the 70 animals examined were described as having macroscopically normal lungs.

Acholeplasma laidlawii has also been isolated from the bovine respiratory tract in U.S.A., Australia and Switzerland (Olson et al., 1960; Cottew, 1970; Nicolet and de Meuron, 1970b; Al-Aubaidi and Fabricant, 1971).

Experimental intratracheal and intranasal inoculation of broth cultures of this organism produced no clinical signs and both macroscopic and microscopic examinations revealed no significant lesions (Trapp et al., 1966). The calves used were conventionally reared and aged three to six months. Pre-inoculation and post mortem microbiological examinations were not carried out.

Infection of foetal bovine tracheal explant cultures with A. laidlawii initiated a multiplication of this organism but no cytopathogenic effects were produced (Thomas and Howard, 1974).

The pathogenicity of bovine mycoplasmas in the respiratory tract has been recently reviewed (Gourlay, Thomas and Howard, 1972; Fabricant, 1973; Gourlay, 1973). In relation to the mycoplasmas associated with calf pneumonia in Great Britain, it was suggested that A. laidlawii and, probably, M. bovirhinis are unimportant, whereas Ureaplasma spp. and M. dispar are undoubtedly pathogenic, being able to produce pneumonia experimentally, although their exact role in the natural disease complex remains undetermined.

APPENDIX 1 : HISTOCHEMICAL STAINS

a. Stains for neutral mucosubstances.

(i) Periodic acid-Schiff Technique.

(ii) Diastase digestion.

b. Stains for acid and neutral mucosubstances.

(i) Alcian blue-periodic acid-Schiff Technique.

c. Stains for sialomucins.

(i) Neuraminidase digestion.

(ii) Acid hydrolysis.

d. Stains for sulphomucins.

(i) Alcian blue (pH 2.6).

(ii) Alcian blue (pH 1.0).

(iii) High-iron diamine.

(iv) High-iron diamine - alcian blue.

e. Stains for hyaluronic acid.

(i) Hyaluronidase digestion.

a. Stains for neutral mucosubstances.

(i) Periodic acid-Schiff (PAS) Technique.

1. Bring section to water.
2. Rinse in 70 per cent alcohol for two minutes.
3. Place in solution A for seven minutes.
4. Rinse in 70 per cent alcohol for two minutes.
5. Place in solution B for two minutes.
6. Rinse in 70 per cent alcohol for two minutes.
7. Wash in water until free of alcohol.
8. Place in solution C for 30 minutes.
9. Wash in water to intensify for five minutes.
10. Stain in Haemalum for one to two minutes.
11. Wash and blue in Scott's Tap Water Substitute.
12. Dehydrate, clean and mount.

Histochemical result : PAS positive substances stained deep red (magenta).

Solution A : Alcohol Periodic acid.

Periodic acid	0.4 g
Distilled H_2O	10 ml
M/5 Sodium acetate buffer	5 ml
Absolute ethanol	35 ml

Solution B : Acid Reducing Rinse.

Potassium iodide	1 g
Sodium thiosulphate	1 g
Distilled H_2O	20 ml
Absolute ethanol	30 ml
2N HCl	0.5 ml

Solution C : Fuchsin sulphite (Schiff's Reagent).

Dissolve 2g basic fuchsin in 400 ml of boiling water. Cool to 50^{O}C and filter. Add to the filtrate 10 ml of 2N HCl and 4g potassium metabisulphite. Stopper and leave in a cool place overnight. Add 1g of decolourizing charcoal and filter promptly. Add up to 10 ml or more of 2N HCl in small amounts until the mixture, when allowed to dry in a thin film on a slide, does not become pink. This solution should be kept in a dark well-stoppered bottle in a dark cupboard. It will keep for two months.

All three solutions are kept in a refridgerator in dark bottles.

(ii) Diastase digestion.

1. Bring two serial sections to water.
2. Rinse in distilled water.
3. Digest one slide in preheated diastase solution at 37^{o}C for 30 minutes.
4. Wash in water for five minutes.
5. Stain both sections with periodic acid-Schiff.

Histochemical result : diastase digestion selectively eliminates PAS staining attributable to glycogen.

Enzyme solution.

A 0.1 per cent solution of malt diastase (mixed α and β amylase, B.D.H.) is prepared in distilled water and used immediately.

b. Stains for acid and neutral mucosubstances.

(i) Alcian blue - periodic acid-Schiff (AB/PAS) Technique.
(modification of Mowry, 1956).

1. Bring section to water.
2. Stain in filtered alcian blue (1 per cent alcian blue in 3 per cent acetic acid) for 30 minutes.
3. Wash in tap water for two minutes.
4. Rinse in distilled water.
5. Place in solution A for seven minutes.
6. Wash in tap water for five minutes.
7. Rinse in 70 per cent alcohol.
8. Stain in solution B for two minutes.
9. Rinse in 70 per cent alcohol.
10. Wash in water until clear of alcohol.
11. Stain in solution C for 30 minutes.
12. Wash in water to intensify for five minutes.
13. Stain in Haemalum for one to two minutes.
14. Wash in Scott's Tap Water Substitute for one to three minutes.
15. Wash in water.
16. Dehydrate, clear through graded alcohols, and mount.

Histochemical result : Acid mucosubstances stain blue, while neutral mucosubstances appear red.

c. Stains for sialomucins.

(i) Neuraminidase digestion (McCarthy and Reid, 1964).

1. Bring two serial sections to water and dry.
2. Flood one section with neuraminidase enzyme solution, cover and incubate at 37°C overnight. Flood control section with four per cent calcium chloride, cover and incubate overnight.
3. Wash carefully in distilled water.
4. Stain both sections by the combined AB/PAS method.

Histochemical result : neuraminidase reacts with neuraminidase-sensitive sialomucins to eliminate metachromasia and alcian blue affinity. Comparison of control and test sections reveals the removal of sensitive sialomucins indicated by a colour change from blue to red with the AB/PAS stain.

Enzyme solution.

Neuraminidase (Wellcome Research Laboratories, Beckenham, Kent) (sialidase, receptor-destroying enzyme) is a filtrate from Vibrio cholerae which is stored in 25 ml bottles at 4°C until required. The working solution is composed of eight parts of enzyme to one part of four per cent calcium chloride solution.

(ii) Acid hydrolysis (Lamb and Reid, 1969).

1. Bring two serial sections to water.
2. Place one section in a Coplin jar containing 0.1 N sulphuric acid at 80°C for one hour.
3. Rinse both sections and stain by the combined AB/PAS method.

Histochemical result : comparison of serial sections reveals loss of staining by all sialic acid groups and resultant loss of alcianophilia. The increase in PAS-staining in the acid hydrolysis section thus represents sites of sialomucin. The remaining alcian blue basophilia is due to other acid mucosubstances i.e., sulphomucins. Comparison of the sections treated with neuraminidase and acid also reveals a loss of alcian blue staining due to the removal of neuraminidase-resistant sialomucins.

d. Stains for sulphomucins.

(i) Alcian blue (pH 2.6) (Spicer, Horn and Leppi, 1966).

1. Bring sections to water.
2. Stain in alcian blue (1 per cent alcian blue in 3 per cent acetic acid) for 30 minutes.
3. Wash in running water for five minutes.
4. Dehydrate in alcohol, clear in xylene, and mount.

Histochemical result : sialomucins, hyaluronic acid and weakly acid sulphated mucosubstances stain dark blue.

(ii) Alcian blue (pH 1.0) (Spicer, Horn and Leppi, 1966).

1. Bring section to water.
2. Stain in alcian blue (1 per cent alcian blue in 0.1 N hydrochloric acid, pH 1.0) for 30 minutes.
3. Blot dry with filter paper without rinsing.
4. Dehydrate in two changes of absolute alcohol and one of equal parts absolute alcohol and xylene, clear in xylene and mount.

Histochemical result : sulphated mucosubstances are selectively stained deep blue.

(iii) High-iron diamine (HID) (Spicer, Horn and Leppi, 1966).

1. Bring section to water.
2. Stain in HID stock solution at room temperature for 24 hours.
3. Rinse quickly in water.
4. Dehydrate, clear and mount.

Histochemical result : sulphated mucosubstances are selectively stained brown-black.

Diamine solution.

Dissolve 120 mg of N, N-dimethyl-m-phenylenediamine-dichloride (Eastman Kodak Co., Rochester, New York) and 20 mg of N, N-dimethyl-p-phenylenediamine-monochloride (Sigma Chemicals, London) simultaneously in 50 ml of distilled water (pH 7.0). When the reagents are dissolved, pour this solution immediately into a Coplin jar containing 0.9 ml of standard ferric chloride solution (60 per cent w/v) and 0.5 ml of concentrated hydrochloric acid. Thus 50 ml of staining solution contains 180 mg of $Fe^{(3+)}$ ions. The pH of the HID solution is approximately 1.7.

(iv) High-iron diamine - alcian blue (HID/AB) (Spicer, 1965).

1. Bring section to water.
2. Stain in fresh diamine solution at room temperature for 24 hours.
3. Rinse quickly in water.
4. Stain in 1 per cent alcian blue in 3 per cent acetic acid (pH 2.5) for 30 minutes.
5. Dehydrate through 95 per cent and absolute alcohol, clear and mount.

Histochemical result : most sulphated mucosubstances are purple-black; acid mucosubstances lacking sulphate esters (i.e., hyaluronic acid and sialomucins) are unstained. The post-staining for 30 minutes in 1 per cent alcian blue in 3 per cent acetic acid colours sialomucins and hyaluronic acid blue.

B. CULTIVATION AND MORPHOLOGICAL CHARACTERISTICS OF FOUR BOVINE MYCOPLASMAS

In chapter one, an outline of the discovery and classification of mycoplasmas was given. This section will initially review the biological features of these organisms as a class and subsequently describe some of the individual features of the four species listed above.

In the latest edition of Bergey's Manual of Determinative Bacteriology, Freundt (1974) defined the class Mollicutes as - .

"procaryotic organisms bounded by a single triple-layered membrane; they lack a true cell wall and are incapable of synthesising cell wall precursors, such as muramic and diaminopimelic acids. The cells are small, sometimes ultramicroscopic (about 200 nm), highly pleomorphic, coccoid to filamentous, with a more or less pronounced tendency of the filaments to produce truly branched myceloid structures. The method of reproduction is controversial, but appears to take place by the development within the filaments of tiny coccoid structures ('elementary bodies') and their subsequent release by fragmentation and disintegration of the filaments, and/or by binary fission; also reproduction through budding may occur. Usually non-motile. No resting stages are known. Gram-negative.

The species recognised thus far can be grown on artificial cell-free media of diverse complexity. Colonies are minute; they have a marked tendency to grow down into solid media and usually have a characteristic 'fried egg' appearance. Most species are completely resistant to penicillin and its analogues. Growth and metabolism is specifically inhibited by antibody. Several of the above-mentioned characteristics of this Class, such as the morphological instability, the inability to retain the dye-iodine complex of the Gram stain, the tendency to penetrate into the depth of solid media, the insusceptibility to penicillin and the susceptibility to antibody, can be attributed to the lack of a cell wall. The mycoplasmas may be saprophytic, parasitic or pathogenic. The pathogens cause diseases of animals and possibly of plants".

e. Stains for hyaluronic acid.

(i) Hyaluronidase digestion (Spicer, Leppi and Stoward, 1965).

1. Bring two serial sections to water.
2. Rinse in distilled water.
3. Incubate one section with hyaluronidase at room temperature for 24 hours. Incubate the other section in buffer solution at room temperature for 24 hours.
4. Wash in running water for five minutes.
5. Stain with 1 per cent alcian blue in 3 per cent acetic acid (pH 2.5) for 30 minutes.
6. Wash, dehydrate, clear and mount.

Histochemical result : basophilia is eliminated by testicular hyaluronidase, indicating the presence of hyaluronic acid.

Enzyme solution.

Dissolve 1,000 units of hyalase (Testicular hyaluronidase, Sigma Chemicals, London) in 100 ml of phosphate-buffered saline (pH 6.9) (Difco).

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The class distinguishing features of the Mollicutes are, in addition to being unicellular and procaryotic, the lack of certain photosynthetic pigments (in contradistinction to Schizophyceae), the lack of a rigid cell wall and cell-wall constituents (in contradistinction to Schizomycetes) and the independence of living cells for growth (in contradistinction to Microtatobiotes) (Wittler, 1973).

Taylor-Robinson (1968) summarised the properties of mycoplasmas in relation to bacteria and viruses (Table 10). Like bacteria, but unlike viruses, mycoplasmas multiplied in a cell-free medium and contained both DNA and RNA. Many mycoplasmas adhered to tissue cells and some possessed a fringe of spikes on the outer surface of the membrane and could be confused with some viruses. Mycoplasmas had several metabolic systems which were usually less complex than those of bacteria. Inhibition of growth by antibody was a feature of mycoplasmas and viruses but not a feature of bacteria generally. From these properties, Taylor-Robinson (1968) speculated that the phylogenetic evolutionary position of mycoplasmas lay between bacteria and viruses.

Many bacteria have the ability to grow as L forms in the presence of various inducing agents, particularly penicillin, which is a common component of mycoplasma media and this caused concern over false identification of mycoplasmas because of the similar morphological features to some L forms of bacteria. The main differences between mycoplasmas and L forms of bacteria are shown in Table 11. L form colonies tend to have a darker centre than mycoplasma colonies and their peripheral portion is lighter with a coarse lace-like structure. Under crowded conditions, the minor morphological differences between the two types of colony are not so evident; subjective morphological criteria can be very misleading, particularly in view of the wide range of colonial morphology seen even within the recognised mycoplasma species. Therefore, suspected mycoplasma colonies should be subcultured without delay onto antibiotic-free medium (as well as serum-free medium), to give an opportunity for the reversion of any L form colonies since the risk of producing stable bacterial L forms increases with passage

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in the presence of the inducing agent.

Wittler (1973) indicated the sophisticated techniques used to circumscribe the order, families, genera and species within the class Mollicutes, techniques which generally rely on genetic, biochemical and chemical properties. Although these methods are reliable, they are unsuitable for routine identification procedures.

The biology of mycoplasmas was reviewed by Smith (1971a), who described the fine structure of a typical mycoplasma cell, average size 300 - 400 nm diameter, as consisting of a trilaminar membrane surrounding a cytoplasm packed with ribosomes, fibrillar DNA, one or more electron dense areas and occasional empty membrane-bound vesicles. The nuclear material consisted of an unbounded fibrillar and granular region; the fibrils were about 3 nm thick and were not membrane-bound. This is reminiscent of the situation in bacteria. The circular chromosome varied from 444 - 790 x 10^6 daltons, which would allow for coding of well over a thousand cistrons. Interestingly, the Mycoplasma spp. have a genome size in the bottom range of the Mollicutes, while the Acholeplasma spp. have a genome size twice as large (Askaa, Christiansen and Ernø, 1973).

The simplicity of these cells has widened the horizons in many fields of scientific research. Geneticists have used the simple chromosome for genetic mapping; cell biologists have studied the basic requirements for cell survival and biochemists have been greatly aided in enzyme research by using the mycoplasma cell membrane, which is readily prepared and purified. Virologists have also become involved in mycoplasmology because mycoplasmas contaminate tissue cultures and form a close association with the tissue culture cells which may alter some of the biochemical and genetical features of the system (Stanbridge, 1971).

Knowledge of the biology of these organisms has increased rapidly in the past few years and the use of more complex media has enabled many new species to be isolated. However, since so many new organisms are being isolated, classification is becoming increasingly difficult.

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For the Mycoplasmatales, presence or absence of a sterol requirement was the fundamental differential character in the dichotomous key which split the order into two families; the Mycoplasmataceae and the Acholeplasmataccae respectively.

The Mycoplasmataceae (as opposed to the acholeplasmas) could be separated into two broad groups, fermentative and non-fermentative, with respect to energy production. Generally, the fermentative group derived its carbon and energy by the dissimilation of hexoses due to the presence of a glucosidase, as seen in M. gallinarum (Henrikson and Smith, 1964), while the non-fermentative mycoplasmas comprised a group capable of fatty acid oxidation, metabolism of short-chain carbon compounds, and amino acid degradation. Most of the non-fermentative organisms examined had arginine deiminase activity which was found in only one of the fermentative species (M. fermentans) (Barile, Schimke and Riggs, 1966). Mycoplasma canis and M. hyorhinis were among the non-fermentative species which lacked this activity.

Mycoplasma isolates from the bovine respiratory tract were separated initially into three groups depending on their biochemical activity. The first of these groups contains the glucose-fermenters, which utilise the Embden-Meyerhof, hexose monophosphate, pyruvate oxidase, and pyruvate dismutase pathways. During these reactions there is a fall in pH due to the accumulation of lactic or pyruvic acids. In the second group are the arginine-splitters which belong to the non-fermentative class and utilise the tri carboxylic acid cycle, glyoxalate, fatty acid oxidative and probably the pyruvic oxidase pathways. Arginine undergoes a quantitative hydrolytic disimidation to citrulline, which in turn is phosphorylated to yield ornithine and carbamyl phosphate. The latter, in the presence of adenine diphosphate, is cleaved to ammonia, carbon dioxide and adenine triphosphate (ATP) and the release of ammonia raises the pH of the culture medium. The third and final group is the urea-splitters which cleave urea to ammonia and carbon dioxide, a process which raises the pH of the medium. The organisms which possess urease are unique and appear to have no fermentative action, although

energy does not appear to evolve during the urealytic breakdown (Hayflick, 1969; Smith, 1971b).

Many problems were encountered in the cultivation of these slow-growing organisms, the major one being overgrowth by bacteria and fungi found in natural materials, although this was overcome to a certain extent by the incorporation of penicillin and thallium acetate into the growth medium. Detection of growth was difficult, since turbidity was not usually produced in broth culture (unlike bacterial cultures) and so mycoplasmologists have relied on either or both of the following methods to detect the growth of mycoplasmas in a broth culture: (i) change in pH of the medium due to the biochemical activity of the organism (detected by the addition of an appropriate indicator) and (ii) subculture onto solid medium, after an adequate incubation period in broth (colony growth being detected by microscopy).

Typical mycoplasma colonies, described as having a 'fried egg' appearance, were umbonate in shape and ranged from ten to 500 μm diameter. The most dense central portion was due in part to penetration of organisms into the interstices of the agar gel and also to heaping up of the cells. As the fluid dried around the single organisms these were drawn by capillary action into the interstices of the agar gel, the plasticity of the individual cells allowing penetration of the agar. There they began to multiply and spread between the agar fibrils and onto the agar surface. The peripheral area, which represented the growing site, was more confined to the surface and therefore less dense. Variations occurred: sometimes no peripheral area was seen and occasional colonies exhibited a lacy network, thought to be the result of lipid accumulation (Morton et al., 1954). However, colony morphology and size were rarely sufficiently constant and distinctive to be used in distinguishing one species from another. The colonial morphology was influenced by crowding, humidity, agar concentration, pH, gaseous environment and temperature (Smith, 1971a).

For the above reasons mycoplasma cultivation and identification can be either very limited or very complex, since routine laboratory workers are confined to the small number of biochemical tests which are available

(and the exacting requirements for mycoplasma growth render most standard bacteriological procedures of little value), while the taxonomists rely on sophisticated genetical and biochemical techniques. In view of these difficulties, many laboratories rely on serological techniques for the identification of mycoplasmas; fortunately, most of these techniques are fairly simple and reliable.

A synopsis of some of the main biological features of the four bovine mycoplasmas is given below.

1. Mycoplasma dispar

This organism was first isolated by Gourlay (1969) from the pneumonic lungs of six months old calves in the South of England. Many of the characteristics of this species were detailed by Gourlay and Leach (1970), who described the complex glucose-serum isolation medium (GS) containing DNA, lactalbumin hydrolysate and foetal bovine serum which was a modification of the medium devised for the isolation and growth of M. suipneumoniae (Goodwin et al., 1965). Later, Andrews et al. (1973) found that the substitution of ampicillin for benzyl penicillin in GS broth enhanced the isolation of M. dispar.

During primary isolation, growth was usually detectable in GS broth within seven to 14 days after inoculation, although laboratory-adapted strains grew faster. Growth was indicated by an acid shift in the medium due to the fermentation of glucose. Gourlay and Leach (1970) reported no visible growth on 5 per cent ox blood agar, although β-haemolysis was detectable. Growth only occurred in media containing serum and at an incubation temperature within the range 33 - 37°C. Growth was found to be variable and altered with different batches of media.

Early subculture onto GS agar and incubation at 37°C in 5 per cent CO_2 in air or nitrogen, produced large colonies about 0.3 mm in diameter after four days, although Freundt (1974) described colonies up to 1.5 mm after one to three days growth in early subcultures, which became smaller and more dense after further subculturing. These colonies were roughly circular and granular, lacked centres and did not penetrate the agar. Later subcultures

produced smaller colonies, approximately 0.1 mm in diameter, with granular centres which penetrated the agar slightly. Storage of M. dispar at - 70°C caused a loss of ability to produce centred colonies, but this was regained after several subcultures (Gourlay and Leach, 1970).

Giemsa smears of centrifuged deposits revealed pleomorphic organisms, the majority of which were ring forms of varying diameter, but there were also many signet-ring and bipolar forms (Gourlay and Leach, 1970). Light microscopy was generally not very suitable, because of the difficulty of distinguishing the mycoplasma cells from precipitated proteins and tissue extracts. The size of the cells varied between 500 and 1,500 nm by electron microscopy; in addition, Gourlay and Leach (1970) described many somewhat smaller bodies and also very small particles about 150 - 200 nm in diameter. All cells possessed a triple-layered cell membrane with no cell wall, although many had an outer 'fringe' of amorphous material which was found to be a capsule following ruthenium red staining (Howard and Gourlay, 1974). This capsule had no obvious structure but extended for 17 to 24 nm beyond the cytoplasmic membrane. Ruthenium red precipitates phospholipids, acid polysaccharides, mucopolysaccharides and fatty acids.

These organisms were unable to pass through Millipore filters with a pore diameter of 450nm without a considerable loss in viability, since these organisms tended to clump during growth in fluid media (Gourlay and Leach, 1970).

Mycoplasma dispar has a DNA base composition of 28.5 per cent guanine-cytosine (Gourlay and Leach, 1970), which falls within the range characteristic of the Mycoplasma genus.

In a serological comparison of 12 strains of M. dispar, using the metabolism inhibition and indirect haemagglutination tests, all strains formed a serologically homogeneous group although they varied in their haemagglutinating activity against erythrocytes from different animal species (Howard, Gourlay and Collins, 1974). The erythrocyte receptor site did not appear to contain sialic acid and was not sensitive to proteolytic enzymes. Two attachment sites have been demonstrated on the mycoplasma cell. One, by

which the cell attached to sheep and bovine erythrocytes, was a protein or, at least, contained a protein moiety. The chemical nature of the other attachment site, by which M. dispar attached to rabbit erythrocytes, has not been determined.

2. Ureaplasma spp.

Ureaplasmas were first recognised and identified in 1954 in primary agar cultures of urethral exudates from male patients with nongonococcal urethritis (Shepard, 1954). They were called 'tiny-form PPLO' (pleuro-pneumonia-like organisms), 'T-form colonies of PPLO' and 'T-strain mycoplasmas', after the minute size, distinctive characteristics and morphology of their agar colonies. Subsequent studies confirmed that T-strains were new, previously-unknown members of the human mycoplasma group and their distinctive morphology and cultural characteristics were soon described and illustrated in detail (Shepard, 1956).

The first bovine ureaplasmas were isolated from the urogenital tract (Taylor-Robinson et al., 1967) and later Gourlay (1968) isolated them from the respiratory tract of calves with pneumonia.

Controversy over the nomenclature of these organisms resulted in the formation of a new genus in the family Mycoplasmataceae to include the T-strains isolated from man and lower animals. The name Ureaplasma was proposed, with the human species U. urealyticum as the type species (Shepard et al., 1974); the characteristics of this genus were described in detail by these workers.

The ureaplasmas were distinguished from all other known mycoplasmas by their production of urease, and, therefore, by their ability to hydrolyze urea (Shepard and Lunceford, 1967). The release of ammonia and the concomitant rise in medium pH were detected by the addition of pH indicator to the medium. Such a colour test medium has been found invaluable for the isolation of ureaplasmas from clinical specimens.

Shephard et al. (1974) stated that optimal growth occurred at pH 6.0 in a medium supplemented with one to ten per cent yeast extract and ten to 20 per cent unheated foetal calf serum (which supplied the essential requirement

for cholesterol) incubated in an atmosphere of five to 15 per cent CO_2 in air or nitrogen. Growth in broth was rapid and maximum titres were attained after only 24 to 36 hours incubation at 37°C, and even after 16 to 20 hours under some circumstances.

Urea did not appear to be an absolute requirement for the growth of all strains, although its presence increased the rate of growth (Shepard et al., 1974). Urea was hydrolyzed to produce CO_2 and ammonia, which raised the pH and quickly killed the cells. The addition of N-2-hydroxyethyl piperazine -N^1-2-ethane sulphonic acid (HEPES) buffer to the medium delayed the loss in viability (Manchee and Taylor-Robinson, 1969a), as did growth in a vacuum-flow system by maintaining a low concentration of ammonia (Hendley and Allred, 1972).

Ureaplasmas did not ferment carbohydrates and phosphatase was the only enzyme other than urease to be identified (Shepard et al., 1974). They haemolyzed guinea-pig erythrocytes and showed variable reactions with human and sheep erythrocytes. Colonies of ureaplasmas adsorbed to HeLa cells (Shepard et al., 1974).

Colonies of Ureaplasma spp. were generally small (20 to 30 μm in diameter) and normally lacked zones of surface growth. Colonies were generally circular in shape, with a characteristic irregular border. Under optimal growth conditions (incorporation of HEPES buffer) zones of surface growth developed and the colonies often had a similar appearance to classical large-colony Mycoplasma species. Other buffering substances, including sodium phosphate buffer and L-histidine, added to the medium, also increased the colony size (Romano et al., 1975).

Ureaplasmas were predominantly coccoid to coccobacillary elements, approximately 300 nm in diameter, which frequently grew in short filaments; they were non-motile, gram-negative and stained well with Giemsa or similar stains (Shepard et al., 1974). They were bounded by a single trilaminar membrane and did not possess an extracellular capsule when stained with ruthenium red and studied by electron microscopy (Howard and Gourlay, 1974). A more recent examination of the ultrastructure of Ureaplasma spp.

recorded individual cells as spherical, 250-1000 nm in diameter, with a bounding trilaminar membrane, 10 nm thick. The cytoplasm contained 7.5 - 12.5 nm 'particles' and the outer surface was covered by a layer of pilus-like projections, five to eight nm long (Whitescarver and Furness, 1975).

The guanine-cytosine content of the DNA from five bovine Ureaplasma spp. was estimated at 29 - 29.8 moles per cent (Howard et al., 1974), which differed sufficiently from the estimated value for human strains, 26.9 - 28.0 moles per cent (Black, Christiansen and Askaa, 1972) to suggest that the bovine and human strains represented two different species or subspecies.

New methods, based upon the demonstration of urease, permit reliable and specific identification of ureaplasma colonies (Shepard, 1973). A spot test employing a combination of enzyme substrate and test reagent was applied directly to 48 hour old agar colonies (Shepard and Howard, 1970). Direct identification of ureaplasma colonies was then made by incorporating in the growth medium a manganese salt which combined with the ammonia produced by the hydrolysis of urea to form a dark-bronze precipitate around the colony (Shepard and Lunceford, 1970).

Bovine Ureaplasma spp. were serologically distinct from all other recognised mycoplasmas and represented a serologically heterogeneous group (Howard and Gourlay, 1972). Howard and Gourlay (1973b) examined eight bovine ureaplasmas serologically, employing the metabolic inhibition, growth inhibition and indirect immunofluorescent tests and proved that the bovine strains were serologically heterogeneous. Strains with common antigens were isolated from different anatomical sites and there was no evidence that any serotype was confined to a particular organ or was associated with any particular pathological condition.

Howard et al. (1973) examined the virulence of bovine Ureaplasma spp. by testing their ability to produce mastitis in cows. The respiratory tract isolates were virulent, but two urogenital tract isolates and strains from other hosts were avirulent. This experimental model is now being used in immunity studies against ureaplasma infections which appear to be strain specific (Howard, Gourlay and Brownlie, 1974).

3. Mycoplasma bovirhinis

This species appears to be a common inhabitant of the respiratory tract of cattle and was first isolated from the respiratory tract of calves with pneumonia (Harbourne et al., 1965). Mycoplasma bovirhinis grew well on Hayflick's conventional mycoplasma media (Hayflick, 1965), either aerobically or under reduced oxygen tension (Cottew and Leach, 1969). It required a source of serum for growth and had an optimum growth temperature of 37°C, although reports of its growth at 25°C and 42°C (Al-Aubaidi and Fabricant, 1971) contradict those of Cottew and Leach (1969) who obtained no growth at 28°C. The latter workers described β-haemolysis on horse and sheep blood agars produced by M. bovirhinis.

This organism fermented glucose, reduced tetrazolium and methylene blue and could grow at pH 5.5 and 9.5 (Al-Aubaidi and Fabricant, 1971). Aluotto et al. (1970) reported the ability of M. bovirhinis to liquify casein and coagulate horse serum, but Freundt (1974) mentioned conflicting reports on these characteristics. Mycoplasma bovirhinis grew to its optimum concentration in GS broth in three to four days, producing lactic and/or pyruvic acids, which caused a drop in the pH of the medium. On subculture onto solid medium, typical mycoplasma 'fried egg' colonies appeared after two to three days incubation in an atmosphere of five per cent CO_2 in air or nitrogen although growth also occurred aerobically. The colonies were large (approximately 0.1 mm) and not pigmented, with a fairly dense centre where the organisms penetrated deep into the agar medium. The periphery of the colony was often wide and had a general lacy appearance.

Many of the biochemical characteristics of this mycoplasma have not been reported and the morphology has not been fully described (Freundt, 1974), although the guanine-cytosine content of the DNA was estimated as 24.5 - 25.4 moles per cent, which is within the range estimated for this genus (Kelton and Mandel, 1969).

Ultrastructurally, M. bovirhinis has been described as being typical of the order Mycoplasmatales, since the cells were pleomorphic, although

mostly spherical in nature and were bounded by a triple-layered cell membrane. Staining cultures with ruthenium red did not demonstrate the presence of any extracellular capsule (Howard and Gourlay, 1974).

Some strains of M. bovirhinis were able to cause cytopathic changes in bovine embryo kidney tissue cultures (Langer and Carmichael, 1963). However, infection of foetal bovine explant cultures produced no cytopathogenic effects, although the organisms multiplied successfully (Thomas and Howard, 1974).

Few serological studies on this organism have been carried out but cross-reactions with other species were not found in one study (Jain, Jasper and Dellinger, 1967).

4. Acholeplasma laidlawii

Laidlaw and Elford (1936) isolated an organism that formed part of a new group of mycoplasmas, the acholeplasmas, by cultivation of the gradocol membrane filtrates of raw London sewage. This group was found to grow in culture medium at room temperature without supplementation. Under anaerobic conditions, the medium became pigmented and it was later shown that this yellow pigmentation was due to the synthesis of carotenoids by the organisms (Smith, 1960). Seiffert (1937a and b) found mycoplasmas of similar nature to the saprophytes of Laidlaw and Elford in soil, compost, leaves and manure. Harbourne et al. (1965) were the first to isolate A. laidlawii from the respiratory tract of cattle, culturing it from nasal swabs of cattle with a respiratory disease.

Acholeplasma laidlawii has become the E. coli of mycoplasmology because of its ubiquitous habitat and rapid growth on simple media. Detailed studies have been made of its biochemical, physical and serological characteristics. It was readily distinguishable from all other bovine mycoplasmas by its ability to grow in indefinite subculture in media lacking serum or other cholesterol-supplying additives and by its ability to grow well at 22°C. The former characteristic was due to its ability, unique among mycoplasmas, to synthesise carotenoids, which appeared to perform some of the functions

normally carried out by added sterols. Mycoplasmas were sensitive to the reagent digitonin, which caused lysis of the cells, because they were unable to anabolise sterols and had a subsequent requirement for sterols in the growth medium. Acholeplasma laidlawii, on the other hand, was resistant to this detergent when grown in minimal medium; however, it was found that this organism became sensitive as soon as it was grown in the presence of cholesterol, indicating the ability of the cells to utilise the sterol when present (Razin and Argaman, 1963). Resistance to digitonin and to sodium-polyanethol-sulphonate, was used routinely to distinguish acholeplasmas from mycoplasmas and was carried out by a simple growth inhibition technique employing paper discs impregnated with one of these compounds (Freundt et al., 1973). In addition, A laidlawii lysed readily in medium of low ionic strength when grown in the absence of cholesterol.

Freundt (1974) described the minimal nutritional requirements of this organism, the only mycoplasma for which a specific medium has been detailed. These requirements include potassium, magnesium and phosphate ions, glucose, 13 amino acids, nucleic acid precursors, vitamin derivatives and fatty acids.

Growth of A. laidlawii was profuse in standard mycoplasma broth occasionally producing a faint turbidity within 24 hours at $37^{o}C$, and after subculture on horse serum agar colonies often exceeding 1 mm in diameter could be seen. Leach (1967) reported that a few newly-isolated strains from bovine sources failed to cause significant fermentation of glucose, but with most well-established strains GS broth was fermented rapidly.

Acholeplasma laidlawii grew with characteristic mycoplasma colonies, up to 1 mm in diameter when not crowded closely together. The central area was clearly demarcated and regular, with the peripheral growing area lacy and vacuolated. However, under crowded conditions this peripheral growth was limited, the colonies were restricted to the central area and in some cases smaller colonies could be seen growing from the large colony.

Ultrastructurally, this organism has been well studied and has been used to identify the typical features of these microorganisms. Weibull and

Lundin (1962 and 1963) studied the morphology of A. laidlawii and described the primary morphological unit of young viable cultures as a coccoid element about 500 nm in diameter. The size of the cell varied with the stage of the life cycle and could range from 100 - 600 nm in diameter. Acholeplasma laidlawii is the model mycoplasma, but it may be atypical in some respects. Rottem, Stein and Razin (1968) demonstrated a cross-striated-type appearance of the membranes, when viewed electron microscopically in this section after negative staining with phosphotungstic acid, a feature not described in any other species.

Unlike the Mycoplasma genus, this organism has a higher guanine-cytosine content in its DNA, estimated at 31.7 - 34.4 moles per cent (Kelton and Mandel, 1969), which falls within the range estimated for other Acholeplasma species.

Acholeplasma laidlawii, as stated previously, has been a model scientific organism and the ultrastructure of the ribosomes, cell membrane and DNA have been studied in detail. The ease with which the cell membrane can be prepared and purified has enabled many biochemical and serological studies to take place. Serologically, all strains of A. laidlawii make up a homologous group (Jain et al., 1967) although Al-Aubaidi and Fabricant (1971) found strains which cross-reacted with M. mycoides var. mycoides and their group E mycoplasma.

Property	Presence (+) or Absence (-) of Property with		
	Mycoplasmas	Bacteria	Viruses
Ubiquitous nature	+	+	+
Growth in cell-free medium	+	+	-
Smallest forms 100 nm or less	+	-	+
Cell wall absent	+	-	+
Contain DNA and RNA	+	+	-
Various Metabolic systems	+	+	-
Growth inhibition by antibody	+	-	+
Growth inhibition by antibiotic	+	+	-

Table 10. Properties of Mycoplasmas in Relation to Bacteria and Viruses (Taylor-Robinson, 1968).

Mycoplasmas	L forms
Occur in nature	Usually laboratory artefacts.
Do not revert to bacterial forms if grown in antibiotic-free media.	Unstable L forms will revert to bacterial form on removal of agent (e.g. antibiotic) used to produce L form.
Limited demonstrable metabolic activity.	L form will often have similar metabolic activity to parent organism.
Not related to bacteria genetically.	Genetically indistinguishable from parent organism.
Guanine-cytosine per cent in DNA bases may be lower than for any known bacteria.	Guanine-cytosine per cent in DNA bases as for parent bacteria and often higher than for mycoplasmas.

Table II. Differences between mycoplasmas and bacterial L forms (modified after Fallon and Whittlestone, 1969).

CHAPTER FOUR

A COMPARISON OF FIVE METHODS FOR THE ISOLATION OF MYCOPLASMAS FROM BOVINE RESPIRATORY TISSUE

A. INTRODUCTION

B. MATERIALS AND METHODS

1. Experiment 1.
2. Experiment 2.

C. RESULTS

1. Experiment 1.
2. Experiment 2.

D. DISCUSSION

A. INTRODUCTION

The recovery rates for mycoplasmas from tissue can be variable. Isolation is considered to be difficult since there are many problems associated with tissue-mycoplasma relationships and the cultural requirements.

A possible cause of low yields from tissue is the attachment of the organisms to cell surfaces. Although mycoplasmas are considered to be extracellular organisms (Hayflick, 1969), ultrastructurally they can be seen in very close contact with cell surfaces; M. gallisepticum, M. pneumoniae and M. mycoides var. mycoides all possess features which would enable specific attachment to other cells (Maniloff and Morowitz, 1967; Maniloff and Quinlan, 1973; Rodwell, Peterson and Rodwell, 1973). Manchee and Taylor-Robinson (1969b) studied the nature of receptors involved in the attachment of mycoplasmas to tissue culture cells and found that N-acetyl neuraminic acid residues on HeLa cells were responsible for the adsorption of M. gallisepticum and M. pneumoniae. Proteolytic receptors on the tissue cells were required for the adsorption of M. hominis and M. salivarium and these were destroyed by trypsin and formalin. These workers also found that neuraminidase treatment of mycoplasmas did not affect their attachment to tissue cells. However, Sobeslavsky, Prescott and Chanock (1968) claimed that adsorption of M. pneumoniae to epithelial cells and erythrocytes was prevented by pretreatment of cells with neuraminidase or Influenza B virus, or pretreatment of the mycoplasma cells with neuraminic acid. More recently, Collier and Baseman (1973) demonstrated M. pneumoniae organisms attaching to both hamster and human respiratory epithelium in organ culture by a specialised terminal structure and causing a cytopathic change in the epithelium. Treatment of mycoplasmas and tissue culture cells with neuraminidase demonstrated that attachment of M. pneumoniae through sialic acid residues was limited to virulent strains only.

Attachment of M. gallisepticum to erythrocytes, lymphocytes and fibroblasts required an ambient temperature and also occurred after the organisms were disrupted by freeze-thawing (Thomas, 1969). Attachment did not occur when heat-killed or trypsin-treated organisms were used or when

tissue cells were treated with neuraminidase. This data suggested that adsorption required a tissue cell sialic acid determinant and a mycoplasma surface -protein component (Thomas, 1969).

Jones, Yeh and Hirsch (1972) found that M. pulmonis bound avidly to mouse peritoneal macrophages. Attachment occurred in media containing no nutritional supplements or divalent cations; attachment was not blocked by proteolytic enzymes, neuraminidase, lysozyme or by exposure of the mycoplasmas to glutaraldehyde, heat or repeated freezing and thawing. Only glutaraldehyde fixation of both the macrophages and the mycoplasmas and the use of a nonionic sucrose medium prevented attachment.

Preliminary studies with M. dispar demonstrated that this organism did not attach to erythrocytes by a sialic acid residue, although specific binding to bovine and sheep erythrocytes was via a protein moiety (Howard et al., 1974). The extracellular capsule of M. dispar, demonstrated ultrastructurally with ruthenium red staining (Howard and Gourlay, 1974), suggests a possible mechanism of attachment to other cells.

In addition to the problems of specific attachment of mycoplasmas to tissue, the inaccessibility of these organisms in the specimen may contribute to the low isolation rates. Many tissues, particularly lung, have a large surface area on which mycoplasmas can exist and this may enhance the difficulty of detecting them. Breakdown of tissue to expose mycoplasmas could, however, release tissue extracts which were mycoplasmacidal in nature. Tully and Rask-Nielson (1967) first noted the inhibitory effects of homogenised tissue, which were later demonstrated in relation to mycoplasmas by Kaklamanis et al. (1969), who suggested that the active tissue extract was a lysolecithin. Mårdh and Taylor-Robinson (1973) found that lysolecithin killed mycoplasma cells while the addition of ammonium reineckate or lysophospholipase to medium containing lysolecithin abolished its activity against M. pulmonis. Commercially available ammonium reineckate allowed the isolation of M. pneumoniae from hamster lung suspensions that would otherwise have inhibited its growth (Mårdh and Taylor-Robinson, 1973).

Other substances, such as lysosomes released from damaged inflammatory cells and local or systemic antibody possibly present in the tissue, may contribute to a low yield of mycoplasmas on culture.

Many pathogenic mycoplasmas are undetected when diseased tissue is examined in routine diagnostic laboratories. Their small size and special requirements for nutrients and culture conditions has led to the development of complex and poorly defined isolation media which may vary in their ability to support the growth of pathogenic mycoplasmas.

Barile (1974) has indicated some of the problems encountered in general isolation procedures for mycoplasmas. Cultural conditions were considered very important, particularly medium constituents, type of agar, choice of antibiotics, pH, atmospheric conditions and temperature, with a slight variation in any factor preventing the optimal isolation frequency of certain mycoplasmas being attained.

Whittlestone (1974) suggested that cultural examinations of animal diseases should be performed on tissue from cases at an early stage of the disease, since the primary pathogens are then usually present in large numbers while contaminant and secondary organisms are often absent or present in only low titres so the inhibitory effects of the products of cell destruction are unlikely to be present.

Several techniques for the isolation of mycoplasmas from the bovine respiratory tract have been reported. Direct smears of lung tissue onto solid agar and the inoculation of minced tissue directly into semi-solid media were the earlier techniques (Harbourne et al., 1965; Davies, 1967). A more recent method involved the inoculation of standard samples of triturated lung tissue into the appropriate mycoplasma broth in serial ten-fold dilutions (Gourlay et al., 1970; Thomas and Smith, 1972). This dilution technique avoided overgrowth with bacteria or secondary mycoplasmas, as the higher dilutions often yielded the pathogenic mycoplasmas. The presence in concentrated tissue suspensions of a mycoplasmacidal factor is an additional reason for using dilute tissue suspensions (Whittlestone, 1974).

Homogenisation of bovine lung has also been used to isolate mycoplasmas, by diluting the supernate of the homogenate serially in broth medium (Livingston, 1972; Shimizu et al., 1975).

Lindsey and Cassell (1973), in pathogenesis experiments with M. pulmonis in mice, obtained good isolation rates by flushing the trachea and bronchi with 0.6 ml of mycoplasma broth and subsequently inoculating 0.1 ml of the aspirate into 3 ml of broth culture.

The present study was undertaken to find the best method of isolating mycoplasmas from bovine respiratory tissue. Initially, five techniques of lung tissue treatment were studied and the isolation rates of M. dispar and Ureaplasma spp. were compared for each method. The three most successful treatments were used in a subsequent experiment on pneumonic tissue from 12 calves, whose lungs had previously been cultivated for mycoplasmas and bacteria, to see which treatment gave the highest isolation rates and titres. An additional study was made of the viability of the organisms after storage at - 70°C for approximately ten months and the efficiency of ampicillin and thallium acetate as bactericidal and fungicidal agents respectively.

B. MATERIALS AND METHODS

1. Experiment 1.

a. Tissue.

Lung tissue from two six months old pneumonic calves was used in this experiment to test for the presence of M. dispar and Ureaplasma spp. respectively. The tissue samples had been stored in PBS, GS and U3 broths at - 70°C for approximately ten months. Duplicate samples of tissue from both cases had been examined culturally for mycoplasmas and bacteria prior to storage.

b. Treatments of tissue samples.

Four treatments (1 - 4) were used, with the original cultivation technique acting as control. The five treatments were:

1. Homogenisation.
2. Light chopping and shaking.
3. Treatment with trypsin.
4. Treatment with neuraminidase.
5. Light chopping and incubation (control).

These were carried out on both samples of lung tissue as follows:

1. Homogenisation. Approximately 1.0 cm^3 of lung tissue was chopped lightly with scissors and homogenised in 1.0 ml of broth for 30 seconds in a Stomacher 80 (Colworth laboratory equipment).

2. Light chopping and shaking. Approximately 1.0 cm^3 of lung tissue was chopped lightly with scissors and shaken in broth on an automatic shaker at 37°C for 30 minutes.

3. Treatment with trypsin (Manchee and Taylor-Robinson, 1969b). A 0.25 per cent solution of trypsin (Difco), diluted in PBS (pH 7.3) containing no Mg^{2+} or Ca^{2+} ions, was prepared. Approximately 1.0 cm^3 of lung tissue was chopped lightly with scissors in 1 ml of the trypsin solution, incubated at 37°C for 30 minutes and shaken vigorously by hand.

4. Treatment with neuraminidase. The enzyme used was a crude receptor destroying enzyme (RDE) from Vibrio cholerae containing 0.02 units per ml (Sigma Chemical Co.). Approximately 1.0 cm^3 of lung tissue was lightly chopped with scissors and incubated at 37°C for one hour in 1 ml of the neuraminidase solution. After incubation the sample was shaken vigorously by hand for several minutes.

5. Light chopping and incubation (control). Approximately 1.0 cm^3 of lung tissue was lightly chopped with scissors and incubated at 37°C for 30 minutes in broth media.

c. Cultural techniques.

Serial ten-fold dilutions, up to 10^{-6} of the original sample, were

carried out on all lung specimens after treatment. This involved the removal of 0.2 ml of the test supernate or homogenate and its transfer into 1.8 ml of the appropriate broth medium. Tissue to be examined for the presence of M. dispar was inoculated into GS broth, while Ureaplasma spp. were detected in U3 broth. The titre of the mycoplasmas was recorded as CCU per 0.2 ml of sample, indicated by the final dilution which gave a colour change after a significant period of incubation. Bacteria present in the lung tissue after treatment were detected by inoculation of an undiluted sample onto horse blood agar, chocolate blood agar and MacConkey agar; anaerobes were isolated by inoculating duplicate horse blood and chocolate blood agar plates and incubating in anaerobic conditions. The average number of bacterial colonies from the inoculated agar plates for each treatment was recorded. Procedures of inoculation, incubation and identification were detailed in chapter two, section C (General Materials and Methods).

2. Experiment 2.

a. Tissue.

Pneumonic lung tissue from 12 calves previously examined for mycoplasmas and bacteria was used. The calves were six months of age at slaughter and were the pneumonic animals from a group of 20 (designated M60 to M79), referred to as Group D in chapter five. The tissue had been stored in GS and U3 broths at -70^{o}C for about ten months. A record was made of the isolation titres of M. dispar, Ureaplasma spp. and the presence or absence of bacteria from the original isolation procedures.

b. Treatments of tissue samples.

For each animal three samples of tissue underwent the following three treatments:

1. Homogenisation.
2. Treatment with neuraminidase.
3. Light chopping and incubation (control).

The treatment procedures used were described above for experiment 1.

c. Cultural techniques.

The tissue for each treatment was examined for the presence of M. dispar, Ureaplasma spp. and for the presence or absence of bacteria as described for experiment 1.

C. RESULTS

1. Experiment 1.

Pneumonic lung tissue was treated in five different ways and subsequently cultured for the presence of M. dispar from one calf and Ureaplasma spp. from the other. The results in Table 12 indicate the day on which the appropriate dilution of GS broth changed colour, indicative of mycoplasmal growth; the organism was subsequently identified by immunofluorescent techniques as M. dispar. The mean bacterial colony number is also given in Table 12, the average number of colonies being taken from all the aerobically incubated plates. The anaerobic plates were not included since very few colonies grew and their numbers were considered insignificant. Four of the five treatments yielded M. dispar on cultivation. Treatment 3, trypsin digestion of lung tissue for 30 minutes, did not yield any organisms, neither mycoplasmas nor bacteria. The neuraminidase digestion of treatment 4 was successful and an end titre of 10^3 CCU per 0.2 ml of sample was found within five days. The control method, treatment 5, chopping and incubating the tissue, allowed the highest recovery of M. dispar, giving a final titre of 10^4 CCU per 0.2 ml of sample, although this titre was not obtained until ten days post inoculation. Treatments 1 and 2 gave similar results with a final titre of 10^3 CCU per 0.2 ml of sample being reached. However, these results were only attained after 17 days incubation.

The recovery of Ureaplasma spp. from pneumonic tissue treated in the same way, is presented in Table 13. The results of isolations of Ureaplasma spp. by the five treatments were different from those of M. dispar, with tissue homogenisation (treatment 1) giving a final titre of 10^3 CCU per

0.2 ml of sample after three days incubation. Bacterial counts were also high after homogenisation. A titre for Ureaplasma spp. of 10^2 CCU per 0.2 ml of sample was attained after two days incubation following treatment 4, which involved neuraminidase digestion of the lung tissue for one hour. Treatments 2, 3 and 5 did not yield significant numbers of Ureaplasma spp..

2. Experiment 2.

In considering the results of M. dispar and Ureaplasma spp. isolations in experiment 1, three tissue treatments were considered superior. These were 1. homogenisation, 2. neuraminidase treatment and 3. lightly chopping and incubation. These three treatments were subsequently used on pneumonic tissue from 12 calves whose lungs had previously been cultivated for mycoplasmas and bacteria, to see which treatment gave the highest isolation rates and titres. These titres were finally compared to the results of the original isolations. The isolation rates and titres of M. dispar and Ureaplasma spp. using the three treatments on the 12 pneumonic calf lungs are set out in Table 14.

Only treatment 3, the control method of chopping lightly and incubating, consistently produced M. dispar on culture. The isolation frequency was higher than that obtained by the original procedure, since nine of the 12 pneumonic cases yielded the organism, compared to six initially. All the cases which were originally found to be infected with this organism still yielded it under these conditions. The isolation titres were much reduced, however and the highest obtained was 10^3 CCU per 0.2 ml of sample, compared with 10^5 CCU per 0.2 ml of sample in the original cultivation.

Treatment of tissue stored in U3 broth at -70°C for ten months prior to cultivation for Ureaplasma spp. gave variable results. In this experiment, the isolation frequency of Ureaplasma spp. fell from 66 per cent by original cultivation to 50 per cent when considering the three techniques together. Three of the six cases from which Ureaplasma spp. were cultivated had not previously yielded these organisms. It is difficult to decide from these results which of the three methods of lung tissue treatment was the most efficient

for the isolation of Ureaplasma spp., as all three methods obtained comparable titres from some of the cases examined. Final titres of organisms were similar to those recorded by the original isolation procedure.

Comparison of the titres obtained by any of the methods employed with those from cultivation on fresh tissue, revealed a loss in viability especially of M. dispar in which a drop of 10^1 to 10^2 CCU per 0.2 ml of sample was generally found.

The lung tissue samples had been stored at -70°C in GS and U3 broths, both of which contained the bactericidal and fungicidal agents ampicillin and thallium acetate respectively. After tissue treatment, these specimens were cultured for the presence of bacteria and the results are also given in Table 14 (a positive result indicated the growth of at least two colonies from one loopful of sample). The results of the bacterial isolations from fresh tissue collected in PBS are also given. These results indicate that the antibiotics present in the media provided a reasonably efficient action; only four of the 12 pneumonic cases retained bacteria after storage and these were present in small numbers, compared to positive bacterial isolations from fresh tissue of ten calves. Fungi were not detected in either the fresh or the stored tissue.

D. DISCUSSION

Many difficulties are encountered when attempting to cultivate mycoplasmas from infected tissue. In experiment 1, pneumonic lung tissue from a calf infected with M. dispar and from one infected with Ureaplasma spp. were treated in five different ways to observe if any of the techniques improved the recovery of these organisms. In considering the recovery rates of both organisms by all five methods, the three methods of homogenisation, neuraminidase treatment and light chopping and incubation were found to be the most successful. Homogenisation yielded the highest titre for Ureaplasma spp. isolation, while the control method, which had been used for the initial isolation, was most successful for the recovery of M. dispar.

Treatment of lung tissue with trypsin was unsuccessful, no mycoplasmas or bacteria were grown from the M. dispar-infected tissue and only a few organisms from the tissue infected with Ureaplasma spp.. This result agrees with the study of Barile (1974), who found trypsinised tissue culture cells were poor specimens for mycoplasma isolations.

In M. dispar infected lung, lightly chopping the tissue followed by shaking for 30 minutes at 37°C (treatment 2) gave a similar result to the homogenisation treatment, but the former technique was not successful in the recovery of Ureaplasma spp.. This result is perhaps a little surprising, since an increase in the number of organisms released would be expected after shaking the tissue in the mycoplasma broth. In addition, it is similar to the technique of incubating the tissue after lightly chopping, which was successful.

Using the three most successful treatments of lung tissue in experiment 1, the isolation frequency of M. dispar and Ureaplasma spp. from pneumonic lung tissue of 12 calves was examined in experiment 2. The tissue had been examined prior to deep-freeze storage and the frequency of isolation of M. dispar and Ureaplasma spp. and the isolation titres were noted and compared with the results after deep-freeze storage (experiment 2). The treatment which had been used on fresh tissue was the most successful for the isolation of M. dispar after deep-freezing, although the titres of the organisms cultivated were reduced. This was not unexpected. An interesting and slightly surprising finding, however, was the increased number of M. dispar isolates from lung tissue which had been stored at -70°C for ten months. All three techniques used for the recovery of Ureaplasma spp. had some success, but the results were variable and there was a decreased isolation frequency when compared to the original.

The results of experiments 1 and 2 suggested that the method of treatment used initially (i.e. chopping lightly and incubating) was the most successful way of attaining optimal recovery of mycoplasmas from bovine lung tissue. Light chopping of the tissue with scissors increases the surface area exposed to culture media and may enhance the release of organisms;

incubation for 30 minutes at 37°C possibly allows the organisms to free themselves from the tissue and begin growth. The presence of ampicillin and/or thallium acetate in the medium reduces the activity of any bacteria present and allows the unrestricted growth of mycoplasmas. However, the destruction of cells by chopping may release some mycoplasmacidal tissue factors and may also release complement, antibody and other inhibitors from severed blood capillaries. In contrast to the homogenisation of tissue, these factors must only be present in small quantities. Homogenisation of tissue was relatively successful in these experiments, although it is not a recommended procedure because of the release of the mycoplasmacidal tissue extracts (Barile, 1974). However, the homogenisation technique used on the lung tissue in this study probably minimises this effect since the action of the stomacher is more of cell dispersal than cell destruction.

Sialic acid residues are not considered important in the attachment of M. dispar to erythrocytes (Howard et al., 1974) and the expense of the neuraminidase solution does not commend this method for the treatment of tissue prior to cultivation.

In experiment 2 there was an increase in the frequency of M. dispar isolations but not of Ureaplasma spp.. The increase in isolation frequency of M. dispar is puzzling, since one would expect a drop in frequency due to loss of viability after deep-freeze storage. It is possible that some of the inherent inhibitors in the tissue specimen may be destroyed or reduced in activity under these storage conditions. The decrease in titres of M. dispar perhaps contradicts this hypothesis, although this reduction may be due to the loss of viability of individual mycoplasma organisms since lack of a cell wall renders them more vulnerable to physical factors. These two features, tissue inhibitors and cell viability factors, may be independent entities which are altered by different conditions. For example, freezing and thawing of stored material may destroy some of the tissue inhibitors present in the fresh specimen at the time of initial culturing which resulted in negative M. dispar isolations. Simultaneously, the titre of this organism may be reduced by

loss of viability, consequently giving an increased isolation frequency of reduced titre.

The results for Ureaplasma spp., however, are unlike those for M. dispar. The overall frequency of isolation of these organisms was reduced from eight to six out of 12 calves by culturing the stored tissue and three of the six isolates were from animals which were negative on initial culture. Titres of Ureaplasma spp. obtained from culturing stored tissue were comparable to the original titres from fresh tissue, again unlike the result for M. dispar. The results of these experiments suggest that the effects of deep-freeze storage vary with the organism under examination; this is supported by recent studies on Ureaplasma spp., which were found to be resistant to freeze-thawing (Whitescarver, Castillo and Furness, 1975).

The tissue samples used in experiment 2 had been stored in medium containing ampicillin and/or thallium acetate and culturing for the presence of bacteria after treatment of the tissue tested the efficiency of these antibiotics. The results indicated that these agents were adequate antibiotics against bacteria and fungi which may have been present in the tissue. This experiment does not indicate whether these agents have an effect on the mycoplasmas themselves, perhaps to lower the final titre. This effect was seen in the early M. dispar isolations when the titre of organisms recovered was increased by substitution of ampicillin for benzyl penicillin in the medium (Andrews et al., 1973). However, the presence of antibiotics is essential since overgrowth by bacteria and fungi would in many cases mask the presence of any mycoplasmas. Ampicillin, which acts on the cell wall components of bacteria, is not known to have any effect on mycoplasmas; thallium acetate, on the other hand, is slightly inhibitory to the growth of Ureaplasma spp. and is generally omitted from U3 broth media, although it is used in low concentrations for primary isolations (Shepard, 1967).

Treatment *	GS broth dilution 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5} 10^{-6} Incubation period (days) to obtain a colour change.						Mean bacterial colony counts.
1	5	10	17	-	-	-	-
2	6	6	17	-	-	-	1
3	-	-	-	-	-	-	-
4	5	5	5	-	-	-	-
5	6	6	6	10	-	-	8

* The five tissue treatments were 1. homogenisation, 2. chop and shake, 3. trypsinisation, 4. neuraminidase digestion and 5. chop and incubate.

Table 12. Length of incubation (days) required to obtain a colour change in GS broth indicating the presence of M. dispar (after treatment of tissue). The mean number of bacterial colonies obtained after each treatment of tissue is also given.

Treatment *	U3 broth dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6} Incubation period (days) to obtain a colour change.	Mean bacterial colony counts
1	1	1	3	-	-	-	50
2	2	-	-	-	-	-	-
3	1	-	-	-	-	-	2
4	1	2	-	-	-	-	-
5	2	-	-	-	-	-	2

*The five tissue treatments were 1. homogenisation, 2. chop and shake, 3. trypsinisation, 4. neuraminidase digestion and 5. chop and incubate.

Table 13. Length of incubation (days) required to obtain a colour change in U3 broth indicating the presence of Ureaplasma spp. (after treatment of tissue). The mean number of bacterial colonies obtained after each tissue treatment is also given.

Calf No.	Titre of M. dispar from lung tissue Treatments *				Titre of Ureaplasma spp. from lung tissue Treatments				Presence (+) or absence(-) of bacteria before and after storage in media	
	Original	1.	2.	3.	Original	1.	2.	3.	Before	After
M60	10^2	-	-	10^2	-	-	10^3	-	+	-
M62	10^1	-	-	10^2	10^1	-	-	-	+	-
M63	10^3	-	-	10^2	-	10^2	-	10^2	+	-
M65	-	-	-	10^2	10^1	-	-	-	-	-
M70	10^5	-	-	10^3	-	-	-	10^2	+	+
M71	-	-	-	-	-	-	-	-	+	-
M72	-	-	-	-	10^1	-	-	-	+	-
M74	-	-	-	10^2	10^2	10^2	10^2	10^2	-	-
M75	-	-	-	10^1	10^2	10^2	-	-	+	+
M77	10^4	-	-	10^2	10^2	-	-	-	+	-
M78	10^4	-	-	10^1	10^1	-	-	-	+	+
M79	-	-	-	-	10^1	-	-	10^3	+	+
Isolation frequency	$^6/_{12}$ (50%)			$^9/_{12}$ (75%)	$^8/_{12}$ (66%)			$^6/_{12}$ (50%)	$^{10}/_{12}$	$^4/_{12}$

* The three treatments of tissue were 1. homogenisation, 2. neuraminidase digestion and 3. chop and incubate.

Table 14. Results of isolation frequencies and titres of M. dispar and Ureaplasma spp. from pneumonic lung tissue following three selected treatments. The bacterial isolations from fresh tissue and tissue stored in medium containing antibiotics are also given.

CHAPTER FIVE

MICROBIOLOGICAL AND PATHOLOGICAL FINDINGS IN FOUR AGE GROUPS OF CALVES

A. INTRODUCTION

B. CALVES LESS THAN ONE MONTH OLD

1. Introduction

2. Materials and methods

   a. Animals.

   b. Post mortem techniques.

3. Results

   a. Mycoplasmal and bacterial isolations.

   b. Pathology.

4. Discussion

C. CALVES ONE TO TWO MONTHS OLD

1. Introduction

2. Materials and methods

   a. Animals.

   b. Post mortem techniques.

3. Results

   a. Mycoplasmal and bacterial isolations.

   b. Pathology.

   c. Immunofluorescence.

   d. Serology.

4. Discussion

D. CALVES THREE TO FOUR MONTHS OLD

1. Introduction

2. Materials and methods

   a. Animals.

   b. Post mortem techniques.

3. Results

   a. Pre mortem examinations.

   b. Mycoplasmal and bacterial isolations.

   c. Pathology.

   d. Immunofluorescence.

   e. Serology.

4. Discussion

E. CALVES SIX MONTHS OLD

1. Introduction

2. Materials and methods

   a. Animals.

   b. Post mortem techniques.

3. Results

   a. Mycoplasmal and bacterial isolations.

   b. Pathology.

   c. Immunofluorescence.

   d. Serology.

4. Discussion

F. DISCUSSION

A. INTRODUCTION

Respiratory disease in housed calves has been an important problem for many decades and the recent intensification in beef husbandry has emphasised its economic implications. The aetiology of calf pneumonia under such conditions is complex and numerous agents have been recovered from infected lungs.

Early reviews on some of the agents isolated from the bovine lung report briefly on the presence of mycoplasmas in the respiratory tract (Omar, 1966; Darbyshire and Roberts, 1968), but extensive research in the past five to eight years has focussed attention on these organisms, particularly in association with pneumonia in calves. Bovine Ureaplasma spp. and M. dispar were first isolated from pneumonic calf lung tissue (Gourlay, 1968 and 1969; Gourlay and Leach, 1970); isolations of both these species from pneumonic calf lungs in Great Britain and abroad have since been reported (Gourlay et al., 1970; Romano et al., 1971; Livingston, 1972; Ruhnke and van Dreumel, 1972; St. George et al., 1973; Ose and Mcunster, 1975; Shimizu et al., 1975; Bitsch, Friis and Krogh, 1976) and preliminary investigations on their pathogenesis suggest that they may play an important role in the calf pneumonia complex. The ability of both species to produce experimental pneumonia in young calves (Gourlay and Thomas, 1969 and 1970; St. George et al., 1973; Gourlay et al., 1976) and their association with reported outbreaks of calf pneumonia supports this suggestion (St. George et al., 1973; Shimuzu et al., 1975). However, Ureaplasma spp. and M. dispar have been isolated from the respiratory tract of calves considered normal (Gourlay and Thomas, 1970; Thomas and Smith, 1972).

Three other species of mycoplasmas have been recovered from pneumonic calf lungs in Great Britain; the species isolated were M. bovirhinis, A. laidlawii and M. agalactiae var. bovis (Langer and Carmichael, 1963; Harbourne et al., 1965; Davies, 1967; Gourlay et al., 1970; Jurmanová and Krejčí, 1971; Thomas et al., 1975). Mycoplasma bovirhinis and A. laidlawii are considered unimportant in the bovine respiratory tract (Gourlay, 1973); the significance of M. agalactiae var. bovis in this site is not established since

it has recently been associated with a respiratory outbreak in calves (Thomas et al., 1975).

Many problems are associated with the detection of mycoplasmas from diseased tissue although the current biological studies of these organisms, particularly of their physical characteristics and metabolic processes, have enabled some of these difficulties to be overcome. Many mycoplasmas are slow-growing and have complex nutritional and atmospheric requirements, consequently overgrowth by bacteria and fungi is a common problem; indirect methods of detection are being developed. However, many mycoplasmas may often be undetected in diseased tissue.

Serological techniques are available for the detection of serum antibodies to mycoplasmas; some methods are more sensitive to certain species of mycoplasmas than others. The metabolic inhibition test is considered an accurate method of detecting antibody to many bovine mycoplasmas and is also used for the species and strain identification of Ureaplasma spp. and M. dispar (Howard and Gourlay, 1973b; Ernø, 1974; Shepard et al., 1974); other techniques such as growth inhibition, latex agglutination and immunofluorescent methods are also widely used.

Bacteria have been recovered from the bovine respiratory tract for many years and their occurrence in calf pneumonia is well established (Omar, 1966); their activity as primary aetiological agents is unclear (Ide, 1970) although in field cases lesions attributable to infection by bacteria, especially Pasteurella spp. are seen. However, many bacteria including Pasteurella spp., are not considered to be capable of damaging the bronchial epithelium and only cause disease after damage, presumed to be caused by the invasion of respiratory viruses (Collier, 1968). Pasteurella spp., Corynebacterium pyogenes and Haemophilus spp. have been considered the most pathogenic species of the bovine respiratory tract, and act as opportunistic invaders in the calf pneumonia complex (Omar, 1966).

Pasteurella spp. were isolated from approximately 30 per cent of 65 pneumonic calf lungs (Gourlay et al., 1970) suggesting that they may be important in pulmonary disease but no association with a specific histological

lesion was found.

In addition to the normal respiratory flora, the presence of potential pathogens in the nasal cavity of healthy calves introduces difficulties in assessing the significance of bacterial isolations from respiratory tissue.

A group of organisms which warrant mention in the calf pneumonia complex is the chlamydiae which occur in the bovine respiratory tract; biologically they are poorly understood because of the problems associated with growing them. An experimental pneumonia has been produced in calves by these organisms suggesting them to be pathogenic. The difficult cultivation of chlamydiae has led to the development of the complement fixation test for the detection of serum antibodies to these organisms and it is used diagnostically in routine laboratories. This serological test was used by Thomas and Collins (1974) who examined over 1000 calves ranging in age from ten days to six months but no relationship between chlamydiae and disease of the lower respiratory tract could be detected.

Viruses have been implicated as playing a primary role in the calf pneumonia complex (Phillip, 1970). Six viruses are considered to be closely associated with the bovine respiratory tract and include (i) IBR virus, (ii) PI3 virus, (iii) adenovirus, (iv) reovirus, (v) rhinovirus and (vi) mucosal disease virus; their pathogenic role has been reviewed several times (Omar, 1966; Darbyshire and Roberts, 1968; Phillip, 1968).

Infectious bovine rhinotracheitis virus and adenovirus have been recognised as agents of specific pneumonias in calves. Parainfluenza-3-virus is considered pathogenic but its importance is in natural outbreaks complicated by superinfections with bacteria such as Pasteurella haemolytica, manifesting a clinically severe disease (Heddleston, Reisinger and Watko, 1962). Clinical disease could not be produced experimentally with either reovirus or mucosal disease virus although pulmonary lesions were noted at post mortem examination; rhinovirus is particularly associated with mild upper respiratory tract infections.

Many of the bovine respiratory viruses are slow-growing and difficult to cultivate consequently a large proportion of viral diagnoses are

based on serological techniques. The presence of maternal antibody resulting in false positives is a disadvantage of the use of serology for antibody detection. This problem can be overcome if paired serum samples are tested when an increase in titre indicates infection with the virus being studied. The presence of maternal antibody and the non-specificity of some of these techniques has made establishment of a significant base-line titre in viral infection difficult. Thomas (1973) claimed that haemagglutination-inhibition serum antibody titres over 1 in 20 to P13 virus gave protection against infection; the criterion of protection was the lack of a further serological response by the individual.

The true role played by bovine respiratory viruses is still unclear and it is becoming increasingly evident from reports throughout the world that certain viruses, currently accepted as being important in the aetiology of calf pneumonia, may be present as subclinical infections in cattle populations (Rosner, 1971; Curtis, 1972; Rossi and Kiesel, 1972; St. George et al., 1972).

In a survey of 27 outbreaks of bovine respiratory disease, Thomas (1973) was unable to correlate certain virus infections with subsequent development of clinical pneumonia. Evidence was strong from his studies that viruses were not important in the acute lower respiratory tract syndrome and recent studies by Thomas and Collins (1974) confirmed this lack of relationship between viruses and lower respiratory tract disease.

In 1956 Jarrett, while studying several hundred calves with pneumonic conditions, classified the pulmonary diseases on a morphological basis into three primary types as illustrated in Table 15. The type of inflammatory reaction (proliferative or exudative) provided the initial subdivision; the third group included diseases of known aetiology.

Exudative pneumonias were generally associated with bacteria (Omar, 1966); purulent lesions were often accompanied by C. pyogenes infection while lesions with a fibrinous exudate were associated with Pasteurella spp., the latter lesion being common in calves with signs of shipping fever. A third type of exudative pneumonia recognised by polymorphonuclear neutrophilic infiltration either of diffuse or focal distribution has been associated with calves with septicaemia (Omar, 1966).

Two types of proliferative pneumonias, lymphoid and epithelial, were recognised in the calf (Table 15). The former type was often called cuffing pneumonia or enzootic pneumonia of calves. The pathology of this pneumonia was described in detail in chapter one; the pathological similarity with EPP and CRD in mice was considered to indicate related aetiological agents for these diseases.

A brief synopsis of the pathological features associated with the four main bovine viral infections will follow; the characteristics of these infections in the calf were detailed in a review article by Darbyshire and Roberts (1968).

Infectious bovine rhinotracheitis is an acute, contagious condition characterised by severe inflammatory changes in the upper respiratory tract including the trachea. The histological changes are predominantly ballooning degeneration of epithelial cells with the formation of transient eosinophilic intranuclear inclusions.

In uncomplicated P13 virus infection alveolar epithelialisation is recognised and bronchiolar epithelial cells may give rise to the formation of giant cell syncytia. In addition, eosinophilic intracytoplasmic and intranuclear inclusions develop in bronchiolar epithelial cells and alveolar epithelial cells.

Adenovirus infection of calves results in proliferative bronchiolitis with necrosis and bronchiolar occlusion leading to alveolar collapse. In addition, intranuclear inclusions can be observed in bronchiolar epithelial cells, septal cells and cells associated with lymph nodes.

Interstitial pneumonia, proliferative bronchiolitis and extensive alveolar epithelialisation are the histological lesions associated with reovirus infections; pseudo-epithelialisation and some intraseptal cell hyperplasia are often seen but these features are not specific.

The third type of pulmonary disease (Table 15) involving specific infections are not common in calves and will not be described.

In all parts of the world economic losses are considerable, particularly

from calves which develop enzootic pneumonia. This disease has a high morbidity but low mortality rate and occurs in calves from one week to six months of age (Jubb and Kennedy, 1970). Infection is often clinically undetectable, the animals improving gradually after a month or two. When pneumonia is detectable clinically, the prognosis is poor; the mortality rate may be high and those that survive remain stunted and unthrifty.

The incidence of pneumonia in calves in Great Britain is not widely reported and recorded mortality rates vary from 9.5 per cent in 1966 (Omar) to five per cent in 1974 (Thomas). Economically, pulmonary disease in calves is important; Maclean (1969) estimated that the loss from pneumonia could be nine per cent of the farm income.

An accurate assessment of the incidence of respiratory disease in calves is difficult to obtain since many of the syndromes are not readily detected clinically and consequently only severe cases are reported. In addition, mortality may be low and consideration of the incidence of deaths from pneumonia may again result in an unrealistic measurement of its prevalence and economic effects within groups of calves.

The objective of the present investigation was to examine the relationship between mycoplasmas and pneumonia in calves by studying groups of calves that had been reared together from the first week of their lives for varying periods of time. Four age groups were studied; respiratory disease in some animals in each group had been detected at some time.

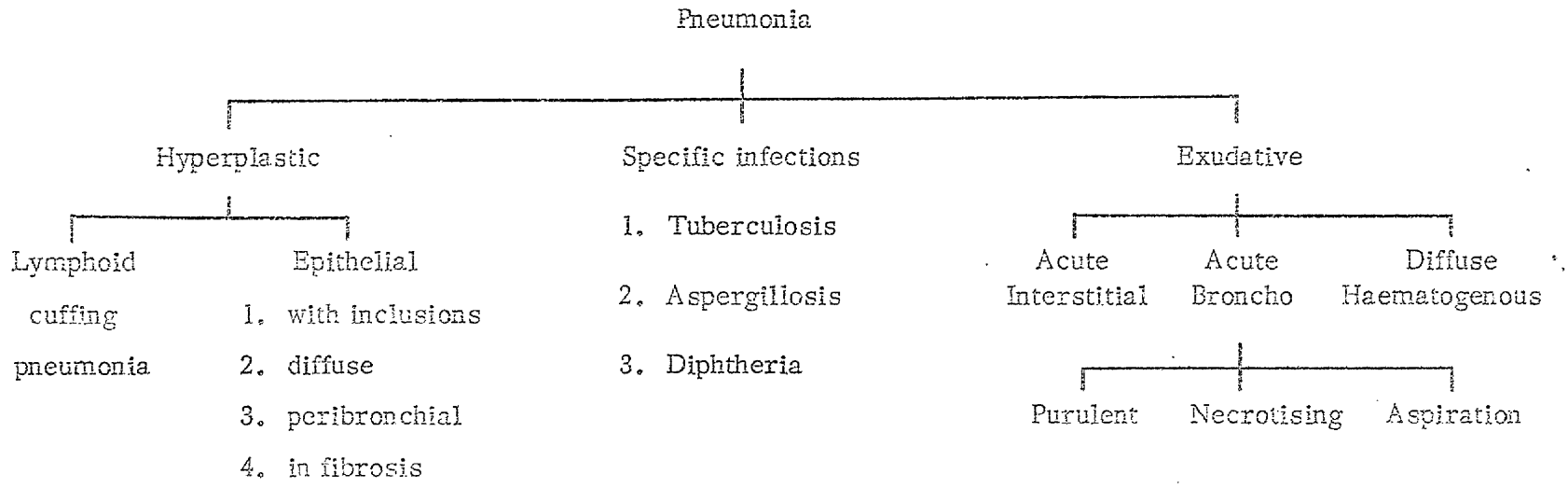


Table 15. A classification of some pulmonary diseases found in the young bovine animal. (modified from Jarrett, 1956).

B. CALVES LESS THAN ONE MONTH OLD

1. Introduction

Respiratory infection is common among young calves and several factors considered important in its development include, (i) colostrum (ii) stress and (iii) intensive housing. The colostrum of the dam is rich in antibody, including antibody against many bovine respiratory viruses and bacteria which may be present in the animal's environment. The second factor includes a wide variety of physical agents which can contribute to the susceptibility of a young calf to disease. The movement of newly born calves between farms and markets is particularly harmful since the animal is exposed to many microbiological agents while in a stressed condition. Although many of these calves receive colostrum their entry into different surroundings often exposes them to microorganisms which their dams may not have experienced. Housing conditions play an important role and large numbers of calves housed in small areas can produce losses in intensive farming. Air conditioning, temperature and humidity are considered to affect the susceptibility of animals to respiratory infections. However, the main problem associated with intensive housing is the rapidity with which a respiratory disease can spread; the introduction of one infected animal can cause maximum loss.

In very young calves many of the deaths are due to calf scour; an exudative pneumonia can accompany this disease following a septicaemic phase (Jarrett, 1956).

This section describes the microbiological and pathological examination of a group of five calves, aged less than one month.

2. Materials and methods

a. Animals.

The five calves examined in this study belonged to a group of 11 animals all of which were purchased at one to two days old from one market during two consecutive days. The calves were housed together in an open pen and fed an adequate diet. The five animals examined all died; four died at two weeks of age and one at one week.

b. Post mortem techniques.

Prior to death nasal and ocular samples were taken from the five calves using sterile swabs, which were placed in sterile PBS immediately after sampling. The lungs were removed from the five calves as soon after death as possible. The trachea and lungs were removed en bloc into polythene bags avoiding contact with foreign surfaces. After macroscopic examination, the right cranial lobe of all calves was sampled into GS, U3 and A broths and into PBS; all specimens were taken in duplicate. In addition, a large piece of bronchus 2 to 3 cm long, was aseptically removed and placed in 1.8 ml of sterile PBS. The epithelial surface of the bronchial tissue was scraped and this material, along with lung tissue samples and the nasal and ocular swabs, were cultured for the presence of glucose-fermenting, arginine-splitting and urea-splitting mycoplasmas, and of aerobic and anaerobic bacteria employing the techniques of cultivation and identification described in section C of chapter two. Blocks of tissue for histological examination were collected from similar sites; fixing and processing of the specimens were carried out as described in section E of chapter two. All blocks of tissue were stained with haematoxylin and eosin (HE) and examined microscopically.

A classification of the bovine lung pathology found in the groups of calves in this study is illustrated in Table 16. Most of the calves examined had characteristic features of one of the types of lesions illustrated; most were proliferative in nature, although additionally calves were found with typical exudative pneumonias, suppurative pneumonias or exudative interstitial pneumonias and when these occurred they were classified as such.

The initial division was based on the presence or absence of macroscopic pulmonary lesions. Microscopical examination of bronchial and lung tissue enabled five subdivisions to be made. Lung tissue with no macroscopic lesions was classified into type A or type B depending on the presence or absence of peribronchiolar lymphoid accumulations (PBLA) and bronchiolitis as shown in Table 16. Lungs with areas of macroscopic pneumonia were allocated C, D or E type lesions. Type C and type D

lesions had bronchitis and bronchiolitis and were further subdivided into (i) and (ii) according to the presence of alveolitis or alveolar collapse respectively. The main difference between type C and type D pneumonia was the arrangement of the lymphocytes in the PBLA, which were present in both. In type C, the PBLA were organised into a follicular (F) pattern and germinal centres were frequently present; this was typical of cuffing pneumonia (Jarrett, 1956). The lymphoid cells encircled more than two thirds of the airway and formed a clearly defined sheath of cells which extended down its length. The lymphocytes of the PBLA in type D lesions did not form cuffs and although present in large numbers around the bronchioles were seen in a diffuse arrangement. A third subtype of the group C lesion was characterised by the presence of alveolitis and alveolar collapse accompanied by pulmonary complications such as bronchial and bronchiolar polyps, abscesses, bronchiectasis and pleurisy. A third type of lesion occasionally seen in calves with macroscopic pneumonia was type E. This was a mild condition typified by the presence of alveolar collapse and bronchiolitis in some cases.

Most calves examined in the following groups were classified with one of the histological types described above.

Table 17 illustrates the nomenclature of lung lobulation employed throughout these studies; the gradation of the lobar lesions, indicating the degree of involvement, is also described.

3. Results

a. Mycoplasmal and bacterial isolations.

Three mycoplasma broths were inoculated with lung tissue samples from each animal. Growth of mycoplasmas was not indicated in either U3 or A broths after incubation for three weeks, or from U3 and A agar plates following blind passage from the broths after three and seven days incubation. A colour change from red to yellow was detected in some of the GS broths after three to four days incubation and subculturing onto GS agar, incubating at 37°C in five per cent CO_2 in air or nitrogen for four to five days, typical 'fried-egg' mycoplasma colonies were seen (Fig. 1).

The unpigmented colonies had a fairly dense core due to the organisms penetrating deep into the agar medium. The periphery of the colony was often wide with a general lacy appearance.

This was the only mycoplasma colony type grown from these calf lung specimens and it was identified by the indirect immunofluorescent technique as M. bovirhinis (Fig. 2). The colony fluoresced green-yellow on a dark background when stained with antiserum to M. bovirhinis.

The results of the isolations of mycoplasmas and bacteria from this group of young calves are illustrated in Table 18. Mycoplasma bovirhinis was recovered from the lung tissue of three of the five calves examined. One calf (M143), which died at one week of age, yielded M. bovirhinis from the bronchial sample; a titre of 10^5 CCU per 0.2 ml of sample was obtained for this organism. Titres of 10^2 and 10^3 CCU of M. bovirhinis per 0.2 ml of sample were obtained from the lung tissue of the remaining three calves. Mycoplasmas were not cultured from either the nasal or ocular swabs from any of the calves.

Eight isolates of bacteria were cultured from four of the five calves; two isolates were identified to their generic level only (Table 18). The youngest calf, M143, had an extensive Escherichia coli infection as judged from the nasal swab cultivation. In addition, Pasteurella multocida was isolated from the lungs of this animal but only in small numbers. Bacteria were cultured in small numbers from the lung tissue of three other calves. The remaining calf, M156, from which no bacteria were isolated was also one of the calves negative on culture, for mycoplasmas.

b. Pathology.

Pulmonary consolidation was not recorded in any of the five calves examined but the lungs of all animals were moderately congested, together with congestion of the broncho-mediastinal lymph nodes in most cases. Mild oedema was recognised in one calf (M152).

All five calves were considered to have a mild acute exudative pneumonia, following microscopic examination. This was recognised by congestion and oedema in all cases with neutrophils infiltrating into the

alveolar air spaces and also into the bronchiolar lumina; the neutrophilic infiltration appeared active although the numbers of neutrophils present in the lung tissue were small. Bacteria were seen in small microcolonies in the exudate in some alveolar air spaces.

4. Discussion

Five calves, less than one month of age, were considered to have died from the 'calf scour' syndrome. Macroscopically extensive congestion and microscopically a very mild acute exudative pneumonia was detected. Several species of bacteria were isolated from the lungs of these animals and were present in small numbers apart from one case from which high numbers of a strain of E. coli were isolated from the nasal cavity.

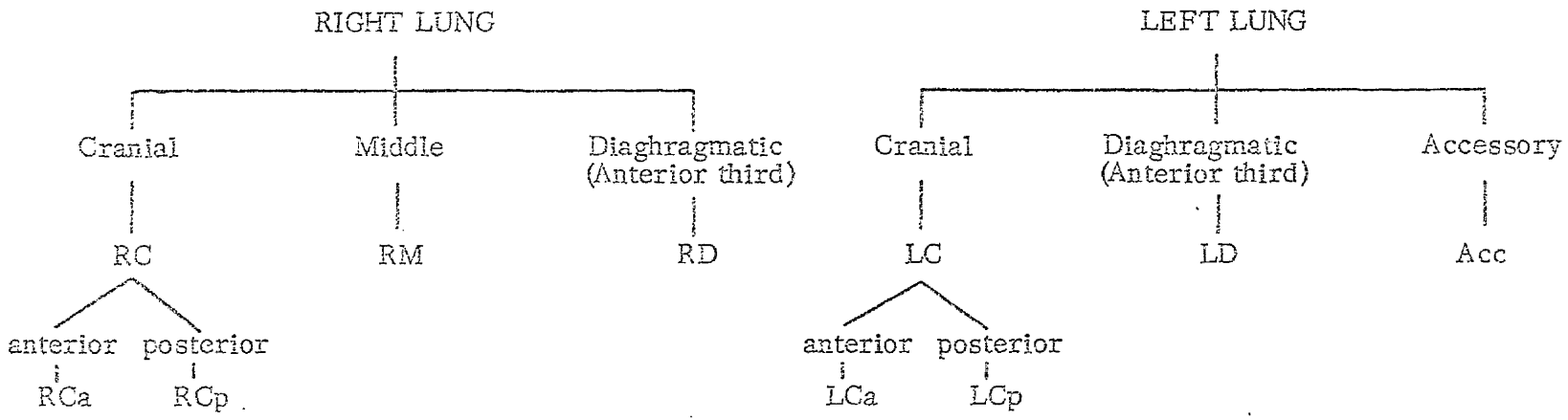
Mycoplasma bovirhinis was the only mycoplasma isolated and was recovered from lung and bronchial tissue in three of the five calves. This organism is considered a common inhabitant of the respiratory tract of normal calves (Davies, 1967), particularly of the upper respiratory tract of very young calves (Thomas and Smith, 1972). However, these animals had little resistance and were stressed from recent movements, and under such conditions, commensals, such as M. bovirhinis are considered capable of invading mucosal tissue, proliferating and producing lesions (Hamdy, 1968).

Escherichia coli is considered to be the most common cause of calf scour and although septicaemia is often part of this disease process, progressing to a lung infection, E. coli was not isolated from the pulmonary tissue in any of these cases. The low resistance of the animals together with the enteric infection probably enabled some of the upper respiratory tract bacteria to proliferate and colonise the lower respiratory tract and thus initiate a bacterial pneumonia, indicated by the neutrophilic infiltration into the alveoli.

Type	NO MACROSCOPIC PNEUMONIA			
A			* PBLA ±	
B	Bronchiolitis ±		PBLA	
	MACROSCOPIC PNEUMONIA PRESENT			
C (i)	Bronchitis Bronchiolitis Alveolitis		PBLA-F	
C (ii)	Bronchitis Bronchiolitis Alveolar collapse		PBLA-F	
C (iii)	Bronchitis Bronchiolitis	Alveolitis Alveolar collapse	PBLA-F	Complications: Polyps, abscesses bronchiectasis.
D (i)	Bronchitis Bronchiolitis Alveolitis		PBLA-D	
D (ii)	Bronchitis Bronchiolitis Alveolar collapse		PBLA-D	
E	Bronchiolitis ± Alveolar collapse			
Others	(i) Acute Exudative pneumonia, (ii) Suppurative pneumonia, and (iii) Exudative Interstitial pneumonia.			

* PBLA : Peribronchiolar lymphoid accumulations; F : Follicular;
D : Diffuse.

Table 16. Lung pathology : a classification based on histological features. Types B - E were considered to represent morphological types of proliferative pneumonia.



DEGREE OF INVOLVEMENT OF PULMONARY LESIONS WITHIN LOBES

\+ : Less than half lobe affected.

++ : Approximately half lobe affected.

+++ : Whole lobe affected.

Table 17. Nomenclature of lung lobulation and indication of lesion involvement used throughout the examinations.

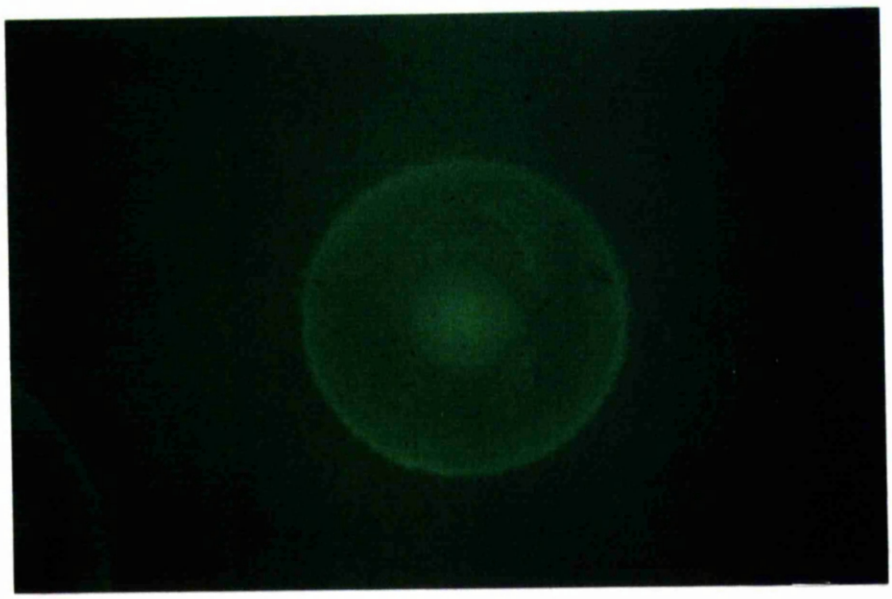
Case No.	Age (weeks)	* Mycoplasma isolations	* Bacterial isolations
MI43	1	M. bovirhinis L, 10^3; B, 10^5	Escherichia coli -N,+++ Pasteurella multocida -L,+
MI48	1 - 2	M. bovirhinis -L, 10^2	Streptococcus pneumoniae) -L,+ Staphylococcus aureus)
MI49	2	M. bovirhinis -L, 10^2	Strep. sp.) Past. haemolytica var. haem.) -L,+
MI52	2	-	Staph. sp.) Strep. mitis) -L,+
MI56	2	-	-

* Ocular (O) and nasal (N) swabs, and tissue from bronchus (B) and lung (L) were examined for the presence of mycoplasmas and bacteria. The titre of mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as ; +++, more than 50 colonies; ++, 20-50 colonies; +, 5-20 colonies.

Table 18. Frequency of isolations of mycoplasmas and bacteria from ocular and nasal swabs, and bronchial and lung tissue from five calves all aged less than one month.

Fig. 1 : Colony of M. bovirhinis showing the typical umbonate (fried egg) shape; the peripheral growing area is wide and has a lacy appearance. Unstained x 200.

Fig. 2 : Colony of M. bovirhinis identified by the indirect immunofluorescent technique x 200.



List of Tables

C. CALVES ONE TO TWO MONTHS OLD

1. Introduction

The main object of this section was to describe the microbiological and pathological findings from three groups of calves aged one to two months. Some animals within the groups underwent additional examinations including microbiological studies of nasal and ocular swabs, immunofluorescent studies of lung and bronchial tissue for the presence of bovine mycoplasmas and serological examinations.

2. Materials and methods

a. Animals.

Three groups of calves, aged between one and two months, were studied; six, five and four calves represented the three Groups A, B and C respectively. At slaughter, the calves in Group A and Group B were one month of age while Group C animals were two months old. The animals within each Group had all been purchased as newborn calves from one market during a two day period. They were reared together from that time, in open pens, and were fed normal diets.

b. Post mortem techniques.

Two calves in Group B died. The remaining calves were all shot and exsanguinated. The animals within each Group were slaughtered during one day. The lungs were removed as soon after death as possible; pulmonary tissue from all animals was sampled for mycoplasmal and bacterial detection and for histological examination. The methods used were described in section B and detailed in chapter two, sections B, C, D and E. Ocular and nasal swabs were taken from Group A calves when three weeks of age, and cultured at that time for the presence of mycoplasmas and bacteria.

Blood samples were collected from some animals in Groups A and B at slaughter. The serum was tested for the presence of anti-mycoplasma antibodies employing the indirect immunofluorescent technique as described in chapter two, section II. Tissue from these two Groups was also examined

for the presence of M. dispar by immunofluorescent methods described in section G of chapter two (Materials and Methods).

3. Results

a. Mycoplasmal and bacterial isolations.

Growth of mycoplasmas was indicated in GS and U3 broths by a change in the colour of the medium to yellow and red respectively; no alteration was found in the A broths from any of the samples examined. A rapid colour change in the GS broth of some cases was noted and slight turbidity was produced within 24 to 48 hours incubation. Large colonies grew after 24 hours incubation at 37°C in an atmosphere of five per cent CO_2 in air or nitrogen after subculture onto GS agar. These colonies had clearly demarcated centres and lacy and vacuolated peripheral growing areas (Fig. 3). However, under crowded conditions the peripheral growth was limited and the colonies were restricted to the central area. In some cases smaller colonies could be seen growing from the peripheral zones of a larger colony (Fig. 4). These colonies were identified as A. laidlawii by the indirect immunofluorescent technique.

In some animals the GS broth colour change was slow, requiring about six days of incubation at 37°C. Subsequent subculture onto GS agar produced atypical mycoplasma colonies after six to seven days incubation in five per cent CO_2 in air or nitrogen. These colonies were small, roughly circular, granular, lacking centres and not penetrating the agar (Fig. 5). Indirect immunofluorescence identified the methanol-fixed colonies as M. dispar.

Additionally, M. bovirhinis was isolated from the GS cultures of some of these animals. The cultural characteristics and colony morphology of this organism were described previously in section B.

A rapid change in colour from yellow to red, generally within 24 to 48 hours of inoculation, occurred in some of the U3 broths. This colour change was due to the hydrolysis of urea releasing ammonia and thus altering the medium pH. This rapid urealytic activity was characteristic of Ureaplasma spp. and the identification of these organisms was confirmed by subculturing

onto U3 agar, which, after incubation at 37°C in five per cent CO_2 in air or nitrogen, produced tiny colonies. The colonies were generally circular with an irregular border and lacked surface growth.

The results of the frequency of isolations of mycoplasmas and bacteria from the calves in Groups A, B and C are illustrated in Tables 19 and 20.

The calves in Group A were four to five weeks of age. Nasal and ocular swabs were collected from these animals at three weeks of age and cultured for mycoplasmas and bacteria; at post mortem examination, lung and bronchial tissue were sampled and studied similarly. Mycoplasmas were recovered from only two of the six animals as seen in Table 19. Mycoplasma bovirhinis was isolated from both calves; it was recovered from the bronchus of one calf while the other calf, M155, yielded it from lung and bronchial tissue and also from the nasal swab. In addition, A. laidlawii was isolated from the lung tissue of this calf. The single isolate of A. laidlawii and the two strains of M. bovirhinis were recovered at titres of 10^3 CCU per 0.2 ml of sample. Bacteria were cultured from four of the six calves in Group A, with isolations from the lung, nose and eye. Six species of bacteria were isolated, and although pathogens such as Corynebacterium pyogenes, Staphylococcus aureus and Pasteurella spp. were recovered, they were cultured in small numbers only.

Of the five calves in Group B, two died from a respiratory disease. The details of the microbiological findings in these calves are given in Table 20. Mycoplasmas were isolated from four of the five calves; Ureaplasma spp. were isolated from three calves while the fourth calf, M98, yielded M. bovirhinis from both the bronchial and lung samples. In one animal ureaplasmas were isolated from both the lung and bronchial specimens; the strains were present at titres of 10^2 and 10^3 CCU per 0.2 ml of sample. Mycoplasma bovirhinis was cultured at a titre of 10^4 CCU per 0.2 ml in both bronchial and lung samples. Bacteriologically four of the five calves were positive, with isolations from the lung tissue only. Six species were recovered and Pasteurella spp. were cultured in high numbers in two calves (M98 and

M100). The calf with a marked Pasteurella sp. infection also yielded high titres of M. bovirhinis from the lung and bronchus.

Four calves, two months of age, constituted Group C and the results of the microbiological isolations are shown in Table 20. Mycoplasmas were cultured from the lung tissue of all four calves; M. dispar was isolated from three of the four animals. A double infection with A. laidlawii was found in two cases and with a Ureaplasma sp. in the third case. A single isolate of M. bovirhinis was obtained from one calf (M92). Mycoplasma dispar and A. laidlawii were present at titres of 10^3 CCU per 0.2 ml of sample, while the other two species were recovered in low numbers. All four calves were positive by bacteriological examination of the lung tissue. Pasteurella haemolytica var. haemolytica was isolated from two calves, but only small numbers were recovered.

The frequency of isolations of mycoplasmas and bacteria from these animals as a single unit is illustrated in Table 21. Fifteen animals were examined from which mycoplasmas were recovered from ten (66 per cent). Mycoplasma dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii were all isolated from some of the 15 calves. These species were recovered at approximately equal rates (about 20 per cent), with their distribution apparently controlled by the calf grouping; for example, M. bovirhinis was present in Group A, Ureaplasma spp. in Group B and M. dispar in Group C. Bacteria were isolated from 12 of the calves with six animals yielding Pasteurella spp.. The distribution of bacterial infections amongst the calves within the three Groups was random.

b. Pathology.

Tables 22 and 23 illustrate the results of the pulmonary pathological examination of the calves in Groups A, B and C.

Extensive macroscopic lesions were seen in the lungs of calves within all three Groups. In many of the cases examined, pneumonic lesions were found in all lobes; the anterior regions of the lungs were the most extensively affected, particularly in the right lung (Fig. 6).

In Group A the lesions were dry, fawn-pink and slightly oedematous, with dry, reddish-purple lesions in the anterior lobes of the severely affected calves. Abscesses were frequent in these areas and adhesive pleurisy was also present in several cases. The smaller, uncomplicated lesions were well-defined and of less than normal volume. In the small bronchi of these calves a thin, grey mucus was present which occasionally extended into the trachea.

Pneumonic lesions of a similar appearance were recorded in the lungs of Group B animals; in three cases (M97, M98 and M100) the severely affected lobes were dark red to purple and small abscesses were seen. One case, M98, was complicated by severe exudative interstitial pneumonia with fibrinous pleurisy.

The pulmonary lesions in the Group C calves were less complicated than those of the other two Groups, being characteristically smooth, dry and pinkish-purple in appearance. The lobules involved had clearly defined edges and were less than normal volume.

The 15 calves, aged between one and two months forming Groups A, B and C, were examined microscopically and some of the histological features of the lesions found in each calf within these Groups are illustrated in Tables 22 and 23.

The calves within Group A had moderate to severe suppurative pneumonia with multifocal abscess formation in four of the six calves examined.

Similar exudative pneumonias were present in three of the calves in Group B (M97, M98 and M99); the first two animals died. The lesions were characterised by the infiltration into the alveolar air spaces and bronchiolar lumina by masses of neutrophils; these cells could also be seen migrating through the bronchial epithelium into the lumen. Bronchitis was evident in some cases with hypertrophy and loss of differentiation of the bronchial epithelium, usually accompanied by hypertrophy of the submucosal glands. Plasma cells were abundant in the lamina propria of the airways and also in the peribronchiolar areas together with some lymphocytes. Most cases had areas of necrosis and one animal (M155) necrotising masses were seen in the

bronchiolar lumina. Bacteria were sometimes distinguishable in these abscesses. The abscesses appeared to consist of a mixture of necrotic debris and inflammatory cells which formed a granular eosinophilic detritis frequently surrounded by fibrinous exudate. Most of the interlobular septa were normal, but in some places there was dilation of the septal lymphatics. In a few areas, particularly in case M98, this process had advanced to the stage of acute interstitial pneumonia with necrosis and inflammation of the lymphatics of the pulmonary septa.

The remaining two animals within Group B (M100 and M101) had a proliferative pneumonia with D type lesions similar to two cases (M92 and M95) in the Group C calves. In addition to the bronchitis and bronchiolitis of the characteristic D type lesion, peribronchiolar lymphoid accumulations in a diffuse form were present around the airways in these cases (Fig. 7). The lymphoid accumulations in these animals were significant but not extensive, consisting of small numbers of cells infiltrating the lamina propria of most bronchioles and frequently displacing the muscularis. Their presence around the larger airways was less obvious but occasionally a discrete aggregate was recognised in the peribronchial region (Fig. 8); the muscularis was not obliterated although some lymphocytes were seen between strands of muscle tissue. In addition, type D lesions were accompanied by an alveolitis or alveolar collapse; alveolitis was the most common feature in the calves in these Groups (B and C) (Fig. 9) and consisted of large numbers of macrophages (Fig. 10) with occasional giant cells. Large, foamy macrophages were present in the alveolar air spaces in many cases and were often mixed with a mucus-like secretion (Fig. 11).

The bronchitis in the majority of these cases was relatively mild (Fig. 12); the epithelial cells were hyperplastic and neutrophils could be seen infiltrating through to the lumen (Fig. 13). The epithelial surface was intact and the cilia were readily visible; this was the usual appearance of the large bronchus among these calves. However in the smaller bronchi, where the surrounding reaction was greater, the epithelial cells had become ragged, torn with a vacuolated appearance (Fig. 14). Plasma cells were usually seen in large

numbers in the lamina propria of the large and small bronchi (Figs. 13 and 14). In addition, the mucous glands were hypertrophied (Fig. 12); the number of mucous tubules increased and were present in clusters on top and at the edge of the cartilage plates. The tubules appeared active, slightly dilated and surrounded by large numbers of plasma cells.

One animal in Group C (M93) was classified with a proliferative pneumonia with a C(i) lesion characterised by the presence of peribronchiolar lymphoid accumulations which were organised around the airways to form cuffs in a follicular pattern. In addition, an alveolitis was present which consisted mostly of macrophages.

Case number M94, in Group C, was classified with a type E proliferative pneumonia, characterised by a slight bronchiolitis accompanied by alveolar collapse.

c. Immunofluorescence.

Tissue samples of lung and bronchus, snap-frozen in acetone and dry ice when fresh, were cryostat-sectioned and examined for the presence of M. dispar by the indirect immunofluorescent technique (IF). The results of this tissue screening are illustrated in Table 24 which also records the species of mycoplasmas recovered from these cases by cultural techniques.

Five of the six animals in Group A were examined and three fluoresced positively. Of these three IF positive cases, mycoplasmas were recovered from only one case which yielded M. bovirhinis and A. laidlawii.

Tissue from Group B was not screened for M. dispar; the four calves in Group C were examined, two of which demonstrated fluorescence to M. dispar in the bronchial samples. All four were culturally positive for mycoplasmas and M. dispar was isolated from three of them; of the two cases positive by IF only one (M94) was also positive culturally for M. dispar.

Bright fluorescence was limited to the luminal surface of the bronchial epithelium of the cases positive (Fig. 15).

Overall, six of the nine calves examined by IF for the presence of M. dispar were culturally positive for mycoplasmas, M. dispar being isolated from three. Five cases were M. dispar positive by IF but only one of these (M94) was also positive by culture.

d. Serology.

Sera collected from four calves in Group A (M141, M153, M154 and M158) and from three calves in Group B (M99, M100 and M101) were tested for the presence of anti-M. dispar antibodies using the indirect immunofluorescent technique. None of the sera were positive. Serological examinations were not carried out on calves in Group C.

4. Discussion

A total of 15 calves, aged one to two months, were examined. Mycoplasmas were recovered in moderately high titres from ten of these calves with approximately equal numbers (20 per cent) of isolations of M. dispar, Ureaplasma spp., M. bovirhinis and A. laidlawii being made. Considering the calves in their three groups, the results of the mycoplasmal isolations illustrated that in each group one species was commonly recovered. In Group A M. bovirhinis was the most frequently isolated mycoplasma; the calves were members of the same group as the five examined at less than one month of age (section B) and these animals also harboured M. bovirhinis in the lower respiratory tract. Lung tissue from the calves in Group B and Group C, on the other hand, were infected with Ureaplasma spp. and M. dispar respectively. Single isolates of mycoplasmas were made from six of the ten calves; A. laidlawii was recovered in three of four double isolations, in combination with any of the other mycoplasma species.

The number of isolations of mycoplasmas from these animals was perhaps lower than first anticipated. The large numbers of neutrophils and macrophages in the lung tissue of many of these cases may account for this since mycoplasmacidal enzymes in the lysosomes can be released from neutrophils and macrophages.

Bacteria were isolated from 12 of the 15 calves; the species were

distributed, more or less equally, between all groups with Pasteurella spp. the most commonly recovered bacteria, found in the lung tissue of six cases. The bacteria were isolated in small numbers except from two calves, in which large numbers of Pasteurella haemolytica var. haemolytica were present in the lung tissue. No association between the species of mycoplasmas and bacteria isolated was apparent.

The macroscopic and microscopic appearance of the lungs within the 15 calves was variable. The animals in Group A had moderate to severe suppurative pneumonias with multifocal abscess formation in four of the six calves examined. These calves were the surviving members of the group which constituted the animals less than one month old which were described in section B. The lesions found in Group A calves could perhaps be recognised as the acute phase of the pulmonary disease initiated in the young animals, some of which had died of calf scour. These pneumonic lesions were most probably caused by the potentially pathogenic organisms isolated from the respiratory tract of four of these animals, although recovered only in small numbers. Interestingly, M. bovirhinis was isolated from the lungs of two of these calves; this mycoplasma was also isolated from the younger calves in this group which had died.

Three of the calves in Group B also had an exudative type of pneumonia similar to the calves in Group A. In these cases, Ureaplasma spp. had become established in the lung tissue. The calves in Group C, however, had an M. dispar infection in their lungs which was associated with the presence of monocytic cells (particularly lymphocytes) around the small airways.

Unfortunately viral infections can not be eliminated from these cases, although pathologically, the lesions do not conform to those of any of the usual bovine respiratory viral infections.

Case No.	Age (weeks)	* Mycoplasma isolations	* Bacterial isolations
M141	4	-	-
M153	4-5	-	Staph. aureus) - 0, + C. pyogenes)
M154	4-5	M. bovirhinis - B, 10^3 , L, 10^3	-
M155	4	M. bovirhinis - B, 10^3 , N, 10^3 A. laidlawii - L, 10^3	Past. multocida) - L, + Staph. epidermidis) Past. haemolytica var. haem. - L, +
M157	4	-	Staph. aureus) Aerococcus viridans) - N, +
M158	4-5	-	Staph. aureus) Staph. epidermidis) - L, +

* Ocular (0) and nasal (N) swabs, and tissue from bronchus (B) and lung (L) were examined for the presence of mycoplasmas and bacteria. The titre of mycoplasmas was recorded as CCU per 0.2 ml of sample, and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++, 20-50 colonies; +, 5-20 colonies.

Table 19. Frequency of isolations of mycoplasmas and bacteria from ocular and nasal swabs, and bronchial and lung tissue from Group A of the one to two months old calves.

Case No.	Age (months)	* Mycoplasma isolations	*Bacterial isolations
M 97	1 (died)	Ureaplasma sp. L, 10^3 / B, 10^3	Strep. faecalis) Neisseria pharyngis) Acinetobacter lwoffi) L, +
M 98	1 (died)	M. bovirhinis L, 10^4 / B, 10^4	Past. haemolytica var haem.) Past multocida) L, ++
M 99	1	Ureaplasma sp. L, 10^2	-
M100	1	Ureaplasma sp. B, 10^3	Past. haemolytica var haem.) Strep. faecalis) L, ++
M101	1	-	Staph. epidermidis L, +
M 92	2	M. bovirhinis - 10^2	Aeromonas formicans +
M 93	2	M. dispar - 10^3 A. laidlawii - 10^3	Past. haemolytica var. haem. +
M 94	2	M. dispar - 10^3 A. laidlawii - 10^3	Aerococcus viridans +
M 95	2	M.dispar - 10^3 Ureaplasma sp. - 10^1	Past. haemolytica var. haem. +

* Bronchial (B) and lung (L) tissue were examined for the presence of mycoplasmas and bacteria. The titre of the mycoplasmas was recorded as CCU per 0.2 ml of sample and the number of bacterial colonies obtained from one loopful of sample assessed as; +++, more than 50 colonies; ++, 20-50 colonies; +, 5-20 colonies.

Table 20. Frequency of isolations of mycoplasmas and bacteria from bronchial and lung tissue from Group B and Group C of the one to two months old calves.

Group	Number calves in Group	Number calves with mycoplasmas	Number of calves with				Number calves with bacteria	Number calves with *Pasteurella* spp.
			M. dispar	*Ureaplasma* spp.	*M. bovirhinis*	*A. laidlawii*		
A	6	2	0	0	2	1	4	2
B	5	4	0	3	1	0	4	2
C	4	4	3	1	1	2	4	2
Total	15	10	3	4	4	3	12	6

Table 21. illustrates the frequency of isolations of mycoplasmas and bacteria from the three Groups A, B and C which include the calves studied in the age range of one to two months.

Case No.	*MACROSCOPIC LESIONS RCa	RCp	RM	RD	LCa	LCp	LD	Acc.	* MICROSCOPIC FINDINGS
M141	+++	++	+	-	+	+	+	+	Exudative pneumonia. Numerous plasma cells. Early areas of necrosis.
M153	++	+	+	+	-	+	+	-	Collapse and a few abscesses.
M154	+++	+++	+++	+++	+++	+++	+++	+++	Suppurative pneumonia. Abscesses. Numerous plasma cells.
M155	+++	+++	+++	+	+++	+++	+	+	Suppurative pneumonia. Abscesses. Numerous plasma cells. Bronchitis.
M157	+++	+++	+++	+	+++	+++	+	+	Suppurative pneumonia. Abscesses.
M158	+++	++	+	-	+	+	-	-	Chronic exudative pneumonia. Bronchitis. Numerous plasma cells. Collapse.

* Based on nomenclature and classification illustrated in Tables 16 and 17 .

Table 22. Pathological findings of pneumonic lungs of six calves in Group A, aged one to two months.

Case No.	*MACROSCOPIC LESIONS								* MICROSCOPIC FINDINGS
	RCa	RCp	RM	RD	LCa	LCp	LD	Acc.	
M97	+	+	+++	+	+	+	+	+	Suppurative pneumonia. Abscesses.
M98	+++	+++	++	+	+++	+++	+	+	Acute exudative interstitial pneumonia.
M99	+	-	-	-	-	-	-	-	Suppurative pneumonia.
M100	+++	+++	++	++	++	+++	++	++	Proliferative D(i) lesion.
M101	+	+	+	+	+	-	+	+	Proliferative D (i) lesion.
M 92	+	+	+	+	+	+	+	+	Proliferative D (ii) lesion, macrophage alveolitis.
M 93	+++	+++	+++	+	-	++	+	++	Proliferative C(i) lesion, macrophage alveolitis.
M 94	+	+	++	+	-	+	+	+	Proliferative E type lesion.
M 95	+++	++	+	+	-	+	-	+	Proliferative D (i) lesion, macrophage alveolitis.

* Based on nomenclature and classification illustrated in Tables 16 and 17 .

Table 23. Pulmonary pathological findings of five calves in Group B, and four calves in Group C, aged between one and two months.

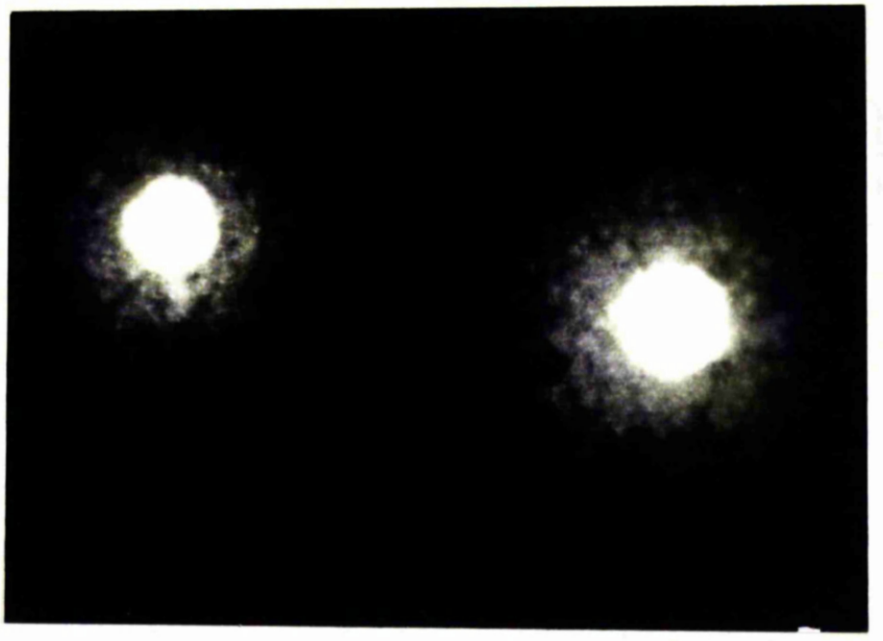
Group	Number calves in Group	Number calves examined IF	Case No.	Mycoplasma isolations	IF +ve or -ve M. dispar
A	6	5	M 153	-	-
			M 154	M. bovirhinis	-
			M 155	M. bovirhinis, A. laidlawii	+
			M 157	-	+
			M 158	-	+
C	4	4	M 92	M. bovirhinis	+
			M 93	M. dispar, A. laidlawii	-
			M 94	M. dispar, A. laidlawii	+
			M 95	M. dispar, Ureaplasma sp.	-
Total	10	9	Positive M. dispar	3 (culture)	5(IF)

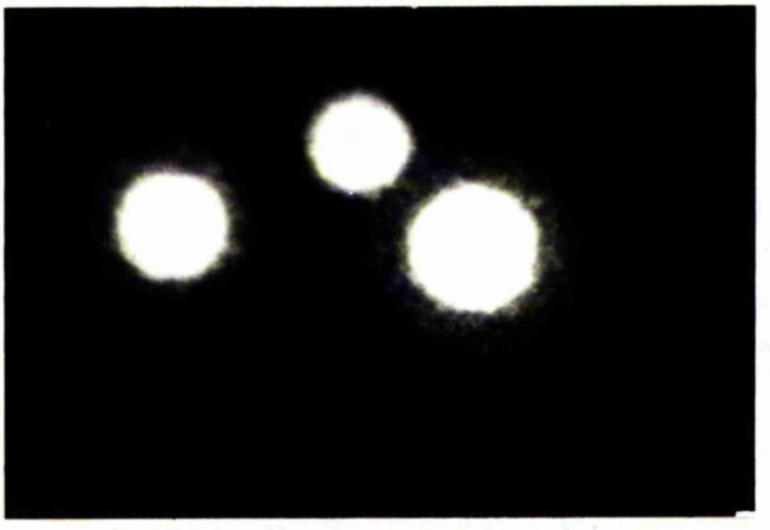
Table 24. The results of the screening of pulmonary tissue from nine calves, aged between one and two months, for M. dispar by the indirect immunofluorescent (IF) technique. The mycoplasmal isolations, by cultural techniques, are also given.

Fig. 3 : Centre-forming colony of A. laidlawii with peripheral growing area becoming lacy and vacuolated. Unstained x 250.

Fig. 4 : Colonies of A. laidlawii grown under crowded conditions; the peripheral zone of growth is reduced. Unstained x 290.

Fig. 5 : Atypical mycoplasma colonies of M. dispar. The colonies are small, roughly circular and do not penetrate the agar. Unstained x 290.





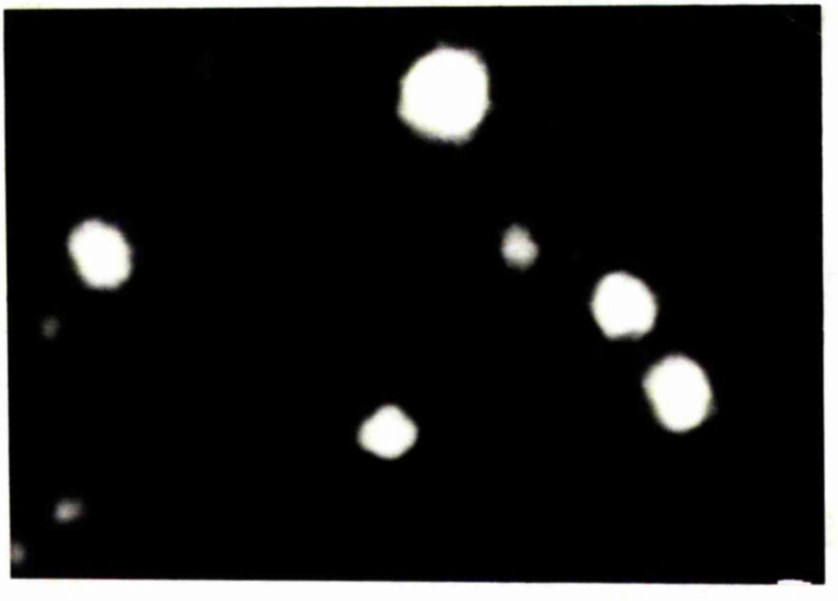
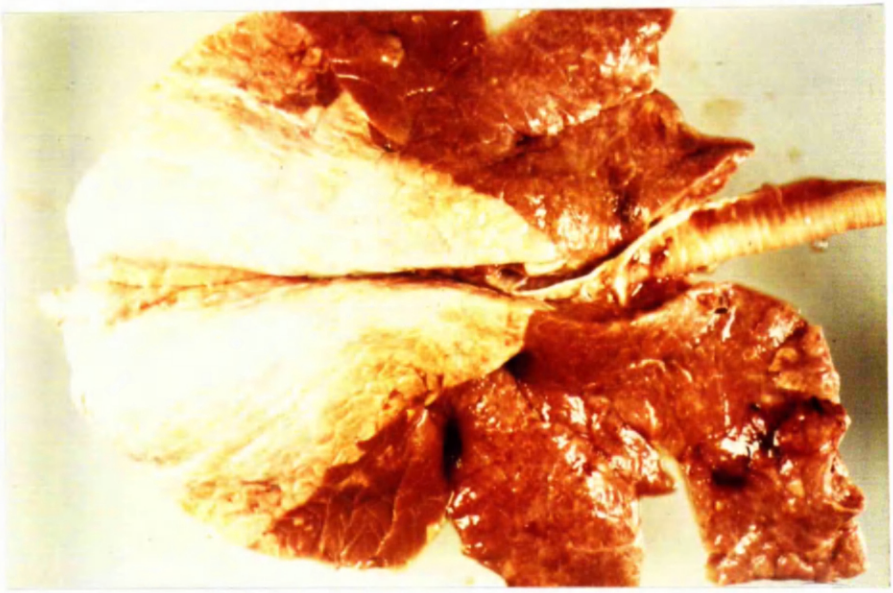


Fig. 6 : One to two months old calf with extensive pneumonic lesions affecting all lobes of the right and left lung. Abscesses are seen in the anterior segments of the lungs which are the most severely affected. The lesions of the diaghragmatic lobes are well-defined and appear uncomplicated.

Fig. 7 : One to two months old calf. Proliferative pneumonia of type D(i) with accumulated lymphocytes around the bronchiole forming a diffuse pattern. The lymphocytes have displaced the muscle layer and infiltrated the lamina propria. An alveolitis is present in the adjacent lung tissue with macrophages and neutrophils in the air spaces. Neutrophils are also present in the lumen of the bronchiole. HE staining, x 120.



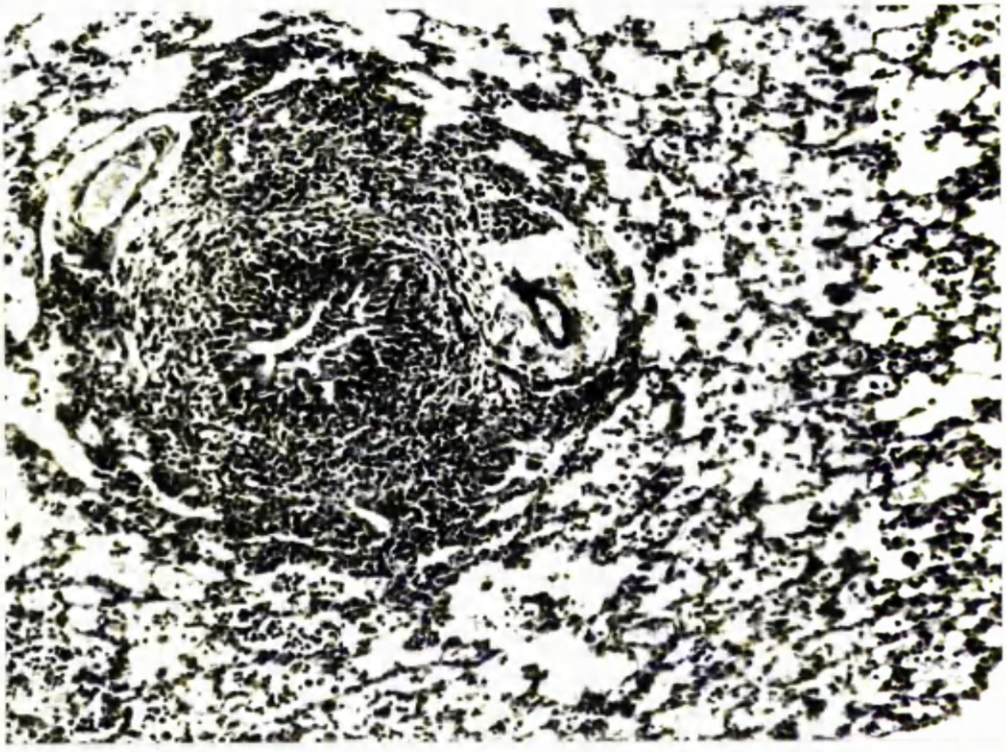


Fig. 8 : One to two months old calf. D(i) pulmonary lesion with a discrete accumulation of lymphocytes in the peribronchial area which has not infiltrated the lamina propria. The epithelium is hyperplastic and vacuolated in appearance; a plug of cellular debris is seen in the bronchial lumen. HE staining, x 120.

Fig. 9 : One to two months old calf. D(i) pulmonary lesion with a bronchiole surrounded by lymphocytes. An alveolitis is present with large numbers of macrophages in the air spaces. HE staining, x 120.