THE TRACHEOBRONCHIAL AIRWAYS OF NORMAL AND PNEUMONIC SHEEP: CYTOLOGY AND CYTOPATHOLOGY

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ABSTRACT

As a basis for subsequent pathological studies the histology, topographical morphology and ultrastructure of the normal ovine tracheobronchial epithelium at five different levels were investigated. In addition,the topographical studies were extended to involve the normal alveolus. The lining epithelium of the trachea and bronchi consisted of pseudostratified ciliated columnar and goblet cells, while from the small bronchi distally, the airways were lined by low columnar or cuboidal ciliated and non-ciliated cells. A slightly lower proportion of mucous cells were present in the upper trachea compared to the lower trachea which also contained Clara cells with PAS-positive granules. Topographically, there was a marked change from a predominance of ciliated cells in the trachea and bronchi to non-ciliated cells in the distal bronchioli. Ten different types of epithelial cell were identified ultrastructurally. These were; two types each of ciliated, goblet and unknown secretory cells together with Clara, brush, basal and intermediate cells. Several cell types of unknown function which have not been previously described were observed. It was concluded that the ovine lung was similar to that of cattle but different from other mammals in two important features. Firstly, interalveolar pores of Kohn were uncommon in young sheep and secondly, there was a relative paucity of alveolar macrophages in alveolar spaces. It is thought that these features may have an influence on the pathophysiology and pathogenesis of pneumonia in sheep and the resistance of the ovine lung to infection.

The pathological changes which occurred in the tracheobronchial epithelium at five different levels were studied in both early and advanced lesions of chronic non-progressive pneumonia (CNP) in lambs 3 to 10 months old. In addition, the alveolar topographical changes were investigated. The most common topographical finding was loss of cilia from the epithelial surface which was more severe in early lesions. The tracheobronchial epithelium in advanced pneumonic lesions showed large areas of squamous metaplasia, while focal areas were observed in early lesions. Extensive inflammatory cell infiltration of the tracheobronchial epithelium was one of the main histological features seen in both early and advanced pneumonic

lesions indicating that active inflammatory changes were occurring at all stages of the disease. Aggregations of lymphoid cells together with submucosal gland hyperplasia and metaplasia were more extensive in advanced cases. Striking changes to Clara cells were observed by scanning electron microscopy in bronchioli in both stages of the disease. Mycoplasmas were commonly found attached by means of pili-like structures to the cilia of epithelial cells of the trachea and bronchi in early lesions and to tracheal and bronchiolar epithelial cilia in advanced lesions. Their presence in early pneumonic lesions suggested that they may compromise the effectiveness of the mucociliary system, allowing other destructive bacteria normally resident in the upper respiratory tract to penetrate into the pulmonary parenchyma and produce more severe lesions.

To quantitate the proliferative changes observed the epithelial and submucosal thicknesses of the tracheobronchial airways of sheep affected with CNP were measured at 6 levels and submucosal gland size and number were measured at 4 levels. The mean thickness of the tracheobronchial mucosal layers of normal sheep decreased regularly from the upper trachea to the distal bronchioli, while in pneumonic lesions the decrease in mucosal thickness was more irregular. Small bronchi and bronchioli were the most severely affected and the percentage increase above normal was 146.5% and 268.2% respectively.Comparative statistical analysis of the results showed that in early lesions the epithelium of the trachea and bronchi were worst affected, whereas in advanced lesions the epithelium of the peripheral airways showed the most severe change. It is thought that the increase in the thickness of the wall of peripheral airways together with the accumulation of inflammatory cells and mucus may result in partial or complete obstruction of the lumen of small airways in affected areas. Statistical analysis of sectional areas of submucosal gland of normal sheep showed that they decreased regularly from the upper trachea to the small bronchi, but this pattern became irregular in the pneumonic lesions. The most significant changes in submucosal gland parameters of early pneumonic sheep occurred in the intrapulmonary bronchi. In sheep with advanced pneumonic lesions changes were most severe in both intrapulmonary and extrapulmonary bronchi. Enlargement of the submucosal glands in pneumonic lesions was found to be due to both hyperplasia and hypertrophy and these changes were more severe in advanced than early lesions.

The histochemistry of the submucosal gland glycoproteins in normal and pneumonic sheep was also studied and statistical analysis of the results showed a change in the types present. It was found that most mucous cells of submucosal gland at all levels of the normal ovine tracheobronchial tree contained either neutral or mixed types of glycoprotein and very few contained the acid type. The submucosal glands of normal bronchi contained significantly more neutral glycoprotein and less mixed and acid glycoproteins than those of the trachea. In pneumonic lungs there were no significant differences in the amount and types of glycoprotein between levels. Comparative statistical analysis showed that in the intrapulmonary bronchi, acid glycoprotein increased and neutral glycoprotein decreased in advanced pneumonic lesions when compared to normal and early pneumonic sheep. It was concluded that the ovine tracheobronchial airways respond to unspecified noxious agents by changing the chemical and physical nature of their mucous secretions.

Ovine tracheal organ cultures were used to investigate the pathogenicity of Mycoplasma ovipneumoniae, Bordetella parapertussis, Pasteurella haemolytica and Neisseria catarrhalis. The ciliary activity, histology, topographical morphology, ultrastructure and microbiology of these experiments are described in detail. Four different titres of each microorganism were used. It was found that all the microorganisms caused cytopathological changes and the ciliostasis produced was dose dependent. Mycoplasma ovipneumoniae and B. parapertussis attached to cilia at 30 min and 1 hr respectively and produced ciliostasis as early as 13 hrs and 1 hr respectively. The means of attachment of both organisms was investigated with both scanning and transmission electron microscopes. A fimbria or pili-like structure was found in close proximity to cilia with both microorganisms. Pasteurella haemolytica and N. catarrhalis failed to attach to cilia but they produced cytopathological changes and the ciliostatic effect was achieved as early as 3 hrs and 4 hrs respectively. Although both of these organisms behaved in a similar manner in organ culture and produced similar cytopathological changes, P. haemolytica was more destructive and produced ciliostatic effects faster than N. catarrhalis. Of the four microorganisms used it was found that only M. ovipneumoniae and B. parapertussis had both an affinity for tracheal epithelial cells and the ability to produce destructive changes in organ culture. On the basis of this work both

M. ovipneumoniae and B. parapertussis could be considered as likely candidates for organisms which in vivo could initiate bronchiolar disease and thus allow the development of CNP. This hypothesis in regard to M. ovipneumoniae is strongly supported by several other workers. The role of B. parapertussis remains to be more fully investigated.

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titres reached a plateau of growth at a level of $\pm 10^8$ CFU/ml 8 hrs post-inoculation.

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INTRODUCTION

Sheep are of special economic importance in New Zealand but like other intensively reared livestock they are prone to respiratory disease. The two principal forms of ovine pneumonia which occur in this country are acute pneumonia (enzootic pneumonia) (Salisbury, 1957) and chronic non-progressive pneumonia (CNP) (Alley, 1975a). It is generally recognised that CNP is one of the most prevalent diseases encountered in sheep both in New Zealand and overseas (Alley, 1975a & Jones; et al. 1976). It is confined to lambs aged 3 to 10 months and affected animals usually exhibit few clinical signs although in severe cases there may be loss of condition, cough and exercise intolerance (Alley 1975a). Recently, it has been shown that experimentally-induced CNP may cause a severe reduction in growth rate resulting in lowered carcass weight (Jones; et al. 1982a & Alley, 1986b).

There has therefore, been widespread interest in this disease and a wide variety of organisms have been isolated from the respiratory tract of pneumonic sheep (Davies, 1985). The aetiological significance of many of these organisms has not been clearly established. Conventional methods of investigation have usually involved the inoculation of a suspected aetiological agent or agents into the respiratory tract of susceptible sheep by intranasal and/or intratracheal routes. Although most of the attempts to reproduce the exact lesions of CNP with microbiological agents have failed, natural and experimental studies undertaken to date have demonstrated a close association between CNP lesions and the presence of Mycoplasma ovipneumoniae and Pasteurella haemolytica microorganisms.

The aim of the present study was to obtain information on the nature and sequence of events involved in the development of CNP in lambs in New Zealand. There was some evidence from previous studies (Alley, 1975a) that the tracheobronchial airways may be involved in the pathogenesis of this disease. The present study has therefore, sought to systematically investigate the morphology, histochemistry and morphometry of the normal and pneumonic ovine tracheobronchial mucosa. In order to help clarify the role of certain microorganisms in respiratory disease of sheep it was also thought appropriate to

study their relationships to tracheobronchial epithelium by using tracheal organ cultures.

CHAPTER 1

GENERAL REVIEW OF LITERATURE

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SECTION 1

THE TRACHEOBRONCHIAL AIRWAYS OF MAMMALS

1.0 STRUCTURE

1.1 GENERAL CHARACTERISTICS

The respiratory system of all vertebrate animals consists of organs that accomplish the primary functions of conduction and exchange of gasses. It may be divided into three main parts (Banks, 1981):

1. The conductive components. These extend from the external nares and include the nasal cavity, paranasal sinuses, nasopharynx, larynx and tracheobronchial airways.

2. The transitional components which are present in some species only and consist of respiratory bronchioli, structures which both conduct and exchange gases.

3. The exchange components, which include alveolar ducts, alveolar sacs and alveoli. Although some conduction occurs within the exchange components, their primary function is gaseous exchange.

The mammalian tracheobronchial tree is generally divided into two parts (Weiss & Greep, 1977; Rhodin, 1974). Firstly the trachea and extrapulmonary bronchi which have a stiff wall of hyaline cartilage in the form of open rings that keep them permanently patent.They are lined by pseudostratified columnar ciliated epithelium containing goblet cells and in the submucosa beneath are numerous sero-mucous glands whose secretion adds to that of the goblet cells (Constantinides, 1974). The second part contains no cartilage and consists of bronchioli. No submucosal glands, basal or goblet cells are present in this region and the luminal surface is lined by

ciliated and Clara cells (Banks, 1981). As one moves towards the alveoli the following changes occur in the wall of the tracheobronchial airway (Constantinides, 1974):

1.In the intrapulmonary bronchi the cartilage becomes divided into multiple plates, joined by connective tissue.

2.In the terminal bronchioli the cartilage, goblet cells and the sero-mucous glands disappear and smooth muscle becomes more prominent.

3.In the short respiratory bronchioli, just before the alveolar ducts, the epithelium becomes simple cuboidal and in most mammals the smooth muscle largely disappears.

Besides the conduction of air, tracheobronchial airways are responsible for clearing the air of particulate matter, humidifying the air and either cooling or warming it before it flows to the exchange part of the respiratory system (Banks, 1981).

1.2 THE BRANCHING OF AIRWAYS

Using epoxy resin casts, the pattern of tracheobronchial branching in humans (Weibel, 1963; Horsfield; et al. 1971), guinea pigs (Kliment; et al. 1972) and hamsters (Kennedy; et al. 1978) has been well documented. The branching pattern of hamsters and guinea pigs was essentially similar to that of man. It has been found that each branch divides into two smaller branches and is thus known as dichotomous branching. Although dichotomy is irregular with regard to the diameter and length of branches, morphometric analysis of resin casts reveals that the progression of airway dimensions from the trachea to the parenchyma of the lungs follows strict laws (Weibel, 1963).

There have been two different approaches to the study of branching of the human tracheobronchial tree. The earliest work was that of Weibel (1963) who followed the branching from the trachea to the lung parenchyma. In this case each bifurcation gave rise to a new generation of airways. The number of branches in each generation is twice that in the parent generation, so that the branching ratio of

dichotomy is 2. Accordingly, the number (N) of branches in each generation (Z) is:

$$N(Z)=2^{Z}$$

As Weibel (1963) demonstrated, within each generation, the lengths and diameters of branches have a characteristic range of sizes but the mean diameter of the airways up to about the sixteenth generation decreases systematically following a simple law:

$$d(Z)=do.2^{-Z/3}$$

where:
d(Z) = the mean diameter of airways generation Z and
do = the diameter of the trachea.
This equation shows that with each generation the airway diameter is reduced by the cube root of the branching ratio 2.

Using this approach, it is possible to construct a model that takes into account irregularities in branching by considering the number of airways of a given diameter, that exist in each generation and the number of lengths of bronchial pathway that intervene between the larynx and a particular airway.

Alternatively, Horsfield; et al. (1971) have used the Strahler system of analysing rivers to follow tracheobronchial branching.In this system branches are grouped into orders by size, beginning with the smallest designated as order 1. Two converging order 1 branches make an order 2 branch, two order 2 branches make an order 3 branch, and so on. This ordering pattern is particularly well adapted to a system of irregular dichotomy because the size of branches in one order varies less than with the other approach. A branching ratio is determined as the ratio of the number of branches in order u to that in order u + 1.

However, both models of approaching airway branching yield basically the same results. The general conclusion drawn from these types of analysis is that the diameter of the tracheobronchial airways are such as to ensure optimal conditions for airflow. Thus, from an engineering point of view, the tracheobronchial airways are well designed (Weibel, 1963; Horsfield; et al. 1971).

The diameter of the most peripheral airways after generation 16 do not follow the law of reduction by the cube root of 2; the diameter of respiratory bronchioli and alveolar ducts change very little with each generation (Weibel, 1963). In the most peripheral airways, mass airflow is part of the means of transporting oxygen, while in the terminal airways oxygen molecules must move into the residual air by diffusion. However, diffusion of oxygen in the gas phase is best served by establishing as large an interface as possible between residual air and the fresh air that flows in from the trachea. Weibel (1963) demonstrated that the airway diameter remains nearly unchanged after generation 16, and thus, the total airway cross section nearly doubles with each generation beyond this point.

1.3 THE STRUCTURE OF THE WALL OF AIRWAYS

The wall of tracheobronchial airways consists of four components (Banks,1981); the epithelium, the lamina propria-tunica submucosa, the tunica muscularis and the adventitia.

The epithelial lining of tracheobronchial airways consists of two main cell types (1) ciliated cells that propel upwards the overlying mucous blanket and (2) mucus producing unicellular glands (goblet cells) (Kuhn, 1976). The number and distribution of the cells in the epithelium are subject to species variation and will be discussed later. In all species, as one moves down the airway tree from the trachea to the terminal bronchioli, the epithelium gradually thins (Weibel, 1980).

The lamina propria-tunica submucosa is made up of areolar connective tissue. Elastic fibres are prominent and are believed to replace the lamina muscularis mucosae in the deeper layer.Mucosal glands of the branched, coiled, tubo-alveolar mucous type are present and extend the depth of the submucosal region adjacent to the inner surface of the cartilage. In the normal bronchus, the glandular acini of these glands are relatively small and are composed of serous and mucous cells (Banks, 1981).

The tunica muscularis is limited to transversely oriented bands of smooth muscle tissue that extend between the open end of the cartilage (Banks, 1981). The smooth muscle bundles form a continuous

sleeve in the connective tissue underlying the epithelial tube that extends from the major bronchi to the respiratory bronchioli. Beyond the respiratory bronchioli, the bundles extend into the wall of alveolar ducts where the muscle fibres lie in rings at the entrance to alveoli. Contraction of these muscles results in a narrowing of the lumen (Cameron & Bateman, 1983).

The tunica adventitia is composed of areolar connective tissue which blends with the surrounding fascia (Banks, 1981).

1.4 THE EPITHELIUM

The mucosa of the mammalian respiratory tract mucosa has been extensively examined by TEM and the cellular details have been defined in number of species including the rat (Rhodin & Dalhamn, 1956),mouse (Karrer, 1956; Hansell & Moretti, 1969; Hama & Nagata, 1970), rabbit (Konradova, 1966), dog (Okano; et al. 1970; Frasca; et al. 1968), man (Frasca; et al. 1967), pig (Baskerville, 1970a & b) and poultry (Walsh & McLelland, 1974). Scanning electronmicroscopy has also been used to study the surface topography of the airways in a variety of animals including the guinea pig (Okada, 1969), horse (Nowell & Tyler, 1971), hamster (Nowell & Tyler, 1971), mouse (Greenwood & Holland, 1972), ox (Mariassy; et al. 1975), rat (Alexander; et al. 1975) and dog (Wright; et al. 1983).

Jeffery (1983) has reviewed the TEM findings to date and tabled at least eight distinct epithelial cell types (Table 1.1). Three of these are thought to be secretory namely; mucous (goblet), serous and non-ciliated bronchiolar epithelial (Clara) cells. The other cell types include ciliated, intermediate, brush, basal and bronchial Kulchitsky cells. In addition to at least two migratory cells and two neural elements have been recognised. Table 1.1 illustrates how the occurrence, frequency and distribution of cell types varies between species. The eight epithelial cell types and their features have been described in considerable detail by Reid and Jones (1979) and Jeffery (1983) has reviewed their occurrence, frequency and distribution in large number of mammals.

Two studies have been made on the frequency and distribution of the cell types at five different levels of the tracheobronchial

TABLE 1.1: NORMAL TRACHEOBRONCHIAL AIRWAY CELL TYPES (JEFFERY, 1983)

MAIN CELL TYPE	SPECIFIC CELL TYPE	HUMAN	MONKEY	DOG	CAT	PIG	COW	RAT	MOUSE	HAMSTER	RABBIT	GUINEA PIG	FERRET	BIRD
EPITHELIAL	CILIATED	+	+	+	+	+	+	+	+	+	+	+	+	+
	MUCOUS	+	+	+	+	+	+	+	+	+	+	+	+	+
	SEROUS	FT	-	-	+	-	-	SPF	-	YA	-	-	-	-
	CLARA	+	+	+	+	+	+	+	+	+	+	+	+	-
	ENDOCRINE	+	+	-	+	-	-	+	+	+	+	+	-	+
	TRANSITIONAL	CMEM	-	-	SM	-	-	SM	CSM	CM	-	-	-	-
	SPECIAL TYPE	-	-	-	+	+	-	-	-	-	-	-	-	-
	BRUSH	-	-	-	+	+	+	+	+	-	+	+	+	-
	INTERMEDIATE	+	-	+	+	+	-	+	+	+	-	-	+	-
	BASAL	+	+	+	+	+	+	+	+	+	+	+	+	+
MIGRATORY	LYMPHOCYTE	+	MC	-	+	-	-	+	+	+	+	-	-	-
	GLOBULE LEUCOCYTE	-	MC	MC	+	-	+	+	-	-	-	-	-	-
	MAST CELL	-	+	MC	-	-	-	-	-	-	-	+	-	-
NEURAL	NEUROEPITHELIAL BODY	+	+	-	+	+	-	+	+	-	+	-	-	+
	NERVE TERMINALS	-	+	-	+	-	-	+	B	+	+	+	+	+

DEFINITION OF ABBREVIATIONS

+ =PRESENT; -=UNIDENTIFIED;FT=FETAL TISSUE;SPF=SPECIFIC PATHOGEN FREE; YA=YOUNG ANIMAL; SM=SEROUSMUCOUS; MC=MIGRATORY CELL; B=BRONCHIOLI ONLY; CMEM=CILIOMUCOUS,MUCOSEROUS,ENDOCRINE-MUCOUS;CSM=CILIOMUCOUS,SEROMUCOUS.

airways. Jeffery and Reid (1975) measured epithelial thickness, the depth of ciliary layer in the rat and described the ultrastructural features of the cells present. They found that the extrapulmonary airways have a pseudostratified epithelium, but their intrapulmonary counterpart had a simple one. The upper tracheal epithelium was thicker than the lower part. They identified ten cell types; Eight were epithelial and two migratory. At all levels, the epithelium consisted of 40 - 50% non-ciliated cells. Clara cells were not restricted to the terminal bronchioli but extended forward as far as extrapulmonary bronchi. In their study, epithelial serous cells were described for the first time in the airway epithelium of sub-human animals. The ultrastructural features of these cells were similar to the serous cells of human submucosal glands (Meyrick & Reid, 1970). Using the same technique, Pack; et al. (1981) studied the tracheobronchial epithelium of mice. Contrary to what was found in the rat and other species, the majority of cells in the mouse airway epithelium were Clara cells. Mucus-producing glands were infrequent throughout the airways, though goblet cells occurred in increased numbers at the carina and in the primary bronchi. These workers provided evidence that Clara cells may undergo both apocrine and merocrine secretion.

Mariassy and Plopper (1983) have recently completed the first systematic study of the epithelial population of tracheobronchial airways of a large mammalian species; the sheep. Based on differences in cell morphology, staining properties and distribution, they recognised and quantified eight major cell groups; four mucous cell categories, ciliated, basal, Clara and serous cells. The last cell type was restricted to submucosal glands. These investigators found that basal cells comprised about 25% of the total epithelial population in the trachea and decreased progressively in more distal generations. The percentage of ciliated cells was between 35 and 60 of the total epithelial population, being lowest in the trachea and the most distal airways and highest in intrapulmonary cartilaginous airways. The proportion of the secretory cells within the epithelial population was approximately the same in all the cartilaginous airways and decreased in the distal non-cartilaginous ones. These workers also found that epithelial cell distribution in sheep does not correlate with airway wall components and that more than one type of secretory epithelial cell can share the lining of the same airway.

However, contrary to what has been found in mice, Clara cell distribution in the sheep is based on airway generation and proximity to alveoli.

When the studies of the tracheobronchial airways of the rat (Jeffery & Reid, 1975), mouse (Pack; et al. 1981) and sheep (Mariassy & Plopper, 1983) are compared, the degree of difference suggests there may be differences in airway function between species. The chemical nature of the mucociliary lining would also appear to be different. Rats (Jeffery & Reid, 1975) and mice (Pack; et al. 1981) have few if any of the airway epithelial cell types which secrete acid glycoproteins and few glands, whereas the sheep (Mariassy & Plopper, 1983) and hamster (Kennedy; et al. 1978) have a large number of these cells in the proximal airways and in addition the sheep has glands in abundance. The variation in numbers of secretory cell types and glands in different airway generations or levels of the same species suggests that the lining varies within the tracheobronchial tree itself.The marked difference in the extent of basal cells within the airways of sheep may indicate a difference in potential for responding to epithelial injury by infectious or toxic agents.

2.0 DEFENCE MECHANISMS

The lung defends against inhaled particles by two mechanisms; clearance by the mucociliary blanket in proximal airways and phagocytosis by alveolar macrophages in distal airways (Green; et al. 1976). Inhaled particles greater than 5 um in diameter are generally trapped on the mucociliary blanket and are then redirected toward the mouth for elimination by swallowing or expectoration. Most particles that are less than 5 um in diameter reach distal airways where they may be engulfed by alveolar macrophages (Daniele, 1980).

However, highly soluble particles dissolve rapidly and are absorbed into the blood stream from the respiratory tract. Their metabolism and fate resembles that of an intravenously injected dose of the same material (Brain, 1980). When normal clearance mechanisms are depressed or absent and when excess secretions are present, a high velocity of airflow may play a very important role in removing inhaled particles. The tracheobronchial epithelium contains cough and irritant receptors, which when stimulated produce a cough reflex.

Coughing creates airway narrowing as well as maximising expiratory flow, thus the high linear velocities created are able to move mucus and debris along the airways (Leith, 1977).

Besides providing a physical lining for the main air ducts, the epithelium is also concerned with; (a) keeping its free surface constantly wet to prevent dehydration, (b) trapping and expelling some of the inhaled particles, (c) offering some preliminary immunological resistance to airborne organisms. The wetting is realised by mucus production from goblet cells and by additional mucus and serous secretion produced by submucosal glands (Constantinides, 1974). The trapping of some airborne particles is probably expedited through their adhesion to the sticky film that forms on the epithelial surface. Their expulsion is due to the wave like propulsive action of the cilia in the direction of the larynx (Cheville, 1976). The primary cell type in the tracheobronchial tract of higher mammals is the ciliated columnar cell, which has numerous cilia extending from its free surface. Krahl (1963) estimated that 270 cilia may be present on each ciliated columnar cell or 1800 million per mm^2 of epithelial surface in the human respiratory tract.

The secretory immune system consisting of lymphocytes and plasma cells within the tracheobronchial submucosa is another important defence mechanism. These cells secrete antibody molecules which are then trasported by epithelial cells into the bronchial lumen (Brain, 1980). The significance of secretory immunoglobulins in the defence of the respiratory tract against inhaled antigens is well documented (Tomas & Bienenstock, 1968; Tomas, 1970; Spiegelberg, 1974). Immunoglobulin A (IgA) has been shown to be the major immunoglobulin in respiratory secretions of man (Keimowitz, 1964; Remington; et al. 1964), cattle (Mach & Pahud. 1971) and sheep (Smith ; et al. 1975; Alley; et al. 1980). Secreted IgA forms a dimer, which combines with secretory component produced by epithelial cells before release into airways. The addition of secretory component to the IgA dimer affords protection from enzymatic digestion by proteolytic enzymes present in bronchial secretions (Reynolds & Newball, 1974) and thus prolongs its activity

A second class of secretory immunoglobulin of importance in the respiratory tract is IgG. Immunoglobulin G is the major

immunoglobulin in newborn animals (Alley; et al. 1980) and the lower respiratory tract of adult humans (Daniele, 1980). Because no secretory immunoglobulins were detected before suckling, it was concluded by Smith; et al. (1976) that the immunoglobulins of newborn lambs were maternally derived. Immunoglobulin M which is also bound to secretory compound before release has been detected in respiratory secretions of man (Kaltreider, 1976) and lambs (Smith; et al. 1976).

Bacteria have been shown to be more effectively phagocytised by alveolar macrophages when coated or opsonised with IgG than with IgA molecules (Reynolds; et al. 1975). However, IgA in bronchial secretions appears to protect the lung by inhibiting viruses from infecting epithelial cells, by promoting clumping and decreasing adherence to mucosal surfaces of certain bacteria such as streptococci and in combination with lysozyme and complement present in bronchial secretions, promoting phagocytosis of IgA-coated particles and bacteria through the alternate pathway (Brain, 1980).

Lysozyme and lactoferrin are secreted by submucosal gland cells of man and some laboratory animals and are present in tracheobronchial secretion in relatively high concentration (Boat & Cheng, 1980). Although, these two proteins have antimicrobial properties, their importance as defence mechanism in the airways has not yet been determined.

3.0 CHANGES IN TRACHEOBRONCHIAL AIRWAYS

3.1 STRUCTURAL CHANGES

Cilia are very sensitive to injury, and disruption of their movement considerably depresses their effectiveness in removing contaminants from the upper respiratory tract (Cheville, 1983). It has been shown that the exposure to gases such as sulphur dioxide, ozone and nitrogen dioxide at low levels damages cilia and increases ciliogenesis in the trachea, bronchi and bronchioli of rats (Stephens; et al. 1974; Schwartz; et al. 1976) and nonhuman primates (Eustis; et al. 1981).

Terminal and respiratory bronchioli and alveolar ducts are

especially equipped to deal with inhaled toxins such as cigarette smoke (Niewoehener; et al. 1974) and coal dust (Heppleston, 1953). Their non-ciliated epithelial (Clara) cells contain large amounts of detoxifying enzymes (Breeze; et al. 1976). When injured, these cells desquamate by the mechanism of reflex airway constriction (Brownstein, 1980) and are replaced by immature forms, which remain attached to the terminal bronchiolar surface (Evans; et al. 1978).

It has been shown that the cilia of the tracheobronchial epithelium disappear rapidly in response to respiratory virus infection.Once viral replication begins inside the ciliated epithelial cells, cilia break away from the surface, leaving only irregular microvilli (Bang & Bang, 1969). Although, most bacteria and mycoplasmas have no ability to invade epithelial cells, many are able to attach to cilia and produce toxins which seep into the epithelium causing cellular degeneration and necrosis. Ultrastructural and biochemical studies of M. hyopneumoniae infection in pigs (Livingstone; et al. 1972), M. pneumoniae in man (Wilson & Collier, 1976) and M. gallisepticum in poultry (Zucker-Franklin; et al. 1966a; b) have demonstrated that these organisms are provided with specific attachment devices which allow them to remain entwined among cilia. Some true bacteria such as Bordetella sp. and Alcaligenes (family Achromobacteraceae) release highly potent toxins which produce rapidly necrotising reactions resulting in cell death and bronchiolitis (Cheville, 1983).

Regardless of the aetiology, destruction of cilia, intercellular oedema, subepithelial capillary dilatation and neutrophil exudation through the epithelium into the lumen are features characteristic of most forms of acute bronchiolitis. Histologically and ultrastructurally, cilia break off and disappear from the surface of epithelial cells and their anchors to basal bodies are disorganised (Dahlgren & Daln, 1972; Giddens & Fairchild, 1972). When bronchiolar disease persists, the ciliated columnar epithelium undergoes metaplasia and basal cell hyperplasia which causes a stratified squamous epithelium to develop (Asmundsson; et al. 1973).

Mucous cell hyperplasia of respiratory epithelium accompanies bronchiolitis in man and the dog. In acute inflammation, mucus formed in epithelial goblet cells and subepithelial mucous glands floods the

damaged epithelial surface. It protects and provides the surface with a tenacious material that, when expectorated, removes much of the debris and irritant. In chronic inflammation, mucous cells may undergo metaplasia but still retain their ability to form mucus (McDowell; et al. 1979).

Bronchiectasis, the dilatation of a segment of a bronchus, results from inflammation of the wall during chronic purulent bronchitis. The process ramifies into the bronchioli, and the dilated airways are filled with mucopurulent exudate. The surrounding lung parenchyma collapses and bronchial walls are destroyed and eventually reconstructed by granulation tissue (Cheville, 1983).

3.2 SECRETORY CHANGES

One of the first events in inflammation of the respiratory system is the exudation of fluid through the tracheobronchial epithelium into the air passages. During influenza-induced inflammation of the respiratory tract in ferrets, Potter; et al. (1972) found that the protein content of respiratory washings markedly increased. These secretions were nonspecific and contained relatively large amounts of plasma proteins which in primary infection, were mostly albumin and globulins. However, unless the animal had previously experienced the disease, specific antibody activity was not seen in the initial outpouring of globulin. Later, specific antibody activity did develop and was measured in the secretions.

Curtain; et al. (1971) and Lieberman; et al. (1971) have demonstrated that after infection, the respiratory secretions contain high concentrations of antibodies which can not be accounted for only by passive transudation from plasma. Most of the antibody activity is associated with IgA. Experimental immunofluorescence studies indicated that IgA antibodies were produced both systemically and locally by immunoblasts and plasmacytes in the lamina propria of the tracheobronchial wall and secreted through the epithelium into the bronchial lumen (Curtain; et al. 1971; Lieberman; et al. 1971). When these workers measured nasal and lung washings for antibody activity and IgA content, they observed that these two factors were closely associated. Although IgE appears in normal tracheobronchial secretions of man, its concentration increases in allergic

subjects(Boat & Cheng, 1980). Bienenstock; et al. (1973) studied the morphological features of the bronchial lymphoid tissue in a large number of mammals including man and revealed that there is an intimate association between the peribronchiolar lymphoid aggregates and the overlying epithelium. These investigators found that the epithelium overlying the lymphoid aggregates is flattened, contains no goblet cells, PAS or AB positive material and is heavily infiltrated with lymphocytes.

The defence mechanisms of the respiratory secretion also include nonspecific humoral factors such as lysozymes and lactoferrin that pour into the airways during early inflammation (Cheville, 1983). Several antiproteases that inhibit trypsin, chymotrypsin and leucocyte proteases appear to be secreted by airway tissues. These protease inhibitors may be important protective agents when leucocyte proteases are released in the course of lung infection, smoking or other inflammatory conditions(Boat & Cheng, 1980).

Polymorphonuclear leucocytes are the dominant antimicrobial factors in the purulent exudate of bacterial infections of the respiratory tract. Brownstein and Johnson (1982) infected by nasal route normal and leucopenic mice with Pseudomonas aeruginosa and observed the disappearance of bacteria in both groups. Most bacteria were cleared rapidly from the nasal cavity of normal mice, whereas they invaded the nasal cavity of the leucopenic animals.

SECTION 2

MUCUS SECRETION IN THE TRACHEOBRONCHIAL AIRWAYS

1.0 INTRODUCTION

Airway secretions are a complex, heterogeneous mixture of substances. Components of these secretions are elaborated and modified by a number of mechanisms and at several levels within the airways. In the absence of disease, small amounts of secretion spread out to form a thin layer over the epithelial surface (Boat & Cheng, 1980).

Mucus has an important protective role in the respiratory system. In acute inflammation, mucus formed in epithelial goblet cells and subepithelial mucous glands, floods the damaged epithelial surface. It protects and provides the surface with the tenacious material that, when expectorated, removes much of the debris and irritants which may be present (Cheville, 1983).

Irritation increases mucus production: arguing teleologically it is presumed that this is to help in dilution, trapping and removal of noxious vapours and particulate agents (Reid, 1978a). The presence of excess mucus has an irritant effect on sensory nerve endings, which induce the cough reflex, a highly important mechanism for removing debris (Cheville, 1983).

Since excessive production of mucus is found in several diseases and is associated with structural changes in the airways, the events of mucus secretion are important both in health and disease.

2.0 SECRETORY CELLS OF AIRWAY EPITHELIUM

The cells that secrete mucous glycoproteins are found in both the submucosal glands and surface epithelium (Reid, 1978a). Submucosal glands are of much more significance than the mucosal goblet cells in production of mucus. It is estimated that goblet cells account for only 1 or 2% of mucus production in the normal

tracheobronchial airways of man (Dunnill, 1982).

The ultrastructure of mucous cells of the surface epithelium and of submucosal glands is similar. The cells are usually packed with secretory granules that are typically electron lucent and confluent.

2.1 MUCOUS CELLS OF SURFACE EPITHELIUM

Mucous, serous and Clara cells have been identified as secretory cells of airway epithelium (Jones & Reid, 1978).

There are eight cell types recognised, which together make up the surface epithelium. Additionally, two migratory cells and two neural elements may either migrate into or pierce the epithelial basement membrane, respectively (Jeffery, 1983). In most mammals, mucous cells are present throughout the airways, while Clara cells are found only in intrapulmonary regions, with the exception of the mouse, where they are found throughout the airways (Table 1.2).The frequency of these cell types has been established in the rat (Jeffery & Reid, 1975), hamster (Kennedy; et al. 1978), mouse (Pack; et al. 1981) and sheep (Mariassy & Plopper, 1983). In normal rat airway, the mucous cell represents less than 1% of the total airway epithelial cell population, while total secretory cells represent 20 - 27% (Jones & Reid, 1978). In hamsters, goblet cells comprise about 39% of the tracheal epithelium (Kennedy; et al. 1978) .

2.2 SUBMUCOSAL GLANDS

The submucosal gland is composed of tubules,each formed either by mucous cells or by serous cells, connected by a duct system to the airway surface (Meyrick; et al. 1969). In humans, the secretions from distally placed serous acini are thus thought to pass through the lumen of more centrally placed mucous tubules and then into the collecting duct where the ionic balance of mixed secretions is modified; its transfer via the ciliated duct, onto surface epithelium then follows (Meyrick; et al. 1969; Meyrick & Reid, 1970). In contrast, the submucosal glands of the opossum often form tubules and acini that are distal to mucous tubules and their ducts. In the later case the hydrotic cells are thought to secrete copious amounts of watery fluid, which presumably is used by the respiratory tract to

TABLE 1.2: NORMAL SECRETORY CELL TYPES OF THE SURFACE EPITHELIUM OF AIRWAYS (JEFFERY, 1983)

ANIMAL SPECIES	CELL TYPES		
	MUCOUS	SEROUS	CLARA
HUMAN	+	FOETUS	+
MONKEY	+	-	+
DOG	+	-	+
CAT	+	-	+
PIG	+	-	+
COW	+	-	+
RAT	+	SPF	+
MOUSE	+	-	+
HAMSTER	+	YOUNG	+
RABBIT	+	-	+
GUINEA PIG	+	-	+
FERRET	+	-	+
BIRD	+	-	-

cool the body by its latent heat of evaporation (Sorokin, 1965).

Many nerve fibres surround the secretory acini in the cat and human, and occasionally they penetrate the acini to lie in juxta position to individual secretory cells (Jeffery, 1983).

The total volume and airway distribution of submucosal glands is species related (Jeffery, 1983) (Table 1.3). Although the cell types of the gland have been well described in the opossum (Sorokin, 1965) and human (Meyrick & Reid, 1970), little is known of their morphology in other species.

The submucosal glands are present in airways that have cartilage. They lie in the submucosa either between the cartilage and the epithelial surface or between and occasionally external to the plates of cartilage (Jones & Reid,1978).

3.0 GLYCOPROTEINS OF MUCUS

Mucoglycoproteins are responsible in large part for the majority of histochemical activity and the gelation of respiratory mucus. These glycoproteins are secreted by mucous cells of submucosal glands and goblet cells of the surface epithelium in the trachea and bronchi (Boat & Cheng, 1980). All the mammalian epithelial glycoproteins are similar in composition. A single peptide chain constitutes up to 20% of the molecule by weight (Roussel; et al. 1978; Boat; et al. 1976). The bulk of the molecule is made up of numerous oligosaccharide chains, 1 - 20 or more sugar residues in length (Roussel; et al. 1975), which are attached to the peptide region by O-glycosidic linkages between N-acetylgalactosamine, and threonine or serine. These oligosaccharide chains contain five sugars; fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine and N-acetylneuraminic acid (Robert, 1974; Boat; et al. 1976; Roussel; et al. 1978). Considerable heterogeneity of oligosaccharide chains has been noted for glycoproteins from a single cellular source and some oligosaccharide chains may be branched (Roussel; et al. 1975).

The special rheologic features of bronchial mucus arise from the epithelial acid glycoproteins. A glycoprotein is a conjugated protein in which a polypeptide core is covalently linked to oligosaccharide

TABLE 1.3: SUBMUCOSAL GLAND DISTRIBUTION THROUGHOUT TRACHEOBRONCHIAL AIRWAYS (JEFFERY, 1983)

SPECIES	TRACHEA	BRONCHUS	BRONCHIOLI
HUMAN	++	++	-
MONKEY	+	+	?
HORSE	+	+	-
PIG	++	+	-
COW	++	+	-
SHEEP	++	+	-
DOG	++	+	-
CAT	++	++	+
RABBIT	+	-	-
GUINEA PIG	-	-	-
FERRET	+	+	+
OPPOSUM	++	++	+
HAMSTER	-	-	-
RAT	+	-	-
MOUSE	+	-	-
BAT	?	+	?

++ = ABUNDANT; + = PRESENT; - = UNIDENTIFIED;
? = UNKNOWN.

side chains, often branched, without a repeating unit and with a relatively low proportion of sugar residues. Three glycoproteins have been described in mucus and are designated sialomucin, sulphomucin and fucomucin. Each contains sialic acid and the first two also contain sulphate (Reid, 1978a).

The type and possibly the amount of glycoprotein secreted by airway mucosal cells is controlled by a series of enzymes at the level of biosynthetic mechanisms. Synthesis of the polypeptide portion of a glycoprotein is initiated in the rough endoplasmic reticulum. The first sugar or sugars are added in the rough or smooth endoplasmic reticulum, and completion of oligosaccharide synthesis occurs in the Golgi apparatus. Growth of the oligosaccharides involves the sequential transfer of individual sugars from sugar nucleotides to the chain. These transfer reactions are catalysed by a series of enzymes collectively called the mutliglycosyltransferase system (Jentoft; et al. 1976). Each glycosyltransferase in the system requires the product of the previous enzymatic reaction as its sugar acceptor and, in turn, generates an acceptor for another transferase. Availability of glycosyltransferase, as well as nucleotide sugars, at any point along the synthetic pathway determines the sequence of sugars and length of the saccharide chain. Addition of sialic acid to a sugar chain terminates chain growth (Carlson; et al. 1970). Sulphate ester is transferred from active sulphate to sugar residues on already growing oligosaccharide chains by sulphotransferases (Silbert, 1967). Heterogeneity of oligosaccharides on each glycoprotein molecule is to be expected because of this imprecise synthetic mechanism (Boat & Cheng, 1980).

4.0 TYPES OF GLYCOPROTEINS

The secretory cells of the rat airway surface may contain only neutral glycoprotein, acid glycoprotein, or a mixture of both (Jones; et al. 1973). In the trachea and main bronchi of normal rats, most cells contain either neutral glycoprotein or neutral mixed with acid glycoprotein, while in the intrapulmonary airways most contain a mixture or acid glycoprotein. After irritation, the regional difference in the secretory cell type is lost and there is a shift in the pattern of synthesis in the extrapulmonary airways to include more acid glycoprotein and in the intrapulmonary airways to include more

neutral secretion.

Sturgess and Reid (1973) observed that a stimulus to the secretory cell population of the airway may change the predominant type of acid group. Isoprenaline sulphate increases both the absolute number of cells containing acid glycoprotein in rat airway epithelium and the proportion of secretory cells containing sulphated glycoprotein.

Changes in the predominant type of acid glycoprotein were also found in mucous cells in hypertrophied bronchial submucosal glands in pigs with experimentally induced enzootic pneumonia (Jones; et al. 1975). Increase in gland size was accompanied by increase in the proportion of area containing acid glycoprotein, with relative increase in the amount of sialidase-sensitive sialomucin and sulphomucin glycoprotein and decrease in the sialidase-resistant sialomucin type.

Jeffery and Reid (1977) have studied ultrastructurally the changes of secretory cell granules. In addition to cells containing electron-dense granules (serous cells) and those containing electron-lucent granules (mucous cells) a cell with "transitional" granules was observed. In the irritated airways these investigators found an increase in the population of the cell third type and they suggested that irritation may cause a transition from one secretory cell type to another; from Clara to serous and from serous to mucous.

An individual cell can quickly "switch on" organelles that lead to development of a secretory cell or "switch over" from the production of one type secretion to another. These changes affect both the original population and new secretory cells that are developing as the result of conversion or mitosis. On withdrawal of the stimulus these steps may be retraced, at various speeds to achieve the original state (Baskerville, 1976). The switch back to the original glycoprotein type seems particularly fast and is even shorter than the epithelial replacement time (Jones & Reid, 1978)

5.0 HISTOCHEMICAL IDENTIFICATION OF GLYCOPROTEINS

Present knowledge of the varieties of glycoproteins synthesised

by secretory cells is based on their intracellular identification by histochemical techniques. Quantitative analysis of glycoprotein staining has added both to our understanding of the population of secretory cells within an organ and of its synthesis within a secretory cell. No abnormal type of glycoprotein is found in disease, but there may be a striking changes in the population of the various cell types (Jones & Reid, 1978).

Four major groups of intracellular glycoproteins have been identified by histochemical methods: (1) neutral glycoprotein, (2) sialic acid-sensitive glycoprotein, (3) sialic acid-resistant glycoprotein and (4) sulphated glycoprotein (Jones & Reid, 1978).

Histochemically, differences in the oligosaccharide side chains of the glycoprotein can be identified. The periodic acid-Schiff (PAS) reaction stains the sugar region of glycoprotein, while Alcian blue (AB) stains the acid radicals (Jones, 1978; Jones & Reid, 1973b). Epithelial glycoproteins may be either sialyated or sulphated. At pH 1, AB stains only the sulphated ester; at pH 2.6, it stains all acid radicals. Sialidase digestion removes the sialidase-susceptible sialic acid and leaves behind the resistant form. By staining a series of consecutive sections with these techniques, the various types of glycoprotein molecules with or without acid radicals can be identified (Jones & Reid, 1973a & b).

Quantification has made it possible to assess the proportion of tracheobronchial glands that make a given type of glycoprotein molecule. In the case of goblet cells the number of secretory cells can be counted and related to the total number of cells present . The various types of secretory cells can be expressed by reference to either total cells or total secretory cells (Reid, 1978b). Lamb and Reid (1972) have used point counting to find the proportion of the gland occupied by various types of cell.

6.0 FACTORS INFLUENCING THE ACID PROPERTIES OF GLYCOPROTEINS

6.1 SOURCE

Glycoproteins secreted by goblet cells appear to be more acid than their counterparts from submucosal glands. Analysis of differences between labelled glycoproteins released by intact and brushed dog tracheal explants have demonstrated that goblet cell mucus is relatively enriched in SO_4-labelled, compared with [3H]-glycosamine-labelled glycoproteins (Stahl & Ellis, 1973). This observation suggests that the composition and perhaps also the properties of goblet cell mucus may differ from gland mucus. However, direct analysis of goblet cell secretions "in situ" is necessary to confirm this. Surface epithelium has been removed from cat and human trachea by exposure to EDTA-containing solution (Tandler; et al. 1979). The epithelium can be maintained in organ culture for 24 or more hours, during which time labelled precursors are incorporated into glycoproteins that are subsequently released onto the surface (Boat & Cheng, 1980). This technique should provide opportunities to collect and analyse goblet cell mucus, until future studies provide better methods to isolate goblet cells and submucosal gland cells individually for assessment of their secretory function and products.

6.2 CHRONIC LUNG DISEASE

The acidic properties of secreted glycoproteins also change in several hypersecretory conditions. Enlargement of the tracheobronchial mucous glands, together with an increase in the number of mucus-secreting cells in the surface epithelium at all levels are the characteristic findings in chronic bronchitis of man (Dunnill, 1982) and enzootic pneumonia of pigs (Jones; et al. 1975). Glycoprotein precursors containing acid radicals are increased in chronic lung disease. In particular, those containing sulphate ester or sialidase-resistant sialic acid are increased in man and sialidase-sensitive sialic acid are increased in the pig (Lamb, 1968; Jones; et al. 1975) .

SECTION 3

CHRONIC NON-PROGRESSIVE PNEUMONIA OF SHEEP IN NEW ZEALAND

1.0 INTRODUCTION

Sheep are of special economic importance to New Zealand, and it is generally recognised that chronic non-progressive pneumonia (CNP) is one of the most prevalent diseases of sheep in this country. Chronic non-progressive pneumonia is a low mortality, high morbidity disease characterised by inadequate weight gain and exercise intolerance (Alley, 1975a & 1985). Therefore, there has been a widespread attention and interest in this disease.

The pathogenesis and aetiology of pneumonia in sheep has been comprehensively discussed by Alley (1975a) and Davies (1985a). The current situation regarding the cause or causes of CNP of young sheep in New Zealand is vague. Several agents have been isolated from cases of naturally occurring pneumonia in young sheep (Carter & Hunter, 1970; Alley, 1975b; Davies & Humphreys, 1977) (Table 1.4), but the aetiological significance of several of these organisms has not yet been determined. Experimental reproduction of the disease has been accomplished by endobronchial or intranasal inoculation both of lung lesion homogenate (Alley & Clarke, 1979) and cultures of *M. ovipneumoniae* either alone or combined with *Pasteurella haemolytica* (Alley & Clarke, 1979; Gilmour; *et al.* 1979 & 1982), PI3 virus (Davies; *et al.* 1981b), or adenovirus (Davies; *et al.* 1982).

The role of these organisms, alone or in combination as a cause of CNP of lambs in New Zealand will be the subject of this review.

TABLE 1.4: ORGANISMS COMMONLY ISOLATED FROM NORMAL AND PNEUMONIC LUNGS OF YOUNG SHEEP IN NEW ZEALAND

ORGANISMS	TYPE OF PNEUMONIA	SITE OF ISOLATION	REFERENCE
MYCOPLASMAS			
M. ovipneumoniae	CNP	LT	Clarke; *et al.* (1974)
M. arginini	"	LT	" " "
GRAM NEGATIVE BACTERIA			
P. haemolytica	"	LT	Alley, (1975)
N. catarrhalis	"	LT	" "
E. coli	"	LT	" "
B. parapertussis	"	LT	Manktelow (1984)
GRAM POSITIVE BACTERIA			
staphyloccoci	"	LT	Alley(1975)
streptoccoci	"	LT	" "
Corynebacterium sp.	"	LT	" "
VIRUSES			
Parainfluenza type 3	AP	NS	Carter and Hunter(1970)
Adenovirus	NORMAL	NS,LT, FAECES	Davies and Humphreys(1977)

DEFINITION OF ABBREVIATIONS

CNP=CHRONIC NON-PROGRESSIVE PNEUMONIA, LT=LUNG TISSUE, NS=NASAL SECRETIONS, AP=ACUTE PNEUMONIA.

2.0 DEFINITION

The common forms of pneumonia of sheep in New Zealand may be divided into two distinct pathological and epidemiological entities; an acute pneumonia affecting sheep of all ages (Salisbury, 1957) and a chronic or subacute pneumonia confined to lambs aged 3 - 10 months (Alley, 1975a). Acute pneumonia is characterised by intense congestion, alveolar haemorrhage, fibrinous exudation and ventral consolidation of both lungs. Salisbury (1957) investigated outbreaks of acute pneumonia in New Zealand and found that the disease affected adults as well as young stock and was often associated with a predisposing environmental stress. Chronic non-progressive pneumonia is characterised by varying degrees of dull red to grey consolidation of the anterior lobes (Alley, 1975a & b). Alley (1975b) divided CNP into four categories on the basis of the gross and histological appearance of the lesions. Chronic and subacute pneumonia is widespread throughout New Zealand and may sometimes affect up to 70 to 80% of some groups of lambs being slaughtered during the March to May period (Alley, 1975a & b). Mortalities attributable directly to CNP are low (Alley, 1975a).

In the United Kingdom, a chronic pneumonia of lambs, similar to CNP but termed atypical pneumonia to distinguish it from the acute enzootic pneumonia has been recognised for more than two decades (Stamp & Nisbet, 1963).

Experimental reproduction of the disease has been accomplished by the endobronchial inoculation both of pneumonic lung homogenates (Alley & Clarke, 1979) and of cultures of *M. ovipneumoniae* either alone or combined with *P. haemolytica*, with and without *M. arginini* (Jones; *et al.* 1978; Gilmour; *et al.* 1979) and also with cultures of PI3 virus combined with *P. haemolytica* (Davies; *et al.* 1977 & 1981a).

3.0 POSSIBLE ROLE OF MYCOPLASMA OVIPNEUMONIAE

This organism was originally isolated in Queensland, Australia by St. George; et al. (1971) and later characterised by Carmichael; et al. (1972). In a series of experiments by these authors, nearly all the animals infected with M. ovipneumoniae by various methods subsequently showed microscopic evidence of a proliferative interstitial pneumonia. Macroscopic lesions occurred in just over half of all experimental animals (St. George; et al. 1971; Sullivan; et al. 1973a & b). However, recovery of mycoplasma from experimental animals was achieved only in preliminary experiments (St. George; et al. 1971). In Scotland, Foggie; et al. (1976) obtained similar results when specific pathogen-free lambs (SPF) were inoculated with M. ovipneumoniae and they recovered the organisms from all infected but from no control animals. In transmission experiments in New Zealand, Alley and Clarke (1979) found the M. ovipneumoniae produced focal and early pneumonic lesions when inoculated intranasally into a group of conventionally reared lambs, but the organisms failed to produce lesions similar to the natural disease. Strains of M. ovipneumoniae isolated from sheep affected with "atypical" pneumonia were inoculated endobronchially into conventionally reared and SPF lambs by Gilmour; et al. (1979). These investigators observed changes resembling those of the naturally occurring disease in about 50% of conventionally reared animals given M. ovipneumoniae alone. Similar but extensive changes were seen in SPF lambs. In this study, M. ovipneumoniae organisms were recovered from the majority of inoculated lambs. Nevertheless, Davies; et al. (1981b) found that when SPF lambs were infected with M. ovipneumoniae alone, no gross lesions or histological changes were produced in the lungs.

The effect of penicillin on CNP transmitted by hemogenates of affected lung of CNP in conventionally reared lambs was investigated by Alley and Clarke, (1980). Although penicillin markedly diminished the severity of lesions in this study, it did not prevent either colonisation of the lung by M. ovipneumoniae or the development of localised minimal lesions that had some features of the natural disease. Later, Jones;et al. (1982a) compared a suspension of ampicillin treated pneumonic lung tissue prepared from naturally occurring cases of chronic pneumonia, with a mixture of six cloned

strains of M. ovipneumoniae for its pathogenicity in both SPF lambs and conventionally reared lambs. All the animals used in this study were treated for the first three days of the experiment with ampicillin. The finding in both groups of the animals were similar and M. ovipneumoniae was the only organism recovered from the respiratory tract of infected animals but was not recovered from the control group. However, histopathologically confirmed proliferative exudative pneumonic lesions were seen in 50% of the lambs receiving pneumonic lung tissue; compared with only 21% of those receiving M. ovipneumoniae. The authors suggested that despite the use of mixed cloned strains cultured in suspension at -40 °C, some degree of attenuation of the mycoplasma had occurred, either by selection for avirulent strains in the act of isolation on artificial medium, or during subculture. Alley and Clarke (1979) also found pneumonic lung homogenate to be more pathogenic than a low passage strain of M. ovipneumoniae. Recently, Thorns and Boughton (1980) have shown that low passage strains of Mycoplasma bovis, but not high passage strains induced a systemic response in mice inoculated into the mammary glands.

More recently Buddle; et al. (1984) compared the virulence of five strains of M. ovipneumoniae in the mouse mammary gland. They found that the three most virulent M. ovipneumoniae isolates were initially recovered from pneumonic ovine lungs. The variation in the ability of M. ovipneumoniae isolates to induce mastitis indicated variation in the virulence for the mouse. If there is a similar variation in virulence in the sheep respiratory tract, then this may explain the inconsistent production of lesions with pure culture of the agent obtained in some studies (Foggie; et al. 1976; Jones; et al. 1978; Alley & Clarke, 1979).

The suggestion by Alley and Clarke (1980) that bacteria are an essential factor in the pathogenesis of moderate and severe forms of CNP in lambs, was refuted by Jones;et al. (1982b), who found that an inoculum which contained no demonstrable organisms other than M. ovipneumoniae produced pneumonic lesions in both SPF and conventionally reared lambs. However, their inoculum contained a variety of strains of M. ovipneumoniae. Jones; et al. (1976) have described serological differences between M. ovipneumoniae isolates, so that variation in pathogenicity might be expected to occur. The

variation in the nature and severity of lesions produced experimentally with M. ovipneumoniae alone by several workers tends to support this hypothesis (Sullivan; et al. 1973b; Foggie; et al. 1976; Alley & Clarke,1979). In considering these results the finding by Jones et al. 1976 & 1982b of differences in pathogenicity of M. ovipneumoniae isolates may be well pertinent.

The results of these experiments have suggested that M. ovipneumoniae alone may be capable of producing chronic pneumonia in young sheep, but under natural conditions, interaction with other pathogens normally occurs.

4.0 POSSIBLE ROLE OF PASTEURELLA HAEMOLYTICA

Although Pasteurella haemolytica has been commonly associated with an acute fibrinous pneumonia (Salisbury, 1957). Alley (1975a & b) found that it was also the most common bacterium recovered from the nasal cavities and lungs of sheep with naturally occurring CNP. Experimental reproduction of pneumonic lesions in conventionally reared lambs with P. haemolytica alone has not been uniformly successful and requires large doses of inoculum (Stamp & Nisbet 1963; Biberstein; et al. 1967 & 1971; Davies; et al. 1981a; b & 1982). Davies et al. (1981c) found that the number of bacteria recovered from inoculated lungs tended to increase between 0 and 4 hr post-inoculation and then declined rapidly over the next 8 hr. These authors found that the rate of clearance was extremely variable and viable bacteria were recovered from animals up to 72 hr post-inoculation. Nevertheless, P. haemolytica organisms persisted and proliferated to produce mild to moderate exudative pneumonic lesions in some conventionally reared animals (Davies; et al. 1982) and more severe lesions in SPF lambs (Gilmour; et al. 1975). Occasionally, proliferative exudative lesions were produced in some SPF lambs by P. haemolytica alone and this was thought to be due to a less specific response by SPF lambs to the bacterium compared with conventionally reared sheep (Gilmour; et al. 1979; Jones; et al. 1982b).

Although it has been proved that P. haemolytica alone is capable of producing exudative pneumonic lesions, its role as a primary aetiological agent in CNP is doubtful because of the very

large number of organisms required to establish an infection.

5.0 POSSIBLE ROLE OF VIRUSES

Parainfluenza type 3 virus (Carter & Hunter, 1970) and adenovirus (Davies & Humphreys, 1977a) are the only viruses isolated from pneumonic lesions of sheep in New Zealand to date. There is also serological evidence of RSV presence in lambs in New Zealand (Davies, 1985b) PI3 and adenovirus have been used alone or in combination with other microbial agent(s) to produce experimental pneumonic lesions.

5.1 OVINE ADENOVIRUS

Two strains of adenovirus were originally isolated from a flock of lambs by Davies and Humphreys (1977a). The isolation could not be correlated with the development of any observed respiratory disease in individual animals in the flock. Davies; et al. (1980) related the appearance of a high level of pneumonia in slaughtered lambs to serological evidence of infection with adenovirus in a flock of lambs. Although no causal relationship could be established from their data they suggested that this virus may have a role in the development of ovine pneumonia .

Experimental inoculation of adenovirus alone into a group of SPF lambs produced areas of epithelial cell proliferation and consolidation of lung tissue (Davies & Humphreys, 1977b, Davies; et al. 1982), however, the virus failed to produce a lesion similar to that of CNP.

5.2 PARAINFLUENZA TYPE 3 VIRUS

Although the New Zealand strain of ovine PI3 virus was originally isolated from cases of mild enzootic pneumonia (Carter & Hunter, 1970), it failed experimentally to produce pneumonic lesions when inoculated alone into a group of lambs (Davies; et al. 1981a). However, Hore and Stevenson (1969) in Scotland and Cultip and Lehmkuhl (1982) in the USA successfully produced lesions by experimental infection of lambs with PI3 alone. The main features described in the experimental disease were similar to uncomplicated natural cases of mild acute pneumonia. Post-mortem examination of the lungs revealed

multifocal areas of consolidation in all lobes. These areas were characterised microscopically by bronchiolitis and interstitial pneumonia (Hore & Stevenson, 1969; Cultip & Lehmkuhl,1982). Lesions produced experimentally by ovine PI3 virus are typically an acute type of lesion, thus the evidence that PI3 virus infection on its own as a major cause of CNP is not strong.

6.0 EVIDENCE FOR MULTIFACTORIAL AETIOLOGY

6.1 VIRUS AND P. HAEMOLYTICA

Experimental reproduction of pneumonia in sheep by inoculating P. haemolytica alone intranasally or intratracheally, as discussed previously, has not been satisfactory. Therefore, a predisposing agent may be necessary for P. haemolytica to become established in the lungs and cause disease (Biberstein; et al. 1967). Initial attempts to produce pneumonia by inoculating lambs with PI3 virus and P. haemolytica were made by Biberstein; et al. (1971). Subsequently, it was shown that animals inoculated with PI3 virus (Davies, 1977; Sharp; et al. 1978; Davies; et al. 1981b & c),bovine syncytial virus (al-Darraji; et al. 1982),adenovirus (Belak; et al. 1976; Davies; et al. 1982) and reovirus (Belak; et al. 1974) were more susceptible to infection with P. haemolytica if the bacteria were inoculated late in the course of the virus infection. The results of these experiments have shown that viruses enhance the pathogenicity of P. haemolytica and that the combined infection was associated mainly with the development of an acute necrotising type of lesion resembling enzootic pneumonia.

Ovine PI3 virus is the virus that has been examined most intensively as a predisposing agent (Davies,1977; Sharp; et al. 1978; Davies; et al. 1981b & c). In SPF lambs, histologically, the lesions produced fell into two categories: an acute necrotic lesion and a milder, purulent bronchopneumonia (Davies; et al. 1981c). Rushton; et al. (1979) and Sharp; et al. (1978) observed occasionally milder chronic pneumonic lesions co-existed with the necrotising lesions. These observations prompted these authors to suggest that there may be long-lasting effects of the combined infection and that there may therefore be a role for viruses in the aetiology of subacute and chronic pneumonias.

6.2 MYCOPLASMA OVIPNEUMONIAE AND P. HAEMOLYTICA

The organisms most commonly associated with CNP are M. ovipneumoniae and P. haemolytica (Alley, 1975a). Inoculation of conventionally reared lambs with lung lesion homogenates from naturally occurring cases, or with cultures of M. ovipneumoniae and P. haemolytica have produced lesions indistinguishable from CNP in most animals (Alley & Clarke, 1979; Jones; et al. 1978; Gilmour; et al. 1979). In contrast, with SPF lambs only, a small proportion developed chronic pneumonia when inoculated with M. ovipneumoniae and P. haemolytica and the extent of the lesions induced was slight (Gilmour; et al. 1979). Since the reasons for this were not clear Jones; et al. (1982c) re-investigated the susceptibility of SPF lambs to infection with these organisms. They used an inoculum composed a mixture of six strains of M. ovipneumoniae to exclude the possibility that strains of low virulence had initially been used. Proliferative lesions similar to chronic pneumonia were found in all the SPF lambs inoculated with these two organisms.

Davies; et al. (1981b) infected SPF lambs with P. haemolytica, M. ovipneumoniae and PI3 virus individually and in combination. No gross or histological changes in the lung of lambs inoculated with M. ovipneumoniae were detected. Where infection was established, it had minimal effect on the course of infection with either PI3 virus or P. haemolytica. However, infection with either PI3 virus or P. haemolytica did not influence the course of infection with M. ovipneumoniae (Davies; et al. 1981b).

7.0 CONCLUSION

The literature indicates that the aetiology of CNP in sheep is likely to be complex. Both experimental studies and the investigation of natural disease have shown that M. ovipneumoniae and P. haemolytica are likely to be major pathogens although it is difficult to reproduce the disease experimentally with pure cultures of these organisms. Viruses, particularly PI3 have been shown experimentally to be triggering agents for bacterial infection but the evidence for their involvement in the natural disease is not strong.

The inconsistent results obtained in some experimental studies using M. ovipneumoniae were perhaps due to variation in virulence of mycoplasma isolates or the use of laboratory attenuated strains of the organisms.

The other organisms isolated from pneumonic lesions appear to be of little significance and they are probably opportunistic invaders.

SECTION 4

TRACHEAL ORGAN CULTURE

1.0 INTRODUCTION

The use of organ culture to provide a differentiated epithelium for observation of the interaction between microbial agent and host cell has only recently begun to be exploited. Organ culture may be described as the maintenance of complete rudiments or fragments of organs in a viable, differentiated,functional condition in a nutrient medium *in vitro* (Collier,1979). Although cell division takes place, the study of growth, in the sense of cell multiplication, is seldom the primary object of the technique; rather, the method is designed to provide an environment which permits differentiated tissues to exercise their normal functions under the closely controlled conditions obtainable. Once this has been achieved for a given tissue, all kinds of experiments become possible which could not be undertaken *in vivo* (Fell, 1976).

The practice of *in vitro* culture extends back as early as 1859 and has been instrumental in the advancement of knowledge in the areas of embryology, endocrinology, pharmacology, genetics, carcinogenesis and infectious disease (Hodges, 1976). The employment of organ culture to study the effects of infectious agents on host tissue was begun in 1957 when Barski; *et al*. (1957) & (1959) used cultures of human and simian tissues of the bronchial and tracheal lining to study the effect of poliovirus and adenovirus on respiratory ciliated epithelium. Hoorn & Tyrrell (1965) used organ culture to examine the effects of respiratory viruses on tracheal epithelium.Since these first reports many infectious agents have been studied in organ cultures from many different host tissues. Tracheal organ cultures in particular have proved extremely useful in studying the pathogenesis of respiratory microbial infections (Collier, 1979).

For studies using organ cultures to be meaningful, at least two criteria must be met. Firstly,the cultured host tissue must be able to be maintained in a state of normal structure and funtional activity

throughout the duration of the experiment. Secondly, the response to the infectious agent by the cultured tissue *in vitro* must be substantially the same as the response of the target tissue in the natural disease *in vivo* (Fell, 1976).

2.0 METHODS OF ORGAN CULTURE PREPARATION AND MAINTENANCE

Three main types of technique have evolved over the last two decades and although all are currently in use the liquid medium technique is more popular than the others.

2.1 CULTURE OF TISSUES ON SOLID SUBSTRATES

The culture of tissues on the surface of a medium was first introduced by Strangeways and Fell (1926a & b) and developed by Fell and Robison (1929). In this technique organ explants are placed on the surface of a coagulum of plasma and embryo extract. The plasma clot is formed in a watch-glass which rests on a layer of cotton wool in a petri dish, the central area of the cotton wool being cut away to allow transillumination for observation purposes. The cotton wool is moistened with distilled water to prevent excessive evaporation of the medium during incubation. The petri dish lid is not sealed and there is free gaseous exchange with the air which results in an initial alkalinity of the medium. Gaillard (1951) used paraffin wax to seal the embryological watch-glass so that carbon dioxide accumulated thus reducing the initial alkaline pH of the medium.

The plasma clot method has several limitations and is not used nowadays in its original form. There are two main disadvantages. Firstly, the digestion and liquefaction of the plasma clot occurs almost invariably in the vicinity of the tissue explants. Secondly, the medium cannot be changed without transplanting the cultures and adequate samples of medium can not be removed or chemicals added to the system. Some of these difficulties have been overcome by Wolff and Haffen (1952) who introduced the concept of media, solidified with 1% agar and Rumery and Balndau (1971) have further modified this system. The agar-medium system has the advantage of not undergoing liquefaction and has properties which minimise cellular outgrowth. Tissues grown on agar substrates tend, however, to contract into a lenticular form becoming encapsulated by a layer of cells. The

resulting increased thickness of the explant and reorganisation of tissue structure can be detrimental to the culture (Hodges, 1976).

2.2 CULTURE OF TISSUE ON FLUID MEDIUM

The adequate sampling and analysis of media is difficult in the solid substrate system and limits the biochemical study of organ cultures. Furthermore, problems of adequate diffusion of metabolites; of local exhaustion of medium; and of accumulation of waste products in the immediate vicinity of the tissue explants, are accentuated in this system. These limitations led to the development of the fluid medium system (Hodges, 1976).

Attempts to grow tissues on a fluid medium were first made by placing explants on "rafts" of lens paper (Chen, 1954) or of siliconised rayon fabric (Shaffer, 1956) which were then floated on the surface of the medium. Trowell (1954) introduced the "grid" technique, whereby tissue explants placed on expanded stainless steel platforms standing in small, flat-bottomed dishes, which were incubated in sealed and humid chambers to prevent excessive evaporation of the medium.

2.3 CULTURE OF TISSUE SUBMERGED IN FLUID MEDIUM

Although the stationary culture systems have been the most extensively used among the submerged systems, they have one major drawback which is that the rate of metabolic and gas exchange in the tissue differs between areas which are either at the tissue-gas or tissue-medium interface. Medium circulation and gas exchange may be improved by agitation of the culture vessel. This approach was introduced initially by Medawar (1948) , and later Petrovic and Huesner (1963) developed a submerged organ culture method in which a continuous flow of gas mixture was passed through the medium, resulting in gentle agitation of the tissue explants. This prevented the tissues from settling on the bottom of the culture vessel with possible cell outgrowth, while gas and metabolite exchange was ensured over the entire surface of the explants. However, New and Mizell (1972) have observed that explanted embryos rotated continuously in roller tubes show more growth than those maintained in stationary cultures. The organ culture fragments can also be placed in closed

tissue culture tubes. The tubes are then incubated on a roller drum rotating at about 15 revolutions per hour (Reed,1976).

Nevertheless, the stationary tracheal organ cultures are still the most extensively used method for studying bacterial disease pathogenesis. In this method, explants are simply placed on small areas of crosshatched scratches on the bottom of a petri dish, and the medium to be used is added to a depth to just cover the tissue. The dish is then placed in a 37°C incubator with a 5 % carbon dioxide atmosphere (Collier, 1979).

3.0 MONITORING THE PATHO-PHYSIOLOGICAL STATE OF THE TRACHEAL EPITHELIUM

3.1 CILIARY ACTIVITY

The trachea is a hollow organ lined by ciliated columnar epithelial cells (Banks, 1981). The cilia of these cells are beating continuously in a wave-like propulsive action through the layer of nonviscous material that exists between the cilia and the mucous layer (Cheville, 1976). In organ cultures of respiratory epithelium, ciliated and mucus-secreting cells are in their normal topological relationship to each other and the underlying tissues, ciliary activity is normal and there is some mucus secretion. But the metabolic rate probably declines sharply after initiation of the culture and mucus secretion and mitotic rate diminish (Reed, 1976). The character and frequency of the ciliary beating in tracheal organ culture rings can be observed by light microscopy at 100X magnification (Collier, 1979). Cilia are very sensitive to injury and disruption of movement considerably reduces their effectiveness in removing microbial agents and particulate material from the upper respiratory tract (Cheville, 1976). Ciliary activity has been shown to correlate with tissue viability in organ culture and has been used as an indication of injury. In studies with tracheal organ culture models various methods have been used to quantitate ciliary activity. Shroyer and Easterday (1968) classified it simply as remaining or abolished. Cherry and Taylor-Robinson (1970a) measured the vigour of ciliary beating and the percentage of tissue surface with ciliary activity present, and Collier and Baseman (1973) measured the frequency of ciliary beating with a stroboscope.

3.2 HISTOLOGY

The histopathological examination of tissue from tracheal organ culture has been frequently used as a method of assessing tissue injury. Tracheal tissue is removed from the culture media, fixed and processed in a similar manner to that used for routine histology (Shroyer & Easterday, 1968). Most of histopathological staining techniques available may be applied to organ culture material (Collier, 1979).

3.3 IMMUNOFLUORESCENCE MICROSCOPY

Immunofluorescence microscopy has been utilised to localise and examine the relationship of organisms to the tissue tracheal organ culture. Indirect fluorescent-antibody staining technique is most commonly used. The tissue rings or fragments are removed from the medium and quickly frozen in optimal cutting temperature compound (O.C.T.) and 4 um sections are prepared using a cryostat microtome. The sections are then exposed to rabbit antiserum against the organism, followed by goat anti-rabbit globulin conjugated with fluorescein isothiocyanate. The sections are then examined using a fluorescence microscope (Collier; et al. 1971). Although the immunological specificity of this method provides localisation of the organisms, which cannot be done with certainty using conventional morphological methods, it does not show the exact relationship between individual organisms and ciliated cells.

3.4 ELECTRON MICROSCOPY

The examination of thin sections by transmission electron microscopy (TEM) is often used with considerable success on tracheal organ cultures. It allows the study of the internal ultrastructure of both organism and host cell as well as the interaction between them. The methods currently in use are described by Collier; et al. (1971). To study the external features of the organism and host tissue and their attachment, scanning electron microscopy (SEM) has been employed. Muse; et al. (1976) have given a detailed account of the methods of preparation of tracheal epithelium for SEM.

3.5 METABOLIC ACTIVITY

In order to gain knowledge of the metabolic activity of the individual cells in tracheal organ culture, Collier and Baseman (1973) examined hamster tracheal rings exposed to [3H] amino acids and [3H] thymidine by radioautography. In studies utilizing radioisotopically labelled carbohydrate, protein, and nucleic acid precursors, Hu; et al (1976) examined the metabolic alterations of hamster tracheal rings infected by Mycoplasma pneumoniae. These authors found that the intimate contact between virulent mycoplasmas and the respiratory epithelium does not alone account for the subsequent interruption of host cell metabolism but must be accompanied by continued multiplication and biochemical function of attached mycoplasmas. In previous studies, Hu; et al. (1975) infected tracheal rings with virulent M. pneumoniae organisms and found an alteration in macromolecular biosynthesis and metabolic activity of the respiratory epithelial cells. There was an initial increase in [14C] galactose uptake followed by a significant decline as infection progressed with parallel decrease in [3H] orotic acid and [3H] amino acid uptake. The course of mycoplasma infection could be interrupted or reversed by the addition of erythromycin to the organ cultures which prevents the onset of abnormal orotic acid uptake. Therefore, Hu; et al. (1975 & 1976) suggested that mediation of host cell injury requires continued protein synthesis by attached mycoplasmas and that the primary effect of mycoplasma infection on tracheal organ culture may be at a transcriptional or translational level.

Tracheal organ cultures have also been shown to consume a measurable amount of oxygen when incubated in a closed micro_chamber, and tracheas infected with M. pneumoniae showed a significantly lower oxygen utilization (Gabridge, 1975). The effect of M. pneumoniae infection on hamster tracheal organ cultures has also been examined with respect to the kinetics of cellular respiration (Gabridge, 1975), dehydrogenase activity (Gabridge & Polisky, 1976) and ATP content (Gabridge & Polisky, 1977). It has been found that all these activities were diminished in infected ciliated respiratory epithelium but not in the controls.

Peroxide production was demonstrated in Mycoplasma mycoides var.capri infected chicken embryo tracheal rings but not in uninfected

cultures (Cherry & Taylor-Robinson, 1970c). Other studies, using bovine tracheal organ cultures infected with BHV1 have successfully demonstrated interferon production (Fulton & Pearson (1980).

4.0 THE USE OF TRACHEAL ORGAN CULTURES FOR STUDYING RESPIRATORY INFECTIONS

Undifferentiated cell culture lines have been used to study alterations produced by infectious microbial agents for many years. However, these cultures may or may not retain the characteristics of differentiated host cells. Organ culture has the advantage of providing differentiated cells thus enabling the interaction between agent and host cell to be fully developed (Collier, 1979). In recent years the techniques of organ culture have been widely applied to microbiological problems. Very significant advances have been made by these methods, and their role in microbiological research has become firmly established (Reed, 1976).

With the organ culture model, tissue from a single animal can be used for control and test groups at the same time. Thus, in addition to being an economic method, tracheal rings can be infected with a specific number of cloned respiratory pathogens, avoiding the effect of secondary infection which commonly occurs in naturally reared and SPF animal experiments (Collier & Baseman, 1973). Ciliary activity permits continuous monitoring of injury and viability of individual ciliated epithelial cells during the experiment and serves as an index of varying degrees of damage.

Organ culture models do not possess a blood supply and therefore do not have systemic host immune, humoral or nutritional factors present as in the intact animal. This probably magnifies the injury patterns that would be seen in the intact host. Therefore, individual cells in this system respond to infection mainly through the mechanisms of hyperplasia and/or necrosis (Collier & Baseman, 1973).

Although, organ culture studies have provided information that could not have been obtained from in vivo animal experiments; it should be remembered that isolated tissue explants can not provide any information on the systemic effect produced by infectious agents (Collier, 1979).

4.1 MYCOPLASMA INFECTION

Most mycoplasma species are extracellular pathogens that have as their target cells the epithelium of mucosal surfaces. Organ cultures permit the maintenance of an organized mucosal surfaces *in vitro* and thus allow direct observation of the interaction of mycoplasmas with these target cells (Collier, 1979).

4.1.1 HUMAN MYCOPLASMAS

One of the most commonly studied organisms using organ culture techniques has been *M. pneumoniae*; the cause of atypical pneumonia in man. This is because the organism has been difficult to study using conventional methods and there is a lack of suitable animal models. The initial studies were made by Butler (1969) who infected human embryo tracheal culture with several species of mycoplasma. In this study Butler (1969) found that tracheal organ cultures infected with *M. pneumoniae* were damaged while the other mycoplasmas used were produced no lesion. At about the same time Collier; *et al*. (1969) found that *M. pneumoniae* produced dramatic cytopathology when inoculated into hamster tracheal organ culture.

The interaction between *M. pneumoniae* and hamster tracheal organ cultures has been studied by Collier; *et al*. (1971). They studied the cytopathology following attachment of infecting organisms and observed ciliocytophthoria (formation of coalescing cytoplasmic vacuoles eventuates in fragmentation and exfoliation of ciliated epithelial cells) and eventual sloughing of the epithelial layer. Collier and Clyde(1970 & 1971a & b) continued their studies using human foetal cultures infected with *M. homonis*, *M. pneumoniae*, *M. fermentans*, *M. salivarium* and *M. orale*. A sequence of cytopathological changes denoting progressive cell injury was observed. Using immunofluorescence and electron microscopy, the *M. pneumoniae* organisms were found concentrated on the luminal surface of the epithelium in an extracellular position. The organisms were filamentous in shape and attached to the epithelial cells by a differentiated portion of the mycoplasma, which served as the means of attachment to host cells. An avirulent *M. pneumoniae* mutant that no longer retained the ability to attach to the epithelial cells produced

no cytopathological changes. No cytopathological changes were seen in human foetal organ cultures inoculated with the other mycoplasma species used in this study.

Attention was first focused on the attachment of M. pneumoniae to the surface of cilia by the studies of Woodruff; et al. (1973). These investigators examined the ultrastructural aspects of surface parasitism of M. pneumoniae in hamster tracheal organ culture. Mucosal cytopathic changes and submucosal necrosis were prominent. Muse; et al. (1976) studied the three dimensional features of the attachment of M. pneumoniae to the epithelial surface of hamster tracheal organ culture. These studies established the filamentous morphology of the M. pneumoniae and the attachment of numerous organisms to individual cells followed by progressive epithelial cell injury. In order to correlate surface with intracellular alterations in respiratory epithelium produced by M. pneumoniae, Murphy; et al. (1980) examined sequentially by TEM & SEM, the attachment of M. pneumoniae to the mucosal surface of hamster tracheal organ culture. In these studies, the organisms were located adjacent to, and on the surface of ciliated cells that exhibited lesions, but they were not intimately associated with the non-ciliated cells. Carson; et al. (1980) utilized freeze-fracture techniques to examine the relationship of M. pneumoniae to epithelial cells in tracheal organ cultures. In this study, membrane-associated particles were observed on all membrane fracture faces of the M. pneumoniae cells, but they were generally more abundant on the convex rather than the concave face of the fractured membrane.

Hu; et al. (1975) presented evidence that M. pneumoniae organisms not only needed to attach to epithelial cells to produce injury but that the organism must also be metabolically active. The course of mycoplasma infection could be interrupted or reversed by erythromycin after the initial mycoplasma-host cell interaction had occurred. The addition of erythromycin 24 hr after infection or earlier prevented the onset of abnormal orotic acid uptake and subsequent cytopathology. Later, Carson; et al. (1980), reported that the morphological alterations they observed in tracheal organ cultures infected with M. pneumoniae might represent a structural analogue to the biological findings of Hu; et al. (1975).

Organ cultures of adult hamster trachea have also been used to evaluate the cytotoxic potential of cell fractions of M. pneumoniae. Gabridge; et al. (1974a & b) reported that M. pneumoniae membranes had cytotoxic effects on tracheal organ cultures producing ciliostasis and epithelial cell cytopathology. Gabridge; et al. (1977) reported that the attachment site of M. pneumoniae membranes to the ciliated epithelium is distinct from the receptor site-mediated attachment of M. pneumoniae cells. These authors suggested that M. pneumoniae membrane, is not entirely responsible for the pathogenicity of these organisms and that other mechanisms might be involved.

In order to study the specific receptor sites of attachment, Collier and Baseman, (1973) treated tracheal rings with neuraminidase and found that the treatment of tracheal rings with the enzyme, lowered the attachment of virulent but not avirulent M. pneumoniae to epithelial cells. In their study, Engelhart and Gabridge (1977), found that the attachment of 14C-labeled M. pneumoniae was more than two fold greater in the normal epithelium and pretreatment of explants with neuraminidase decreased attachment to both squamous and pseudostratified epithelial surfaces to a similar level.

To examine the biochemical basis of changes in epithelial host cells produced by M. pneumoniae, an assessment of carbohydrate, ribonucliec acid and protein biosynthesis and metabolism was achieved by Hu; et al. (1975 & 1976). After the initial mycoplasma-host cell interaction, tracheal organ cultures were treated with erythromycin to stop protein synthesis by the mycoplasmas and then the up take and incorporation of [14C] amino acid by the cells of tracheal organ culture was measured. In these experiments, inhibition of host cell ribonucleic acid and protein synthesis was evident within 24 hr after infection of tracheal rings with virulent mycoplasmas and this damage preceded any histological changes in the tissue.

Gabridge (1975) demonstrated that hamster tracheal organ cultures were consuming measurable amounts of oxygen when incubated in a closed micro-chamber. Tracheas infected with M. pneumoniae showed a significantly lower oxygen utilization than did uninfected controls and the effect was dose dependent. Virulent mycoplasmas caused a significant decrease in relative ciliary activity and oxygen utilization, whereas attenuated mycoplasmas reduced ciliary activity

and oxygen utilization to a lesser extent. Gabridge and Polisky (1977) measured the amount of adenosine triphosphate (ATP) in hamster tracheal organ cultures. They found that in organ cultures infected with virulent M. pneumoniae or M. pneumoniae membranes there was a decrease in both ciliary activity and ATP content. When tracheal cultures were infected with attenuated or nonvirulent mycoplasmas, ciliary activity was only slightly decreased, while ATP rose slightly. Wolffing and Gabridge (1979) reported that in tracheal organ cultures infected with M. pneumoniae there was a significant decrease in ciliary activity, but a slight decline in cyclic adenosine monophosphoric acid.

The attachment of radioisotopic-labelled M. pneumoniae to hamster tracheal organ culture has been examined by Powell; et al. (1976) using radioautography and liquid scintillation counting. With these techniques they demonstrated that [3H] thymidine-labelled virulent M. pneumoniae organisms attached whereas an isotopic-labelled avirulent strain did not. These authors concluded that the viability of the mycoplasmas metabolic integrity of the tracheal organ culture were important for optimal attachment. Therefore when they pretreated the organ culture with neuraminidase or sodium periodate, attachment of organisms was significantly impaired because of the change in the specific site of attachment.

Identification of the attachment factor on virulent M. pneumoniae organisms which permits surface parasitism of respiratory epithelium was attempted by Hu; et al. (1977). They demonstrated that virulent M. pneumoniae must possess an external membrane protein in order to attach to the respiratory epithelium. This protein is trypsin-sensitive and, when the M. pneumoniae organisms were treated with protease, attachment was impaired without reducing viability of the organisms. However, when protease treated organisms were incubated in mycoplasma medium, the protein regenerated and the ability of attachment was restored. Krause; et al. (1982) and Hansen; et al. (1981) demonstrated that the mechanisms employed for haemadsorption and attachment to respiratory epithelium were similar.

Chandler; et al. (1982) studied the effect of the age of the tracheal organ culture on attachment. In this study they found that attachment of M. pneumoniae to the tracheal organ cultures increased

with the age of the culture.

4.1.2 OTHER MAMMALIAN MYCOPLASMAS

Tracheal organ cultures have been used to study the pathogenicity of several species of non-human mycoplasmas. The first studies were those of Butler (1969) and Butler and Ellaway (1971) who examined the growth and cytopathic effect of M. mycoides var.capri, M. mycoides var.mycoides, M. agalactiae and several other mycoplasmas. The Growth of each species was demonstrated except for M. mycoides var.mycoides. However, in these studies only M. mycoides var.capri produced cessation of ciliary activity and epithelial cell damage. These changes were dose dependent.

One of the main contributions in this area has been made by Cherry and Taylor-Robinson. The growth and cilia stopping effect of 24 Mycoplasma strains were examined by Cherry and Taylor-Robinson (1970a). They found that 17 strains multiplied in the organ culture, and 7 strains including M. mycoides var.capri inhibited ciliary activity.

In studies utilizing chicken embryo tracheal organ cultures Cherry and Taylor-Robinson (1970b & c) measured and compared peroxide production by several mycoplasmas and correlated the findings with damage to the ciliated epithelium. Peroxide production was demonstrated in tracheal rings infected with M. mycoides var.capri infected tracheal rings but not in uninfected cultures. The rapidity of the cilia-stopping effect resulting from M. mycoides var.capri was related to concentration of the organisms in the culture and the production of peroxide was an important virulence factor in M. mycoides var.capri infection. In 1973, these authors published further data on the growth and cilia-stopping effect of several mycoplasmas in 5 tracheal organ culture systems. They found that M. mycoides, M. pulmonis, M. neurolyticum, M. hyorhinis, M. meleagridis and M. agalactiae adversely affected ciliary activity. Mycoplasma mycoides var.capri grew and had a rapid cilia-stoping effect in chicken tracheal organ cultures, whereas the growth of M. mycoides var.mycoides was erratic and the loss of ciliary activity was very slow. In addition, M. mycoides var.mycoides was more fastidious, in that it did not grow in all cultures but in general it

was less damaging to chicken respiratory epithelium than M. mycoides var.capri.

Organ cultures of porcine and bovine trachea were used by Reed (1971) to study the interaction between influenza viruses and mycoplasmas. She found that M. hyorhinis produced minimal histopathological changes in the epithelium and swine influenza virus produced moderate damage, while a double infection with both agents increased the destruction markedly. Titres of virus in the organ culture fluid were unaltered by the presence of concurrent infection with M. hyorhinis, but growth of the mycoplasma was enhanced. Reed (1972 & 1976) reported that prior infection of tracheal organ cultures with bovine rhinovirus or parainfluenza virus type 3, both of which were cytopathic for the tracheal epithelium, enhanced the growth of M. hyorhinis and M. donetta by damaging the epithelium and releasing a conditioning factor into the medium.

Additional studies on the pathogenicity of M. hyopneumoniae, M. hyorhinis, M. granularum and M. hyosynoviae in porcine tracheal organ cultures was made by Pijoan; et al. (1972) and Pijoan (1974). In these experiments, both serum-resistant and serum-sensitive strains of M. hyorhinis had a marked effect on ciliary activity and caused necrosis of the epithelium. Mycoplasma granularum and M. hyosynoviae caused cessation of ciliary movement, but to lesser extent, and necrosis did not occur. With M. hyopneumoniae, the infected organ cultures survived longer than the control cultures.

Further studies of bovine tracheal organ cultures were made by Howard and Thomas (1974) who studied the effect of M. dispar infection. They presented evidence that the injury produced by M. dispar was dependent on the presence of serum in the organ culture medium. Thomas and Howard (1974) examined the ciliary motility, mycoplasma growth curves and histopathology in bovine foetal tracheal organ cultures inoculated with M. dispar, M. bovirhinis, Acholeplasma laidlawii and "T" mycoplasma. In these studies, they found that mycoplasmas multiplied in the organ cultures, but only M. dispar produced cytopathic effects on ciliated epithelium, and only in the presence of large numbers of mycoplasmas.

A limited number of mycoplasmas have been studied in rat and

mouse tracheal organ cultures. Westerberg; et al. (1972a & b) studied the effects produced by single and mixed infections with M. pulmonis and influenza A virus. In this study, they found that M. pulmonis multiplied, producing inhibition of ciliary activity and histopathological damage. However, the organism grew in close association with the cell membranes but did not attach directly to the membranes or the cilia. Influenza A virus replicated in organ cultures and produced ciliary inhibition with extensive cytopathologic changes. Simultaneous infection of the cultures with M. pulmonis and Influenza A virus resulted in more rapid ciliary inhibition and greater tissue damage than that occurring with either agents alone, a finding which confirmed the work of Reed (1971). These investigators noted that the presence of the virus had no effect on the growth of mycoplasmas, but mycoplasmas produced a decrease in the virus titre in the medium.

Recently attention has focused on the effects of Mycoplasma ovipneumoniae and Mycoplasma arginini on the respiratory epithelium by the study of Jones; et al. (1985). These investigators examined the topographical aspects of surface parasitism of M. ovipneumoniae and M. arginini in ovine and caprine tracheal organ cultures. They found that M. ovipneumoniae produced sudden ciliostasis and profound loss of cilia after 3 days in lamb explants while these effects were gradual and less pronounced in caprine explants. Mycoplasma arginini also induced ciliostasis and loss of cilia in both caprine and ovine explants. Although the onset at 2 days was more rapid, there was evidence of recovery after 6 days with apparent regeneration of cilia.

4.1.3 AVIAN MYCOPLASMAS

Organ cultures have proved useful in the study of many avian infectious agents and have been specifically employed to evaluate the effect of avian mycoplasmas.

The spectrum of growth and cilia-stopping effects of M. gallisepticum and M. gallinarum in chicken tracheal organ culture was first studied by Cherry and Taylor-Robinson (1970a). They found that both mycoplasmas, multiplied in organ culture but only M. gallisepticum inhibited ciliary activity. Later these workers (Cherry & Taylor-Robinson, 1970b & 1971a & b) measured and compared peroxide

production by M. gallinarum in organ culture and correlated the findings with histopathological damage to the ciliated epithelium. Although M. gallisepticum caused rapid ciliary damage to chicken tracheal organ cultures the organism failed to produce a measurable amount of peroxide. However, when M. gallisepticum was examined for virulence factor, neither peroxide production (Cherry & Taylor-Robinson, 1970b) nor cytoadsorption(Cherry & Taylor-Robinson, 1973) appeared to play an important role. A toxic substance other than peroxide was identified by Cherry and Taylor-Robinson (1973) on medium from M. gallisepticum infected organ cultures which was mildly toxic to the ciliated epithelium.

Mycoplasma gallinarum, which is considered to be nonpathogenic in natural avian infections, grew in chicken tracheal organ cultures but did not stop ciliary activity. Surprisingly, it was found that cultures infected with M. gallinarum lost ciliary activity less rapidly than the control cultures(Cherry & Taylor-Robinson, 1973). Taylor-Robinson and Cherry (1972) suggested that the cilia-sparing effect of M. gallinarum might be due to the production by this mycoplasma of a substance that destroys peroxide in the organ cultures. Moreover, the ciliated epithelium of the organ culture system can be protected from the adverse effects of M. gallisepticum by prior infection with M. gallinarum organisms (Cherry & Taylor-Robinson, 1973).

The histopathological changes in tracheal organ cultures infected with M. gallisepticum were studied by Rathore; et al. (1979). The infected tracheal rings showed nuclear swelling, cytoplasmic vacuolation and patchy loss of cilia from the lining epithelial cells.

Takagi and Arakawa (1980), presented evidence, that metabolic products from chicken tracheal organ cultures play an important role in stimulating the growth of M. gallisepticum. Additionally, these authors found no evidence of local resistance to the infection in tracheal cultures obtained from chickens immunized experimentally against M. gallisepticum to the infection.

4.2 BACTERIAL INFECTION

Although the technique of tracheal organ culture has been applied less to studying bacteria than the virological and mycoplasma research, several significant studies have been made and the value of the technique in this area is firmly established. One of the limiting factors in its use has been the need to control contamination without using antibiotics.

Some of the first studies involved the attachment of Escherichia coli and Staphylococcus aureus to the epithelial surface of rabbit tracheal organ cultures maintained on solid agar, utilizing a point inoculation technique (Matsuyama , 1974). After point inoculation, the behaviour of the bacteria, the development of ciliostasis and the histological changes on the epithelial surface were examined. Initially, the bacteria were localised on the epithelial surface without disturbing the mucociliary system. Thereafter, surface infection and histopathological changes developed gradually on the in vitro mucous membrane.

A useful contribution to the understanding of Haemophilus influenza infection in man was made by Denny (1974) who showed that either viable H. influenza organisms or sterile supernatant fluid from infected cultures caused ciliostasis and epithelial damage in organ cultures of respiratory tract mucosa. Using scanning electronmicroscopy, the interaction between H. influenza and rat tracheal organ cultures was examined by Johnson; et al. (1983). Organ cultures of rat trachea inoculated with either type b or the noncapsulated strain of H. influenza showed loss of ciliary activity and disruption of the mucosal surface. Examination of tissue pieces by SEM showed that mucosal damage was due to the sloughing of epithelial cells. Bacteria associated with the intact epithelial surface were seen infrequently, and the sloughed epithelial cells were also free of adherent bacteria.

Bordetella bronchiseptica which is believed to be the aetiological agent of swine atrophic rhinitis and also causes pneumonia in piglets has also been studied in tracheal organ culture. This organism was first isolated from a case of canine distemper and is frequently found in the respiratory tract of dogs, pigs and other

animals, either as a member of the normal flora or associated with respiratory disease (Soltys, 1979). It has been demonstrated experimentally using canine tracheal explants that B. bronchiseptica has a strong affinity for the canine respiratory tract and there are similarities between the respiratory infections produced by B. bronchiseptica and S. aureus which has no special predilection for the respiratory tract (Bemis; et al. 1977). Scanning electronmicroscopy has been used by Matsuyama and Takino (1980) to observe infections of the rabbit tracheal mucosa with B. bronchiseptica and S. aureus. Mucous membrane infected with B. bronchiseptica was partly denuded of cilia and tufts of remaining cilia were entangled with large numbers of bacteria. Organ cultures infected with S. aureus showed partial disappearance of cilia with mild exfoliation of epithelial cells. However, it was extremely difficult to find bacteria on the damaged areas and most of the cilia were free from bacteria. Bemis and Kennedy (1981) studied the effect of B. bronchiseptica on the ciliary activity of canine tracheal epithelial cells using a sensitive system for quantitating the ciliary beat frequencies of an individual cell. They found that phase I and intermediate phase of B. bronchiseptica isolates produced significant reductions in ciliary beat frequencies within 5 min. and nearly complete ciliostasis within 3 hr. A rough-phase isolate of this organism had no effect. Phase-I and intermediate-phase isolates attached to cilia, whereas the rough-phase isolate did not.

The effect of diphtheria toxin on guinea pig tracheal organ culture was studied by Baseman and Collier (1974). Exposure of individual tracheal rings to toxins resulted in progressive inhibition of protein synthesis as well as the development of cytopathology. Iida and Ajiki (1974) examined the growth characteristics of B. pertussis in chicken tracheal organ culture, and they observed marked growth on the tracheal fragments. However, they demonstrated that serum-free or conditioned medium alone as well as an emulsion of tracheal tissues prepared in the conditioned medium supported little growth of the bacteria indicating that an intact epithelium was necessary for growth. Fluorescence microscopy showed that the principal site of bacterial growth is the epithelial surface. The inhibitory effect of 2,4 dinitrophenol on the growth of B. pertussis in chicken tracheal organ culture was also observed by these authors . Although the effect of 2,4 dinitrophenol was reversible in the early

stage of infection longer treatment with this inhibitor resulted in an irreversible inhibition of bacterial growth due to secondary damage of the tracheal fragments, thus confirming the necessity of intact epithelium for this organisms growth. Matsuyama (1977) examined the resistance of B. pertussis to mucociliary clearance by rabbit tracheal organ culture. In these studies, cultivated mucous membrane of rabbit trachea was point inoculated with B. pertussis phase-I or phase-III. Phase-I (virulent) bacteria were found to be infective at the point inoculated site, but phase-III (avirulent) bacteria rarely showed such behavior. Muse; et al. (1977) utilized the scanning electronmicroscopy technique to study the infection of hamster tracheal organ cultures with B. pertussis. They proved that B. pertussis organisms attached only to the ciliated epithelial cells and a sequence of events involving the injury, expulsion and destruction of these differentiated cells then occurred.

4.3 VIRUSES

The techniques of tracheal organ culture have been broadly applied to virological problems. Most of the recent work on growth of viruses in tracheal organ culture has been promoted by the development of simple methods of culturing respiratory epithelium and maintaining it in a state suitable for virological studies (Hoorn & Tyrrell, 1965; Tyrrell & Hoorn, 1965). The developments in this field up to 1969, have been comprehensively reviewed by Hoorn and Tyrrell, (1969). The present review deals mainly with work carried out since then involving the use of this technique for studying the mechanism of infection and related morphological changes in vitro.

The specificity of certain viruses for respiratory epithelium has been studied by Toms; et al. (1974) who used ferret organ cultures to quantitate the growth of influenza virus in a range of respiratory and other tissues. Nasal epithelium was more sensitive to influenza virus than other tissues used in these experiments. The ability of a range of human and animal influenza viruses to infect tissues of different species was studied by Schmidt; et al. (1974). In these experiments influenza viruses grew best in organ cultures of their homologous species, but human and swine virus also grew well in heterologous cultures. By somewhat similar methods, Henderson; et al. (1978) demonstrated that synthesis of respiratory syncytial virus

occurred only in a population of ciliated epithelial cells of human trachea. While in ferret trachea, virus growth occurred only in fibroblasts of the lamina propria and serosa. Cytomegalovirus (Mantyjarvi; et al. (1977), human parainfluenza type 3 virus (Klein & Collier, 1974) and bovine adenovirus type 3 (Bouffard & Derbyshire, 1978) all were shown to have a close association with epithelial cells only.

The changes which occur in epithelial cells when they are prepared for tracheal organ cultures apparently do not hamper the growth of many viruses and many of the responses which normally affect growth of organisms in vivo are absent. This makes it possible to study the direct effect of the organisms on the tissue and to determine the role of individual defensive reactions (Reed, 1976). In the absence of circulation, all blood-borne factors including serum antibody and cellular responses are limited, as are the effects of changes in vascular permeability (Finkelstein; et al. 1972). However, organ cultures are able to respond to virus infection as normal tissue may do in vivo, by producing interferon (Somordintsev, 1968; Reed, 1969; Fulton & Pearson, 1980).

Experience varies regarding the secretion of locally produced antibody by tracheal organ cultures following virus infection. Cesario; et al. (1970) found that organ cultures made from the tracheas of rats which had recently recovered from an active infection of parainfluenza 1 virus (Sendai strain), and which might be expected to show some immunity, were fully susceptible to a small dose of the homologous virus. Similarly, work with Newcastle disease virus produced no evidence to implicate antibody as a regulatory factor in the establishment or maintenance of persistence infection of embryonic chicken tracheal organ cultures (Chummiskey; et al. 1973). On the other hand, Heuschele and Easterday (1970); Finkelstein; et al. (1972); Schmidt and Massab, (1974) and Schmidt; et al.(1974) established that tracheal cultures from chickens inoculated with influenza A virus secreted antibody and were resistant to homologous virus infection.

4.3.1 MAMMALIAN VIRUSES

Much of work with mammalian viruses in recent years has involved

the use of laboratory animal organ cultures to study the pathogenesis of human respiratory disease. Utilizing transmission electronmicroscopy, Westerberg; et al. (1972a) studied the pathogenesis of influenza virus infection in mouse tracheal organ cultures. Influenza virus was shown to replicate and produce ciliostasis with progressive damage to the epithelium which resulted in complete desquamation. Immunofluorescnce combined with light and electron microscopy have been used to study human parainfluenza virus infection in hamster tracheal organ cultures. The organ cultures clearly supported replication of the virus and infected tracheal explants exhibited specific cytopathological changes (Klein & Collier, 1974). Mantyjarvi; et al. (1977) assessed the pathogenicity of murine cytomegalovirus in mouse tracheal organ cultures. In these studies cytomegalovirus produced cytopathologic changes with characteristic cytoplasmic inclusions. Nedrud; et al. (1979) successfully demonstrated the cellular basis for susceptibility to mouse cytomegalovirus using tracheal organ cultures. When cultures from susceptible mice were infected with this virus, characteristic cytopathic effects developed, while, tracheal organ cultures produced from genetically resistant mice consistently failed to develop significant virus cytopathic effects.

Studies of the effects of respiratory syncytial virus on ferret and foetal human tracheal organ cultures were made by Henderson; et al. (1978). In these experiments, the sites and morphologic consequences of virus replication differed markedly in these two different species. In human tracheal cultures, synthesis of respiratory syncytial virus occurred in a population of ciliated epithelial cells where the virus produced its cytopathic changes. In ferret tracheal cultures virus growth and cytopathic effects occurred in fibroblasts of the lamina propria and serosa.

A limited number of studies of ruminant viruses have been made using tracheal organ cultures. Bouffard and Derbyshire (1978) examined the effect of bovine adenovirus type 3 (BAV-3) in this system. The growth curve of BAV-3 in bovine tracheal organ cultures showed that the virus replicated, was mainly cell-associated and persisted in the cultures. It produced histopathological changes in the epithelium including intranuclear inclusions. The direct immunoperoxidase reaction demonstrated that the virus replication site

was only in epithelial cells. Ultrastructural studies demonstrated that BAV-3 induced three different types of intranuclear inclusions. A highly electron-dense, granular material of low electron-density and small circular very dense bodies. Campbell; et al. (1979) infected foetal lamb tracheal organ cultures with ovine adenovirus type 2. This virus produced marked effects on ciliary activity and infected explants developed epithelial hyperplasia, focal erosions and intranuclear inclusions.

Fluorescent-labelled antibody techniques have been used by Chia and Savan (1974) to study the pathogenesis of infectious bovine rhinotracheitis virus infection in bovine foetal tracheal organ cultures. A decrease in ciliary activity and cytopathologic effects were the main findings in this study.

Ssentongo; et al. (1980) studied bovine viral diarrhoea-mucosal disease virus infection in bovine foetal tracheal organ cultures. The effect of the virus was assessed by observing ciliary activity and histopathological changes. There was progressive decrease in ciliary activity and the epithelial degeneration progressed to complete destruction.

4.3.2 AVIAN VIRUSES

Avian infectious bronchitis virus (IBV) has been the most commonly studied avian virus using the organ culture technique. Colwell and Lukert (1969) examined the effects of several different serotypes of IBV in cultures of chicken trachea. Extensive cytopathology was observed including rounding and sloughing of ciliated epithelial cells and there was complete cessation of ciliary movement. In this study, the specificity of infection was confirmed by fluorescent antibody technique. Darbyshire (1980), examined the effects of seven strains of IBV on tracheal cultures obtained from vaccinated and unvaccinated birds. He observed that there were no differences in susceptibility between the different levels of the trachea in any individual chicken.

The influence of temperature of incubation on chicken embryo tracheal organ cultures infected with strains of IBV were examined by Yachida; et al. (1981a). In these experiments some IBV strains were

slightly more ciliostatic at 40°C than at 37°C.

Dutta (1975) utilized scanning electron microscopy to examine the morphological changes with IBV. He noted that the columnar epithelial cells lining the tracheal rings lost their cilia and rounded up. There were numerous elevations and holes on the infected cell surface and subsequently the cells sloughed.

One of the important studies using chicken tracheal organ cultures was made by Cook; et al.. (1976). These authors assayed IBV in embryonated eggs and chicken embryo tracheal organ cultures and found that the titres obtained were very similar. Because ciliostasis is an excellent marker of infectivity, these investigators suggested that chicken embryo tracheal organ cultures could offer a reliable alternative system to embryonated eggs in assaying IBV.

The infectivity of strains of IBV, infectious laryngotracheitis, Newcastle disease virus, avian adenovirus and influenza virus were tested in tracheal explants by Butler and Ellaway (1972). Most of the virus strains halted the action of the cilia and the authors concluded that ciliostasis could be used as a measure of the infectivity of the virus pool. Recently, another comparative study was made by Yachida; et al. (1981b), who examined the effects of IBV, Newcastle disease virus, and infectious laryngotracheitis virus, on the ciliostasis patterns of tracheal organ cultures. However, the results obtained from these studies were rather conflicting. Viruses which are known to be non-pathogenic such as adenovirus had only a mild effect on the ciliated epithelium; while some strains of infectious laryngotracheitis virus were ciliostatic even although they were known to be non-pathogenic Cook; et al. (1976). Thus, the use of tracheal organ culture techniques in assessing the virulence of different viruses seems unreliable although its use in assaying different strains of one virus has been accepted.

Duck tracheal organ cultures have also been used to study avian respiratory viruses. Kocan; et al. (1978) used light microscopy and transmission electron microscopy to observe the cytopathological changes associated with type-A influenza virus in Mallard duck tracheal organ cultures.

Chicken tracheal organ culture has recently been used for the detection of IBV as a contaminant of vaccine (Nicholas; et al. 1983). Results showed that the techniques capable of detecting both strains of IBV were, in order of sensitivity; the fluorescence antibody test, ciliostasis of tracheal organ cultures and direct electron microscopy.

CHAPTER 2

THE MORPHOLOGY OF THE TRACHEOBRONCHIAL EPITHELIUM IN NORMAL SHEEP

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1.0 INTRODUCTION

Although the basic structure of the mammalian tracheobronchial epithelium and lamina propria has been known for the last century, considerable advances in understanding have been made in the last three decades, with the introduction of the transmission electron microscope (TEM) and, latterly, the scanning electron microscope (SEM).

Eleven different cell types, nine epithelial and two mesenchymal have now been identified in the tracheobronchial epithelium of mammals. The ultrastructural differences between these cell types in various species has been reviewed by Breeze; et al. (1976). Only three studies (Jeffery and Reid, 1975; Kennedy; et al. 1978; Pack; et al. 1981) have systematically examined pulmonary airway epithelial morphology and the distribution of cell types within the tracheobronchial tree. These studies were conducted on the lung of laboratory animals using light microscopy and TEM.

The normal mammalian respiratory system has proved to be an excellent subject for morphological and topographical studies using the SEM. Again the investigations were mainly on laboratory animals, including the hamster (Nowell and Tyler, 1971), rat (Kuhn and Finke, 1972), mouse (Greenwood and Holland,1972; Kuhn and Finke, 1972; Andrews, 1974), rabbit (Wang; et al. 1973), and non-human primates (Castleman; et al. 1975), although the dog (Groniowski; et al. 1972), man (Wang and Thurlbeck, 1970) and cattle (Mariassy; et al. 1975) have also been studied.

The purpose of the present study was to systematically examine the tracheobronchial airways of normal sheep at five different levels. It aimed to determine the cell types present at each level and find their distribution and frequency. The cell types were characterised

by the light microscope and TEM. In addition, the SEM was used to study surface morphology and investigate three dimensional cellular relationships. Since no SEM studies of the ovine alveolus have previously been reported, the present study of topographic morphology was also extended to this region.

2.0 MATERIALS AND METHODS

2.1 COLLECTION OF LUNGS

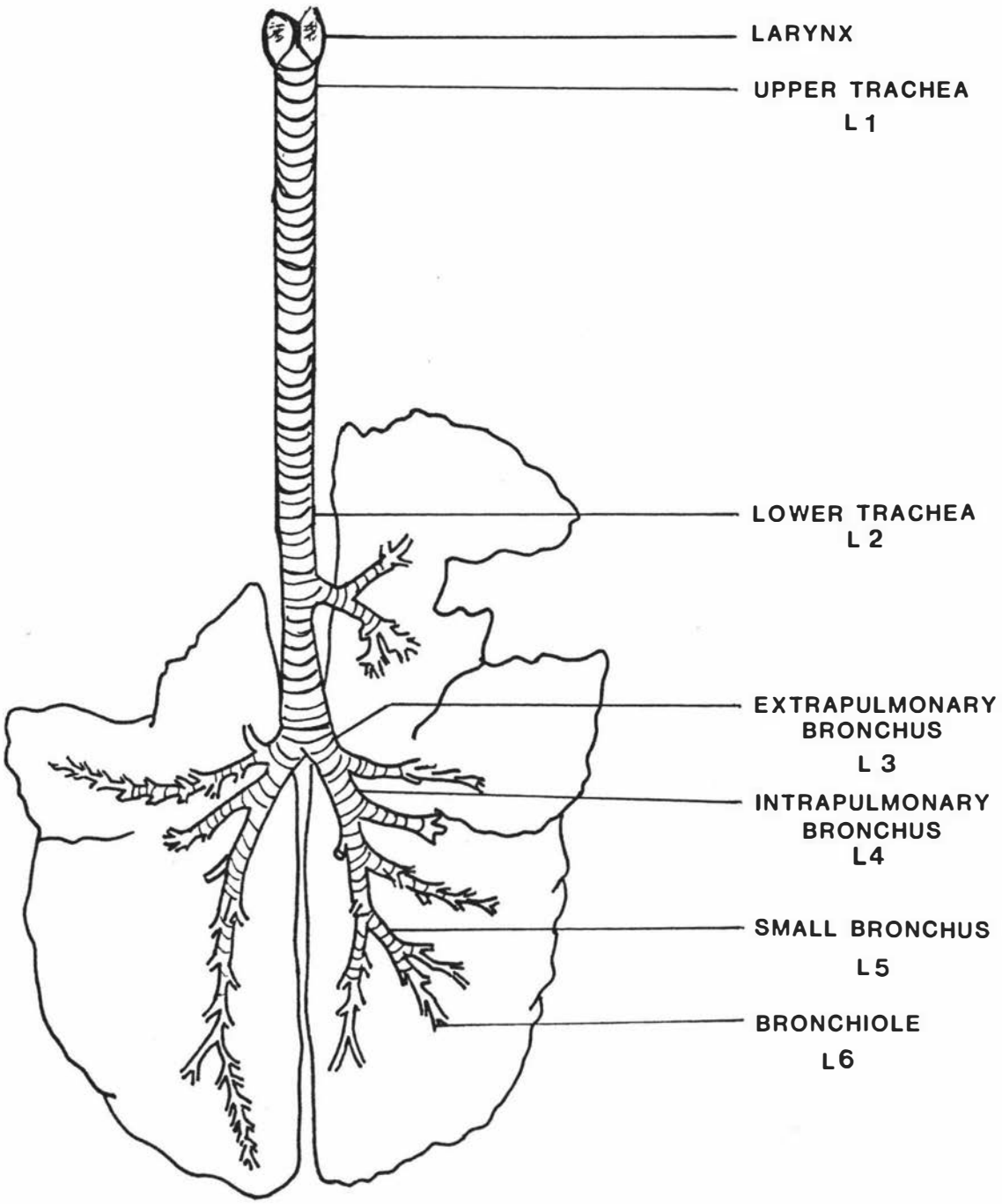
Lungs together with the trachea were collected from six, 5 to 10 month old lambs slaughtered at a local meat works. Each lung was examined macroscopically and all fulfilled the requirement for normal lungs and trachea. An intratracheal tube, modified by connection of a plastic adapter to the cuff end, was introduced as far as the bronchial bifurcation. One of the main bronchi was clamped with 2 pairs of artery forceps, an incision was made between these and the lung was removed. The trachea and attached lung were filled immediately with approximately 800 ml of 1% Karnovsky's fixative (Appendix 2.1) using a syringe until all the lung margins became distended with fixative. They were then fixed by immersion for a further 12 hrs. Tissues were selected from five levels of tracheobronchial airway and lung parenchyma (Fig. 2.1). These were: The first and/or second rings of the upper trachea (Level 1); the last two rings before tracheal bifurcation (Level 2); the extrapulmonary bronchi (Level 3); the intrapulmonary bronchi (Level 4) and a piece of parenchymal tissue for examination of small bronchi, bronchioli and alveoli (Level 5). These were then placed in fixative and cut into smaller sizes suitable for light, TEM and SEM. They were washed in phosphate buffered saline (PBS) and processed as described below.

2.2 LIGHT MICROSCOPY

The parenchymal tissues were processed in the usual manner and embedded in paraffin. They were cut at 4 - 5 um and routinely stained with periodic acid-Schiff (PAS) techniques to study the reaction of Clara cells to PAS. The histological sections were prepared as discribed in TEM .

Figure 2.1

Diagrammatic representation of the tracheobronchial airways of sheep showing the sites selected for study.


LARYNX
UPPER TRACHEA
L 1
LOWER TRACHEA
L 2
EXTRAPULMONARY
BRONCHUS
L 3
INTRAPULMONARY
BRONCHUS
L4
SMALL BRONCHUS
L5
BRONCHIOLE
L6

2.3 SCANNING ELECTRONMICROSCOPY

After further washing in two changes of cold, 0.1M PBS, pH 7.2 at 4°C for one hour, the tissues were cut into approximately 4 mm^2 and dehydrated in graded alcohol solutions of 25, 50, 75, 95 and twice in 100% at room temperature for 20 min each. The dehydrated tissue was dried in a critical drying apparatus (Polaron E3000/Series 2). All the tissue samples were mounted on aluminium stubs with silver conducting paint, coated with 200 A gold by routine methods and examined under a Cwikscan/100 field emission scanning electron microscope. The tracheobronchial specimens (first four levels) were mounted, epithelium face up, while the parenchymal tissue was fractured with a blunt knife and the small pieces mounted.

2.4 TRANSMISSION ELECTRONMICROSCOPY

Specimens for TEM were washed and fixed in similar manner for SEM, then cut into 1 mm^2 pieces and post-fixed in 1% osmium tetroxide (Appendix 2.2) in 0.1M PBS, at pH 7.2 for one hour at 4°C. After washing and dehydrating in alcohol in the same manner as used for SEM, the tissues were infiltrated with propylene oxide for 20 min. and then placed for 12 hr in propylene oxide with 25% resin on a stirrer. The tissues were then embedded in fresh prepared 100% epoxy resin and left for 48 - 72 hr at 60°C to polymerise. One to two micron sections were cut from each block and picked dry off the glass knife with an eyelash. They were transferred onto a drop of water on a glass slide and then heated on a hot plate at 80°C until the water evaporated. The thick sections were immediately covered with a drop of 1% toluidine blue and heated on hot plate at 80°C until the drop of stain evaporated at the edges. After this they were washed thoroughly with distilled water, dried and mounted in Depex. These thick sections were then examined under the light microscope both to evaluate the histological features of normal structure and determine areas of interest for electronmicroscopy. Following this the block face was trimmed down to an area of interest for ultra-thin sectioning. Thin sections were cut on a LKB III ultramicrotome, stained with aqueous uranyl acetate and lead citrate (Appendix 2.3), for 10 min. each and examined on a Philips EM201C transmission electron microscope.

3.0 RESULTS

3.1 HISTOLOGY

Light microscopy showed that the lining epithelium of the tracheal and bronchial airways consisted of pseudostratified ciliated columnar and goblet cells. In 1 um thick sections, all epithelial cells at all tracheobronchial levels stained with toluidine blue, but with varying intensity; the cytoplasm of ciliated cells stained more weakly than that of non-ciliated and basal cells. From the small bronchi distally, the airways were lined by low columnar or cuboidal ciliated and non-ciliated cells with only occasional basal cells observed. The various levels examined are described below.

3.1.1 TRACHEA (LEVELS 1 & 2)

Two types of ciliated cell,differing in the length of their luminal cell border, and the nature of their nucleus and cytoplasm were identified. The first type, which was the most common, had a narrow luminal border and the cytoplasm stained light blue and contained a large number of vacuoles . The nucleus of the first type was larger than the second type. It was eccentrically placed and contained a prominent nucleolus, or occasionally two nucleoli. The second type had a wide luminal border and the cytoplasm stained more darkly with toluidine blue than the first type.It contained fewer cytoplasmic vacuoles and these were found mainly at the base of the cell. The nucleus was rounded, centrally placed and its nucleolus was less prominent (Fig.2.2).

There were approximately five ciliated cells to every goblet cell in the upper trachea or one to four in the lower trachea. Goblet cells stained dark blue with toluidine blue and contained a large number of faint blue granules which varied in size and colour density.

Two groups of cells were found attached to the basal lamina but did not extend to the luminal surface. The first type were identified as basal cells by their small, triangular to oblong shape, small oval nucleus and dark blue cytoplasm. The second category included a small number of cells which could not be clearly identified as belonging to

any definite group. They varied in the density of cytoplasm and shape of the nucleus. Some had a light cytoplasm and an oval nucleus. They were wedged between ciliated cells and thus resembled intermediate cells. Others had a dark cytoplasm with dark granules and resembled secretory cells (Fig. 2.2).

In the lower trachea, the differences between the two types of ciliated cell were less prominent than in the upper trachea. All ciliated cells stained a similar colour density. The globules of the goblet cells in this region were more prominent and stained darker than in the upper trachea.

3.1.2 BRONCHI (LEVELS 3 & 4)

Both the extra- and intrapulmonary bronchial epithelium consisted of ciliated, goblet, intermediate and basal cells, which appeared histologically the same as those in the lower trachea. However, the number of basal and intermediate cells and the height of the epithelium were reduced (Fig. 2.3).

There were approximately five ciliated cells for every goblet cell in extrapulmonary bronchi and seven ciliated cells for every goblet cell in the small bronchi. The goblet cells at these two levels had more granules than at levels 1 & 2, but they were of similar size and variable colour density (Fig. 2.3).

3.1.3 BRONCHIOLI (LEVEL 5)

The epithelium of the primary bronchiole was characterised by a single low columnar to cuboidal layer of cells and basal cells were no longer observed. Three cell types were recognised; ciliated, mucous and Clara cells. Clara cells which were cuboidal in shape with a centrally placed nucleus, projected their apices into the airway lumen above the apices of the ciliated cells. Their cytoplasm stained more densely than that of ciliated cells and the apex and lateral portions of the cell were filled with variable numbers of small, dark blue granules which were considerably smaller than those of mucous cells and they were PAS positive. The epithelium of the terminal bronchioli consisted of only two cell types; Clara and ciliated cells. It was estimated that approximately one Clara cell was present for every

three ciliated bronchiolar epithelial cells (Fig. 2.4).

3.2 TOPOGRAPHICAL MORPHOLOGY

3.2.1 TRACHEA (LEVELS 1 & 2)

Ciliated cells predominated over the majority of the tracheal surface, with a slightly greater density in the upper trachea. Among the cilia were interspersed single non-ciliated cells particularly in the lower trachea (Fig. 2.5). The mucosal surface of the non-ciliated cells was covered by short microvilli and did not protrude into the lumen (Fig. 2.5). Secretory openings of submucosal glands were observed especially at the upper trachea (Fig. 2.5). Because of the high density of the cilia it was difficult to clearly observe goblet cells on the mucosal surface (Fig. 2.5)

3.2.2 BRONCHI (LEVELS 3 & 4)

As in the trachea, ciliated cells predominated over the majority of the surface but to a lesser extent and their ciliary density was greater in the larger bronchi (Fig. 2.6). Single non-ciliated cells were scattered throughout the ciliary carpet (Fig. 2.6). The luminal surface of the non-ciliated cells had very few microvilli and showed large pits or pores opening onto the surface (Fig. 2.7).

3.2.3 BRONCHIOLI (LEVEL 5)

In contrast to the bronchi, the bronchiolar mucosa was composed of approximately equal numbers of ciliated and non-ciliated cells (Fig. 2.8). The ciliated cells had slightly shorter cilia than those in the bronchi and trachea. They also differed from their bronchial counterparts by having shorter and more numerous microvilli (Fig. 2.8). However, the number of ciliated cells decreased gradually toward the alveolar duct. Three types of non-ciliated cells were recognised. The most common type had a granular surface with few very short microvilli. The central portion of the cell occasionally protruded into the airway (Fig. 2.8). The second type of non-ciliated cell was smooth over most of its surface and protruded into the airway. It had a very few short, microvilli and possessed pits and pores opening onto the surface (Fig. 2.8). Secretory

Figure 2.2

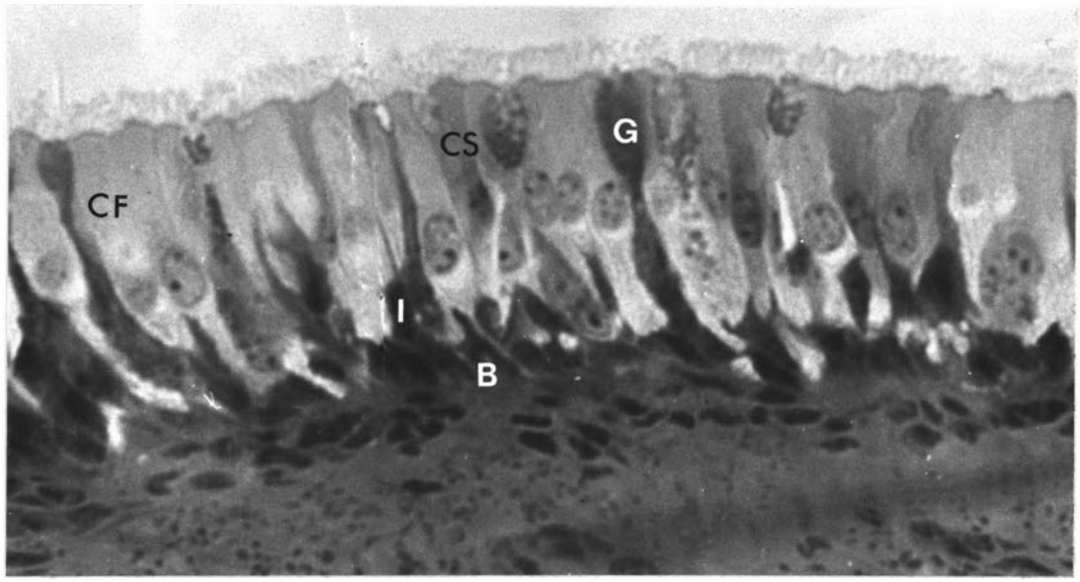
The epithelial surface of normal trachea stained with toluidine blue. Two types of ciliated cell are present. The first type (CF) has a narrow luminal border and the cytoplasm stains light blue. The second type (CS) has a wide luminal border and the cytoplasm stains more darkly. Goblet cells (G) stain dark blue and contain a large number of faint blue granules. Basal cells (B) and intermediate cells (I) are attached to the basal lamina but do not extend to the luminal surface. Toluidine blue. X 312.

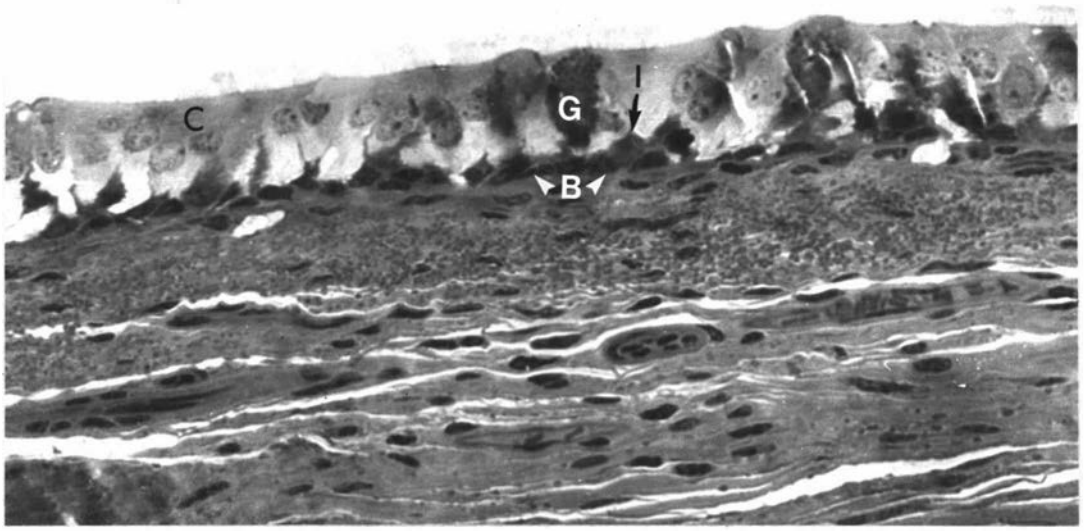
Figure 2.3

Bronchial epithelium consisting of ciliated cells (C), goblet cells (G), intermediate cells (I) and basal cells (B). It is histologically similar to the lower trachea although the height of the epithelium is reduced. Toluidine blue. X 312.

Figure 2.4

Bronchiolar epithelium of normal lung was composed of three cell types; ciliated (C), mucous (M) and Clara (Cl). Toluidine blue. X 1250.


CF
CS
G
I
B


C
G
I
B

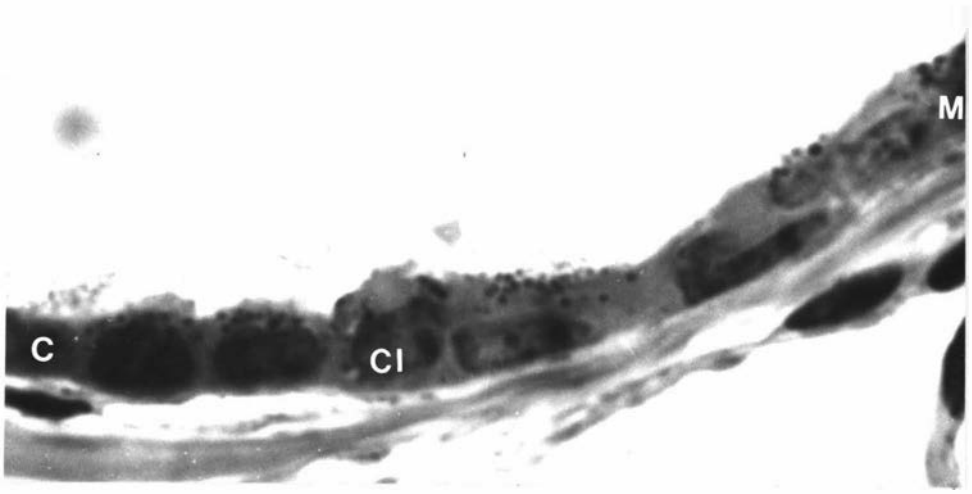
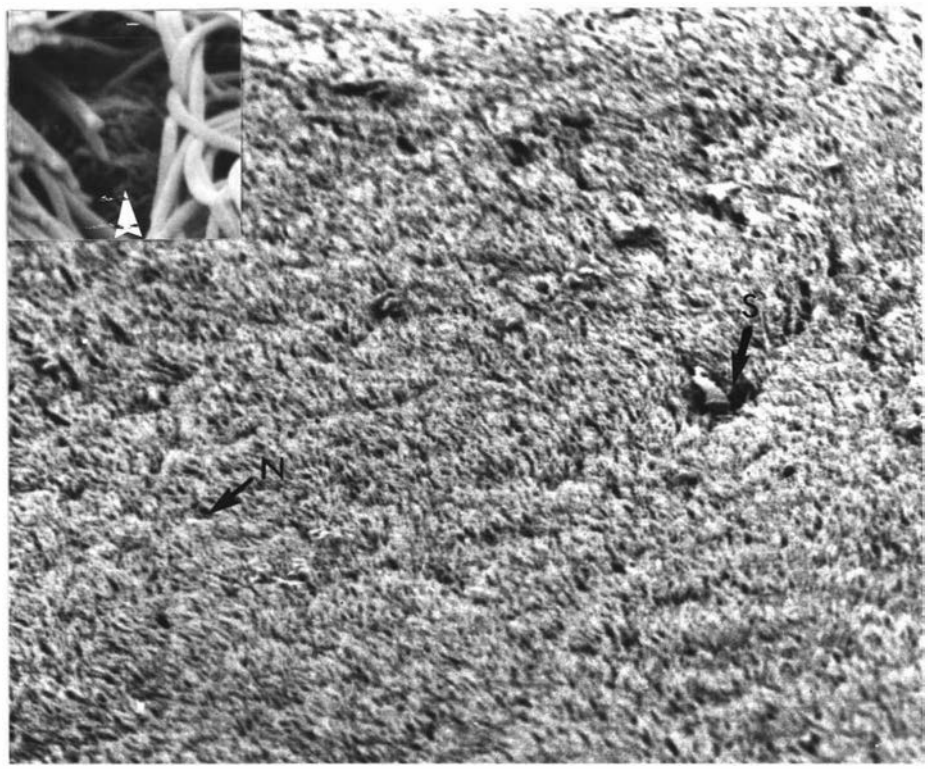

M
C
CI

Figure 2.5

The epithelial surface of normal trachea. Dense cilia cover most of the surface, but among them are interspersed single non-ciliated cells (N) and secretory openings (S) of submucosal glands. SEM. X 670. The inset figure shows the mucosal surface of a non-ciliated cell covered by short microvilli (arrow). SEM. X 8000.

Figure 2.6

The epithelial surface of a normal bronchus. Ciliated cells predominate over the majority of the surface but a large number of non-ciliated cells (arrows) are also present. SEM. X 1335.


S
N

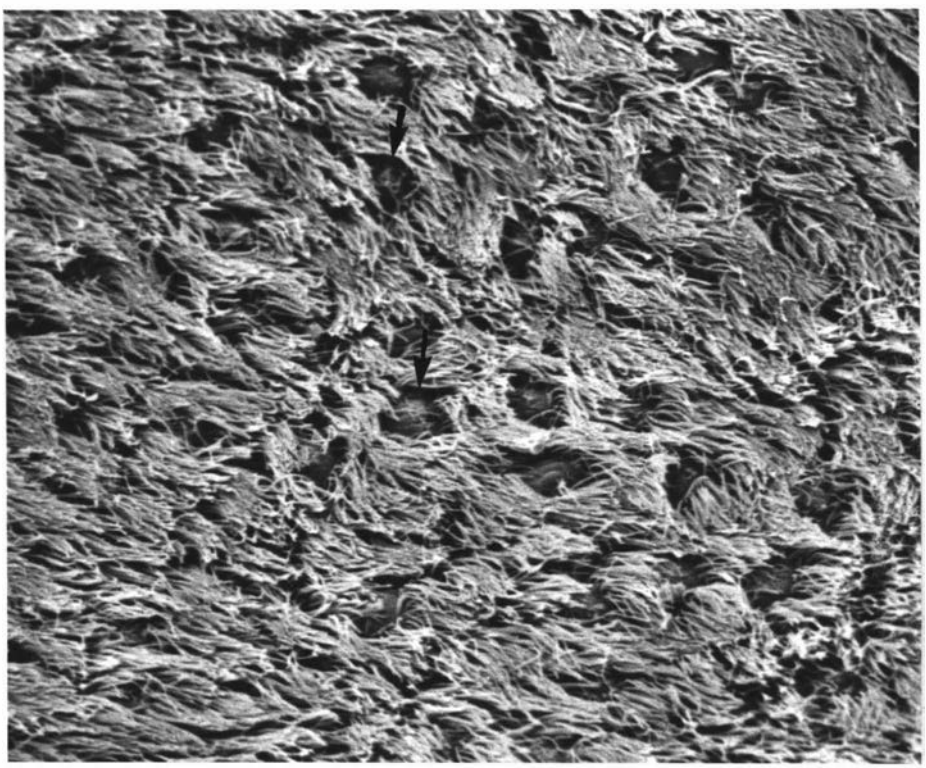
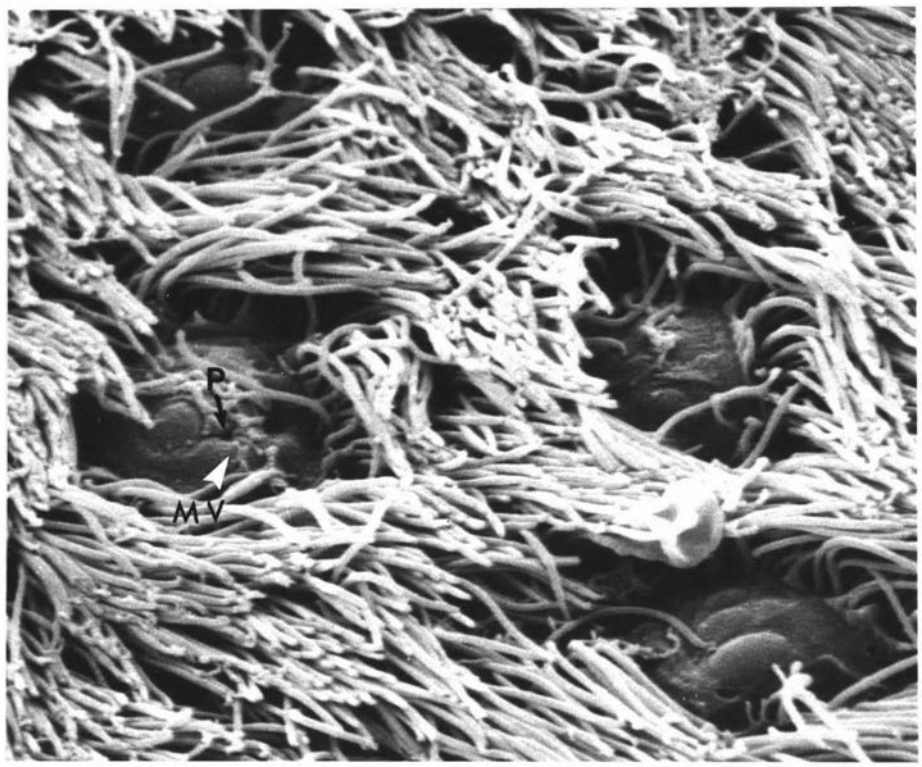


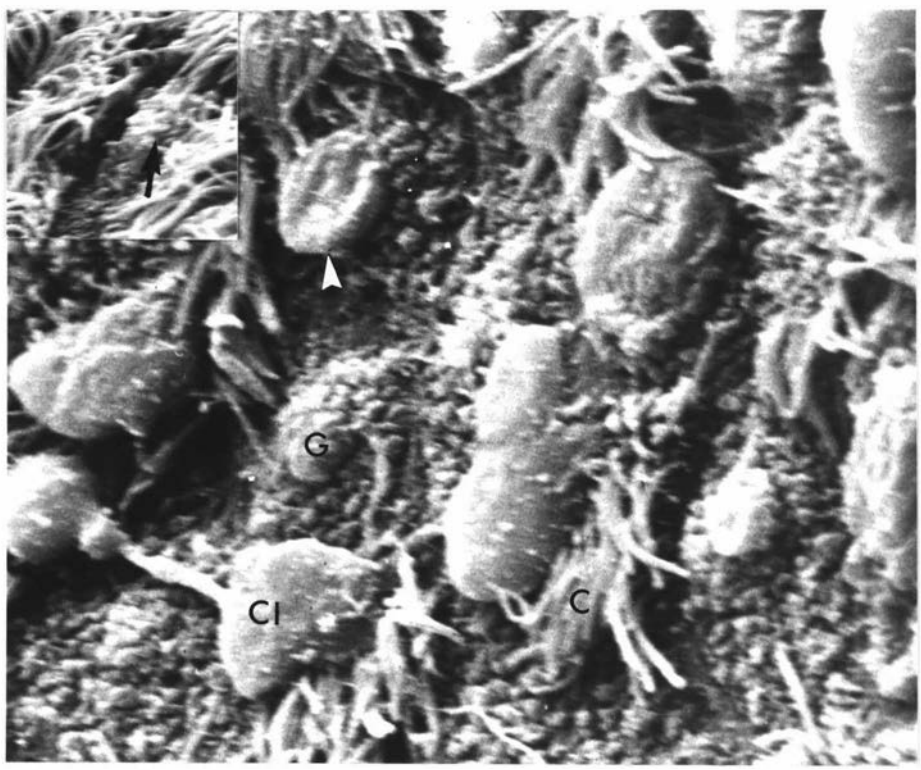
Figure 2.7

The surface of non-ciliated cells. Very few microvilli are present (MV) and large pits or pores (P) open onto the surface. SEM. X 5700.

Figure 2.8

The epithelial surface of a normal bronchiole. A mixture of ciliated cells (C), Clara cells (Cl), cells with a granular surface (G) and cells with microvilli on their luminal surface (black arrow in inset figure) can be seen. The empty bladder-like structure may be an evacuated Clara cell (arrows). SEM. X 6000 (Inset X 4000).


P
MV


G
CI
C

material fixed *in situ* was sometimes attached to the surface of these cells (Fig. 2.8). A moderate number of these cells appeared to be evacuated and had an empty bladder-like appearance (Fig. 2.8). The morphology of these cells was consistent with that of Clara cells. The third non-ciliated cell type had microvilli which completely covered its mucosal surface. Cells of this type were only occasionally seen in any given area (Fig. 2.8).

3.2.4 ALVEOLI

Three cell types were observed in the alveoli. The majority of the alveolar surface was covered by flattened, smooth-surfaced squamous (type I) epithelial cells (Fig. 2.9). Small projections were sometimes seen irregularly distributed over the surface of these cells.

The second type of epithelial cell, the granular (type II) pneumocyte, usually lined only a small portion of the septum. Its surface protruded into the alveolar lumen and was covered by many microvilli (Fig. 2.9). The third type of cells, the alveolar mononuclear phagocyte, was rarely observed.

A notable characteristic of the ovine alveolar wall was the paucity of interalveolar pores of Kohn (Fig. 2.9). Their frequency was approximately one per twelve alveoli examined.

3.3 CELL TYPES AND ULTRASTRUCTURE

Ten different epithelial cell types were identified in the ovine tracheobronchial epithelium. All cells were attached to the basement membrane, but not all reached the airway lumen. The ultrastructural features of these cells are summarised in Table 2.1.

3.3.1 CILIATED CELLS

Ciliated cells were roughly columnar in shape except in small bronchi and bronchioli where they became low columnar or cuboidal. They were approximately 20 um long and 7 um wide, tapering towards the base where they were attached to the basement membrane (Fig. 2.10). The base lateral cell surfaces formed interdigitations with adjacent

TABLE 2.1: SUMMARY OF THE ULTRASTRUCTURAL FEATURES OF TRACHEOBRONCHIAL EPITHELIAL CELL TYPES IN NORMAL SHEEP

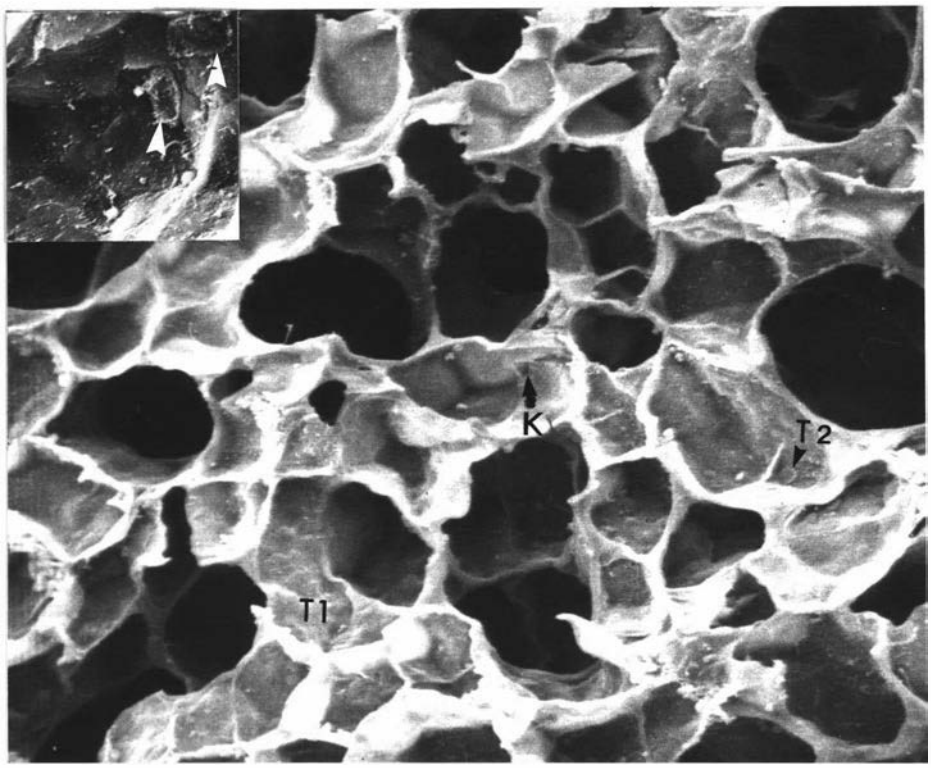
CELL TYPE	SITE	CELL SHAPE	CYTOPLASM	NUCLEUS	VESICLES OR VACUOLES	LUMINAL SURFACE
CILIATED CELLS						
FIRST TYPE	L1-L5	Columnar and low columnar or cuboidal at small bronchi and bronchioli.	Electron-dense, compared to the second type.	Rounded or oval with prominent large nucleolus.	Numerous vesicles in the lower part of the cell.	More cilia and less microvilli than second type.
SECOND TYPE	L1&L2	As for first type	Electron-lucent compared to the first type.	Same as first type	Not common	More microvilli but less cilia than first type. Atypical cilia were common.
NON-CILIATED CELLS						
SECRETORY CELLS						
GOBLET CELLS						
FIRST TYPE	L1-L5	Columnar to low columnar	Contain small homogenised electron-dense granules.	Irregular or oval with small nucleolus.	Not common	Few microvilli with large secretory openings in the middle of the luminal surface.
SECOND TYPE	L1&L2	As for first type	Granules less electron-dense than first type.	Same as first type	Not common	Same as first type.
UNKNOWN SECRETORY CELLS						
FIRST TYPE	L3	Columnar	Electron-dense containing mucus-like granules.	Rounded	Not common	Moderate number of microvilli.
SECOND TYPE	L3&L4	Tall, thin and irregular	Electron-dense.	Not identified	Variable number of vacuoles.	Few microvilli.
CLARA CELLS	L5	Low columnar to cuboidal.	Electron-lucent with large amount of SER.	Flattened or rounded	Variable number of vacuoles	Protruded to the lumen.
NON-SECRETORY CELLS						
BRUSH CELLS						
TRACHEAL	L1&L2	Columnar	Electron-dense.	Rounded or oval	Few vacuoles	Few microvilli.
BRONCHIAL	L4&L5	Columnar or low columnar	Moderately Electron-dense.	Rounded or oval	Not common	Large number of microvilli.
BASAL CELLS	L1-L5	Irregular	Moderately electron-dense.	Large and indented	Not common	No contact with the luminal surface.
INTERMEDIATE CELLS	L1-L4	Spindle	Same as basal cells.	Rounded or oval	Not common	Same as basal cells.

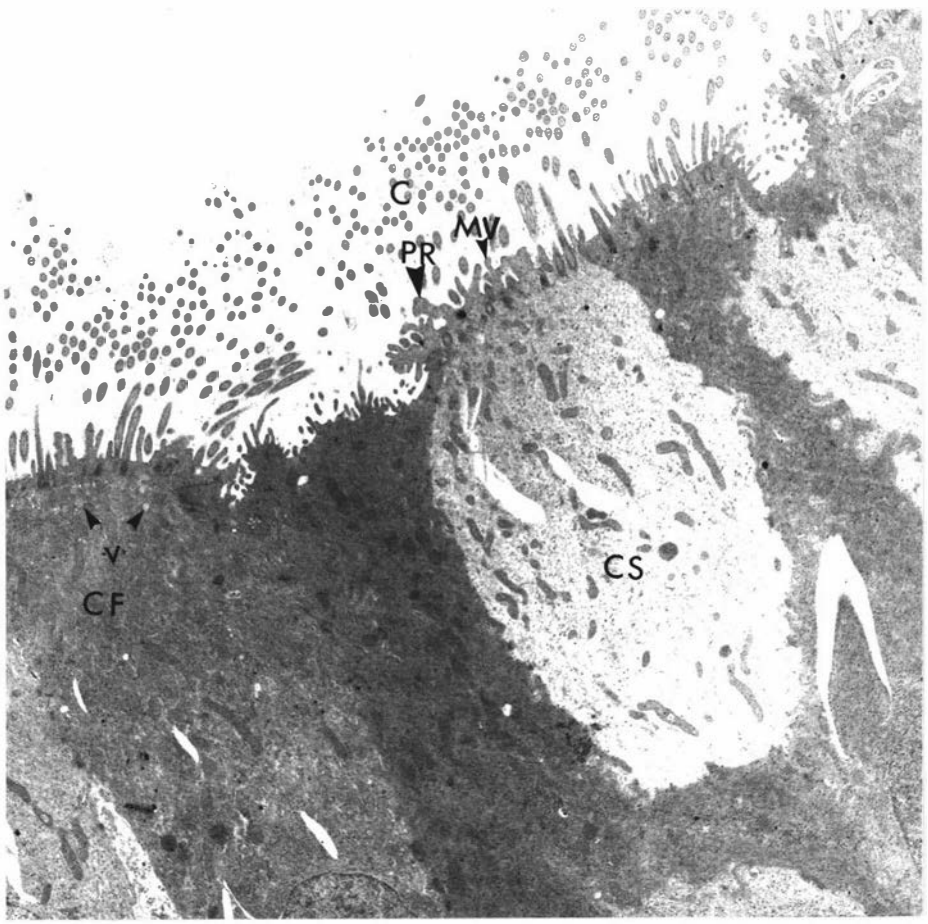
Figure 2.9

Parenchyma of normal lung observed by SEM. Three cell types can be seen within alveoli. These are smooth surfaced type I epithelial cells (T1), granular or type II pneumocytes (T2) and alveolar macrophages (AM). X 340. The inset figure shows a higher magnification of the type II pneumocytes (arrows). Note the low number of interalveolar pores of Kohn (K). SEM. X 1000.

Figure 2.10

Ciliated cells from a tracheobronchial airway in normal lung. Two types can be recognised. The first type (CF) has numerous smooth vesicles (V) in the upper part of the cell. In the second type (CS) the cytoplasm is more electron-lucent and the luminal surface has less cilia (C) but more cytoplasmic processes (PR) and microvilli (M) than the first. TEM. X 5440.


K
T2
T1


C
MV
PR
V
CF
CS

cells, to which attachments were made by desmosomes.

An irregular intercellular space surrounded the ciliated cells except at the luminal surface where tight junctions were found (Fig. 2.11). The cytoplasm was more electron-lucent than that of most non-ciliated epithelial cells (Fig. 2.10). In the lower part of the cell there were scattered profiles of rough endoplasmic reticulum (RER), free ribosomes, a few tonofilaments, smooth surfaced vesicles, lysosomes and glycogen (Fig. 2.12). The Golgi apparatus was usually situated below the nucleus, which was rounded or oval in shape and contained a prominent nucleolus (Fig.2.12). Most of the mitochondria were found in the upper part of the cell, between the basal bodies and the nucleus (Fig. 2.10 & 2.11). A large number of cilia were found on the luminal surface of each cell, and interspersed between them were microvilli and fine cytoplasmic processes. The free part of each cilium was covered by cell membrane and contained an axial filament complex composed of nine microtubular doublets arranged in a ring around a central doublet, and each cilium was anchored to the cell cytoplasm by a basal body (Fig. 2.13).

Two types of ciliated cell were recognised which corresponded to those seen with the light microscope. The first cell type usually had numerous smooth surfaced vesicles in the upper part of the cell, just below the apical row of basal bodies. The vesicles contained an amorphous material (Fig. 2.11). In the second type the cytoplasm was more electron-lucent particularly in the lower part of the cell. It had less RER, more lysosomes and more mitochondria. The luminal surface had more microvilli and cytoplasmic processes and less cilia than the first cell type (Fig. 2.10). These two types of cell were observed mostly in the epithelium of the upper trachea and were not seen in the bronchi and bronchioli. However, the first cell type was more common than the second by a ratio of approximately 6:1.

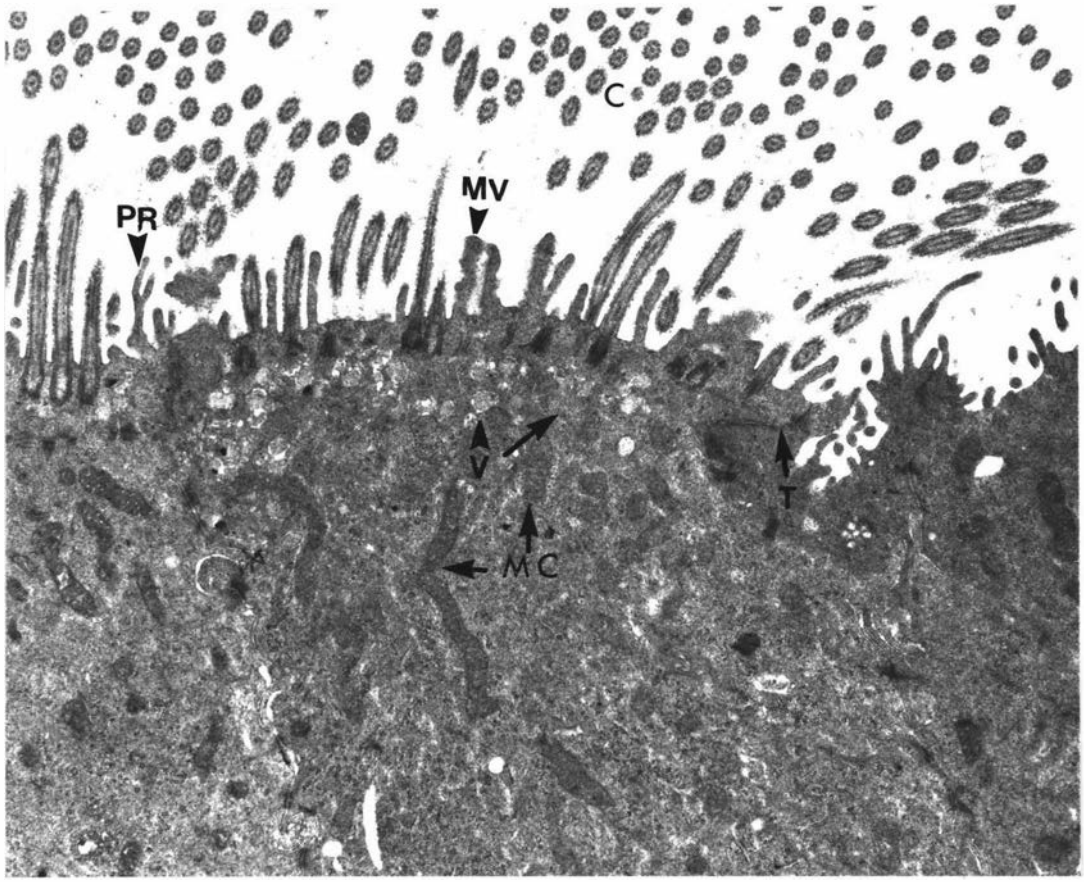
Atypical cilia were observed occasionally in the tracheobronchial epithelium. Usually, these consisted of two, three or more true cilia which had fused to form giant bizarre structures, enveloped by the outer cell membrane (Fig. 2.13). Atypical cilia were observed more frequently in the second type of ciliated cell.

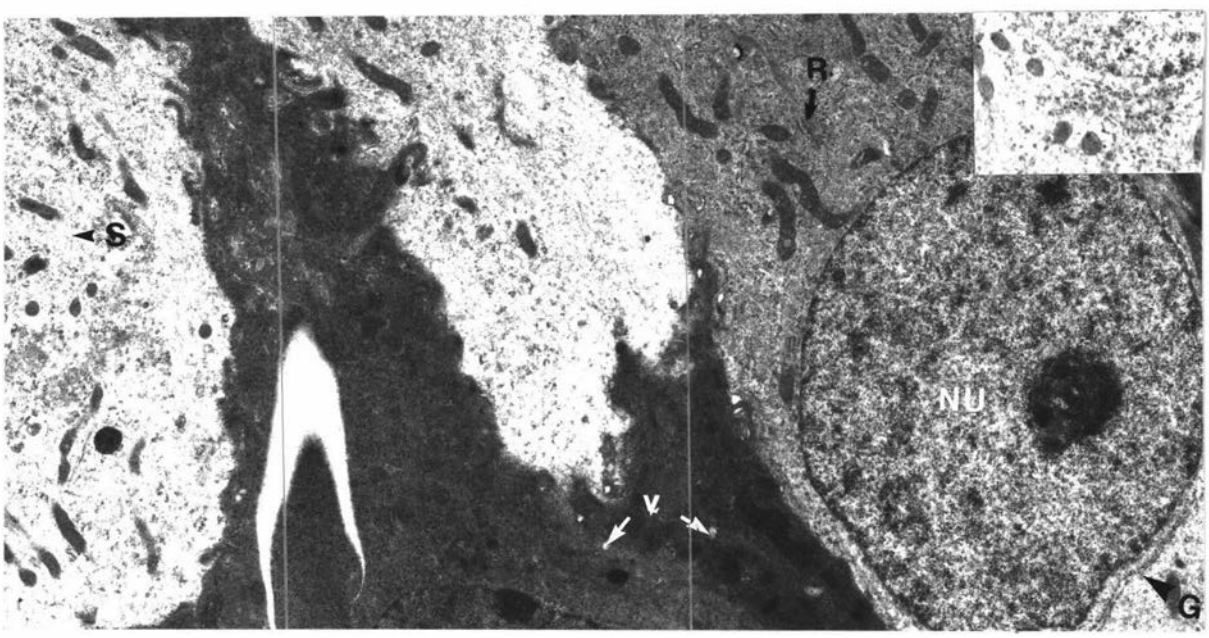
Figure 2.11

High magnification of the apical part of a ciliated epithelial cell. Most of the mitochondria (MC) have accumulated near the apex. A large number of cilia (C) are found on the luminal surface and interspersed between them are microvilli (MV) and fine cytoplasmic processes (PR). An irregular intercellular space is present between these cells except at the apical part where tight junctions (T) are observed. Large numbers of vesicles (V) containing an amorphous material are present near the surface. TEM. X 14,360.

Figure 2.12

High magnification of the lower part of a group of ciliated cells. There are scattered profiles of rough (R) and smooth (S) endoplasmic reticulum, free ribosomes, smooth surfaced vesicles (V), lysosomes and glycogen particles (arrows). The Golgi apparatus (GA) is situated below the nucleus (NU) which contains a prominent nucleolus. TEM. X 7,800. The inset figure shows a higher magnification of the Golgi apparatus. TEM. X 7,800.


C
MV
PR
V
T
MC


R
S
NU
V
G

3.3.2 NON-CILIATED CELLS

3.3.2.1 SECRETORY CELLS

3.3.2.1.1 GOBLET CELLS

Goblet cells differed markedly in the size and content of their mucous granules. One type contained homogeneous small electron-dense granules with a small number of ribosomes (Fig. 2.14). A second type contained larger and less electron-dense granules with more ribosomes than the first (Fig. 2.15).

Regardless of their type of granules, the goblet cells had a relatively electron-dense cytoplasm, compressed into the lower cell by the many mucous granules and ribosomes in the apical cytoplasm (Fig. 2.14). The presence of a large number of mucous granules in the cytoplasm give the mature cell its characteristic goblet shape. However, only a narrow part of the tapered basal cytoplasm was attached to the basement membrane (Fig. 2.14). The basal part of the cell formed interdigitations and desmosome attachments with adjacent cells. Individual goblet cells were surrounded by an irregular intercellular space and closed at the luminal surface by tight junctions (Fig. 2.14). An irregular oval nucleus with a small nucleolus, was found at the base of the cell together with a few mitochondria. The Golgi apparatus was well developed and usually found above the nucleus, alongside the extensive RER (Fig. 2.15). The apical cell cytoplasm contained many electron-dense mucous granules which were of different sizes, bound by a thin membrane and whose content was granular, or homogeneous (Fig. 2.14). These cells were found most frequently in the trachea and bronchial regions.

3.3.2.1.2 UNKNOWN SECRETORY CELLS

Two cell types of unknown secretory cells were identified ultrastructurally.

FIRST CELL TYPE

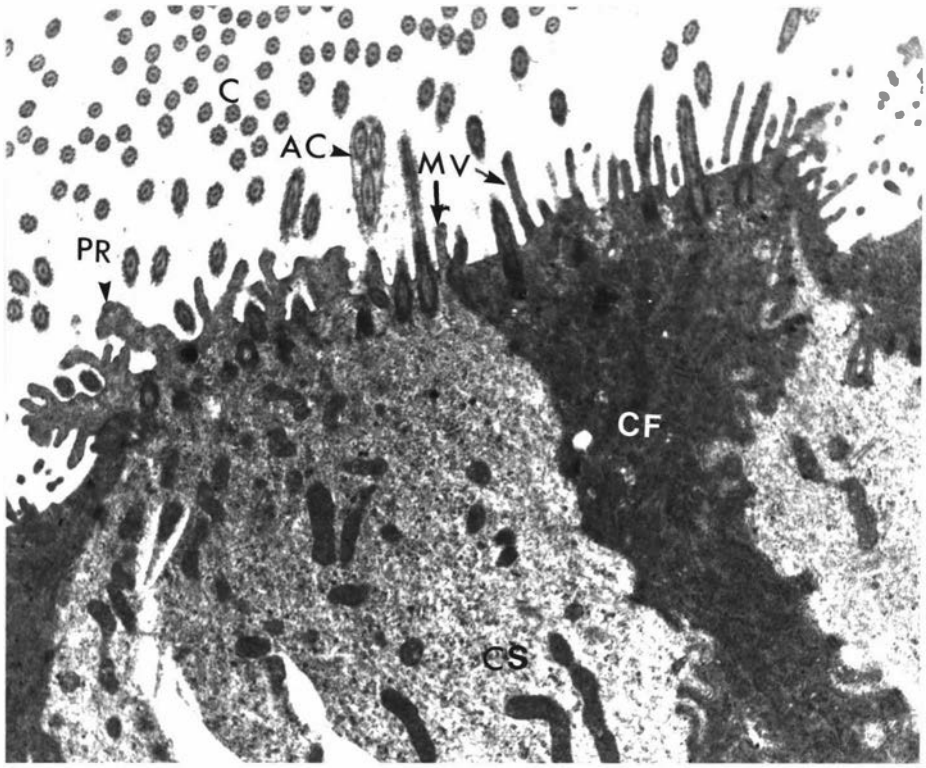
These cells had a dense, electron-opaque cytoplasm with few relatively large mucus-like granules in their apical cytoplasm. These

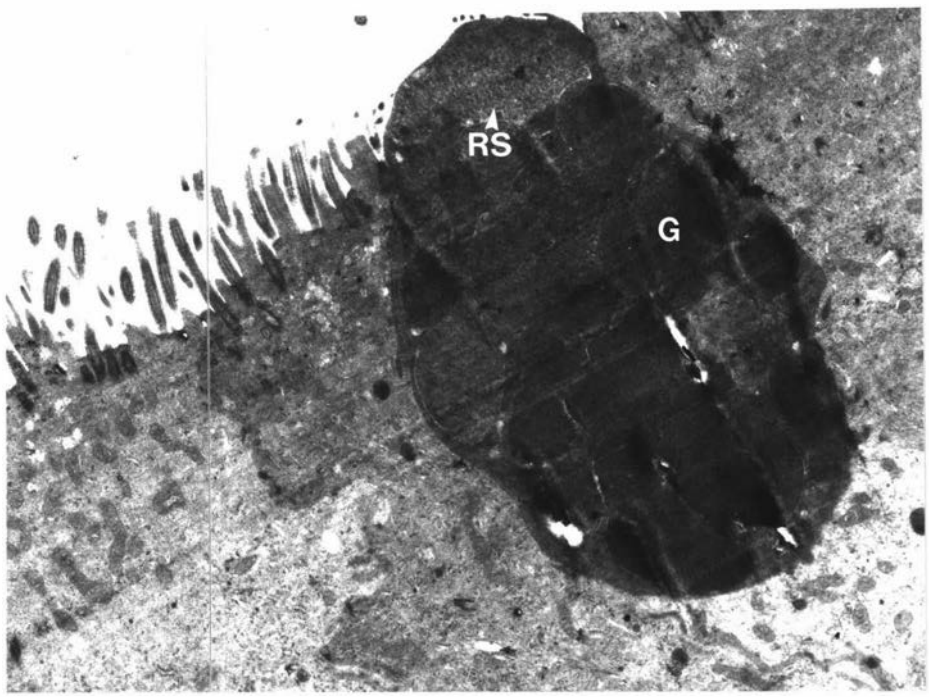
Figure 2.13

High magnification of the upper part of both the first (CF) and second (CS) types of ciliated cell. A large number of cilia (C), and microvilli (MV) are found on the luminal surface of both cell types. Atypical or compound cilia (AC) and cytoplasmic processes (PR) are observed mostly on the luminal surface of the second type of ciliated cell. TEM. X 11,010.

Figure 2.14

The first cell type of goblet cell. It contains homogeneous small electron-dense granules (G) and a small number of ribosomes (RS). TEM. X 8,540.


C
AC
MV
PR
CF
CS


RS
G

granules contained fibrillar, low density material and were bound by a thin continuous membrane. Relatively, long microvillous projections were present on the free or luminal border of these cells (Fig. 2.16). This type of cell was observed mainly in the intrapulmonary bronchi.

SECOND CELL TYPE

This cell type was tall, very irregular, narrow and shrunken in appearance with a dense cytoplasm, few mitochondria and variable numbers of small spherical vacuoles (Fig. 2.17). The vacuoles contained granular amorphous material which in some cases could be seen emptying onto the surface of epithelium (Fig. 2.17). A few, relatively long microvillous projections were seen on the mucosal surface of these cells. This cell type was found mostly in extra- and intrapulmonary bronchi and was more frequent than the first type. Because the cytoplasm of these cells was tapering at the lower part, the nucleus of these cells were difficult to identify.

3.3.2.1.3 CLARA CELLS

Clara cells were low columnar to cuboidal in shape and protruded beyond the ciliated cells (Fig. 2.18). The apical cell cytoplasm contained profuse smooth endoplasmic reticulum (SER) while the mitochondria in this zone were virtually devoid of cristae. Regular, electro-lucent, heterogeneous, membrane-bound inclusions were observed in the apical cytoplasm (Fig. 2.18). The nucleus was found in the central part of the cell. There was also a well developed Golgi apparatus, RER and numerous mitochondria (Fig. 2.18). Clara cells always rested on the basement membrane. Their lateral cell walls invariably showed numerous interdigitations, joined by tight cell junctions or desmosomes to adjacent epithelial cells (Fig. 2.18)

3.3.2.2 NON-SECRETORY CELLS

3.3.2.2.1 BRUSH CELLS .

Two types of brush cell were recognised.

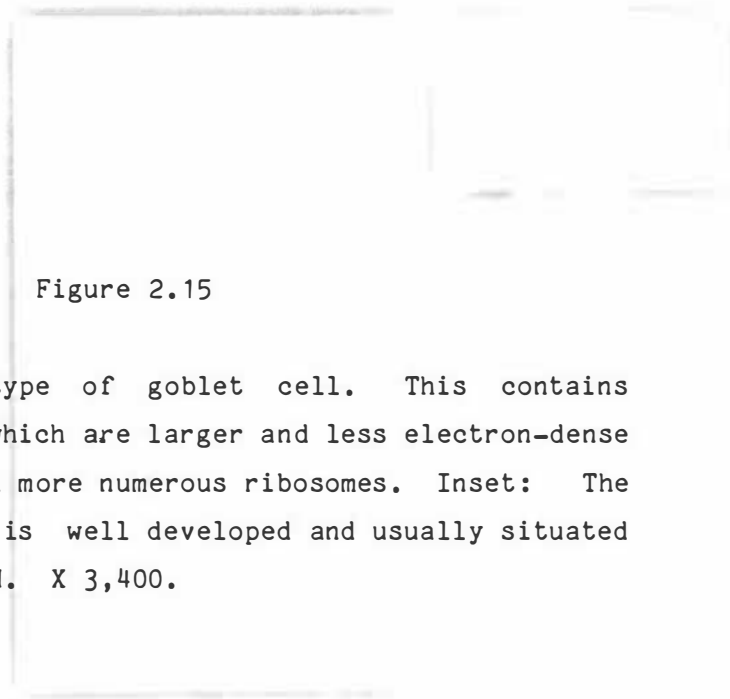
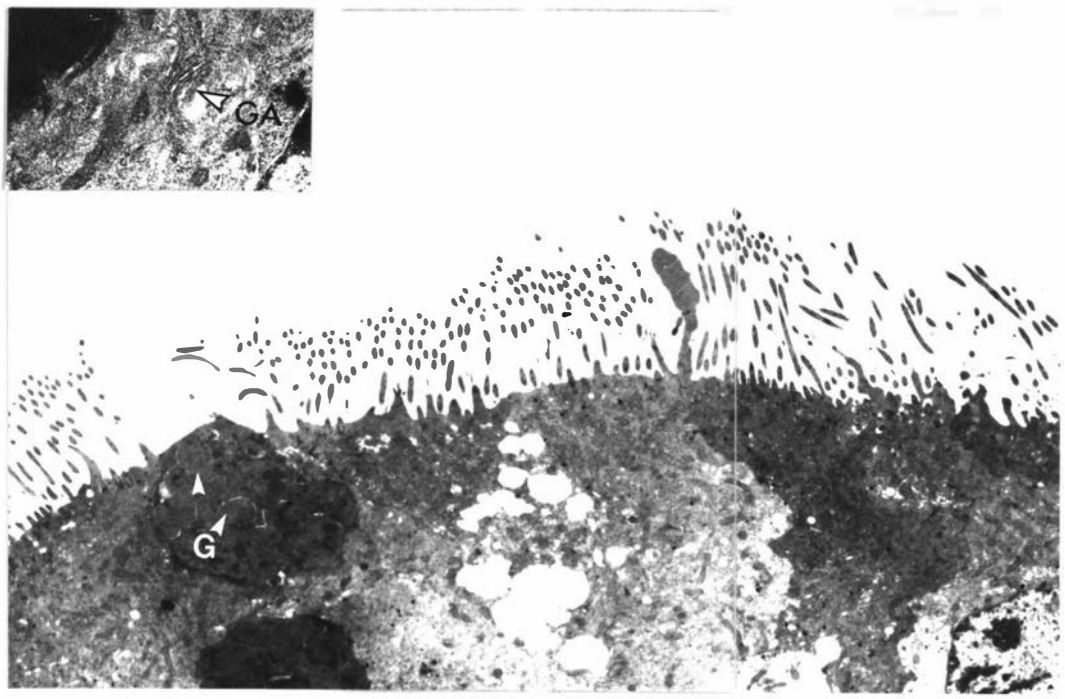


Figure 2.15

The second cell type of goblet cell. This contains mucous granules (G) which are larger and less electron-dense than the first type and more numerous ribosomes. Inset: The Golgi apparatus (GA) is well developed and usually situated above the nucleus. TEM. X 3,400.

Figure 2.16

High magnification of the apical part of the first type of unknown secretory cell. The cytoplasm of the cell is electron-dense and contains a few mucus-like granules ((G). The luminal surface shows relatively few long microvilli (MV). TEM. X 15,300.


GA
G

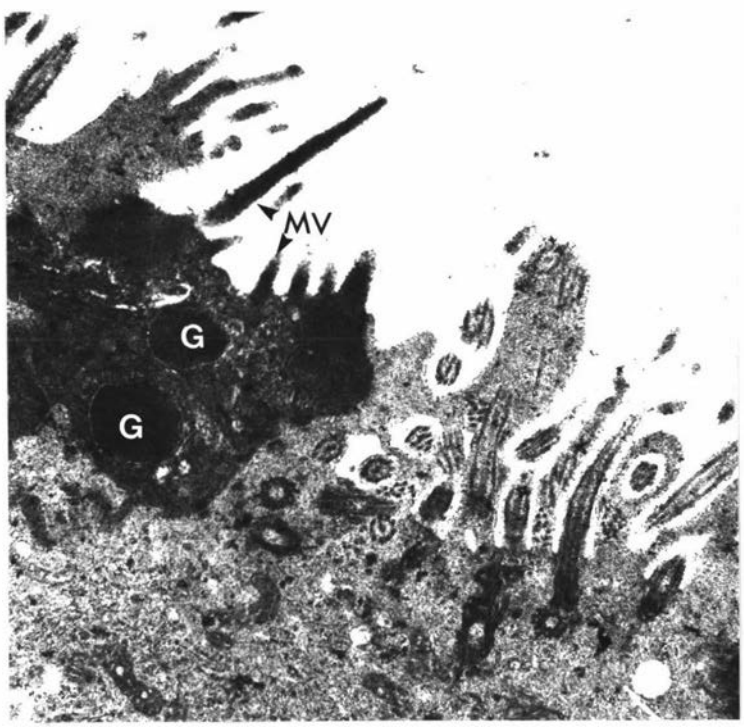
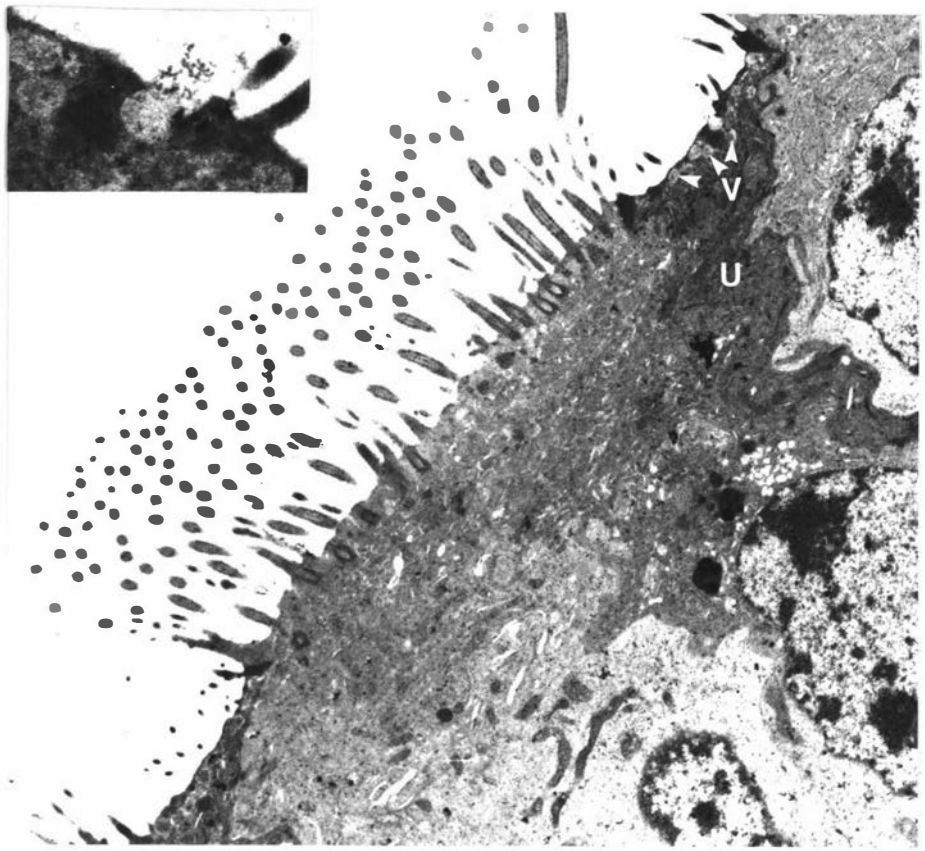

MV
G
G

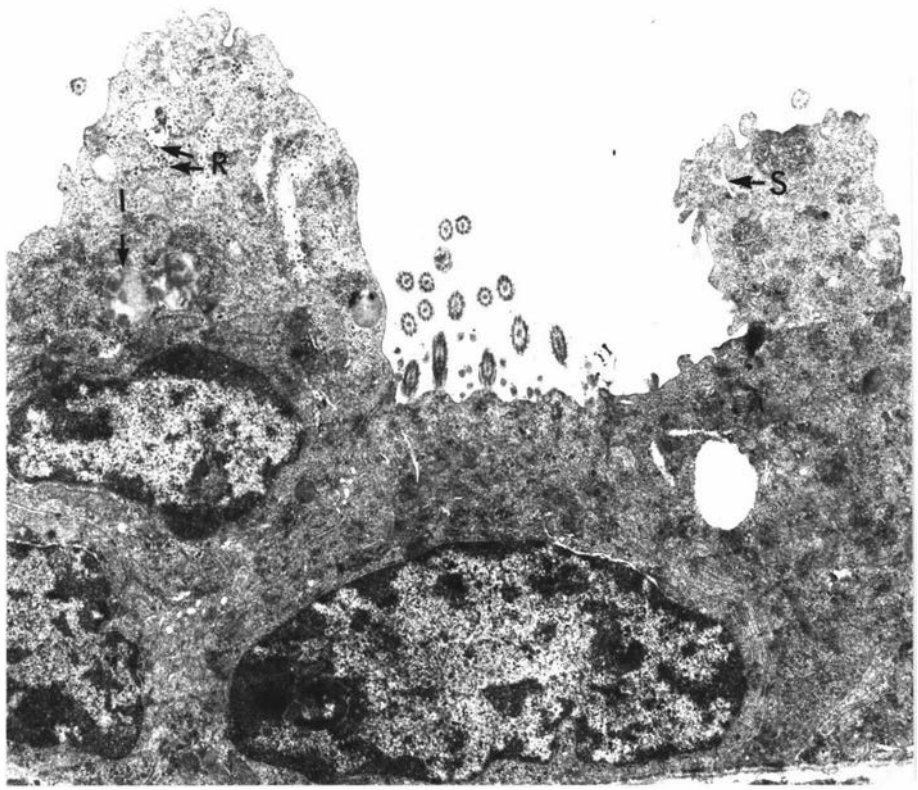
Figure 2.17

The second type of unknown secretory cell (U). These tall and irregular cells have variable numbers of small spherical vacuoles (V) near their surface. TEM. X 8,900. The inset figure shows some of the vacuoles which appear to be emptying onto the surface. TEM. X 28, 000.

Figure 2.18

Clara cell showing apical cytoplasm containing large numbers of free ribosomes (R) and abundant SER (S). The mitochondria are virtually devoid of cristae. Electron-lucent, heterogeneous, membrane-bound inclusions (I) are stained incompletely. TEM. X 12,150.


V
U


R
S

TRACHEAL BRUSH CELLS

Occasional non-ciliated cells without secretory granules and with a brush border of microvilli were found in the trachea. The cytoplasm was electron-dense, contained moderate numbers of mitochondria, a large amount of RER and SER and a few empty vacuoles (Fig. 2.19).

BRONCHIAL BRUSH CELLS

These showed a more typical brush cell morphology than their tracheal counterparts. They were columnar to low columnar in shape, rested on the basement membrane and reached the airway surface. The nucleus was not lobulated and was found in the lower part of the cell. The cytoplasm was of moderate electron-density and contained abundant RER, numerous mitochondria and a large number of free ribosomes. The filament bundles, which are a characteristic feature of brush cells, were found in the apical part of the cell. The second distinguishing feature of the brush cell, the dense population of microvilli on the luminal surface, was also obvious and appeared to be continuous with the filament bundles in the cytoplasm (Fig. 2.20).

3.3.2.2.2 BASAL CELLS

Basal cells were found in the ovine tracheobronchial epithelium as far distally as the bronchioli, although they were more numerous in the trachea and extrapulmonary bronchi. There was a wide, irregular intercellular space around each cell and this was often crossed by long cytoplasmic processes which contacted adjacent cells. They did not extend to the luminal surface. The nucleus was large and indented occupying a large area of the cell. Large numbers of ribosomes and tonofilaments were found in the cytoplasm, which also contained a few mitochondria and large amounts of RER (Fig. 2.21).

3.3.2.2.3 INTERMEDIATE CELLS

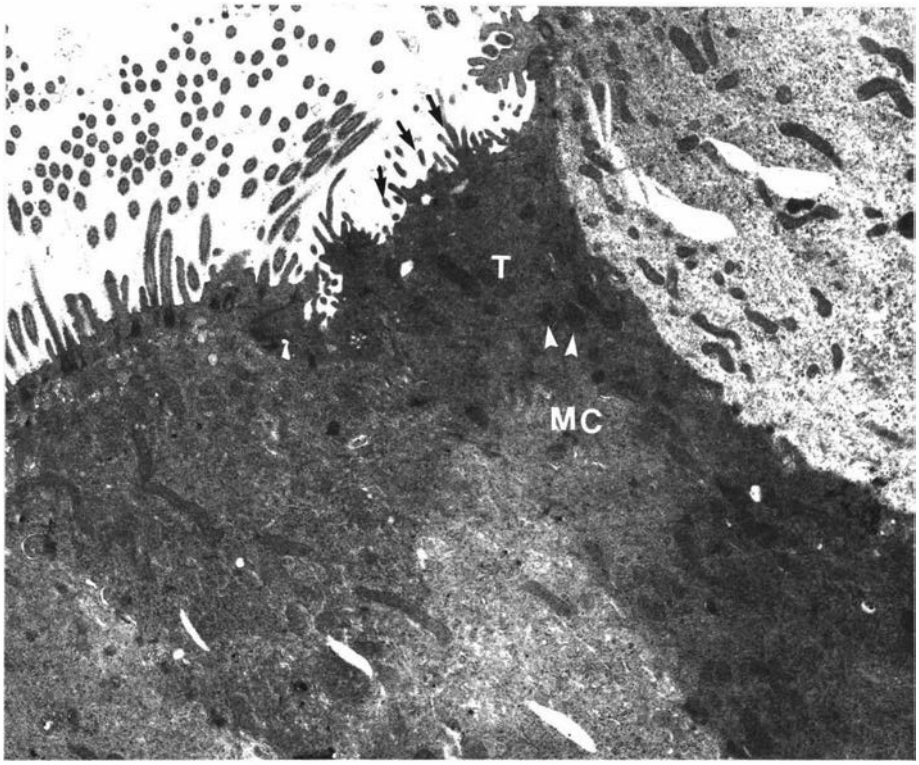
Intermediate cells formed a poorly defined layer just above the basal cells, from which they could be distinguished by their spindle shape. Although in close proximity to the lumen, they did not usually reach the surface. They had an abundant cytoplasm containing

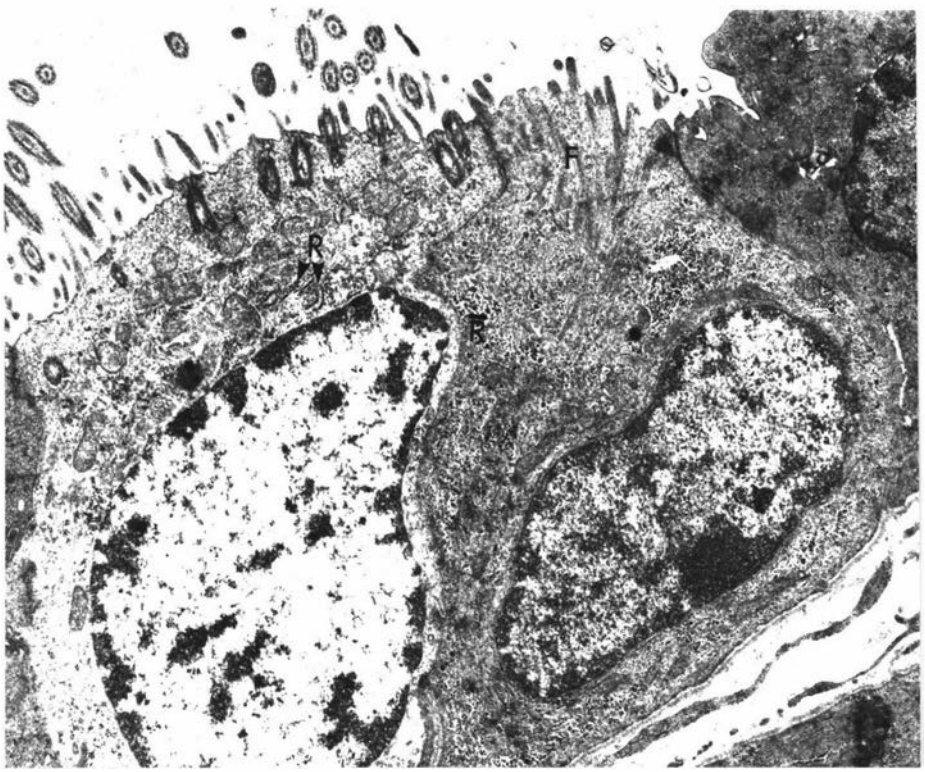
Figure 2.19

A tracheal brush cell (T) showing its luminal brush border of microvilli (arrows). The cytoplasm is electron-dense and contains a moderate number of mitochondria (MC), a large amount of RER , SER and a few empty vacuoles . TEM. X 7,900.

Figure 2.20

A bronchial brush cell showing the typical luminal brush border. Its electron-dense cytoplasm contains abundant RER (R), numerous mitochondria and a large number of ribosomes (R̃). The filament bundles (F) can be seen in the apical part of the cell. TEM. X 1,340.


T
MC


F
R
B

mitochondria, RER, and a few tonofilaments and their nucleus was large and oval. Cytoplasmic projections contacted other cells and sometimes formed desmosomes, although these were less frequent than in the basal cell (Fig. 2.21).

4.0 DISCUSSION

4.1 HISTOLOGY AND TOPOGRAPHICAL MORPHOLOGY

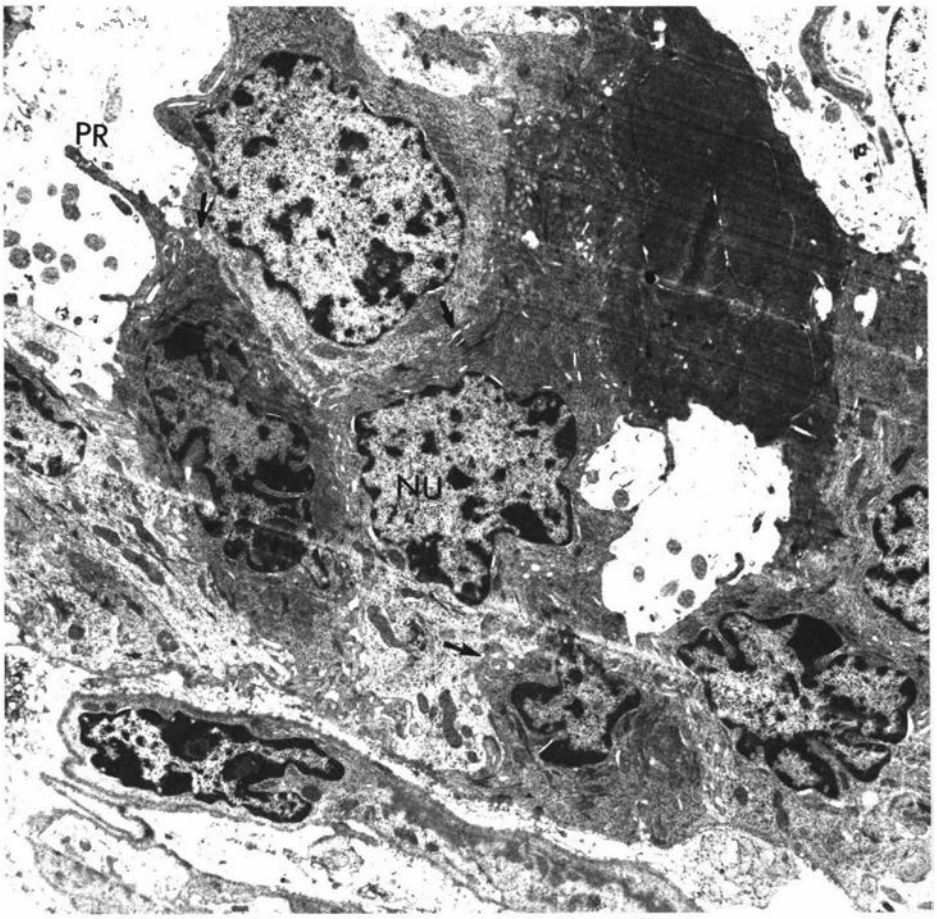
It has been apparent since early studies employing light microscopy that the respiratory tract mucosal surface is composed of ciliated and non-ciliated epithelium. However, the cellular orientation, relative distribution of the various cell types, and surface topography have been difficult to define clearly by either light or transmission electron microscopy. The use of SEM in this study has provided reliable information on the structural features of the epithelium which could not be obtained by other means.

In general, this study has demonstrated that the ovine tracheobronchial surface morphology and structure resemble those of other mammalian species previously reported. Histologically, it has been shown that a slightly lower proportion of mucous cells are present in the upper trachea compared to the lower trachea and that the ciliated cells were of two types. No other studies of mucosal cell distribution at different levels of the trachea have been reported in the literature.

Topographically, the epithelium of the ovine airways was a mixture of ciliated and non-ciliated cells, with the former predominating in the trachea, bronchi and the latter in the bronchioli. In the trachea, ciliated cells were ubiquitous, which differs from descriptions of trachea of the rat (Alexander; et al. 1975), mouse (Greenwood and Holland, 1972) and hamster (Kennedy; et al. 1978) where the ciliated cells were arranged in groups interspersed between numerous non-ciliated cells. However, they were similar to the trachea of adult dogs (Wright; et al. 1983). In the bronchi, the goblet cells had short microvilli similar to those in rodents (Greenwood and Holland, 1972), macaque monkeys (Castleman; et al. 1975) and cattle (Mariassy; et al. 1975).

Figure 2.21

Basal and intermediate cells. There is a wide, irregular intercellular space (arrows) around both cells and they have a large number of cytoplasmic processes (PR) on their surface. The intermediate cells have a large nucleus (NU) and an electron-lucent cytoplasm. TEM. X 8,400.


PR
NU

Brush cells were observed in the trachea, bronchi and bronchioli.They have now been found in several species including the bronchioli of the man (Watson and Brinkman, 1964), and the mouse (Hama and Nagata, 1970), the trachea of guinea pigs (Inoue and Hogg, 1974),the proximal bronchioli of the pig (Baskerville, 1970b) and in the upper and lower airways and alveoli of hamsters (Kennedy; et al. 1978) and rats (Jeffery and Reid, 1975). Although Mariassy; et al. (1975) did not observe brush cells in bovine airways these authors used only light microscopy and were not able to exclude their presence.

The bronchiolar epithelial admixture of ciliated and non-ciliated cells seems to be a common feature of all mammalian lungs thus far described. The function of ovine bronchiolar non-ciliated cells remains to be determined but their close resemblance to human Clara cells suggests that they may have a secretory role (Cutz and Conen,1971).

Interalveolar pores of Kohn and alveolar mononuclear phagocytes were uncommon in the lungs of the sheep examined in this study. Similar observations have been reported in cattle (Mariassy; et al. 1975), suggesting that this is a characteristic feature of the ruminant lung. In addition, recent studies in monkeys have shown that the frequency of pores of Kohn are age related in primates (Shimura; et al. 1986). Although, more extensive studies will be needed before the present findings can be confirmed, they have important implications regarding the pathophysiology and pathogenesis of pneumonia in ruminants. Van Allen et al. (1931) have shown that collateral ventilation within and among pulmonary lobules is severely limited by a scarcity of pores of Kohn and the completeness of interlobular septa. These investigators also showed that focal pulmonary atelectasis was more prone to occur in the absence of collateral ventilation. Lack of collateral ventilation is also likely both to hamper compensation for inequalities of ventilation associated with obstruction of small airways and to reduce the efficiency of mechanical expulsion of an intraluminal obstruction (Mariassy; et al. 1975). Consequences of the latter in the case of intrabronchiolar accumulations of exudate would be to impair the combating of an incipient bronchopneumonia or the resolution of a fully developed inflammatory process. Bronchiolar-alveolar anastomoses have been

reported in the ovine lung (Krahl, 1956) and these may limit the occurrence of the alveolar collapse, although little is known of their relative frequency in different species.

The low numbers of alveolar mononuclear phagocytes found in normal lungs would also appear to put the ovine lung at a disadvantage with respect to resistance to aerogenous bacterial infections since maintenance of bacterial sterility of alveolar parenchyma is primarily ascribed to these cells (Tizard, 1977). This could also reflect exposure to a relatively pathogen and particle-free environment. Since the animals used in this study were young, this could perhaps be another contributing factor for the paucity of alveolar macrophages.

4.2 CELL TYPES AND ULTRASTRUCTURE

The present study of the ultrastructure of the surface of the respiratory epithelium at five levels is the first reported in non-laboratory animals. Nevertheless, the findings at the levels investigated were similar to those in the rat (Jeffery and Reid, 1975) and mouse (Pack; et al. 1981). Ten morphologically distinct epithelial cell types were identified. This does not imply that one cell type may not, under changed environmental or developmental conditions, be transformed into another.

4.2.1 CILIATED CELLS

Two morphologically distinct ciliated epithelial cells were recognised in the ovine tracheobronchial airways. The first type was morphologically and ultrastructurally similar to that commonly reported in other species including the dog (Frasca; et al. 1968), pig (Baskerville, 1970a), man (Rhodin, 1966), guinea pig (Inoue and Hogg, 1974), rat (Jeffery and Reid, 1975), mouse (Pack; et al. 1981) and hamster (Kennedy; et al. 1978). The second cell type seen in this study has not been described previously. It is possible that both cells represent different stages of ciliated cell maturation. Because the second cell type has electron-lucent cytoplasm and large number of mitochondria and it is the less common type it is more likely that this type is the immature stage of the first type. Nevertheless, the possibility that they are two distinct cell population can not be excluded as they were both histologically and

ultrastructurally different.

The atypical cilia observed in the ciliated tracheal cells were similar to those seen in man (Alisby and Ghadially, 1972; Clarke; et al. 1981), normal dogs (Wilsman; et al. 1982) and hamsters exposed to respiratory carcinogens (Harris; et al. 1974). Alisby and Ghadially (1972) found atypical cilia in the respiratory epithelium of man with bronchial carcinoma and speculated that they were a feature of a bronchial epithelium with impaired clearance and may have been associated with a history of cigarette smoking. In the hamsters exposed to carcinogens by intratracheal injection; the cilia were discovered intracellulary and intercellularly as well as on the luminal surface. Harris; et al. (1974) and Wilsman; et al. (1982) have studied the incidence of abnormal tracheal cilia in healthy dogs. They estimated that abnormal numbers of central microtubules and abnormal numbers of peripheral microtubules occur in about 2% of all cilia. In the current study compound cilia were consistently observed only in the upper trachea. It is difficult to establish a good reason for the occurrence of atypical or compound cilia and it is unlikely that the sheep used were exposed to any noxious agents. It seems likely therefore that they are normal structures. This possibility is reinforced by the observation that they occur mainly in the second type of ciliated cell.

4.2.2 GOBLET CELLS

Two types of goblet cell were identified in this study. The first type had granules which were smaller and more electron-dense than those of the second type which had granules similar to those commonly reported in other species including the rat (Jeffery and Reid, 1975), human (Watson and Brinkman, 1964), dog (Frasca; et al. 1968) and pig (Baskerville, 1970a). Using a methylene blue-Azure stain, Mariassy and Plopper (1983) distinguished three types of mucous cells in the ovine tracheobronchial epithelium. These two cell types were similar in structure apart from their granules. The first cell type had confluent blue staining granules, while the second type had granules of slightly larger size which stained variable shades of bright lavender. These observations with the light microscope are supported by the ultrastructural findings of the present study, thus confirming that there are two distinct types of mucous goblet cell in

the tracheobronchial epithelium of sheep.

The finding that goblet cells were slightly more numerous in the lower tracheal epithelium than in the upper trachea was not unexpected in view of the observations of human trachea by other workers (Ellefsen and Tos 1972). Goblet cells were numerous in the tracheobronchial airway epithelium of man (Rhodin,1963), dogs (Frasca; et al. 1968) and the pig (Baskerville, 1970a). However, only Jeffery and Reid, (1975) have made quantitative studies and found that goblet cells make up less than 1% of the total epithelial cells of SPF rats at all levels of the trachea and bronchi.

4.2.3 UNKNOWN SECRETORY CELLS

Two secretory cells not previously described were identified ultrastructurally in this study. The first type contained small mucus-like granules in its apical cytoplasm. It had relatively, long microvilli on the mucosal surface of the cell. Greenwood and Holland (1972) have observed that both immature and discharged goblet cells have more numerous microvilli than mature cells. Although this cell could be considered to be an immature or discharged goblet cell, its shape was irregular rather than slender or columnar which is the usual shape for both immature and discharged cells (Breeze; et al. 1976). The second type of unknown secretory cell was distinctly different from the first. It contained small granules in the apical part of the cytoplasm which evacuated a finely granular material. The content of these granules was distinct and consistently different ultrastructurally from that of mucous granules, which usually resemble ribosome particles. Histologically, this cell resembled the third type of secretory mucosal cell described by Mariassy and Plopper (1983). However, the present study has shown that ultrastructurally, these were not typical mucous granules.

4.2.4 CLARA CELLS

The secretory granules of the ovine Clara cells were morphologically different from those described in other mammals. The granules of Clara cells of the pig (Baskerville, 1970b), rabbit, man (Cutz and Conen, 1971), rat (Jeffery and Reid, 1975), hamster (Kennedy; et al. 1978) and mouse (Pack; et al. 1981) are

characteristically irregular, electron-dense and homogeneous, while in sheep these granules were even more irregular, and contained areas of both electron-dense and electron-lucent material.

There is much disagreement in the literature as to the reaction of Clara cells to the PAS stain. This study and others have demonstrated PAS-positive granules in Clara cells (Azzopardi and Thurlbeck,1969; Cutz and Conen, 1971; Pack; et al. 1981) while others have not (Kuhn; et al. 1974; Niden, 1967). This controversy may in part be explained by species differences and variations in fixation or embedding procedures (Cutz and Conen,1971). The lack of specificity of the PAS technique itself may also be a contributing factor.

One of the most consistent ultrastructural features of the Clara cell is its abundance of SER. In this respect it resembles certain steroid producing cells (Volk and Scarpelli,1964). Several studies have suggested that Clara cells may undergo apocrine activity (Etherton; et al. 1973; 1979). Profiles have been observed in section where the "apical cap" of the Clara cell appeared to be budding from the body of the cell (Jeffery and Reid, 1975). Jeffery and Reid (1975) have suggested that this "apical cytoplasmic bleb" is a fixation artifact. However, Etherton; et al. (1979) used several fixation techniques and indicated that these profiles may genuinely represent apocrine secretion. To date, there is no direct experimental evidence to explain the nature of the secretory product of Clara cells.

4.2.5 BRUSH CELLS

The brush cells found in the ovine bronchi and bronchioli in this study, resemble those previously described in the airways and alveoli of the rat (Jeffery and Reid,1975), mouse (Hama and Nagata, 1970), guinea pig trachea (Inoue and Hogg,1974) and pig bronchioli (Baskerville, 1970b).

In addition to the typical brush cells found in this study, an atypical type was also observed. Microvillus projections similar to those seen on the atypical cells have been observed on the mucosal surface of goblet cells and potential ciliated cells in rats (Andrew,

1974), but the cells found in this study lacked any secretory granules or basal bodies.

The function of brush cells is not yet known. The presence of many pinocytotic vesicles at their luminal edge, similar to brush border cells of the alimentary tract, has led some investigators to postulate an absorptive function (Jeffery and Reid, 1975). The filament bundle content present in the cytoplasm of these cells has led Meyrick and Reid, (1968) to suggest their role is that of a stretch receptor.

4.2.6 BASAL CELLS

The morphology of basal cells found in this investigation resembles that previously described in rats (Jeffery and Reid, 1975), man (Rhodin, 1963; 1966), dogs (Frasca; et al. 1968), pigs (Baskerville, 1970a) and the hamster (Kennedy; et al. 1978). Basal cells were found in the ovine epithelium as distally as the bronchioli, although they were more numerous in the trachea and extrapulmonary bronchi. This distribution is similar to that which occurs in the rat (Jeffery and Reid,1975).

Jeffery and Reid,(1975) pointed out the existence of two epithelial compartments in the trachea and bronchi: a basal layer involved with division and a superficial layer concerned chiefly with differentiation and maturation. Basal cells proliferated in response to experimental mechanical damage involving destruction of the most differentiated cells. This produced a multilayered undifferentiated epithelium (Lane and Gordon, 1974). Lane and Gordon (1974) observed that their speed of differentiation is rapid and basal bodies were present in some differentiated cells and others contained mucous granules at 60 hr after injury. Well developed ciliated cells or goblet cells were present by 90 hr after injury.

4.2.7 INTERMEDIATE CELLS

Intermediate cells formed a poorly defined layer just above the basal cells, which they closely resembled, although they were more spindled in shape and extended further toward the lumen. Cells of this type have been described in the tracheal epithelium of the rat

(Jeffery and Reid, 1975), rabbit (Konradova, 1966) and man (Rhodin, 1966) and in the bronchus of the pig (Baskerville, 1970a). In the present study, these cells did not usually reach the airway lumen; a finding which is similar to the situation in rabbits (Konradova, 1966), man (Rhodin,1966) and pigs (Baskerville, 1970a) but different from the rat (Jeffery and Reid, 1975). In the dog (Frasca; et al. 1968) and foetal rat (Sorokin,1968), intermediate cells differentiate and become more electron-dense as they start to either accumulate mucous granules or begin ciliogenesis, but this was not seen in the present study.

2.5 CONCLUSION

The present work could have been improved by morphometric quantitation of the different cell types. However, because of the large number of cell types recognised and the five levels of respiratory tract examined, such an undertaking would be particularly time consuming since many of the cell types could only be identified accurately by the TEM.

The present study of 5 different levels of the tracheobronchial epithelium of normal sheep will serve as a useful base for future studies of ovine respiratory pathology. Although the airway epithelium generally resembles that of the higher mammals, two important morphologic features of the parenchymal tissue of ovine lung are in sharp contrast with the pulmonary parenchyma of other mammals, apart from cattle. Firstly, interalveolar pores of Kohn are uncommon in the lungs of young sheep. Secondly there is a relative rarity of alveolar mononuclear phagocytes in alveolar spaces. These observations suggest there are differences in lung function between species which may have implications for the susceptibility of sheep to pneumonia. In addition, the investigation has identified several cell types not previously described and whose function is not clearly understood. Studies of the normal ovine lung will therefore continue to be a fruitful exercise.

CAPTER 3

THE MORPHOLOGY OF THE TRACHEOBRONCHIAL EPITHELIUM IN OVINE CHRONIC NON-PROGRESSIVE PNEUMONIA

CHAPTER 3

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THE MORPHOLOGY OF THE TRACHEOBRONCHIAL EPITHELIUM IN OVINE CHRONIC NON-PROGRESSIVE PNEUMONIA

1.0 INTRODUCTION

There are a considerable number of descriptions of the histopathology of both naturally occurring and experimentally produced CNP of lambs in New Zealand and the equivalent diseases (atypical or proliferative exudative pneumonia) overseas. However, surprisingly little work has been carried out on the ultrastructural and topographical changes which occur in the lungs and respiratory airways of these diseases. The only detailed electron microscopic investigation was carried out by Alley (1975a) which dealt predominantly with ultrastructural changes occurring in the pulmonary parenchyma in sheep in New Zealand with naturally occurring CNP.

No work has been reported on the tracheobronchial epithelium in pneumonic sheep. However, there is some evidence from previous studies in sheep (Alley, 1975a) and pigs (Baskerville, 1972) that bronchial and bronchiolar changes may be important in the pathogenesis of chronic pneumonia. In order to follow the early changes and their development to advanced pneumonic lesions, two groups of lungs were collected for study. The first group consisted of early pneumonic lesions and the second group advanced pneumonic lesions. This chapter presents the light microscopic, TEM and SEM features of the lesions at five different levels of the tracheobronchial airways in sheep affected with naturally occurring CNP.

Because there have been no studies of surface morphology of alveoli in ovine pneumonia, the present SEM study was extended to these airspaces also.

2.0 MATERIALS AND METHODS

The lungs together with their attached trachea, were collected from 12, 5 to 9 month old lambs slaughtered at a local meat works;

six showed early pneumonic lesions and six had advanced lesions of CNP. The early lesions had a gross appearance similar to the type I category described by Alley (1975b) and the advanced lesions were similar to category IV. They were collected on several different occasions and originated from different farms. Each lung was examined grossly and all fulfilled the requirements for each group.

The selection of levels, fixation, TEM and SEM techniques, preparation and staining were similar to those described previously (Chapter 2). Sections for light microscopy were routinely stained with haematoxylin and eosin (H & E) and the technique of AB-PAS staining is described in Appendix 5.1.

3.0 RESULTS

3.1 EARLY PNEUMONIC LESIONS

3.1.1 HISTOLOGY

3.1.1.1 TRACHEA

In all cases the epithelium was severely hyperplastic and showed various degrees of degenerative change including mild to severe loss of cilia and focal areas of squamous metaplasia of epithelial cells. Small numbers of macrophages and neutrophils were present in the lamina propria and large numbers of neutrophils were found between epithelial cells. Submucosal glands exhibited a very mild inflammatory changes, including mild swelling of the gland and mild to moderate neutrophil infiltration (Fig. 3.1).

The mucous granules of goblet cells either showed less colour density than those of normal sheep or contained foamy heterogeneous material when stained with AB-PAS. There was also an increase in the number of discharged goblet cells and cells containing acid glycoprotein were prominent.

3.1.1.2 MAIN BRONCHI

In general, the extrapulmonary bronchi were less affected than

the intrapulmonary bronchi. There was mild to moderate hyperplasia in both the bronchial and submucosal gland epithelium, with occasional polypoid structures which occurred only on the luminal surface of the epithelium. Moderate numbers of neutrophils were found between the epithelial cells and early lymphoid aggregations were present in the lamina propria, immediately beneath the epithelial layer and around submucosal glands. These lymphoid aggregations also contained a small number of neutrophils. The lumen of the submucosal gland acini contained variable amounts of material which included mucus, moderate number of neutrophils, cellular debris and a few macrophages (Fig. 3.2).

The number of goblet cells containing neutral glycoproteins in the main bronchi was similar to or slightly greater than these containing mixed glycoproteins. A few intra-epithelial cysts containing mucus were observed.

3.1.1.3 SMALL BRONCHI

Lesions at this level were of similar type but were more extensive than those in the main bronchi.

3.1.1.4 BRONCHIOLI

Moderate to mild epithelial hyperplasia and peribronchiolar lymphoid aggregations were consistently present. The lumen of a large number of bronchioli contained variable amounts of mucus, cellular debris and inflammatory cells, mainly neutrophils (Fig. 3.3). Alcian blue-PAS revealed a marked change in the frequency and distribution of both Clara and goblet cells at this level, most of which had discharged their secretion. Those which had not discharged contained mainly neutral glycoproteins.

3.1.2 TOPOGRAPHICAL MORPHOLOGY

3.1.2.1 TRACHEA

The luminal surface of the epithelium was irregular and composed of equal numbers of ciliated and non-ciliated cells (Fig. 3.4).

Figure 3.1

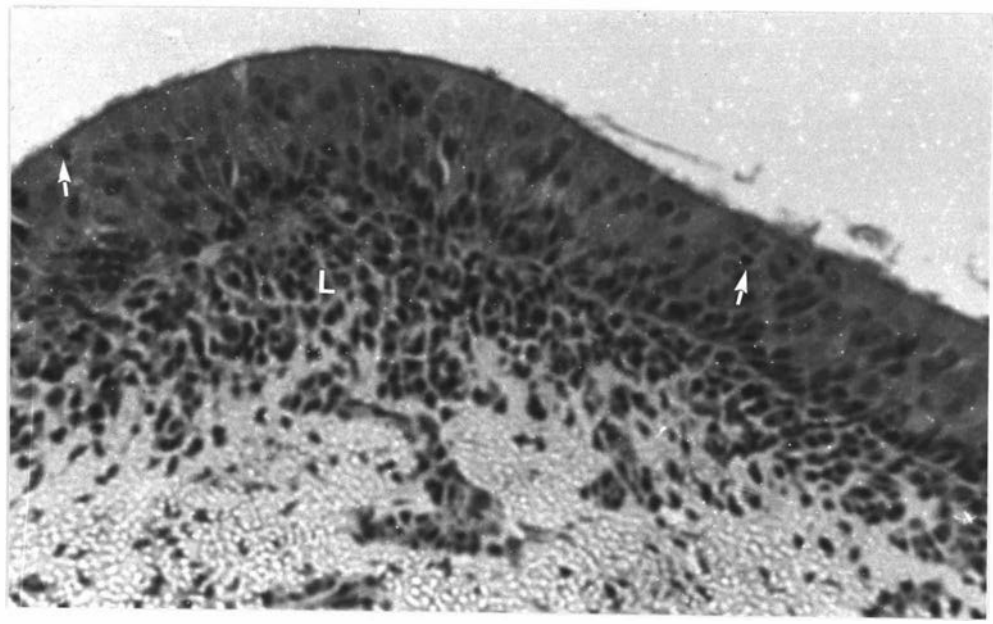
The epithelium of early pneumonic trachea showing moderate hyperplasia. The luminal border has lost most of its cilia. Moderate numbers of neutrophils and macrophages are present in the lamina propria (L) and large numbers of neutrophils (arrows) are found between epithelial cells. H&E. X 156.

Figure 3.2

Bronchial epithelium of an early pneumonic lesion. There are moderate numbers of neutrophils (arrows) between the epithelial cells with early lymphoid aggregations immediately beneath the epithelial layer. H&E. X 312.

Figure 3.3

Bronchiole from an early pneumonic lesion showing moderate epithelial hyperplasia and mild peribronchiolar lymphoid aggregations. The lumen contains necrotic material and inflammatory cells. H&E. X 156.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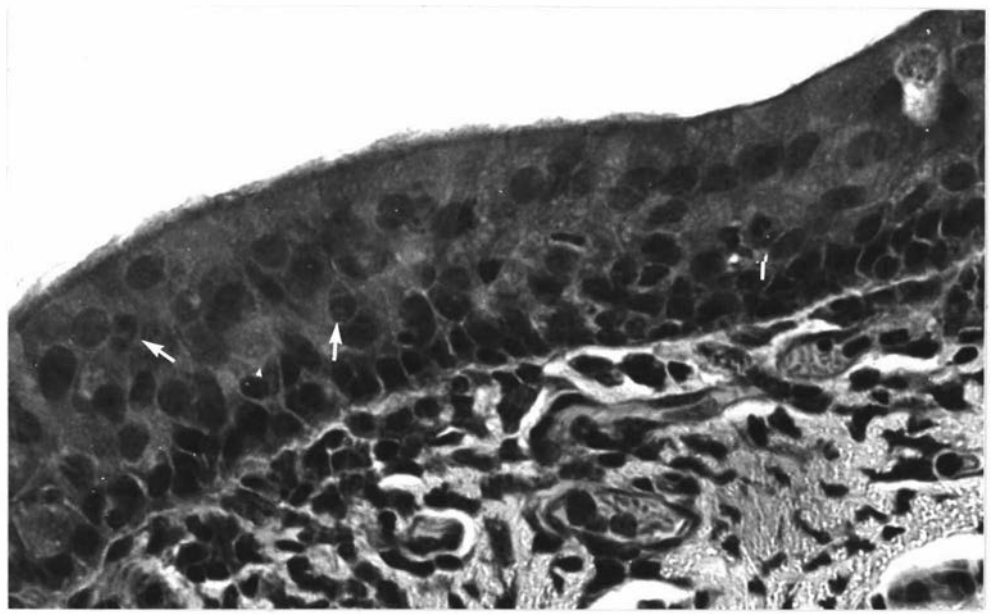
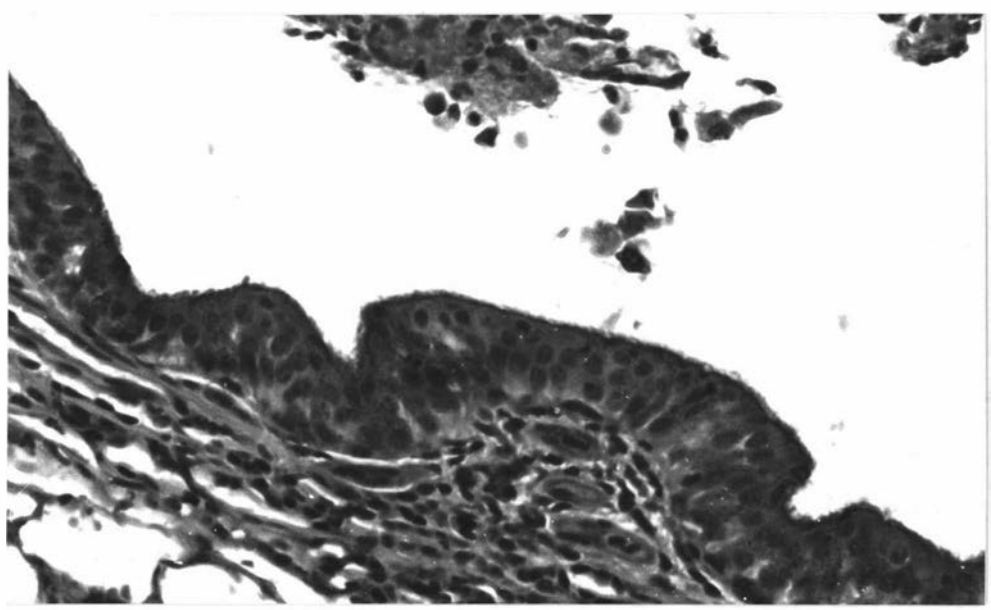




Three types of non-ciliated cell were observed. The mucosal surface of the first type was covered by a large number of microvillous projections. In the second type the luminal surface was devoid of both microvilli and cilia. This may have represented the metaplastic changes observed using the light microscope. The third type consisted of goblet cells, which formed small pits on the surface epithelium. Submucosal gland surface openings were frequent and showed lack of cilia. Although the goblet cells and submucosal gland openings were clear of mucus secretion, some cellular debris and inflammatory cells were observed in close proximity (Fig. 3.4). Most of the epithelial surface of the upper trachea was clear of mucus, but at the lower part of the trachea, the ciliated surface was covered by a thick layer of adherent secretion containing occasional inflammatory cells (Fig. 3.5). Although there were inflammatory cells and cellular debris on the non-ciliated surface, it was not covered by a mucus blanket. A large number of bacteria were trapped between the cilia, but no organisms were observed on the non-ciliated surface. The bacteria were of various morphological types and a large number of organisms attached firmly to the bottom of the cilia were characteristically mycoplasma-like in appearance. These organisms were polymorphic and had an indentation on one side of their flat surface, similar to the morphology of M ovipneumoniae observed by Al-Kaissi & Alley (1983) (Fig. 3.5).

The cilia of the epithelial surface were shorter than those of normal sheep and varied in shape. Most were broken and entangled with one another (Fig. 3.4).

3.1.2.2 BRONCHI

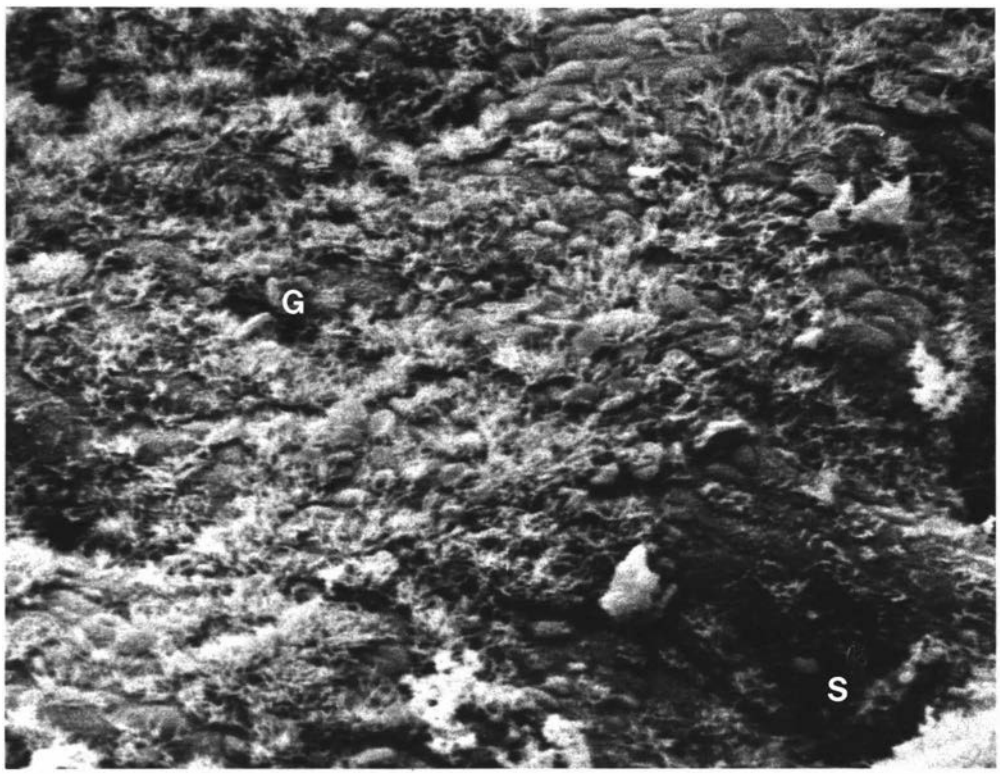
Almost equal numbers of ciliated and non-ciliated cells were present on the epithelial surface (Fig. 3.6). These cells demonstrated two types of pi ts and holes; Large size pits with a regular outline were common and these resembled the openings of goblet cells. Smaller size holes with an irregular outline were also seen on many cells and these probably represented sites of cellular membrane damage. The epithelial surface was clear of mucus but a moderate number of neutrophils together with occasional macrophages, epithelial cells and fragments of cell debris were observed. Areas of squamous cells were seen more often than in the trachea of pneumonic sheep

Figure 3.4

The tracheal epithelial surface in early pneumonia. The mucosal surface contains approximately equal numbers of ciliated and non-ciliated cells. Observe the goblet cell openings (G) and submucosal gland opening (S). SEM. X 800.

Figure 3.5

The luminal surface of the lower trachea in early pneumonia showing a thick layer of mucus secretion (arrows) which covers most of the ciliated but not the non-ciliated surface. SEM. X 10,000. The inset micrograph shows some of the large number of mycoplasma-like organisms (M) which are attached firmly to the bottom of the cilia. SEM. X 2,500.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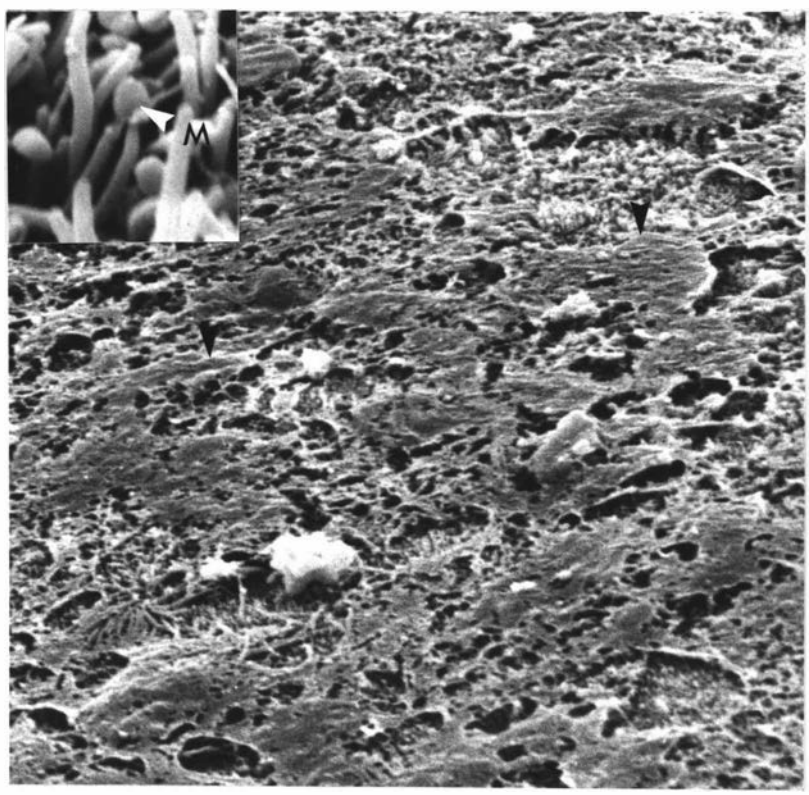
S


M

(Fig. 3.6).

The cilia of the bronchial epithelial cells showed the same morphological changes and presence of organisms as noted in the trachea (Fig. 3.7).

Bronchial submucosal gland openings were denuded of cilia and showed increased numbers of squamoid cells. The secretions of some glands were fixed in situ and contained a large number of polymorphs, mucous strands and globules (Fig. 3.8).

3.1.2.3 BRONCHIOLI

The bronchioli from the affected part of the lung had thickened walls and the lumen contained a variable amount of necrotic exudate, containing mucus, polymorphs and a few macrophages, some of which were attached to the surface epithelium (Fig. 3.9 & 3.10). Three main types of cell were present in the epithelium. The first type were ciliated cells which had long and short cilia and microvillus projections on their surface. The second type were brush cells which had mostly microvillus projections and occasional cilia on the surface. The third type of cell protruded from the surface like Clara cells and had small microvillous projections on their surface (Fig. 3.11). In the less severely affected bronchioli, the number of Clara cells were greatly increased and their luminal surface exhibited morphological changes including the appearance of microvillus-like projections and cilia (Fig. 3.12).

3.1.2.4 ALVEOLI

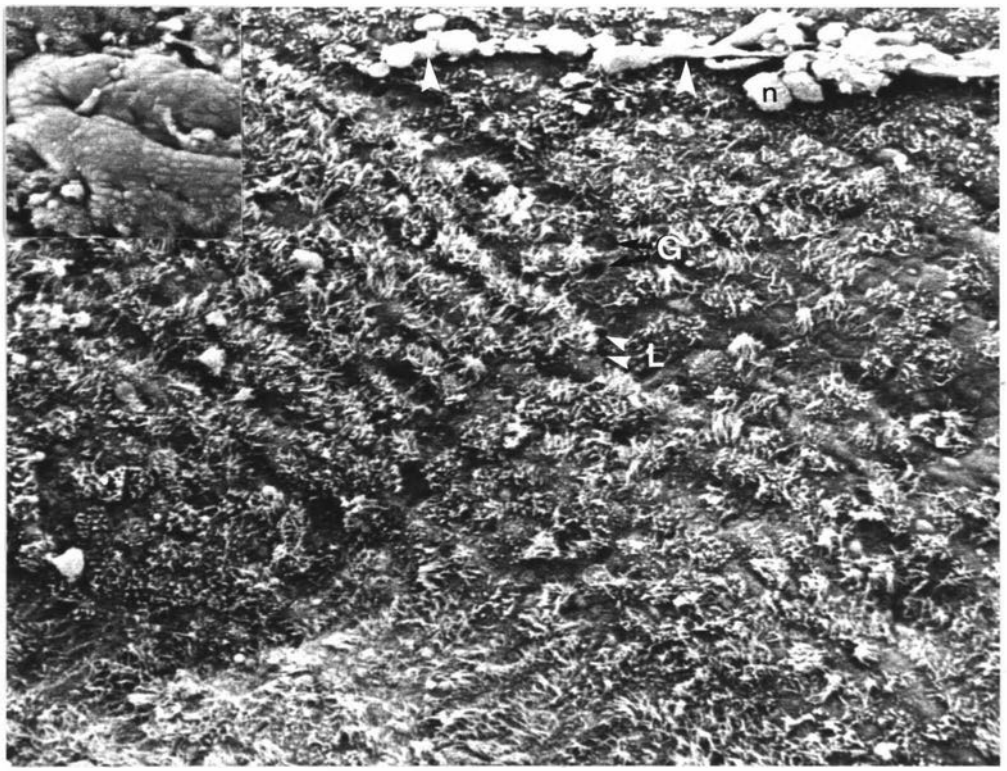
The affected alveoli showed two characteristic changes; an increase in the thickness of the alveolar septa and an accumulation of cells within the alveolar space (Fig. 3.13). The cells found in the alveoli were mainly polymorphs with digestion vacuoles although some macrophages were seen. The macrophages had the morphology characteristic of pulmonary macrophages, with long cytoplasmic extensions. The alveolar epithelial surface showed ruffling and budding of the plasma membrane which also contained small pits and holes. Large numbers of granules were also seen lying on the surface of the epithelium (Fig. 3.13). Other changes included localised

Figure 3.6

The bronchial surface epithelium in an early pneumonic lesion. The large holes (G) are goblet cell openings and the small holes (L) may be due to damage to the cell membrane. Large amounts of cellular debri (arrows) and neutrophils (n) are present on the surface. SEM. X 660. The inset figure illustrates a focal area of squamous cell metaplasia (arrows). SEM. X 300.

Figure 3.7

A large number of microorganisms attached to the top of the bronchial cilia in an early pneumonic lesion (large arrows). The morphological features of many of the attached organisms were similar to those of mycoplasmas (small arrows). SEM. X 10,000.


n
G
L

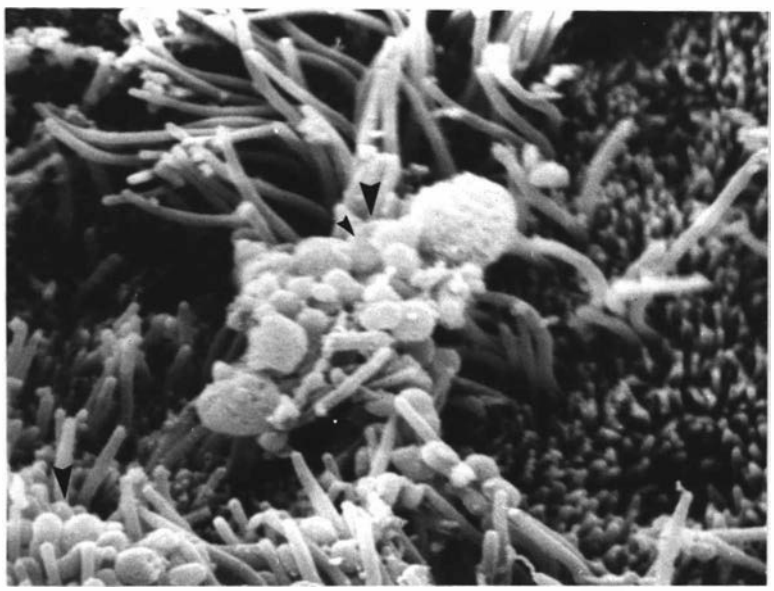


Figure 3.8

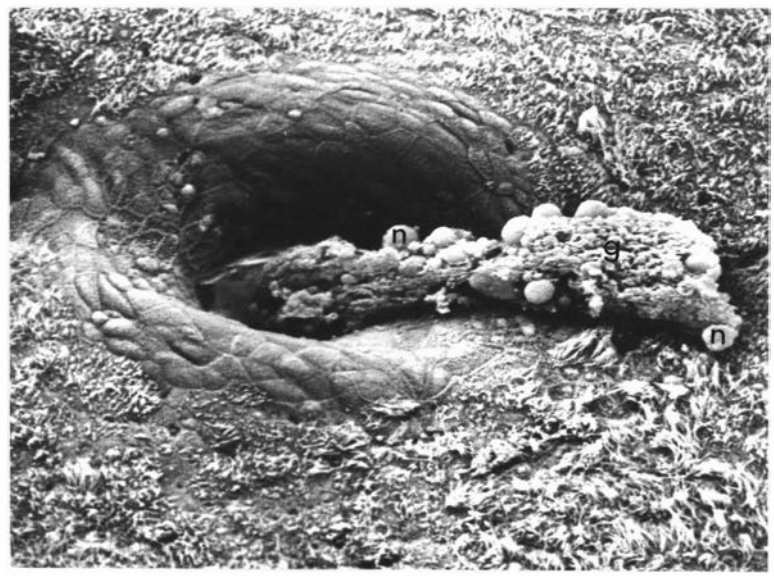
A submucosal gland opening denuded of cilia and showing squamous metaplasia. The secretions which are fixed *in situ* contain a large number of neutrophils (n) mixed with mucosal strands and granules (g). SEM. X 700.

Figure 3.9

An affected bronchiole which has a thickened wall (arrows). Its lumen contains large amounts of necrotic exudate consisting mainly of neutrophils . SEM. X 400.

Figure 3.10

The luminal surface of an affected bronchiole showing a few macrophages (arrow) attached to epithelial cells. SEM. X 1600.


n
g
n

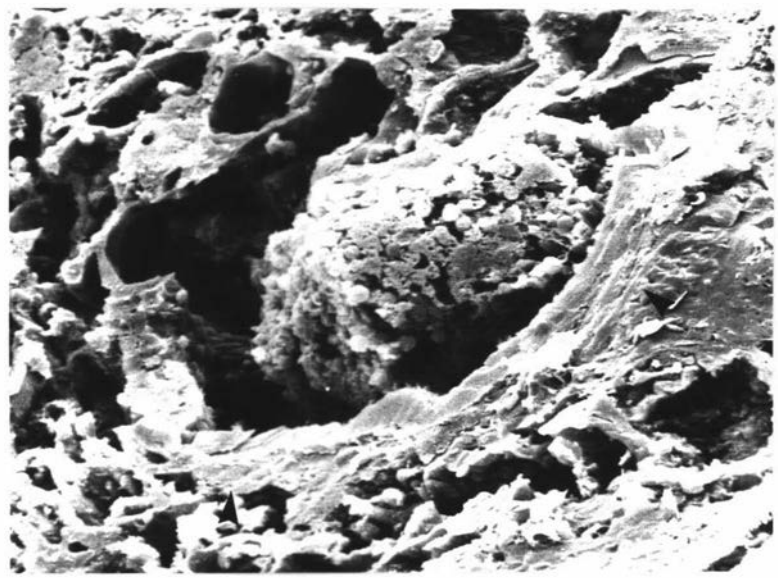


Figure 3.11 (Left)

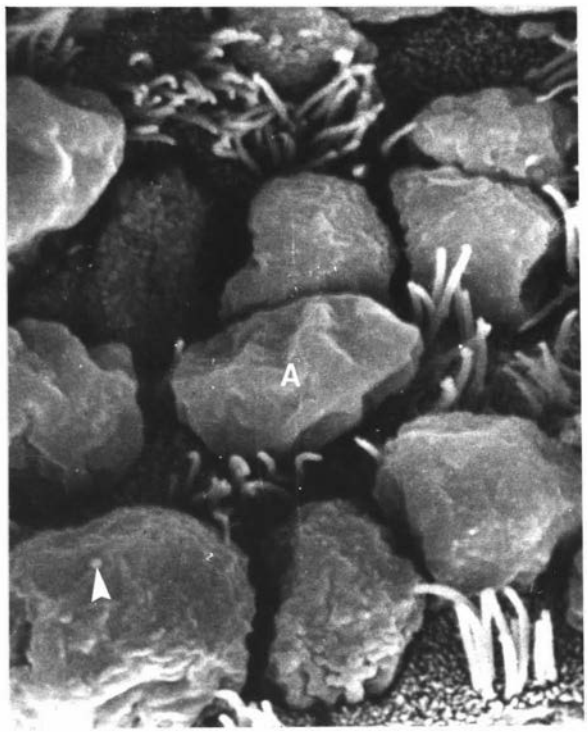
High magnification of the bronchiolar epithelial surface from a severely affected area. The surface consists of ciliated, brush and Clara-like cells. SEM. X 5080.

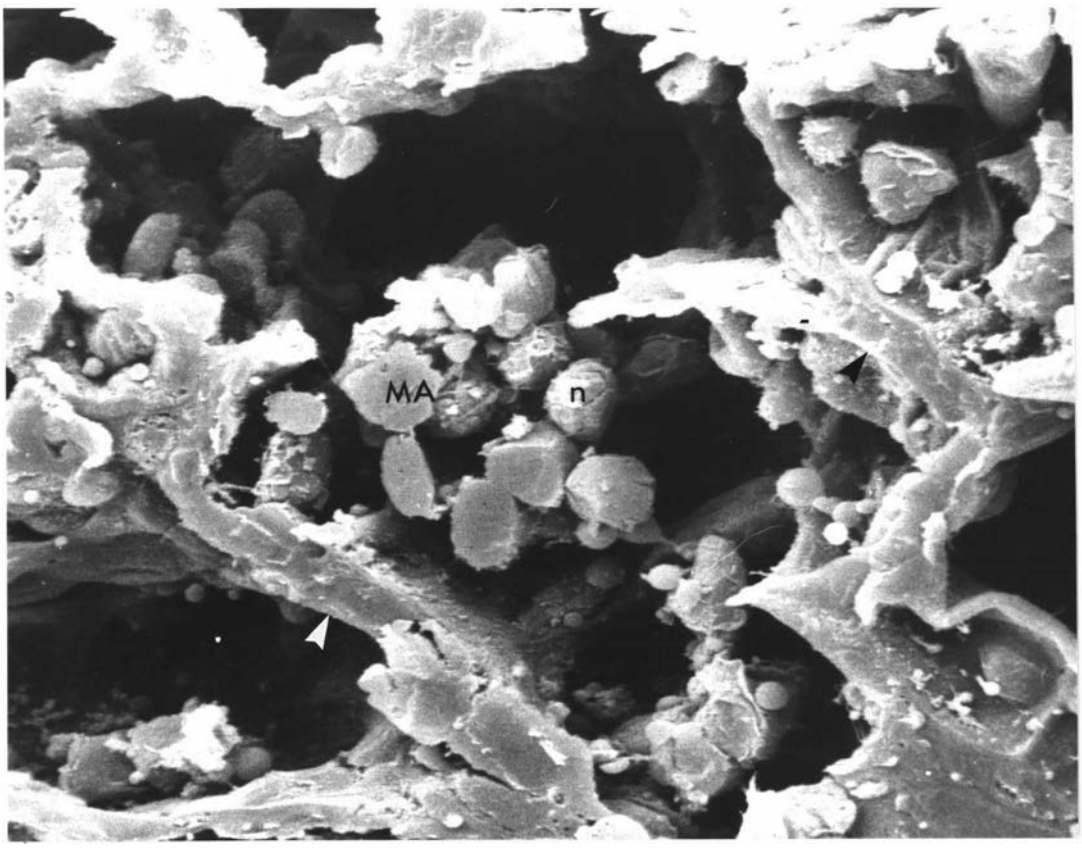
Figure 3.12 (Right)

High magnification of the bronchiolar epithelial surface from a less severely affected area. Clara cells (A) exhibit small microvillus-like projections (arrows). SEM. X 6000.

Figure 3.13

The alveolar region in early pneumonia showing thickening of the alveolar septa (arrows) and accumulation of neutrophils (n) and a few macrophages (MA) within the alveolar space. SEM. X 1,470.


A


MA
n

increases in the number of alveolar type II cells.

3.1.3 ULTRASTRUCTURE

3.1.3.1 TRACHEA

Most of the epithelial surface, particularly in the lower part of the trachea, was covered by variable amounts of moderately electron-dense material (Fig. 3.14). This consisted of mucus, cellular debris and organisms of variable shape and number embedded in amorphous material (Fig. 3.15). A large number of cilia in mucus areas covered exhibited a variable degree of ciliogenesis. The organisms were most frequent free between the surfaces of the amorphous material and cilia. They were not seen in direct contact with the cytoplasm of either ciliated or non-ciliated cells (Fig. 3.15). A few of these organisms resembled mycoplasmas in structure with a round or oval shape bounded by a three layered plasma membrane (Major; et al. (1978)(Fig. 3.14). The majority of the other organisms resembled bacteria and had a rounded cross section, a cocco-bacilli shape in longitudinal section and a densely stained cytoplasm(Fig. 3.15).

Fewer cilia than normal were found on the luminal epithelial surface and a larger number of microvillus projections were present (Fig. 3.14). Small cytoplasmic projections were seen protruding from the surface of some epithelial cells. Ciliated and non-ciliated cells contained a large number of mitochondria, lysozymes and glycogen granules. Large numbers of neutrophils and a few plasma cells were seen between the epithelial cells (Fig. 3.14).

3.1.3.2 BRONCHI

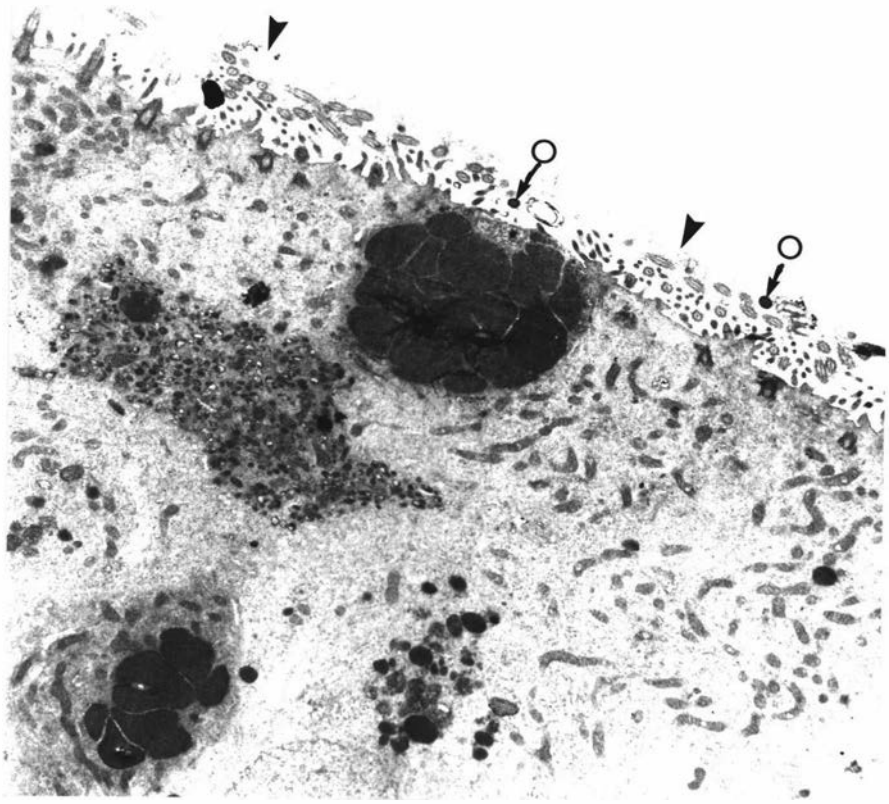
The most striking feature seen in the bronchi was the infiltration of the epithelium with moderate numbers of neutrophils, lymphocytes and a few macrophages (Fig. 3.16). • In some sections, mononuclear cells formed a row underneath the basement membrane. Large numbers of mycoplasmas and bacteria were found between the cilia and the microvillous projections of the bronchial epithelium (Fig. 3.17). The epithelial surface close to these small microcolonies was

Figure 3.14

The tracheal epithelium from an early pneumonic lesion showing electron-dense material (arrows) covering the surface. A few microorganisms (O) are embedded in the amorphous material. There is neutrophil infiltration between underlying cells. TEM. X 7,640.

Figure 3.15

High magnification of the surface material showing the bacteria (O) which are not in direct contact with cilia. TEM. X 31,800.



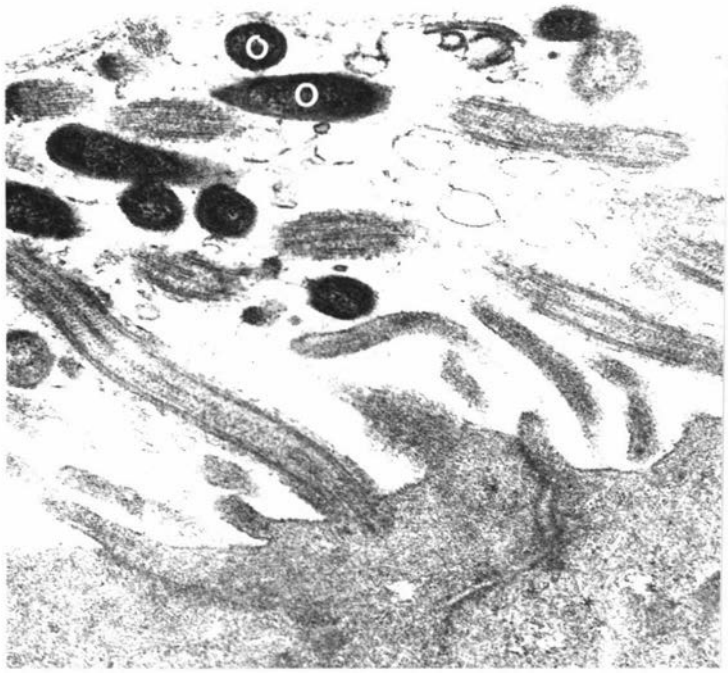


Figure 3.16

The bronchial epithelium in an early pneumonic lesion showing neutrophils (n) between the epithelial cells. TEM. X 5.660.

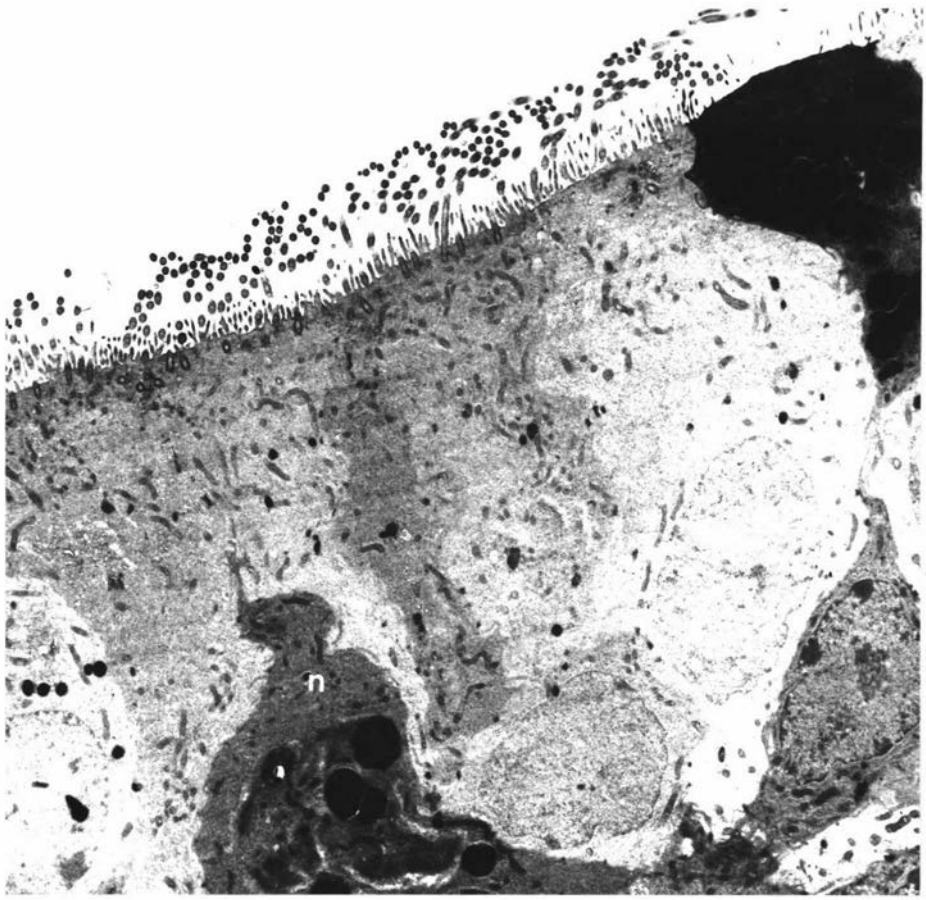
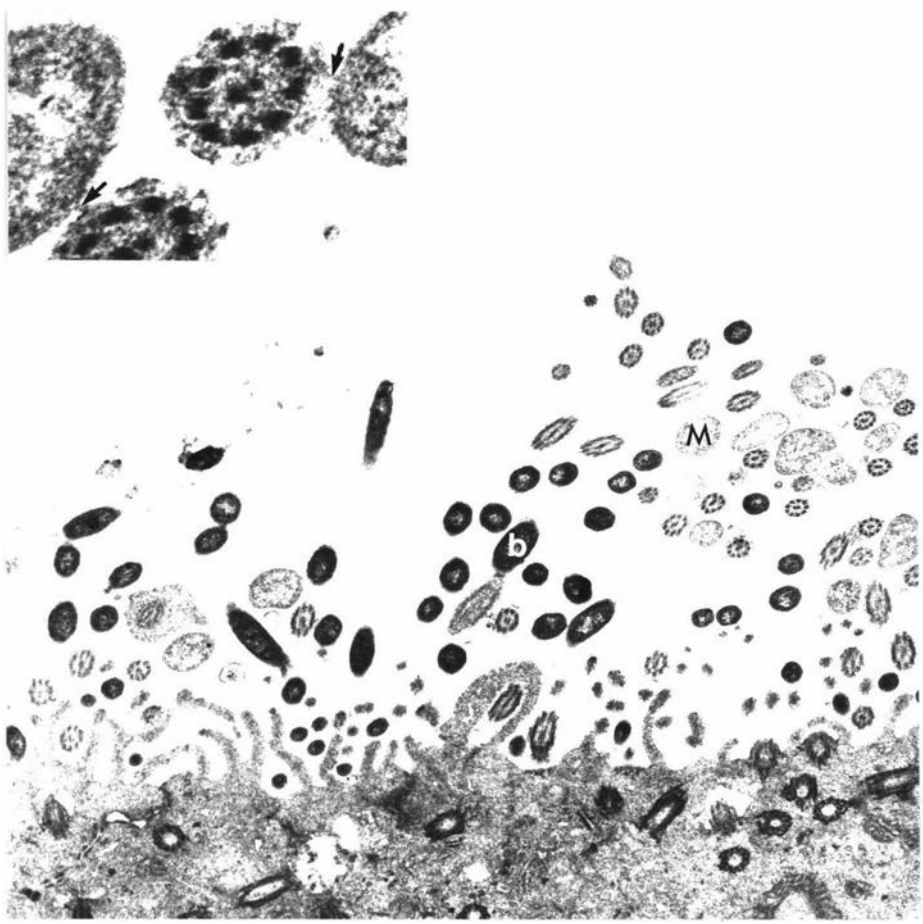

n

Figure 3.17

The bronchial epithelial surface showing a large number of mycoplasmas (M) and bacteria (b) between cilia. TEM. X 14,900. The inset micrograph illustrates the tubular organelles (arrows) which may be the means of attachment between mycoplasmas and cilia. TEM. X 72,100.


M
b

almost denuded of cilia (Fig. 3.17). Apart from the presence of occasional vacuoles, the cytoplasm of the epithelial cells showed little degenerative change (Fig. 3.16).

Mycoplasmas were seen more frequently at this level than in the trachea, but were never seen in close contact with the external cell surfaces or within digestive vacuoles of polymorphs or other phagocytes (Fig. 3.17). These organisms were attached to the cilia by means of tubular organelles which originated from the cytoplasm and were anchored to the plasma membrane of the cilium. At the attachment sites on some cilia, the plasma membrane was absent (Fig. 3.17). The bacteria were variable in size and the smallest of these were in close proximity to the cell membrane of the bronchial epithelium. Although small bacteria were in close contact with cilia, microvilli and cell membranes no means of attachment was observed. Large bacteria were observed between cilia and microvilli, but the majority of these were not in close contact with cilia (Fig. 3.17).

3.1.3.3 BRONCHIOLI

The epithelium of many bronchioli showed moderate hyperplasia and was composed of three or more layers of cells. The surface cells were differentiated into the normal ciliated and non-ciliated types but the underlying cells were irregular in shape although their cytoplasm was of similar density (Fig. 3.18). In some regions a distinct basal layer was observed, composed of irregularly round cells with round nuclei and relatively little cytoplasm (Fig. 3.19). In other airways ciliated cells had lost most of their cilia and the apical plasma membrane of some of these cells had ruptured, releasing cell contents into the bronchiolar lumen (Fig. 3.20). The other common change in the epithelium was the presence of large projections of cytoplasm from the apex of ciliated cells and non-ciliated cells. These protrusions occupied large areas of the cell apex and were frequently longer than the cilia. The cytoplasm of these extensions contained clumps of ribosomes sparsely scattered throughout but little endoplasmic reticulum or other organelles. The remainder of the apex of affected cells had few or no cilia (Fig. 3.20).

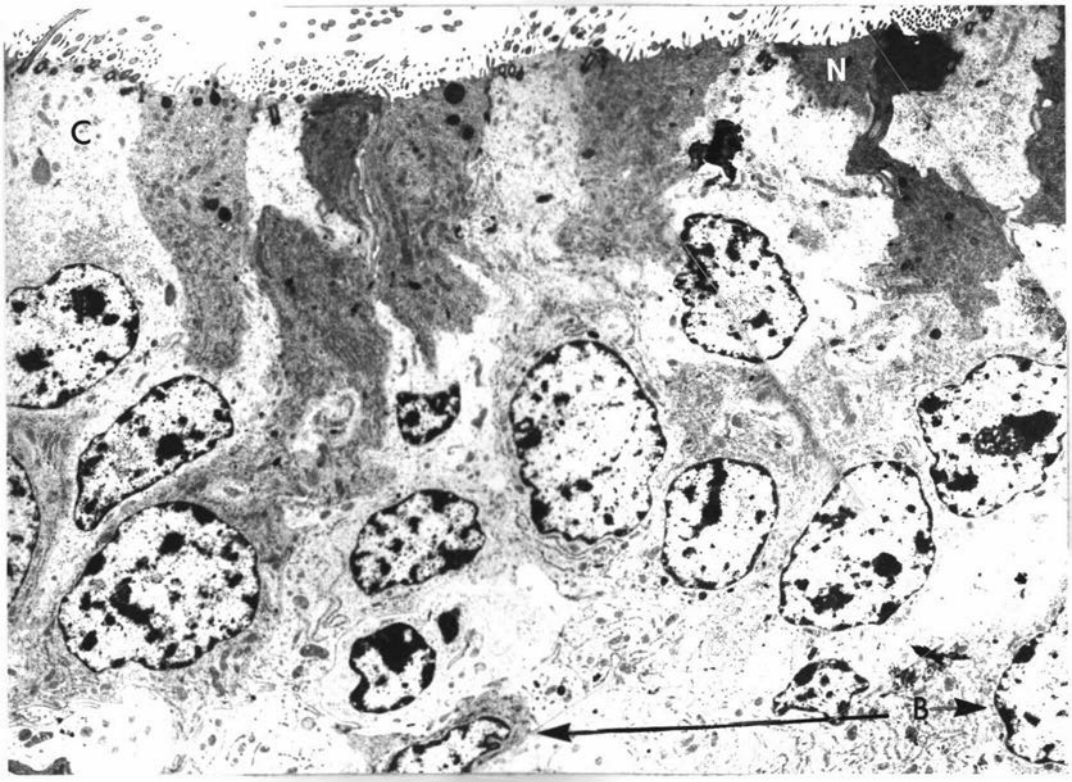
The lumen of most airways contained amorphous material, cellular debris, neutrophils and a few macrophages (Fig. 3.21). Mycoplasmas

Figure 3.18

Ultrastructure of the bronchiolar epithelium in an early pneumonic lesion. Both ciliated (C) and non-ciliated cells (N) are present together with distinct basal cells (B). TEM. X 4,300.

Figure 3.19

High magnification of the distinct basal layer (arrows) which was observed in some pneumonic bronchioli. TEM. X 5,200.


N
C
B

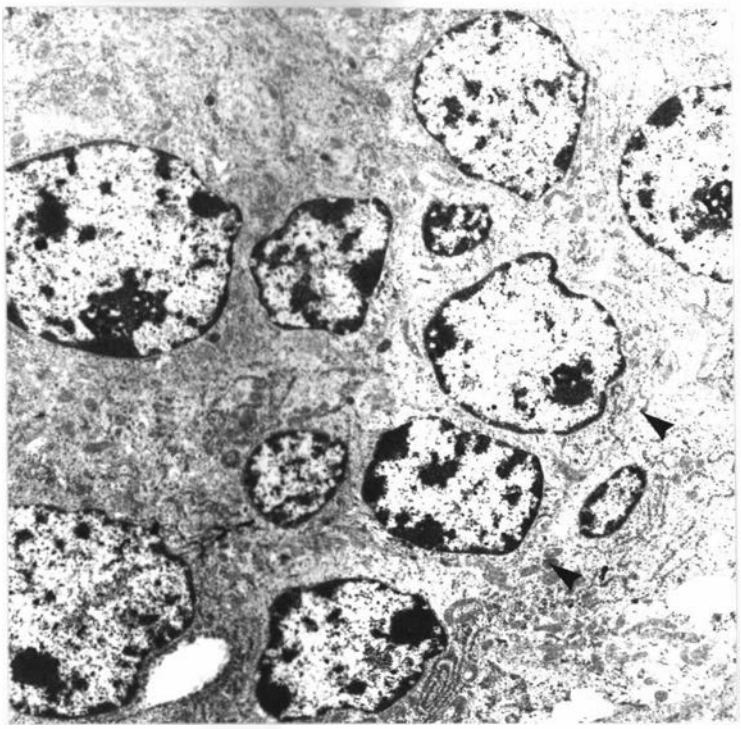
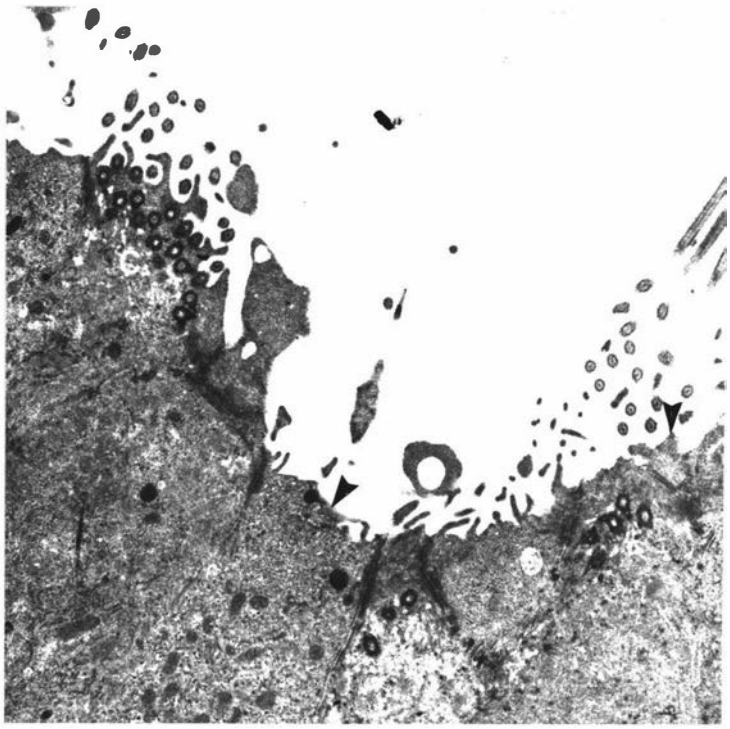


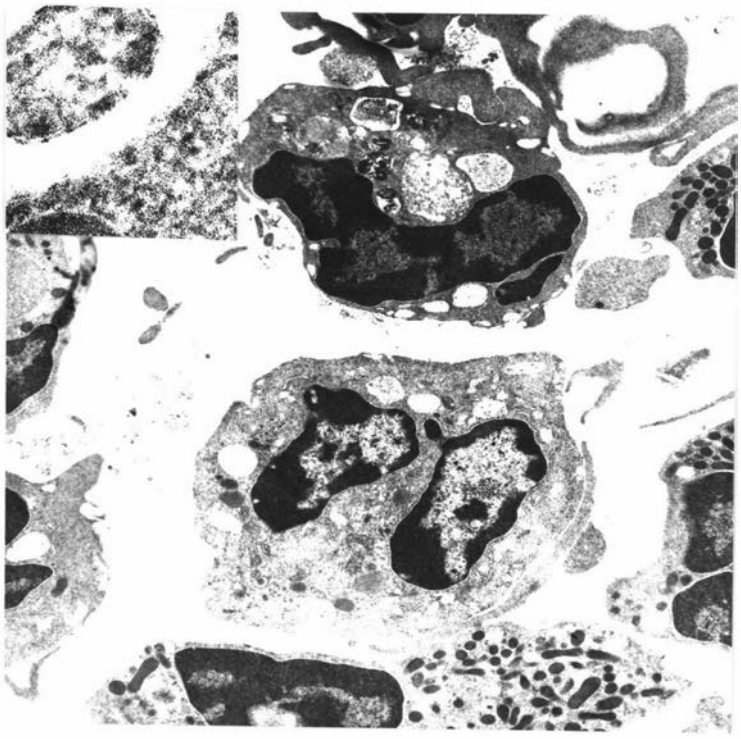
Figure 3.20

A severely affected bronchiole showing ciliated cells (C) which have lost their cilia and show rupture of the apical plasma membrane (arrows). TEM. X 7,800.

Figure 3.21

The bronchiolar luminal contents in early pneumonic lesion. Cellular debris, neutrophils and macrophages are present. TEM. X 7,800. The inset micrograph shows a mycoplasma-like organism observed inside the digestive vacuole of a neutrophil. TEM. X 103,600.





were observed occasionally inside digestive vacuoles of neutrophils. No bacteria were seen in either the lumen or in contact with bronchiolar epithelial cells.

3.2 ADVANCED PNEUMONIC LESIONS

3.2.1 HISTOLOGY

3.2.1.1 TRACHEA

Extensive areas of the epithelium showed squamous metaplasia and moderate to mild hyperplasia. A large to moderate number of neutrophils and mononuclear cells were present in the lamina propria together with small aggregations of lymphoid cells. Neutrophils were commonly observed between epithelial cells. The submucosal glands showed a variable degree of hyperplasia and moderate number of mononuclear and neutrophil cells had infiltrated into their lumina (Fig. 3.22).

Fully mature goblet cells were less numerous than normal and most of these contained only a few mucous granules which were mainly neutral glycoproteins. A few intra-epithelial cysts containing mucus were observed.

3.2.1.2 MAIN BRONCHI

Mild to moderate epithelial and submucosal hyperplastic changes were commonly seen and a large number of neutrophils were found between the epithelial cells. Lymphocytes, macrophages and neutrophils were invariably present in large numbers in the lamina propria. The bronchial lumen usually contained a small amount of exudate consisting of cellular debris, large numbers of neutrophils and a few mononuclear cells. Lymphoid cells aggregates were particularly common around the submucosal glands, and frequently extended into the muscularis mucosae and the lamina propria as far as the epithelial basement membrane (Fig. 3.23).

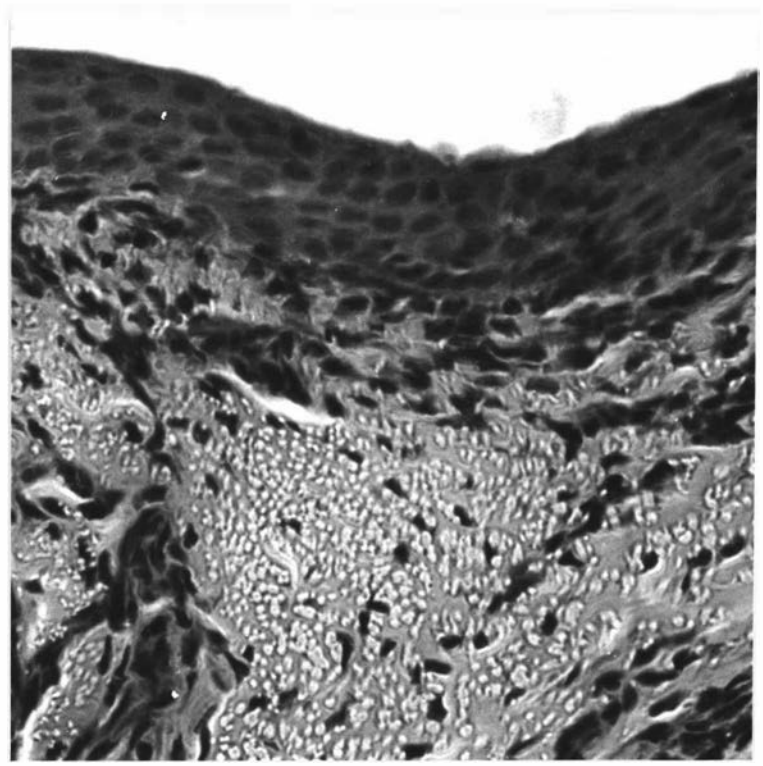
With AB-PAS stain, most goblet cells were either empty or contained small numbers of neutral glycoprotein mucous granules. A

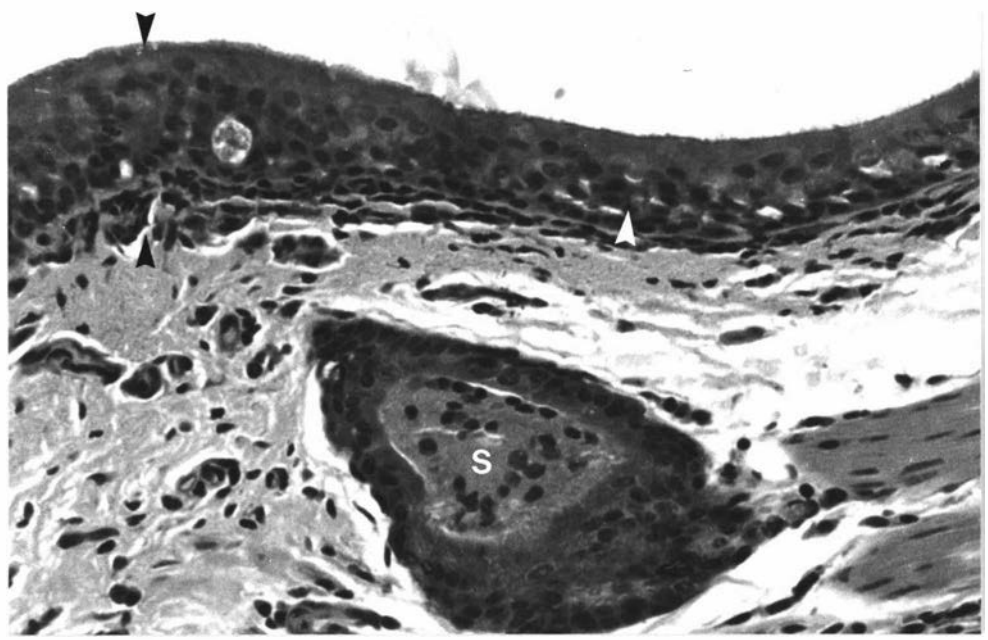
Figure 3.22

Tracheal epithelial surface of an advanced pneumonic lesion showing severe squamous metaplasia. H&E. X 312.

Figure 3.23

The bronchial epithelial surface of an advanced pneumonic lesion showing moderate hyperplasia (large arrows) and metaplasia (white arrows). Large numbers of neutrophils are present between the epithelial cells (small arrows). The lumen of the submucosal gland (S), is full of mucus mixed with large numbers of neutrophils and macrophages. H&E. X 156.




S

few goblet cells contained acid glycoproteins. Intra-epithelial cysts were occasionally observed.

3.2.1.3 SMALL BRONCHI

Moderate to severe hyperplasia of epithelial mucosa and submucosal glands was present which resulted in the development of epithelial polyp-like structures in some areas. The lymphoid cells aggregations in the submucosa were more extensive than those seen in the early pneumonic lesions. The lumina of bronchi and submucosal gland acini contained a moderate amount of cellular debris, neutrophils and mononuclear cells (Fig. 3.24). The AB-PAS sections of these airways were similar to that observed in the main bronchi.

3.2.1.4 BRONCHIOLI

This level showed the most marked hyperplasia of epithelium, peribronchiolar and perivascular lymphoid tissue. The lumen of the airways usually contained a large amount of mucus, mixed with cellular debri and inflammatory cells. Large numbers of neutrophils and mononuclear cells were commonly observed within the epithelium. An increase in numbers of goblet and ciliated cells and decrease in Clara cells were observed in the epithelium of primary bronchioli, which also showed various degrees of metaplasia (Fig. 3.25).

Goblet cells containing neutral glycoprotein were completely absent from the epithelium and only those containing acid glycoprotein remained. Epithelial secretory cells similar to goblet cells which contained acid glycoprotein were present in the epithelium of secondary and terminal bronchioli. The mucus present in the lumen or the epithelial surface was of the acid type.

3.2.2 TOPOGRAPHICAL MORPHOLOGY

3.2.2.1 TRACHEA

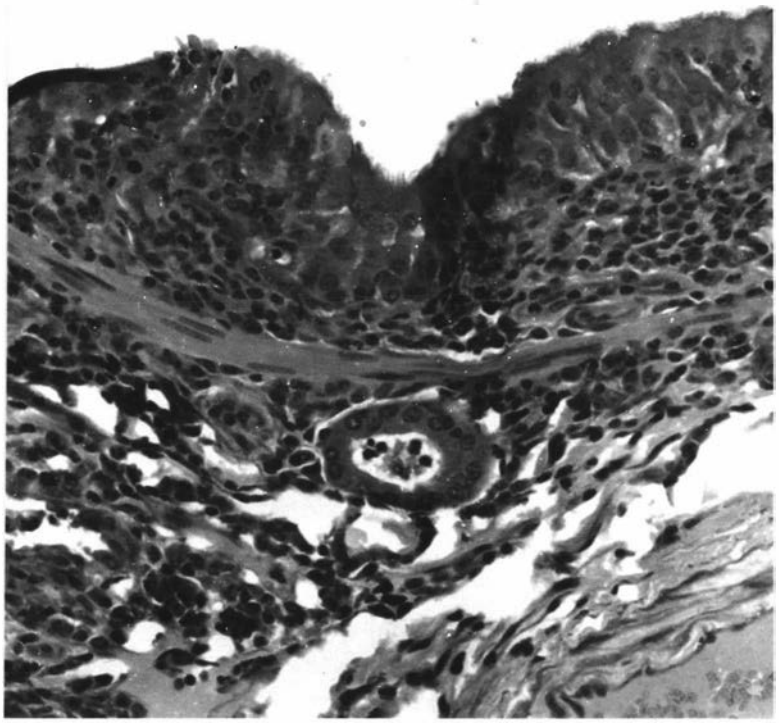
The loss of cilia was less severe than that of the early pneumonic lesions, and occasionally, cellular debris and inflammatory cells were observed on the luminal surface. The epithelium was

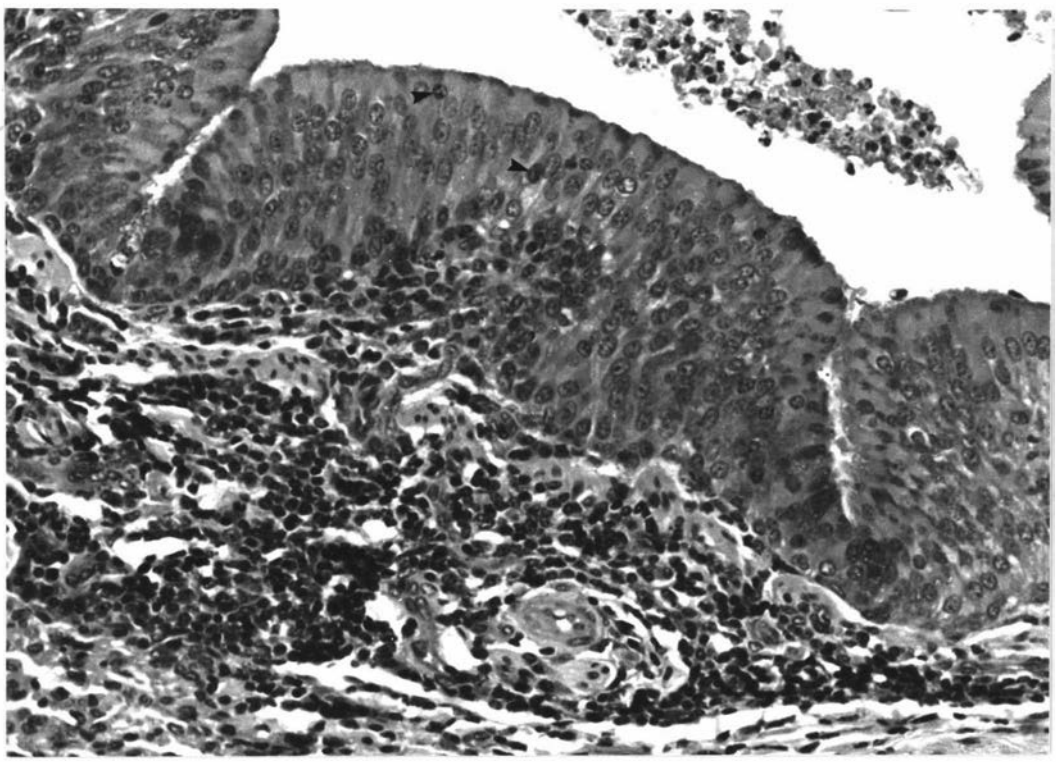
Figure 3.24

The epithelium of a small bronchus from an advanced pneumonic lesion showing moderate hyperplasia. The lymphoid aggregations in the submucosa are more extensive than those seen in early lesions. The lumen of the submucosal gland contains small numbers of neutrophils and macrophages. H&E. X 312.

Figure 3.25

A bronchiole from an advanced pneumonic lesion showing marked epithelial hyperplasia and peribronchiolar lymphoid aggregations. Large numbers of neutrophils and mononuclear cells are found within the epithelium (arrows). The lumen contains mucus, neutrophils and macrophages. H&E. X 156.





composed of ciliated and non-ciliated cells; and although ciliated cells predominated over the majority of the surface, among them were interspersed single non-ciliated cells (Fig. 3.26). Comparatively large areas of cells without cilia were observed. The mucosal surface of the non-ciliated cells was covered by short microvilli and did not protrude into the lumen (Fig. 3.27). The remaining cilia were shorter than those of normal sheep and exhibited variable degrees of ciliogenesis, similar to that seen in early pneumonic lesions. Most of the cilia were tufted and showed plasma membrane alterations, including budding and sequestration. A large number of organisms were entangled between the cilia (Fig. 3.27). These organisms were similar to the morphology of M. ovipneumoniae described by Al-Kaissi and Alley (1983).

3.2.2.2 BRONCHI

As in the trachea, the loss of cilia was less severe than that observed in the early pneumonic lesions but a large number of neutrophils and mononuclear cells were found on the surface (Fig. 3.28). Ciliated cells predominated over the other types of cells. Most of the non-ciliated cells were goblet cells (Fig. 3.28).

3.2.2.3 BRONCHIOLI

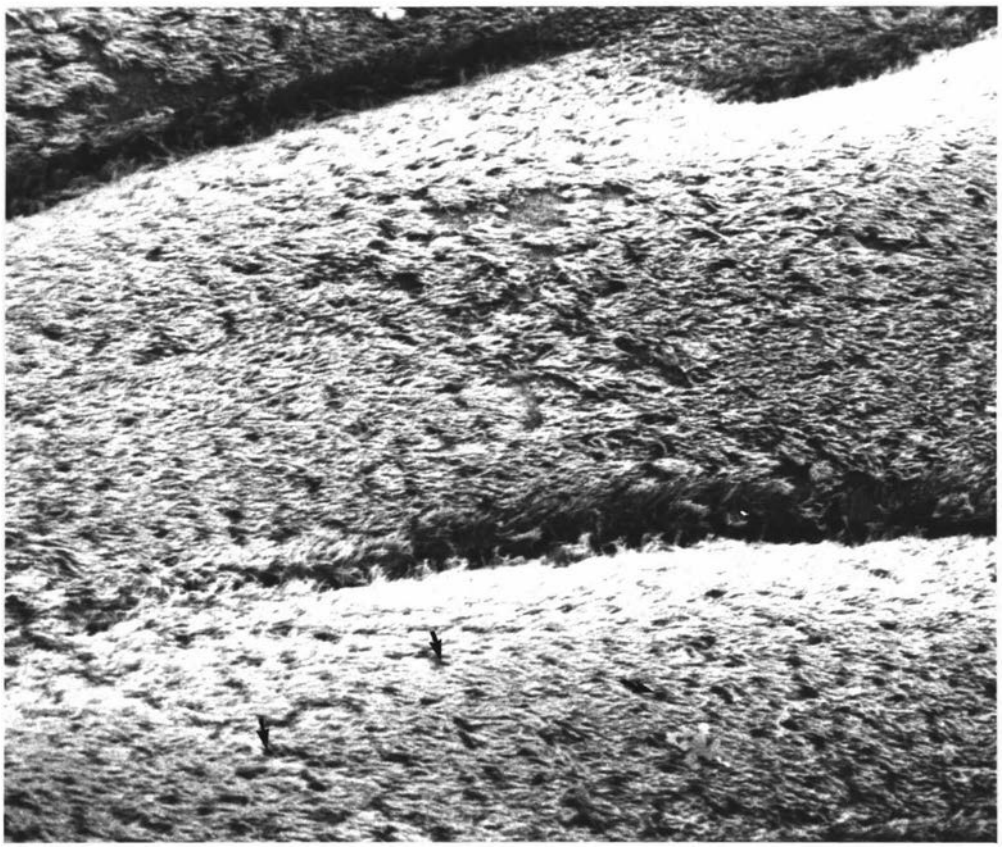
Two types of bronchiole, differing primarily in their luminal surface structures were observed. One type had non-ciliated cells with a large number of microvillus-like projections, Clara cells and a small of number of ciliated cells. In these the majority of cells had pits on their plasma membrane, which varied in size and shape(Fig. 3.29). The epithelial surface of the other type of bronchiole consisted of Clara cells, cells with microvillus projections on the surface and a cell type which appeared to be intermediate between these two. The intermediate cell type protruded from the surface in a similar manner to Clara cells and had a large number of microvillus projections on its luminal surface (Fig. 3.30). In contrast to the first type, this bronchiole had only occasional pits and these were seen only on the surface of Clara cells. Some Clara cells exhibited surface features similar to type II alveolar epithelial cells (Fig. 3.30).

Figure 3.26

Tracheal luminal surface from an advanced pneumonic lesion showing single non-ciliated cells (arrows) together with irregular areas of cilia loss. SEM. X 600.

Figure 3.27

High magnification of the ciliated surface showing a large number of mixed microorganisms (arrows) between the cilia. Many of the cilia are entangled and shortened. SEM. X 1,300.



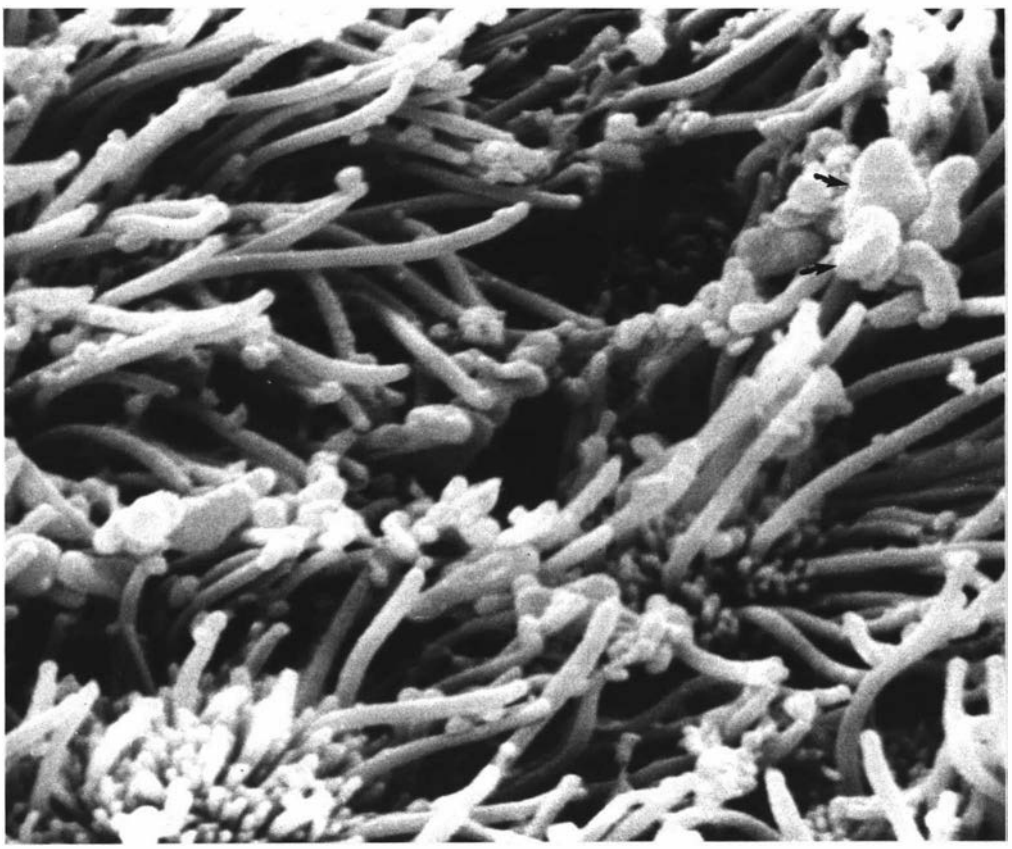


Figure 3.28

The bronchial luminal surface from an advanced lesion. Ciliated cells predominate over other types which are mostly goblet cells (arrows). A large number of neutrophils and macrophages were observed on the epithelial surface. SEM. X 680.

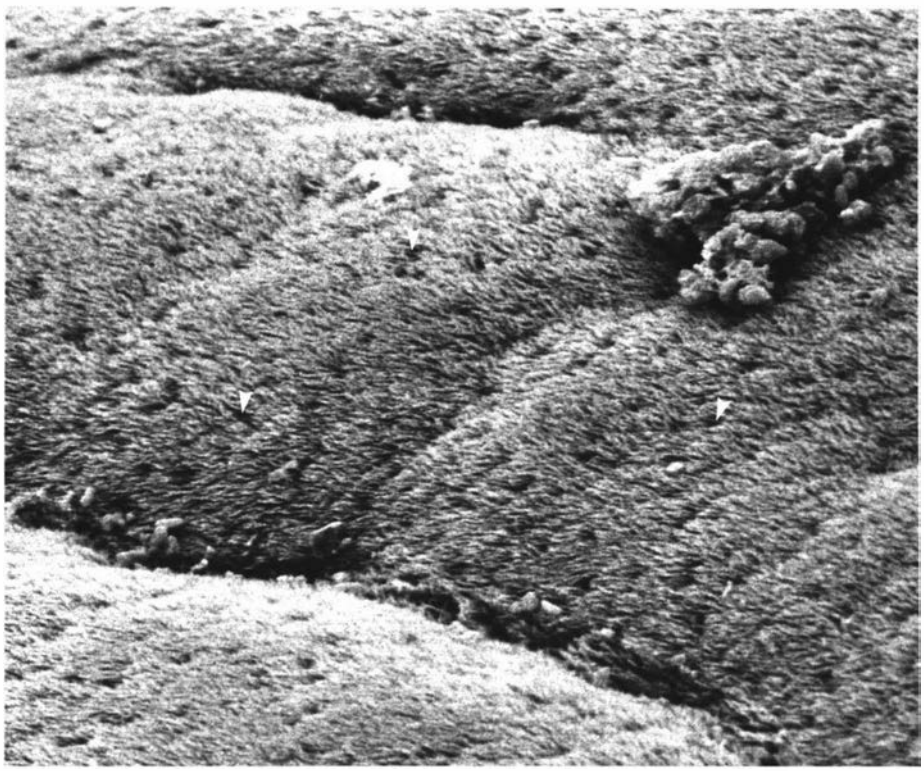
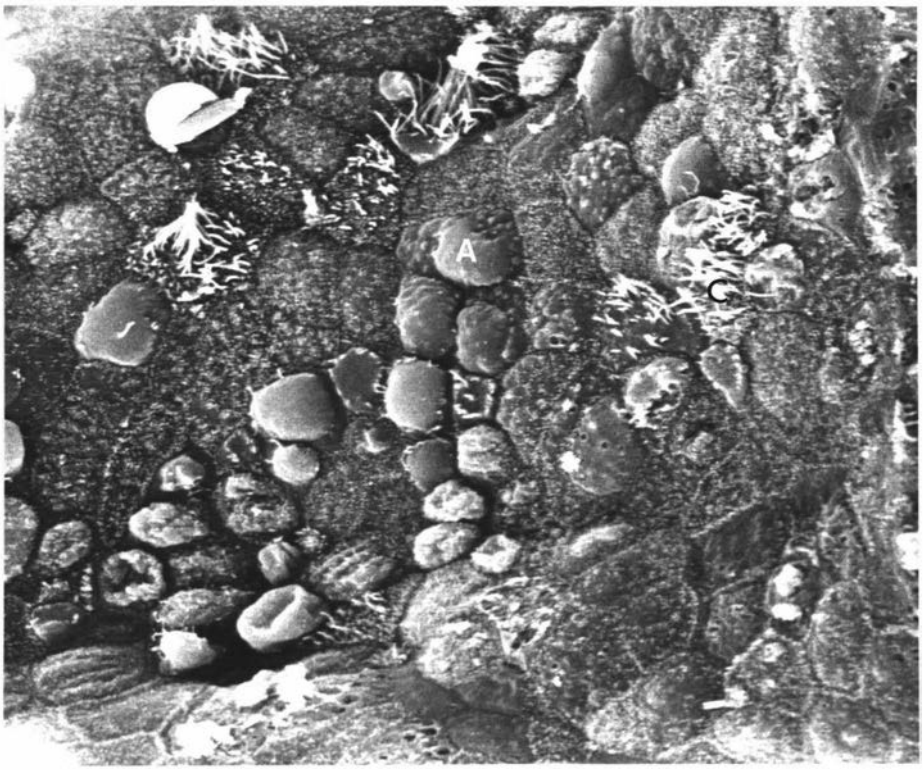


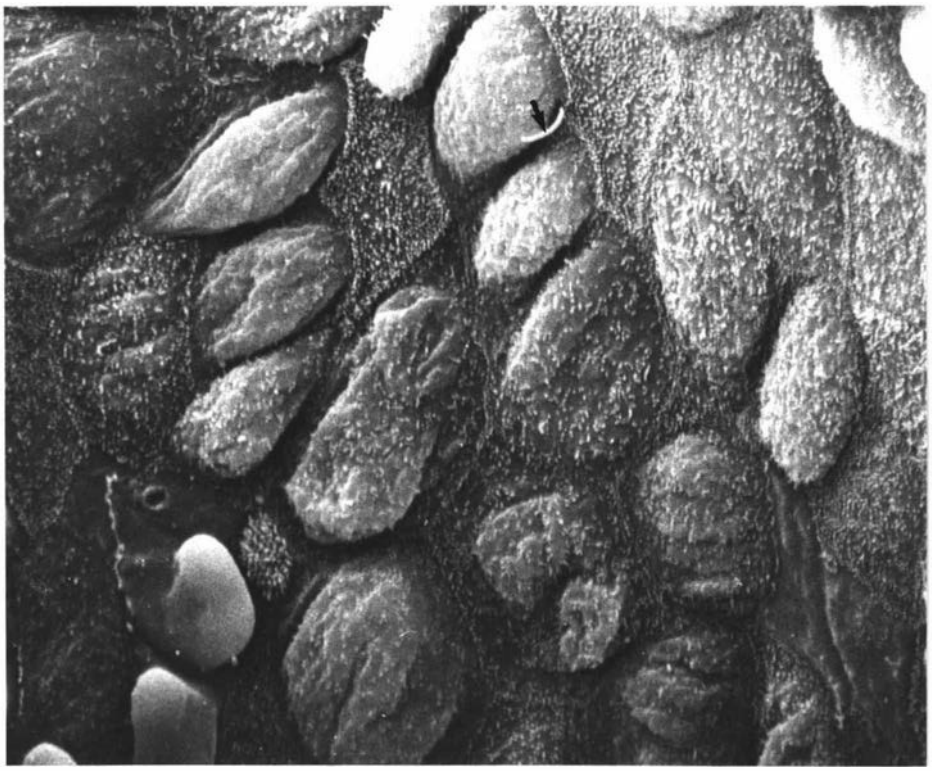
Figure 3.29

A bronchiole covered by cells with a large number of microvillus-like projections. Clara cells (A) and a small number of ciliated cells (C) were also common. SEM. X 1980.

Figure 3.30

Another type of bronchiole containing no ciliated cells. An occasional single cilium can be observed (arrow) attached to a Clara-like cell. SEM. X 2,860.


A
C



The ciliated cells were either devoid of most of their cilia or the cilia present were tufted together. Some ciliated cells occasionally had microvillus-like projections together with cilia and very smooth areas on their luminal surface (Fig. 3.29). Other ciliated cells had a smooth luminal surface with few cilia in the middle. Further types had similarly placed cilia with microvillus-like projections on the boundaries of the cell surface. A few cells had a blebbing of the cell membrane with microvilli scattered between these blebs (Fig. 3.29).

Bronchioli from severely affected parts of the lung showed a markedly thickened mucosal wall and their lumina were occluded by large numbers of neutrophils,macrophages, mucus and necrotic cellular debris (Fig. 3.31 & 3.32).

3.2.2.4 ALVEOLI

The most striking features of the pneumonic alveoli were a marked increase in thickness of the alveolar septa and the presence of large numbers of cells lying within the alveolar spaces (Fig. 3.33). The cells remaining adherent to the alveolar walls were mainly neutrophils, although a variable number of macrophages and type II alveolar epithelial cells could also be seen. The macrophages had the usual morphology of pulmonary macrophages with long cytoplasmic extensions and invaginations of the plasma membrane. Increased numbers of type II alveolar cells were seen in some alveoli and occasionally they almost covered the entire surface (Fig. 3.33). The surface of these cells protruded into the alveolar space and was covered by numerous microvilli (Fig. 3.34). Openings or pits on the surface of type II alveolar cells were only seen on the surface of protruded bubbles. The alveolar type I cells exhibited morphological changes characterised by small pits and ruffling of the cell membrane (Fig. 3.34).

3.2.3 ULTRASTRUCTURE

3.2.3.1 TRACHEA

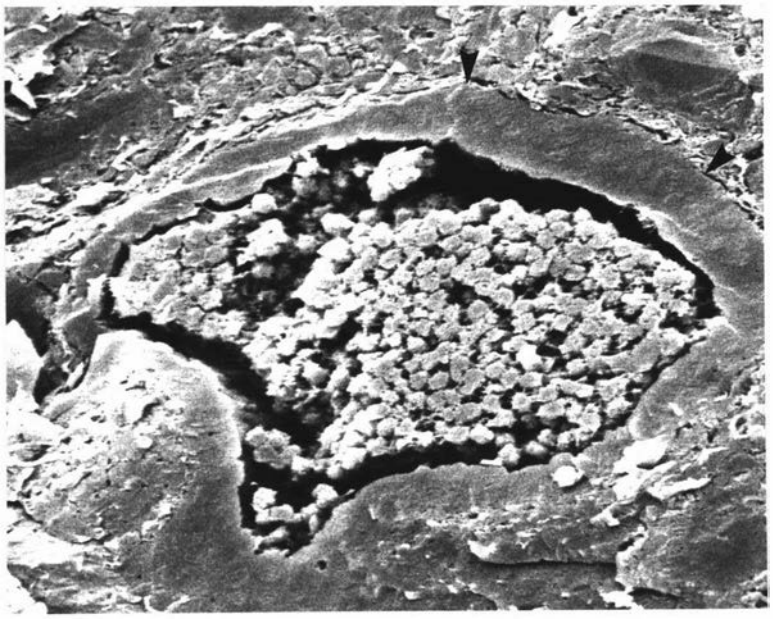
Large areas of the epithelium exhibited a variable degree of

Figure 3.31

A bronchiole from a severely affected part of the lung showing a markedly thickened wall (arrow) and lumen occluded by neutrophils and macrophages. SEM. X 500.

Figure 3.32

High magnification of the luminal contents of the above showing numerous neutrophils and macrophages together with mucous granules and strands. SEM. X 2,400.



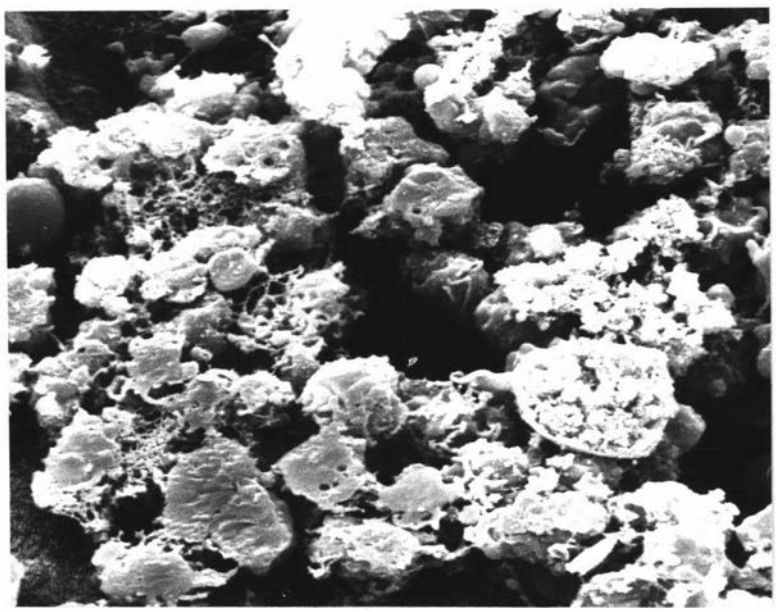
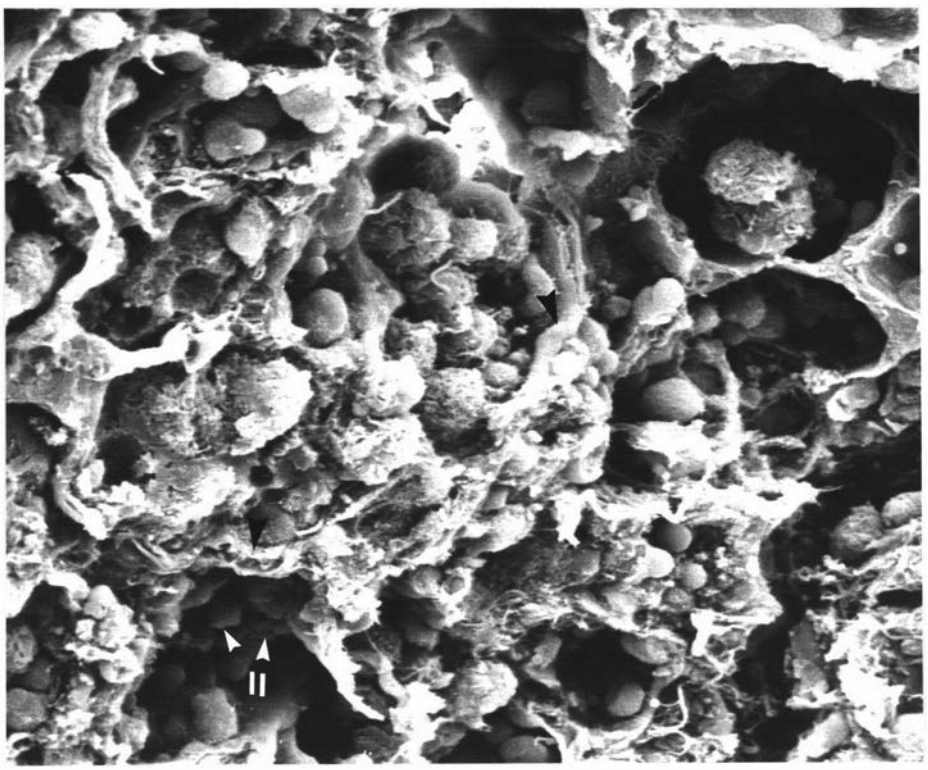


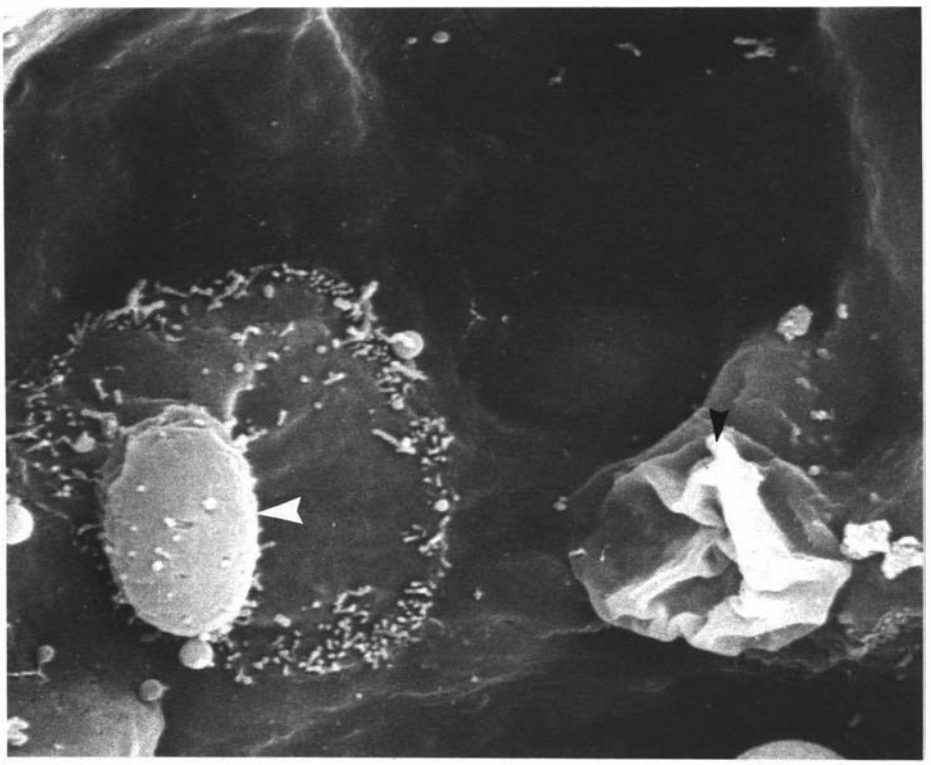
Figure 3.33

Alveoli from a severely affected part of lung showing marked thickening of the alveolar septa (arrows). Alveolar spaces contain both neutrophils and macrophages. Increased numbers of type II epithelial cells can be seen in some alveoli (II). SEM. X 1000.

Figure 3.34

High magnification of two, type II alveolar epithelial cells. The one on the left shows a secretory bubble or cytoplasmic bleb (arrow) protruding from its surface and the one on the right shows an evacuated bubble (arrow). SEM. X 5,800.





metaplastic change. In these regions epithelial cells were either low and irregular columnar (Fig. 3.35) or squamoid (Fig. 3.36). The luminal surface of the former bore a small number of cilia and a large number of microvilli (Fig. 3.35). The apical part of the cytoplasm of these cells contained moderate to large numbers of basal bodies. The nucleus was small and irregular in shape and the cytoplasm contained a variable number of small glycogen particles and large vacuoles. The mitochondria were either small, electron-dense and of ill-defined structure (Fig. 3.35) or clear with disrupted cristae (Fig. 3.37). The luminal surface of the squamoid cells bore only microvillus-like projections and the cell cytoplasm contained no basal bodies (Fig. 3.36). The nucleus was either rounded or flattened and the cytoplasm contained few mitochondria which were small and contained clear cristae (Fig. 3.36).

A lack of goblet cells was one of the main features of the tracheal epithelium of advanced pneumonic lesions. Small cytoplasmic projections protruded from all the remaining epithelial cells and these contained a variable number of small vacuoles (Fig. 3.36). A large number of neutrophils and a moderate number of mononuclear cells were seen between epithelial cells (Fig. 3.35 & 3.37). No bacteria were observed in direct contact with cilia or the plasma membrane of epithelial cells.

3.2.3.2 BRONCHI

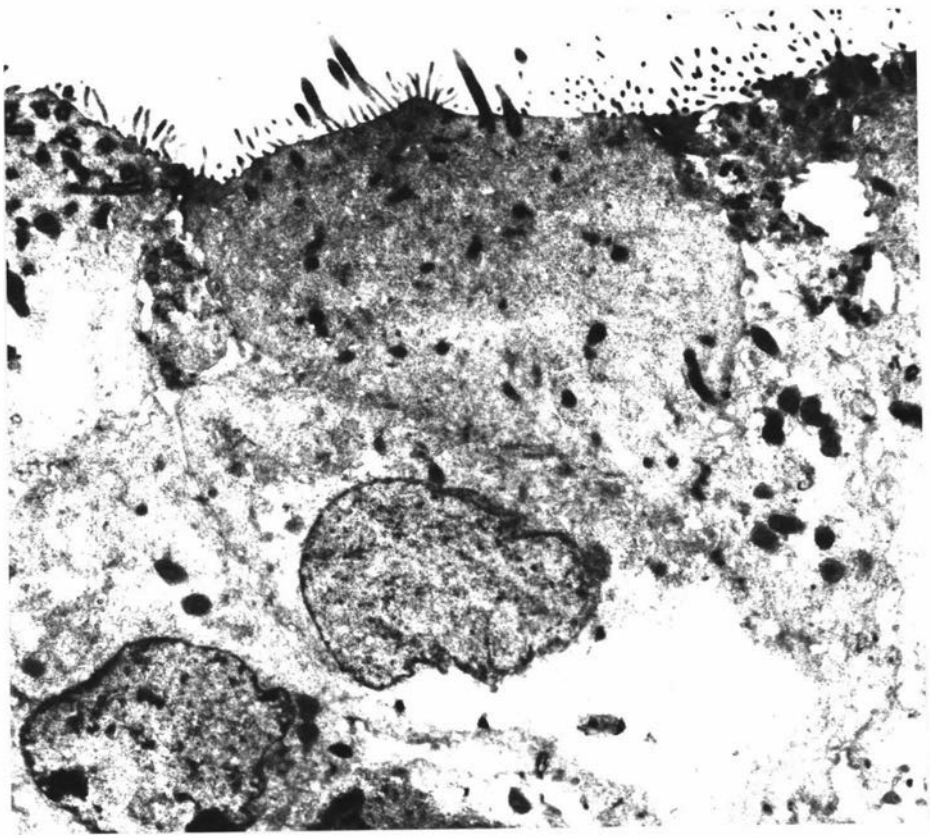
Many areas of the bronchial epithelium were hyperplastic and composed of low columnar cells. In these regions the epithelial cells were flattened or compressed and contained large and elongated nuclei and relatively little cytoplasm (Fig. 3.38). The luminal surface showed reduction in numbers of cilia and epithelial cells showed mild cytoplasmic vacuolation but no distortion or swelling of subcellular organelles was observed. A few bacteria were seen in close association with cilia but none were seen intracellularly or in close contact with the plasma membrane. Empty goblet cells were frequently encountered on the epithelial surface. Occasionally, intra-epithelial cysts containing large numbers of mucous granules or globules, mitochondria were observed. Large numbers of mononuclear phagocytes and neutrophils were found between the epithelial cells (Fig. 3.38).

Figure 3.35

Ultrastructure of the tracheal epithelium from an advanced pneumonic lesion showing variable degrees of metaplastic change. Most of cells in this region were irregular columnar in shape. TEM. X 6,930.

Figure 3.36

Another area of tracheal epithelium from an advanced lesion showing severe metaplastic change. Most of cells in this region are squamoid in shape. TEM. X 8,150.



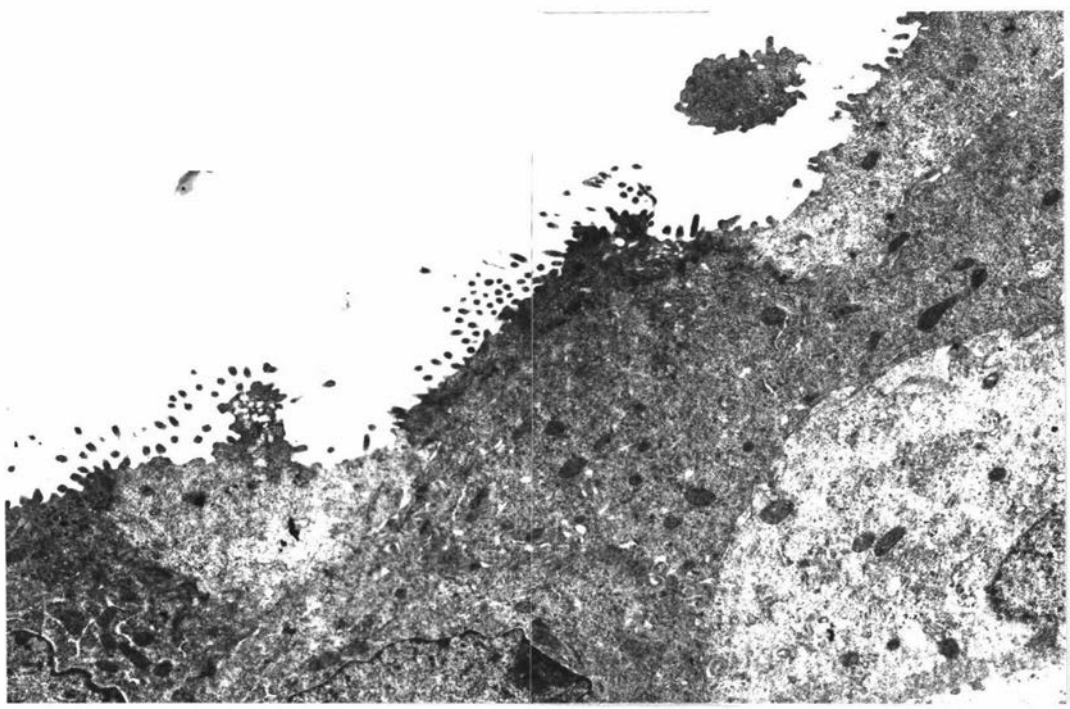


Figure 3.37

The cytoplasmic contents of damaged tracheal epithelial cells. The mitochondria were either small, electron-dense or clear with disrupted cristae. Observe the neutrophil between the cells (arrows). TEM. X 10,400.

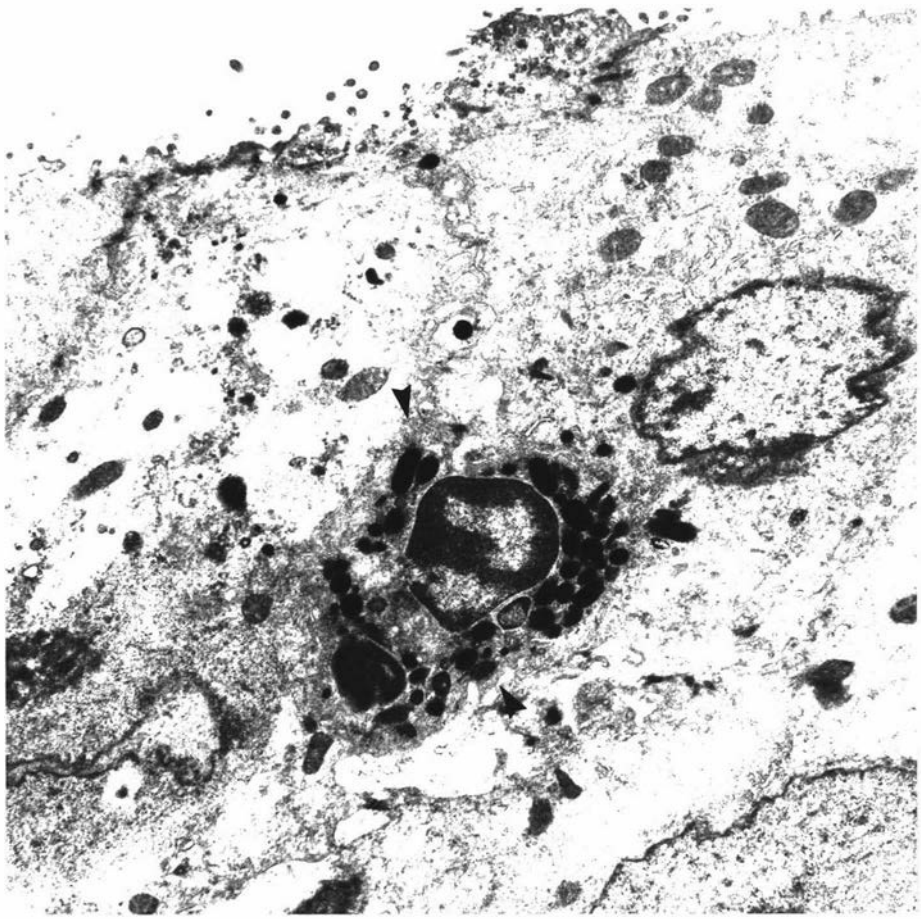
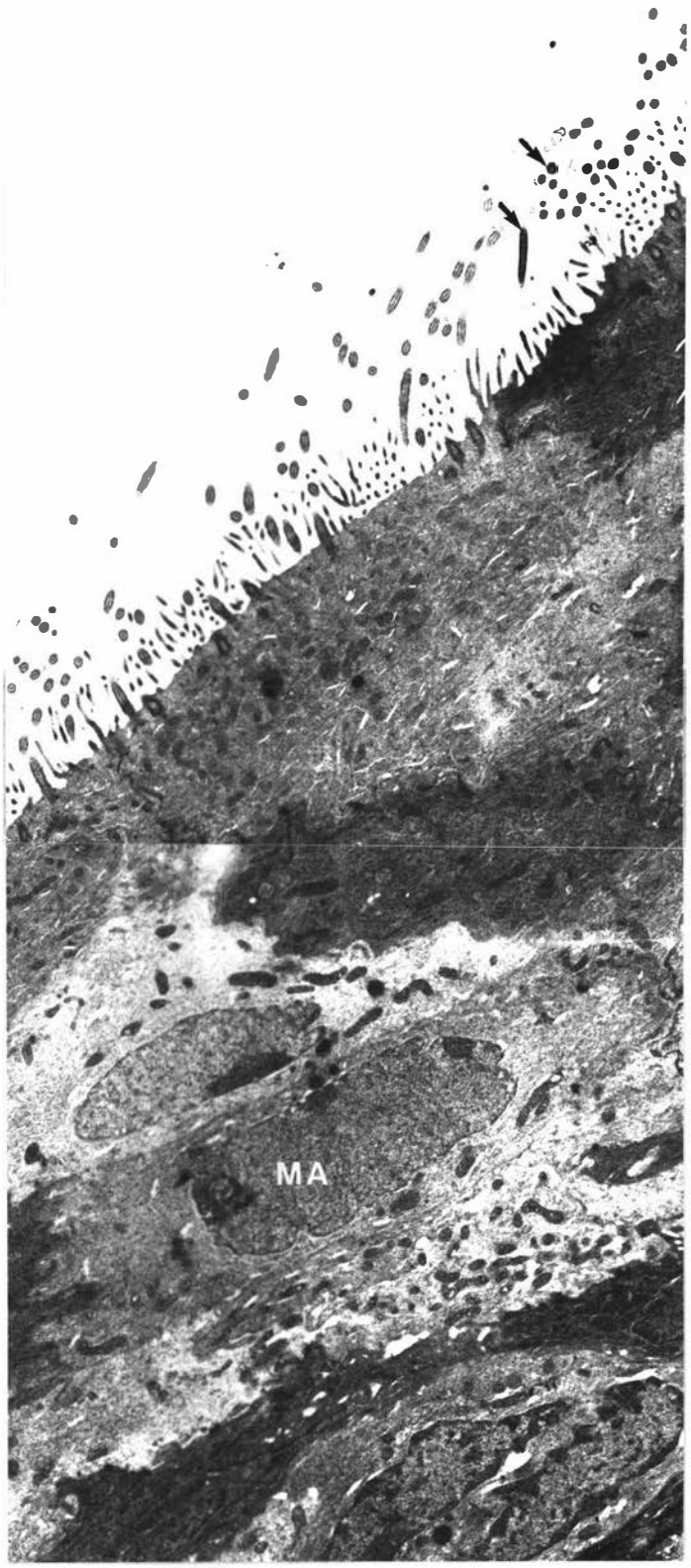


Figure 3.38

The bronchial epithelium from an advanced pneumonic lesion showing flattened, compressed epithelial cells. Goblet cells are empty. A few bacteria (arrows) are closely associated with cilia. A macrophage (MA) can be seen between the cells. TEM. X 6,880.


MA

3.2.3.3 BRONCHIOLI

The epithelium of affected bronchioli was invariably hyperplastic and consisted of more than one layer of cells. The first layer had differentiated into typical columnar, ciliated and non-ciliated cells which were similar to the types seen in normal bronchi (Fig. 3.39). The cytoplasm of these cells was densely vacuolated and contained secretory granules some of which could be seen in the process of being released from the luminal surface. The majority of ciliated cells had lost most of their cilia and the apical plasma membrane of some of both ciliated and non-ciliated cells had ruptured, releasing cell contents into the lumen (Fig. 3.39). Another frequently observed change was the protrusion of large cytoplasmic projections from the luminal surface of ciliated cells (Fig. 3.40). These projections occupied large areas of the cell surface and were commonly larger than the cilia. The cytoplasm of these protrusions was more electron-dense than that of the cell and contained only ribosomes but no endoplasmic reticulum or other organelles. The remainder of the surface of the affected cells had few or no cilia but the cytoplasm contained basal bodies (Fig. 3.40).

The lumen of most affected bronchioli was filled with amorphous material, cellular debri, neutrophils and a variety of mononuclear phagocytes (Fig. 3.41). Few bacteria or mycoplasmas were observed in the amorphous material (Fig. 3.39) although structures resembling mycoplasmas were seen entangled in some cilia (Fig. 3.42). No bacteria or mycoplasmas were seen inside neutrophils or mononuclear phagocytes.

4.0 DISCUSSION

Since this investigation was probably the first systematic study of the surface epithelium of conducting pneumonic airways of a large mammalian species it was difficult to compare the present findings with other similar studies in other species.

One of the main histological features seen in both early and advanced pneumonic lesions was inflammatory cell infiltration of the epithelium.Neutrophils and macrophages were commonly seen between the

Figure 3.39

The epithelium from a severely affected bronchiole showing differentiation of the first layer into typical columnar epithelial cells. The majority of cells have a vacuolated cytoplasm and some ciliated cells have lost their cilia. The apical plasma membrane of some cells has ruptured (arrows), releasing cell contents into the lumen. Mycoplasma-like organisms (M) can be observed in close association with ciliated cells. TEM. X 138,100.

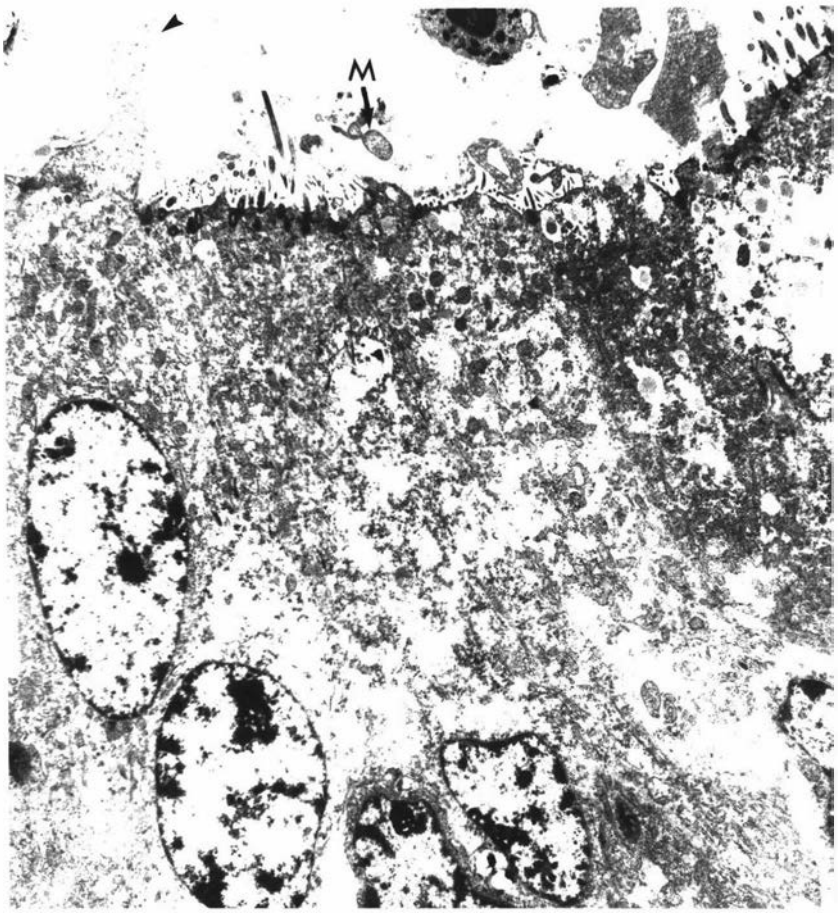
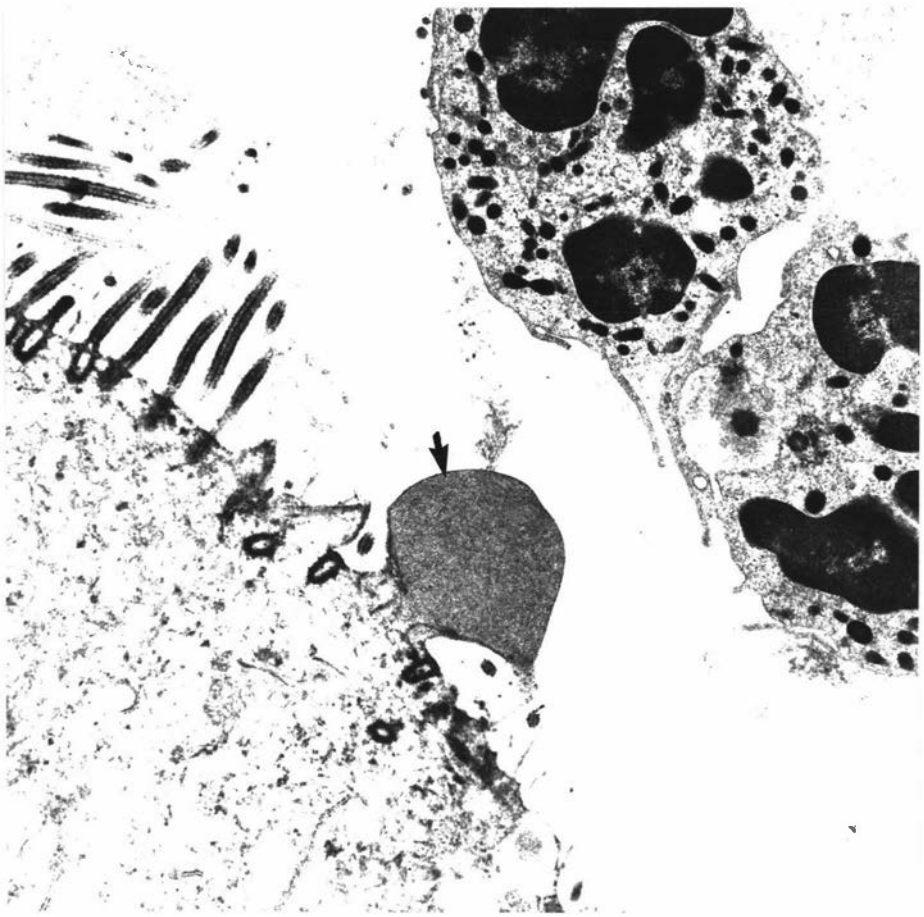

M

Figure 3.40

Bronchiolar epithelium from severely affected lung showing a cytoplasmic projection (arrow) protruding from the luminal surface of a ciliated cell. TEM. X 10,670.

Figure 3.41

The lumen contents of a severely affected bronchiole. It consists of amorphous material, cellular debris, neutrophils. TEM. 4,700.



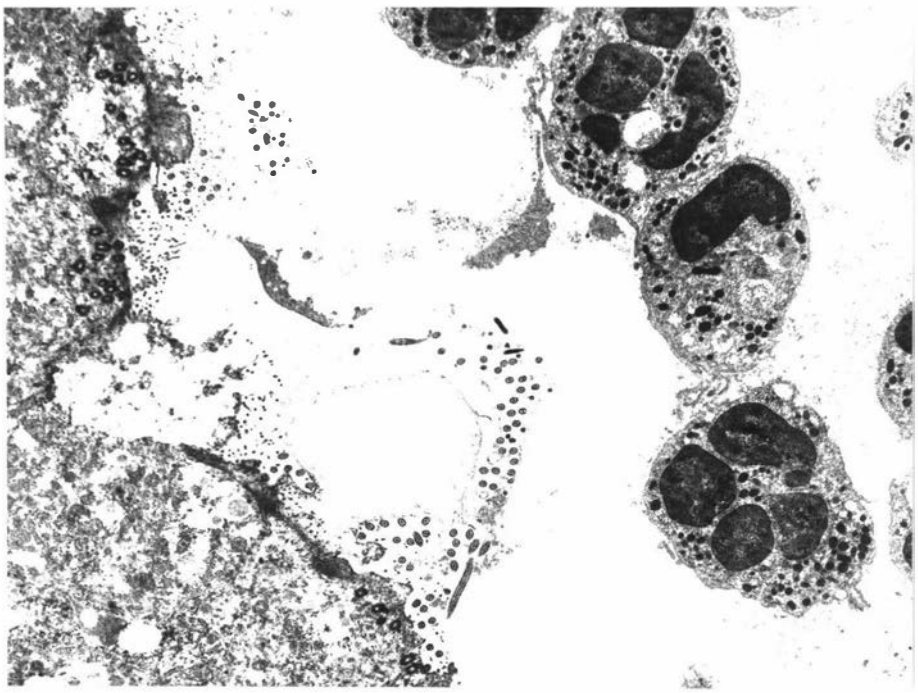
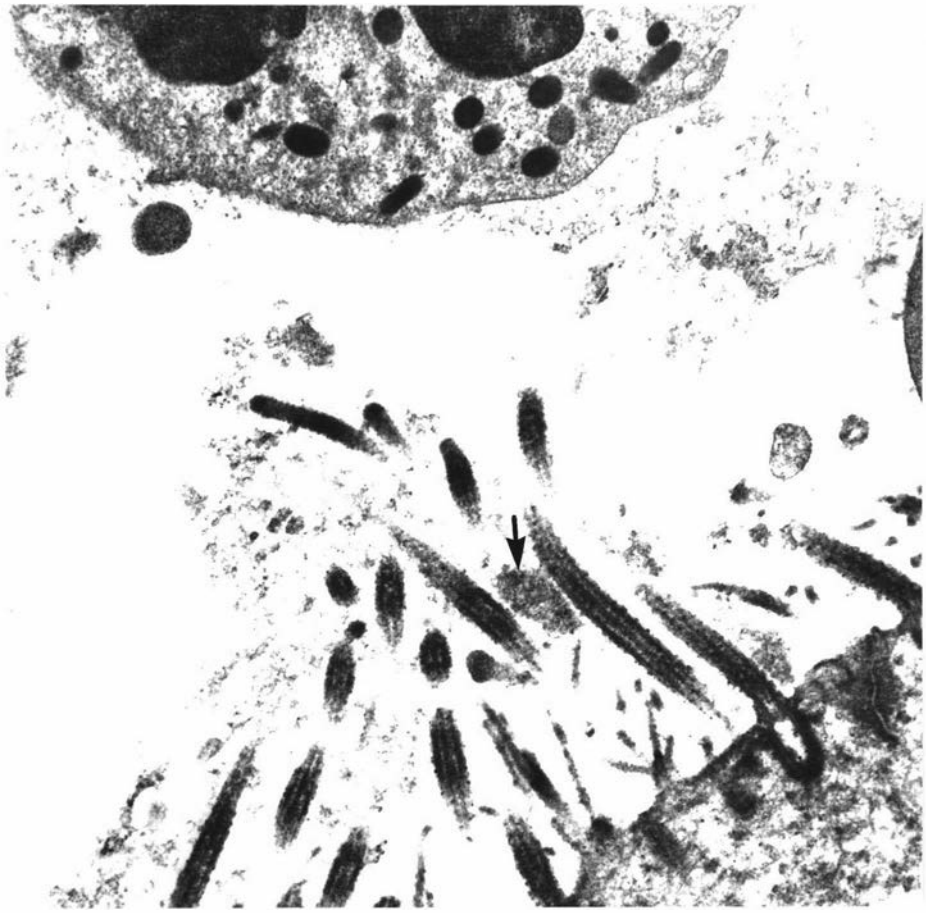


Figure 3.42

Microorganisms resembling mycoplasmas (arrows) closely associated with the cilia of affected bronchiolar epithelial cells. TEM. X 38,160.



epithelial cells in large numbers indicating that active inflammatory changes is occurring in the tracheobronchial epithelium at all stages of the disease.

The tracheobronchial epithelium in advanced pneumonic lesions showed large areas of squamous metaplasia with mild to moderate hyperplasia particularly in the extrapulmonary airways, while in early pneumonic lesions there were focal areas of metaplasia and moderate to severe hyperplastic changes in intrapulmonary airways. There were two other major differences between these two stages of pneumonia. Firstly, the aggregations of lymphoid cells were more extensive in advanced pneumonic lesions and secondly the bronchial submucosal glands of advanced pneumonic lesions showed more hyperplasia and metaplasia than those of the early pneumonic group. Some of these proliferative changes will be reported on in more detail in Chapter 4. In general however, they tend to confirm that the early pneumonic lesions do, in fact, progress to advanced pneumonic lesions and are unlikely to be separate disease entities

Gilmour and Brotherston (1963) who studied an outbreak of CNP (atypical pneumonia) in young sheep in Scotland were unable to correlate the severity of clinical signs they observed with the macroscopic lesions in affected lungs examined some 6 months later. Stamp and Nisbet (1963) described the histopathological changes in atypical pneumonia and they classified these changes into interstitial pneumonia and lymphoid hyperplasia, either or both of which could be present in any animal. In New Zealand Alley (1975b) examined 246 lungs with lesions of naturally-occurring CNP and divided the changes he observed into four categories (I to IV) on the basis of the gross and histological severity of the lesions. The most prominent histological feature Alley observed in category 1 was alveolar collapse; and in category 2, severe infiltration of neutrophils and proliferative changes including early bronchiolar hyperplasia and peribronchiolar lymphoid hyperplasia. In category 3, proliferative changes predominated over exudative changes. In category 4, chronic proliferative changes predominated microscopically. More recently Gilmour; et al. (1982) studied the long-term pathological progress in experimentally produced (using endotracheal tube) CNP (proliferative-exudative pneumonia). These authors presented evidence that their experimental pneumonia started to wane within 28 weeks

postinfection and that a neutrophilic exudate was not seen within the alveoli after 11 weeks pi. Bronchial epithelial hyperplasia was only mild at 24 and 28 weeks pi but there was severe nodular lymphoid hyperplasia at 15, 18 and 24 weeks pi. The lesions observed in the present study support the conclusion that CNP is likely to persist over a period of several months starting from lesions of early red consolidation, and progressing through regression phases where lymphoid hyperplasia is the principle feature (Alley, 1975b), to a recovery stage at which the alveoli are expanded and largely free of exudate (Gilmour; et al. 1982).

There were at least two major differences between the tracheobronchial epithelium of early and advanced pneumonic lesions observed by SEM. Firstly, loss of cilia was more severe at all levels in early pneumonic lesions than in advanced where at least half of the epithelial surface remained ciliated. Due to the high density of cilia, goblet cell openings were not commonly observed in normal tracheobronchial epithelium. In both early and advanced pneumonic lesions these openings were commonly seen as large pits on the epithelial surface perhaps because of the loss of cilia. Secondly, the metaplastic changes were more pronounced in the advanced pneumonic epithelium than in the early pneumonic lesions. However, once again these changes merely reflect the chronic proliferative nature of the disease since metaplastic changes occur more commonly with chronic irritation (Jones;et al. 1973).

Mycoplasma-like structures were found attached firmly to the cilia of epithelial cells of the trachea and bronchi in early pneumonic lesions and to tracheal epithelial cilia in the advanced lesions. Other studies have shown that M. hyorhinis, a cause of enzootic pneumonia in pigs, colonises airways but not alveoli, the inference being that the alveolar region is unfavourable for the growth of these organisms (Gois; et al. 1971; Baskerville, 1972). The finding of large numbers of mycoplasmas in the trachea and bronchi of early pneumonic lesions gives weight to the hypothesis that under certain conditions M. ovipneumoniae may be responsible for a low grade tracheitis, bronchitis and bronchiolitis which, in turn, may result in sufficient disturbance to clearance mechanisms to allow the invasion of the lower respiratory tract by destructive bacteria which are normally resident in the upper respiratory tract (Alley & Clarke,

1979).

The absence of mycoplasmas from the bronchi in advanced pneumonic lesions may perhaps be due to changes in the microclimate of the lower respiratory tract making it unfavourable for mycoplasma growth. In addition, antimycoplasma antibody activity in the bronchial secretions which includes IgA, IgG and IgM may reach effective concentrations at later stages of the infection. Holmgren (1974) detected indirect haemagglutinating antibodies in bronchial secretions of disease-free pigs inoculated intranasally with Mycoplasma hyopneumoniae within 2 weeks of inoculation. These peaked between 8 and 9 weeks, and persisted for over 13 weeks.

The presence of mycoplasmas within neutrophils in only the early pneumonic lesions could perhaps be nonspecific phagocytosis. In general it appeared that mycoplasmas resist degradation and digestion by phagocytes in the absence of specific antiserum (Simberkoff & Elsbach, 1971; Al-Kaissi & Alley, 1983). Simberkoff & Elsbach (1971) observed that E. coli ingestion and killing by neutrophils were reduced in the presence of Mycoplasma hominis and Mycoplasma arthritidis organisms or supernatant fluid from a mycoplasma culture. If this is an analogue for what happens in vivo then the secondary bacterial infection in CNP may be an important contributing factor to the severity and duration of pneumonic lesions as had been suggested by Alley (1975b).

In the bronchioli of both early and advanced pneumonic lesions, one of the most striking features observed by SEM was the surface morphological changes to Clara cells. These changes have been reported previously with Clara cells of rats where they were attributed to cell injury (Kuhn; et al. 1974) and/or fixation artifact (Jeffery and Reid, 1975).

Three distinct morphological features distinguished the alveoli of advanced pneumonic lesions from those of early lesions. Firstly, there were many more mononuclear phagocytes in the alveolar spaces. Secondly, the alveolar septa was considerably thicker. In experimental enzootic pneumonia of pigs, Baskerville and Wright (1973) also observed ultrastructurally that the majority of interalveolar septa were greatly thickened by accumulation of lymphocytes,

macrophages and plasma cells. Thirdly, there was an increase in the number and activity of type II epithelial cells. Increased numbers of type II epithelial cells in the lungs of sheep with advanced pneumonic lesions have been observed by other workers (Alley, 1975a). Type II epithelial cells are difficult to distinguish from macrophages under the light microscope, but SEM provided an excellent means of visualising this change.

The ultrastructural changes in the trachea and bronchi of early pneumonic lesions were markedly different from those observed in advanced lesions. The most obvious change was the degree of epithelial metaplasia and the relative paucity of bacteria seen in advanced lesions when compared to early lesions. The epithelium of the trachea and bronchi of early pneumonic lesions showed acute changes such as broken cilia and the presence of large amounts of mucus on the luminal surface. A striking feature observed in early lesions was the large number of inflammatory cells observed migrating between epithelial cells and the presence of large numbers of mycoplasmas and bacteria on the ciliary carpet. The mycoplasma organisms were attached firmly to the cilia in early and less commonly in advanced lesions by means of tubular structures which pentrated the plasma membrane of the cilia. They were found in greater numbers in the bronchi than in the trachea.

The present study suggests that the attachment of mycoplasma organisms to the tracheal and bronchial cilia may reduce the ciliary activity. Since this is the most effective mechanism of clearance in the tracheobronchial airways (Green; *et al.* 1976), other more destructive organisms such as *P. haemolytica* which have no ability to attach to cilia may thus have an opportunity to penetrate into the pulmonary parenchyma and produce more severe lesions. This is supported by the experiments of Alley and Clarke (1980) who suggested that bacteria are an essential factor in the pathogenesis of moderate and severe forms of CNP. An alternative hypothesis is that the mycoplasmas may produce a primary bronchitis during attachment and the other bacteria present were merely secondary invaders. This hypothesis is backed by the work of Jones; *et al.* (1978) and Gilmour; *et al.* (1979). These authors produced lesions resembling those of naturally-occurring CNP in over most lambs (over 90%) inoculated with *M. ovipneumoniae* alone.

All goblet cells in the bronchioli of both stages of chronic pneumonia contained acid glycoprotein. However, in normal tracheobronchial airways, goblet cells extend distally as far as primary bronchioli, while in advanced pneumonic lesions the goblet cells or cells containing acid glycoprotein extended down to the terminal bronchioli. Baskerville (1970) and Baskerville & Wright (1973) observed similar changes which involved the extension of goblet cells to the bronchioli of pigs with experimentally induced enzootic pneumonia. In the present study, there were fewer goblet cells present in the trachea and bronchi. These seemed depleted of secretion and most of them contained neutral glycoprotein. These findings represent severe structural and functional changes in the epithelium of peripheral airways which could have significant effects on both colonising microorganisms and the hosts respiratory efficiency.

Although the distribution of various types of glycoprotein within goblet cells has not been quantitated in the present study, the limited histochemical and morphological observations made suggest that there were changes in the proportion of the various types of glycoproteins. Similar changes have been reported previously in rat respiratory airways after exposure to sulphur dioxide, tobacco smoke (Lamb & Reid, 1968 & 1969b) and isoprenaline (Sturgess & Reid, 1973), and also in pig respiratory tract with enzootic pneumonia (Jones; et al. 1975). Change in the proportions of glycoproteins would seem a sensitive marker of damage to the respiratory epithelium since it may also occur with cell hypertrophy but without goblet cell increase (Jones; et al. 1972 & 1973).

The major ultrastructural changes which occurred in the bronchiolar epithelium of both early and advanced pneumonic lesions were; (1) the development of blebs in the apical cytoplasm of many ciliated cells, and (2) severe degenerative changes of the superficial epithelial cell layer which were particularly marked in advanced lesions. The cytoplasmic projections are not a feature of normal ciliated bronchiolar cells in sheep (Chapter 2). Similar though more extensive bleb formation has been described in ciliated cells of the bronchi of dogs following prolonged exposure to tobacco smoke (Frasca; et al. 1968) and in pigs infected experimentally with M. hyorhinis (Baskerville and Wright, 1973). The production of blebs by such

diverse methods suggests they may be nonspecific changes induced by irritation mediated by a number of different agents. The projections in both the present study and those reported by Baskerville and Wright, (1973) were longer than cilia and present on a large number of bronchiolar cells. It therefore seems probable that they could impede the motility of the cilia and consequently interfere with clearance mechanisms in small airways.

Because of the detailed nature of the current investigation involving SEM and TEM preparations of 5 different levels of the respiratory tract, only a relatively small number of animals could be examined. Although they were obtained from several different farms, all the animals came from the Manawatu district so it is uncertain whether the lesions observed represent a typical sample of naturally-occurring CNP in New Zealand. Nevertheless the differences observed between individuals were not great and were a matter of degree of severity rather than differences in the nature of the lesions.

This study has demonstrated that the tracheobronchial epithelium of sheep affected with either early or advanced pneumonic lesions undergoes extensive histological, ultrastructural and topographical changes. As expected the pathological changes were more severe in advanced than early pneumonic lesions, nevertheless, significant bronchiolitis and hyperplastic changes were evident in the early lesions. This suggests that CNP may begin as a tracheobronchitis, which with the accumulation of intrabronchiolar exudate and mucus may result in the obstruction of small airways. This in turn, may cause alveolar collapse, eventually leading to the development of pneumonia as discussed later in Chapter 7.

CAPTER 4

MORPHOMETRIC STUDIES OF THE TRACHEOBRONCHIAL AIRWAYS

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1.0 INTRODUCTION

The mucosal thickness of the tracheobronchial airways and anatomical features of the submucosal glands have not been investigated in either normal or pneumonic sheep. Apart from limited studies in man (Reid, 1960) and pigs (Baskerville, 1972) little quantitative data is available on the changes which occur at different levels of the tracheobronchial tree during the development of pneumonic lesions. Many of the respiratory pathogens in sheep are likely to inhabit the tracheobronchial airways (Chapter 3). It is important therefore, to acquire a knowledge of the environment these organisms inhabit and the changes which occur following infection, in order to gain a better understanding of the pathogenesis of chronic pneumonia.

Two important morphological features of the tracheobronchial airways are amenable to quantitative investigation. These are:

1. The thickness of the various layers of the epithelial mucosa.

2. The size and number of the submucosal glands.

The main aim of the present study was to compare the mucosal thickness at six levels and submucosal gland area at four levels of tracheobronchial airway of normal sheep and to find how this differs in sheep affected by early and advanced CNP.

2.0 MATERIALS AND METHODS

2.1 TRACHEOBRONCHIAL SPECIMENS

The collection technique and number of lungs used were the same

as previously described in Chapter 3. Tracheobronchial rings were selected in the manner described previously, with the addition of two more levels for study of the epithelial mucosal thickness. The two additional levels studied were the small bronchi and bronchioli (Levels 5 & 6). Because of the small size of the small bronchi, the rings of these structures were embedded as a single piece of tissue. In order to study primary bronchioli, a piece of lung tissue was embedded. Four tissue blocks were obtained from each level and two consecutive sections were cut from each block. The sections were cut at 4 - 5 um in thickness and stained with either H & E or PAS techniques. The mucosal thickness was measured using H & E stained sections, while both stains were used for submucosal gland morphometry. Consecutive sections of the same glands stained with PAS were used as duplicates for the H & E sections.

2.2 SELECTION OF SUBMUCOSAL GLANDS

Since the tracheobronchial sections contained a variable number of submucosal glands, a random method was developed to select three submucosal glands from each H & E section for analysis. Glass marbles were numbered and set in numerical order on a wooden rack, so that their numbers were visible.

(1) Using a projection light microscope [Leitz, Wetzlar, Micropromar, Germany], the section was viewed on a piece of tracing paper and each submucosal gland in the section was systematically numbered. Any group of three acini or more were counted as an individual gland.

(2) The corresponding number of marbles were picked from the rack, placed in a bowl, mixed and three were drawn to represent the submucosal glands of that particular section.

(3) The selected glands were then traced onto tracing paper. An outline of the whole gland and each acinus inside was recorded.

(4) After the glands in the H & E section were traced, the corresponding glands on the PAS duplicate slide were also traced.

Thus, 12 glands were traced in duplicate from each tracheobronchial level giving a total of 48 paired tracing's from each animal.

2.3 QUANTITATION

2.3.1 MUCOSAL THICKNESS

Four random sets of measurements were taken from different areas of each section using a light microscope and micrometer integrating eyepiece and the mean of each section was recorded. These were:

1. The epithelial thickness, measured from the basement membrane to the apical surface of a ciliated cell.

2. The mucosal thickness, measured from the luminal margin of the cartilaginous ring to the apical surface of a ciliated cell.

The difference between these two measurements was recorded as the submucosal layer. All the measurements were read in micrometers (um).

2.3.2 SUBMUCOSAL GLANDS

The dimensions of each gland were measured using a Summagraphic digitiser connected to a Hitachi MBE 16002 microcomputer. A computer programme developed by Massey University Computer Centre was used to calculate the total gland area, the total acinar area , the number of acini per gland and the ratio between the area of each gland and the area of its acini. Additionally, the non-acinar area and the mean of a single acinus were estimated from the data.

2.4 ANALYSIS OF DATA

2.4.1 UNIVARIATE ANALYSIS

The data was analysed using a Generalized Linear Models Computing Package (REG), (Gilmour, 1983).

Tracheobronchial tissues were classified into three groups on

the basis of their gross and histological features. These were:

1. Normal, or control group.
2. Early pneumonic group.
3. Advanced pneumonic group.

The general form of the linear model used to analyse the data was:

$$Y_{ijk} = u + A_i + B_j + (AB)_{ij} + e_{ijk}$$

where:

Y_{ijk} = the kth observations in the ith group in the jth level

u = the overall mean

A_i = the effect of ith group

B_j = the effect of jth level

$(AB)_{ij}$ = the effect of the ith group and jth level

2.4.2 DUNCAN'S NEW MULTIPLE-RANGE TEST

This test, while not as discerning as other tests such as Student-Newman-Keuls test, has the advantage of simplicity (Steel and Torrie,1981). It uses multiple ranges for testing and is result guided. Confidence intervals are not appropriate; the notion of confidence is replaced by that of protection levels against finding false significant differences at various stages of testing. The test uses a variable level dependent on the number of means involved at any stage. It is based on the idea that as the number of means under test increases, the probability that they will all be alike becomes smaller.

3.0 RESULTS

3.1 TRACHEOBRONCHIAL MUCOSAL THICKNESS

The results are presented as histograms of mean thicknesses. The tables on which they based are listed in the Appendix together with Duncan's test of significant differences.

3.1.1 NORMAL SHEEP

The mean thicknesses of the tracheobronchial mucosa of normal sheep are shown in (Fig. 4.1A). Statistical analyses of the data showed that there were significant ($P < 0.001$) differences in the epithelial, submucosal and mucosal thickness at different levels (Appendix 4.1). Duncan's test showed that the epithelium of the upper trachea was significantly ($P < 0.05$) thicker than that of the rest of the tracheobronchial airway. The lower tracheal epithelium was also significantly ($P < 0.01$) thicker than the more peripheral airways, but the thicknesses of extrapulmonary, intrapulmonary and small bronchi were not significantly different from one another. However, the epithelial thickness of all tracheobronchial airways examined was significantly ($P < 0.01$) thicker than that of the bronchioli (Appendix 4.2).

The mucosa and submucosa of the upper trachea were not significantly thicker than those of lower trachea but they were significantly ($P < 0.01$) thicker than those of the rest of the tracheobronchial airways. There were no significant differences between the thicknesses of extra- and intrapulmonary bronchi or between small bronchi and bronchioli. However, there were significant ($P < 0.05$) differences between these two main groups of airway levels.

The ratio between the mucosal layer and epithelium began at 11.1:1 in the upper trachea but decreased gradually until it became 2.2:1 in the primary bronchioli. At the same time the ratio between mucosa and submucosa increased distally from 1.1:1 in the upper trachea to 1.8:1 in the primary bronchioli. The ratio between submucosa and epithelial layers was almost equal at the level of primary bronchioli (1.2:1) but enlarged toward the upper trachea where it became as large as 10.1:1 (Table 4.1).

3.1.2 EARLY PNEUMONIC LESIONS

The mean thicknesses of the tracheobronchial mucosa of early pneumonic sheep are presented in Figure 4.1B. Analysis of the data obtained revealed that epithelium of the upper trachea was significantly ($P < 0.01$) thicker than that of the rest of the airway

levels (Appendix 4.3). The mucosa and submucosa of the upper trachea were also significantly ($P < 0.01$) thicker than those of the rest of the tracheobronchial airways. Although there were no significant differences between the lower trachea and extrapulmonary bronchi, they were both significantly ($P < 0.01$) thicker than the more peripheral airways. Statistical analysis showed that there were no significant differences between thicknesses in the intrapulmonary bronchi, small bronchi and bronchioli (Appendices 4.3 & 4.4).

The ratio between the mucosal and epithelial layers of the upper trachea (7.9:1) was smaller than that of the lower trachea (9.6:1) and extrapulmonary bronchi (8.3:1). The lower tracheal ratio was greater than that of extrapulmonary bronchi, thereafter, it decreased distally until it became (2.3 :1) at the primary bronchioli. However, the ratio between mucosa and submucosa in the upper trachea (1.2:1) was similar to that of the intrapulmonary bronchi. This ratio decreased between upper trachea and extrapulmonary bronchi but thereafter it increased distally . The ratio between submucosa and epithelium was similar to the pattern which occurred with the mucosa to submucosa ratios (Table 4.2).

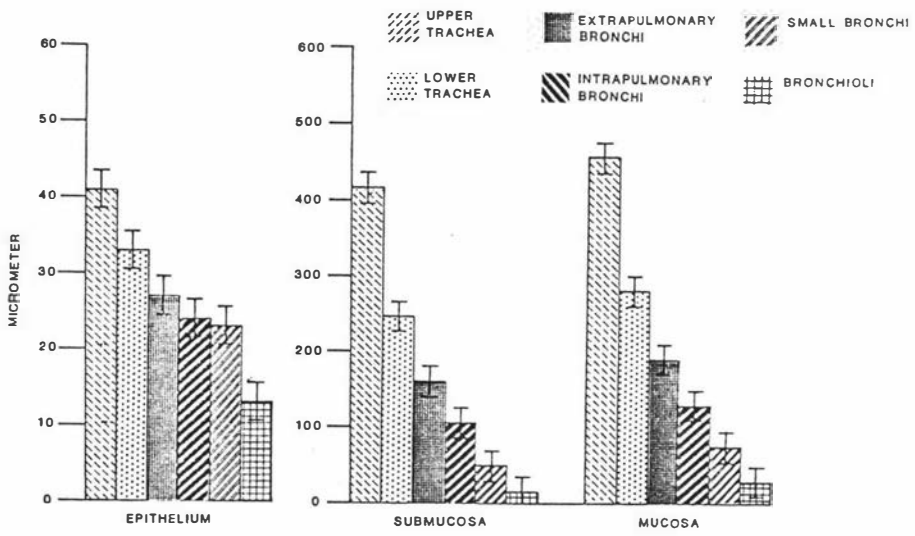
3.1.3 ADVANCED PNEUMONIC LESIONS

The mean thicknesses of the tracheobronchial mucosa of advanced pneumonia are shown in Figure 4.1C. Statistical analysis of the epithelial measurements showed there were no significant differences in thickness between the upper trachea, intrapulmonary bronchi, small bronchi and bronchioli. However, these four airways were significantly ($P < 0.05$) thicker than the lower trachea and extrapulmonary bronchi, which in turn, were not significantly different from one another (Appendices 4.5 & 4.6). The mucosa and submucosa of the upper trachea was significantly ($P < 0.01$) thicker than other parts of the tracheobronchial tract. Although there were no significant differences between lower trachea, extra- and intrapulmonary bronchi they were significantly ($P < 0.01$) thicker than the small bronchi. No significance differences were observed between small bronchi and bronchioli (Fig. 4.1C & Appendices 4.5 & 4.6).

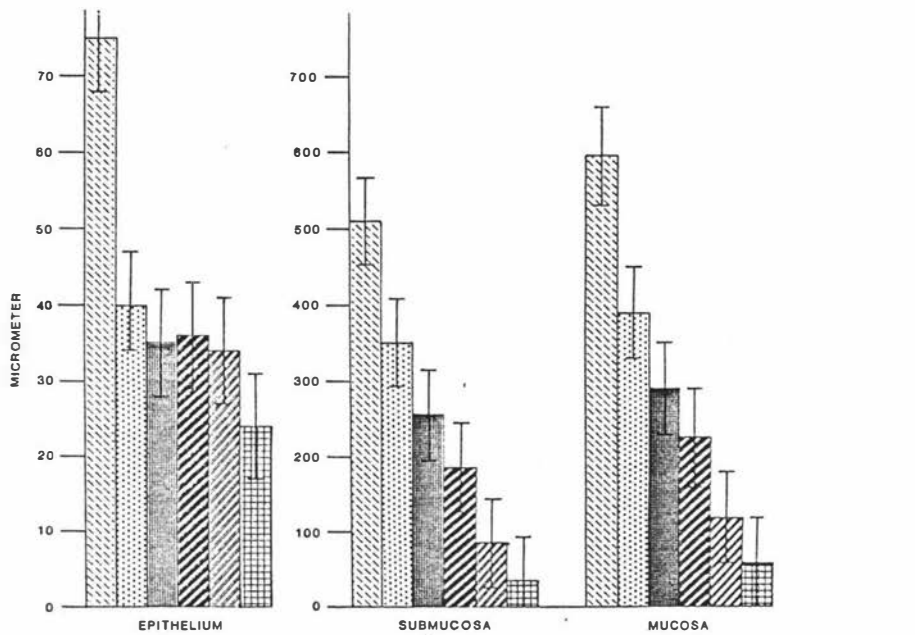
The ratio between mucosa and epithelium decreased irregularly in a distal direction from upper trachea (13.2:1) to primary bronchioli

Figure 4.1

The mean thicknesses (with standard errors) of the tracheobronchial epithelium, submucosa and mucosa at six levels of normal lungs (A), early pneumonic lesions (B) and advanced pneumonic lesions (C).


UPPER TRACHEA
LOWER TRACHEA
EXTRAPULMONARY BRONCHI
INTRAPULMONARY BRONCHI
SMALL BRONCHI
BRONCHIOLI
MICROMETER
0
10
20
30
40
50
60
100
200
300
400
500
600
EPITHELIUM
SUBMUCOSA
MUCOSA

A


MICROMETER
0
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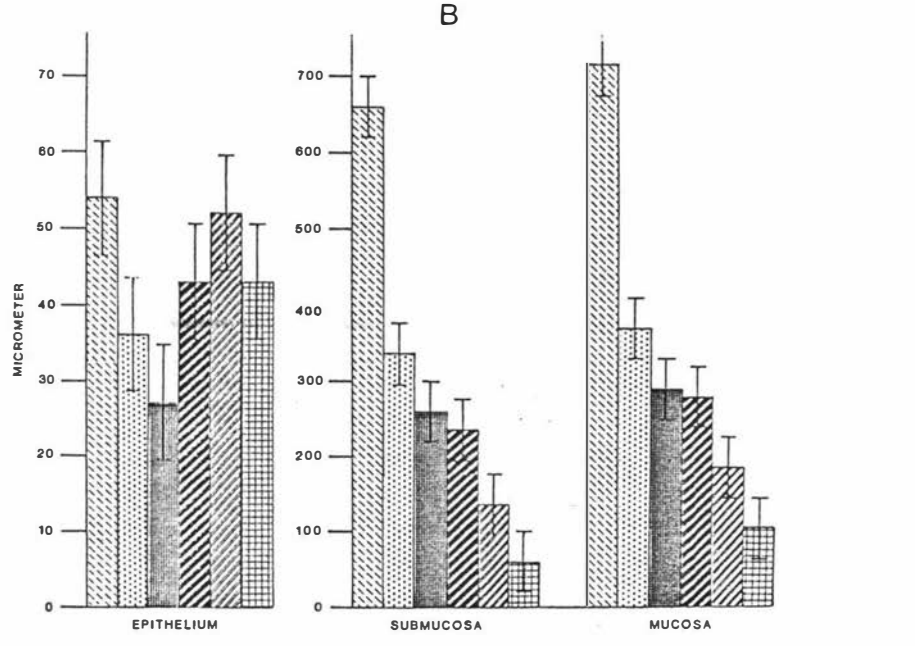
EPITHELIUM
SUBMUCOSA
MUCOSA

B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EPITHELIUM
SUBMUCOSA
MUCOSA

C

(2.4:1). At the extrapulmonary bronchi it was similar to the ratio in the lower trachea but at the intrapulmonary bronchi level it was markedly smaller than the ratio of the higher tracheobronchial airways. The ratio between mucosa and submucosa increased distally in an uneven manner. It was similar at the upper trachea, lower trachea and extrapulmonary bronchi levels, but increased thereafter to 1.7:1. The ratio between the submucosa and epithelium decreased distally. Although the ratio at the extrapulmonary bronchi was similar to that at the lower trachea and that of the intrapulmonary bronchi, it was much smaller than at higher levels (Table 4.3).

3.1.4 THE COMPARATIVE STUDY OF MUCOSAL THICKNESS

3.1.4.1 EPITHELIUM

Statistical analysis showed that there were significant differences in the thickness of the epithelium of the small bronchi ($P < 0.05$) and bronchioli ($P < 0.01$) between the three groups (Fig. 4.2A & Appendix 4.7). Duncan's test showed that at the level of the upper trachea, the epithelium of early pneumonic animals was significantly ($P < 0.05$) thicker than that of both normal and advanced pneumonic groups, but there were no significant differences between the last two categories. At small bronchi and bronchioli levels , the epithelium of advanced pneumonic lesions was significantly ($P < 0.05$ and $P < 0.01$ respectively) thicker than that of both normal and early pneumonic groups, but no significant differences were observed between the early pneumonia and normal sheep (Fig. 4.2A & Appendix 4.8). No significant differences between the epithelium of normal, early and advanced pneumonic animals were observed at the lower trachea, extra- and intrapulmonary bronchi.

The tracheobronchial epithelium increased more variably along the airways in both early and advanced pneumonic groups than in normal lungs (Fig. 3 & Appendix 4.13). In both early and advanced lesions, however, the primary bronchioli were the most severely affected region. The increase in epithelial thickness of this region was 93% and 234.6% respectively above that of normal sheep.

TABLE 4.1: THE RATIO BETWEEN THE LAYERS OF THE TRACHEOBRONCHIAL MUCOSA OF NORMAL SHEEP AT DIFFERENT LEVELS

MUCOSA LAYERS	THE RATIO AT EACH LEVEL					
	L1	L2	L3	L4	L5	L6
MUCOSA:EPITHELIUM	11.1:1	8.3:1	6.9:1	5.4:1	3.2:1	2.2:1
MUCOSA:SUBMUCOSA	1.1:1	1.1:1	1.2:1	1.2:1	1.5:1	1.8:1
SUBMUCOSA:EPITHELIUM	10.1:1	7.3:1	5.9:1	4.4:1	2.2:1	1.2:1

TABLE 4.2: THE RATIO BETWEEN THE LAYERS OF THE TRACHEOBRONCHIAL MUCOSA OF EARLY PNEUMONIC LESIONS AT DIFFERENT LEVELS

MUCOSAL LAYERS	THE RATIO AT EACH LEVEL					
	L1	L2	L3	L4	L5	L6
MUCOSA:EPITHELIUM	7.9:1	9.6:1	8.3:1	6.2:1	3.4:1	2.3:1
MUCOSA:SUBMUCOSA	1.2:1	1.1:1	1.1:1	1.2:1	1.4:1	1.7:1
SUBMUCOSA:EPITHELIUM	6.9:1	8.6:1	7.3:1	5.2:1	2.4:1	1.4:1

TABLE 4.3: THE RATIO BETWEEN THE LAYERS OF THE TRACHEOBRONCHIAL MUCOSA OF ADVANCED PNEUMONIC LESIONS AT DIFFERENT LEVELS

MUCOSAL LAYERS	THE RATIO AT EACH LEVEL					
	L1	L2	L3	L4	L5	L6
MUCOSA:EPITHELIUM	13.2:1	10.2:1	10.5:1	6.3:1	3.5:1	2.4:1
MUCOSA:SUBMUCOSA	1.1:1	1.1:1	1.1:1	1.2:1	1.4:1	1.7:1
SUBMUCOSA:EPITHELIUM	12.2:1	9.2:1	9.5:1	5.3:1	2.5:1	1.4:1

N.B

L1 = UPPER TRACHEA, L2 = LOWER TRACHEA, L3 = EXTRAPULMONARY BRONCHI, L4 = INTRAPULMONARY BRONCHI, L5 = PRIMARY BRONCHI, L6 = BRONCHIOLI.

3.1.4.2 SUBMUCOSA

Statistical analysis of the measurements of this layer showed there were significant differences between groups in the thickness of the intrapulmonary bronchi ($P < 0.05$), small bronchi ($P < 0.01$) and bronchioli ($P < 0.01$) (Fig. 4.2B & Appendix 4.9). However, no significance differences were observed between the trachea and extrapulmonary bronchi. Duncan's test showed that the submucosa of the animals with advanced pneumonia was significantly ($P < 0.05$) thicker than normal but not thicker than early pneumonic animals at the intrapulmonary and small bronchi levels. At the bronchioli, the submucosa was significantly ($P < 0.01$) thicker than that of normal but not early pneumonic sheep (Appendix 4.10).

There was a variable increase in the thickness of submucosa at all tracheobronchial levels in both early and advanced pneumonic lesions when compared to normal lungs (Fig. 4.3 & Appendix 4.13). In both early and advanced lesions, the primary bronchioli were most severely affected and the increase in the thickness being 118.6% and 298.7% respectively.

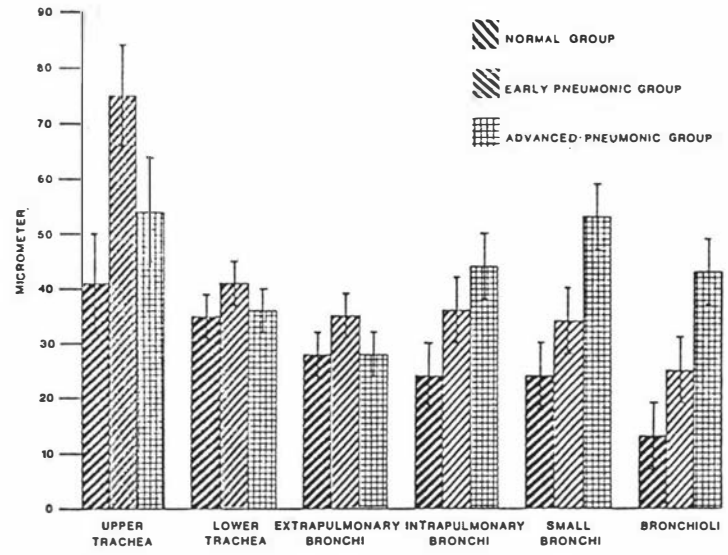
3.1.4.3 MUCOSA

Analyses of the data obtained for the thickness of the mucosal layer as whole, showed significant differences ($P < 0.01$) at intrapulmonary bronchial, small bronchial and bronchiolar levels, but no significant differences at the tracheal and extrapulmonary bronchial levels (Fig. 4.2C & Appendix 4.11). Duncan's test showed that only advanced pneumonic lesions were significantly thicker than normal. This was true at the levels of the upper trachea ($P < 0.05$), intrapulmonary bronchi ($P < 0.05$), small bronchi ($P < 0.01$) and bronchioli ($P < 0.01$) (Appendix 4.12).

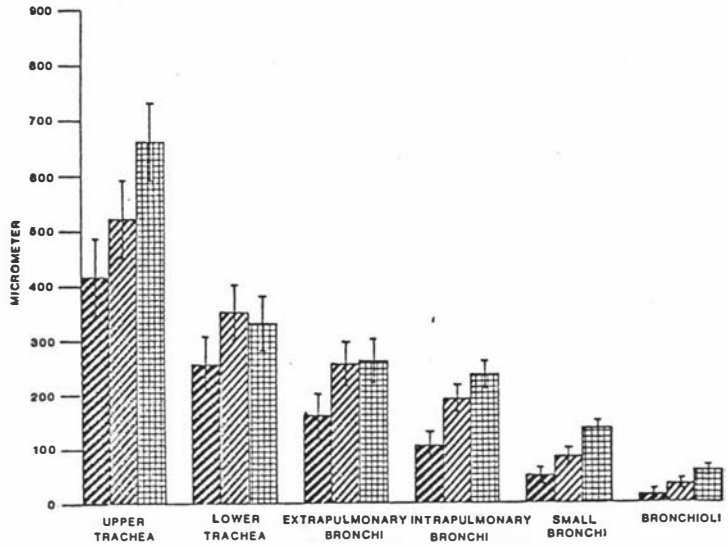
The percentage increase above normal in the mucosal thickeness along the tracheobronchial tract was variable. Small bronchi and primary bronchioli were the most severely affected with increases of 146.5% and 268.2% respectively (Fig. 4.3 & Appendix 4.13).

Figure 4.2

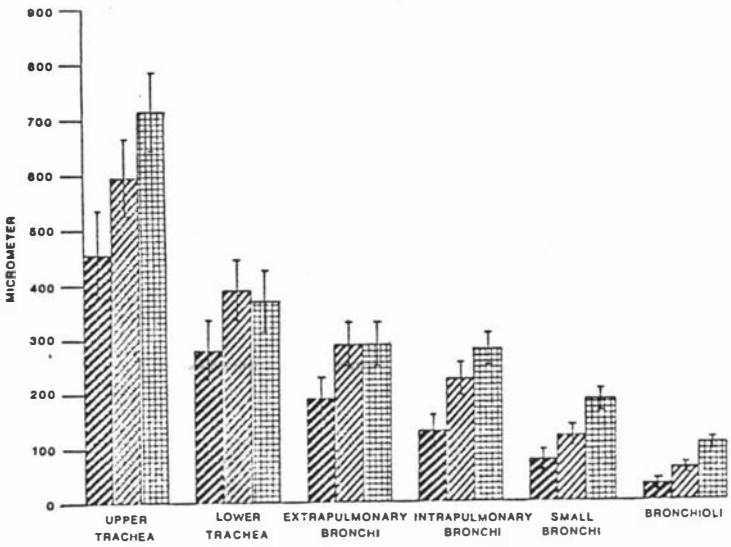
Differences in the mean thickness of the epithelium (A), submucosa (B) and mucosa (C) between normal, early pneumonic and advanced pneumonic groups.


NORMAL GROUP
EARLY PNEUMONIC GROUP
ADVANCED PNEUMONIC GROUP
MICROMETER
0
10
20
30
40
50
60
70
80
90
UPPER TRACHEA
LOWER TRACHEA
EXTRAPULMONARY BRONCHI
INTRAPULMONARY BRONCHI
SMALL BRONCHI
BRONCHIOLI

A


MICROMETER
0
100
200
300
400
500
600
700
800
900
UPPER TRACHEA
LOWER TRACHEA
EXTRAPULMONARY BRONCHI
INTRAPULMONARY BRONCHI
SMALL BRONCHI
BRONCHIOLI

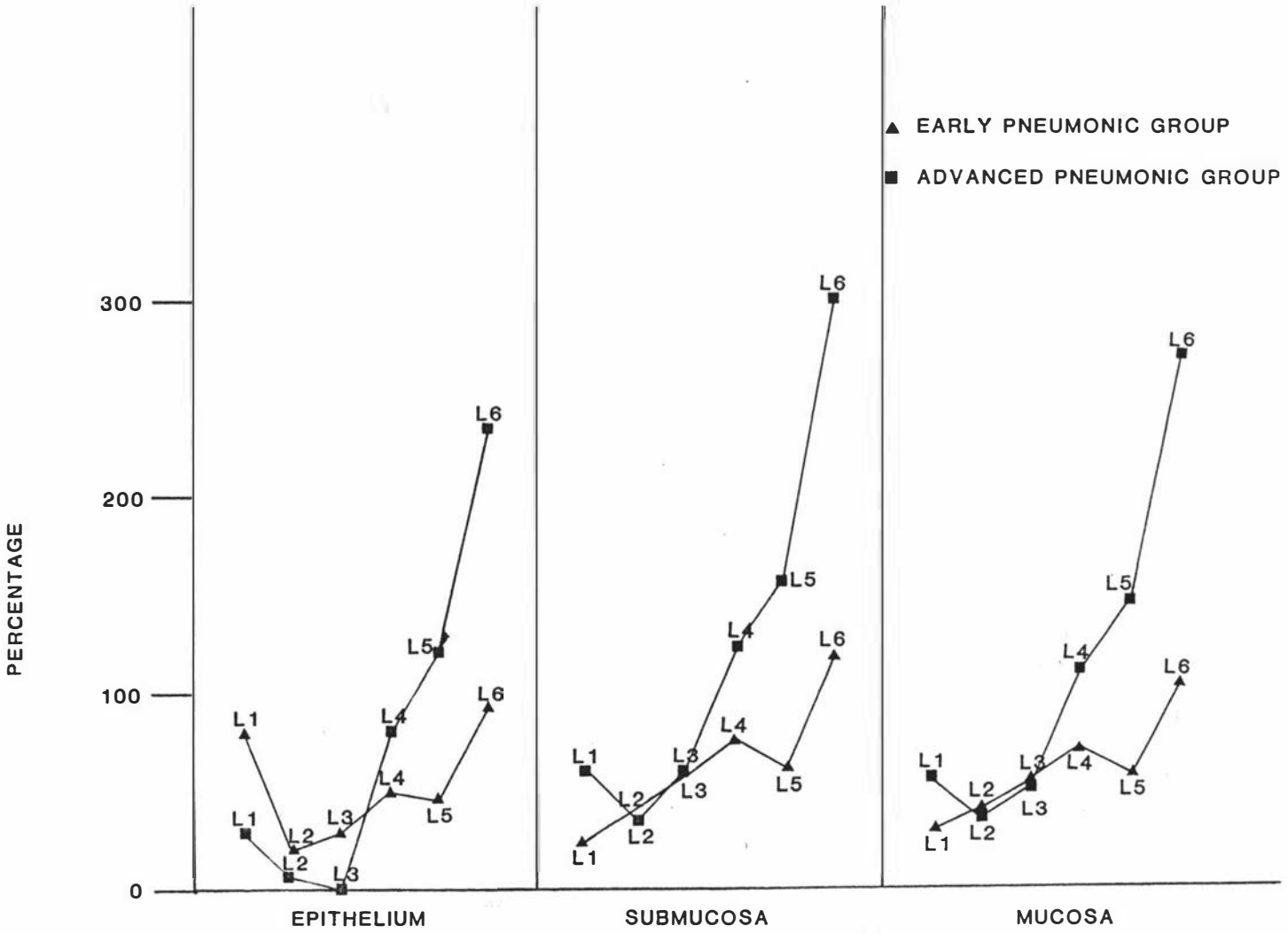
B


MICROMETER
0
100
200
300
400
500
600
700
800
900
UPPER TRACHEA
LOWER TRACHEA
EXTRAPULMONARY BRONCHI
INTRAPULMONARY BRONCHI
SMALL BRONCHI
BRONCHIOLI

C

Figure 4.3

The percentage increase in mean thickness of epithelium, submucosa and mucosa in early and advanced pneumonic groups compared to normal lungs.


▲ EARLY PNEUMONIC GROUP
■ ADVANCED PNEUMONIC GROUP
PERCENTAGE
300
200
100
0
EPITHELIUM
SUBMUCOSA
MUCOSA
L1
L2
L3
L4
L5
L6

3.2 SUBMUCOSAL GLANDS

3.2.1 NORMAL SHEEP

When the data obtained from normal sheep was analysed statistically, significant differences in the number of acini ($P < 0.001$), gland area ($P < 0.01$), total acinar area ($P < 0.01$), non-acinar area ($P < 0.01$) and gland area to acinar area ratio ($P < 0.05$) were found at different levels of the airways (Fig. 4.4A & Appendix 4.14). However, there were no significant differences in the area of single acini within submucosal glands at different airway levels. Using Duncan's test for further analyses the number of acini, gland area, total acinar area and non-acinar area of submucosal glands in the upper trachea were found to be significantly ($P < 0.01$) higher than those in other parts of the airway (Appendix 4.15). All the parameters measured in the lower trachea, except non-acinar area were significantly ($P < 0.05$) higher than those in the intrapulmonary bronchi (Appendix 4.15). The non-acinar area of the lower trachea was significantly ($P < 0.05$) higher than at of both extra- and intrapulmonary bronchi but no significant differences were observed between the submucosal gland parameters of extra- and intrapulmonary bronchi. In addition, there was no significant difference between single acinus area at different levels.

3.2.2 EARLY PNEUMONIC LESIONS

A summary of the data obtained from the submucosal glands of sheep with early pneumonic lesions is given in Figure 4.4B and Appendix 4.16. No significant differences were found in the number of acini, gland area, acinar area, non-acinar area, acinus area and gland area to acinar area ratios between the various tracheobronchial levels.

3.2.3 ADVANCED PNEUMONIC LESIONS

A summary of the data obtained from the submucosal glands of advanced pneumonic lesions is given in Figure 4.4C and Appendix 4.17. Statistical analyses of the data showed that the only significant ($P < 0.01$) difference between tracheobronchial levels was in the number of acini. Using Duncan's test for further analyses, the number of acini

in the upper tracheal submucosal glands were found to be significantly ($P < 0.01$) higher than those in the extra- and intrapulmonary bronchi but not significantly different from those of lower trachea (Fig. 4.4C & Appendix 4.18). The number of acini in the lower trachea were significantly ($P < 0.05$) higher than those in the intrapulmonary bronchi, but there were no significant differences between extra- and intrapulmonary bronchi (Fig. 4.4C & Appendix 4.18). Although, the single acinar area showed only a marginally significant difference ($P < 0.07$) between levels, further statistical analyses, using Duncan's test revealed that the single acinar area at the level of the intrapulmonary bronchi was significantly ($P < 0.05$) larger than that of upper trachea and extrapulmonary bronchi. However, there was no significance difference from that of lower trachea (Fig. 4.4C & Appendix 4.18).

3.2.4 COMPARATIVE STUDIES OF THE SUBMUCOSAL GLANDS

3.2.4.1 NORMAL LUNGS AND EARLY PNEUMONIC LESIONS

A comparative statistical analysis of the submucosal glands of normal and early pneumonic sheep revealed that there were no significant differences at either upper (Fig. 4.5A & Appendix 4.19) and lower tracheal levels (Fig. 4.5B & Appendix 4.20). Significant changes were first seen at the level of the extrapulmonary bronchi where the number of acini per gland increased significantly ($P < 0.05$) (Fig. 4.5C & Appendix 4.21). There were no significant differences in the total gland, acinar, non-acinar and single acinar areas or gland area to acinar area ratio.

The submucosal glands of intrapulmonary bronchi in early lesions showed highly significant changes when compared to normal animals. There was a significant increase ($P < 0.01$) in the number of acini, as well as an increase in the acinar, non-acinar and total gland areas. In addition, the gland area to acinar area ratio was significantly increased ($P < 0.05$). No significant difference was found in single acinus area between pneumonic and normal sheep (Fig. 4.5D & Appendix 4.22)

Figure 4.4

The parameters of the submucosal glands in normal (A), early pneumonic (B) and advanced pneumonic (C) groups.

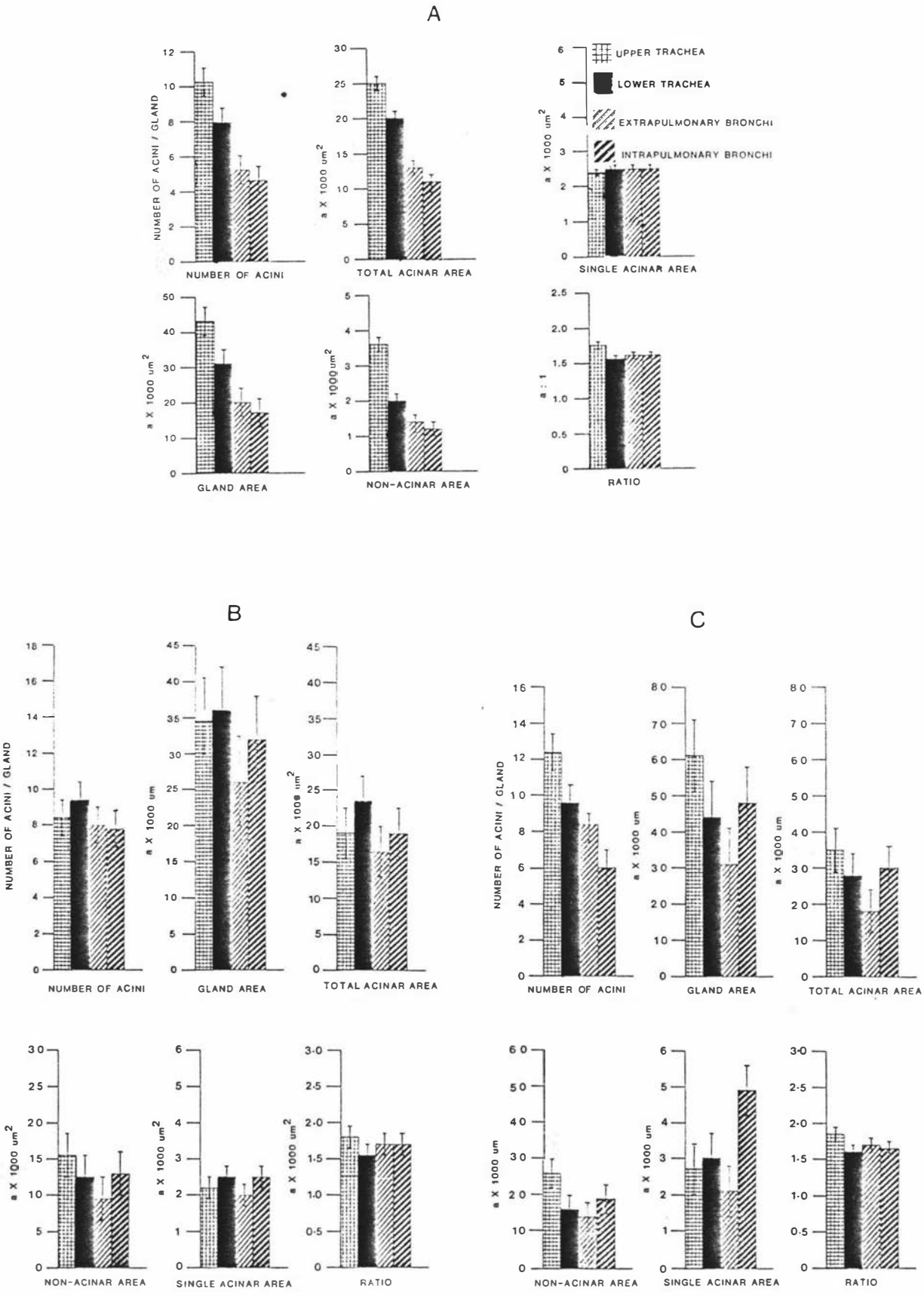
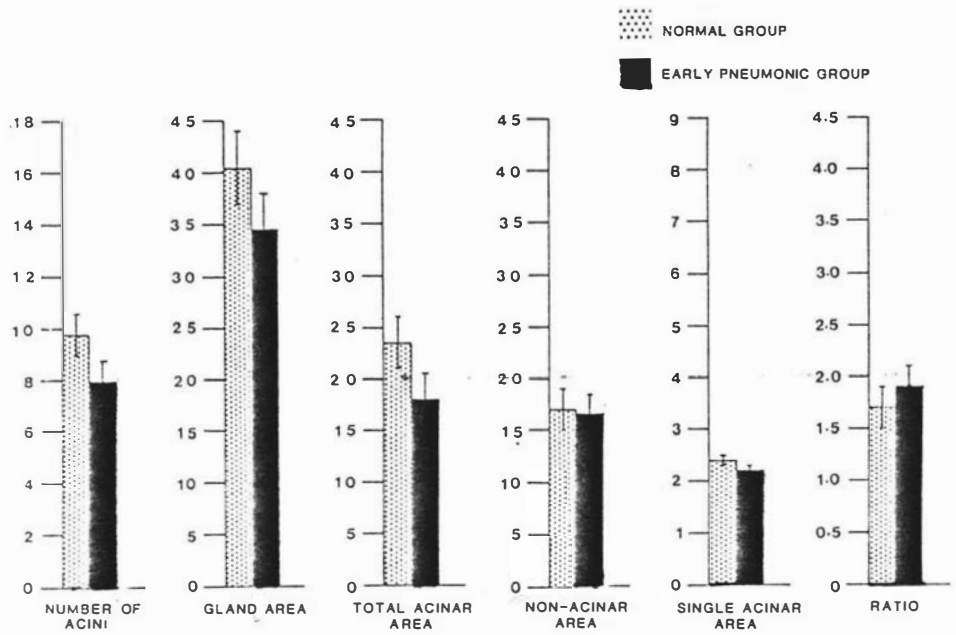
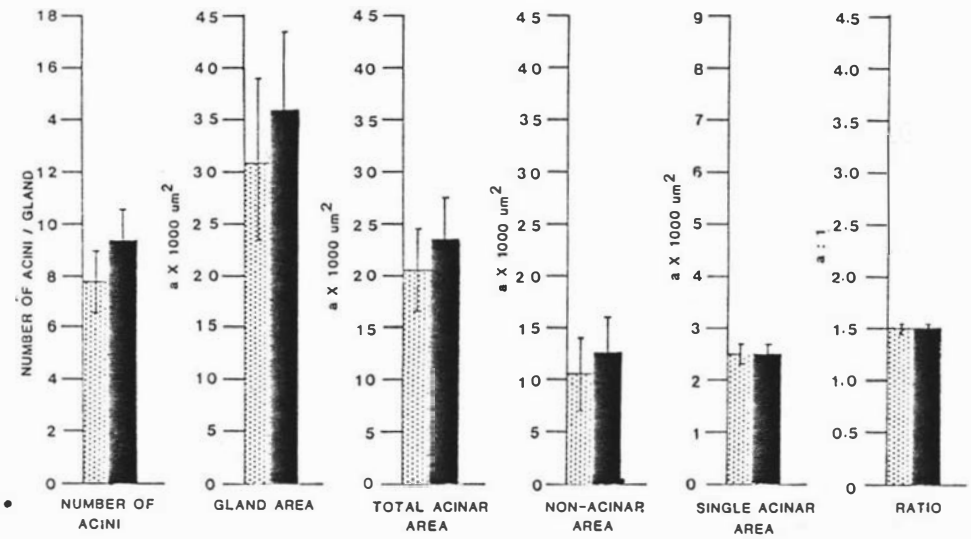

A
NUMBER OF ACINI / GLAND
NUMBER OF ACINI
a X 1000 um2
TOTAL ACINAR AREA
UPPER TRACHEA
LOWER TRACHEA
EXTRAPULMONARY BRONCHI
INTRAPULMONARY BRONCHI
SINGLE ACINAR AREA
GLAND AREA
NON-ACINAR AREA
a : 1
RATIO
B
C
a X 1000 um
NON-ACINAR AREA
SINGLE ACINAR AREA

Figure 4.5

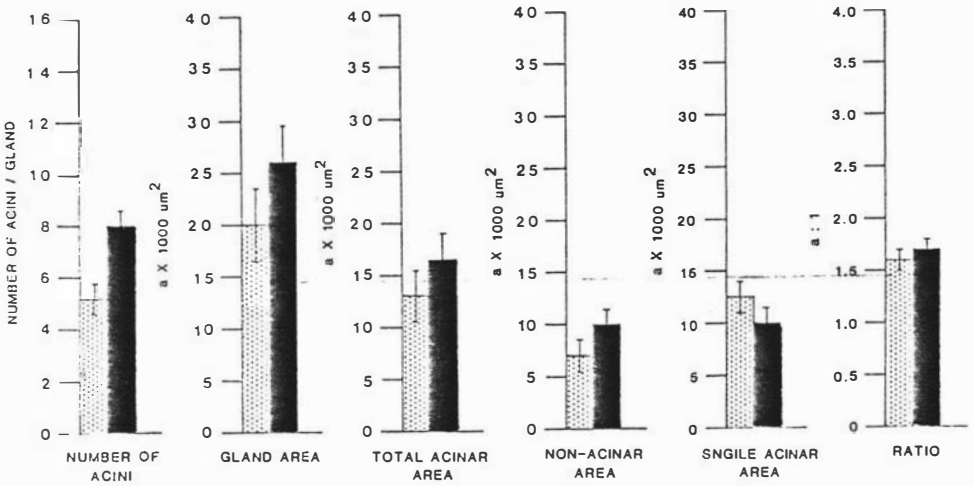
A comparative statistical analysis of submucosal gland parameters of normal and early pneumonic groups at the upper trachea (A), lower trachea (B), extrapulmonary bronchi (C) and intrapulmonary bronchi (D).


NORMAL GROUP
EARLY PNEUMONIC GROUP
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

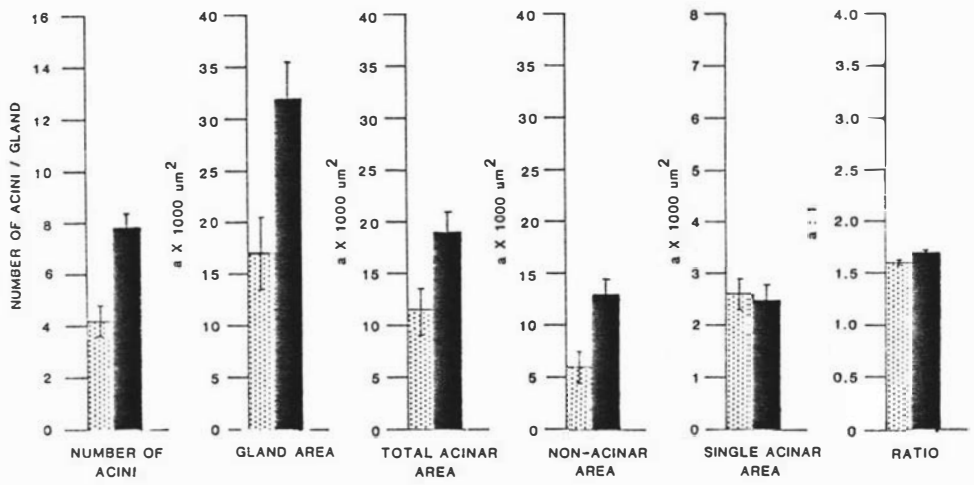
A


NUMBER OF ACINI / GLAND
a X 1000 um2
a X 1000 um2
a X 1000 um2
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

B


NUMBER OF ACINI / GLAND
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SNGILE ACINAR AREA
RATIO

C


NUMBER OF ACINI / GLAND
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

D

3.2.4.2 NORMAL LUNGS AND ADVANCED PNEUMONIC LESIONS

A comparison between the submucosal glands of the upper and lower trachea of normal sheep (Fig. 4.6A & Appendix 4.23) and advanced pneumonic lesions (Fig.4.6B & Appendix 4.24) showed that there were no significant differences between the two groups. At the level of the extrapulmonary bronchi, however, analysis showed that the number of acini increased significantly ($P < 0.001$) in pneumonic sheep. There was also a significant ($P < 0.05$) increase in the area of total gland and non-acinar area of the gland ($P < 0.01$) (Fig. 4.6C & Appendix 4.25). The mucosal glands of the intrapulmonary bronchi of advanced lesions showed a significant increase in the number of acini ($P < 0.01$), gland area ($P < 0.06$) and acinar area($P < 0.06$). There were no significant differences in the non-acinar area, single acinar area or gland area to acinar ratio (Fig. 4.6D & Appendix 4.26).

4.0 DISCUSSION

4.1 MUCOSAL THICKNESS

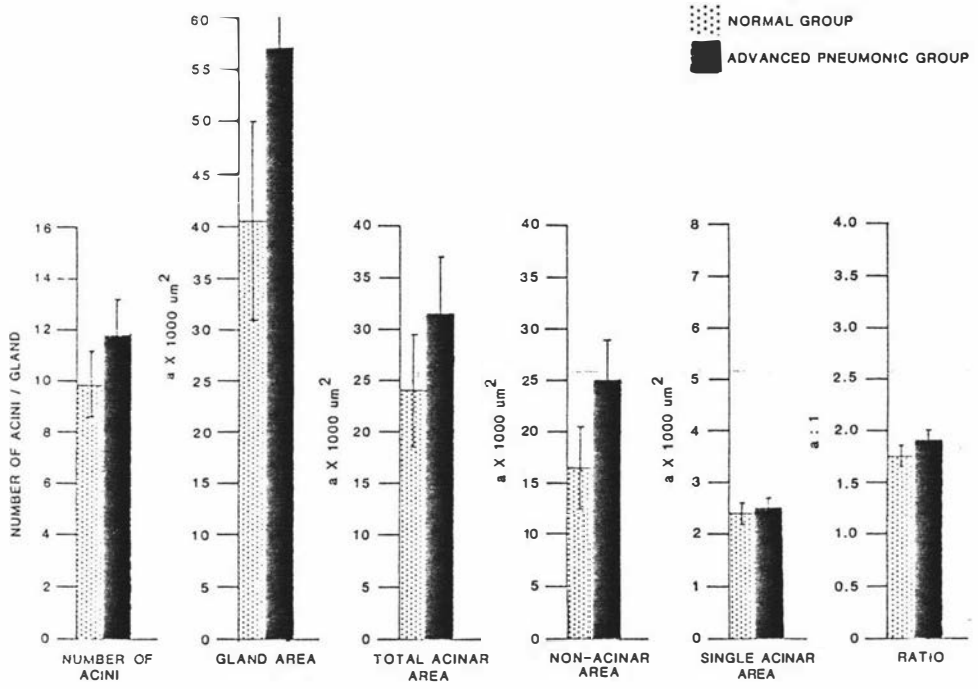
The present study has succeeded in quantifying the proliferative changes observed in the mucosa of the tracheobronchial airways of sheep with CNP. It involved detailed measurement and statistical analysis of six different levels of normal lungs and comparison with similar sites in early and advanced pneumonic lesions.

Although the methods used in measurement were relatively simple, the interpretation of the data obtained would not have been possible without the use of a computer based analytical system. The computer statistical model (REG) used was generalized linear model program developed by an agricultural statistical group. It fits any linear model to unbalanced univariate and multivariate data (Gilmour, 1983).

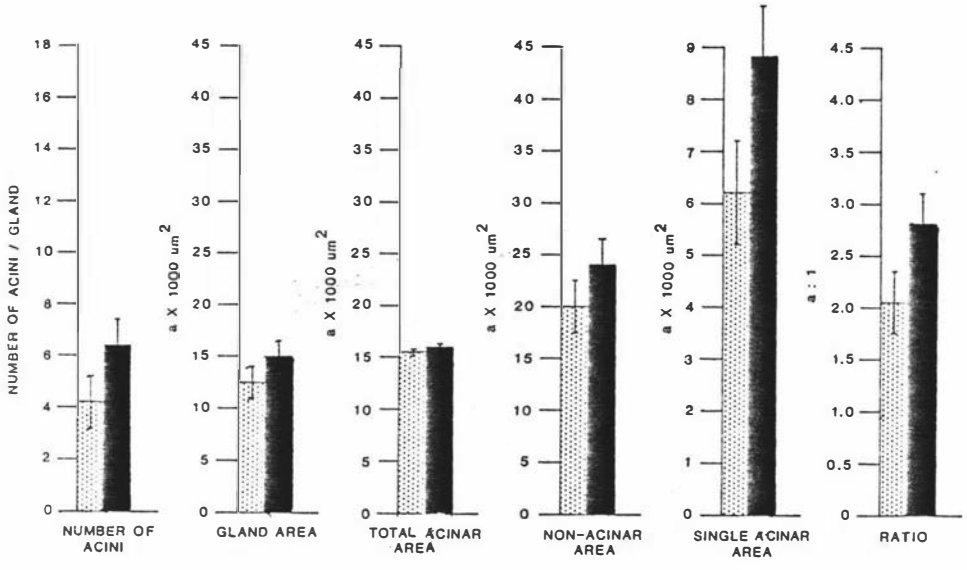
Previous studies of thickness of layers of the tracheobronchial mucosa has been confined mainly to human pathology where it has been used as a diagnostic aid in chronic bronchitis. In 1960, Reid developed a technique (later known as the Reid Index) to quantify mucous gland changes by measuring the ratio of the thickness of the gland layer to the thickness of the bronchial wall in main bronchi.

Figure 4.6

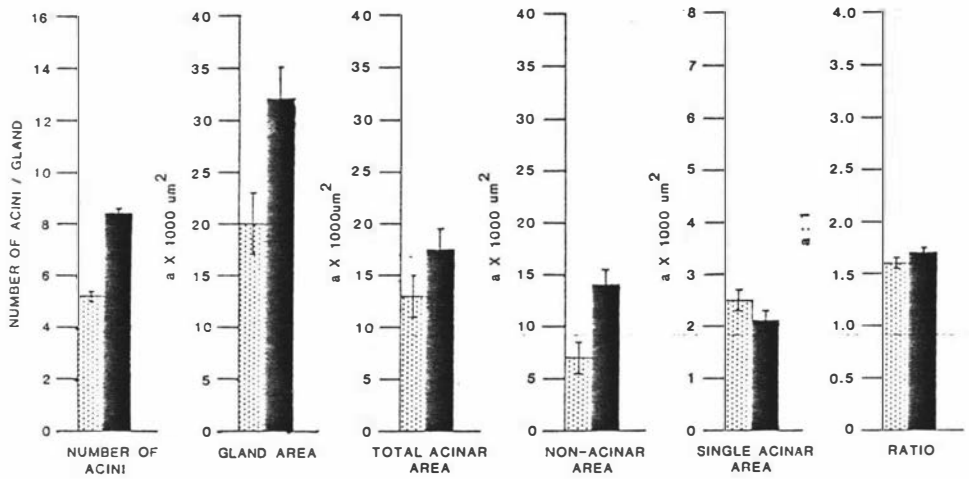
A comparative statistical analysis of submucosal gland parameters of normal and advanced pneumonic groups at the upper trachea (A), lower trachea (B), extrapulmonary bronchi (C) and intrapulmonary bronchi (D).


NORMAL GROUP
ADVANCED PNEUMONIC GROUP
NUMBER OF ACINI / GLAND
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

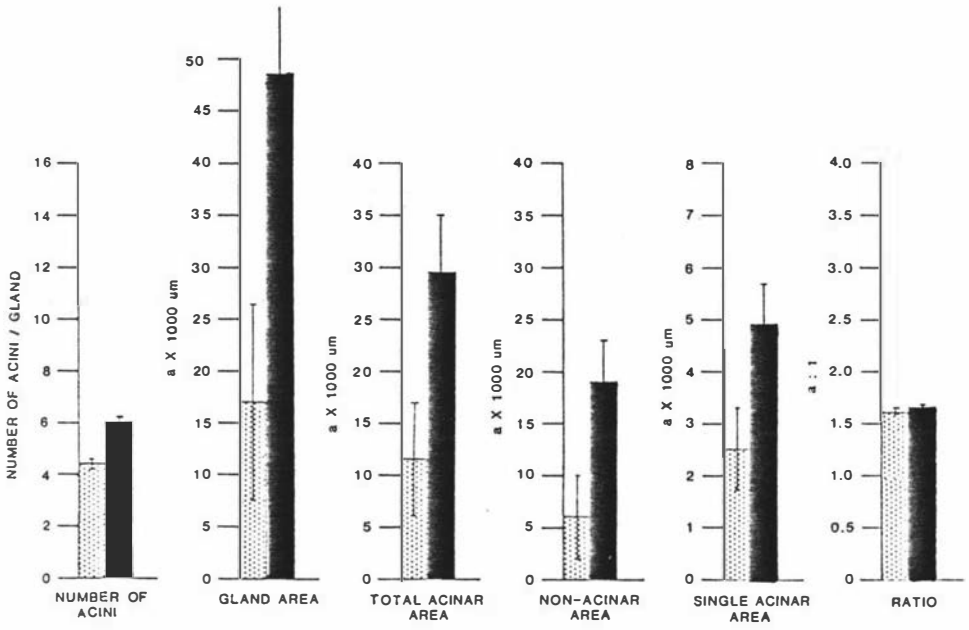
A


NUMBER OF ACINI / GLAND
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

B


NUMBER OF ACINI / GLAND
a X 1000 um2
a X 1000um2
a X 1000 um2
a X 1000 um2
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

C


NUMBER OF ACINI / GLAND
a X 1000 um
a X 1000 um
a X 1000 um
a X 1000 um
a : 1
NUMBER OF ACINI
GLAND AREA
TOTAL ACINAR AREA
NON-ACINAR AREA
SINGLE ACINAR AREA
RATIO

D

Since then the Reid index has been criticised on several grounds (Dunnill, 1982), and this led to the development of other techniques aimed at measuring the total percentage of bronchial wall occupied by glandular tissue (Restrepo and Heard 1963a & b; Dunnill; et al. 1969). Since submucosal gland hypertrophy has an important pathological counterpart in human chronic bronchitis, all of the above mentioned techniques were interested to establish the percentage volume or ratio of the bronchial wall occupied by mucous glands. Because the aim of the present investigation differed from these previous studies, a more appropriate method of measurement was developed by which the thickness of the epithelial and submucosal layers could be estimated separately.

In their studies of tracheobronchial airway morphology Mariassy and Plopper (1983) found that normal sheep followed the conventional classification into cartilaginous and non-cartilaginous regions (Weiss and Geep, 1977). They showed that the epithelial thickness of cartilaginous airways was greater than that of non-cartilaginous and this finding coincided with the disappearance of the mucosal glands. These authors gave no indication of the method and unit of measurement used or details of the relative thickness of the different levels.

The morphometric method used in the present study has confirmed Mariassy and Plopper's (1983) observations and provided a great deal of additional information on the airways of normal sheep. It has been shown that the epithelium thins progressively from the upper trachea to the primary bronchioli and there is a positive correlation between epithelial and submucosal thickness. As the epithelium decreases in thickness, the submucosa follows the same pattern.

The thinning pattern of ovine tracheobronchial airway epithelium observed in this study was different from that of the rat. Although they made no mention of the method and unit of measurement, Jeffery and Reid (1975) estimated the thickness of normal rat epithelium at five levels and observed a progressive thinning from upper to lower trachea. The epithelium of the lower trachea was thicker than at the more peripheral airway levels, but all the remainder were of similar thickness.

In the pneumonic lesions currently studied, a progressive

mucosal thinning from upper trachea to primary bronchioli was still apparent with most of the thinning of the mucosa occurring between the upper and lower trachea. However, the thinning pattern seen in the advanced pneumonic lesions was more irregular than that in early pneumonia. Earlier morphological studies (Chapter 3) have shown that the increased thickness is caused by oedema, cellular infiltration and gland hypertrophy in the submucosa together with oedema and hypertrophy of the epithelium.

An increase in the wall thickness of peripheral airways during the development of CNP together with the accumulation of inflammatory cells and mucus must result in partial obstruction of the airway lumen of the affected part of the respiratory tract. These changes are likely to radically alter the ventilation of the affected area. Histologically, some of the small airways were found to be completely obstructed particularly at the small bronchi and bronchioli levels (Chapter 3). These findings are similar to those described in human chronic bronchitis (Dunnill, 1982) and enzootic pneumonia in pigs (Baskerville, 1972).

The narrowing of the airways (particularly small bronchi and primary bronchioli) in early lesions may also be a contributing factor to the reflux and accumulation of mucus in the alveoli, observed by Alley and Clarke, (1979) in experimentally and naturally infected sheep.

The current study has clearly demonstrated that small bronchi and primary bronchioli of both early and advanced pneumonic lesions showed proportionally the greatest increase in thickness and were significantly thicker than that of control animals. The effects of this change are likely to be a maldistribution of ventilation or hypoventilation due to changes in luminal volume. Maldistribution of ventilation is the result of localised uneven changes in lung compliance and airway resistance. Alveoli with decreased compliance will take a smaller volume of gas than normal alveoli. If severe, these functional disturbances may be sufficient to impair gaseous exchange, leading to hypoxemia (Robinson and Gillespie, 1975). Williams (1971) demonstrated that hypoxia can be caused by hypoventilation and that abnormal ventilation-perfusion relationships are the most important cause of hypoxemia. When the total lung

capacity and functional residual capacity are reduced the affected animal will need a larger amount of muscular effort to overcome increased lung elastic recoil during breathing (Robinson and Gillespie, 1975). The increased respiratory work required by animals with these changes may be a major contributing factor to poor growth rates and decreased food conversion efficiecy observed by Jones; et al. (1982a) and Alley, (1985).

4.2 SUBMUCOSAL GLANDS

Little attention has been previously paid to changes in the tracheobronchial airways and submucosal glands of sheep with CNP. Nevertheless, submucosal gland enlargement is considerable (Chapter 3) and it is possible that increased mucus production plays a role in the pathology and clinical syndromes of the disease (Alley and Clarke, 1979; Pirie, 1979).

Calculation of the ratio of mucous gland thickness to bronchial wall depth has been accomplished in porcine enzootic pneumonia (Baskerville, 1972) and human chronic bronchitis (Reid, 1960). In both these investigations, measurement was made by use of the Reid index. This technique can be criticised on several grounds: (1) It will be influenced by crenation of the tracheobronchial mucosa which is frequently present. (2) There are very few places where mucosa and cartilage are parallel. (3) It takes no account of the large quantity of glandular tissue situated deep or lateral to the cartilage (Dunnill, 1982). (4) It provides no information on whether the increase was in the actual glandular acini or in the non-acinar interstitial tissue of the gland.

It was for these reasons that more detailed methods of measurement were developed by Restrepo and Heard, (1963); Hale; et al. (1968); Dunnill; et al. (1969) which aimed at measuring the total percentage of bronchial wall occupied by glandular tissue. These methods require that a standard bronchus be used for each measurement and that the adventitial margin of the bronchus be clearly defined. Dunnill; et al. (1969) used a point counting method to measure the proportion of various structures within the bronchial wall.

The present investigation aimed not only at assessing the presence and degree of submucosal gland enlargement but sought to identify the type of tissue involved and its distribution at various levels of the tracheobronchial airways. To collect this amount of information it was necessary to use a summagraphic digitiser and a microcomputer to process and store the data.

The submucosal glands of the respiratory airways are of much greater significance than mucosal goblet cells in producing mucus. It has been estimated that normal human goblet cells account for only 2% of mucus production in the tracheobronchial tree (Dunnill, 1982). Submucosal gland hypertrophy has been reported in chronic bronchitis and cystic fibrosis of man (Reid, 1978b) and experimental enzootic pneumonia of pigs (Baskerville, 1972). It has also been produced experimentally in a number of laboratory animals as a model for human disease such as chronic bronchitis and cystic fibrosis (Reid, 1978).

The results obtained in the present study have shown that in normal sheep the number of acini together with the area of the total gland and both its acinar and non-acinar components decreased gradually from the upper trachea to the intrapulmonary bronchi. This indicates that the glands gradually became smaller and contained fewer acini as the peripheral airways were approached. Despite this, the submucosal glands at all levels had the same proportion of gland area to acinar area with the exception of the upper trachea where the glands had more non-acinar tissue than elsewhere. Because the total acinar area of the upper trachea submucosal glands was significantly larger than elsewhere, it could be expected that each submucosal gland in the upper trachea would produce a greater volume of mucus than the lower parts of the bronchial tree. Since the trachea is the closest part to the external environment, it is likely to be subjected to more irritants than the peripheral parts. A large volume of mucus may therefore be necessary to trap and dilute noxious particles which have an irritant effect on sensory nerve endings(Cheville, 1983). The stimulation of the tracheal parasympathetic nerve endings induces the cough reflex, which removes debris by forcing it to the pharynx where it will be swallowed.

Although, the present study has shown that normal submucosal glands in the sheep have variable numbers of acini at different

tracheobronchial levels, the area means of a single acinus at any level were similar. In sheep with early pneumonic lesions, however, there was no significant difference in the number of acini in the submucosal glands at different levels. Detailed examination of the data revealed that this was due to a decrease in the number of acini in the upper tracheal glands and concomitant increase in the number of acini in the glands at all the lower levels. Further confirmation of hyperplastic and perhaps hypertrophic changes in the submucosal glands was provided by the data showing an increase in the total gland acinar and non-acinar areas. These areas increased to a point at which there were no significant differences between levels as there was in normal animals. An exception to this occurred in the upper trachea where there was a slight but nonsignificant decrease in gland area compared to normal sheep.

The data obtained from advanced pneumonic lesions showed there was severe submucosal gland enlargement at all levels. The enlargement in the gland area was due to both hypertrophy of the submucosal glands and an increase in the number of acini. These changes occurred at every level, including the upper trachea.

The comparative statistical analyses of submucosal gland parameters showed that the most significant changes in tracheobronchial airways of early pneumonic sheep occurred in the intrapulmonary bronchi. In this region there were increases in the number of acini,the total gland area, and both acinar and non-acinar components. The area occupied by submucosal glands and glandular acini in early pneumonic sheep was twice that of normal animals. These two observations were enough to confirm that hyperplastic and hypertrophic changes were occurring simultaneously. These changes were more severe in sheep with advanced lesions where they also involved extrapulmonary bronchi.

The role of infection as an initiator of chronic bronchitis in man is uncertain and a direct causal relationship between infection and bronchial gland hypertrophy has never been established in either humans or experimental animals. In the pig, however, it has been found that experimental enzootic pneumonia is associated with submucosal gland hypertrophy (Baskerville, 1972). This was the first naturally occurring lung disease in animals in which the gland changes

of chronic bronchitis were recognised as being related to infection. The current quantitative investigation and the previous morphological evidence (Chapter 3) have confirmed that similar changes occur in sheep and that these are related to the presence of infectious agents.

The nature of the mechanism by which submucosal gland enlargement occurs remains uncertain. The possibility of a direct irritant effect producing hyperplasia seems unlikely as there was no evidence of invasion of the gland or the respiratory mucosa by organisms. Jones; et al. (1975) suggested that bronchial gland hypertrophy which occurred in the experimental enzootic pneumonia of the pig may be a reflex response to a local stimulus acting either directly on the overlying bronchial epithelium or in the peripheral region of the lung. Since it is now well established that enzootic pneumonia in pig is caused by Mycoplasma hyorhinis, these authors suggested that hydrogen peroxide produced by these organisms may act as an irritant to receptors within the airways. It has also been observed that the administration of chemicals such as isoprenaline to rats may cause tracheal submucosal gland hypertrophy (Sturgess and Reid, 1973). Jones; et al. (1975) proposed that an increase in activity, from any cause, leads to an increase in the size of the gland, in other words "work" hypertrophy may lead to cell hypertrophy and eventually to cell hyperplasia.

The present study represents the first quantitative experimental work carried out in sheep respiratory tract. It has succeeded in measuring and statistically assessing the differences in the thickness of mucosal layers and submucosal gland areas. In addition, detailed information on the differences which occurred in early and advanced pneumonic lesions has been obtained. Although the methods used in the present study were relatively sophisticated , tracing the submucosal glands twice onto paper in addition to the delination by summagraphic digitiser was time consuming and the use of more advanced methods of staining and image analysis are recommended for future studies of this type.

CHAPTER 5

TYPES AND DISTRIBUTION OF GLYCOPROTEINS IN THE TRACHEOBRONCHIAL SUBMUCOSAL GLANDS

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1.0 INTRODUCTION

Respiratory mucus may come from two anatomical sources; the secretory cells of the surface epithelium and those of the submucosal glands (Reid, 1963; Jones; et al. 1975). The majority of mucus, however, comes from submucosal glands (Chapter 4).

Present knowledge of the varieties of glycoprotein synthesised by secretory cells is based on their intracellular identification by histochemical techniques. No abnormal type of glycoprotein is found in disease, but there may be striking changes in the proportion of the various secretory cell types. Histochemical analysis has provided information on the changes which occur in diseases such as chronic bronchitis in man (Dunnill, 1982) and enzootic pneumonia in the pig (Jones; et al. 1975). These changes may also be induced experimentally in response to irritation (Lamb and Reid, 1968; Jones; et al. 1973), certain drugs (Sturgess and Reid, 1973; Baskerville, 1976; Kleinerman; et al. 1976) or enzymes such as pancreatopeptidase (elastase) (Christensen; et al. 1977). Such experiments were undertaken to provide animal models for human disease and allow the study of gland and goblet cell changes experimentally.

The work presented in this chapter is the result of investigations of the histochemical nature of glycoproteins in the tracheobronchial submucosal glands of normal sheep and sheep affected with early and advanced stages of CNP.

2.0 MATERIALS AND METHODS

2.1 COLLECTION OF TRACHEOBRONCHIAL SPECIMENS

The lungs and trachea were collected from 18, 5 to 9 month old lambs slaughtered at a local meat works: six were normal, six showed

early CNP lesions and six showed advanced CNP lesions. Each lung was subsequently examined histologically and all fulfilled the requirement for each group (Chapter 3). Fixation and selection of levels for examination were similar to those described previously (Chapter 2), although only the first 4 levels were involved in this study. Each tracheal ring was cut into four pieces, washed in PBS, processed and embedded routinely in paraffin. They were then cut at 4 to 5 um and stained with H&E and AB pH 2.6-PAS techniques (AB-PAS) (Appendix 5.1). Because of the variable distribution of secretory cell types in the ovine tracheobronchial airways four different levels of airway were selected for study.

2.2 STAINING TECHNIQUES

Alcian blue at pH 2.6 periodic acid Schiff (AB-PAS) was used as the basic histochemical reaction. Alcian blue(a water soluble copper thalocyanin) stains acidic, weakly sulphated or acidic, non-sulphated mucoglycoproteins, when used at pH 2.6-3.0 (Bancroft and Stevens, 1975; Culling, 1974). The PAS reaction stains aldehydes as a result of oxidation; a red colour (Culling, 1974). By the combination of these two techniques in one method (Appendix 5.1), carboxylated acid mucopolysaccharides, neutral and mixed mucins can be differentiated (Lamb and Reid, 1972; Jones and Reid, 1973a & b).

Five categories of staining response were recognised within cells; blue, blue-red, red, red-blue and purple. These colours indicated a different intensity of staining and were used to delineate the area distribution of each type of glycoprotein. Two cell types stained with only one of the two stains used,either blue or red, and the intermediate groups were then subdivided according to which of the two stains predominated.The interpretation of the intermediate staining response was similar to that described by Jones; et al. (1975). Briefly, after AB pH 2.6-PAS, the blue region of the gland contains acid glycoprotein only; the blue-red region virtually all acid glycoprotein; the red region contained neutral glycoprotein only and red-blue practically all neutral glycoprotein. In the blue-red area a trace of redness showed the presence of a few neutral radicles, but since the amount was minimal it was accepted that most of a cell staining in this way contained the acid group. The same procedure was followed in the red-blue area but in this case it was assumed that

most of a cell staining red-blue contained the neutral group. The fifth colour category; purple, was assumed to contain equal amounts of acid and neutral glycoprotein mixed together in one glandular cell (Bancroft and Stevens, 1975).

2.3 QUANTITATION

The proportion of the differentially staining submucosal glands were estimated by a point-counting technique using a Zeiss integrating eyepiece with 25 points (Dunnill, 1968). The position of each point in the eyepiece was recorded according to the constituent in which it lay on the histological section. A hand-operated counting device of the type used for differential blood counts was used to record the results of each estimate. To more accurately differentiate the histochemically produced colours, an oil immersion high power objective (X100) and(X10) ocular were used. Sixty four fields giving 1600 possible hits were surveyed for each level. The area of each field was 0.06 mm^2 and the total area surveyed for each level was 3.84 mm^2. The result for any stain or colour was expressed as a percentage of the total area of mucous cells examined and analysed statistically by the same methods previously used to analyse morphometric data (Chapter 4).

3.0 RESULTS

3.1 TYPE AND DISTRIBUTION OF GLYCOPROTEINS AT EACH LEVEL

3.1.1 NORMAL SHEEP

Most mucous cells in the normal ovine tracheobronchial submucosal glands stained either red-blue or purple. Very few cells stained blue, blue-red or red with the combined AB-PAS technique. It was concluded that they were mostly non-alcianophilic to some degree and thus contained neutral glycoprotein. With these techniques, the ovine tracheobronchial mucosal glands presented a variegated appearance due to different types of glycoprotein. Cells with similar staining features were often grouped together, although adjacent cells sometimes differed widely.

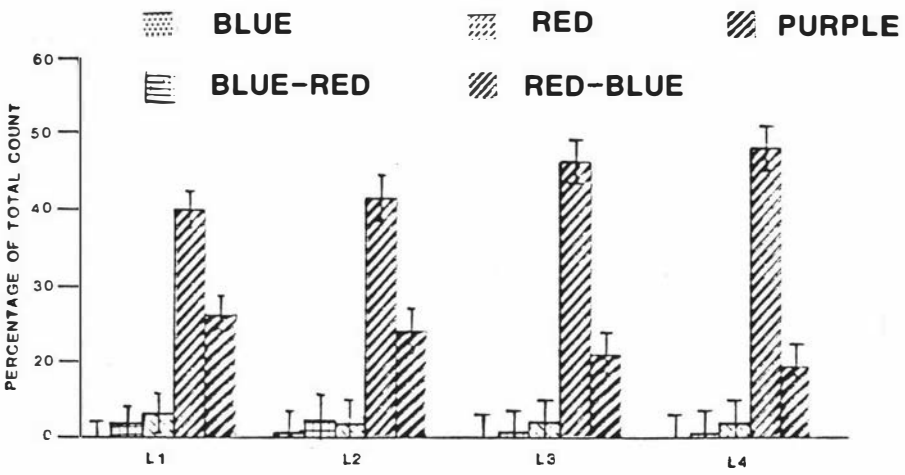
Statistical analyses of the number of positive counts at each level revealed that the red-blue and purple stained glycoprotein content of submucosal glands were significantly ($P < 0.01$ and $P < 0.05$ respectively) greater than other glycoproteins at each level. There were no significant differences between the blue, blue-red and red stained glycoproteins (Fig. 5.1A & Appendix 5.2). The red-blue stained glycoprotein comprised 60-70 % and the purple 19-24 % of total glycoproteins in the glands at each level (Fig. 5.1B).

When the data for each type of the glycoprotein was analysed statistically, it was found that the red-blue and purple group varied most significantly ($P < 0.001$ and $P < 0.01$ respectively) between levels (Fig. 5.1B & Appendix 5.3). Duncan's test showed that the blue and blue-red stained glycoproteins were significantly ($P < 0.05$) greater in the submucosal glands of the lower trachea than of other levels, while the red group was not significantly different. The submucosal glands of bronchi contained significantly ($P > 0.05$) more red-blue and less purple than the upper trachea which in turn contained significantly ($P > 0.05$) more purple than the other glycoproteins (Fig. 5.1B & Appendix 5.4). Figure 5.1C illustrates how the red-blue stained glycoproteins increased gradually and the purple stained group decreased gradually from the upper trachea to the intrapulmonary bronchi.

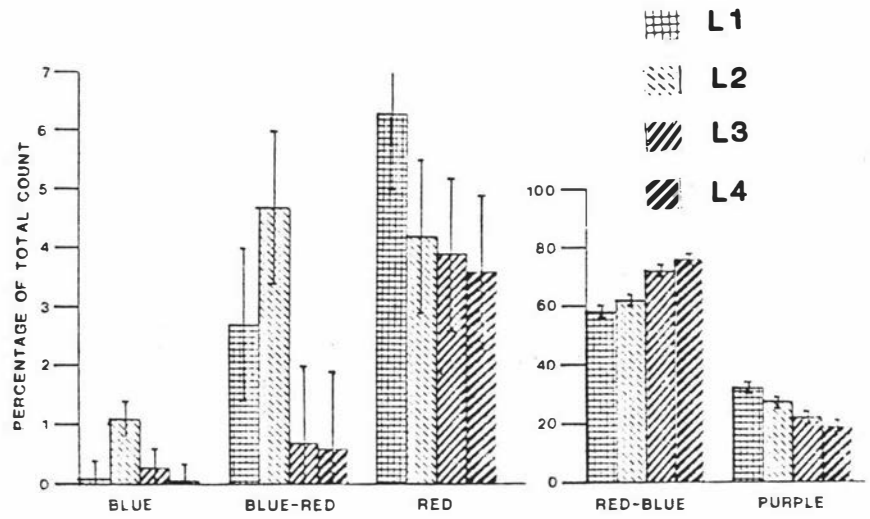
In order to simplify the analysis, the glycoproteins were divided into 3 groups. The blue and blue-red staining groups were added together (acid glycoproteins), the red group was added to the red-blue (neutral glycoprotein). These were compared statistically with the purple group (mixed glycoprotein). This analysis showed that there were significant variations between levels (Fig.5.1C & Appendix 5.5). The submucosal glands of the bronchi contained significantly ($P < 0.05$) more neutral glycoprotein than those of the trachea. In contrast the mixed glycoproteins were significantly ($P < 0.05$) decreased in the submucosal glands of bronchi when compared to the trachea. The submucosal glands of the lower trachea contained significantly ($P < 0.05$) more acid glycoprotein than those of the intrapulmonary bronchi (Fig. 5.1C & Appendix 5.3).

Figure 5.1

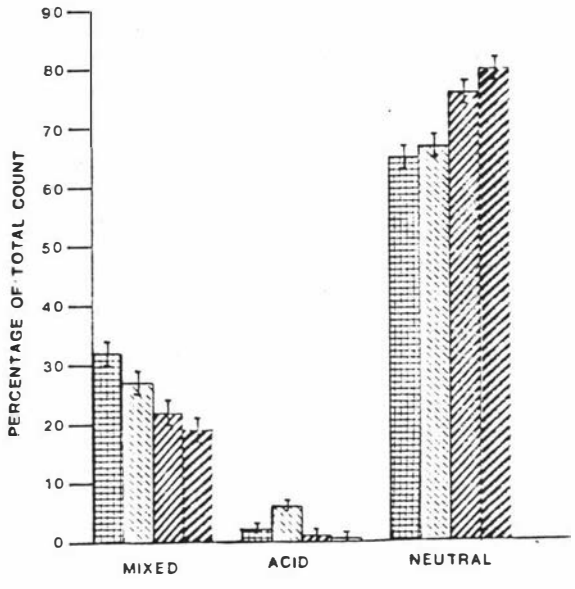
Statistical analysis of the different types of glycoprotein at each level (A) and between levels (B) of normal tracheobronchial airways. In C the categories of glycoprotein are simplified into mixed, acid and neutral.


BLUE
RED
PURPLE
BLUE-RED
RED-BLUE
PERCENTAGE OF TOTAL COUNT
60
50
40
30
20
0
L1
L2
L3
L4

A


L1
L2
L3
L4
PERCENTAGE OF TOTAL COUNT
7
6
5
4
3
2
1
0
100
80
60
40
20
0
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE

B


PERCENTAGE OF TOTAL COUNT
90
80
70
60
50
40
30
20
10
0
MIXED
ACID
NEUTRAL

C

3.1.2 EARLY PNEUMONIC LESIONS

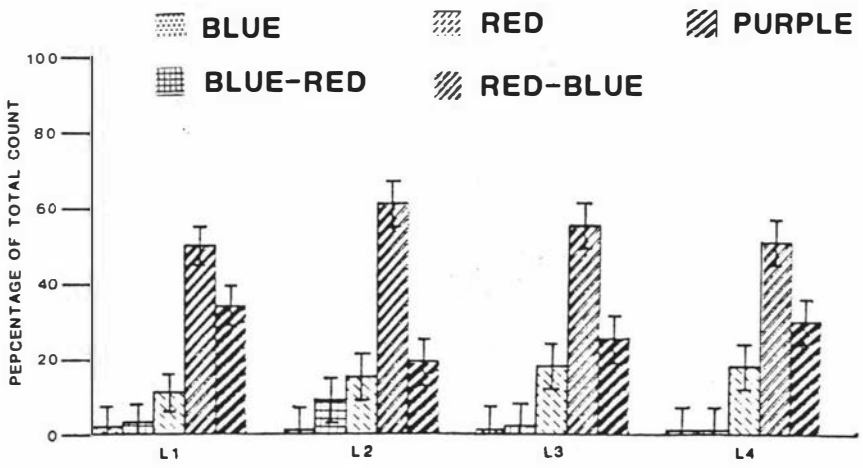
There was a highly significant ($P < 0.001$) overall relationship between different types of glycoprotein at each level throughout the tracheobronchial tract of sheep with early pneumonic lesions (Fig. 5.2A). Duncan's test indicated that in the upper trachea the red-blue and purple stained glycoproteins groups were present in significantly ($P < 0.05$ and $P < 0.01$) larger amounts than the other glycoproteins. No significant differences was found between the remaining groups (Fig. 5.2A & Appendix 5.6). In the lower trachea, the red-blue staining glycoprotein group was once again present in signfficantly ($P < 0.01$) greater amounts than the others. The purple group was significantly ($P < 0.05$) greater in amount than the blue group which in turn showed no significant difference from the red and blue-red stained groups (Fig. 5.2A & Appendix 5.6). At the extra- and intrapulmonary bronchi the submucosal glands contained significantly ($P < 0.01$) greater amount of red-blue staining glycoprotein while the purple group was significantly ($P < 0.05$) more extensive than the blue and blue-red groups (Fig. 5.2A & Appendix 5.6).

When the amount of each glycoprotein was compared between levels, there were no significant differences (Appendix 5.7). However, Duncan's test showed that the submucosal glands of the lower trachea contained significantly ($P > 0.05$) more blue-red stained glycoprotein than the upper trachea. The upper trachea in turn contained significantly ($P < 0.05$) more purple glycoprotein than the lower trachea and extrapulmonary bronchi (Fig. 5.2B).

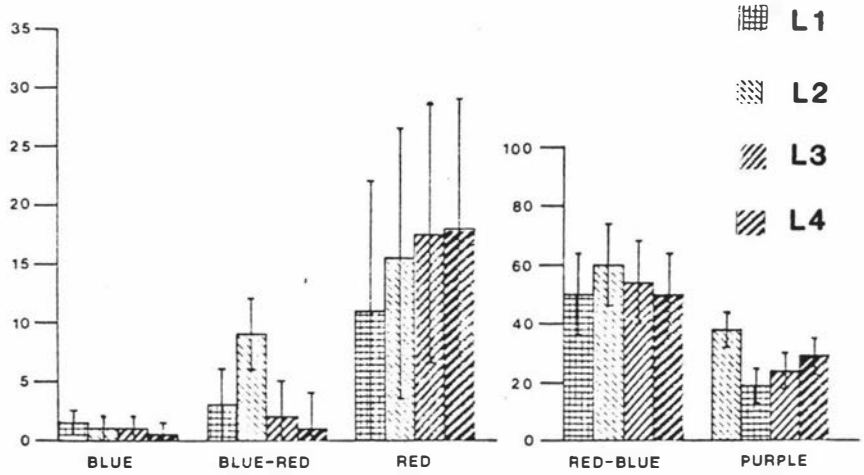
However, when the categories of glycoproteins were simplified into acid (blue and blue-red), neutral (red and red-blue) and mixed (purple), it was found that there were no significant differences between the amount present in each of these groups at different levels (Fig. 5.2C & Appendix 5.8). Duncan's test indicated that the submucosal glands of the lower trachea contained significantly ($P < 0.05$) more neutral glycoprotein than the upper trachea and that of the upper trachea contained significantly ($P < 0.05$) more purple glycoprotein than the lower trachea (Fig. 5.2C).

Figure 5.2

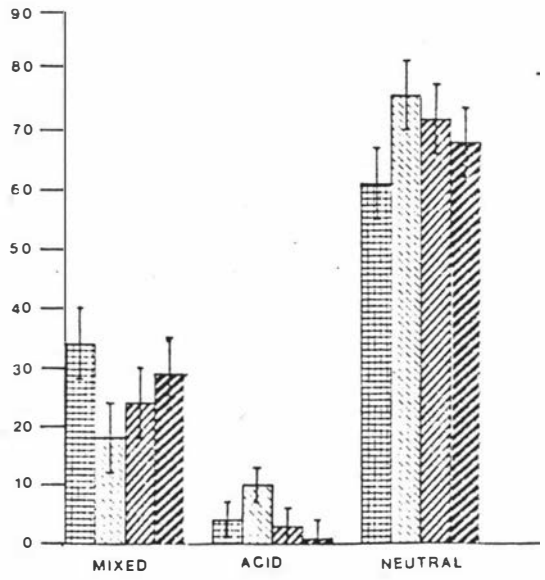
Statistical analysis of the different types of glycoprotein at each level (A) and between levels (B) of the tracheobronchial airways of the early pneumonic group. In C the categories of glycoprotein are simplified into mixed, acid and neutral.


BLUE
RED
PURPLE
BLUE-RED
RED-BLUE
PEPCENTAGE OF TOTAL COUNT
100
80
60
40
20
0
L1
L2
L3
L4

A


L1
L2
L3
L4
35
30
25
20
15
10
5
0
100
80
60
40
20
0
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE

B


90
80
70
60
50
40
30
20
10
0
MIXED
ACID
NEUTRAL

C

3.1.3 ADVANCED PNEUMONIC LESIONS

Statistical analysis of the data obtained from advanced pneumonic lesions revealed that red-blue and purple staining glycoproteins were present in significantly ($P < 0.01$) greater amounts than other glycoproteins. All other types of glycoprotein showed no significant differences at any level. However, the red-blue group was significantly ($P < 0.01$) more widely distributed than the purple glycoprotein (Fig. 5.3A & Appendix 5.9).

When the amount of each type of glycoprotein present was compared between levels, it was found that there were no significant differences (Appendix 5.10). Further statistical analysis using Duncan's test found that the submucosal glands of the upper trachea contained significantly ($P < 0.05$) more blue-red glycoprotein than the other levels. In addition, the glands of the lower trachea contained more red glycoprotein than the intrapulmonary submucosal glands (Fig. 5.3B).

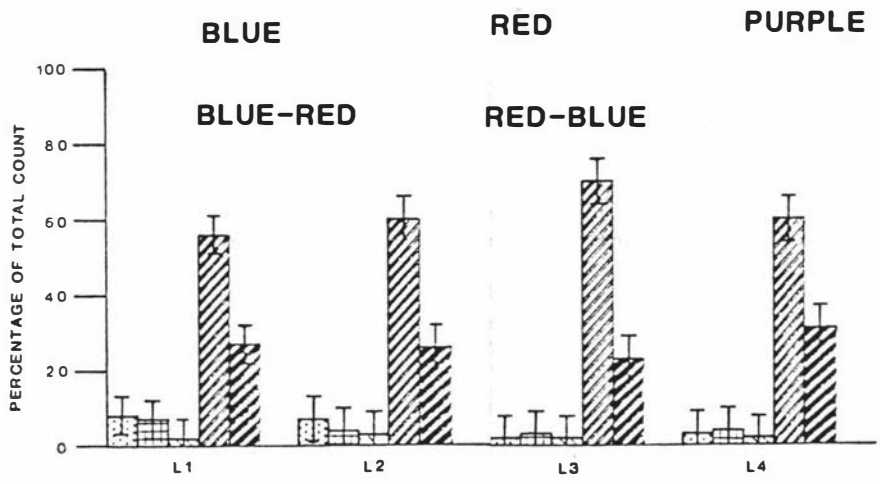
Simplification of categories into acid (blue and blue-red), neutral (red and red-blue) and mixed (purple) revealed that there were no significant differences in the amount of each type of glycoprotein present between different levels (Fig. 5.3C & Appendix 5.11).

3.2 COMPARATIVE STATISTICAL ANALYSIS

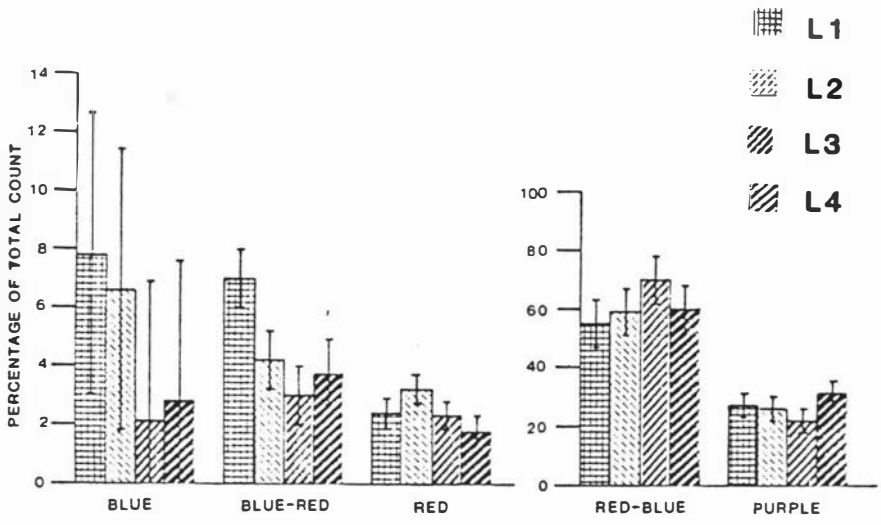
When the glycoprotein content of the submucosal glands of normal lungs were compared to the lungs from sheep with early and advanced CNP lesions, the results showed that there were no significant differences between the groups at the upper trachea, lower trachea and extrapulmonary bronchi levels (Fig. 5.4A,B,C & Appendix 5.12). Further statistical analysis using Duncan's test showed that the submucosal glands of the upper trachea from early pneumonic sheep contained significantly ($P < 0.05$) more red glycoprotein than normal lungs and advanced pneumonic lesions (Fig. 5.4A). At the intrapulmonary bronchi, the submucosal glands of early and advanced pneumonic lesions contained significantly ($P < 0.05$) more blue-red glycoprotein than that of normal lungs (Appendix 5.12). In addition, it was found that purple glycoprotein was present in significantly (P

Figure 5.3

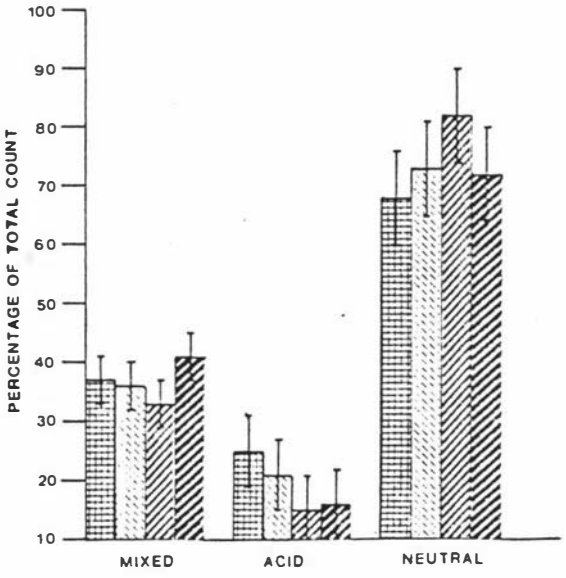
Statistical analysis of the different types of glycoprotein at each level (A) and between levels (B) of the tracheobronchial airways of the advanced pneumonic groups. In C the categories of glycoproteins are simplified into mixed, acid and neutral.


BLUE
RED
PURPLE
BLUE-RED
RED-BLUE
PERCENTAGE OF TOTAL COUNT
100
80
60
40
20
0
L1
L2
L3
L4

A


L1
L2
L3
L4
PERCENTAGE OF TOTAL COUNT
14
12
10
8
6
4
2
0
100
80
60
40
20
0
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE

B


PERCENTAGE OF TOTAL COUNT
100
90
80
70
60
50
40
30
20
10
MIXED
ACID
NEUTRAL

C

< 0.05) greater amounts in both early and advanced CNP lesions when compared to normal lungs (Fig. 5.4D).

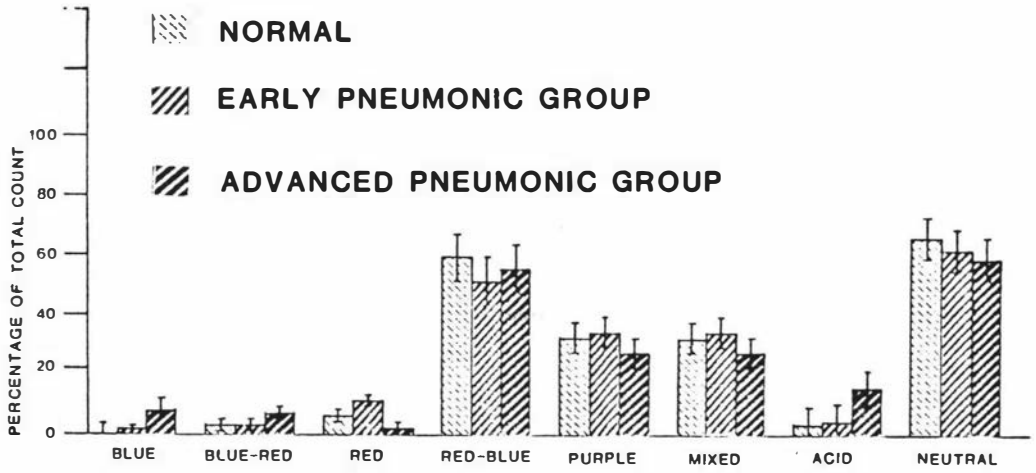
Grouping of glycoproteins into acid (blue and blue-red), neutral (red and red-blue) and mixed (purple) showed that in the intrapulmonary bronchi the acid glycoprotein was significantly ($P < 0.05$) more widely distributed, while neutral glycoprotein was significantly ($P < 0.05$) decreased in advanced lesions compared to normal and early pneumonic animals (Fig. 5.4D & Appendix 5.13). Using Duncan's test for further analysis it was found that the mixed glycoprotein from glands of both early and advanced CNP lesions were significantly ($P < 0.05$) increased when compared to normal lungs (Fig. 5.4D).

Analysis of the total number of positive reactions of the glycoproteins between levels indicated that the level had significant effect ($P < 0.01$) on the number of cells in the normal group. However, there was no indication of similar effects in either the early or advanced CNP groups. Duncan's test showed that the total number of active cells in submucosal glands of the upper trachea was significantly ($P < 0.05$) higher than that from other levels. In the normal sheep there was a progressive decrease in the total active cells per gland area from the upper trachea to the intrapulmonary bronchi in the normal sheep (Appendix 5.12) but no such pattern was observed in the early and advanced pneumonic groups.

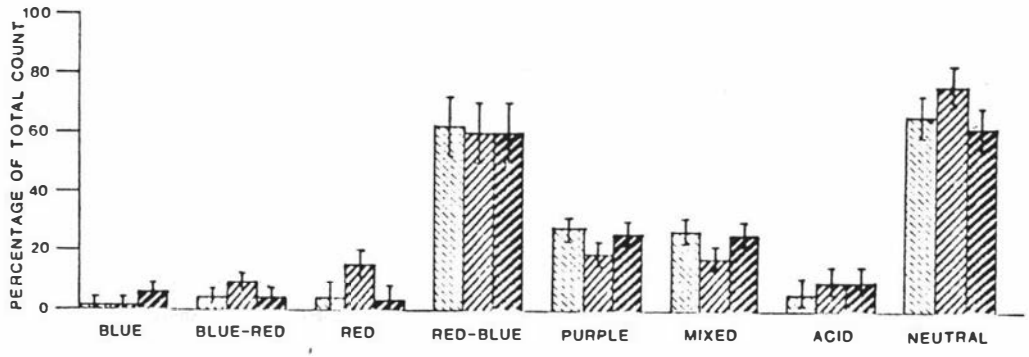
To study the total active cell numbers per level between normal and pneumonic groups , the data from the three groups was fitted into the computer model. The results indicated that there were significant differences between the groups at the upper trachea ($P < 0.01$) and intrapulmonary bronchi levels ($P < 0.001$). However, Duncan's test showed that the upper trachea submucosal glands of the normal and the advanced pneumonic animals contained significantly ($P < 0.05$) more active cells per gland area than those of the early pneumonic group. In the intrapulmonary bronchi, submucosal glands from both the early and advanced CNP lesions contained significantly ($P < 0.05$) more active cells per gland area than those of normal sheep (Appendix 5.12).

Figure 5.4

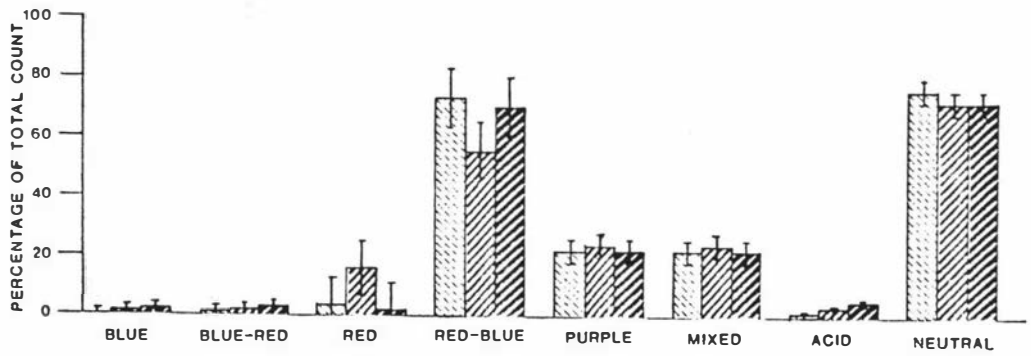
A comparative statistical analysis of the different types of glycoprotein in normal, early pneumonic and advanced pneumonic groups at upper trachea (A), lower trachea (B), extrapulmonary bronchi (C) and intrapulmonary bronchi (D).


NORMAL
EARLY PNEUMONIC GROUP
ADVANCED PNEUMONIC GROUP
PERCENTAGE OF TOTAL COUNT
0
20
40
60
80
100
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE
MIXED
ACID
NEUTRAL

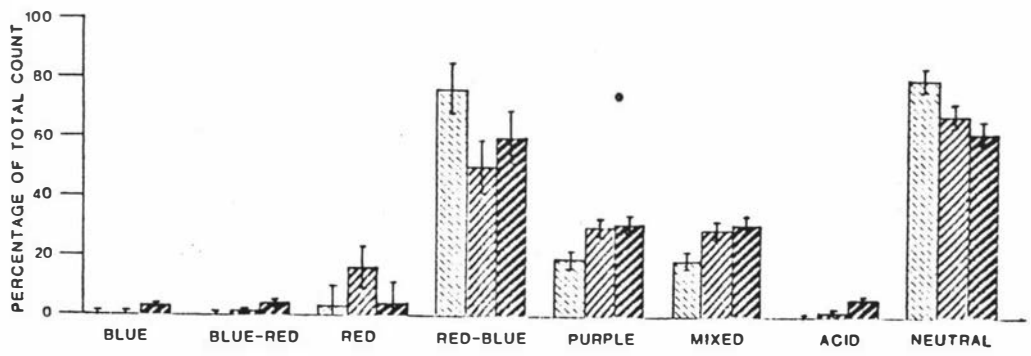
A


PERCENTAGE OF TOTAL COUNT
0
20
40
60
80
100
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE
MIXED
ACID
NEUTRAL

B


PERCENTAGE OF TOTAL COUNT
0
20
40
60
80
100
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE
MIXED
ACID
NEUTRAL

C


PERCENTAGE OF TOTAL COUNT
0
20
40
60
80
100
BLUE
BLUE-RED
RED
RED-BLUE
PURPLE
MIXED
ACID
NEUTRAL

D

4.0 DISCUSSION

Since no detailed histochemical studies of the glycoproteins in tracheobronchial submucosal glands of sheep have previously been reported, the current investigation was concerned primarily with the distribution of the three main types; acid, mixed and neutral. Acid glycoprotein was present in very small amounts, while the neutral group was the most abundant in normal and pneumonic sheep. The histochemical technique(AB pH 2.6-PAS) used in this study has been used previously to identify glycoproteins in a variety of epithelial tissue sites (Jones and Reid, 1973 a; b; Jones; et al. 1975). It offers a relatively simple means of indentifing and analysing changes in the distribution of glycoproteins, both within a cell and cell population (Jones; et al. 1973).

The mixed glycoprotein was classified as a group in the present study because it stained a distinctively homogeneous purple colour, differentiating it from the blue-red or red-blue groups. This glycoprotein was found in every level of normal and pneumonic ovine tracheobronchial airways, in proportionally higher amounts than acid glycoprotein. At only one level of the pneumonic tracheobronchial tract it was found in significantly greater amounts than normal; the intrapulmonary bronchi.

The types of glycoprotein found in the submucosal glands of the normal ovine tracheobronchial airways were similar to those of both man (Lamb and Reid, 1969b) and the pig (Jones; et al. 1975), with the exception that the glands from sheep contained mixed glycoprotein and more neutral glycoproteins. The study of the distribution of these substances at different levels of the tracheobronchial tree of normal sheep showed that towards the peripheral airways neutral glycoprotein increased to more that 80% of the total, while acid and mixed types decreased.

In pneumonic sheep the total glycoprotein content of the submucosal glands decreased slightly below normal in the upper trachea but increased greatly above normal in the intrapulmonary bronchi. The most likely explanation for these changes is that they were caused by mucosal cell enlargement and hyperplasia or hypertrophy of submucosal glands. The distribution of the glycoprotein types in glands from

pneumonic sheep was different from that of the control animals. Quantitative analysis of the mucous cells showed that in the glands from pneumonic sheep the percentage of the acid glycoprotein was increased, when compared to normal sheep.

Change in the proportion of cells producing acid and neutral glycoproteins has been reported with goblet cell hypertrophy and hyperplasia in rat respiratory airways after approximately 3 weeks exposure to irritants such as sulphur dioxide (Reid,1963; Lamb and Reid, 1968) and tobacco smoke (Lamb and Reid, 1969a). Secretory cells of the rat airway surface may contain granules of neutral glycoprotein or of acid glycoprotein, or a mixture of these granules (Jones; et al. 1973). When these authors exposed rat airways to tobacco smoke, the regional difference in the secretory cell types observed in normal rats was lost. They found that within 24 hr of exposure to the irritant the cell type containing a mixture of acid and neutral granules was predominant throughout the airways. However, after six weeks of exposure most cells contained acid glycoprotein throughout the airways. Other workers have found that a chemical stimulus to the secretory cell population may also change the predominant acid glycoprotein cell type. Thus isoprenaline sulphate was found to increase the absolute number of cells containing acid glycoprotein in rat airway epithelium (Sturgess and Reid, 1973). Change from the predominant acid group has also been found in mucous cells in the hypertrophied bronchial submucosal glands of pigs with enzootic pneumonia experimentally induced by intranasal inoculation of M. hyorhinis (Jones; et al. 1975). Jones; et al. (1975) observed that the increase in gland size was accompanied by increase in the proportion of the area containing acid glycoprotein. In the present study the acid glycoprotein increased and neutral glycoprotein decreased significantly in submucosal glands of intrapulmonary bronchi from sheep with CNP lesions.

Change in the proportion of the glycoproteins is not clearly understood but it would seem to be a sensitive marker of damage to respiratory epithelium, since it may also occur in goblet cell hypertrophy without any increase in cell numbers (Jones; et al. 1973). However, Jones and Reid (1978) postulated that in the bronchioli, irritation causes a transition from one secretory cell type to another; from Clara to serous and from serous to mucous

cells. An individual cell can quickly "switch on" organelles that lead to development of a secretory cell or "switch over" from the production of one glycoprotein type to another. These changes affect both the original population and new secretory cells that are developing and may result from conversion or mitosis (Jones and Reid, 1978). Baskerville (1976) and Jones (1978) observed that on withdrawal of the stimulus these steps may be retraced at various speeds, depending on the time of exposure, to return to the original state.

The reason for change in the proportion of glycoprotein types within a cell population is not clearly understood (Jones; et al. 1975). A cell may contain the enzymes necessary for the production of a wide range of glycoproteins. Quantitative studies in the rat have shown that a single goblet cell may produce both neutral and acid glycoproteins (Jones; et al. 1973). The same is true of mucous and serous cells of the human bronchial glands (Lamb and Reid, 1969c; 1970).

The proportion of glycosyltransferases within mucous secreting cells is thought to be an important factor governing glycoprotein synthesis (Boat & Cheng, 1980). Baker; et al. (1975) demonstrated an increase in glycosyltransferases in microsomal fractions from cells prepared from respiratory tract tissue showing goblet cell and submucosal gland hyperplasia, in dogs exposed to sulphur dioxide. Degand; et al. (1973) suggested that an increase in galactosamine (a carbohydrate side chain of the glycoprotein) leads to an increase in the length of the glycan chains and thus to a possible increase in the number of sites available for sulphation of the glycoprotein. It has also been proposed that the increase in sulphomucin observed in the cells of the hypertrophied bronchial glands of the pig is secondary to the synthesis of galactosamine (Jones; et al. 1975).

While a change in the type of glycoproteins may be the result of an increase in the rate of biosynthesis, any change would appear to be independent of its rate of discharge from cells. It seems that where there is no stimulus to increase production, discharging cells produce an acid glycoprotein but where there is such a stimulus, production of neutral glycoprotein is favoured (Jones; et al. 1975). In the rat, it has been observed that there is a shift to sulphated glycoprotein

without an increase in the rate of mucous discharge from goblet cells, after exposure to tobacco smoke to which phenylmethyloxadiasole (PMO) [an anti-inflammatory agent] has been added (Jones; et al. 1973c) or after isoprenaline (an antispasmodic agent) (Jones; et al. 1972) was added. However, organ culture studies have shown that, unlike isoprenaline; pilocaprine (cholinergic and parasympathominetic agent) increases secretion from bronchial glands (Sturgess and Reid, 1972a & b). In in vivo studies in the rat, epithelial goblet cells appeared to be empty of secretion and increased in number following pilocaprine administration. The increased number of cells contained both neutral and acid glycoproteins (Sturgess and Reid, 1973). After the administration of benzylamine (Bisolvon) a loss of goblet cells secreting acid glycoprotein, particularly sulphomucin, has been associated with an increase in the rate of mucous cell discharge (Janatuinen and Korhonen, 1969). Infections which lead to bronchial hypertrophy such as experimentally produced porcine enzootic pneumonia, also appear to stimulate goblet cell discharge with a reduction in acid glycoprotein and an increase in neutral glycoprotein production (Jones; et al. 1975).

Although no attempt was made in the present study to identify the types of acid glycoprotein, the distribution of the histochemical types of mucin were similar throughout the ovine tracheobronchial airway. This finding is similar to that in man (Lamb and Reid, 1970; Jones and Reid, 1969c) and the pig (Jones; et al. 1975).

The normal function of the mucociliary system relies on the integrity and activity of the cilia, the presence of a preciliary layer and the physical properties of the tracheobronchial mucus layer (Lopez-Vidriero and Reid, 1978). The visco-elastic properties of mucus are determined by the structure of bronchial glycoprotein and the concentration of the various types of macromolecules and their interaction (Boat and Cheng, 1980). The increase in viscosity of mucus in humans with chronic bronchitis has been related to an increase in N-acetylneuraminic acid content (Keal and Reid, 1972). Other macromolecules (such as lysozyme, albumin and IgA) and the fucose content of mucus, are also correlated with its viscosity (gelation) (Boat and Cheng, 1980). Increase in the number of mucous cells, their extension to peripheral airways and gland hypertrophy are the characteristic pathological changes found in chronic bronchitis of

man (Dunnill, 1982) and experimentally induced porcine enzootic pneumonia of pigs (Baskerville, 1972). Extension of epithelial mucous cells to more peripheral airways than normal and submucosal gland hypertrophy have also been observed in lambs with CNP (Chapter 3 and 4). Experimental studies with rigid branching tubes and various liquids having different visco-elastic properties have shown that the thickness of the liquid layer and its visco-elastic properties influence the resistance to airflow (Clarke, 1973). These experimental models represent a version of the in vivo condition and help in understanding the complexity of the dynamics of airflow and the effect of mucus and its viscosity on the airway performance.

It has been suggested that disulphide bonds of the glycoprotein play an important role in the rheological properties of the tracheobronchial mucus (Roberts, 1976). However, it has been established that the rheological properties of a substance are mainly responsible for transport thus glycoproteins with a high degree of cross-linking (disulphide bonds) may result in a decrease in the rate of mucus transport (Sade; et al. 1975).

The observations made on humans with airway obstruction and mucus hypersecretion have shown that the rate of the clearance of radioactive materials is increased when compared with the clearance rate of normal subjects (Thomson and Short, 1969). Excessive mucus in the trachea and large airways entraps more particles, and by increasing turbulence of the airflow, may also reduce penetration of particles into the lower respiratory tract. The rate of mucus transport is faster in large airways and the trachea than in peripheral airways (Lopez-Vidriero and Reid, 1978).

Variation in acidic properties not only influences the physical properties of the mucus as discussed earlier, but it also affects the virus-binding properties of mucus. Usually glycoproteins which are of a molecular weight larger than 60,000 and which contain more than 3.5% sialic acid will bind to influenza A or influenza B viruses and cause virus haemagglutination inhibition (Springer; et al. 1969). The shift to more acid glycoprotein production seen in the pneumonic sheep may therefore result in a more effective means of handling respiratory virus infections. However, Boat and Cheng (1976) have found that although sialic acid is required, acid content is not the sole

determinant of the virus haemagglutination inhibition properties of the respiratory mucus glycoproteins.

It can be concluded from this and previous studies that the tracheobronchial tree responds to various noxious agents by increasing the volume and changing the physical and chemical nature of its protective barrier; mucus. This protective barrier may ultimately become a cause of additional pathological problems (particularly in chronic disease). Reflux of mucus into alveoli may produce an irritant effect on the blood-air barrier, reduce gaseous exchange and perhaps, disturb the surface tension reducing properties of surfactant resulting in alveolar collapse. Excess mucus may also be an important contributing factor to airway obstruction, particularly in small bronchioli.

CHAPTER 6

OVINE TRACHEAL ORGAN CULTURE STUDIES

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1.0 INTRODUCTION

Many potential pathogens have been isolated from natural cases of CNP in sheep but little is known about their relative importance and the mechanisms by which these organisms mediate host cell injury in respiratory tissue.

A better understanding of the cellular and subcellular events which accompany infections with these organisms requires the establishment of experimental models which permit monitoring of the infectious process under carefully controlled conditions. The technique of tracheal organ culture allows this, and at the same time provides respiratory epithelial cells in a viable and differentiated state, thus serving as a highly sensitive host indicator system.

This chapter will examine the pathogenesis of M. ovipneumoniae, P. haemolytica, B. parapertussis and N. catarrhalis organisms on ovine tracheal organ cultures. All the strains of organism used were originally isolated from natural cases of CNP of lambs in New Zealand. Observation of the infected tracheal organ cultures was initially made with inverted light microscope to observe ciliary activity. Sequential histopathological examinations were subsequently made by light, transmission and scanning electron microscopes to correlate surface morphology with intracellular changes and to observe the attachment of the organisms. Sequential microbiological studies were also carried out to follow the growth of each organism in organ culture and correlate this with the ciliary and histological alterations.

2.0 MATERIALS AND METHODS

2.1 COLLECTION AND PREPARATION OF TRACHEAL RINGS

Ovine foeti varying in age from 4 to 5 months of gestation were

obtained from healthy, mature Romney and Romney-cross breeds of sheep killed by electrical stunning at a local meat works. The foeti were transported to the laboratory within 30 min. of collection while remaining in the excised uterus.The uteri were washed with 70% alcohol after which an incision was made and the foeti were dislodged from their amniotic sacs. Because of the necessity to avoid contamination careful preparation of the rings was critical, and, the protocol is therefore described in detail. The skin over the neck area was clipped finely with surgical scissors and an incision was made from the larynx to the sternum. Fat, connective tissue and muscle were cut and removed to expose the trachea and esophagus. The thin serosal membrane covering the trachea was cut and stripped and the trachea was gently lifted while a scalpel blade was slid beneath it to completely free it from the esophagus. As much of adventitia as possible was removed in situ, and the trachea then severed just below the larynx and immediately above the carina tracheae. The severed trachea was placed directly into a bottle containing prewarmed phosphate buffer saline (PBS) and moved to a laminar flow hood for further manipulation with sterile instruments and full aseptic technique.

The intact trachea was then removed from the PBS, placed in a 85 X 12 mm petri dish containing prewarmed culture medium, and stripped of remaining connective tissue with fine forceps and scissors. It was than placed in another 85 X 12 mm petri dish containing fresh prewarmed culture medium. One end of the trachea was clamped with artery forceps which were placed at an acute angle so that the trachea was held inside the open petri dish of medium. The loose end of the trachea was grasped with forceps and gently stretched to separate the cartilaginous rings. A single cut was made between, and parallel to, the first two cartilage rings with a no.20 scalpel blade. This left a free tracheal ring in the forceps, which in turn were used to place it in culture medium. This sequence was repeated until the entire trachea was sectioned into rings approximately 4 mm thick; each trachea yielding 20 - 28 usable rings. The rings were placed in a bottle containing culture medium and then shaken vigorously to release the mucus trapped on the mucosal surface. They were then removed and placed in 85 X 12 mm petri dishes to which was added culture medium. The rings were examined with an inverted microscope to ensure that the lumen was not occluded with tissue, mucus or cells and that the cilia were beating normally. Groups of two rings were then placed in 35 X

10 mm plastic petri dishes containing 3 ml of culture medium and maintained for 24 hr at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. The ciliary action was then observed again with an inverted, phase microscope and those tracheal rings showing strong ciliary activity were marked, regrouped and subsequently evaluated for changes in ciliary activity.

2.2 MEDIA

2.2.1 BLOOD AGAR MEDIUM

This consisted of two layers:

1. The salt base was prepared by dissolving 15 gm of Bacto-Agar (Difco) in 1 l of 0.5% sodium chloride solution. This was autoclaved for 15 minutes and 10 ml was poured into each 85 X 10 mm plastic petri dish.

2. The blood base prepared by dissolving 44 gm of sterilised Bacto-Columbia blood agar base (Difco) in 1 l of distilled water. When cooled to 45°C, 5% sterile defibrinated blood was mixed well with it and then 10 - 15 ml was poured over the salt base.

This medium was used for growing P. haemolytica, N. catarrhalis and B. parapertussis.

2.2.2 T199 MEDIUM

This consisted of 10 gm of TC 45 Tissue Culture Medium 199 (Wellcome) dissolved in 950 ml of deionised water. The medium was sterilised by filtration through a membrane with a pore size of 0.22 um. Sodium bicarbonate at the rate of 1.5 gm and 18 gm of tricine buffer was added. The medium was then left for 24 hr at 37°C to ensure that it was free of contamination. If any turbidity was observed the medium was discarded. This medium was used for all tracheal organ culture experiments with the exception of those involving M. ovipneumoniae.

2.2.3 MODIFIED FM4 LIQUID MEDIUM

The liquid modified FM4 medium in which M. ovipneumoniae was cultured was originally formulated by Frey; et al. (1968) and is detailed in Appendix 6.1. The yeast autolysate was omitted from the medium and swine serum and phyton were replaced to avoid cross-reaction with the organism (Major, 1976). The dry ingredients were dissolved in 1 litre of distilled water and supplemented with Eagle's vitamin solution. The medium was clarified by filtration through 0.45 um and 0.22 um pore size filters and subsequently sterilised by filtration through a sterile 0.22 um filter.This medium was used to grow M. ovipneumoniae but when used as an organ culture medium, serum was not added and thallium acetate was omitted from the preparation because of its toxicity to organ culture cells.

2.2.4 MODIFIED FM4 SOLID MEDIUM

To prepare FM4 solid medium, 1.9 gm of Bacto-agar (Difco) were added to 42 ml of brain-heart infusion broth (Difco). The mixture was autoclaved, cooled to 50°C and mixed with 200 ml of standard modified FM4 liquid medium.

Approximately 4 ml aliquots were then placed into 50 X 9 mm plastic petri dishes with tightly fitting lids. The solidified medium was either used immediatelly or stored at 4°C for use within 3 days. The stored medium was prewarmed for 1 - 2 hr at 37°C before use.

2.3 MICROORGANISMS

2.3.1 MYCOPLASMA OVIPNEUMONIAE

The isolate of M. ovipneumoniae used was strain 5 of the New Zealand isolates, kindly provided by Dr J.K.Clarke (Dept. of Microbiology and Genetics, Massey University). It was originally isolated from the lung of lambs with CNP in the Manawatu district. A Universal bottle containing approximately 10^9 CFU/ml organisms in 10 ml of modified FM4 medium was mixed with 1 litre of freshly prepared liquid medium and incubated at 37°C on a rotary shaker. When the colour of the phenol red indicator in the medium changed from red to turbid orange or turbid yellowish-orange this indicated that growth of

the culture had occurred. The mycoplasma suspension was then centrifuged at 16,000 R/sec in RC-5 superspeed refrigerated centrifuge (SORVALL) for 10 min. The pellets were washed twice and resuspended in liquid modified FM4 medium, free of thallium acetate to produce a mycoplasmal suspension of titre 10^7 CFU/ml. Opacity tubes OT01 (Wellcome) were used to standardise the titre of this suspension. Four dilutions, each 20-fold different were then made in liquid medium, free of thallium acetate and 4 ml of this were used to infect each tracheal organ culture.

2.3.2 BORDETELLA PARAPERTUSSIS

Six isolates of B. parapertussis were obtained from bronchial washings of sheep lungs affected with lesions of CNP. These were plated on blood agar and incubated at 37°C for 3 days.

A plate with well grown and isolated colonies was selected and a sterile loop was used to remove colonies from the surface of the medium. These were placed in a bottle containing 10 mls PBS, and shaken vigorously to suspend the bacteria. Opacity tubes OT01 were used to standardise the titre of bacterial suspension and the procedure was repeated until a titre of 10^7 CFU/ml was obtained. Four dilutions of 20-fold difference were made in PBS and one ml of each dilution was mixed in 3 ml T199 medium and used to infect the tracheal organ cultures.

2.3.3 NEISSERIA CATARRHALIS AND PASTEURELLA HAEMOLYTICA

In both cases, the isolates of these organisms were originally derived from sheep lungs with lesions of CNP. The procedure was the same as with B. parapertussis with only two exceptions. Firstly, the organisms were harvested after 24 to 48 hr of incubation and secondly, titres were 10^8 CFU/ml of obtained for both organisms.

2.4 ASSAY OF COLONY FORMING UNITS

2.4.1 MYCOPLASMA OVIPNEUMONIAE

Serial 10 fold dilutions of M. ovipneumoniae were initially

made by transferring 0.2 ml of the culture into 1.8 ml aliquots of liquid FM4 medium.

Using an automatic pipette with a disposable tip, 0.05 ml samples of the appropriate dilutions were plated onto the surface of FM4 agar medium in 50 X 9 mm plastic petri dishes with tightly fitting lids. This procedure had the advantage that it was not necessary to keep the plates in a humidified box to prevent drying of the agar medium.

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

2.4.2 PASTEURELLA HAEMOLYTICA NEISSERIA CATARRHALIS AND BORDETELLA PARAPERTUSSIS

Serial 10 fold dilutions of these organisms were made by trasferring 0.1 ml of the culture into 9.9 aliquots of PBS for the first two dilutions then by transferring 1 ml of the previous bacterial suspension into 9 ml of PBS up to the 10^8 CFU/ml.

Using an automatic pipette with a disposable tip, two, 0.1 ml samples of appropriate dilutions were each plated onto the surface of blood agar medium in two 85 X 10 mm plastic petri dishes.The plates were incubated at 37°C for 24 to 72 hr and the colonies were counted macroscopically.

2.5 CILIARY ACTIVITY

The ciliary activity of the respiratory epithelium was determined by observing the inner surface of the tracheal organ cultures through the floor of their petri dishes using an inverted microscope (WILD M40 - Heerbrugg, Switzerland) at X100 magnification. Tracheal rings were observed to determine the cilia-stopping effect of each titre for each organism. The speed of ciliary beating could also be determined from the flash frequency, which gave the illusion of arrested ciliary motion.

When ciliary beating ceased, the tissues were removed, washed in

PBS and processed for light,scanning and transmission electron microscopy.

2.6 HISTOPATHOLOGY

Specimens were fixed in 10% formol-saline, processed in the usual manner and embedded in paraffin. They were sectioned at 4 - 5 um and routinely stained with haematoxylin and eosin (HE) and gram Twort (Appendix 6.2).

2.7 SCANNING ELECTRONMICROSCOPY

For SEM the tracheal organ cultures were washed three times in PBS to remove mucus, and immediately fixed in modified Karnovsky's fixative (Appendix 2.1) in 0.1 M PBS, pH 7.2 at $4^{o}C$ for 2 hr. After further washing in two changes of cold, 0.1 M PBS, pH 7.2 at $4^{o}C$ for 1 hr each, the tracheal organ rings were cut into 2 mm^3 pieces and processed as described previously (Chapter 2).

2.8 TRANSMISSION ELECTRONMICROSCOPY

Specimens for TEM were washed and fixed in a similar manner to that used for SEM, then cut into 2 mm^3 pieces, then processed, cut and stained as described in Chapter 2.

2.9 EXPERIMENTAL DESIGN

When each of the above organisms were studied, tracheal organ cultures were freshly prepared as described above. On each occasion rings were subdivided into five groups, each group containing 16 rings. Two rings were placed in each of the labeled 35 X 10 mm plastic petri dishes containing 3 ml of medium. Four of the groups of tracheal organ cultures were each incubated with one of each dilution of the organism under study. The fifth group was left as a control and the ciliary activity of all cultures was observed with an inverted microscope• twice during the first half hour, then half hourly for the 6 hr and once every 2 - 3 hr thereafter. When ciliary beating in any group of cultures stopped, the time was recorded and two petri dishes from this group, as well as two from each of the other groups were removed. Slices of tracheal organ rings were prepared for SEM, TEM

and light microscopy from each group as previously described. A bacterial viable count was made on the medium from each of the petri dishes removed from the incubator. The remaining dishes were left incubating and observed until ciliary action in the next group of organ cultures ceased. The procedure was then repeated and a viable count made. This system was continued until the last of the cultures stopped beating.

3.0 RESULTS

3.1 MYCOPLASMA OVIPNEUMONIAE

3.1.1 OBSERVATION ON CILIARY ACTIVITY

3.1.1.1 IN FM4 MEDIUM

Uninfected ovine tracheal organ cultures remained viable, as indicated by continuation of ciliary activity, for up to 36 hr in FM4 medium.

The time required for slowing and disappearance of ciliary activity and its rapidity of onset was related inversely to the initial number of M. ovipneumoniae organisms placed in the organ cultures. The highest titres of $\pm 10^8$ CFU/ml of M. ovipneumoniae produced slowing of ciliary activity as early as 6 hr post inoculation (pi) and complete cessation was observed 7 hr later (Table 6.1 & 6.2).

Cessation of ciliary activity developed more slowly and irregularly in organ cultures infected with the lower mycoplasmal titres. The pattern of the inactivation of cilia of the lowest three titres was basically similar, but the times taken to reach the ciliostatic effect were different. Ciliary activity ceased completely after 20.5 - 36 hr pi, when titres of $\pm 10^5$ - $\pm 10^7$ CFU/ml were attained (Table 6.1).

3.1.1.2 IN T199 MEDIUM

Control tracheal organ cultures maintained for 53 hr in T199 medium, remained viable as indicated by continuation of ciliary

TABLE 6.1: THE RELATION BETWEEN TIME - STOPPING EFFECT AND NUMBER OF M OVIPNEUMONIAE ORGANISMS (CFU/ML) IN FM4 MEDIUM

GROUP NO.	ORIGINAL INOCULUM	NUMBER OF ORGANISMS (CFU/ML) PI (HR)			
		13	20.5	27.5	36
01	$\pm 10^8$	$\pm 10^{7*}$	$\pm 10^7$	$\pm 10^6$	$\pm 10^6$
02	$\pm 10^6$	$\pm 10^6$	$\pm 10^{6*}$	$\pm 10^6$	$\pm 10^6$
03	$\pm 10^4$	$\pm 10^8$	$\pm 10^8$	$\pm 10^{7*}$	$\pm 10^5$
04	$\pm 10^2$	$\pm 10^{10}$	$\pm 10^9$	$\pm 10^9$	$\pm 10^{5*}$

* TIME - STOPPING EFFECT COMPLETED.

TABLE 6.2: THE EFFECT OF FOUR M. OVIPNEUMONIAE TITRES ON THE CILIARY ACTIVITY OF TRACHEAL ORGAN CULTURES IN FM4 MEDIUM

CFU/ML	CILIARY ACTIVITY AFTER INOCULATION (HR)									
	0-6	6-9	9-12	13	14-19	20.5	21-24	27.5	28-33	36
$\pm 10^8$	+++	++	+	-	-	-	-	-	-	-
$\pm 10^6$	+++	+++	++	++	+	-	-	-	-	-
$\pm 10^4$	+++	+++	+++	+++	++	+	+	-	-	-
$\pm 10^2$	+++	+++	+++	+++	+++	++	++	++	+	-
CONTROL	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

activity.

The time required for slowing ciliary activity and ciliostasis was also related to the initial number of M. ovipneumoniae organisms inoculated to the organ culture. The mycoplasmal titre of $\pm 10^8$ CFU/ml produced slowing of ciliary activity as early as 12 hr pi and complete ciliostasis was achieved 18 hr later (Table 6.3).

The development of ciliostasis was slower and more irregular in organ cultures inoculated with the three lowest mycoplasmal titres. Complete ciliostasis was observed 32 - 53 hr pi (Table 6.3). However, inoculated M. ovipneumoniae organisms were not recovered from all the organ cultures maintained in T199 medium.

In general, M. ovipneumoniae induced significant change in the ciliary activity in both FM4 and T199 media. Microscopically, the organisms produced an accumulation of mucous globules and cells on the epithelial surface or free in the lumen of the rings and there was a reduction in the vigor of ciliary beating as well as the number of epithelial areas showing activity.

3.1.2 HISTOLOGY

The cellular morphology of epithelium from uninoculated control cultures remained healthy in appearance and well differentiated until the end of the experiment in both FM4 and T199 medium (Fig. 6.1). Features of the tissue which could be identified were the pseudostratified ciliated columnar epithelium, the lamina propria with numerous submucosal glands, cartilage and serosa.

Ovine tracheal organ cultures infected with $\pm 10^2$ CFU/ml exhibited mild damage to the epithelial layer, at 13 and 30 hr pi in FM4 and T199 medium respectively. These changes included epithelial cell cytoplasmic eosinophilia and vacuolation, nuclear swelling and chromatin margination. There was very mild epithelial exfoliation and areas covered with bistratified cuboidal epithelial cells were evident (Fig. 6.2).

Organ cultures inoculated with $\pm 10^4$ CFU/ml showed more severity of damage to the epithelium. There was moderate epithelial cell

exfoliation, cytoplasmic vacuolation and moderate to severe loss of cilia (Fig. 6.3). Metaplastic change to stratified cuboidal and columnar epithelium was seen in some areas. Following inoculation with $\pm 10^6$ CFU/ml the architecture of the epithelial layer of organ cultures was disorganised, nuclei appeared pyknotic and vacuolation was apparent in most of the cells (Fig. 6.4). Epithelial exfoliation and loss of cilia was severe and part of the remaining epithelium took on a squamoid-like appearance,while other areas were bistratified cuboidal or bistratified columnar (Fig. 6.5).

Ovine tracheal organ cultures exposed to the highest mycoplasma titres ($\pm 10^8$ CFU/ml), which were achieved only in FM4 medium showed evidence of moderate damage to the epithelial layer as early as 30 min. pi (Fig. 6.5). The epithelial architecture was moderately well preserved with moderate exfoliation of epithelium. The earliest histopathological changes included epithelial cell cytoplasmic eosinophilia and vacuolation, nuclear swelling and chromatin margination. In addition, many cells appeared to have lost their cilia.

When the tracheal rings were fixed after ciliary activity ceased at 13 hr (FM4 medium) and 30 hr (T199 medium), they exhibited severe epithelial damage regardless of the medium used. The epithelial architecture was completely lost, nuclei appeared pyknotic and cytoplasmic vacuolation was severe with complete loss of cilia from most cells (Fig. 6.6). Other cells protruded into the lumen, showed distortion of the cilia and the entire epithelial layer became disorganised with loss of polarity of individual cells. The remaining epithelium took on a squamous, bistratified cuboidal or irregular relaxed transitional appearance (Fig. 6.7) (Table 6.4).

3.1.3 TOPOGRAPHICAL MORPHOLOGY

Examination of uninfected tracheal cultures maintained in FM4 medium for 36 hr showed that the epithelial surface was composed predominantly of ciliated cells. Other than occasional thin strands, no mucous blanket was apparent on the surface (Fig. 6.8).

Because no growth of *M. ovipneumoniae* occurred in T199 medium, examination of infected tracheal cultures with SEM and TEM was

TABLE 6.3: THE EFFECT OF FOUR M. OVIPNEUMONIAE TITRES ON THE CILIARY ACTIVITY OF TRACHEAL ORGAN CULTURES IN T199 MEDIUM

ORIGINAL	CILIARY ACTIVITY AFTER INOCULATION/HR									
INOCULUM	0-12	18	24	30	32	32-38	38-44	44-49	50	53
$\pm 10^8$	+++	++	+	-	-	-	-	-	-	-
$\pm 10^6$	+++	+++	++	+	-	-	-	-	-	-
$\pm 10^4$	+++	+++	+++	++	++	++	+	+	-	-
$\pm 10^2$	+++	+++	+++	+++	+	++	++	+	+	-
CONTROL	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

TABLE 6.4: CYTOPATHOLOGICAL CHANGES IN EPITHELIAL CELLS OF TRACHEAL ORGAN CULTURES INOCULATED WITH M. OVIPNEUMONIAE

ORIGINAL	CYTOPATHOLOGICAL CHANGES			
INOCULUM	ENLARGMENT	CYTOPLASMIC	EPITHELIAL	EPITHELIAL
CFU/ML	OF NUCLEI	VACUOLATION	EXFOLIATION	METAPLASIA
$\pm 10^8$	SEVERE	SEVERE	SEVERE	NIL
$\pm 10^6$	MODERATE	MODERATE	MODERATE	PRESENT
$\pm 10^4$	MILD	MILD	MILD	PRESENT
$\pm 10^2$	NIL	MILD	VERY MILD	NIL

Figure 6.1

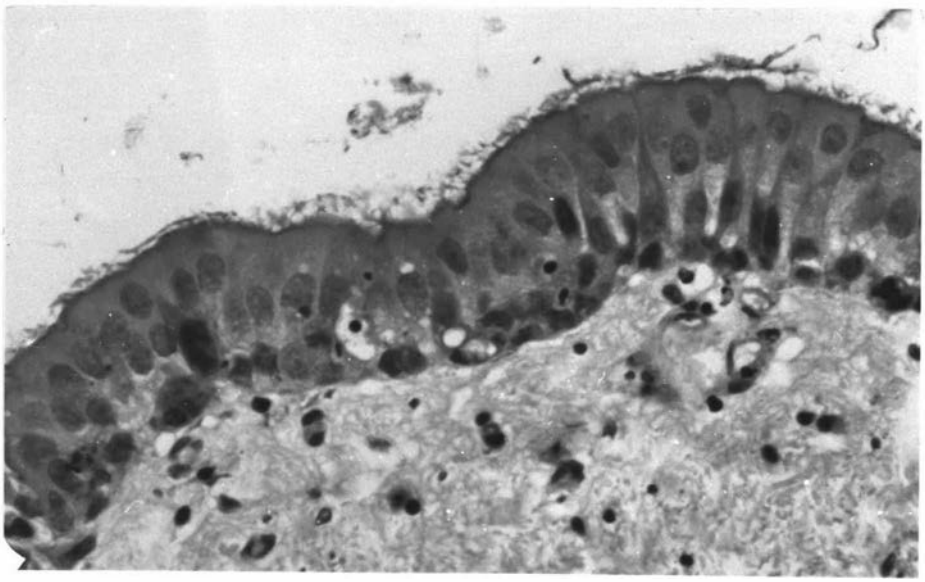
Epithelium of uninoculated control cultures maintained for 13 hrs in FM4 medium. The cells are healthy and well differentiated. H&E. X 312.

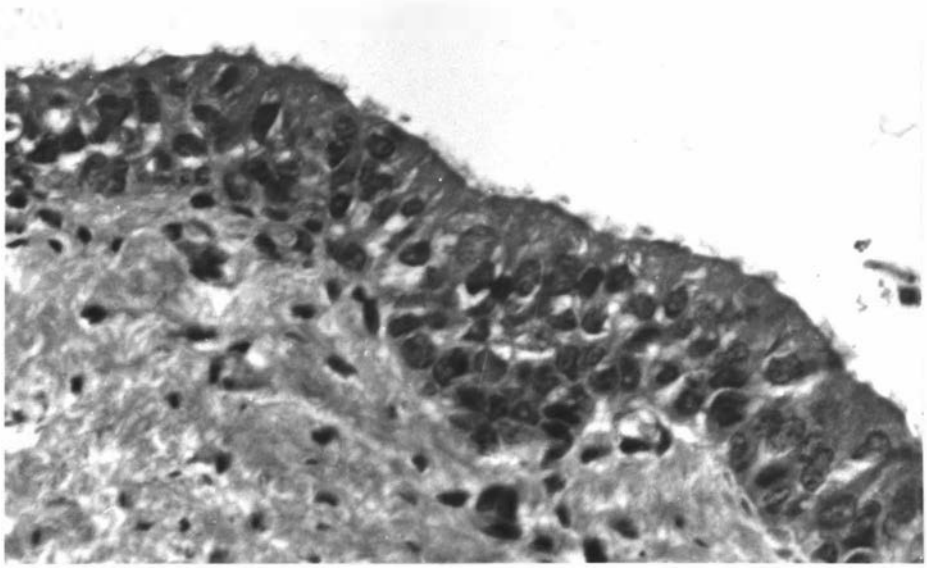
Figure 6.2

Epithelium of tracheal organ culture infected with $\pm 10^2$ CFU/ml *M. ovipneumoniae* in FM4 medium for 13 hrs. There is epithelial cell cytoplasmic vacuolation, nuclear swelling and chromatin margination. H&E. X 312.

Figure 6.3

Epithelium of tracheal organ culture inoculated with $\pm 10^4$ CFU/ml *M. ovipneumoniae* in FM4 medium for 13 hrs. There is cytoplasmic vacuolation and moderate loss of cilia. Metaplastic change is evident in some areas (arrow). H&E. X 312.





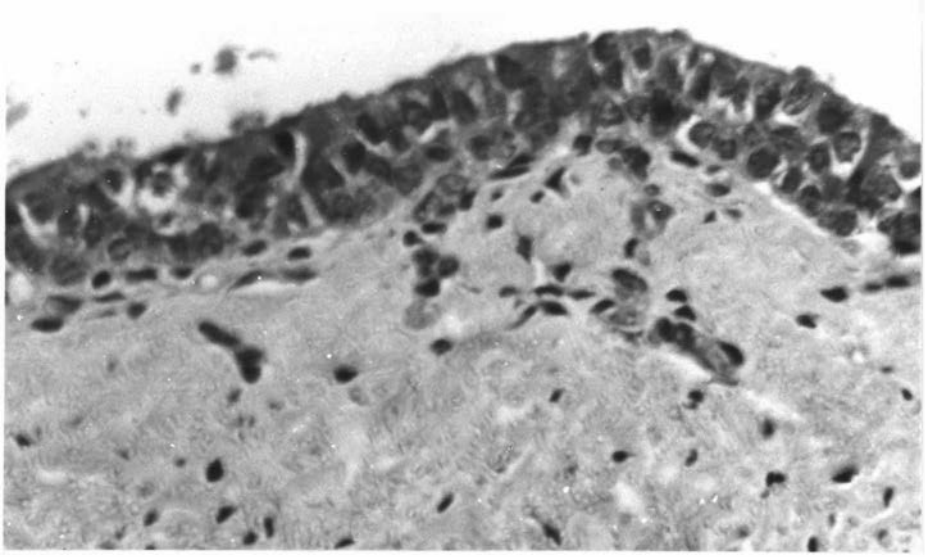
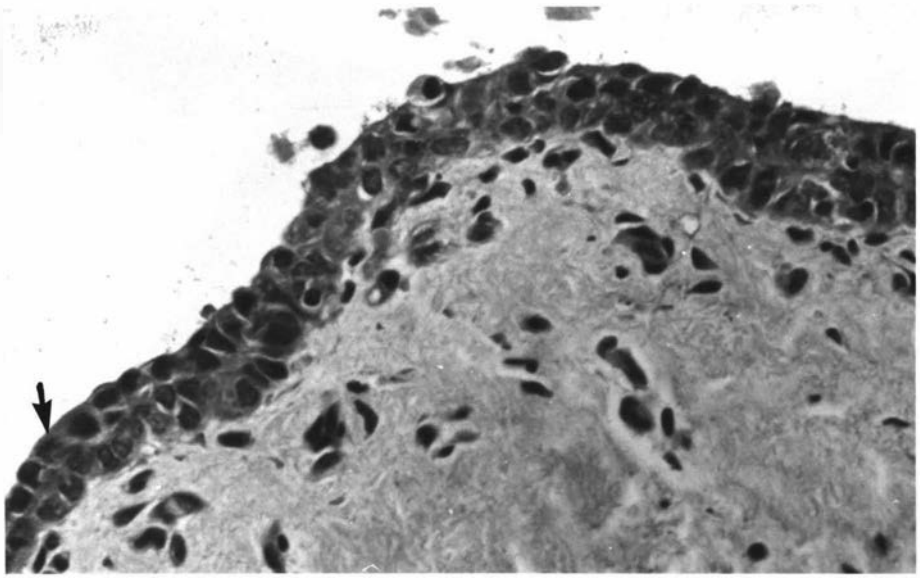


Figure 6.4

A tracheal organ culture inoculated with $\pm 10^6$ M. ovipneumoniae and maintained in FM4 medium for 13 hrs. The epithelium in disorganised with severe epithelial cell exfoliation and loss of cilia. The remaining epithelium has a squamous-like appearance (arrows). H&E. X 312.

Figure 6.5

Tracheal organ culture infected with $\pm 10^8$ CFU/ml M. ovipneumoniae and maintained in FM4 medium for 30 min. The epithelial architecture is slightly disrupted. H&E. X 312.



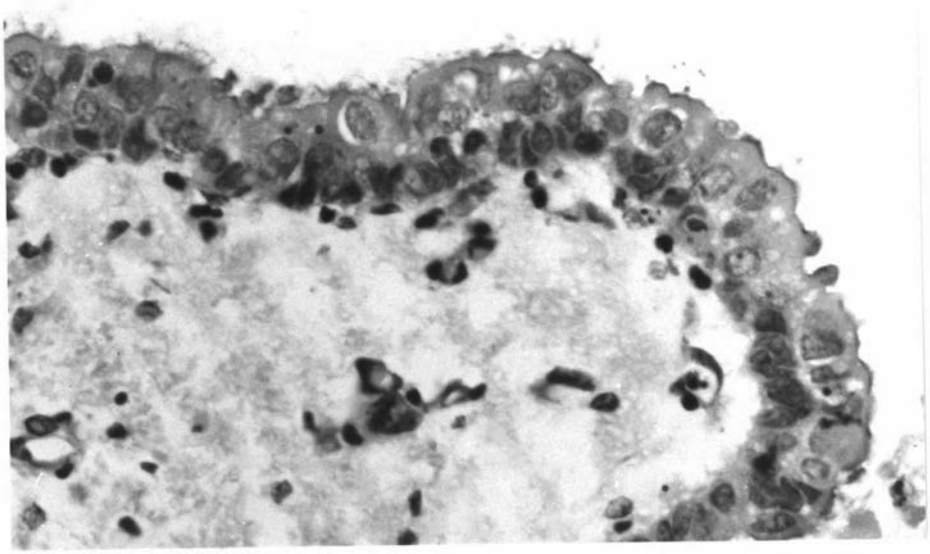
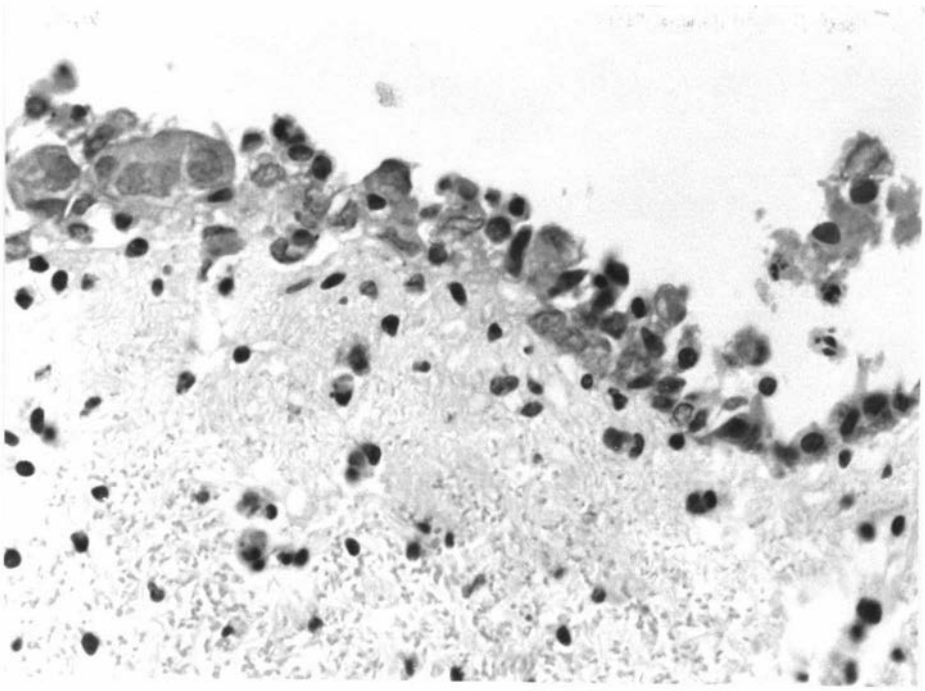


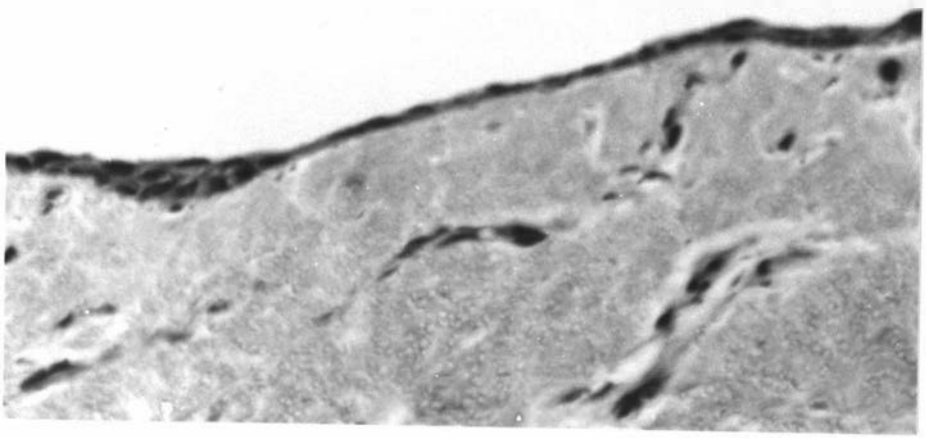
Figure 6.6

Tracheal culture inoculated with $\pm 10^8$ CFU/ml M. ovipneumoniae and maintained in FM4 medium for 13 hrs. The epithelium is severely damaged and the nuclei appear pyknotic. H&E. X 312.

Figure 6.7

A different area from the above culture showing a squamous, bistratified epithelium. H&E. X 312.





confined to samples maintained in FM4 medium only.

Tracheal rings examined 13 hr after inoculation with $\pm 10^2$ CFU/ml showed loss of cilia and ciliated cells (Fig. 6.9) when compared with uninfected controls. With high magnification, structures recognisable as surface-attached mycoplasmas were observed mostly at the base of the cilia (Fig. 6.9). The organisms measured approximately 700 - 900 nm in diameter, showed invagination and possessed fine projections attaching them to the cilia. No organisms were attached or in direct contact with non-ciliated cells.

The luminal surface of tracheal cultures infected with the titres of $\pm 10^4$ and $\pm 10^6$ CFU/ml for 13 hr, showed exfoliation of ciliated cells measuring 4 - 5 um in diameter. Cell membrane fragments were present on the epithelial surface (Fig. 6.10). More severe reduction in the ciliary density was observed, but cilia that remained attached appeared morphologically unaltered. The non-ciliated surfaces appeared flattened with numerous knob-like microvillous projections (Fig. 6.11). At this titre of infection, organisms with characteristic mycoplasma morphology were evenly distributed over and between the top of cilia although a few were also present at the base. At very high magnification, M. ovipneumoniae organisms appeared to be attached to the cilia by means of fine and short projections or pili-like structures (Fig. 6.11). No mycoplasma organisms were seen on non-ciliated surfaces.

To explore the early phases of colonisation and attachment of M. ovipneumoniae, tracheal organ cultures at 30 min and 1 hr pi with $\pm 10^8$ CFU/ml were also examined by SEM.

At 30 min pi, the epithelial layer exhibited severe damage, including loss of cilia and extrusion of epithelial cells. The surface of non-ciliated cells was rounded and protruded to the luminal surface and showed numerous, knob-like microvillous projections (Fig. 6.12). Even at this early stage most of the ciliary carpet was covered by single layer of M. ovipneumoniae (Fig. 6.12). At 1 hr after infection, the epithelial surface showed severe sloughing of ciliated cells with a further reduction in the ciliary carpet density (Fig. 6.13). Cilia of the intact epithelial cells appeared to be unchanged and mycoplasma organisms were entangled between the tips of

Figure 6.8

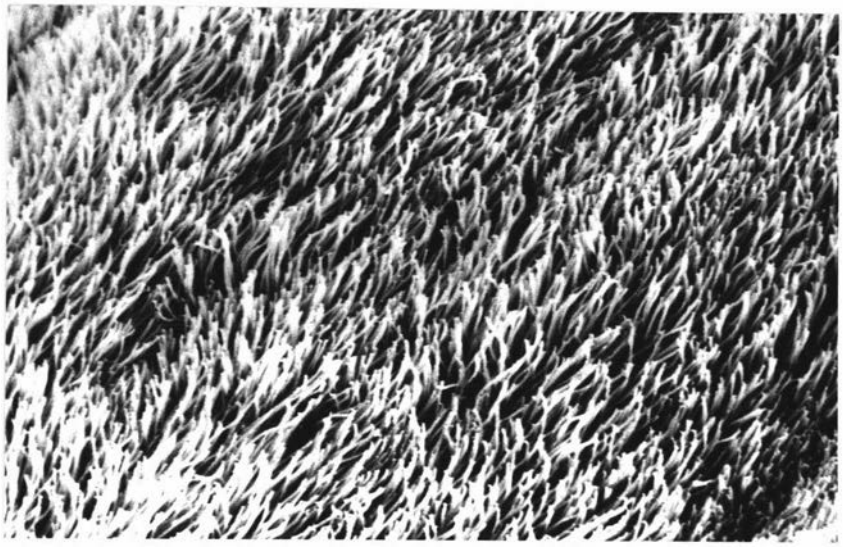
Uninfected tracheal epithelium maintained in FM4 medium for 36 hr. It is composed almost entirely of ciliated cells. SEM. X 2000.

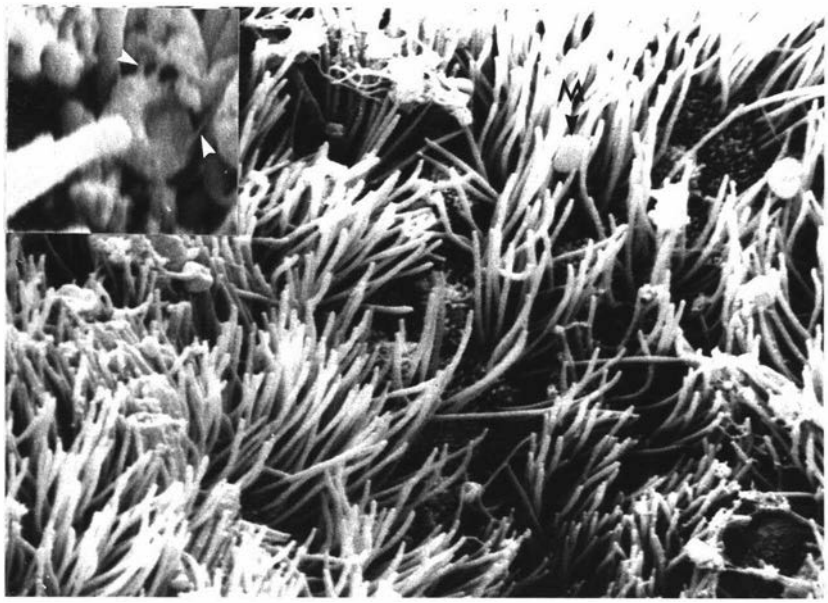
Figure 6.9

Tracheal epithelium 13 hr after inoculation with $\pm 10^2$ CFU/ml of M. ovipneumoniae. There is loss of cilia and ciliated cells and a large number of mycoplasmas (M) are attached to the cilia. SEM. X 4000. The inset micrograph shows the mycoplasmas at high magnification attached by fine projections (arrow). SEM. X 16,000.

Figure 6.10

The surface epithelium of a tracheal organ culture infected with $\pm 10^4$ CFU/ml of M. ovipneumoniae for 13 hr. It shows exfoliation of ciliated cells (C) and fragments (F). A large number of mycoplasmas (M) are distributed over and between the tips of the cilia. SEM. X 2000.




M


F
C
M

Figure 6.11

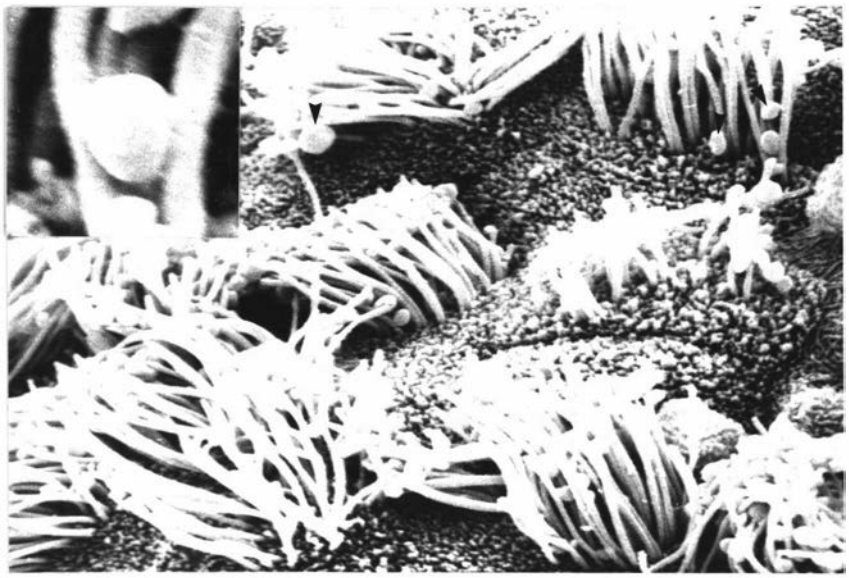
Tracheal epithelium infected with $\pm 10^6$ CFU/ml of M. ovipneumoniae for 13 hr. There is loss of a large number of ciliated cells. Numerous mycoplasmas (arrows) are distributed between the cilia. SEM. X 4000. The inset micrograph shows the firm attachment of mycoplasma to cilia. SEM. X 20,000.

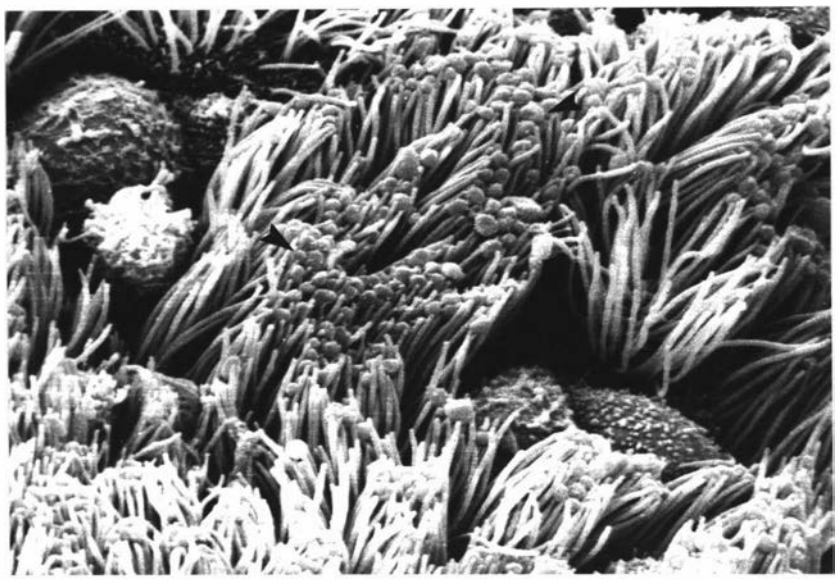
Figure 6.12

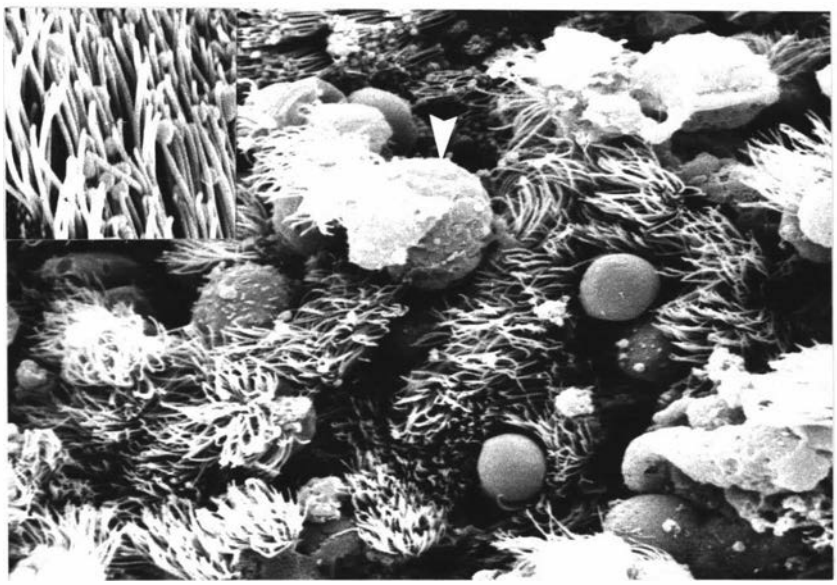
Tracheal epithelial surface 30 min post-inoculation with $\pm 10^8$ CFU/ml of M. ovipneumoniae. It shows severe damage and most of the ciliary carpet is covered by a single layer of mycoplasmas (arrow). SEM. X 4000.

Figure 6.13

One hr post-inoculation with $\pm 10^8$ CFU/ml of M ovipneumoniae. The epithelial surface shows severe sloughing of ciliated cells (arrow). Mycoplasmas are visible between the tips of the remaining cilia. SEM. X 1600. The inset micrograph shows a higher magnification of attached mycoplasmas. SEM. X 4000.







cilia (Fig. 6.13).

At 13 hr pi with $\pm 10^8$ CFU/ml, the epithelial surface showed very severe damage and most of the ciliary carpet had disappeared. The luminal surface was covered with extruded cells and cellular fragments. Few ciliated cells remained (Fig. 6.14). Mycoplasma ovipneumoniae organisms were attached and entangled between the remaining cilia and tufted together (Fig. 6.15). These organisms were intact, exhibited normal M. ovipneumoniae morphology and were not located on non-ciliated surfaces.

3.1.4 ULTRASTRUCTURE

The morphology of tracheal tissue from uninoculated organ cultures resembled that of the normal adult ovine trachea (Banks, 1982). The epithelium was layered three to five cells deep. The fibrillar structure of the cilia, numerous branching microvilli, mitochondria, and endoplasmic reticulum were all well preserved, and a few cytoplasmic vacuoles and lysosomes were present on epithelial cells. Ciliated cells were frequent, often being adjacent to one another and comprised at least half the epithelial surface (Fig. 6.16).

Tracheal organ cultures maintained in FM4 medium and infected with $\pm 10^2$ CFU/ml M. ovipneumoniae, had good structural integrity and closely resembled the controls. Ciliated cells were almost as numerous as in control groups (Fig. 6.17). However, the cytoplasm of epithelial cells regardless of the cell type, contained more vacuoles than were present in the controls. These were greatest in number in non-ciliated cells. The largest of these vacuoles contained amorphous material and some of them contained mitochondria (Fig. 6.17). Lysosomes were not prominent and mitochondria were generally intact. No organisms were found attached to or in close contact with cilia.

The epithelial architecture of tracheal cultures maintained for 13 hr in FM4 medium and infected with titres of $\pm 10^4$ CFU/ml was disorganised and showed marked destruction of subcellular organelles. A few ciliated cells contained large vacuoles (Fig. 6.18). A few M. ovipneumoniae organisms were attached to the cilia of epithelial cells (Fig. 6.18).

Figure 6.14

A tracheal organ culture 13 hr post-inoculation with $\pm 10^8$ CFU/ml of M. ovipneumoniae. There is very severe damage and most of the ciliary carpet has disappeared. The surface is covered with extruded cells and cellular fragments with only a few ciliated cells remaining (arrows). SEM. X 1000.

Figure 6.15

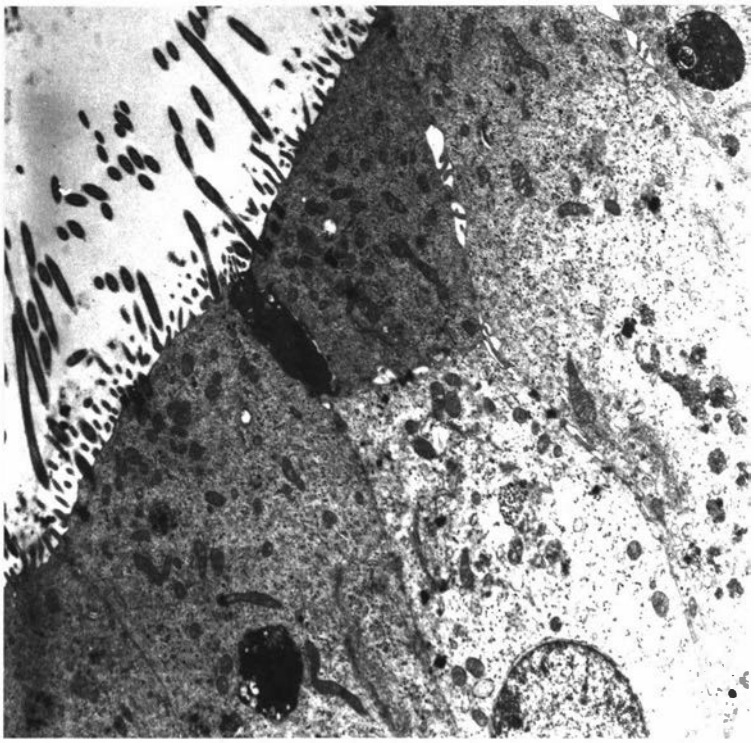
Higher magnification of the surface of the above culture showing attachment of numerous M. ovipneumoniae organisms to cilia on the remaining cells. SEM. X 8000.

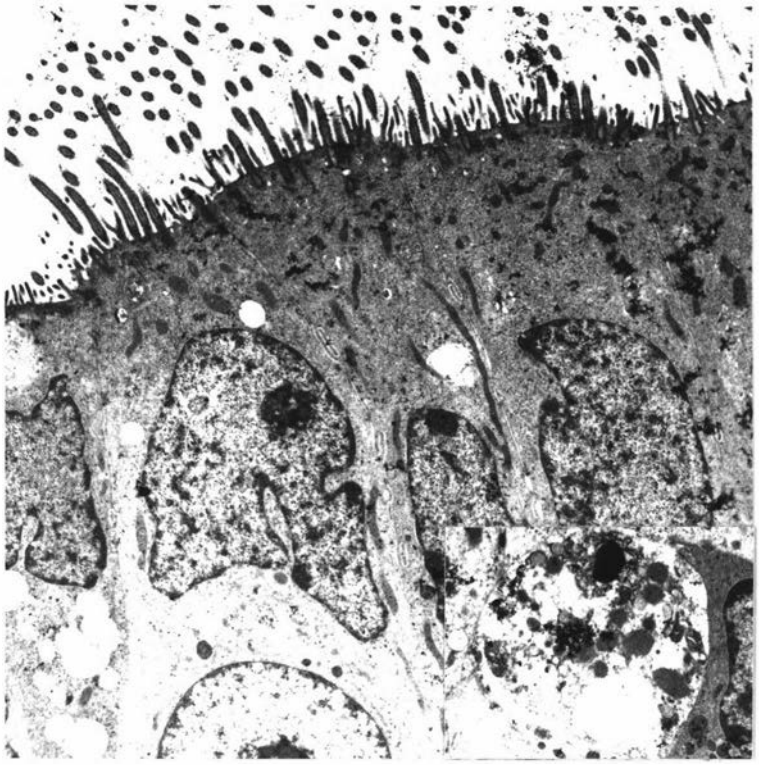
Figure 6.16

Uninoculated tracheal epithelium maintained for 36 hr in FM4 medium. There is good preservation of the ultrastructure and differentiation into ciliated cells. TEM. X 5,000.

Figure 6.17

A tracheal organ culture maintained in FM4 medium infected with $\pm 10^2$ CFU/ml of M. ovipneumoniae showing vacuolation of some cells. TEM. X 6,300 . The inset micrograph shows higher magnification of the large vacuoles which contain amorphous material and mitochondria. TEM. X 6,300.





These changes were more severe and widespread in organ cultures infected with titres of $\pm 10^6$ and 10^8 CFU/ml *M. ovipneumoniae* (Fig. 6.19). Following inoculation with these titres, cytoplasmic vacuolation was observed distorting the cytoplasm of the ciliated and non-ciliated cells and electron dense material was seen deposited on the luminal cellular membrane. *Mycoplasma ovipneumoniae* were found to be in intimate contact with cilia (Fig. 6.19).

Organ cultures infected with the highest titres ($\pm 10^8$ CFU/ml) of *M. ovipneumoniae* were examined by TEM at 30 min and 1 hr pi. At this stage the epithelial layer showed significant ultrastructural changes. These changes included moderate to severe loss of cilia and extrusion of ciliated epithelial cells (Fig. 6.20). Some surface epithelial cells were flattened and extended to cover a wide area. These cells contained flattened nuclei, laminated inclusions, short cilia and numerous microvilli (Fig. 6.21). Ciliated epithelial cells manifested a focal intracellular oedema, and the cytoplasm contained moderate numbers of vacuoles together with a few lysosomes (Fig. 6.20). Numerous non-capsulated organisms recognisable as *M. ovipneumoniae* were found in close contact with the cilia ((Fig. 6.22) but no organisms were found inter- or intracellularly.

Close examination of the organisms attached to cilia revealed a distinctive morphological feature extending from the cytoplasm. It consisted of a fimbria or pili-like structure measuring approximately 83 nm in diameter. This portion of the *M. ovipneumoniae* organism was always found in close proximity to cilia and it appeared that structure was anchoring the organism to the cell membrane thus acting as a means of attachment. A sagittal section of an organism attached to a cilium is shown in figure 6.23. The organism measured 700 - 900 nm in diameter. The specialised pili appeared to originate from within the mycoplasmal cell and extend through its membrane into the cilium. Examination of a series of sections from several attached organisms indicated that each organism had more than one specialised pilus. Each of these structures consisted of an electron-dense core surrounded by a lucent area.

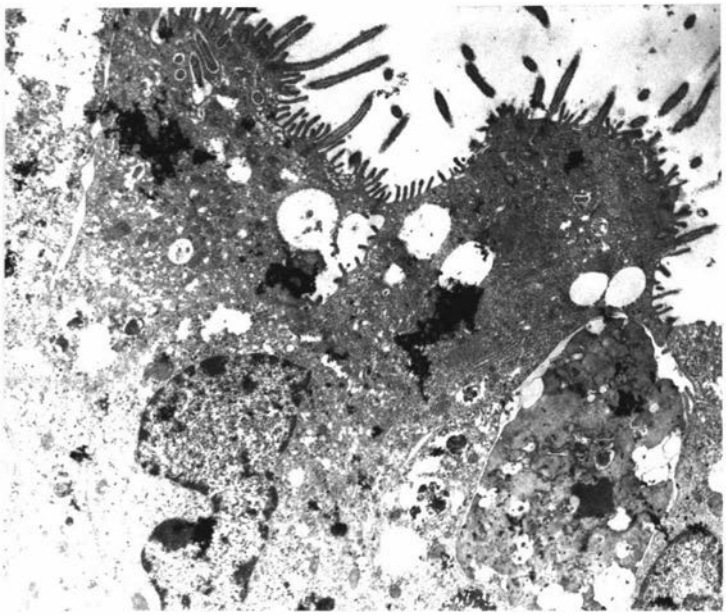
At 13 hr pi, infected tracheal cultures exhibited very severe epithelial disorganisation and intracellular changes (Fig. 6.24). These changes included, severe loss of cilia and cell extrusions.

Figure 6.18

A tracheal culture maintained for 13 hr in FM4 medium and infected with $\pm 10^4$ CFU/ml of M. ovipneumoniae. The epithelial cells are disorganised and show marked destruction of subcellular organelles. TEM. X 5,000.

Figure 6.19

The luminal surface of a tracheal culture infected with $\pm 10^8$ CFU/ml of M. ovipneumoniae. There is very severe damage including the formation of a large number of cytoplasmic vacuoles (V). Electron-dense material has been deposited on the luminal surface (arrows). A few M. ovipneumoniae organisms (M) are in intimate contact with the remaining cilia. TEM. X 8,200.



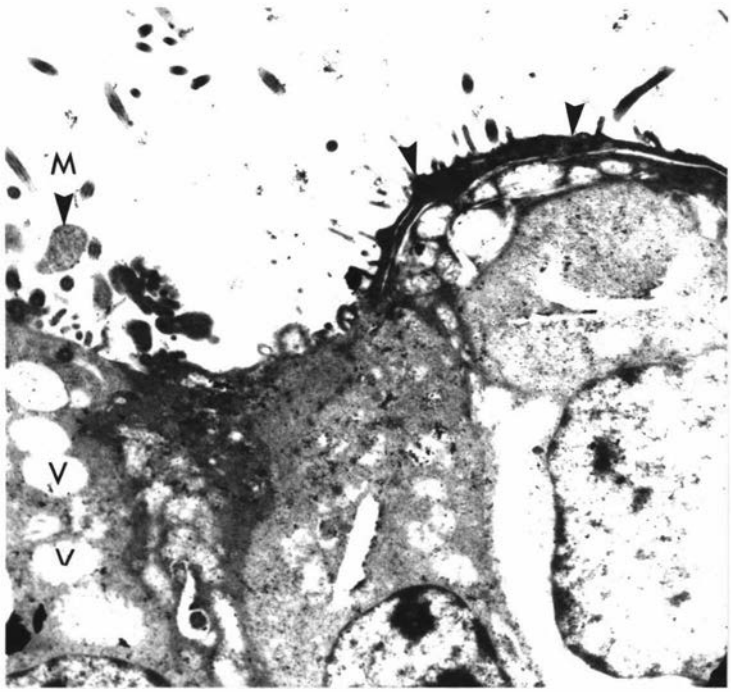
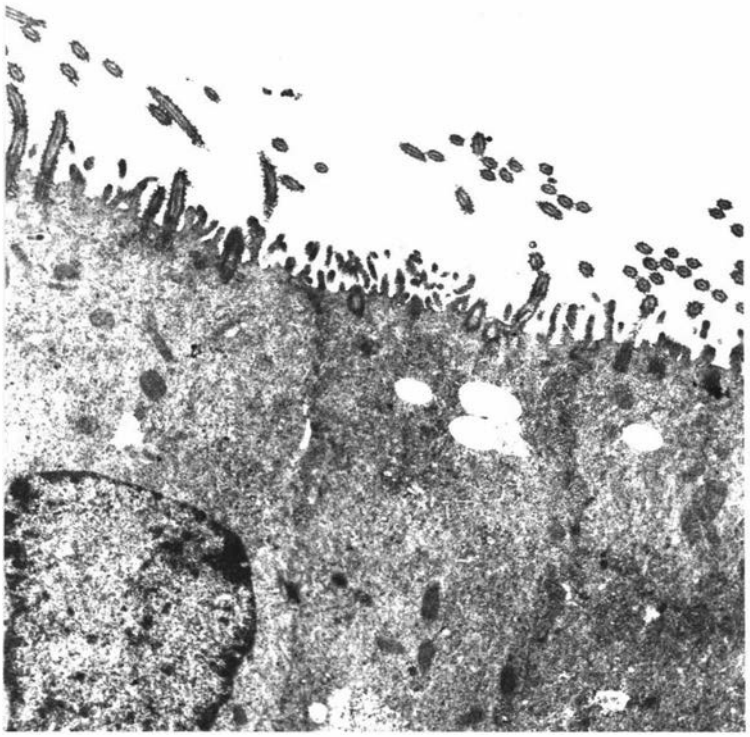

M
V
V

Figure 6.20

The epithelial surface of an organ culture infected with $\pm 10^8$ CFU/ml of M. ovipneumoniae for 30 min. The luminal surface has less cilia than the controls and the epithelial cell cytoplasm contains a moderate number of vacuoles. TEM. X 8,200.

Figure 6.21

Flattened surface epithelial cells from a similar culture to the above. These cells had an extended cytoplasm and contained flattened nuclei (N), laminated inclusions (L) and numerous microvilli (MV). TEM. X 19,200.



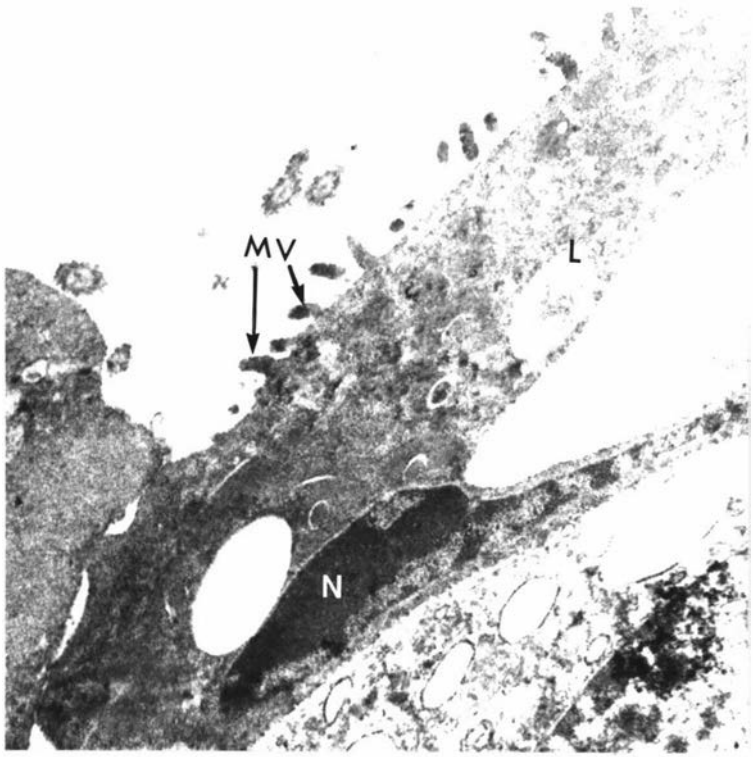
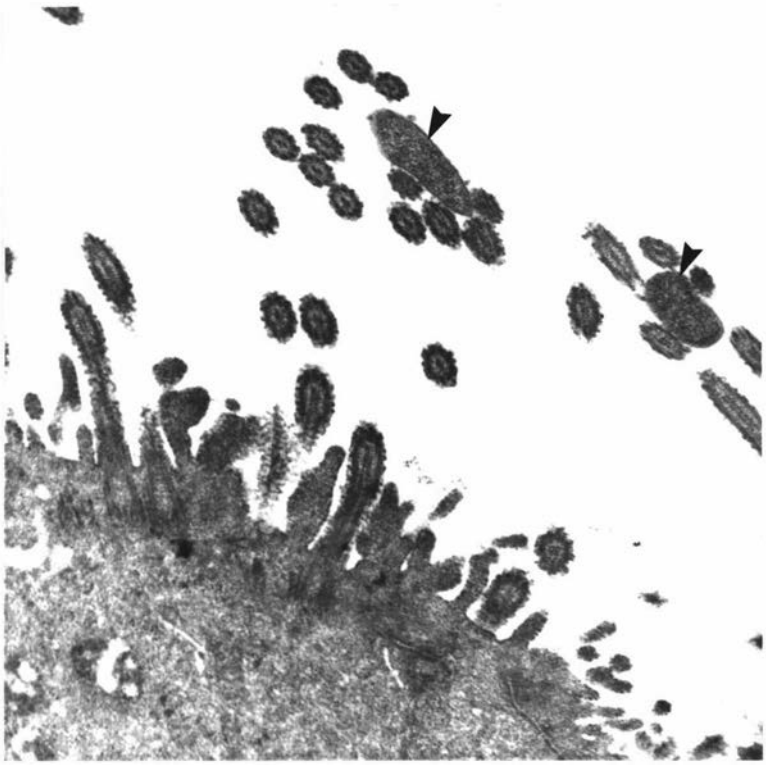

MV
L
N

Figure 6.22

Mycoplasma ovipneumoniae organisms (arrow) in close contact with the cilia of a tracheal organ culture infected with $\pm 10^8$ CFU/ml for 30 min. TEM. X 19,200.

Figure 6.23

High magnification of an M. ovipneumoniae organism from the above culture revealing a distinctive pili-like structure (arrow) attaching it to the cilia. TEM. X 160,100.



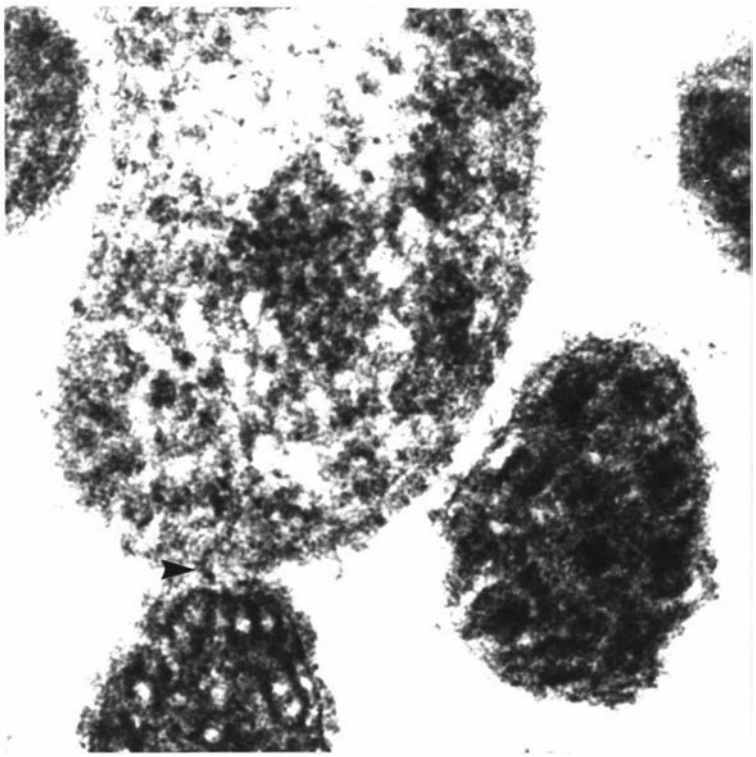
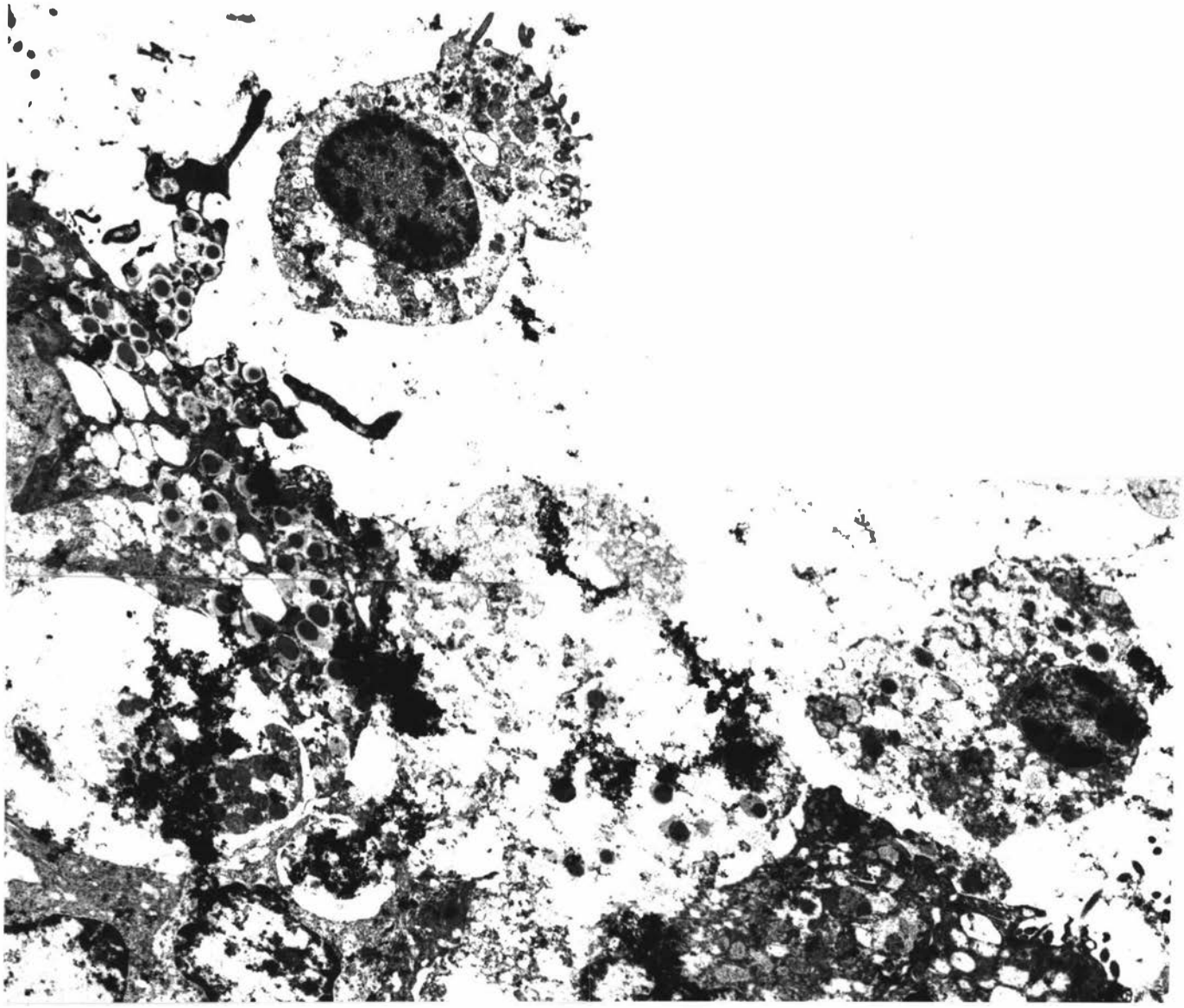


Figure 6.24

A tracheal culture infected with $\pm 10^8$ CFU/ml of M. ovipneumoniae for 13 hr exhibiting very severe disorganisation and intracellular change. TEM. X 6,300.



Epithelial cells were severely vacuolated and contained a large number of lysosomes. The vacuoles contained dark electron dense material. No organisms were seen attached to the severely damaged epithelium.

3.1.5 MYCOPLASMAL GROWTH CURVES IN ORGAN CULTURE

The growth of M. ovipneumoniae was studied in FM4 and T199 medium containing tracheal rings and compared to that in FM4 medium alone. All titres of M. ovipneumoniae grew best in the ciliated epithelium of ovine tracheal organ cultures maintained in FM4 medium (Fig. 6.25).

The numbers of mycoplasmas in the organ cultures maintained in FM4 medium was in general greater in FM4 medium alone or in organ cultures maintained in T199 medium. In organ cultures maintained in FM4 medium, titres did not exceed $\pm 10^7$ CFU/ml for the highest titre inoculated, and $\pm 10^{10}$ CFU/ml for the lowest titre. Mutiplication could only be demonstrated if the inoculum was below a titre of $\pm 10^6$ CFU/ml (Fig. 6.26). Mycoplasma ovipneumoniae did not grow in tracheal organ culture maintained in T199 medium and organism viable count in relation to 24 hours post inoculation was higher in organ cultures maintained in FM4 medium ($\pm 10^5$ CFU/ml) than in FM4 medium alone ($\pm 10^1$ CFU/ml) (Fig. 6.25).

3.2 BORDETELLA PARAPERTUSSIS

3.2.1 OBSERVATION OF CILIARY FUNCTION

Uninfected ovine trachea in organ cultures remained viable, differentiated, and organised with ciliary activity present throughout the duration of the experiment (7.5 hr).

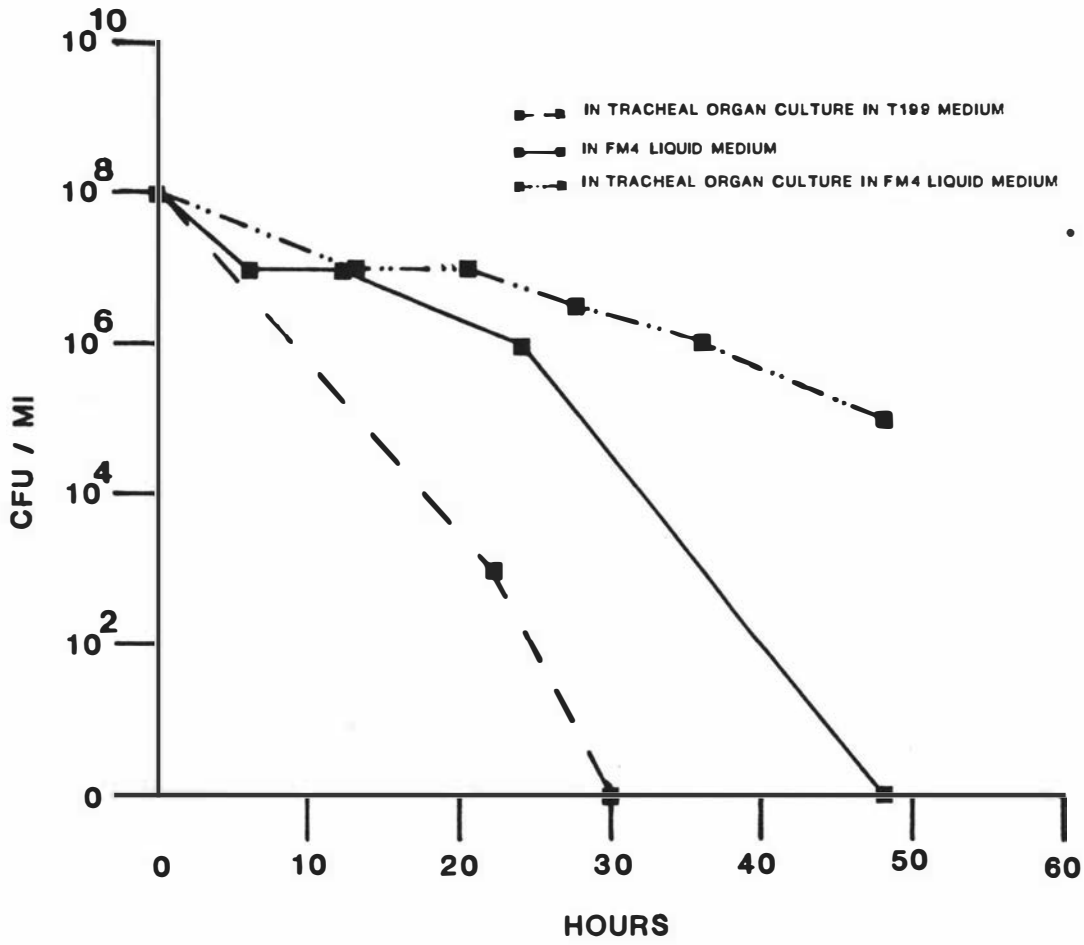
Consistent inhibition of ciliary activity was produced by B. parapertussis organisms and its rapidity of onset was related directly to the concentration of organisms in the culture (Table 6.5 & 6.6). The earliest effect observed was seen 5 min pi with the bacterial titre of $\pm 10^7$ CFU/ml. Single spherical cells containing bubble-like structures appeared on the ciliary surface. This was accompanied by a

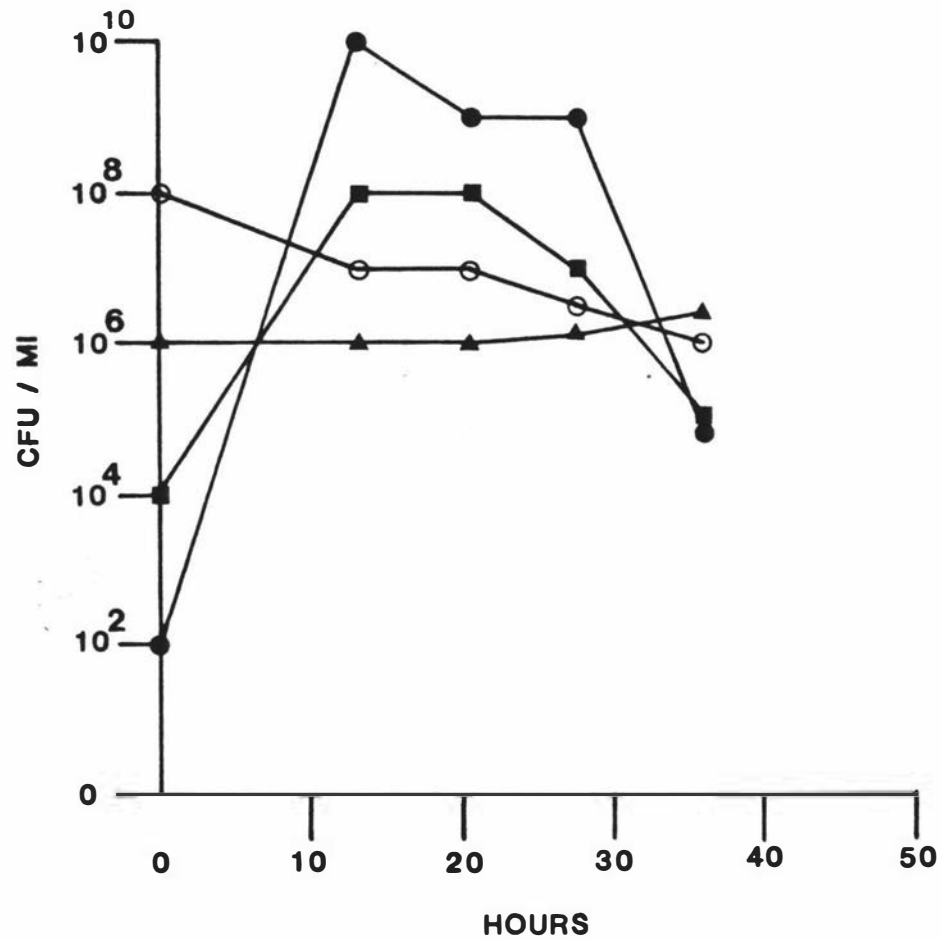
Figure 6.25

Mycoplasma ovipneumoniae growth curves in three different culture systems.

Figure 6.26

The growth curves of four titres of M. ovipneumoniae maintained in liquid FM4 medium. Multiplication of the microorganisms only occurred if the inoculum was below a titre of $\pm 10^6$ CFU/ml.


IN TRACHEAL ORGAN CULTURE IN T199 MEDIUM
IN FM4 LIQUID MEDIUM
IN TRACHEAL ORGAN CULTURE IN FM4 LIQUID MEDIUM
CFU / MI
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
0
0
10
20
30
40
50
60
HOURS


CFU / MI
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
0
0
10
20
30
40
50
HOURS

significant reduction in ciliary beating. Following this there was severe desquamation of epithelial cells which began at 15 min pi. Complete loss of ciliary activity occurred at about 1 hr after incubation. In addition to the slowing of beating frequency, all of the cilia of an individual cell appeared to clump together and beat in a less coordinated manner with greater amplitude than normal.

After incubation with bacterial titres of $\pm 10^5$ CFU/ml, ciliostasis developed more slowly and irregularly but there was no essential difference in the pattern of changes observed. Ciliostasis was invariably complete after 4 - 5 hr when titres of $\pm 10^7$ and $\pm 10^8$ CFU/ml were attained (Table 6.5 & 6.6).

3.2.2 HISTOLOGY

The uninoculated ovine tracheal organ cultures, maintained for 1 hr in T199 medium *in vitro*, showed similar histological appearance to those described earlier (Fig. 6.1).

The severity of changes in the inoculated cultures showed a direct correlation with the number of organisms inoculated (Table 6.7). When ciliostasis occurred following inoculation with the bacterial titres of $\pm 10^1$ and $\pm 10^3$ CFU/ml, the epithelial cells showed cytoplasmic eosinophilia and vacuolation together with swelling of the nucleus and margination of chromatin. Some individual epithelial cells protruded from the surface into the lumen suggesting a process of extrusion was occurring (Fig. 6.27). Very few gram negative cocco-bacilli organisms were observed on the epithelial surface. Cultures inoculated with a bacterial titre of $\pm 10^5$ CFU/ml, showed complete loss of cilia from many epithelial cells (Fig. 6.28). In these cultures Gram negative organisms were observed on the remaining ciliated surface. The most severe changes were seen in those cultures inoculated with the highest bacterial titre ($\pm 10^7$ CFU/ml). Here the entire epithelial layer became disorganised and showed severe loss of ciliated cells. Other changes included severe cytoplasmic vacuolation, and nuclear swelling. The remaining epithelium took on a bistratified columnar or squamous appearance (Fig. 6.29). The cytoplasm of some epithelial cells spread extensively over the underlying cuboidal cells. Gram negative cocco-bacilli were located on the cilia of the surviving ciliated epithelial cells.

TABLE 6.5: THE RELATION BETWEEN TIME-STOPPING EFFECT AND NUMBER OF B. PARAPERTUSSIS ORGANISMS (CFU/ML)

GROUP CODE NO.	ORIGINAL INOCULUM CFU/ML	VIABLE NUMBER OF ORGANISMS CFU/ML PI AT DIFFERENT TIMES (HR)			
		1	3.5	6.5	7.5
1	$\pm 10^7$	$\pm 10^{7*}$	$\pm 10^5$	$\pm 10^6$	$\pm 10^6$
2	$\pm 10^5$	$\pm 10^5$	$\pm 10^{6*}$	$\pm 10^7$	$\pm 10^7$
3	$\pm 10^3$	$\pm 10^5$	$\pm 10^6$	$\pm 10^{7*}$	$\pm 10^8$
4	$\pm 10^1$	$\pm 10^5$	$\pm 10^6$	$\pm 10^8$	$\pm 10^{8*}$

* TIME - STOPPING EFFECT COMPLETED.

TABLE 6.6: EFFECT OF FOUR B. PARAPERTUSSIS TITRES ON THE CILIARY ACTIVITY OF OVINE FOETAL TRACHEAL ORGAN CULTURE

ORIGINAL INOCULUM CFU/ML	CILIARY ACTIVITY PI HR									
	0	1/4	1/2	1	2	3	4	5	6	7.5
$\pm 10^7$	+++	++	+	-	-	-	-	-	-	-
$\pm 10^5$	+++	+++	+++	++	+	-	-	-	-	-
$\pm 10^3$	+++	+++	+++	+++	+++	++	++	+	-	-
$\pm 10^1$	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
CONTROL	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

TABLE 6.7: CYTOPATHOLOGICAL CHANGES IN EPITHELIAL CELLS OF TRACHEAL ORGAN CULTURES INOCULATED WITH *B. PARAPERTUSSIS*

ORIGINAL INOCULUM CFU/ML	CYTOPATHOLOGICAL CHANGES				NUMBER OF BACTERIA ON LUMINAL SURFACE
	ENLARGEMENT OF NUCLEI	CYTOPLASMIC VACUOLATION	EPITHELIAL EXFOLIATION	EPITHELIAL METAPLASIA	
$\pm 10^7$	SEVERE	SEVERE	SEVERE	PRESENT	LARGE
$\pm 10^5$	SEVERE	MODERATE	MODERATE	NIL	MODERATE
$\pm 10^3$	MODERATE	MILD	MILD	NIL	FEW
$\pm 10^1$	MODERATE	MILD	MILD	NIL	FEW

Figure 6.27

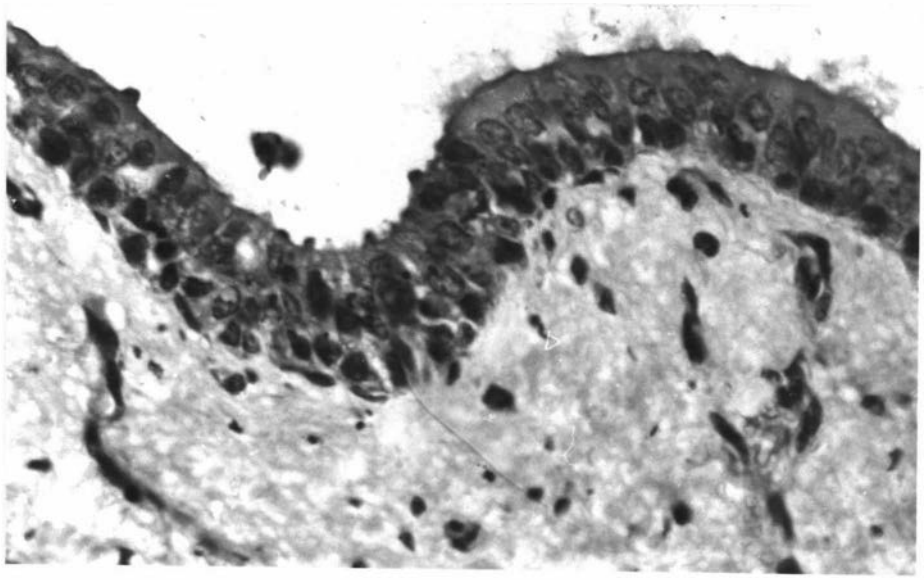
A tracheal organ culture inoculated with $\pm 10^1$ CFU/ml *B. parapertussis* for 1 hr and maintained in T199 medium. The epithelial cells show mild cytoplasmic vacuolation and loss of cilia from their luminal border. H&E. X 312.

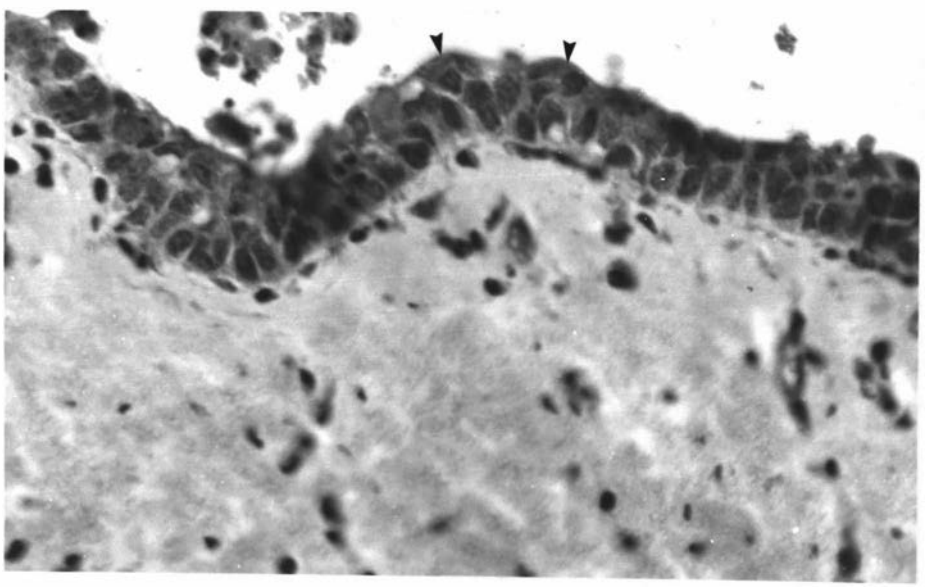
Figure 6.28

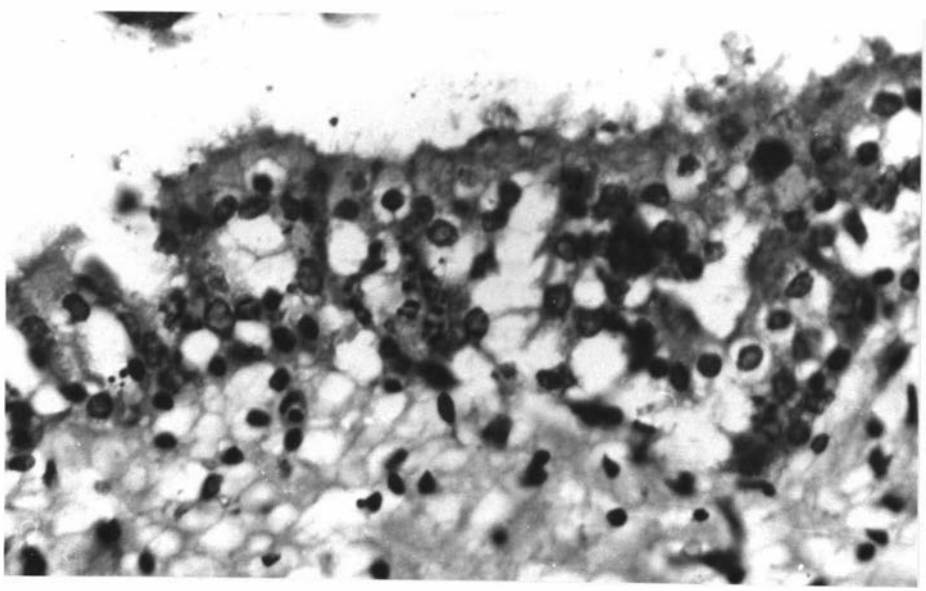
An organ culture inoculated with $\pm 10^5$ CFU/ml *B. parapertussis* for 1 hr and maintained in T199 medium. There is complete loss of cilia from many epithelial cells (arrows). H&E. X 312.

Figure 6.29

A tracheal culture infected with $\pm 10^7$ CFU/ml *B. parapertussis* in T199 medium.The entire epithelial layer is disorganised and shows severe loss of ciliated cells. The remaining cells show severe cytoplasmic vacuolation and the nuclei appear pyknotic. H&E. X 312.







3.2.3 TOPOGRAPHICAL MORPHOLOGY

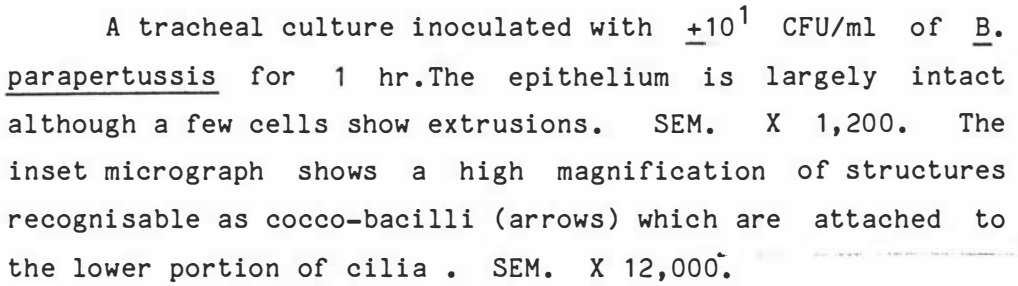
The luminal surface of uninfected tracheal organ cultures fixed 1 hr pi showed good preservation of the respiratory epithelium when examined by SEM. The mucosal surface consisted of both ciliated, non-ciliated and goblet cells and the boundaries between adjacent non-ciliated and goblet cells were easily discerned similar to that described in the earlier controls (Fig. 6.8).

In cultures inoculated with the bacterial titres of $\pm 10^1$ CFU/ml, most of the epithelium was intact although a few ciliated epithelial cells showed mild extrusion from the mucosal surface (Fig. 6.30). At high magnification, structures recognisable as cocco-bacilli were found attached to the lower portion of cilia (Fig. 6.30). Epithelial cell extrusions were more marked in the cultures inoculated with $\pm 10^3$ CFU/ml bacterial titre (Fig. 6.31) and single bacteria were found between the tips of the cilia in intact areas (Fig. 6.31).

Most of the cilia still remained intact in organ cultures inoculated with a $\pm 10^5$ CFU/ml bacterial titre (Fig. 6.32) and there was moderate extrusion of epithelial cells. Bacterial microcolonies were observed on the top of the ciliary carpet (Fig. 6.33) and a few single organisms were found between the cilia (Fig. 6.33).

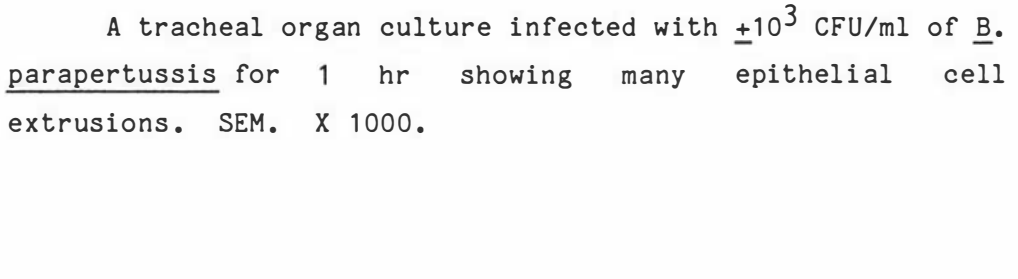
The mucous membrane infected with a bacterial titre of $\pm 10^7$ CFU/ml was largely denuded of cilia (Fig. 6.34), and underlying non-ciliated cells occupied the spaces left by expelled ciliated cells. At high magnification, the predilection of *B. parapertussis* for ciliated cells was readily demonstrated, and the organisms were found entangled within tufts of the remaining disordered cilia. Attachment appeared to be predominantly to the upper half of the cilia (Fig. 6.35) and no bacteria were seen on the luminal surface of non-ciliated cells. Most of the non-ciliated epithelial cells were swollen and protruded into the lumen. Their surface was irregular, bulbous and devoid of bacteria (Fig. 6.35). Multiple attachment of a single bacterium to more than one cillium and attachment of several organisms to the same cillium was common.

Figure 6.30



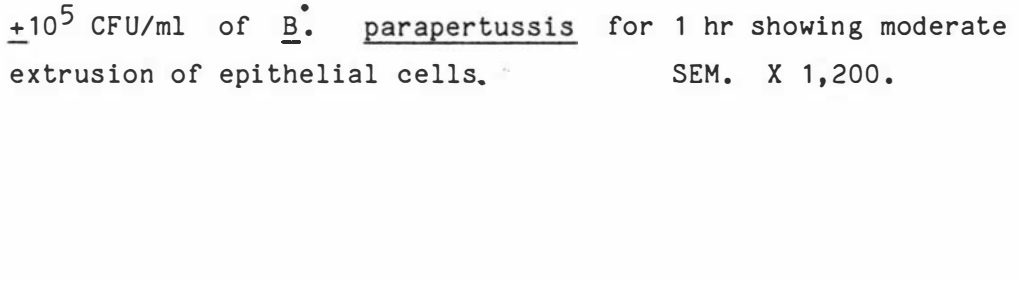
A tracheal culture inoculated with $\pm 10^1$ CFU/ml of B. parapertussis for 1 hr.The epithelium is largely intact although a few cells show extrusions. SEM. X 1,200. The inset micrograph shows a high magnification of structures recognisable as cocco-bacilli (arrows) which are attached to the lower portion of cilia . SEM. X 12,000.

Figure 6.31



A tracheal organ culture infected with $\pm 10^3$ CFU/ml of B. parapertussis for 1 hr showing many epithelial cell extrusions. SEM. X 1000.

Figure 6.32



The epithelium of a tracheal culture inoculated with $\pm 10^5$ CFU/ml of B. parapertussis for 1 hr showing moderate extrusion of epithelial cells. SEM. X 1,200.

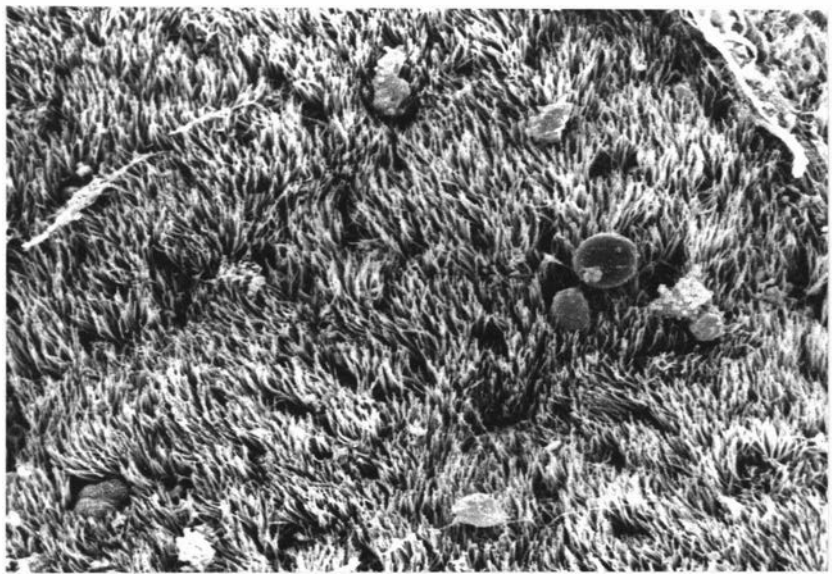


Figure 6.33

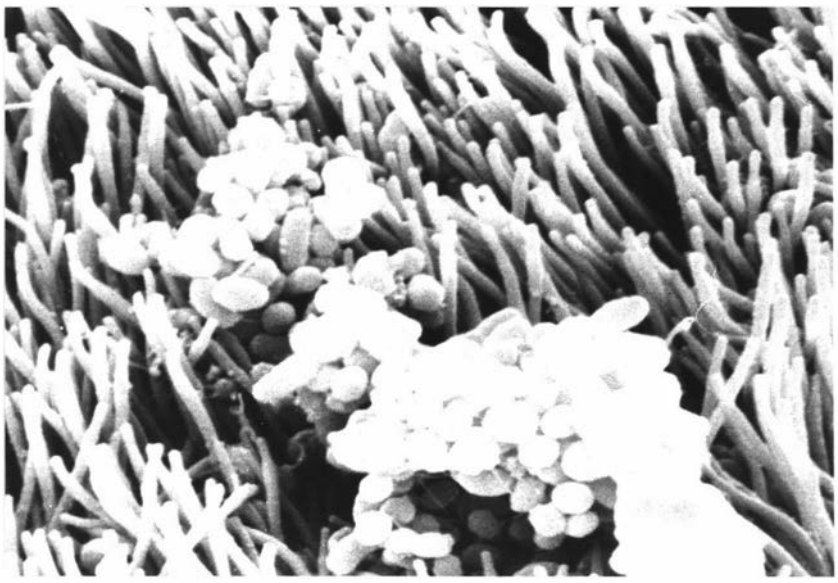
Bacterial microcolonies observed on the top of the ciliary carpet of tracheal cultures inoculated with $\pm 10^5$ CFU/ml of B parapertussis for 1 hr. SEM. X 8000.

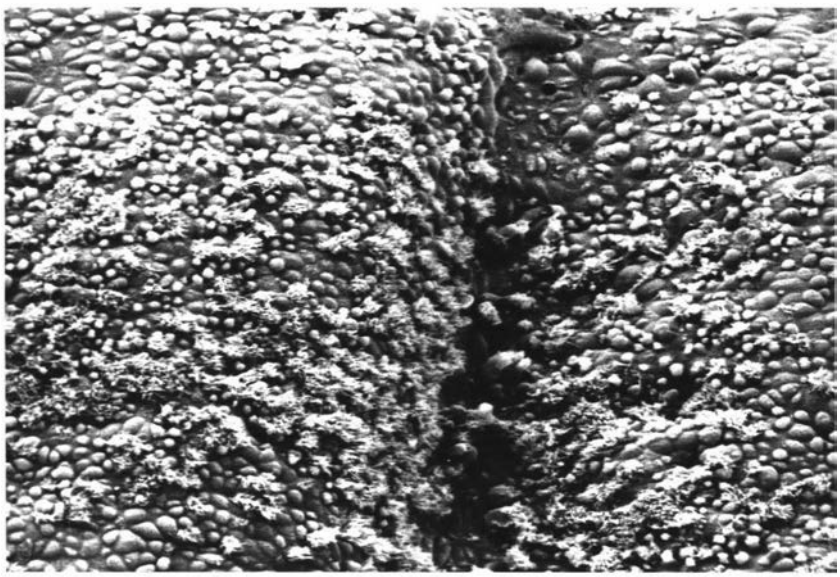
Figure 6.34

The mucus membrane of a tracheal culture inoculated with $\pm 10^7$ CFU/ml of B. parapertussis for 1 hr showing severe loss of cilia. SEM. X 400.

Figure 6.35

High magnification of the epithelium of a tracheal culture infected with $\pm 10^7$ CFU/ml of B. parapertussis. Most of the non-ciliated cells are swollen and protrude into the lumen. SEM. X 2000. The inset micrograph illustrates the close association of B. parapertussis organisms (arrows) with the cilia. SEM. X 10,000.





3.2.4 ULTRASTRUCTURE

The ultrastructural features of uninoculated ovine tracheal organ cultures were similar to those described earlier (Fig. 6.16).

Mild cellular degenerative changes including slight swelling of the nucleus and margination of chromatin were seen in cultures inoculated with bacterial titres of $\pm 10^1$ and $\pm 10^3$ CFU/ml and occasionally bacteria were found among cilia (Fig. 6.36). At 1 hr pi with $\pm 10^5$ CFU/ml titre, multiple encapsulated bacteria could be seen on the luminal border of ciliated cells (Fig. 6.37). Numerous organisms were found among the cilia and up to six organisms could be found between cilia across the width of a single epithelial cell (Fig. 6.37). High magnification revealed that the organisms were surrounded by a dense, fuzzy, pili-like structures (Fig. 6.38), which appeared to mediate the attachment of the bacteria to the cilia. The extracellular membrane of the cilia at the site of the attachment was absent (Fig. 6.38), but bacteria remained in an extracellular location, with no evidence of inter- or intracellular invasion. Adjacent non-ciliated cells had no organisms attached and appeared to be ultrastructurally normal.

Examination of organ cultures 1 hr after inoculation with the highest bacterial titre ($\pm 10^7$ CFU/ml) showed severe damage and exfoliation of most of the ciliated and intermediate cells of the epithelial surface (Fig. 6.39). The epithelium was replaced by extensively flattened elongated cells covering undifferentiated basal cells which formed a continuous monolayer overlying the basement membrane. The cytoplasm of these cells contained prominent supra- and sub-nuclear amorphous material within vacuoles which resembled lysosomes. The interdigitation of the lateral surfaces of the adjacent basal cells were loose. No organisms were seen in close association with the epithelium, but very occasionally single bacteria were found a few microns above the luminal surface of the epithelium.

3.2.5 BACTERIAL GROWTH CURVES IN ORGAN CULTURE

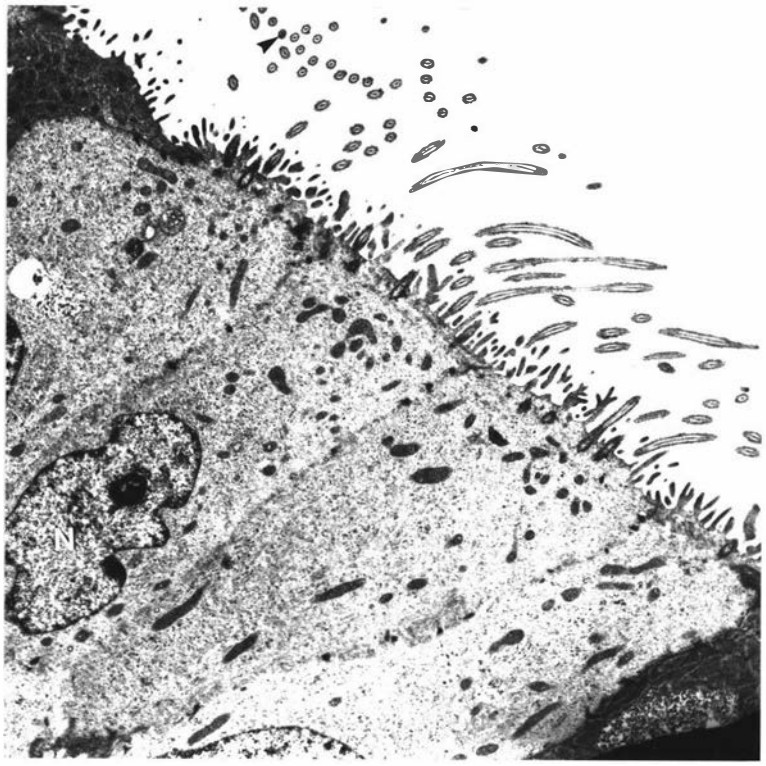
Tracheal organ cultures infected with *B. parapertussis* consistently yielded the organisms at the time of harvest (up to 7.5 hr pi).

Figure 6.36

The epithelium of an organ culture infected with $\pm 10^3$ CFU/ml of B. parapertussis for 1 hr. It exhibits mild cellular degeneration including slight swelling of the nucleus (N) and margination of chromatin. Occasionally bacteria (arrows) were found among the cilia. TEM. X 6,300.

Figure 6.37

A large number of B. parapertussis organisms (Arrows) are present among the cilia of tracheal cultures infected with $\pm 10^5$ CFU/ml for 1 hr but cell damage is minimal. TEM. X 5,000.


N

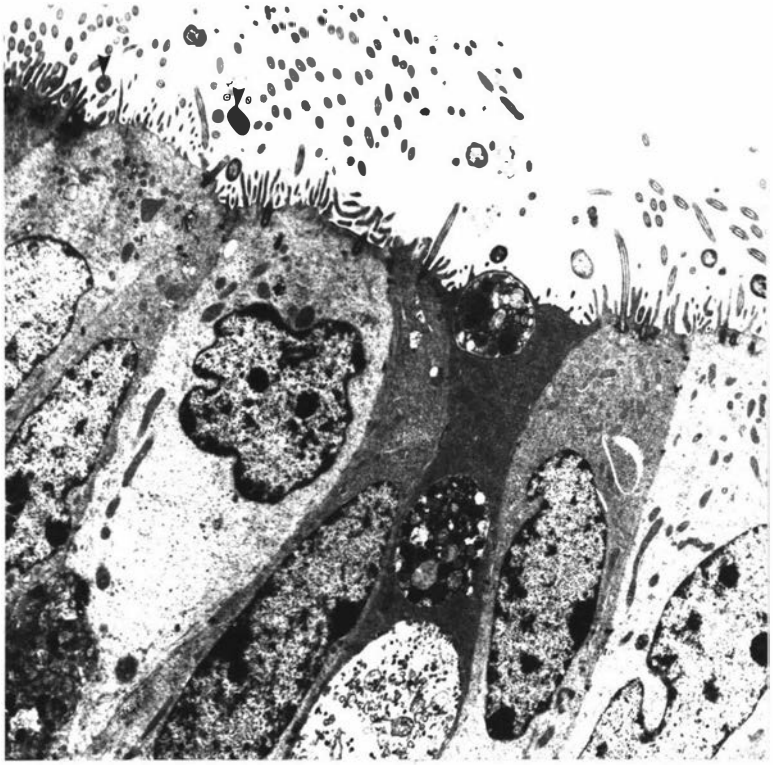
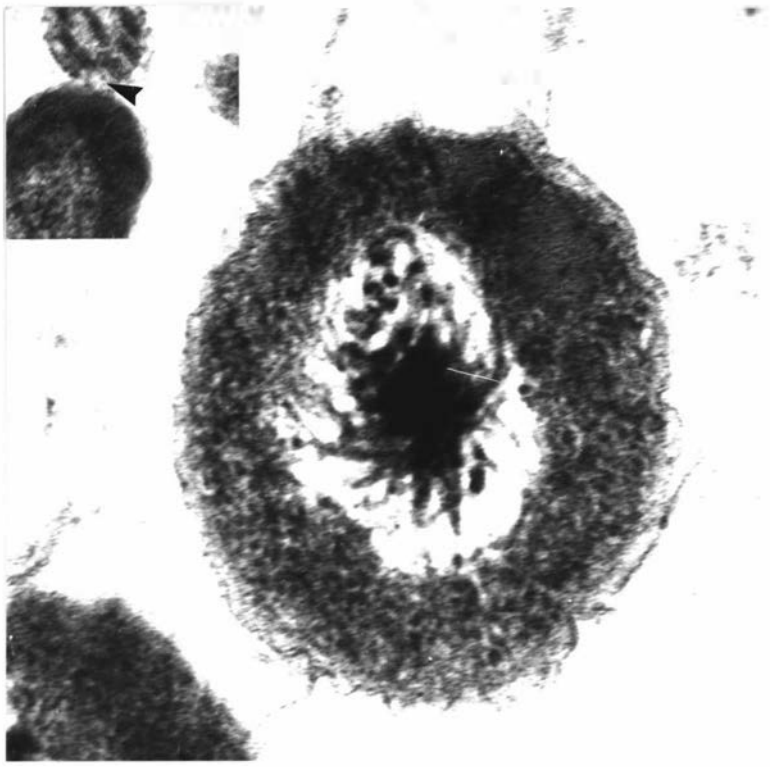


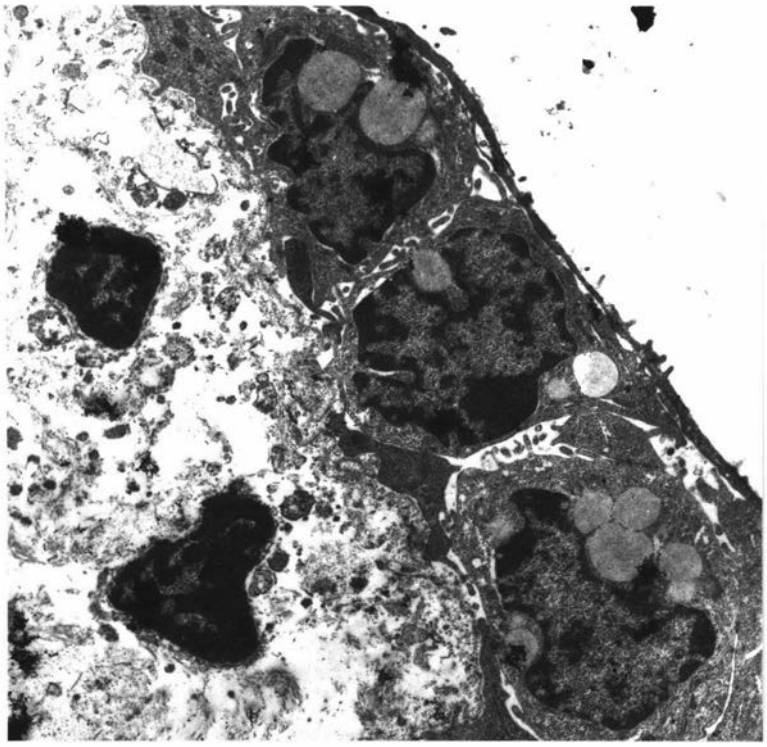
Figure 6.38

High magnification of a B. parapertussis organism showing the dense, fuzzy, pili-like structures on its surface. TEM. X 85,500. The inset micrograph shows that the extracellular membrane of the cilia at the site of bacterial attachment is absent (arrow). TEM. X 64,100.

Figure 6.39

A tracheal organ culture infected with $\pm 10^7$ CFU/ml of B. parapertussis for 1 hr showing severe damage and exfoliation of most of the upper and intermediate cells leaving only a layer of basal cells remaining. TEM. X 8,200.





The points in Figure 6.40 represent the cilia-stopping effect for each titre at the intervals shown. Growth curves observed after the incubation of viable bacteria at titres ranging from $\pm10^1$ - $\pm10^7$ CFU/ml all reached a platau (lag phase) at the level of approximately $\pm10^8$ CFU/ml. Viable numbers reached between $\pm10^6$ - $\pm10^8$ CFU/ml on the seventh hour after inoculation even with titres as low as $\pm10^1$ CFU/ml (Fig. 6.40). Ciliary beating tended to disappear at the time when the viable number of organisms reached $\pm10^6$ - $\pm10^8$ CFU/ml. The time of ciliostasis was also dependent upon the initial titre of the inoculum used (Table 6.5).

3.3 PASTEURELLA HAEMOLYTICA

3.3.1 OBSERVATION OF CILIARY ACTIVITY

Uninoculated tracheal organ cultures, maintained in T199 medium for 3 hr displayed an intact epithelium with vigorously beating cilia throughout the 7 hr of the observation period.

Viable P. haemolytica organisms at $\pm10^9$ CFU/ml, initially produced a significant reduction in ciliary beating within the first hour of exposure, with massive epithelial cell desquamation. Complete inhibition of ciliary activity was achieved by 3 hr (Table 6.8 & 6.9). The other groups of tracheal cultures inoculated with P. haemolytica titres were slower in reducing ciliary activity. The ciliary inactivation patterns were basically similar, but the time taken to reach the various levels of lowered activity increased by 1 - 1.5 hr for each 20-fold decrease in viable organisms inoculated (Table 6.8).

3.3.2 HISTOLOGY

Tracheal tissue maintained in T199 medium for 3 hr retained good preservation of cellular morphology, similar to that described earlier (Fig. 6.1).

Organ cultures inoculated with the two lowest titres of P. haemolytica ($\pm10^3$ CFU/ml and $\pm10^5$ CFU/ml)showed mild to moderate changes at 3 hr pi. These changes included mild nuclear swelling and

TABLE 6.8: THE RELATION BETWEEN TIME - STOPPING EFFECT AND NUMBER OF *P. HAEMOLYTICA* ORGANISMS (CFU/ML)

GROUP CODE NO.	ORIGINAL INOCULUM CFU/ML	NUMBER OF ORGANISMS (CFU/ML) PI AT DIFFERENT TIMES (HR)			
		3	4	5.5	7
01	$\pm 10^9$	$\pm 10^{9*}$	$\pm 10^9$	$\pm 10^9$	$\pm 10^8$
02	$\pm 10^7$	$\pm 10^8$	$\pm 10^{9*}$	$\pm 10^9$	$\pm 10^8$
03	$\pm 10^5$	$\pm 10^8$	$\pm 10^8$	$\pm 10^{9*}$	$\pm 10^9$
04	$\pm 10^3$	$\pm 10^7$	$\pm 10^7$	$\pm 10^8$	$\pm 10^{8*}$

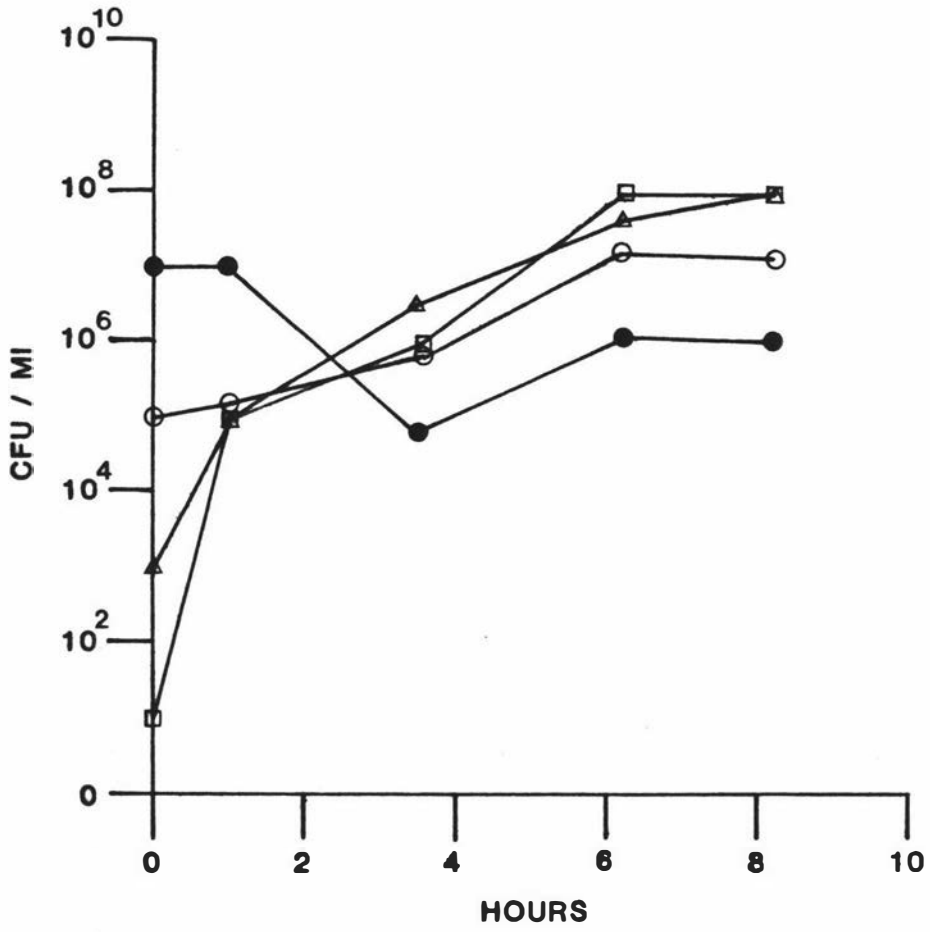
* TIME - STOPPING EFFECT COMPLETED.

TABLE 6.9: EFFECT OF FOUR *P. HAEMOLYTICA* TITRES ON CILIARY ACTIVITY OF TRACHEAL ORGAN CULTURES

ORIGINAL INOCULUM CFU/ML	CILIARY ACTIVITY POST INOCULATION (HR)							
	0	1/2	1	2	3	4	5.5	7
$\pm 10^9$	+++	+++	++	+	-	-	-	-
$\pm 10^7$	+++	+++	+++	+	+	-	-	-
$\pm 10^5$	+++	+++	+++	++	+	+	-	-
$\pm 10^3$	+++	+++	+++	+++	++	+	+	-
CONTROL	+++	+++	+++	+++	+++	+++	+++	+++

Figure 6.40

Bordetella parapertussis growth curves in tracheal organ culture maintained in T199 medium showing that all titres reached a plateau (lag phase) at the level of $\pm 10^6$ to $\pm 10^8$ CFU/ml in the seventh hour post-inoculation.


10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
0
CFU / MI
0
2
4
6
8
10
HOURS

chromatin margination, moderate cytoplasmic vacuolation and loss of cilia from some epithelial cells. There was moderate exfoliation of ciliated epithelial cells and occasional gram negative organisms were present on the remaining cells (Fig. 6.41).

Cellular morphology of tracheal organ cultures at 3 hr after infection with P. haemolytica at titres of $\pm 10^7$ CFU/ml demonstrated severe exfoliation of ciliated and non-ciliated epithelial cells and the remaining epithelial cells exhibited moderate nuclear swelling and chromatin margination, cytoplasmic vacuolation and moderate loss of cilia. Moderate numbers of gram negative organisms were seen on the luminal surface (Fig. 6.42).

The most striking cellular changes in this group of experiments occurred in the tracheal organ cultures inoculated with the highest titres ($\pm 10^9$ CFU/ml) fixed at the cessation of ciliary beating (3 hr, pi) . The epithelial layer was severely damaged and disorganised with severe exfoliation of both ciliated and non-ciliated cells. This involved the whole epithelial thickness leaving large denuded areas (Fig. 6.43). The intact ciliated epithelial cells demonstrated nuclear swelling and chromatin margination, severe cytoplasmic vacuolation in subnuclear areas and loss of cilia. Large areas of ciliated epithelial cells exhibited metaplastic change to cuboidal cells. No organisms were found on the epithelial surface. Table 6.10 summarises the cytopathological changes seen.

3.3.3 TOPOGRAPHICAL MORPHOLOGY

Uninoculated tracheal organ cultures maintained for 3 hr in T199 medium exhibited normal surface morphology which was similar to that described earlier (Fig. 6.8).

The epithelial surface of organ cultures examined 3 hr after inoculation with P. haemolytica at $\pm 10^3$ CFU/ml and $\pm 10^5$ CFU/ML titres showed mild to moderate loss of cilia when compared with the uninfected controls. This was accompanied by the appearance of extruded fragments of ciliated cells which left behind a smooth-surfaced membrane. The cilia showed an obvious lack of rigidity and some lay across the non-ciliated surface , while others were tufted together(Fig. 6.44). The non-ciliated surface appeared

TABLE 6.10: CYTOPATHOLOGICAL CHANGES IN EPITHELIAL CELLS OF TRACHEAL ORGAN CULTURES INOCULATED WITH *P. HAEMOLYTICA*

ORIGINAL INOCULUM CFU/ML	CYTOPATHOLOGICAL CHANGES				NUMBER OF BACTERIA ON LUMINAL SURFACE
	ENLARGMENT OF NUCLEI	CYTOPLASMIC VACUOLATION	EPITHELIAL EXFOLIATION	EPITHELIAL METAPLASIA	
$\pm 10^9$	SEVERE	SEVERE	VERY SEVERE	NIL	OCCASIONAL
$\pm 10^7$	SEVERE	MODERATE	SEVERE	NIL	FEW
$\pm 10^5$	MODERATE	MODERATE	MILD	NIL	MODERATE
$\pm 10^3$	MILD	MODERATE	MILD	PRESENT	MODERATE

Figure 6.41

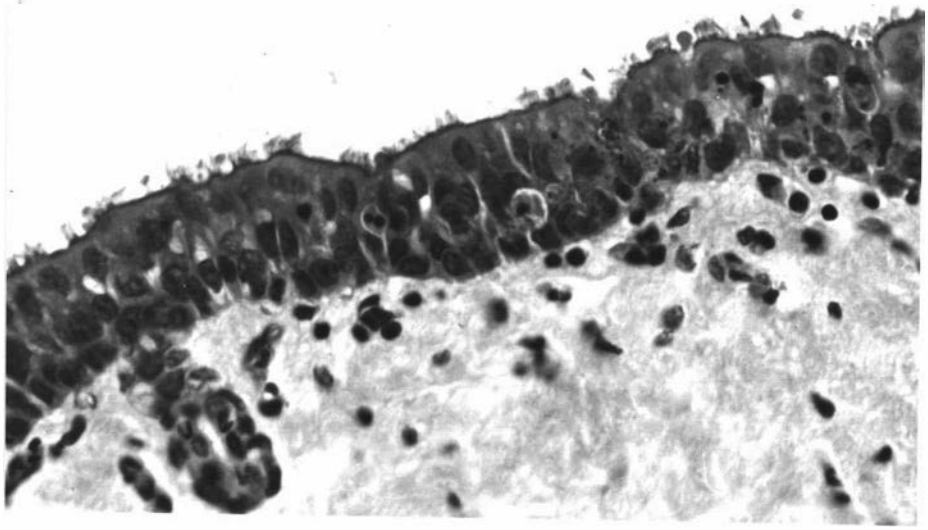
A tracheal organ culture inoculated with $\pm 10^3$ CFU/ml of P. haemolytica for 3 hrs in T199 medium. The epithelial cells show mild nuclear swelling, vacuolation and loss of cilia. H&E. X 312.

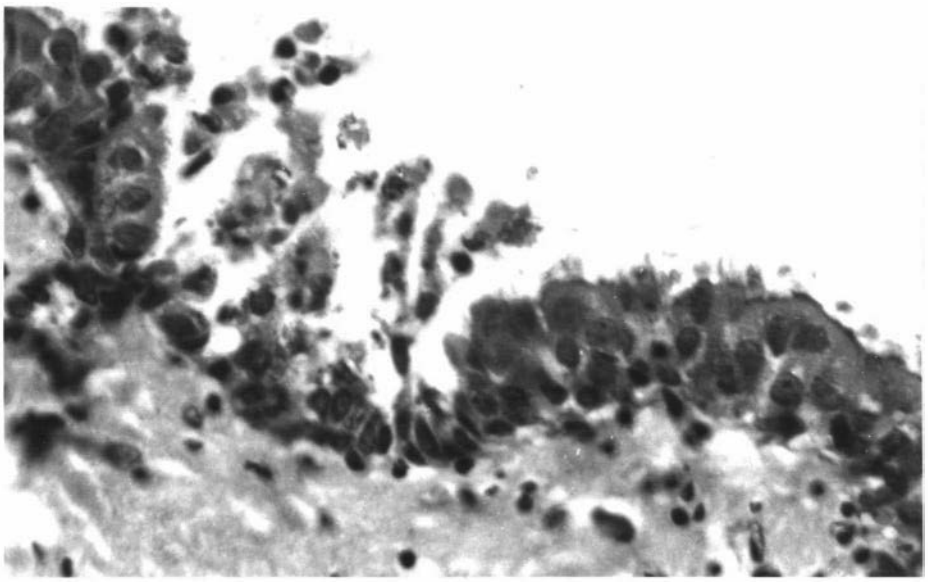
Figure 6.42

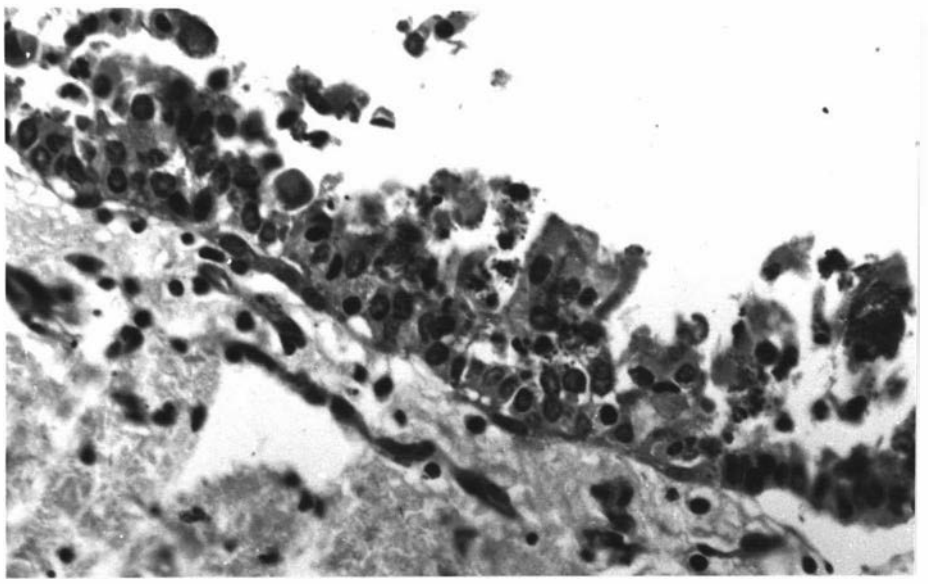
An organ culture infected with $\pm 10^7$ CFU/ml of P. haemolytica for 3 hrs and maintained in T199 medium.There is severe exfoliation of ciliated and non-ciliated epithelial cells (arrows). H&E. X 312.

Figure 6.43

A tracheal organ culture inoculated with $\pm 10^9$ CFU/ml of P. haemolytica for 3 hrs and maintained in T199 medium. The epithelial layer is severely disorganised with severe exfoliation of both ciliated and non-ciliated cells. H&E. X 312.







flattened with numerous microvilli. No organisms were found attached to the epithelial surface ciliated or non-ciliated cells (Fig. 6.44).

Severe epithelial damage was seen in tracheal rings inoculated with P. haemolytica at titres of $\pm 10^7$ CFU/ml. The exfoliated cells covered the entire luminal surface (Fig. 6.45) and most were swollen and exhibited a variety of morphological changes. The extruded cells consisted of both ciliated and non-ciliated cells together with cellular fragments. The plasma membrane of these cells was either roughened or smooth with numerous holes and pits (Fig. 6.45). No organisms were seen attached to any epithelial surfaces in these cultures.

Tracheal cultures examined 3 hr pi with the highest titres of P. haemolytica ($\pm 10^9$ CFU/ml) showed extensive and severe exfoliation of the epithelium with complete loss of intact cells (Fig. 6.46). The exfoliated cells were characterised by a smooth-surfaced membrane which was in most instances was severely damaged by holes and pits (Fig. 6.47). The plasma membrane of most of the exfoliated cells was shrunken. Structures recognisable as capsulated bacilli-like organisms were found occasionally but were not in close contact with the cellular membrane and tended to form small microcolonies trapped between the fragments of cellular debri (Fig. 6.47).

3.3.4 ULTRASTRUCTURE

There was good preservation of ciliated epithelium in the uninfected organ cultures which resembled the controls of earlier experiments (Fig. 6.16).

Organ cultures maintained in T199 medium and examined 3 hr after inoculation with $\pm 10^3$ CFU/ml of P. haemolytica were markedly different from controls. There were fewer ciliated cells and those present contained less cilia. The epithelial cells were swollen and their cytoplasm contained numerous vacuoles. Some vacuoles were large and contained an amorphous, dark material resembling lipid. Lysosomes were also prominent (Fig. 6.48). These changes were more pronounced and widespread in the organ cultures infected with bacterial titres of $\pm 10^5$ CFU/ml for 3 hr. The most prominent changes seen in this group were subnuclear vacuolation and the development of

Figure 6.44

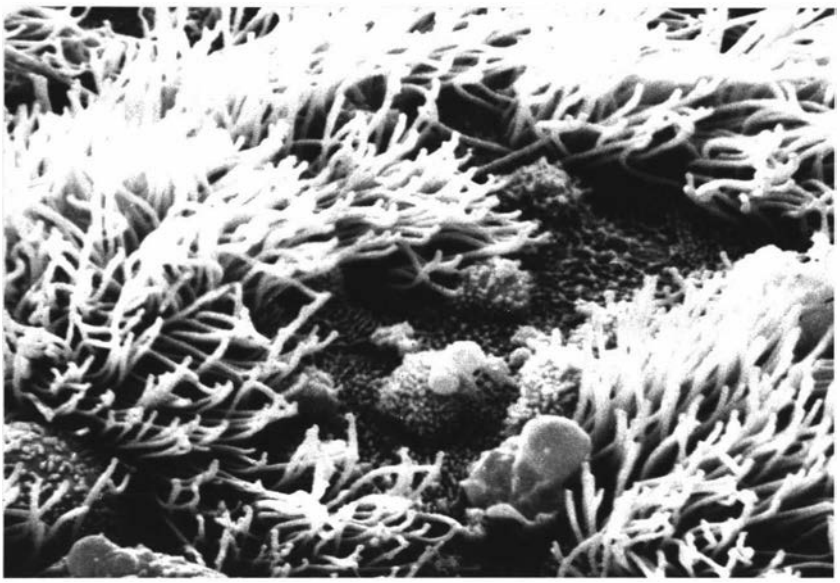
The epithelial surface of an organ culture inoculated with $\pm 10^3$ CFU/ml of P. haemolytica for 3 hrs showing moderate loss of cilia. The cilia show an obvious lack of rigidity and some lie across the non-ciliated surface. The non-ciliated surface appears flattened and shows numerous microvilli. SEM. X 4,000.

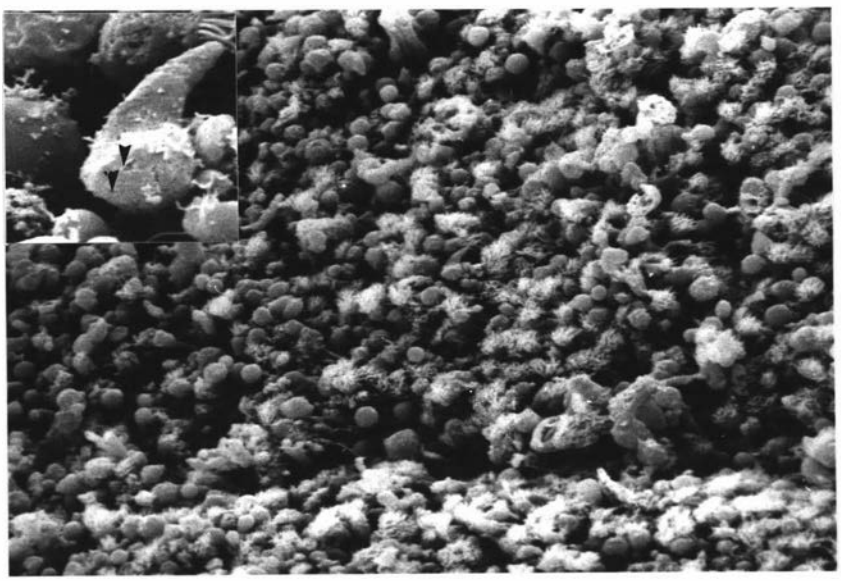
Figure 6.45

An organ culture infected with $\pm 10^7$ CFU/ml of P. haemolytica for 3 hrs showing severe epithelial damage. Exfoliated cells cover the entire luminal surface. SEM. X 400. The inset micrograph shows the plasma membrane of an exfoliated cell which is roughened and contains numerous holes and pits (arrows). SEM. 2,000.

Figure 6.46

A tracheal organ culture examined 3 hrs post-inoculation with $\pm 10^9$ CFU/ml of P. haemolytica showing extensive and severe exfoliation of the epithelium with complete loss of cilia. SEM. X 400.





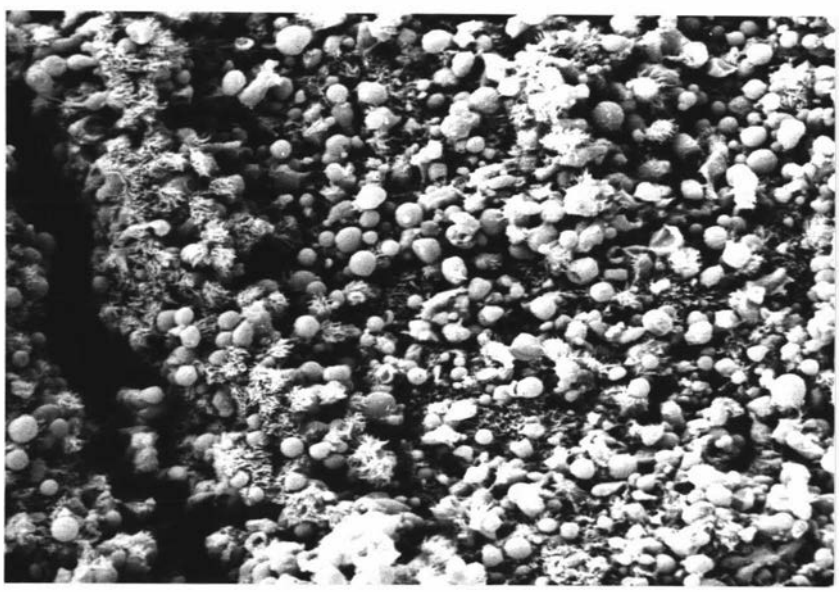
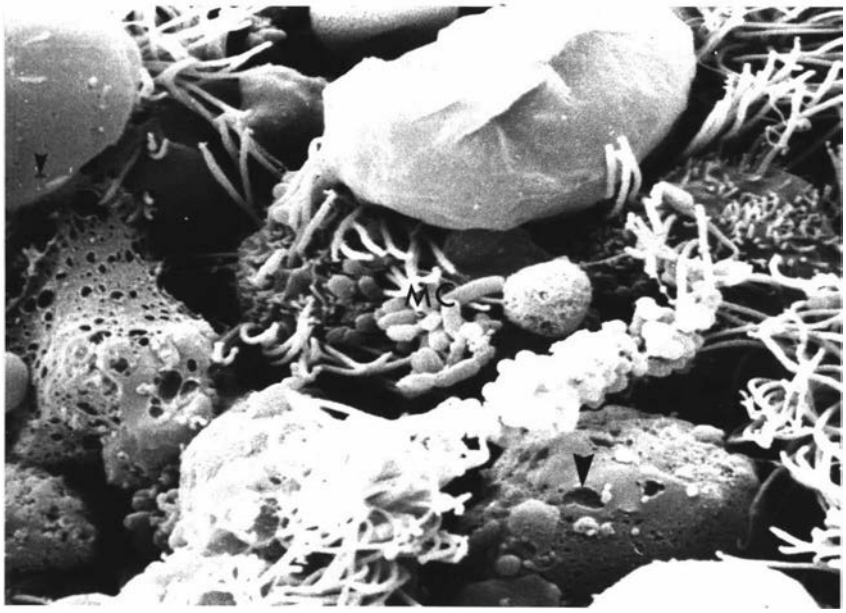


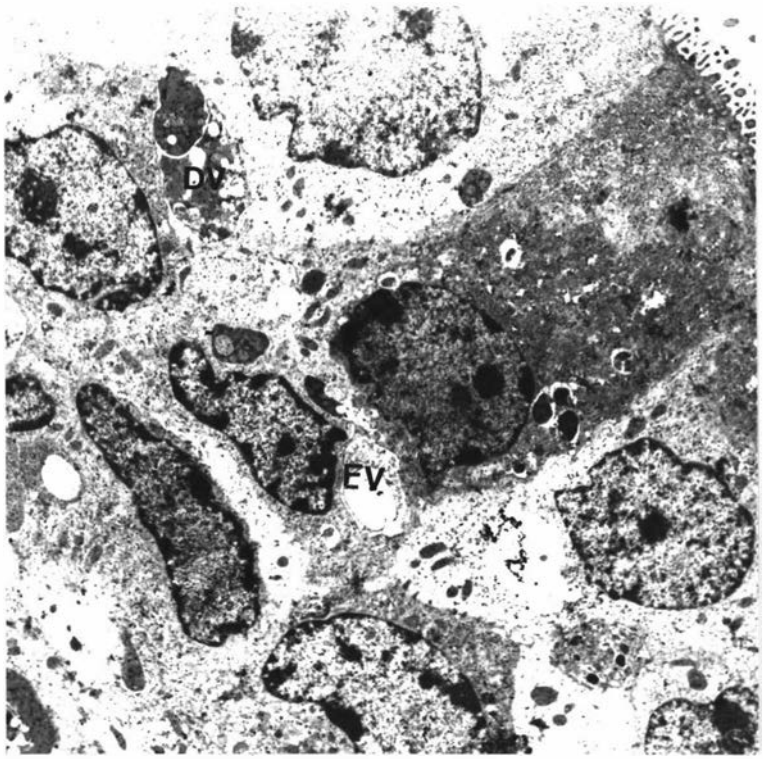
Figure 6.47

Exfoliated cells from organ cultures infected with $\pm 10^9$ CFU/ml of P. haemolytica for 3 hrs. They were characterised by a smooth-surfaced membrane which is severely damaged by holes and pits (arrows). Capsulated bacilli-like organisms were observed in small microcolonies (MC) between the fragments of cellular debri. SEM. X 4,000.

Figure 6.48

The ultrastructure of a tracheal culture infected with $\pm 10^3$ CFU/ml of P. haemolytica and examined 3 hrs post-inoculation. The epithelial cells are swollen and their cytoplasm contains numerous empty vacuoles (EV). Some vacuoles are large and contain an amorphous, dark material (DV). TEM. X 5,000.


MC


DV
EV

translucent areas in the subnuclear cytoplasm (Fig. 6.49).

The intact epithelial layer of cultures inoculated with $\pm 10^7$ CFU/ml, showed severe morphological changes. Many cells displayed loss of cilia and nuclear enlargement. The subnuclear cytoplasm was translucent having lost most of its ultrastructural constituents. Most of the RER was absent and the mitochondria appeared enlarged with disrupted cristae (Fig. 6.50). These changes became more severe and widespread in the epithelium of tracheal organ cultures inoculated with $\pm 10^9$ CFU/ml, especially the cytoplasmic and surface changes. The epithelial layer was highly disorganised and many cells showed nuclear enlargement with chromatin margination. The luminal surface was markedly uneven due to cytoplasmic budding and swelling of microvilli. The cytoplasmic vacuolation was very severe and involved both supranuclear and subnuclear areas (Fig. 6.51). In addition, the mitochondria were enlarged and most contained disrupted cristae (Fig. 6.51).

3.3.5 BACTERIAL GROWTH CURVES IN ORGAN CULTURE

Pasteurella haemolytica organisms multiplied readily in ovine tracheal organ cultures maintained in T199 medium. Growth curves observed after inoculation of viable organisms at titres between $\pm 10^3$ to $\pm 10^9$ CFU/ml showed plateaus (lag phase) of growth at a level of $\pm 10^9$ CFU/ml. This titre was reached with all inocula on about the seventh hour of infection (Fig. 6.52). Bacterial titres of $\pm 10^3$ CFU/ml increased five fold within 7 hr; titres of $\pm 10^5$ and $\pm 10^7$ CFU/ml increased four and two fold respectively within 5.5 hr pi and titres $\pm 10^9$ CFU/ml remained stable in lag phase up to 5.5 hr before declining (Fig. 6.52).

3.4 NEISSERIA CATARRHALIS

3.4.1 OBSERVATION ON CILIARY ACTIVITY

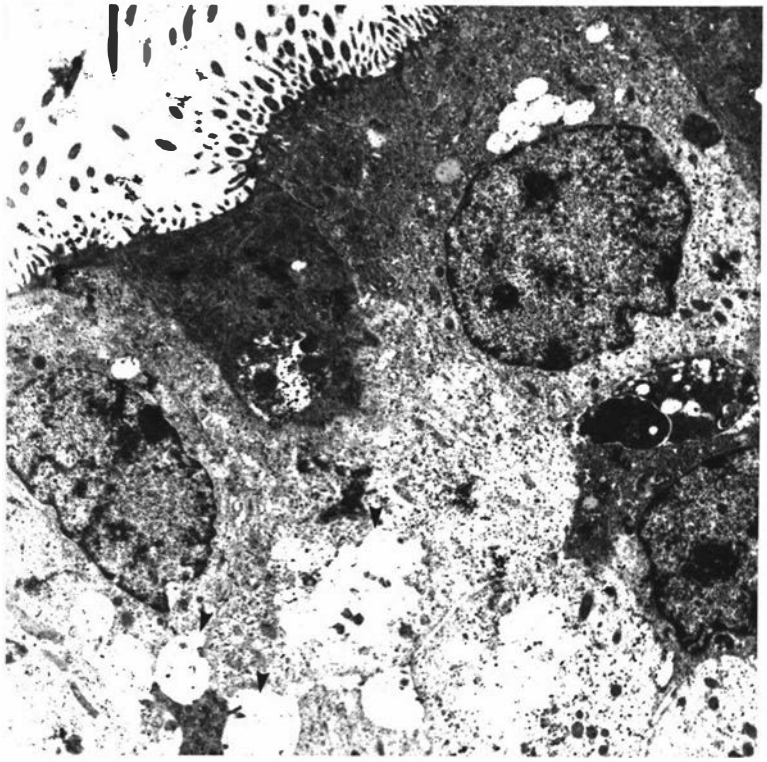
Ciliary beating of uninfected tracheal organ cultures remained strong for up to 9 hr after preparation.(Table 6.11).

Figure 6.49

A tracheal organ culture infected with $\pm 10^5$ CFU/ml of P. haemolytica for 3 hrs. Subnuclear vacuolation (arrows) was the most prominent change observed in the epithelial cells. TEM. X 5,000.

Figure 6.50

Epithelial cells of a tracheal organ culture infected with $\pm 10^7$ CFU/ml of P. haemolytica for 3 hrs. There is severe loss of cilia (arrows) and nuclear enlargement (N). The subnuclear cytoplasm is translucent having lost most of its ultrastructural constituents. TEM. X 5,000.



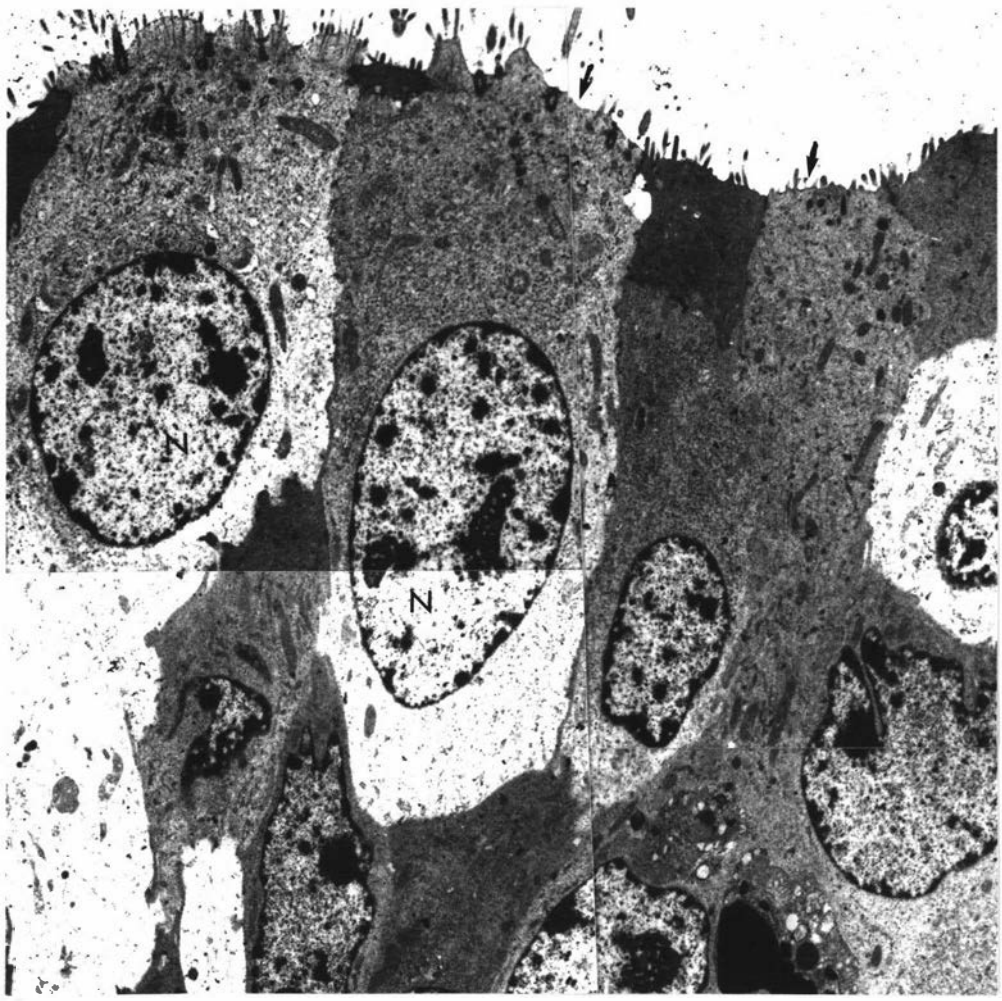

N
N

Figure 6.51

The luminal surface of a tracheal culture infected with $\pm 10^9$ CFU/ml of P. haemolytica for 3 hrs. It is markedly uneven due to cytoplasmic budding and swelling of microvilli. The cytoplasm contains numerous vacuoles in both supranuclear and subnuclear areas. TEM. X 2,000.

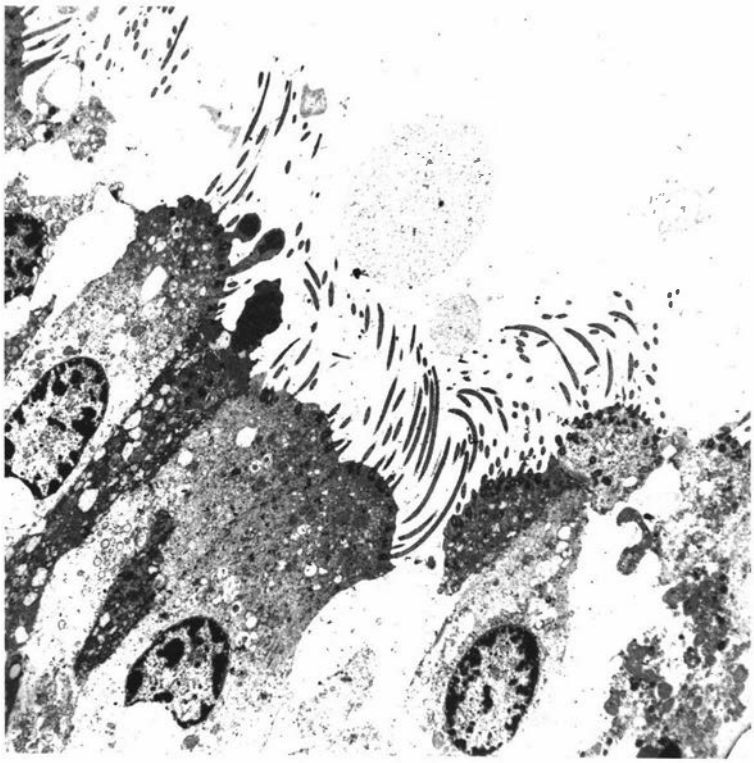
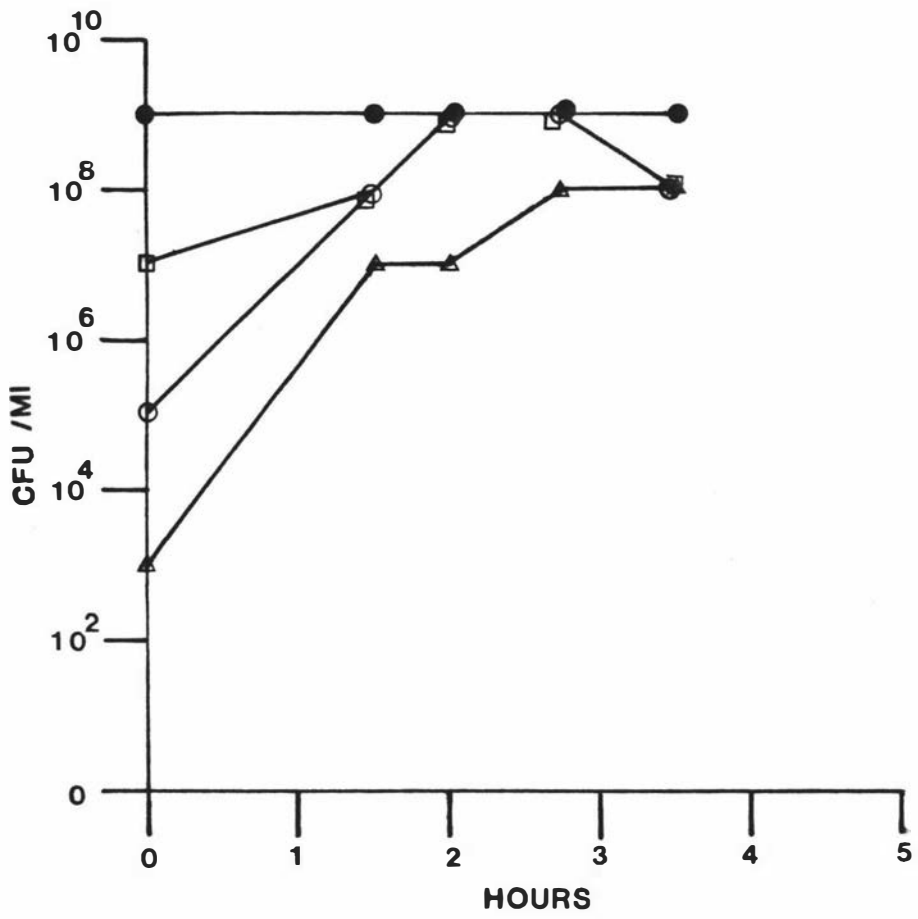


Figure 6.52

Pasteurella haemolytica growth curves in tracheal organ culture maintained in T199 medium showing that all titres reached a plateau of growth at a level of $\pm 10^8$ to $\pm 10^9$ CFU/ml 7hrs post-inoculation.


10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
0
CFU /Ml
0
1
2
3
4
5
HOURS

When tracheal cultures were infected with N. catarrhalis at high titres ($\pm 10^8$ CFU/ml) there was slowing of the ciliary activity 2 hr pi followed by complete ciliostasis 2 hr later (Table 6.12). The viable number of N. catarrhalis for each inoculum at the time of ciliostasis at the intervals shown are given in Table 6.11. Cessation of ciliary activity occurred when each inoculum reached $\pm 10^8$ CFU/ml at times of 4, 5, 6 and 9 hr. At lower bacterial titres there was no essential difference in the pattern of development of ciliostasis although it developed more slowly.

3.4.2 HISTOLOGY

Uninfected tracheal tissue cultures retained a comparatively good ciliated epithelium when fixed at 4 hours and showed similar histological appearance to those described earlier (Fig. 6.1).

Tracheal rings infected with the lowest bacterial titres ($\pm 10^2$ CFU/ml) showed mild nuclear swelling and cytoplasmic vacuolation. There was moderate loss of cilia and mild epithelial cell exfoliation. In some areas, the epithelial cells took on a cuboidal appearance. Sections stained with gram Twort showed gram negative diplococci organisms between cellular debri but no organisms were found on the intact epithelial surface (Fig. 6.53).

Organ cultures infected with bacterial titres of $\pm 10^4$ CFU/ml showed similar but slightly more severe histopathological changes and moderate epithelial exfoliation. Cytoplasmic vacuolation and early squamous metaplasia were the most prominent changes (Fig. 6.54). No organisms were seen in sections stained with gram Twort technique.

The cellular architecture of the epithelium of rings infected with bacterial titres $\pm 10^6$ CFU/ml was disorganised and the nuclei appeared pyknotic. Vacuolation was severe and present in most cells. In addition, most cells appeared to have lost their cilia and there was mild epithelial exfoliation involving both ciliated and intermediate layers. Neither metaplastic changes nor N. catarrhalis organisms were observed (Fig. 6.55).

Tracheal tissue in organ cultures exposed to the highest N

TABLE 6.11: THE RELATION BETWEEN TIME-STOPPING EFFECT AND NUMBER OF N. CATARRHALIS ORGANISMS (CFU/ML)

GROUP CODE NO.	ORIGINAL INOCULUM CFU/ML	VIABLE NUMBER OF ORGANISMS CFU/ML PI (HR)			
		4	5	6	8
01	$\pm 10^8$	$\pm 10^{8*}$	$\pm 10^8$	$\pm 10^8$	$\pm 10^8$
02	$\pm 10^6$	$\pm 10^8$	$\pm 10^{8*}$	$\pm 10^8$	$\pm 10^8$
03	$\pm 10^4$	$\pm 19^8$	$\pm 10^8$	$\pm 10^{8*}$	$\pm 10^8$
04	$\pm 10^2$	$\pm 10^8$	$\pm 10^8$	$\pm 10^8$	$\pm 10^{8*}$

* TIME - STOPPING EFFECT COMPLETED.

TABLE 6.12: EFFECT OF FOUR N. CATARRHALIS TITRES ON THE CILIARY ACTIVITY OF TRACHEAL ORGAN CULTURES

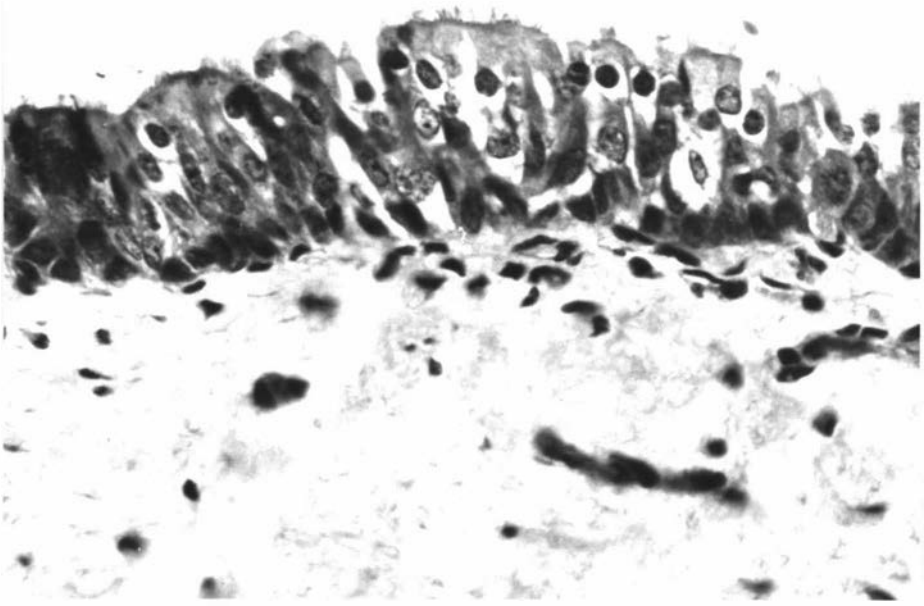
ORIGINAL INOCULUM	CILIARY ACTIVITY INOCULATION (HR)								
	0	1/2	1	3	4	5	6	8	9
$\pm 10^8$	+++	+++	++	+	-	-	-	-	-
$\pm 10^6$	+++	+++	++	+	+	-	-	-	-
$\pm 10^4$	+++	+++	+++	++	+	+	-	-	-
$\pm 10^2$	+++	+++	+++	+++	++	++	+	+	-
CONTROL	+++	+++	+++	+++	+++	+++	+++	+++	+++

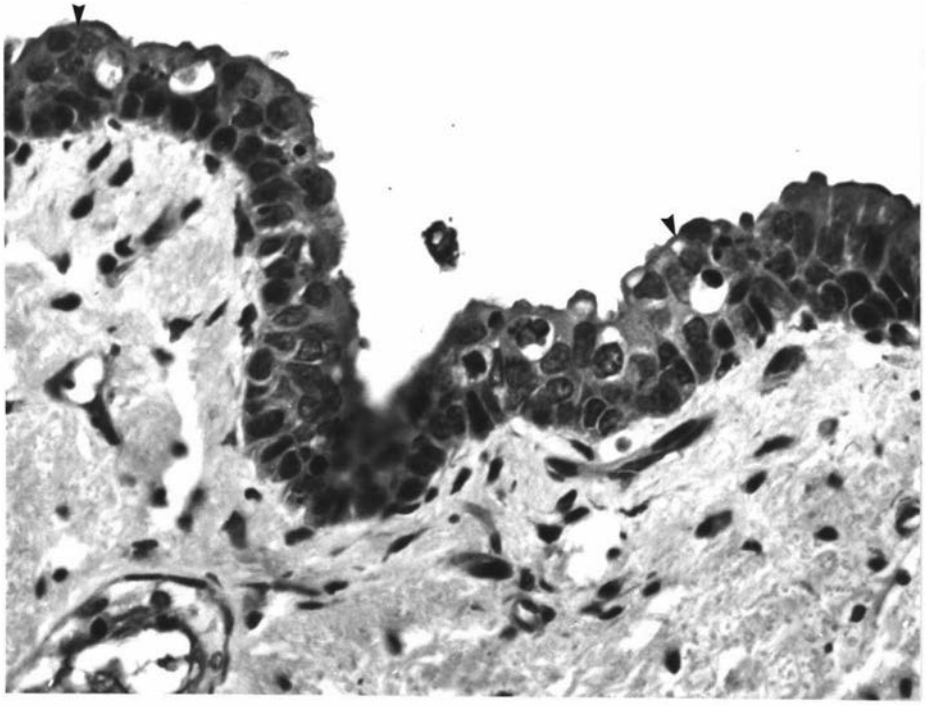
Figure 6.53

A tracheal ring infected with $\pm$ 10^2 CFU/ml *N. catarrhalis* for 4 hrs and maintained in T199 medium. There is moderate loss of cilia and mild epithelial cell exfoliation. H&E. X 312.

Figure 6.54

An organ culture infected with $\pm 10^4$ CFU/ml *N. catarrhalis* for 4 hrs and maintained in T199 medium. The cytoplasmic vacuolation of epithelial cells and early squamous metaplasia (arrows) are the main features. H&E. X 312.





catarrhalis titres ($\pm 10^8$ CFU/ML) showed evidence of severe damage to the epithelial layer.There was moderate exfoliation, severe loss of cilia and severe cytoplasmic vacuolation. The exposed epithelial surface took on an appearance of squamous metaplasia (Fig. 6.56) but no attached organisms were demonstrated. Table 6.13 summarizes the cytopathological changes which occurred.

3.4.3 TOPOGRAPHICAL MORPHOLOGY

The mucosal surface of uninoculated organ cultures appeared generally intact and was similar in appearance to those described earlier (Fig. 6.8), although occasionally a few sloughed cells were observed.

Organ cultures infected with the lowest bacterial titres ($\pm 10^2$ CFU/ml) showed slight epithelial disruption with mild loss of cilia and sloughing of a few epithelial cells (Fig. 6.57). The epithelial surface appeared highly ciliated and the sloughed epithelial cells also remained predominantly ciliated. Neisseria catarrhalis organisms were not seen associated or attached to the epithelial surface.

The luminal surface of tracheal rings inoculated with $\pm 10^4$ CFU/ml showed a greater loss of cilia. This loss was accompanied by the appearance of bulbous bulges on the epithelial surface. There were distinct boundaries between the bulges and the rest of epithelium suggesting that the bulges originated from cells in the inferior epithelial layer. The surface of these bulges varied from rough to microvillus like in appearance. The cilia of the intact ciliated cells showed loss of rigidity and regularity (Fig. 6.58).

Loss of cilia was more pronounced in organ cultures infected with $\pm 10^6$ CFU/ml and there was moderate exfoliation of epithelial cells. Although there was an increase in the non-ciliated surface the number of bulges or protruded cells was fewer than at lower titres. The cilia of intact ciliated cells appeared entangled and showed loss of rigidity (Fig. 6.59).

The epithelial surface of tracheal cultures infected with $\pm 10^8$ CFU/ml showed severe loss of cilia. The remaining non-ciliated surface was uneven and rough with numerous knob-like projections (Fig.

TABLE 6.13: CYTOPATHOLOGICAL CHANGES IN EPITHELIAL CELLS OF TRACHEAL ORGAN CULTURES INOCULATED WITH *N. CATARRHALIS*

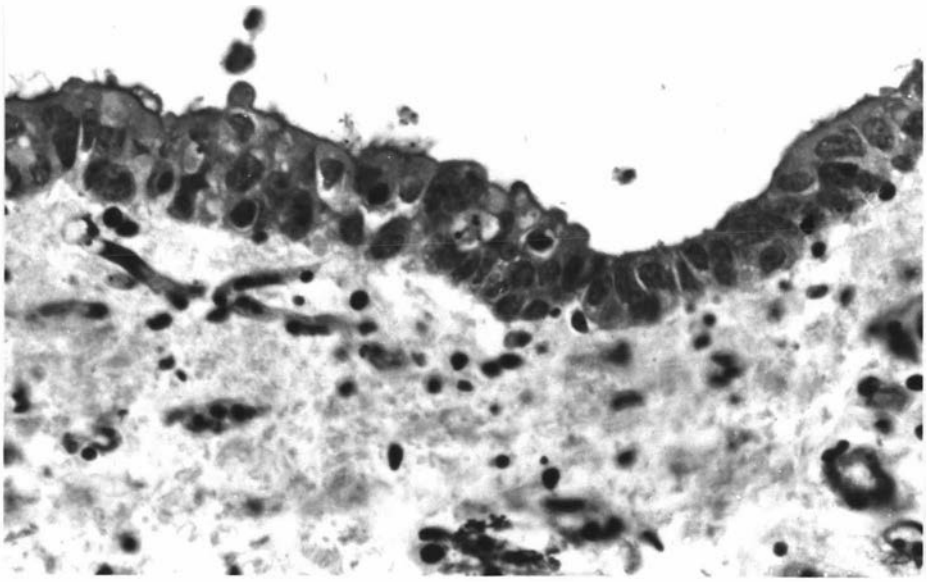
ORIGINAL INOCULUM CFU/ML	CYTOPATHOLOGICAL CHANGES				NUMBER OF BACTERIA ON LUMINAL SURFACE
	ENLARGMENT OF NUCLEI	CYTOPLASMIC VACUOLATION	EPITHELIAL EXFOLIATION	EPITHELIAL METAPLASIA	
$\pm 19^8$	SEVERE	SEVERE	SEVERE	PRESENT	NIL
$\pm 10^6$	SEVERE	SEVERE	SEVERE	PRESENT	NIL
$\pm 10^4$	MODERATE	MODERATE	MODERATE	PRESENT	NIL
$\pm 10^2$	MILD	MILD	MILD	PRESENT	NIL

Figure 6.55

An organ culture inoculated with $\pm 10^6$ CFU/ml *N. catarrhalis* for 4 hrs and maintained in T199 medium. The epithelial layer is disorganised and there is moderate loss of cilia as well as mild epithelial exfoliation. H&E. X 312.

Figure 6.56

A tracheal culture infected with $\pm 10^8$ CFU/ml *N. catarrhalis* for 4 hrs and maintained in T199 medium. There is severe loss of cilia and ciliated cells. In some areas the remaining epithelium shows squamous metaplasia. H&E. X 312.



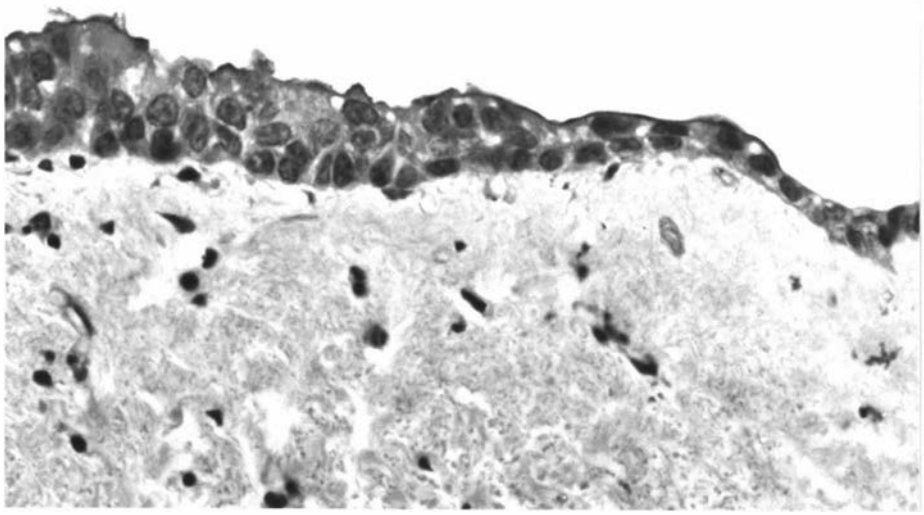


Figure 6.57

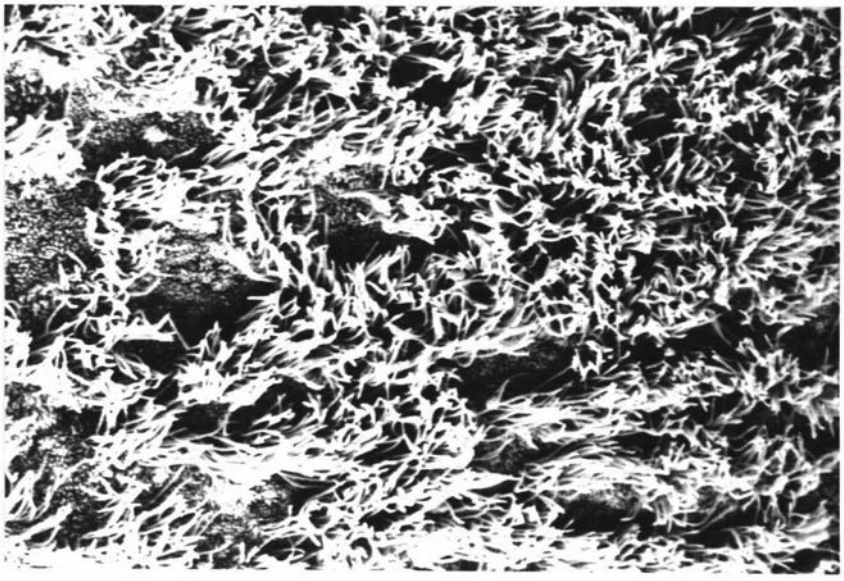
The epithelium of an organ culture infected with $\pm 10^2$ CFU/ml N. catarrhalis for 4 hrs showing mild loss of cilia and sloughing of a few epithelial cells. SEM. X 2,000.

Figure 6.58

The luminal surface of a tracheal organ culture inoculated with $\pm 10^4$ CFU/ml of N. catarrhalis for 4 hrs showing numerous bulbous projections. The surface of these varies from rough to microvillus-like in appearance. SEM. X 2,000.

Figure 6.59

The surface epithelium of an organ culture infected with $\pm 10^6$ CFU/ml of N. catarrhalis for 4 hrs. Although there is an increase in non-ciliated cells (arrows) the number of bulges and protruding cells in less than at lower titres. SEM. X 2,000.



6.60). Some cells had roughened apical surfaces marginated by linear structures, resembling of the bases of previously fragmented epithelial cells. Other cells appeared enlarged and swollen and many had acquired a ruffled plasma membrane which protruded from the luminal surface (Fig. 6.60).

3.4.4 ULTRASTRUCTURE

The ultrastructural morphology of tissue from uninoculated control organ cultures was healthy in appearance and the epithelium remained comparatively well differentiated throughout the experiment (9 hr), which was similar to earlier findings (Fig. 6.16).

Mild loss of cilia and focal cytoplasmic alterations were found in the tracheal rings inoculated with $\pm 10^2$ CFU/ml of N catarrhalis (Fig. 6.61). These changes were more prominent and widespread in the tracheal cultures inoculated with $\pm 10^4$ CFU/ml. At this titre there was prominent subnuclear vacuolation, moderate numbers of lysosomes, mitochondrial enlargement and nuclear margination. Moderate number of sloughed epithelial cells or cell fragments were observed on the luminal surface (Fig. 6.62).

The epithelial layer of tracheal cultures infected with $\pm 10^6$ CFU/ml mainly consisted of only two layers; most of ciliated epithelial cells having sloughed. The upper layer was composed of very flattened cells with few microvilli, while the lower layer contained almost cuboidal cells. Both sloughed and intact epithelial cells exhibited severe vacuolation, nuclear margination of chromatin, and a loss in intercellular digitation (Fig. 6.63). At this titre the nuclei of sloughed cells or cells undergoing exfoliation showed no discrete nuclei and the mitochondria were electron-dense (Fig. 6.64). These changes were more severe in the tracheal epithelium inoculated with $\pm 10^8$ CFU/ml, and there was loss of microvilli from the luminal surface (Fig. 6.65). There was no evidence of N. catarrhalis organisms attached to the cilia or penetrating the epithelium in any of the inoculated cultures.

3.4.5 BACTERIAL GROWTH CURVES IN ORGAN CULTURE

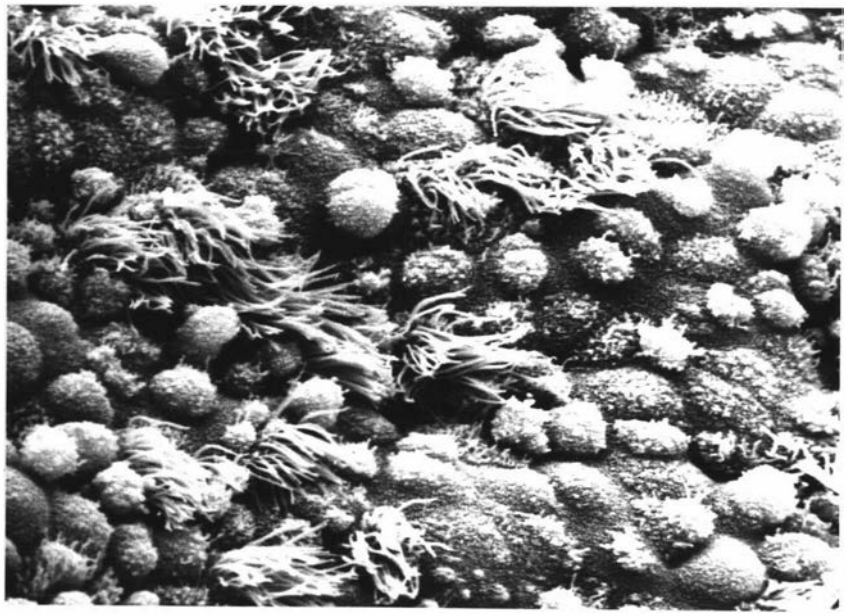
NEISSERIA CATARRHALIS organisms multiplied rapidly in ovine

Figure 6.60

The epithelial surface of a tracheal culture infected with $\pm 10^8$ CFU/ml of N. catarrhalis for 4 hrs showing severe loss of cilia. The non-ciliated surface in uneven and rough with numerous knob-like projections. Other cells are enlarged and swollen and many have a ruffled plasma membrane. SEM. X 4,000.

Figure 6.61

An organ culture infected with $\pm 10^2$ CFU/ml of N. catarrhalis showing mild loss of cilia only. TEM. X 6,300.



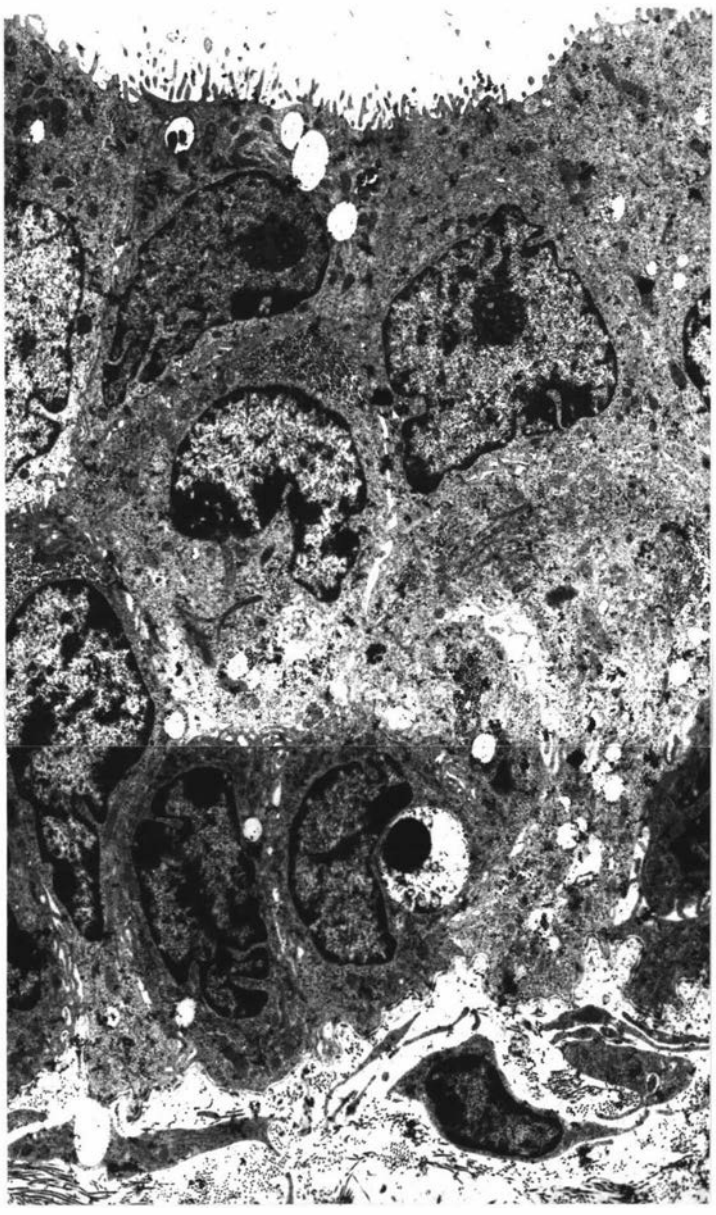
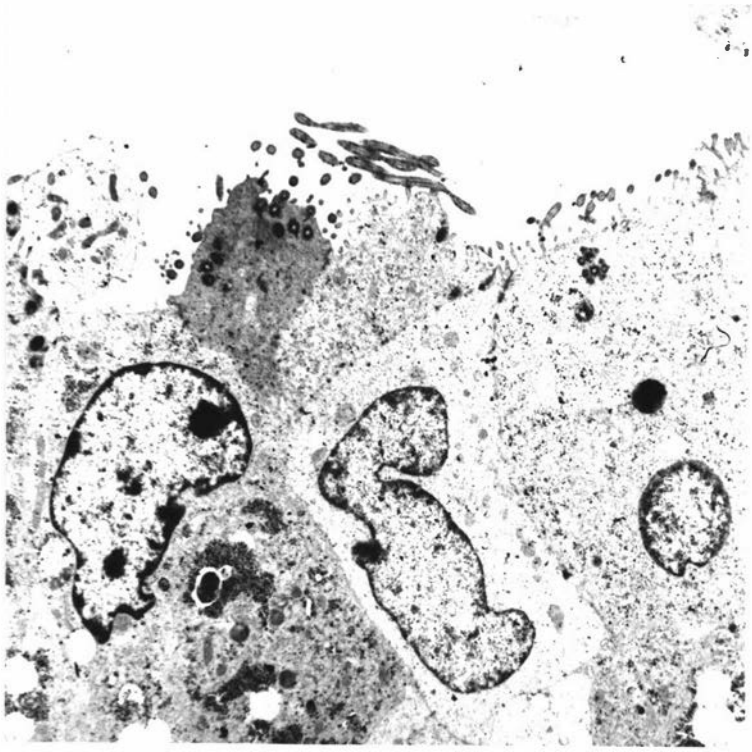


Figure 6.62

A tracheal culture infected with $\pm 10^4$ CFU/ml of N. catarrhalis for 4 hrs showing prominent subnuclear vacuolation and nuclear margination of some epithelial cells. TEM. X 5,000.

Figure 6.63

The epithelium of a tracheal organ culture infected with $\pm 10^6$ CFU/ml of N. catarrhalis for 4 hrs. It consists of an upper layer which is composed of flattened cells with few microvilli and a lower layer containing almost cuboidal cells. Both sloughed and intact epithelial cells exhibit severe vacuolation (V) and loss of intercellular digitation. TEM. X 3,200.



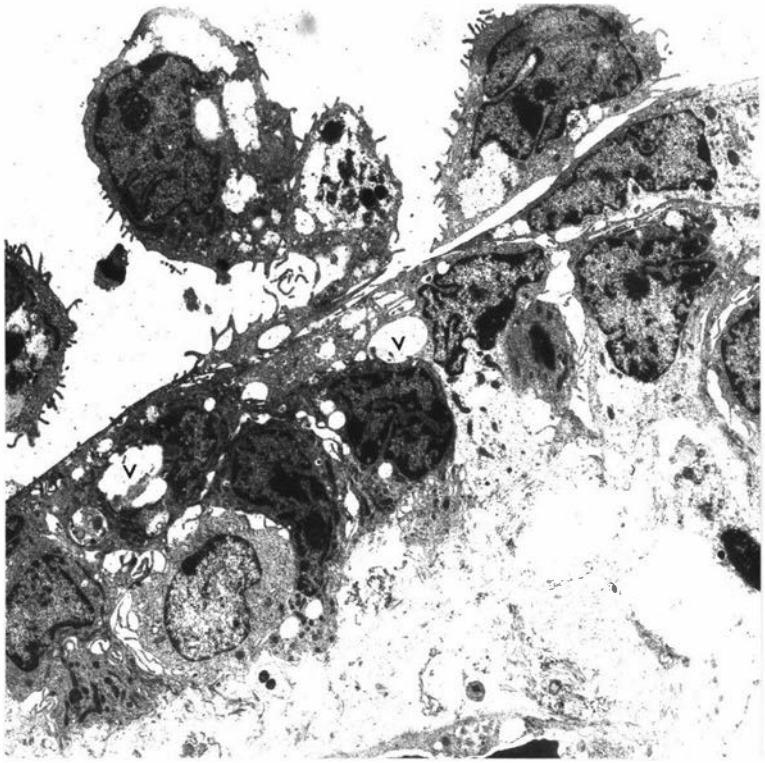
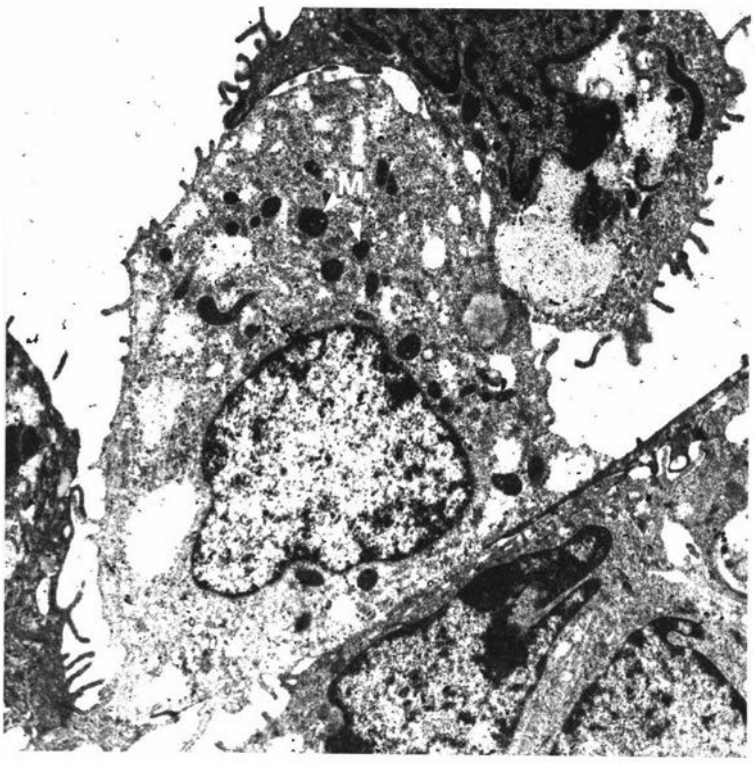

V
V

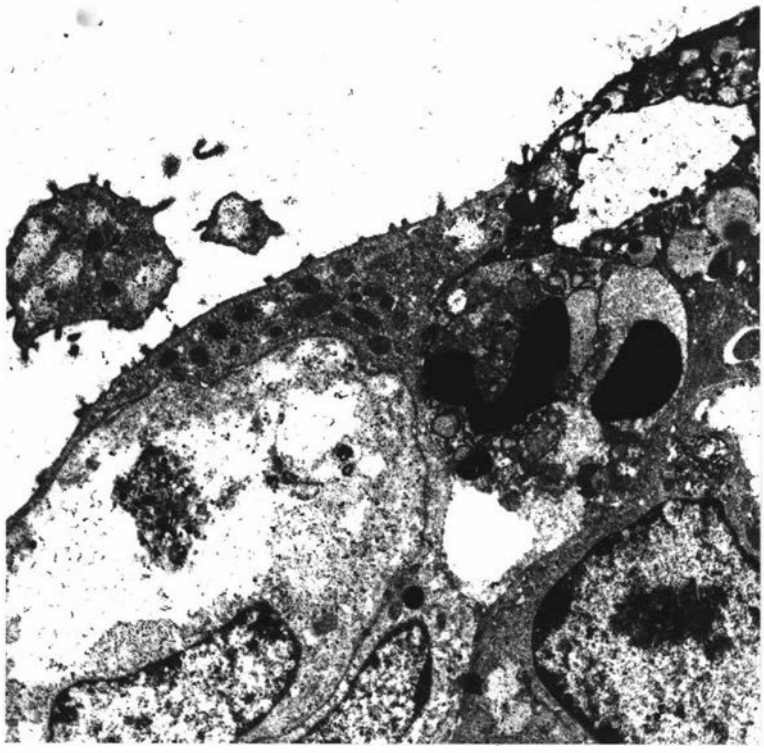
Figure 6.64

AN epithelial cell undergoing exfoliation from a tracheal culture infected with $\pm 10^6$ CFU/ml of N. catarrhalis for 4 hrs. It has no discrete nuclear membrane and the mitochondria (M) are electron-dense. TEM. X 8,200.

Figure 6.65

A tracheal organ culture infected with $\pm 10^8$ CFU/ml of N. catarrhalis for 4 hrs showing severe damage to its luminal surface with complete loss of microvilli and cilia. TEM. X 8,200.


M



tracheal organ cultures maintained in T199 medium. The lag phase of the growth curve occurred at $\pm 10^8$ CFU/ml and viable numbers reached similar levels 4 hr after inoculation irrespective of the initial titre of the inoculum (Fig. 6.66). The duration of the lag phase was up to 9 hr.

Figure 6.66 also illustrates the increase in viable number of N. catarrhalis relative to the original inoculum; bacterial titres of $\pm 10^2$ CFU/ml increased 6 fold, $\pm 10^4$ CFU/ml increased 4 fold and $\pm 10^6$ CFU/ml increased 2 fold , while titre of $\pm 10^8$ CFU/ml showed no increase and remained at the initial level of $\pm 10^8$ CFU/ml for up to 9 hours.

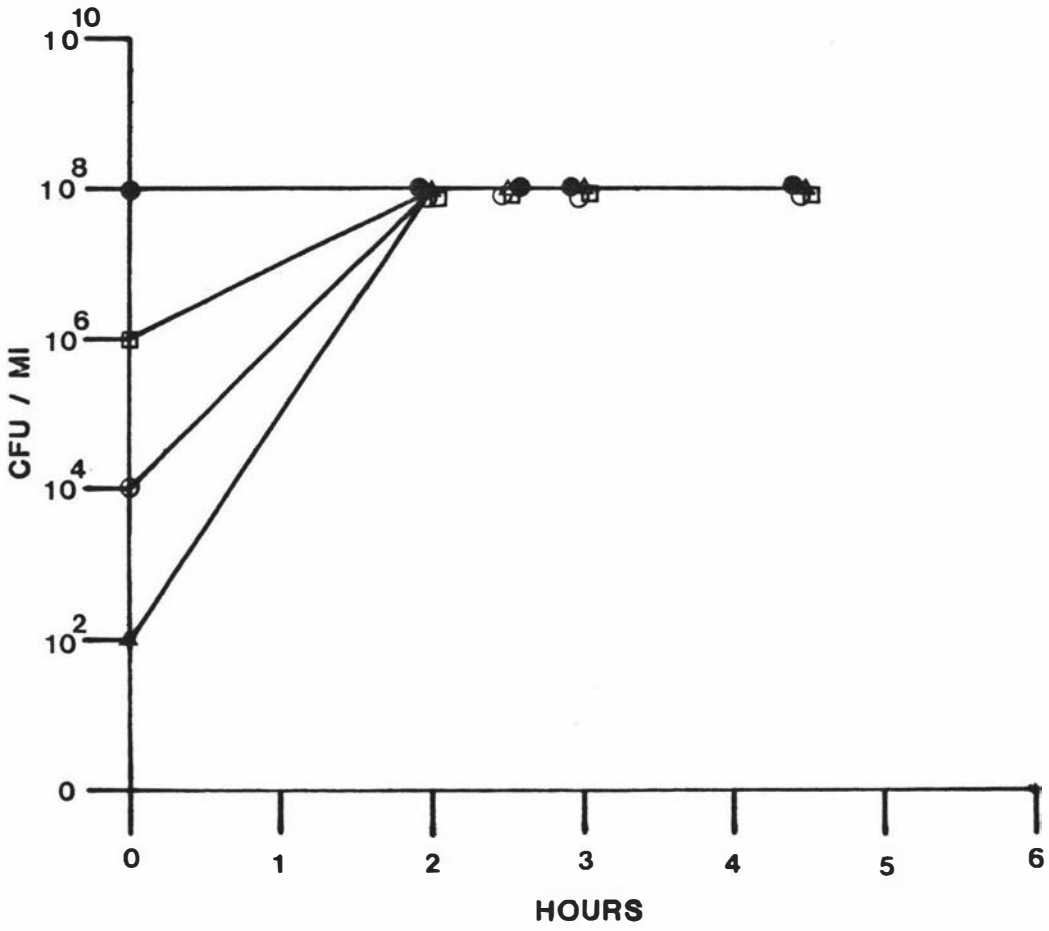
4.0 DISCUSSION

Studies of the pathogensis of CNP in sheep have been faced by a number of difficulties. One of the most important of these is the need to assess the relative importance of the ever increasing number of potential pathogens which have been isolated from the ovine respiratory tract. Ideally, pathogenicity should be measured in the natural host under natural (ie. field) conditions, however, the widespread infection in sheep in New Zealand and its seasonal nature makes this impossible. The use of SPF lambs for intranasal aerosol inoculation would provide the next most useful means of investigation, however the production and maintenance of such lambs is extremely expensive and time consuming.

Organ culture systems have the advantage of providing specialised and differentiated host tissue to study the effects of different organisms in vitro. With these models, tissue from a single animal can be used for control and test groups, thus biological variation between individuals is eliminated. Tracheal cultures may be infected with a specific number of cloned respiratory pathogens, thus avoiding the effect of secondary invaders. Observation of ciliary activity permits continuous monitoring of injury and viability of individual ciliated epithelial cells during the experiment serves as an index of varying degrees of damage.

Figure 6.66

Neisseria catarrhalis growth curves in tracheal organ culture maintained in T199 medium. All titres reached a plateau of growth at a level of $\pm 10^8$ CFU/ml 8 hrs post-inoculation.


10^10
10^8
10^6
10^4
10^2
0
CFU / MI
0
1
2
3
4
5
6
HOURS

Organ culture models do not possess a blood supply and therefore do not have systemic host immune, hormonal, or nutritional factors present in the intact animal. This probably magnifies the injury patterns that would be seen in the intact host. Individual cells in this system can respond to infection mainly through the mechanism of hyperplasia, metaplasia and/or necrosis.

The present studies, have shown the value of an organ system for comparing pathogenicity of microorganisms. The effect of B. parapertussis, M. ovipneumoniae, P. haemolytica and N. catarrhalis infections could be observed at the cellular level. Use of this system provides an indication of the direct pathogenicity of the organisms by revealing (i) interference with ciliary activity and synchrony, (ii) a sequential pattern of cytopathological changes, and (iii) disruption of normal tissue architecture.

Although all the organisms used in this study produced, variably, ciliary inhibition and epithelial cell injury, the results have shown that the organisms can be divided into two groups. The first group include B. parapertussis and M. ovipneumoniae which have the ability to attach to cilia. The second group include P. haemolytica and N. catarrhalis which failed to attach. Electronmicroscopy showed that numerous B. parapertussis and M. ovipneumoniae organisms were located between the cilia and on the surface of ciliated cells. The organisms were not immediately associated with non-ciliated cells although these cells also manifested cytopathological changes.

The close association of B. parapertussis and M. ovipneumoniae organisms with ciliated epithelial cells is consistent with the observations made of other recognised respiratory pathogens such as Bordetella pertussis (Collier; et al. 1977) and Bordetella bronchiseptica (Bemis and Kennedy, 1981), Haemophilus influenza (Denny, 1974), Corynebacterium diphtheria (Baseman and Collier, 1974), Mycoplasma pneumoniae (Hu; et al. 1976), and Mycoplasma gallisepticum (Takagi and Arakawa, 1980) and recently M. ovipneumoniae (Jones; et al. 1985). Mycoplasma cell extract (Chandler and Barile, 1980), Neisseria gonorrhoeae and gonococcal endotoxin (Mardh; et al. 1979), certain chemicals (Asmundsson; et al. 1973; Dahlgren and Dalen, 1972; Mossman;et al. 1977) and avian

'infectious bronchitis virus (Yachida; et al. 1978) have also been observed to inhibit ciliary activity of respiratory epithelial cells in vitro. However, to exert their ciliostatic effects in vivo, they either must be present in large preformed quantities or must first overcome the cleansing effect of the mucociliary apparatus and replicate to a density that is sufficient to produce the ciliostatic effect. In vitro experiments have shown that epithelial attachment is not necessarily a precondition to ciliostasis, although the later condition may be promoted by bacterial attachment to the cilia (Bemis and Kennedy, 1981). Among the above mentioned organisms, only M. pneumoniae, M. gallisepticum, B. pertussis and B. bronchiseptica have been shown to have the ability to attach to the ciliated part of the respiratory epithelium by a specialised structure. The results of the present studies have shown that both B. parapertussis and M. ovipneumoniae are capable of attaching to cilia and producing ciliostatic effects in the ovine respiratory tract.

Pasteurella haemolytica and N. catarrhalis have also been commonly isolated from the naturally-occurring cases of CNP in sheep (Alley, 1975b; Jones; et al. 1978). In the present studies both organisms produced ciliary inhibition and epithelial cell injury of ovine tracheal cultures although both organisms failed to attach to the ciliary system. The degree of ciliostasis and epithelial cell injury observed was closely related to the viable numbers of these two organisms in the organ culture medium and occurred when P. haemolytica and N. catarrhalis attained titres of $\pm 10^9$ CFU/ml and $\pm 10^8$ CFU/ml respectively. Although both of these organisms behaved in similar manner in organ culture and produced similar cytopathological changes, P. haemolytica was more destructive than N. catarrhalis and produced a complete loss of the epithelial layer, exposing the basement membrane beneath. In both cases the mucosal damage occurred as a result of the extrusion of epithelial cells. Sloughed cells were observed to be free of adherent bacteria, which indicated that direct association between bacteria and cells was not necessary for sloughing to occur.

These results are similar to the finding of Denny (1974) and Johnson; et al. (1983) who studied Haemophilus influenza infection in vitro. Denny (1974) noted that sterile supernatant fluids from infected organ cultures contained a soluble factor that caused loss of

ciliary activity when transferred to fresh organ cultures. He partially characterised the soluble toxic factor present and suggested that it might be H. influenza endotoxin. It is interesting to note that sterile supernatant fluid from fallopian tube organ cultures infected with Neisseria gonorrhoeae similarly cause loss of ciliary activity and sloughing of epithelial cells when transferred to fresh organ cultures (Melly; et al. 1981). Gregg; et al. (1981) have shown that the active factor in such supernatant fluid is gonococcal lipopolysaccharide endotoxin.

In the present study, B. parapertussis was the organism which produced the earliest ciliostatic effect. Bacterial titres of $\pm 10^5$ and $\pm 10^7$ CFU/ml produced rapid attachment and marked ciliary inhibition within 1 to 3 hr of incubation. Lower titres showed very little ciliary attachment although they did produce ciliostatic effect within 6 to 7 hours after incubation. Cytopathic changes due to B. parapertussis at high titres ($\pm 10^7$ CFU/ml) were detected as early as 5 min pi and complete ciliostasis as early as 1 hr. The earliest ciliostatic effects previously reported in the literature occurred 3 hr after incubation with B. bronchiseptica in canine tracheal organ cultures, although this organism produced some detectable changes in the ciliary activity within 5 min (Bemis and Kennedy, 1981). Muse; et al. (1979) have found that ciliary inactivation with B. pertussis is dose dependent although after 12 hr pi there was little difference between the effects of inocula containing $\pm 10^5$ or $\pm 10^7$ CFU/ml. The mechanism responsible for producing ciliostasis in bordetella infection is as yet unknown. It is possible that the ciliostatic properties of the Bordetella organisms are an energy dependent parasitism. Ciliary activity is an energy dependent mechanism and the enzymes responsible for the energizing process are highly membrane associated (Bemis and Kennedy, 1981). It is possible that the energy required for bacterial growth could be derived from the cellular metabolism of ciliated cells (Iida and Ajiki, 1975). The release of large amounts of extracellular adenyl cyclase (Hewlett; et al. 1977; Endoh; et al. 1980) from ciliated cells closely associated with Bordetella organisms would tend to support this hypothesis. This would explain why the ciliostatic effect in the present study was directly proportional to the viable number of organisms in the medium. Attachment may still occur without ciliostasis and Bemis and Kennedy (1981) observed that heat and formalin killed B. pertussis organisms

attached to cilia of the canine tracheal epithelium. These inactivated organisms and their endotoxin failed to cause ciliostasis or a significant reduction of beating frequency.

If, as the present study suggests, B. parapertussis organisms readily produce pathological changes in the ovine respiratory tract, it is possible that these organisms may have a role in the aetiology of CNP in sheep. The organism has not been previously recognised as a pathogen of the ovine respiratory tract either in New Zealand or overseas. One of the reasons for this has been the difficulty in isolating the organism from the lung parenchyma. The present study showed that it is a specific coloniser of ciliated epithelial cells and may therefore have a role in reducing mucociliary clearance and allowing colonisation of the lower respiratory tract by more destructive organism such as P. haemolytica. Alternatively the organism may have a role in prolonging existing respiratory infection by interfering with lung clearance mechanisms. It has been suggested that the prolonged colonisation of the respiratory tract by B. bronchiseptica is due to specific attachment to the cilia (Bemis and Kennedy, 1981). Further work on the pathogenesis of B. parapertussis in the respiratory tract of sheep is therefore necessary together with information on the prevalence of the organism in both normal and pneumonic sheep.

Mycoplasma ovipneumoniae titres of $\pm 10^4$, $\pm 10^6$, $\pm 10^8$ CFU/ml established attachment to cilia within 1 hr pi and produced ciliostatic effect 13, 20 and 27 hr respectively after attachment. The lowest titres of $\pm 10^2$ CFU/ml showed no ciliary attachment although they produced ciliostasis 36 hr after inoculation. These results clearly demonstrate the abilities of M. ovipneumoniae to produce both tissue injury and ciliostatic effects. Similar effects have recently been observed with M. ovipneumoniae in caprine and ovine tracheal organ culltures by Jones; et al. (1985) although these authors did not describe ultrastructural studies. In the present investigation it was found that the organism attached to cilia by means of specialised pili-like structures within 1 hr of incubation and produced complete ciliostasis as early as 13 hr in FM4 medium and 30 hours in T199. The earliest ciliostatic effects previously reported with other mycoplasmas occurred 48 - 72 hr after inoculation with M. pneumoniae (Collier; et al. 1969). Observations on M. pneumoniae infection in

vitro strongly suggested that the ability of this organism to attach to respiratory epithelial cells is a prerequisite for disease production (Collier and Baseman, 1973). Hu; et al. (1976) have published data indicating that intimate contact between M. pneumoniae and the respiratory epithelium alone does not account for the subsequent interruption of host cell macromolecular synthesis and the resulting tissue cytopathology. Their observations support the concept that M. pneumoniae infection is a two-step process; internal and specific attachment. The metabolic alterations and other cytopathology that follow the initial parasite-host cell interaction appear to result from the multiplication of mycoplasmas accompanied by possible membrane perturbation, nutritional parasitism, and/or introduction of certain toxic factors into sensitive cells. The last point is substantiated by the observation that decreased up take of metabolic precursors; such as galactose, orotic acid and amino acids; by host cells can be prevented by addition of erythromycin, which inhibit protein sysnthesis by mycoplasmas (Hu; et al. 1975) or by shifting tracheal rings to a nonpermissive medium at early and intermediate stage of infection (Hu; et al. 1976).

Although M. ovipneumoniae was the slowest of the four organisms tested in producing a pathogenic effect, it nevertheless attached strongly to the cilia of the tracheal epithelium within 1 hr of inoculation. This suggests the organism may have a particular affinity for ciliated epithelium and may explain why it is the most common organism isolated from ovine lungs and a rapid coloniser of the nasal cavity (Alley and Clarke, 1979).

Exfoliation of ciliated epithelial cells was seen in the later stages of infection with all four organisms used in this investigation, This has been described previously in infections with M. pneumoniae (Muse; et al. 1976; Murphy; et al. 1980),B. pertussis (Muse; et al. 1977),rhinovirus and parainfluenza virus (Reed and Boyde, 1972). In the present study exfoliation appeared to arise mainly from loss of attachment between cells lateral or beneath the affected cell. The damaged cells showed swelling of organelles and extruded into the lumen, leaving a space which was covered by elongation of remaining cells. This mechanism is different from the process of ciliocytophthoria which has been described previously with M. pneumoniae (Muse; et al. 1976; Murphy; et al. 1980) and some

chemicals (Asmundsson; et al. 1973; Dahlgren and Dalen, 1972; Mossman; et al. 1977). Although the main mechanism of cell loss was extrusion a process similar to ciliocytophthoria was observed to some extent in P. haemolytica and N. catarrhalis infections.

The cytopathological changes demonstrated in the different surface epithelial cells were not present in exposed basal cells. In this and previous studies, it has been noticed that basal cells of tracheal epithelium take on a flattened, squamous appearance after sloughing of surface epithelial cells (Murphy; et al. 1980). Basal cells of the respiratory epithelium are known to be resistant to virus (Craighead,1968) and particulate materials (Mossman, 1977), suggesting that this process may be a protective mechanism.

The present study confirms the value of the tracheal organ culture system as a means of assessing the relative pathogenicity of organisms which colonise the ovine respiratory tract. It has shown that both M. ovipneumoniae and B. parapertussis act by initially attaching to cilia and then producing cytopathological lesions. Although P. haemolytica and N. catarrhalis do not attach, they can nevertheless produce severe destructive changes in epithelial cells. These investigations have not provided information on the mechanisms of mixed infections or interactions if any which may occur between organisms in the ovine respiratory tract. The techniques used however, would be highly suitable for future studies of this type.

It is clear that current organ culture methods have been refined to the point where the data obtained can provide valuable analyses of the parameters which govern the properties of tissues. Nevertheless, it is evident from the foregoing discussions that further research is necessary before organ culture can be used with maximum efficiency within the intrinsic limitations of the system.

CHAPTER 7

GENERAL DISCUSSION

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The response of the respiratory tract to injury and the resulting patterns of disease are determined largely by the normal structure and function of the tissue in the species involved. The morphological study of normal ovine lung undertaken in Chapter 2 will serve as a useful base for future studies of respiratory pathology in sheep. The normal ovine lung was shown to have two important features which may affect its susceptibility to injury. These are; a relative paucity of the pores of Kohn and the presence of smaller numbers of alveolar macrophages than that observed in other species.

In the ovine lung, collateral ventilation is likely to be severely limited because of the scarcity of interalveolar pores. This feature of ovine lung may be associated with the commonly observed areas of peribronchiolar alveolar collapse which are characteristic of early cases of subacute and chronic pneumonia (Alley, 1975b & Pfeffer; et al. 1983). If a small airway is blocked and the gas distal to it is no longer in continuity with the inspired gas, the air space will collapse. The degree to which this occurs will depend on the site of the obstruction and the number of bronchiolar-alveolar anastomoses present in the affected area. Vascular perfusion to such an area will be reduced due to a physiological shunt (Woolcock & Macklem, 1971) resulting in poor oxygenation of tissues which may make them suitable for microorganism proliferation. The degree of collateral ventilation shows marked species variation depending on the development of interalveolar pores and interlobular septa. The lungs of pigs (Woolcock & Macklem, 1971) and cattle (Mariassy; et al. 1975) are highly lobulated with few interalveolar pores and there is therefore a limited collateral ventilation (Woolcock & Macklem, 1971). Ovine lung appears to follow closely the design of bovine lung.

Whether or not alveolar collapse follows obstruction will depend on the size of the airway obstructed and the degree of collateral ventilation. Complete blockage of lobar or segmental bronchi is necessary for alveolar collapse in the dog (Woolcock & Macklem, 1971) and cat (Howard & Ryan, 1982), where collateral ventilation is

extensive. Blockage of small bronchi or even bronchioli can result in alveolar collapse in pigs(Woolcock & Macklem, 1971) and cattle (Mariassy; et al. 1975) where there is insignificant collateral ventilation. The lungs of sheep are recognised as being particularly prone to alveolar collapse (Dungworth, 1985). Alveolar collapse is more likely to develop in dependent lung regions where experimentally produced CNP lesions are observed (Alley & Clarke, 1979).

The second significant morphological feature observed was the rarity of macrophages in alveolar spaces. Similar observations have been reported in cattle (Mariassy; et al. 1975; Rybicka; et al. 1974a), and as previously suggested this could be a characteristic feature of the ruminant lung. Rybicka; et al (1974a & b) observed numerous cells in the pulmonary capillaries of normal calves which they suggested, on the basis of ultrastructual characteristics and evidence of phagocytic capability, were "intravascular macrophages" rather than monocytes. Mariassy; et al. (1975) speculated that the relationship between the low numbers of alveolar macrophages and abundance of cells having the features of macrophages within pulmonary capillaries in cattle was not fortuitous. That is to say, the intravascular cells might represent a recruitable pool of macrophages able to compensate for the scarcity of resident alveolar macrophages. Although this feature has yet to be investigated in the ovine lung, the intravascular macrophages described by Rybicka; et al (1974a) could represent the intermediate lung maturation compartment proposed by Bowden (1971) in which monocytes undergo differentiation into functional alveolar macrophages.

Alveolar macrophages in other species, apart of cattle and sheep commonly line alveoli and are therefore the first immunologically competent cells to encounter inhaled pathogens. Although the actual physical removal of particles from alveoli is relatively inefficient, compared to particle removal following deposition on the mucociliary blanket, it is well known that the sterility of alveoli is maintained by the ability of alveolar macrophages to kill ingested bacteria and to secrete interferon (Dungworth, 1985). The limitation of collateral ventilation and the rarity of alveolar macrophages would therefore put the ovine lung in a disadvantageous position with respect to alveolar collapse and to resistance to aerogenous bacterial infection.

The morphological and morphometrical studies described in Chapter 3 and 4 respectively confirm that the tracheobronchial epithelium of sheep affected with CNP undergoes extensive structural changes. The most striking changes were the severe damage to the ciliary blanket and the increase in epithelial thickness throughout the tracheobronchial airways. Although these changes were most severe in peripheral airways in advanced lesions, early lesions showed marked changes in the upper tracheobronchial airways. In subacute and chronic pneumonia the earliest changes observed by Alley (1975a) were peribronchiolar alveolar collapse, the accumulation of small numbers of macrophages in alveolar spaces and mild thickening of alveolar septa with mononuclear cells. He noted that these changes were often accompanied by mild hyperplasia of the terminal bronchiolar epithelium and the infiltration of small numbers of neutrophils into the lumen of bronchioli and alveolar ducts. Although Alley (1975a) pointed out the importance of the tracheobronchial tree in influencing the course of subacute and chronic pneumonia, he did not examine the tracheobronchial airways in detail. Pfeffer; et al. (1983) examined the lungs from lambs selected at random from a property in Hawkes Bay and found small lesions consisting mainly of alveolar collapse in the anterior lobes. They were increasingly common from November onwards and occured well before the onset of CNP in March. Although Pfeffer; et al. (1983) failed to isolate viruses, mycoplasmas or bacteria from these lesions it is possible that this early alveolar collapse may be due to a previously existing tracheobronchitis, since these airways were not examined. The present study has shown there is severe involvement of the tracheal epithelium from an early stage. This suggests that CNP may begin as a tracheobronchitis before extending to involve more peripheral airways.

Little attention has previously been paid to the submucosal glands of the tracheobronchial airways in normal and pneumonic lambs. The present investigation aimed not only at assessing the presence and degree of submucosal gland enlargement but also at identifying the structure involved and its distribution at various levels of the tracheobronchial airways (Chapter 4). The data obtained from pneumonic lesions showed there was submucosal gland enlargement at all levels of the tracheobronchial airways. Similar changes have been observed in pigs with enzootic pneumonia experimentally induced by inoculation of Mycoplasma hyorhinis (Baskerville, 1972). In the

present study it was found that gland enlargement was due to both hypertrophic changes and an increase in the number of acini per gland. This submucosal gland enlargement was more severe in advanced than early pneumonic lesions. These changes and differences between the two stages of pneumonic lesion reflect the chronic proliferative nature of CNP, since hypertrophic changes occur more commonly with chronic respiratory diseases such as enzootic pneumonia in pigs (Baskerville, 1972) and chronic bronchitis in man (Dunnill, 1982). The submucosal gland glycoproteins were also identified histochemically (Chapter 5). Quantitative analysis of the mucus cells showed that in the glands from pneumonic sheep, the percentage of the acid glycoprotein was increased when compared to normal sheep. This change may be advantageous and help in the resistance and inhibition of viral infections of the respiratory tract as previously discussed (Chapter 5).

The role of cilia in pulmonary clearance mechanisms and defense of the respiratory tract against infectious disease is well known (Chapter 1). In the present study it was found that loss of cilia in the early pneumonic lesions was more severe than that observed in advanced lesions (Chapter 3). This damage and loss of cilia would be expected to decrease markedly the resistance of the respiratory tract to pathogenic microorganisms and effectively depress the removal of particles and mucus accumulated in the tracheobronchial lumen. These changes together with the increased production of mucus by hypertrophic bronchial submucosal glands (Chapter 4) may well be responsible for the blockage of the bronchiolar lumen observed in both the histological and SEM studies of advanced pneumonic lesions. The possible scarcity of pores of Kohn in the ovine lung together with blockage of bronchiolar lumina are likely to both hamper compensation for inequalities of ventilation and reduce the efficiency of mechanical expulsion of exudate. Accumulation of exudate may either impair the combating of an incipient bronchopneumonia or prolong the resolution of a fully developed inflammatory process with the consequent production of a long standing proliferative lesion typical of naturally-occurring CNP.

Although large numbers of bacteria were found in close proximity to cilia in both early and advanced stages of CNP, mycoplasma were more regularly found amongst the cilia particularly in early lesions.

This confirms recent work by Jones et al. (1985) who found that all tracheal organ cultures infected with M. ovipneumoniae had clusters of organisms in close contact with their cilia. In the present study however, M. ovipneumoniae organisms were found to attach to cilia by means of pili-like structures in both naturally occurring cases of CNP (Chapter 3) and tracheal organ cultures (Chapter 6). Whether the pili-like structures have a function other than attachment remains to be determined. The mechanism by which mycoplasma may exert their effects on cells from an extracellular location is not fully understood. It has been suggested that the generation of peroxide in close proximity to the cell membrane is of primary importance (Cohen & Somerson, 1967) and organ culture experiments indicate that at least some mycoplasmas inhibit ciliary activity and can cause cellular damage, possibly as a result of peroxide production (Chapter 1).

The common clinical manifestations of CNP, include symptoms of trachitis or tracheobronchitis which implies that the ciliated respiratory epithelium is intimately involved in the disease process from an early stage. Animal models provide the specialsied mucosal cells needed to study the host-pathogen relationship at a cellular level, but analysis is complicated by a lack of direct control over the microclimate. The organ culture system used in the present studies maintained viable ciliated tracheal epithelium in which the effect of M. ovipneumoniae, P. haemolytica, B. parapertussis and N. catarrhalis infection could be observed in detail with both SEM and TEM.

Of the four organisms used it was shown that only M. ovipneumoniae and B. parapertussis have an affinity for ovine tracheal epithelial cells. In organ cultures they were able to attach to cilia and produce cytopathological lesions. Pasteurella haemolytica and N. catarrhalis produced destructive changes but failed to attach to cilia. None of the large numbers of bacteria observed on the ciliated epithelial surface in the tracheobronchial airways of natural cases of CNP, had the dense, fuzzy, pili-like structures on the surface which were the typical of B. parapertussis in organ culture. The role of B. parapertussis in the ovine respiratory tract has not yet been established, but preliminary work has shown it is difficult to isolate B. parapertussis from pneumonic lung and it is not commonly present in the nasal cavity of adult sheep

(Alley, 1986a). Nevertheless, the present study has demonstrated that it has the potential to be pathogenic in ovine tracheobronchial airways. Both P. haemolytica and N. catarrhalis produced lesions in tracheal organ cultures although P. haemolytica was more destructive. Both organisms failed to show any affinity for ciliated cells.

In general, the current studies have tended to reaffirm the importance of M. ovipneumoniae as a respiratory pathogen in sheep. The only organisms which had both an affinity for cilia and the ability to produce destructive changes in the respiratory epithelial cells were M. ovipneumoniae and B. parapertussis and of these only M. ovipneumoniae has been demonstrated in pneumonic lung in large numbers. The common arguement against M. ovipneumoniae as a cause of CNP in lambs, is its isolation from normal as well as from pneumonic sheep lungs(Ionas; et al. 1985 & Pfeffer; et al. (1983). This could be explained by the finding that many strains of M. ovipneumoniae exist and these may have different pathogenicities (Jones; et al. 1982b; Ionas; et al. 1985 & Mew; et al. 1985). An analogous situation may exist with regard to Mycoplasma hyopneumoniae which is now recognised as the main aetiological agent of chronic enzootic pneumonia in pigs (Baskerville & Wright, 1973). This organism has recently been isolated from (12%) of normal pig lungs in New Zealand (Macpherson & Hodges, 1985).

Epidemiologic and experimental evidence indicates that the important infectious bronchopneumonias of animals usually develop only when the balance is tipped in favour of disease by an increase in number of pathogenic micoorganisms reaching vulnerable bronchiolar-alveolar regions of the lung or when pulmonary defense mechanisms are impaired (Dungworth, 1985). It has been shown that both M. ovipneumoniae and P. haemolytica organisms reside in the nasal cavity of normal sheep, although younger lambs are not carriers (Alley, 1975b; Alley; et al. 1975 & Ionas; et al. 1985). The finding by both Alley (1975a) and Pfeffer; et al. (1983) that the small areas of alveolar collapse seen in lamb lungs from as early as 3 months of age often fail to yeild microorganisms is of interest in this regard. It has been suggested by both these authors that non-infectious agents may be responsible for these lesions which subsequently become invaded by nasal organisms.

Viruses have been neglected in the present study. Parainfluenza type 3 virus, adenovirus and RSV have been isolated from sheep in New Zealand and overseas (Chapter 1). Their use as initiating agents for acute exudative pneumonia in sheep is well established, however evidence of direct viral involvement in CNP of lambs is minimal. In the present study a large number of thin sections from different levels of tracheobronchial epithelium were examined by TEM, but no pathological evidence of virus infection, such as intracellular inclusion bodies were observed.

In the light of these possibilities, future studies on CNP in New Zealand might be profitably directed toward the interaction of different strains of M. ovipneumoniae, P. haemolytica and B. parapertussis in tracheal organ culture. By combining the information obtained from organ culture, experimental animals and observation of the natural disease, the chances of gaining the information needed to understand the pathogenesis of CNP appear to be good. There is still an obvious need for information on the nature and distribution of the microorganisms in the respiratory system of sheep and the present study suggests that trachea and bronchi of early pneumonic lesions and the bronchioli of advanced lesions are areas worthy of special attention.

The pathogenesis of CNP in lambs in New Zealand remains incompletely understood, due in part to the limited information available on the biology of the major microorganisms involved. The application of experimental models to analyse of the host-pathogen interaction will therefore continue to be a fruitful means of investigation.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ailsby, R.L. & Ghadially, F.N. (1972): Atypical cilia in human bronchial mucosa. J. Pathol. 109: 75.

Al-Darraji, A.M.; Cutlip, R.C.; Lehmkuhl, H.D. & Graham, D.L. (1982): Experimental infection of lambs with bovine respiratory syncytial virus and Pasteurella haemolytica: pathologic studies. Am. J. vet. Res. 42: 224.

Alexander, I.; Ritchie, B.C.; Maloney, J.E. & Hunter, C.R. (1975): Epithelial surfaces of the trachea and principal bronchi in the rats. Thorax. 30: 171.

Al-Kaissi, A. & Alley, M.R. (1983): Electronmicroscopic studies of the interaction between ovine alveolar macrophages and Mycoplasma ovipneumoniae. Vet. Microbiol. 8: 571.

Alley, M.R. (1986a): Personal communication.

Alley, M.R. (1986b): The effect of chronic non-progressive pneumonia on weight gain of pasture fed lambs. N. Z. vet. J. on print.

Alley, M.R. (1985): Personal communication.

Alley, M.R. (1975a): The pathogenesis of pneumonia in sheep. Ph.D. thesis. Massey University. New zealand.

Alley, M.R. (1975b): The bacterial flora of the respiratory tract of normal and pneumonic sheep. N. Z. vet. J. 23: 113.

Alley, M.R. & Clarke, J.K. (1980): The effect of chemotheraputic agents on the transmission of ovine chronic non-progressive pneumonia. N. Z. vet. J. 28: 77.

Alley, M.R. & Clarke, J.K. (1979): The experimental transmission of ovine chronic non-progressive pneumonia. N. Z. vet. J. 27: 217.

Alley, M.R.; Quinlan, J.R. & Clarke, J.K. (1975): The prevalance of Mycoplasma ovipneumoniae and Mycoplasma arginini in the respiratory tract of sheep. N. Z. vet. J. 23: 137.

Alley, M.R. ; Wells, P.W.; Smith, W.D. & Gardiner, A.C. (1980): The distribution of immunoglobulin in the respiratory tract of sheep. Vet. Pathol. 17: 372.

Al-Rawi, K.M. & Khalf-Allah, A.M. (1980): Design and analysis of agricultural experiments. Musal University. Iraq. pp. 73 - 77.

Andrews, A. (1974): Further evidence that enterochromaffin cells are not derived from the neural crest. J. Embryol. exp. Morphol. 31: 589.

Asmundsson, T.; Kilburn, K.H. & McKenzie, W.N. (1973): Injury and metaplasia of airway cell due to SO_2. Lab. Invest. 29: 41.

Azzopardi,A. & Thurlbeck, W.M. (1969): The histochemistry of the nonciliated bronchiolar epithelial cells. Am. Rev. res. Dis. 99: 516.

Baker, A.P.; Charkin, L.W. & Wardell, J.R. (1975): Chronic cholinergic stimulation of canine respiratory tissue. Am. Rev. resp. Dis. 111: 423.

Bancroft, J.D. & Stevens, A. (1975): Histopathological stains and their diagnostic uses. Churchill livingstone. London. pp. 11.

Banks, W.J. (1981): Applied veterinary histology. Williams & Wilkins. London. pp. 440

Bang, F.B. & Bang, M.A. (1969): Experimentally induced changes in nasal mucous secretory system and their effect on virus infection in chickens. II. Effects on adsorption of Newcastle disease virus. J. exp. Pathol. 130: 121.

Barski, G.; Cornefert, Fr. & Wallace, R.E. (1959): Response of ciliated epithelia of different histological origin to virus infection in vitro. Proc. Soc. exp. Biol. Med. N.Y. 100: 407.

Barski, G.; Kourilsky, R. & Cornefert, Fr. (1957): Resistance of respiratory ciliated epithelium to action of polio and adeno viruses, in vitro. Pro. Soc. exp. Biol. Med. 96: 386.

Baseman, J.B. & Collier, A.M. (1974) Diphtheria pathogenesis in guinea pig tracheal organ culture. Infec. Immun. 10: 1146.

Baskerville, A. (1976): The development and resistence of bronchial gland hypertrohpy and goblet cell hyperplasia in the pig after injection of isoprenalin. J. Pathol. 119: 35.

Baskerville, A. (1972): Development of the early lesions in experimental enzootic pneumonia of pigs: an ultrastrucrutal and histological study. Res. vet. Sci. 13: 570.

Baskerville, A. (1970a): Ultrastructure of the bronchial epithelium of the pig. Zentralbl. Veterinaermed. Reihe A. 17: 796. Cited by Breeze; et al. (1976).

Baskerville, A. (1970b): Ultrastructural studies of the normal pulmonary tissue of the pig. Res. vet. Sci. 11: 150.

Baskerville, A. & Wright, C.L. (1973): Ultrastructural changes in experimental enzootic pneumonia in pigs. Res. vet. Sci. 14: 155.

Belak, S.; Palfi, N. & Huszar, E. (1974): Baranyok reovirus okozta

Tomeges Legzoes emesztoszervi megbetegedese. Magy. Allatorv. Lapja. 29: 14. Cited by Al-Darraji; et al. (1982).

Belak, S.; Palfi, V. & Palya, V. (1976): Adenovirus infection in lambs. I. Epizootiology of the disease. Zentlbl. vet. Med 23 [B]: 320. Cited by Al-Darraji; et al. (1982).

Bemis, D.A.; Greisen, H.A. & Appel, M.J.G. (1977): Pathogenesis of canine bordetellosis. J. infec. Dis. 135: 753.

Bemis, D.A. & Kennedy, J.R. (1981): An improved system for studing the effect of Bordetella bronchiseptica on the ciliary activity of canine tracheal epithelial cells. J. infec. Dis. 144: 349.

Biberstein, E.L.; Nisbet, D.I. & Thompson, D.A. (1967): Experimental pneumonia in sheep. J. comp. Pathol. 77: 181.

Biberstein, E.L.; Shreeve, B.J. & Augus, K.W. (1971): Experimental pneumonia of sheep. Clinical, microbiological and pathological responses to infection with myxovirus parainfluenza 3 and Pasteurella haemolytica. J. comp. Pathol. 81: 339.

Biennstock, J.; Johnston, N. & Perrey, D.Y.E. (1973): Bronchial lymphoid tissue. I. Morphologic characteristics. Lab. Invest. 28: 686.

Boat, T.F. & Cheng, P.W. (1980): Biochemistry of airway mucus secretion. Fed. Proc. 39: 3067.

Boat, T.E.; Cheng, P.W.; Lyer, R.N.; Carlson, D.M. & Polony, I. (1976): Human respiratory tract secretions. Mucous glycoproteins of nonpurulent tracheobronchial secretions and sputum of patients with bronchitis and cystic fibrosis. Arch. Biochem. Biophys. 177: 95.

Bouffard, A. & Derbyshire, J.B. (1978): Cultivation of bovine adenovirus type 3 in tracheal organ cultures. Vet. Microbiol. 3: 3.

Bowden, D.H. (1971): The alveolar macrophage. current top. Pathol. 55: 1. Cited by Mariassy et al. (1975).

Bradbury, J.M. (1979): Phosphate activity in avian mycoplasmas. Res. vet. Sci. 27: 386.

Brain, J.D. (1980): Nonimmunologic defense mechanisms. In "Pulmonary diseases and disorders". By Fishman, A.P. (ed). McGraw-Hill Book Company. N.Y. pp. 633.

Breeze, R.G.; Wheeldon, E.B. & Pirie, H.M. (1976): Cell structure and function in the mammalian lung, the trachea, bronchi and bronchioles. Vet. Bull. 46: 319.

Browstein, D.G. (1980): Reflex-mediated desquamation of bronchiolar epithelium in guinea pigs exposed acutely to sulfuric acid aerosol. Am. J. Pathol. 98: 577.

Brownstein, D.G. & Johnson, E. (1982): Experimental nasal infection of normal and leukopenic mice with Pseudomonas aeruginosa. Vet. Pathol. 19: 169.

Buddle, B.M.; Herceg, M. & Davies, D.H. (1984): Comparison of virulence of ovine respiratory mycoplasmas in the mouse mammary glands. Vet. Microbiol. 9: 367.

Butler, M. (1969): Isolation and growth of mycoplasma in human embryo trachea cultures. Nature (London) 224: 605.

Butler, M. & Ellaway, W.J. (1972): Compararive studies on the infectivity of avian respiratory viruses for eggs, cell cultures and tracheal explants. J. comp. Pathol. 82: 327

Butler, M. & Ellaway, W.J. (1971): Growth and cytopathology of mycoplasma in human and chicken trachea explants. J. comp. Pathol. 81: 359.

Cameron, I. R. & Bateman, N. T. (1983): Respiratory disorders. Edward Arnold. Great Britain. pp 12 - 28.

Campbell, R. S. F.; Rae, A.; Sharp, J. M. & Smith, W. (1979): Infection of tracheal organ cultures with ovine adenovirus type 2. J. comp. Pathol. 89: 107.

Carlson, D. M.; Lyer, R. N. & Mayo, J. (1970): "Blood and tissue antigen". Aminoff, D. (ed.) N. Y. Academic Press. pp. 229 247.

Carmichael, N. E.; St. George, T. D.; Sullivan, N. D. & Horsfall, N. (1972): Isolation, propagation and characterisation studies of an ovine mycoplasma responsible for proliferative interstitial pneumonia. Cornell Vet. 62: 654.

Carson, J. L.; Collier, A. M. & Hu, S-C. S. (1980): Ultrastructural observations on cellular and sub-cellular aspects of experimental Mycoplasma pneumoniae disease. Infec. Immun. 29: 1117.

Carter, M. E. & Hunter, R. (1970): Isolation of parainfluenza type 3 virus from sheep in New Zealand. N. Z. vet. J. 18: 226.

Castelman, W. L.; Dungworth, D. L. & Tyler, W. S. (1975): Intrapulmonary airway morphology in three species of monkeys. A correlated scanning and transmission electron microscopic study. Am. J. Anat. 142: 107.

Cesario, T. C.; Caldwell, G. G. & Chin, T. D. Y. (1970):

Studies on the susceptibility of organ cultures to infection and on the localization of the virus in the cultures. J. Infec. Dis. 121: 70.

Chandler, D.K. & Barile, M.F. (1980): Ciliostatic haemagglutinating and proteolytic activities in a cell extract of Mycoplasma pneumoniae. Infec. Immun. 29: 1111.

Chandler, D. K. F.; Collier, A. M. & Barile, M. F. (1982a): Attachment of Mycoplasma pneumoniae to hamster tracheal organ cultures, tracheal outgrowth mono layers, human erythrocytes and human tissue carcinoma WiDr. Infec. Immoun. 35: 937.

Chen, J.M. (1954): The cultivation in fluid medium of organised liver, pancears and other tissues of foetal. Exp. Cell Res. 7: 518.

Cherry, J. D. & Taylor-Robinson, D. (1973): Mycoplasama pathogenicity studies in organ cultures. Ann. N.Y. Acad. Sci. 225: 290.

Cherry , J. D. & Taylor-Robinson, D. (1971a): Growth and pathogenecity studies of Mycoplasma gallisepticum in chicken tracheal organ cultures. J. med. Microbiol. 4: 441.

Cherry J. D. & Taylor-Robinson, D. (1971b): Mycoplasma pathogenicity studies in chicken tracheal organ cultures. J. Pediatr. 78: 1064.

Cherry, J. D. & Taylor-Robinson, D. (1970a): Large quantity production of chicken embryo tracheal organ cultures and use in virus and mycoplasma studies. Appl. Microbiol. 19: 658.

Cherry, J. D. & Taylor-Robinson, D. (1970b): Growth and pathogenesis of Mycoplasma mycoides var capri in chicken embryo tracheal organ cultures. Infec. Immun. 2: 431.

Cherry, J. D. & Taylor-Robinson, D. (1970c): Peroxide production by mycoplsama in chicken tracheal organ cultures. Nature (London). 228: 1099.

Cheville, N.F. (1983): Cell Pathology. Iowa State University Press. pp. 530-558.

Cheville, N.F. (1976): Cell Pathology. Iowa State University Press. pp. 323 - 325.

Chia, W-K & Savan, M. (1974): Pathogenesis of infectious bovine rhinotracheitis virus infection in bovine fetal tracheal organ cultures. Can. J. Microbiol. 20: 839.

Christensen, T. G.; Korthy, A. L.; Snider, G. L. & Hayes, J. A. (1977): Irreversible bronchial goblet cell metaplasia in hamster

with elastase-induced paracinar emphysema. J. clin. Invest. 59: 397.

Chummiskey, J. F.; Hallum, J. V.; Skinner, M. S. & Leslie, G. A. (1973): Persistent Newcastle disease virus infection in embryonic chicken tracheal organ cultures. Infec. Immun. 8: 657.

Clarke, S. W. (1973): Bull. Eru. Physiopathol. Respir. 9: 359. Cited by Lopez-Vidriero and Reid, (1978).

Clarke, J. K.; Brown, J. G. & Alley, M. R. (1974): Isolation and identification of mycoplasmas from the respiratory tract of sheep in New Zealand. N. Z. vet. J. 22: 117.

Clarke, A. W.; Lopez-Vidriero, M. T.; Pavia, D. & Lewin, D. (1981): To the Editor: Abnormal cilia in Polynesians with bronchiectasis. Am. Res. resp. Dis. 123: 141.

Cohen, G. & Somerson, N.L. (1967): Mycoplasma pneumoniae: hydrogen peroxide secretion and its possible role in virulence. Ann. N.Y. Acad. Sci. 143: 85.

Collier, A.M. (1979): Mycoplasma in organ culture. In "The Mycoplasma, Vol. II, Human and Animals Mycoplasmas". By Tully and Whitcomb (eds.). Academic Press. N.Y. pp. 475-493.

Collier, A. M.; & Baseman, J. B. (1973): Organ culture techniques with mycoplasmas. Ann. N. Y. Acad. Sci. 225: 277.

Collier, A. M. & Clyde, W, A. JR. (1971a): Nature of human respiratory epithelium infection by Mycoplasma pneumoniae. Bacteriol. Proc. 71: 76.

Collier, A. M. & Clyde, W. A. JR. (1971b): Relationships between Mycoplasma pneumoniae and human respiratory epithelium. Infec. Immun. 3: 694.

Collier, A. M. & Clyde, W. A. JR. (1970): Patho physiology of Mycoplasma pneumoniae infection in human fetal tracheal organ culture. Pediat. Res. 4: 460.

Collier, A. M. & Clyde, W. A. JR. (1969): Mycoplasma pneumoniae disease , pathogenesis studied in tracheal organ culture , Abstract hamster pneumonia. Fed. Proc. 28: 616.

Collier, A. M.; Clyde, W. A. JR. & Denny, F. W. (1971): Mycoplasma pneumoniae in hamster tracheal organ culture, Immuno fluorescent and electron microscopic studies. Proc. Soc. exp. Biol. Med. 136: 569.

Collier , A. M.; Clyde, W. A. JR. & Denny, F. W. (1969): Biologic effects of Mycoplasma pneumoniae and other mycoplasmas

from man on hamster tracheal ortgan culture. Proc. Soc. exp. Biol. Med. 132: 1153.

Collier, A. M.; Peterson, L. P. & Baseman, J. B. (1977): Pathogenesis of infection with Bordetella pertussis in hamster tracheal organ culture. J. infec. Dis. 136: S196.

Colwell, W. M. & Lukert, P. D. (1969): Effects of avian infectious bronchitis virus IBV on tracheal organ cultures. Avian Dis. 13: 888.

Constantinides, P. (1974): Functional electronic histology. Elsevier Scientific Publication Company. Oxford. pp. 97.

Cook, J. K. A.; Darbyshire, J. H. & Peters, R. W. (1976): The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 50: 109.

Craighead, J.E. (1968): Cytopathic effects of parainfluenza type 3 in organ cultures of human respiratory tract tissue. Am. J. Pathol. 52: 287.

Culling, C. F. A. (1974): Handbook of histopathological and histochemical techniques. Butterworths. Trowbridge. pp. 276.

Curtain, C. C.; Clarke, B. L. & Dufty, J. H. (1971): The origins of the immunoglobulins in the mucous secretions of cattle. Clin. exp. Immunol. 8: 335.

Cutlip, R. C. & Lehmkuhl, H. D. (1982): Experimentally induced parainfluenza type 3 virus infection in young lambs: Pathologic response . Am. J. vet. Res. 43: 2101.

Cutz, E. & Conen, P. E. (1971): Ultrastructure and cytochemistry of Clara cells. Am. J. Pathol. 62: 127.

Dahlgren,S. E. & Dalen, H. (1972): Ultrastructural observations on chemically induced inflammation in guinea pig trachea. Virc. Arch. B. 11: 211.

Daniele, R. P. (1980): Immune defenses of the lung. In "Pulmonary diseases and disorders". By Fishman, A. P. (ed.). McGraw-Hil Company. pp. 624 - 632.

Darbyshire, J. H. (1980): Assessment of cross immunity in chickens to strains of avian infectious bronchitis virus using trahceal organ cultures. Avian pathol. 9: 179.

Davies, D. H. (1985a): Aetiology of pneumonias in young sheep. Prog. vet. Microbiol. Immunol. 1: 229.

Davies, D.H. (1985b): Personal communication.

Davies, D. H. (1977): Ovine pneumonia. N. Z. vet. J. 25: 297

Davies, D. H.; Dungworth, D. L.; Humphreys, S. & Johnson, A.J. (1977): Concurrent infection of lambs with parainfluenza virus type 3 and Pasteurella haemolytica . N. Z. vet. J. 25: 263.

Davies, D.H.; Herceg, M.; Jones, B.A. & Thurley, D.C. (1981a): The pathogenesis of sequential infection with parainfluenza virus and Pasteurella haemolytica in sheep. Vet. Microbiol. 6: 173.

Davies, D. H.; Herceg, M. & Thurley, D. C. (1982): Experimental infection of lambs with an adenovirus followed by Pasteurella haemolytica . Vet. Microbiol. 7: 369.

Davies, D. H. & Humphreys, S. (1977a): Characterisation of two strains of adenovirus isolated from New Zealand sheep. Vet. Microbiol. 2: 97.

Davies, D.H. & Humphreys, S. (1977b): Experimental infection of lambs with an adenovirus of ovine origin. Vet Microbiol. 2: 67.

Davies, D. H.; Jones, B. A. H. & Thurley, D. C. (1981b): Infection of specific-pathogen-free lambs with parainfluenza virus type 3, Pasteurella haemolytica and Mycoplasma ovipneumoniae. Vet. Microbiol. 6: 295.

Davies, D. H. ; McCarthy, A. R. & Penwarden, R. A. (1980): The effect of vaccination of lambs with live parainfluenza virus type 3 on pneumonia produced by parainfluenza virus type 3 and Pasteurella haemolytica. N. Z. vet. J. 28: 201.

Davies , D. H.; Rosemary, A. & Penwarden, A. (1981a): The phagocytic cell response of the ovine lung to Pasteurella haemolytica . Vet. Microbiol. 6: 183.

Degand, P.; Roussel, P.; Lambin, G. & Harez, R. (1973) Purification et etude des mucines de Kystes broncho-geniques. Biochem. Biophys. Acta. 320: 318.

Denny, F. W. (1974): Effect of a toxin produced by Haemophilus influenzae on ciliated respiratory epithelium. J. infec. Dis. 129: 93.

Dungworth, D.L. (1985): The respiratory system. In "Pathology of Domestic Animals". 3rd edd. By Jubb, K.V.E.; Kennedy, P.C. & Palmer, N. (eds.). Academic Press Inc. London. pp. 413 - 556.

Dunnill, M. S. (1982): Pulmonary Pathology. Churchill Livingstone, Edinburgh. pp. 33 - 49.

Dunnill, M. S. (1968): Quantitative methods in histology . In "Recent Advances in clinical Pathology". Series 5. Dyke, S.C.

(ed.). London. Churchill. pp. 407.

Dunnill, M. S.; Massarella, G. R. & Anderson, J. A. (1969): A comparison of the quantitative anatomy of the bronchi in normal subjets, in status asthmaticus, in chronic bronchitis and in emphysema. Thorax. 24: 176.

Dutta, S. K. (1975): Morphological changes of chicken tracheas and tracheal organ cultures infected with avian infectious bronchitis virus studies in scanning electron microscope. Avian Dis. 19: 429.

Ellefsen, P. & Tos, M. (1972): Goblet cells in the human trachea. Quantitative studies of a pathological biopsy material. Arch. Otolar. 95: 547. cited by Breeze; et al. (1976).

Endoh, M.; Takezawa, T. & Nakase, Y. (1980): Adenylate cyclase activity of Bordetella organisms. I. It production in liquid medium. Microbiol. Immunol. 24: 95.

Engelhardt, J. A. & Gabridge, M. G. (1977): Effect of squamous metaplasia on infection of hamster trachea organ cultures with Mycoplasma pneumoniae. Infec. Immun. 15: 647.

Etherton, J. E.; Conning, D. M. & Corrin, B. (1973): Autoradiographical and morphological evidence for apocrine secretion of dipalamitoyl lecthin in the terminal bronchiole of mouse lung. Am. J. Anat. 138: 11.

Etherton, J. E.; Purchase, I. F. H. & Corrin, B. (1979): Apocrine secretion in the terminal bronchiole of mouse lung. J. Anat. 129: 305.

Eustis, S. L.; Schwartz, L. W.; Kosch, P. C. & Dungworth, D. L. (1981): Chronic bronchiolitis in nonhuman primates after prolonged ozone exposure. Am. J. Pathol. 105: 121.

Evans, M. J.; Cabral-Anderson, L. J. & Freman, G. (1978): Role of the Clara cell in renewal of the bronchiolar epithelium. Lab. Invest. 6: 648.

Fell, H. B. (1976): The development of organ culture. In "Organ Culture in biomedical Research". By Balls, M. & Monnickendam, M. (eds.). Cambridge University Press. Cambridge. pp 1-13.

Fell, H. B. & Robison, R. (1929): The growth, development and phosphatase activity of embryonic avian femora and limb-buds cultivated in vitro. Biochem. J. 23: 767.

Finkelstein, M. S.; McWilliams, M. & Huizenga, C. G. (1972): Use of chicken tracheal organ culture for studing resistence of respiratory epithelium to viral infection. J. Immunol. 108:

183.

Fishman, A. P. (ed.) (1980): Pulmonary diseases and disorders. McGraw-Hill Book Commpany. New York.

Florey, H.; Carleton, H. M. & Wells, A. Q. (1932): Mucus secretion in the trachea. Br. J. exp. Pathol. 13: 269.

Foggie, A.; Jones, G. E. & Buxton, D. (1976): The experimental infection of specific pathogen free lambs with Mycoplasma ovipneumoniae. Res. vet. Sci. 21: 28.

Frasca, J. M.; Auerbach, O. & Parks. V. R. (1967): Electron microscope observation of bronchial epithelium. I. Annulate lamellae. Exp. mol. Pathol. 6: 261.

Frasca, J. M.; Auerbach, O.; Parks, V. R. & Jamieson, J. D. (1968): Electron microscope observations of bronchial epithelium of dogs. 1. Control dogs. Exp. mol. Pathol. 9: 363.

Frey, M.C.; Hanson, R.P. & Anderson, D.P. (1968): A medium for the isolation of avian mycoplasmas. Am. J. vet. Res. 29: 2163.

Fulton, R. W. & Pearson, N. J. (1980): Interferon production by bovine tracheal organ cultures infected with Bovid herpesvirus 1 strains. Can. J. comp. Med. 44: 447.

Gabridge, M.G. (1975): Oxygen consumption by trachea organ cultures infected with Mycoplasma pneumoniae. Infec. Immun. 12: 544.

Gabridge, M.G.; Barden-Stahl, Y.D.; Polisky, R.B. & Engelhardt, J.A. (1977): Differences in the attachment of Mycoplasma pneumoniae cells and membranes to tracheal epithelium. Infec. Immun.. 16: 766.

Gabridge, M.G.; Johnson, C.K. & Cameron , A.M. (1974a): Cytotoxicity of Mycoplasma pneumoniae membranes. Infec. Immun.. 10: 1127.

Gabridge, M.G.; Keady, M.C. & Johnson, C.K. (1974b): Use hamster tracheal organ cultures in the identification of membrane associated toxins from Mycoplasma pneumoniae. In Vitro. 9: 383.

Gabridge, M.G. & Polisky, R.B. (1977): Intracellular levels of ATP in hamster tracheal organ cultures exposed to Mycoplasma pneumoniae cells or membranes. In Vitro. 13: 510.

Gabridge , M.G. & Polisky, R.B. (1976): Quantitative reduction of 2 3 4 tri phenyl tetrazolium chloride by hamster trachea organ cultures effects of Mycoplasma pneumoniae cells and membranes. Infec. Immun.. 13: 84.

Gaillard, P.L. (1951): Organ culture technique using embryologic

watch glasses. In "Methods in medical Research" by Visscher, M.R.(ed.). Year Book Publishers. Chicago. pp. 241. Cited by Hodges (1976).

Giddens, W.E. & Fairchild, G.A. (1972): Effects of sulfur dioxide on the nasal mucosa of mice . Arch. Env. He. 25: 166.

Gilmour, A.R. (1983): REG-Generalized linear models programme. New South Wales, Dept. of Agriculture.

Gilmour, N.J.L. & Brotherston, J.G. (1963): Clinical and pathological findings in an outbreak of pneumonia in sheep. J. comp. Pathol. 73: 329.

Glimour, J.S.; Jones, G.E.; Keir, W.A. & Rae, A.G. (1982): Long term pathological and microbiological progress in sheep of experimental disease resemlbling atypical pneumoniae. J. comp. Pathol. 92: 229.

Gilmour, J.S.; Jones, G.E. & Rae,A.G. (1979): Experimental studies of chronic pneumonia of sheep. Comp. Immun. Microbiol. infect. Dis. 1: 285.

Gilmour, N.J.L.; Thompson, D.A.; Smith, W.D. & Angus, K.W. (1975): Experimental infection of lambs with an aerosol of Pasteurella haemolytica. Res. vet. Sci. 18: 340.

Gois, M.; Pospisil, Z.; Cerny, M. & Mrva, V. (1971): Production of pneumonia after intranasal inoculation of gnotobiotic piglets with 3 strains of Mycoplasma hyorhinis. J. comp. Pathol. 81: 401.

Green, G.M.; Jacob, G.J.; Low, R.B. & Davis, G.S. (1976): Defense mechanisms of the respiratory membrane. Am. Rev. resp. Dis. 115: 479.

Greenwood, M.F. & Holland, P. (1972): The mammalian respiratory tract surface. A scanning electron microscopic study. Lab. Invest. 27: 296.

Gregg, C.R.; Melly, M.A.; Hellerqvist, C.G.; Coniglio, J.G. & McGee, Z.A. (1981): Toxic activity of purified lipopolysaccharide of Neisseria gonorrhoeae on human fallopian tube mucosa. J. infec. Dis. 143: 432.

Groniowski, J. ; Walski, M. & Biczuysko, W. (1972): Application of scanning electron microscopy for studies of the lung parenchyma. J. Ultrastruct. Res. 38: 473.

Hale, F.C.; Olsen, C.K. & Mickey, M.R. (1968): The measurement of bronchial wall components . Am. Rev. resp. Dis. 98: 978.

Hama, K. & Nagata, C.F. (1970): A steroscopic observation of

tracheal epithelium of the mouse by means of the high voltage electron microscope. J. cell Biol. 45: 654.

Hansell, M.M. & Moretti, R.L. (1969): Ultrastructure of mouse tracheal epithelium. J. Morphol. 128: 159.

Hansen, E.J.; Wilson, R.M.; Clyde, W.A. & Baseman, J.B. (1981): Characterisation of haemadsorption-negative mutante of Mycoplasma pneumoniae. Infec. Immun. 32: 127.

Harris, C.C.; Kanfman, D.G.; Jackson, F.; Smith, J.M.; Dedick, P. and Saffiotti, U. (1974): Atypical cilia in the tracheobronchial epithelium of the hamster during respiratory carcinogenesis. J. Pathol. 114: 17.

Henderson, F.W.; Hu, S.C. & Collier, R.M. (1978): Pathogenesis of respiratory syncytial virus infection in ferret and fetal human tracheas in organ culture. Am. Rev. resp. Dis. 118: 29.

Heppleston, A.G. (1953): The pathological anatomy of simple pneumokoniosis in coal workers. J. Pathol. Bacteriol. 66: 235.

Heuschele, W.P. & Easterday, B.C. (1970): Local immunity and persistence of virus in the tracheas of chickens following infection with Newcastle disease virus.I. Organ culture studies. J . infec. Dis. 121: 486.

Hewlett, E.L.; Manclark, C.R. & Wolff, J. (1977): Adenyl cyclase in Bordetella pertussis vaccines. J. infec. Dis. 136 (Suppl.): S216.

Hodges, M. (1976): A review of methology in organ culture. In "Organ Culture in biomedical Research". By Balls, M. & Monnickendam, M.(eds.). Cambridge University Press. Cambridge. pp. 15

Holmgren, N. (1974): On immune response in porcine serum and tracheobronchial secretions following experimental infection with Mycoplasma hypopneumoniae. Zentralbl. veterinaermed. Reihe B. 21: 188.

Hoorn, B. & Tyrrell, D.A. (1969): Organ cultures in virology . Prog. med. Virol. 11: 408:.

Hoorn, B. & Tyrrell, D.A. (1965): On the growth of certain newer respiratory viruses in organ cultures. Br. J. exp. Pathol. 46: 109.

Hore, D.E. & Stevenson, R.G. (1969): Respiratory infection of lambs with an ovine strain of parainfluenza virus type 3. Res. vet. Sci. 10: 342.

Horsfield, K.; Dart, G.,; Olsen, D.E.; Filley, G.F. & Cumming, G. (1971): Model of the human bronchial tree. J. appl. Physiol. 31: 207.

Howard, E.B. & Ryan, C.P. (1982): Chronic obstructive pulmonary disease in the domestic cat. Calf. Vet. 36: 7. Cited by Dungworth (1985).

Howard, C.J. & Thomas, L.H. (1974): Inhibition by Mycoplasma dispar of ciliary activity in tracheal organ culture. Infec. Immun. 10: 405.

Hu, P.C.; Collier, A.M. & Baseman, J.B. (1977): Surface parasitism of Mycoplasma pneumoniae of respiratory epithelium. J. exp. Med. 145: 1328.

Hu, P.C.; Collier, A.M. & Baseman, J.B. (1976): Interaction of virulent Mycoplasma pneumoniae with hamster tracheal organ cultures. Infec. Immun.. 14: 217.

Hu, P.C.; Collier, A.M. & Baseman, J.B. (1975): Alterations in the metabolism of hamster tracheas in organ culture after infection by virulent Mycoplasma pneumoniae. Infec. Immun. 11: 704.

Iida, T. & Ajiki, Y. (1975): The effect of 2, 4-dinitrophenol on the growth of Bordetella pertussis in chick tracheal organ culture. Jap. J. Microbiol. 19: 381.

Iida, T. & Ajiki, Y. (1974): Growth characteristic of Bordetella pertussis in the chick tracheal organ culture. Jap. J. Microbiol. 18: 119.

Ionas, G.; Mew, A.J.; Alley, M.R.; Clarke, J.K.; Robinson, A.J. & Marshall, R.B. (1985): Colonisation of the respiratory tract of lambs by strains of Mycoplasma ovipneumoniae. Vet. Microbiol. 10: 533.

Inoue, S. & Hogg, J.C. (1974): Intercellular junctions of the tracheal epithelium in guinea pig. Lab. Invest. 31: 68.

Janatuinen, M. & Korhonen, L.K. (1969): The effect of substituted Benzylamine (Bisolvon) on mucosubstance production. Sonderdruck aus "Naunyn-Schmiedebergs Archiv Fur Pharmakologie". 265: 112. Cited by Jones; et al. (1975).

Jeffery, P.K. (1983): Morphological features of airways surface epithelial cells and glands. Am. Rev. resp. Dis. 128: 14.

Jeffery, P.K. & Reid, L. (1977): Lung biology in health and disease. In "Respiratory Defense Mechanism" by Brain, J.D. and & Proctor, D.E.(eds.). Vol. 5. dekker, N.Y. pp. 193

Jeffery, P.K. & Reid, L. (1975): New observations of rat airway

epithelium : a quantitative and electron microscopic study. J. Anat. 120: 295.

Jentoft, N.; Cheng, P.W. & Carlson, D.M. (1976): Glycosyltransferases and glycoprotein biosynthesis. In "The enzymes of biological membranes". By Martonos. A. (ed.). N.Y. Plenum Press. pp. 343.

Johnson, A.P. ; Clark, J.B. & Osborn, M.F. (1983): Scanning electron microscopy of the interaction between Haemophilus influenza and organ cultures of rat trachea. J. med. Microbiol. 16: 477.

Jones, R. (1978): The glycoproteins of secretory cells in airway epithelium. In "Respiratory Tract Mucus". Ciba Found. Symp. 54: pp. 175 - 188.

Jones, R.; Baskerville, A. & Reid, L. (1975): Histochemical identification of glycoproteins in pig bronchial epithelium: (a) Normal and (b) hypertrophied from enzootic pneumonia. J. Pathol. 116: 1.

Jones, R.; Bolduc, P. & Reid, L. (1973): Goblet cell glycoprotein and tracheal gland hypertrophy in the rat airways. The effect of tobacco smoke with or without the antinflammatory agent phenylmethyloxadiazole. Br. J. exp. Pathol. 54: 229.

Jones, R.; Bolduc, P. and Reid, L. (1972): Protection of rat bronchial epithelium against tobacco smoke. Br. med. J. 2: 142.

Jones, G.E.; Buxton, D. & Harker, D.B. (1979): Respiratory infections in housed sheep with particular reference to mycoplasmas. Vet. Microbiol. 4: 47.

Jones, G.E.; Field, A.G.; Gilmour, J.S.; Rae, A.G.; Nettleton, P.F. & McLauchlan, M. (1982a) Effects of experimental chronic pneumonia on bodyweight feed intake and carcase composition of lambs. Vet. Rec. 110: 168.

Jones, G.E.; Foggie, A.; Mould, D.L & Livitt, S. (1976): The comparison and characterisation of glycolytic mycoplasmas isolated from the respiratory tract of sheep. J. med. Microbiol. 9: 39.

Jones, G.E.; Gilmour, J.S. & Rae, A.G. (1982b): The effect of different strains of Mycoplasma ovipneumoniae on specific pathogen-free lambs and conventionally-reared lambs. J. comp. Pathol. 92: 267.

Jones, G.E.; Gilmour, J.S. & Rae, A.G. (1982c): The effect of

Mycoplasma ovipneumoniae and Pasteurella haemplytica on specific pathogen-free lambs. J. comp. Pathol. 92: 261.

Jones, G.E.; Gilmour, J.S. & Rae, A. (1978): Endobronchial inoculation of sheep with pneumonic lung-tissue suspensions and with the bacteria and mycoplasma isolated from them. J. comp. Pathol. 88: 85.

Jones, G.E.; Keir, W.A. & Gilmour, J.S. (1985): The pathogenicity of Mycoplasma ovipneumoniae and Mycoplasma arginini in ovine and caprine tracheal organ cultures. J. comp. Pathol. 95: 477.

Jones, R. & Reid, L. (1978): Secretory cells and their glycoproteins in health and disease. Br. med. Bull. 34: 9.

Jones, R. & Reid, L. (1973a): The effect of pH on Alcian blue staining of epithelial acid glycoproteins I. Sialomucins and sulphomucins (singly or in single combinations). Histochem. J. 5: 9.

Jones, R. & Reid, L. (1973b): The effect of pH on Alcian blue staining of epithelial acid glycoproteins II. Human bronchial submucosal gland. Histochem. J. 5: 19.

Kaltreider, H.B. (1976): State of the art: expression of immune mechanisms in the lung. Am. Rev. resp. Dis. 113: 347.

Karrer, K.E. (1956): Electronmicroscopic study of bronchiolar epithelium of normal mouse lung. Exp. Cell Res. 10: 237.

Kazemi, H. (1976): Disorders of the respiratory system. Grune and Strattion. London. pp. 2 - 3.

Keal, E.E. & Reid, L. (1972): Thorax. 27: 643. Cited by Lopez-Vidreiro and Reid, (1978).

Keimowitz, R.K. (1964): Immunoglobulins in normal human tracheobronchial washings. A qualitative and quantitative study. J. Lab. clin. Med. 63: 54.

Kennedy, A.R.; Desrosiers, A.; Terzaghi, M. & Little, J.B. (1978): Morphometric and histological analysis of lungs of syrian golden hamsters. J. Anat. 125: 527.

Klein, J.D. & Collier, A.M. (1974): Pathogenesis of human parainfluenza type 3 virus infection in hamster tracheal organ culture. Infec. Immun. 10: 883.

Kliment, V.; Libich, J. & Kaudersova, V. (1972): Geometry of guinea pig respiratory tract and application of Landahl`s model of deposition of aerosol particles. J. Hyg. Ep. Mi. 16: 107.

Kocan, A.A.; Daubney, G.A. & Kocan, K.M. (1978): Structural

changes associated with type A influenza in Mallard duck tracheal organ culture. Avian Dis. 22: 535.

Kohn, D.F. & Chinookoswong, N. (1980): Pathogenicity of Mycoplasma pulmonis in ependymal organ culture. Infec. Immun.. 28: 601.

Konradova, V. (1966): The ultrastructure of tracheal epithelium in rabbit. Folia morphol. 14: 210.

Krahl, V.E. (1963): Microstructure of the lung. Arch. Env. He. 6: 43.

Krahl, V.E. (1959): Microscopic anatomy of the lungs. Am. Rev. resp. Dis. 80: 22.

Krause, D.C.; Leith, D.K.; Wilson, R.M. & Baseman, J.B. (1982): Identification of Mycoplasma pneumoniae proteins associated with hemadsorption and virulence. Infec. Immun. 35: 809.

Kuhn, C. III. (1976): The cells of the lung and their organelles. In "Lung Biology in Health and Disease". Vol. 2. The biochemical basis of pulmonary function". By Crystal, R.G. (ed.). N.Y. pp. 3 - 48.

Kuhn, C. III. & Finke, E.H. (1972): The topography of the pulmonary alveolus: scanning electron microscopy using different fixations. J. Ultrastruct. Res. 38: 161.

Kuhn, C.; Callaway, L.A. & Askin, F.B. (1974): The formation of granules in the bronchiolar Clara cells of the rat. I. Electron microscopy. J. Ultrastrut. Res. 49: 387.

Lamb, D. (1968): Mucous secretion in hypersecretory state. Bronches. 18: 453. Cited by Jones, (1978).

Lamb, D. & Reid, L. (1972): Quantitative distribution of various types of acid glycoprotein in mucous of human bronchi. Histochem. J. 4: 91.

Lamb, D. & Reid, L. (1970): Histochemical and autoradiographic investigation of the serous cells of the man bronchial glands. J. Pathol. 100: 127.

Lamb, D. & Reid, L. (1969a): Goblet cell increase in rat bronchial epithelium after exposure to cigarette and cigar tobacco smoke. Br. med. J. 1: 33.

Lamb, D. & Reid, L. (1969b): Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man. J. Pathol. 98: 213.

Lamb, D. & Reid, L. (1968): Mitotic rates, goblet cell increase and histochemical changes in mucus in rat bronchial epithelium during exposure to sulphur dioxide. J. Pathol. Bacteriol. 96: 97.

Lane, B.P. & Gordon, R. (1974): Regeneration of rat tracheal epithelium after michanical injury. 1. The relationship between mitotic activity and cellular differentiation. Proc. Soc. exp. Biol. Med. 145: 1139.

Leith, D.E. (1977): Cough. In "Lung biology in health and disease". Part II. Vol. 5. "Respiratory defense mechanisms". Brain, J.D.; Proctor, D.F. & Reid, L. N.Y. pp. 545 - 592.

Lieberman, P.; Patterson, R.; Petersen, V.; Chakrin, L.W.; Wardell, J.R.J.R. & Ricks, J. (1971): Effect of antigen variation on production of antibody in canine tracheal secretions. J. Immunol. 107: 1349.

Livingstone, C.W.; Stair, E.L.; Underdahl, N.R. & Mebus, C.A. (1972): Pathogenesis of mycoplasmal pneumonia in swine. Am. J. vet. Res. 33: 2249.

Lopez-Vidriero, M.T. & Reid, L. (1978): Bronchial mucus in health and disease. Br. med. Bull. 34: 63.

Mach, J.P. & Pahud, J.J. (1971): Secretory IgA, a major immunoglobulin in most bovine external secretions. J. Immunol. 106: 552.

Macpherson, M.R. & Hodges, R.T. (1985): The occurrence of mycoplasmas in the lung of pigs in New Zealand. N. Z. vet. J. 33: 194.

Major, R.M.; Clarke, J.K. & Alley, M.R. (1978): An in vitro study of the replication of Mycoplasma ovipneumoniae. N. Z. vet. J. 27: 58.

Mankelow, B.W. (1984): The veterinary handbook. Massey University. Palmerston North. New Zealand. pp. 57.

Mantyjarvi, R.A.; Selgrade, M.J.K.; Collier, A.M.; Hu, S.C. & Paguno, J. (1977): Murine cytomegalovirus infection of epithelial cells in mouse tracheal ring organ culture. J. infect. Dis. 136: 444.

Mardh, P.A.; Baldetrop, B.; Hakansson, C.H.; Fritz, H. & Westrom, L. (1979): Studies of ciliated epithelia of the human genital tract. 3. Mucociliary wave activity in organ cultures of human fallopian tubes challenged with Neisseria gonorrhoeae and gonococcal endotoxine. Br. J. vener. Dis. 55: 256.

Mariassy, A.T. & Plopper, C. (1983): Tracheobronchial epithelium of the sheep: I. Quantitative light-microscopic study of epithelial cell abundance and distribution. Anat. Rec. 205: 263.

Mariassy, A.T.; Plopper, C.G. & Dungworth, D.L. (1975): Characteristic of bovine lung as observed by scanning electron microscopy. Anat. Rec. 183: 13.

Matsuyama, T. (1977): Resistance of Bordetella pertussis phase I to mucociliary clearance by rabbit tracheal mucous membrane. J. infec. Dis. 136: 609.

Matsuyama, T. (1974): Point inoculation of cultivated tracheal mucous membrane with bacteria. J. infec. Dis. 130: 508.

Matsuyama, T. & Takino, T. (1980): Scanning electronmicroscopic studies of Bordetella bronchiseptica on the rabbit tracheal mucosa. J. med. Microbiol. 13: 159.

McDowell, E.M.; Becci, P.J.; Schurch, W. & Trump, B.F. (1979): The respiratory epithelium. VII. Epidermoid metaplasia of hamster tracheal epithelium during regeneration following mechanical injury. National cancer Institute J. 62: 995.

Medawar, P.B. (1948): The cultivation of adult mammalian skin epithelium in vitro. Q. J. microsc. Sci. 89: 187.

Melly, M.A.; Gregg, C.R. & McGee, Z.A. (1981): Studies of toxicity of Neisseria gonorrhoeae for human fallopian tube mucosa. J. infec. Dis. 143: 423.

Mew, A.J.; Ionas, G.; Clarke, J.K.; Robinson, A.J. & Marshall, R.B. (1985): Comparison of Mycoplasma ovipneumoniae isolates using bacterial restriction endonuclease DNA analysis and SDS-PAGE. Vet. Microbiol. 10: 541.

Meyrick, B. & Reid, L. (1970): Ultrastructure of cells in the human bronchial submucosal glands. J. Anat. 107: 281.

Meyrick, B. & Reid, L. (1968): The alveolar brush cell in rat lung - a third pneumonocyte. J. Ultrastruct. Res. 23: 71.

Meyrick, B.; Sturgess, J. & Reid, L. (1969): Reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. Thorax. 24: 729.

Mossman, B.T.; Kessler, J.B.; Ley, B.W. & Craighead, J.E. (1977): Interaction of crocidlite asbestos with hamster respiratory mucosa in organ culture. Lab. Invest. 36: 131.

Murphy, G.F.; Brody, A.R. & Craighead, J.E. (1980): Exfoliation of respiratory epithelium in hamster tracheal organ cultures infected with Mycoplasma pneumoniae. Virchows Arch. A Pathol. Anat. Histol. 389: 93.

Muse, K.E.; Collier, A.M. & Baseman, J.B. (1977): Scanning electron microscopic study of hamster tracheal organ cultures

infected with Bordetella pertussis. J. infec. Dis. 136: 768.

Muse, K.E.; Findley, D.; Allen, L. & Collier, A.M. (1979): In vitro model of Bordetella pertussis infection: pathogenic and microbicidal interactions. In C.R. Manclark and J.C. Hill (eds.). International symposium on pertussis. U.S Government Printing Office. Washington, D.C. pp. 41-50. Cited by Bemis & Kennedy (1981).

Muse, K.E.; Powell, D.A. & Collier, A.M. (1976): Mycoplasma pneumoniae in hamster tracheal organ culture studied by scanning electron microscopy. Infec. Immun.. 13: 229.

Nedrud, J.G.; Collier, A.M. & Pagano, J.S. (1979): Cellular basis for susceptibility to mouse cytomegalovirus evidence from tracheal organ culture. J. gen. Virol. 45: 737.

New, D.A.T. & Mizell, M. (1972): Opossum fetuses grown in culture. Science. N.Y. 175: 533.

Nicholas, R.A.J.; Wood, G.W. & Thornton, D.H. (1983): Comparison of techniques for the detection of avian infectious bronchitis virus as a contaminant of vaccines. J. biol. Stand. 11: 75.

Niden, A.H. (1967): Bronchiolar and large alveolar cell in pulmonary phospholipid metabolism. Science. 158: 1322.

Niewoehner, D.E.; Kleinerman, J. & Rice, D.B. (1974): Pathologic changes in the peripheral airways of young cigarette smokers. N. Engl. J. Med. 291: 755.

Nowell, J.A. & Tyler, W.S. (1971): Scanning electron microscopy of the surface morphology of mammalian lungs. Am. Rev. resp. Dis. 103: 313.

Okada, Y. (1969): The ultrastructure of the Clara cells in the bronchiolar epithelium. Bull. Chest. Dis. Res. Inst. Kyoto Univ. 3: 1. Cited by Breeze; et al. (1976).

Okano, M.; Weber, A.F. & Frommes, S.P. (1970): Electron microscopic studies of the distal border of the canine olfactory epithelium. J. Ultrastruct. Res. 17: 487.

Pack, R.J.; Al-Ugaily, L.H. & Morris, G. (1981): The cells of the tracheobronchial epithelium of the mouse: A quantitative light and electron microscopic study. J. Anat. 132: 71.

Pearse, A.G.E. (1972): Histochemistry: Theoretical and applied. 3rd edit. Churchill Livingstone, London.

Pfeffer, A.; Thurley, D.C.; Boyes, B.W.; Davies, D.H.; Davis, G.B. & Price, M.C. (1983): The prevlence and microbiology of pneumonia in a flock of lambs. N. Z. vet. J. 31: 196.

Petrovic, A. & Huesner, A. (1963): Culture Organotypique prolongee de rate, de thymus et de ganglion lymphatique de Souris adulte en milieu semi-synthetique liquide continuellement oxygene. Comptes Rendues de l' Academie des Sciences, Paris. 257: 4050. Cited by Hodges (1976).

Pijoan, C. (1974): The effect of Mycoplasma hyorhinis strain S-7 on pig tracheal organ cultures. Br. vet. J. 130: 593.

Pijoan, C.; Roberts, D.H. & Harding, J.D.J. (1972): The effect of porcine Mycoplasmata on pig tracheal organ cultures. J. appl. Bacteriol. 35: 361.

Potter, C.W.; Shore, S.L. & McLaren, C. (1972): Immunity to influenza in ferrets. III. Proteins in nasal secretions. J. infec. Dis. 126: 387.

Powell, D.A.; Hu, P.C.; Wilson, M.; Collier, A.M. & Baseman, J.B. (1976): Attachment of Mycoplasma pneumoniae to respiratory epithelium. Infec. Immun. 13: 959.

Rathore, B.S.; Mohanty, G.C. & Rajya, B.S. (1979): Pathogenesis of Mycoplasma gallisepticum infection in the reproductive and respiratory tracts of chickens in vivo and in vitro studies. Indian J. Anim. Sci. 49: 932.

Reed, S. (1976): Organ cultures for studies of viruses and mycoplasmas. In "Organ Culture in Biomedical Research". By Balls, M. & Monnickendam, M (eds.) Cambridge University Press. London. pp515 - 532.

Reed, S,E, (1972): Viral enhancement of mycoplasma growth in tracheal organ cultures. J. comp. Pathol. 82: 267.

Reed, S.E. (1971): The interaction of Mycoplasmata and influenza viruses in tracheal organ cultures. J. infec. Dis. 124: 18.

Reed, S.E. (1969): Persistent respiratory virus infection in tracheal organ cultures. Br. J. exp. Pathol. 50: 378.

Reed, S.E. & Boyde, A. (1972): Organ cultures of respiratory epithelium infected with rhinovirus or parainfluenza virus studied in a scanning electron microscope. Infec. Immun. 6: 68.

Reid, L. (1978a): The cell biology of mucus secretion in the lung. In "The lung Structure, Function and Disease".by Thurlbeck, W.M. & Abell, M.R. (eds.). The Williams and Wilkins Company. Baltimore. pp. 138 - 150.

Reid, L. (1978b): Animal models in clinical disease. In "Respiratory Tract Mucus". Ciba found. Symp. 54: 297.

Rhodin, J.A.G. & Dalham, A. (1956): Electron microscopy of the tracheal ciliated mucosa in rat. Zelforsch. mikrosk. Anat. 44: 345.

Reid, L. (1963): An experimental study of hypersecretion of mucus in the bronchial tree. Br. J. exp. Pathol. 44: 437.

Reid, L. (1960): Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis. Thorax. 15: 132.

Reid, L. & Jones, R. (1979): Bronchial mucosal cells. Fed. Proc. 38: 191.

Remington, J.S.; Vosti, K.L.; Lietze, A. & Zimmerman, A.L. (1964): Serum proteins and antibody activity in human nasal secretions. J. clin. Invest. 43: 1613.

Restrepo, G. & Heard, B.E. (1963a): The size of the bronchial glands in chronic bronchitis. J. Pathol. Bacteriol. 85: 305.

Restrepo, G. & Heard, B.E. (1963b): Mucous gland enlargement in chronic bronchitis: extent of enlargement in the tracheobronchial tree. Thorax. 18: 334.

Reynolds, H.Y.; Kazmierowski, J.A. & Newball, H.H. (1975): Specificity of opsonic antibodies to enhance phagocytosis of Pseudomonas aeruginosa by human alveolar macrophages. J. clin. Invest. 56: 376.

Reynolds, H.Y. & Newball, H.H. (1974): Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. J. Lab. clin. Med. 84: 559.

Rhodin, J.A.G. (1974): Histology. Oxford University Press. N.Y. pp. 607 - 646.

Rhodin, J.A.G. (1966): The ciliated cell. Ultrastructure and function of the human tracheal mucosa. Am. Rev. resp. Dis. 93: Supp. 1.

Rhodin, J.A.G. (1963): An atlas of ultrastructure. Saunders. London.

Robert, G.P. (1976): The role of disulfide bonds in maintaining the gel structure of bronchial mucus, Arch. Biochem. Biophys. 173: 528.

Robert, G.P. (1974): Isolation characterization of glycoproteins from sputum. Eur. J. Biochem. 50: 265.

Robinson, N.E. & Gillespie, J.R. (1975): Physiology of the respiratory system. In "Textbook of veterinary internal Medicine" by Ettinger, S. (ed). W.B. Saunders Company. London. pp. 527 - 543.

Roussel, P.; Degand, P.; Lamblin, G.; Laine, A. & La Fitte, J.J. (1978): Biochemical definition of human tracheobronchial mucus. Lung. 154: 241.

Roussel, P.; Lamblin, G.; Degand, P.; Walker-Nasir, E. & Jeanloz, R.W. (1975): Heterogenicity of the carbohydrate chains of sulfated bronchial glycoproteins isolated from a patient suffering from cystic fibrosis. J. biol. Chem. 250: 2114.

Rumery, E.P. & Blandau, R.L. (1971): Culture of mammalian embryonic ovaries and oviducts. In "Methods in Mammalian Embryology. Daniel, J.C. (ed). W.H. Freeman. San Francisco. pp. 472. Cited by Hodges (1976).

Rushton, B.; Sharp, J.M.; Gilmour, N.J.L. & Thompson, D.A. (1979): Pathology of an experimental infection of specific pathogen-free lambs with parainfluenza virus type 3 and Pasteurella haemolytica. J. comp. Pathol. 89: 321.

Rybicka, K.; Daly, D.T.; Migliore, J.J. & Norman, J.C. (1974a): Ultrastructure of pulmonary alveoli of the calf. Am. J. vet. Res. 35: 213.

Rybicka, K.; Daly D.T.; Migliore, J.J. & Norman, J.C. (1974b): Intravascular macrophages in normal calf lung. An electron microscopic study. Am. J. Anat. 139: 353.

Sade, J.; Meyer, F.A.; King, M. & Silberberg, A. (1975): Acta Otolaryngol. 79: 277. Cited by Lopez-Vidriero & Reid (1978).

Salisbury, R.M. (1957): Enzootic pneumonia of sheep in New Zealand. N. Z. vet. J. 5: 124.

Schmidt, R.C. & Massab, H.F. (1974): Local immunity to influenza virus in chicken trachea organ cultures. J. infec. Dis. 129: 637.

Schmidt, R.C.; Massab, H.F. & Davenport, F.M. (1974): Infection by influenza A viruses of tracheal organ cultures derived from homologous and heterologous hosts. J. infec. Dis. 129: 28.

Schwartz, L.W.; Dungworth, D.L.; Mustafa, M.G.; Tarkington, B.K. & Tyler, W.S. (1976): Pulmonary responses of rats to ambient levels of ozone. Lab. Invest. 34: 565.

Shaffer, B.M. (1956): The culture of organs from the embryonic chick on cellulose-acetate fabric. Exp. Cell Res. 11: 244.

Sharp, J.M.; Gilmour, N.J.L.; Thompson, D.A. & Rushton, B. (1978): Experimental infection of specific pathogen-free lambs with parainfluenza virus type 3 and Pasteurella haemolytica. J. comp. Pathol. 88: 237.

Shimura, S.; Boatman, E.S. & Martin, C.J. (1986): Effects of ageing on the alveolar pores of Kohn and on the cytoplasmic components of alveolar type II cells in monkey lungs. J.

Pathol. 148: 1.

Shroyer, E.L. & Easterday, B.C. (1968): Growth of infectious bovine rhinotrachitis virus in organ cultures. Am. J. vet. Res. 29: 1355.

Silbert, J.E. (1967): Biosynthesis of heparin. III. Formation of a sulfated glycosaminoglycan with a microsomal preparation from mast cell tumors. J. biol. Chem. 242: 5146.

Simberkoff, M.S. & Elsbach, P. (1971): The interaction in vitro between polymorphonuclear leukocytes and mycoplasma. J. exp. Med. 134: 1417.

Smith, W.D.; Dawson, A.M.; Wells, P.W. & Burrells, C. (1975): Immunoglobulin concentration in sheep body fluids. Res. vet. Sci. 19: 189.

Smith, W.D.; Wells, P.W.; Burrells, C. & Dawson, A.M. (1976): Immunoglobulin concentration and parainfluenza 3 virus inhibitors in the nasal and lachrymal secretions and serum of newborn lambs. Clin. exp. Immunol. 23: 544.

Soltys, M.A. (1979): Introduction to Veterinary Microbiology. Penebit Universiti Pertanian Malaysia. Serdang. Selangor. pp. 172.

Somordintsev, A.A. (1968): The production and effect of interferon in organ cultures of calf trachea. Br. J. exp. Pathol. 49: 511.

Sorokin, S.P. (1968): Reconstructions of centriole formation and ciliogenesis in mammalian lungs. J. Cell. Sci. 3: 207.

Sorokin, S.P. (1965): On the cytology and cytochemistry of the opossum's bronchial glands. Am. J. Anat. 117: 311.

Spiegelberg, J.T. (1974): Biological activities of immunoglobulins of different classes and subclasses. Adv. Immunol. 19: 259.

Springer, G.F.; Schwick, H.G. & Fletcher, M.A. (1969): The relationship of the influenza virus inhibitory activity of glycoproteins to their molecular size and sialic acid content. Proc. Nat. Acad. Sci. USA. 64: 634.

Ssentongo, Y.K.; Campbell, R.S.F. & Johnson, R.H. (1980): Pathogenesis of bovine viral diarrhea mucosal disease virus infection in tracheal organ cultures. Vet. Microbiol. 5: 257.

Stahl, G.H. & Ellis, D.B. (1973): Biosynthesis of respiratory tract mucins: a comparison of canine epithelial goblet cell and submucosal gland secretions. Biochem.J. 163: 845.

Stamp, J.T. & Nisbet, D.I. (1963): Pneumonia in sheep. J. comp.

Pathol. 73: 319.

Steel, R.G.D. & Torrie, J.H. (1981): Principles and Procedures of Statistics. A biometrical Approach. 2nd edd. International student edition. Auckland. pp. 187.

Stephens, R.J.; Sloan, M.F.; Evans, M.J. & Freeman, G. (1974): Early exposure of lung to low levels of ozone. Am. J. Pathol. 74: 31.

St. George, T.D.; Sullivan, N.D.; Love, J.A. & Horsfall, N. (1971): Experimental transmission of pneumonia in sheep with a mycoplasma isolated from pneumonic sheep lung. Aust. vet. J. 47: 282.

Strangeways, T.S.P. & Fell, H.B. (1926a): Experimental studies on the differentiation of embryonic tissues growing in vivo and in vitro. I. Proc. Roy. Soc. B. 99: 340. Cited by Fell (1976).

Strangeways, T.S.P. & Fell, H.B. (1926b): Experimental studies of embryonic tissues growing in vivo and in Vitro. II. Proc. Roy. Soc. B. 100: 273. Cited by Fell (1976).

Sturgess, J. & Reid, L. (1973): The effect of isoprenaline and pilocaprine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver and spleen. Br. J. exp. Pathol. 54: 388.

Sturgess, J. & Reid, L. (1972a): An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland. Clin. Sci. 43: 533.

Sturgess, J. & Reid, L. (1972b): The secretory activity of the human bronchial mucous glands in vitro. Exp. mol. Pathol. 16: 362.

Sullivan, N.D.; St. George, T.D. & Horsfall, N. (1973a): A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 1. Natural history of the disease in a flock. Aust. vet. J. 49: 57.

Sullivan, N.D.; St. George, T.D. & Horsfall, N. (1973b): A proliferative interstitial pneumonia of sheep associated with mycoplasma infection: 2. The experimental exposure of young lambs to infection. Aust. vet. J. 49: 63.

Takagi, H. & Arakawa, A. (1980): The growth and cilia stopping effect of Mycoplasma gallisepticum 1RF in chicken tracheal organ cultures. Res. vet. Sci. 28: 80.

Tandler, B.; Sherman, J. & Boat, T.F. (1979): EDTA-mediated

separation of lining epithelium from the cat trachea. Anat. Rec. 193: 700.

Taylor-Robinson, D. & Cherry, D.A.J. (1972): A nonpathogenic mycoplasma inhibiting the effect of a pathogenic mycoplasma in organ culture. J. med. Microbiol. 5: 291.

Thomas, L.H. & Howard, G.J. (1974): Effect of Mycoplasma dispar, Mycoplasma bovirhinis, Acholeplasma laidlawi and T- Mycoplasmas on explant cultures of bovine trachea. J. comp. Pathol. 84: 193.

Thomson, M.L. & Short, M.D. (1969): Mucociliary function in health, chronic obstructive disease and asbestosis. J. appl. Physiol. 26: 535.

Thorns, C.J. & Boughton, E. (1980): Effect of serial passages through liquid medium on the virulence of Mycoplasma bovis for the mouse mammary gland. Res. vet. Sci. 29: 328.

Tizard, I. (1977): An Introduction to veterinary Immunology. W.B. Saunders Company. London. pp. 146.

Tomas, T.B. (1970): Structure and function of mucosal antibodies. Annu. Rev. Med. 21: 218.

Tomas, T.B. & Bienenstock, J. (1968): Secretory immunoglobulins. Adv. Immunol. 9: 1.

Toms, G.; Rosztoczy, I. & Smith H. (1974): The localization of influenza virus: minimal infectious dose determinations and single cycle kinetic studies on organ cultures of respiratory and other ferret tissues. Br. J. exp. Pathol. 55: 116.

Trowell, O.A. (1954): A modified technique for organ culture in vitro. Exp. Cell Res. 6: 246.

Tyrrell, D.A.J. & Hoorn, B. (1965): The growth of some myxoviruses in organ cultres. Br. J. exp. Pathol. 46: 514.

van Allen, C.M.; Lindskog, G.E. & Richter, H.G. (1931): Collateral respiration. Transfer of air collaterally between pulmonary lobules. J. clin. Invest. 10: 559.

Volk, T.L. & Scarpelli, D.G. (1964): Alterations of fine structure of the rat adrenal cortex after the administration of triparanol. Lab. Invest. 13: 1205.

Walsh, C. & McLelland, J. (1974): Intraepithelial axons in avian trachea. Z. Zellforsch mikrosk. Anat. 147: 209.

Wang, N.S.; Taeusch, H.W.; Thurlbeck, W.M. & Avery, M.E. (1973): A combined scanning and transmission electron microscopic study of alveolar epithelial development of the fetal rabbit lung. Am.

J. Pathol. 73: 365.

Wang, N.S. & Thurlbeck, W.M. (1970): Scanning electron microscopy of the lung. Human Pathol. 1: 227.

Watson, J.H.L. & Brinkman, G.L. (1964): Electron microscopy of the epithelial cells of normal and bronchitic human bronchus. Am. Rev. resp. Dis. 90: 851.

Weibel, E.R. (1980): Design and structure of the human lung. In "Pulmonary Diseases and Disorders" by Fishman, A.P. (ed.) McGraw-Hill. N.Y. pp. 227 - 232.

Weibel, E.R. (1963): Morphometry of the human lung. Heidelberg. Springer. Cited by Weibel (1980).

Weiss, L. & Greep, R.O. (1977): Histology. McGraw-Hill. N.Y. pp. 765 - 830.

Wells, H. (1963): The neural regulation of salivary gland growth. In "Mucous secretion". Ann. N.Y. Acad. Sci. 106: 654.

Westerberg, S.C.; Smith, C.B.; Wiley, B.B. & Jensen, C.G. (1972a): Pathogenesis of influenza virus in mouse tracheal organ cultures. Proc. Sco. exp. Biol. Med. 140: 846.

Westerberg, D.C.; Smith, C.B.; Wiley, B.B. & Jensen, C. (1972b): Mycoplasma-virus interrelationship in mouse tracheal organ cultures. Infec. Immun.. 5: 840.

Williams, M.M. (1971): Hypoxemia. In "Cecil-loeb textbook of medicine" By Beeson, P.B. & McDermott, W. (eds.). 13th ed. Philadelphia. W.B. Saunders Company.

Wilsman, N.J.; Farnum, C.E. & Reed, D.K. (1982): Variability of ciliary ultrastructure in normal dogs. Am. J. Anat. 164: 343.

Wilson, M.H. & Collier, A.M. (1976): Ultrastructural study of Mycoplasma pneumoniae in organ culture. J. Bacteriol. 125: 332.

Wolff, E. & Haffen, K. (1952): Sur une methode de culture d'organes embryonnaires in vitro. Texas reports of biology and medicine. 10: 463. Cited by Hodges (1976).

Wolffing, B.K. & Gabridge, M.G. (1979): Effect of in vitro cultivation and Mycoplasma pneumoniae infection on intracellular cyclic AMP levels in hamster tracheal organ cultures. In Vitro 15: 308.

Woodruff, K.H.; Schneider, E.; Unger, L. & Coalson, J.J. (1973): Ultrastructural changes in hamster tracheal ring cultures exposed to Mycoplasma pneumoniae. Am. J. Pathol. 72: 91.

Woolcock, A.J. & Macklem, P.T. (1971): Mechanical factors influencing collateral ventilation in human, dog and pig lung. J. appl. Physiol. 30: 99.

Wright, N.G.; Brown, R.M.H.; McCandlish, I.A.P.; Thompson, N. & Cornwell, H.J.C. (1983): Patterns of cilia formation in the lower respiratory tract of the dog: a scanning electron microscopic study. Res. vet. Sci. 34: 340.

Yachida, S.; Aoyama, S.; Takahashi, N.; Iritani, Y.; and Katagiri, K. (1981a): Influence of temperature of incubation on chicken embryo tracheal organ cultures and chick embryos infected with strains of avian infectious bronchitis virus. Res. vet. Sci. 31: 14.

Yachida, S.; Aoyama, S.; Takhashi, N.; Sawaguchi, K. and Iritani, Y. (1981b): Effects of three avian respiratory viruses on pathogenicity in tracheal organ cultures. Jap. J. vet. Sci. 43: 491.

Yachida, S.; Aoyama, S.; Takahashi, N.; Iritani, Y. & Katagiri, K. (1978): Plastic multiwell plates to assay avian infectious bronchitis virus in organ cultures of chicken embryo trachea. J. clin. Microbiol. 8: 380.

Zucker-Franklin, D.; Davidson, M. & Thomas, L. (1966a): The interaction of mycoplasmas with mammalian cells. I. HeLA cells, neutrophils and eosinophils. J. exp. Med. 124: 521.

Zucker-Franklin, D.; Davidson, M. & Thomas, L. (1966b): The interaction of mycoplasmas with mammalian cells. II. Monocytes and lymphocytes. J. exp. Med. 124: 533.

APPENDIX 2.1

MODIFIED KARNOVSKY'S FIXATIVE

Paraformaldehyde	2.0 gm
Gluteraldehyde	12.0 ml
$Na_2HPO_4.12H_2O$	2.51 gm
KH_2PO_4	0.41 gm

METHOD

A) Heat the paraformaldehyde (2.0 g in 80 mls distilled water) to between 60 and 80°C.

B) Slowly add 1.0N NaOH, dropwise until the solution clears.

C) Add the buffer salts ($Na_2 HPO_4.12H_2O$) and gluteraldehyde.

D) Make up the solution to 100 mls at 4°C .

APPENDIX 2.2

OSMIUM TETROXIDE

Osmium tetroxide was made up as a 1% aqueous solution in 0.1M phosphate buffer and kept in a dark bottle.

APPENDIX 2.3

STAINING TECHNIQUE FOR TEM SECTIONS

SOLUTIONS

URANYL ACETATE

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

LEAD CITRATE

$Pb(NO_3)_2$	1.33 gm
Trisodium citrate	1.76 gm
Distilled water	30.00 mls, then 50 mls

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

METHOD

A) Using a pipette, place in a petri dish as many drops of the uranyl acetate and lead citrate solutions as there are grids.
B) Rinse the grid with 50% alcohol.
C) Float the grid with section side down on a drop of uranyl acetate for 6 min.
D) Transfer the grid to a dish containing 50% alcohol, rinse thoroughly and gently with 50% alcohol and then distilled water.
E) Float the grid with section side down on a drop of lead citrate for 6 min.
F) Rinse the grid with distilled water, dry and store for TEM examination.

LIST OF THE ABBREVIATIONS USED IN THE
STATISTICAL ANALYSIS APPENDICES

B = BLUE
BR = BLUE-RED
EP = EXTRAPULMONARY
F-R = F-RATIO
G1 = NORMAL ANIMAL GROUP
G2 = EARLY PNEUMONIC GROUP
G3 = ADVANCED PNEUMONIC GROUP
GP = GLYCOPROTEIN
IP = INTRAPULMONARY
L1 = UPPER TRACHEA
L2 = LOWER TRACHEA
L3 = EXTRAPULMONARY BRONCHI
L4 = INTRAPULMONARY BRONCHI
L5 = SMALL BRONCHI
L6 = BRONCHIOLI
NS = NOT SIGNIFICANT
P = PURPLE
P< = PROBABILITIES
R = RED
RB = RED-BLUE
SE = STANDARD ERROR

THE KEY FOR DUNCAN'S ANALYSIS: NO SIGNIFICANT DIFFERENCES
BETWEEN THE UNDERLINED VALUES

APPENDIX 4.1: MEAN THICKNESSES OF THE NORMAL OVINE TRACHEOBRONCHIAL MUCOSAL LAYERS

MUCOSAL LAYERS	MEAN THICKNESS IN MICROMETRES(um) L1	L2	L3	L4	L5	L6	SE	F-R	P<
EPITHELIUM	41.3	33.8	27.6	24.4	23.8	13.0	2.5	15.3	0.001
SUBMUCOSA	415.5	246.3	163.0	106.6	51.7	15.6	20.2	52.9	0.001
MUCOSA	456.8	280.1	190.6	131.2	75.5	28.6	19.9	61.3	0.001

APPENDIX 4.2: DUNCAN'S STATISTICAL ANALYSIS OF THE TRACHEOBRONCHIAL MUCOSAL THICKNESS AT DIFFERENT LEVELS OF NORMAL SHEEP LUNGS

AT 5% PROTECTION LEVEL

EPITHELIUM

L6	L5	L4	L3	L2	L1
13.0	23.8	24.4	27.6	33.7	41.3

SUBMUCOSA

L6	L5	L4	L3	L2	L1
15.6	51.7	106.9	163.0	246.3	415.5

MUCOSA

L6	L5	L4	L3	L2	L1
28.6	75.5	131.2	190.6	280.1	456.8

AT 1% PROTECTION LEVEL

EPITHELIUM

L6	L5	L4	L3	L2	L1
13.0	23.8	24.4	27.6	33.7	41.3

SUBMUCOSA

L6	L5	L4	L3	L2	L1
15.6	51.7	106.9	163.0	246.3	415.5

MUCOSA

L6	L5	L4	L3	L2	L1
28.6	75.5	131.2	190.6	280.1	456.8

APPENDIX 4.3: DUNCAN'S STATISTICAL ANALYSIS OF THE TRACHEOBRONCHIAL MUCOSAL THICKNESS AT DIFFERENT LEVELS OF EARLY PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

EPITHELIUM

L6	L5	L3	L4	L2	L1
24.9	34.4	35.3	36.3	40.7	75.0

SUBMUCOSA

L6	L5	L4	L3	L2	L1
34.1	84.0	187.9	256.2	349.7	517.9

MUCOSA

L6	L5	L4	L3	L2	L1
58.4	118.4	224.0	291.5	389.6	594.5

AT 1% PROTECTION LEVEL

EPITHELIUM

L6	L5	L4	L3	L2	L1
24.9	34.4	35.3	36.3	40.7	75.0

SUBMUCOSA

L6	L5	L4	L3	L2	L1
34.1	84.0	187.9	256.2	349.7	517.9

MUCOSA

L6	L5	L4	L3	L2	L1
58.4	118.4	224.0	291.5	289.6	594.5

APPENDIX 4.4: MEAN THICKNESSES OF THE TRACHEOBRONCHIAL MUCOSAL LAYERS OF EARLY PNEUMONIC LESIONS

MUCOSAL LAYERS	MEAN THICKNESS IN MICROMETRES (um) L1	L2	L3	L4	L5	L6	SE	F-R	P<
EPITHELIUM	75.0	40.7	35.3	36.3	34.4	24.9	6.8	6.5	0.001
SUBMUCOSA	517.9	349.7	256.2	187.9	84.0	34.1	59.9	9.5	0.001
MUCOSA	594.5	389.6	291.5	224.0	118.4	58.4	62.7	9.6	0.001

APPENDIX 4.5: DUNCAN'S STATISTICAL ANALYSIS OF THE TRACHEOBRONCHIAL MUCOSAL THICKNESSES AT DIFFERENT LEVELS OF ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

EPITHELIUM

L3	L2	L6	L4	L5	L1
27.5	36.3	43.3	44.0	52.6	54.3

SUBMUCOSA

L6	L5	L4	L3	L2	L1
62.0	133.5	234.2	261.5	333.5	661.8

MUCOSA

L6	L5	L4	L3	L2	L1
105.3	186.1	278.1	289.0	369.7	716.8

AT 1% PROTECTION LEVEL

EPITHELIUM

L3	L2	L6	L4	L5	L1
27.5	36.3	43.3	44.0	52.6	54.3

SUBMUCOSA

L6	L5	L4	L3	L2	L1
62.0	133.5	234.2	261.5	333.5	661.8

MUCOSA

L6	L5	L4	L3	L2	L1
105.3	186.1	278.1	289.0	369.7	716.8

APPENDIX 4.6: THE MEAN THICKNESSES OF THE TRACHEOBRONCHIAL MUCOSAL LAYERS OF ADVANCED PNEUMONIC LESIONS

MUCOSAL LAYERS	MEAN THICKNESS IN MICROMETRES (um) L1	L2	L3	L4	L5	L6	SE	F-R	P<
EPITELIUM	54.3	36.3	27.5	44.0	52.6	43.3	7.7	1.7	NS
SUBMUCOSA	661.8	333.5	261.5	234.2	133.5	62.0	39.5	28.1	0.001
MUCOSA	716.8	369.7	289.0	278.1	186.1	105.3	39.0	29.7	0.001

APPENDIX 4.7: COMPARATIVE STATISTICAL ANALYSIS OF THE EPITHELIAL THICKNESS BETWEEN NORMAL, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

AIRWAY LEVEL	THICKNESS IN MICROMETRES(um) NORMAL	EARLY PNEUMONIA	ADVANCED PNEUMONIA	SE	F-R	P<
UPPER TRACHEA	41.3	75.0	54.3	9.2	3.4	NS
LOWER TRACHEA	33.8	40.7	36.3	4.6	0.6	NS
EP BRONCHI	27.6	35.3	27.5	4.3	1.1	NS
IP BRONCHI	24.4	36.3	43.9	6.0	2.7	NS
SMALL BRONCHI	23.8	34.4	52.6	5.6	6.7	0.05
BRONCHIOLI	130	24.9	43.3	5.6	7.5	0.01

APPENDIX 4.8: DUNCAN'S STATISTICAL ANALYSIS OF THE EPITHELIAL THICKNESS OF NORMAL LUNGS, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

AT 5% PRTECTION LEVEL

UPPER TRACHEA	G1	G3	G2
	41.3	54.3	75.0
LOWER TRACHEA	G1	G3	G2
	38	36.3	40.4
EXTRAPULMONARY BRONCHI	G3	G1	G2
	27.5	27.6	35.3
INTRAPULMONARY BRONCHI	G1	G2	G3
	24.4	36.3	44.0
SMALL BRONCHI	G1	G2	G3
	23.8	34.4,	52.6
BRONCHIOLI*	G1	G2	G3
	13.0	24.9	43.3

* = AT 1% AS WELL AS 5% PROTECTION LEVEL

APPENDIX 4.9: COMPARATIVE ANALYSIS OF THE SUBMUCOSAL THICKNESS BETWEEN NORMAL LUNGS, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

	THICKNESS IN MICROMETRES(um)					
AIRWAY LEVEL	NORMAL	EARLY PNEUMONIA	ADVANCED PNEUMONIA	SE	F-R	P<
UPPER TRACHEA	415.5	517.9	661.8	70.5	3.2	NS
LOWER TRACHEA	246.4	349.7	333.5	56.6	1.1	NS
EP BRONCHI	163.0	256.2	261.6	39.6	1.8	NS
IP BRONCHI	106.9	187.9	234.2	24.1	6.2	0.05
SMALL BRONCHI	51.7	84.0	133.5	15.6	8.1	0.01
BRONCHIOLI	15.6	34.1	62.0	8.6	10.8	0.01

APPENDIX 4.10: DUNCAN'S STATISTICAL ANALYSIS OF THE SUBMUCOSAL THICKNESS AT DIFFERENT LEVELS OF NORMAL LUNGS, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

UPPER TRACHEA	G1	G2	G3
	415.5	517.9	661.8
LOWER TRACHEA	G1	G3	G2
	246.3	333.5	349.7
EXTRAPULMONARY BRONCHI	G1	G2	G3
	163.0	256.2	261.5
INTRAPULMONARY BRONCHI	G1	G2	G3
	106.9	187.9	234.2
SMALL BRONCHI	G1	G2	G3
	51.7	84.0	133.5
BRONCHIOLI*	G1	G2	G3
	15.6	34.1	62.0

* = AT 1% AND 5% PROTECTION LEVEL

APPENDIX 4.11: COMPARATIVE STATISTICAL ANALYSIS OF THE MUCOSAL THICKNESS BETWEEN NORMAL LUNGS, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

	THICKNESS IN MICROMETRES(um)					
AIRWAY LEVEL	NORMAL	EARLY PNEUMONIA	ADVANCED PNEUMONIA	SE	F-R	P<
UPPER TRACHEA	456.8	594.5	716.8	72.8	3.1	NS
LOWER TRACHEA	280.1	389.6	369.7	56.5	1.0	NS
EP BRONCHI	190.6	291.5	289.0	42.4	2.0	NS
IP BRONCHI	131.2	224.0	278.1	29.8	7.1	0.01
SMALL BRONCHI	75.5	118.4	186.1	19.6	7.0	0.01
BRONCHIOLI	28.6	58.4	105.3	11.8	7.3	0.01

APPENDIX 4.12: DUNCAN'S STATISTICAL ANALYSIS OF THE MUCOSAL THICKNESS AT DIFFERENT LEVELS OF NORMAL LUNGS, EARLY PNEUMONIC AND ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

UPPER TRACHEA	G1	G2	G3
	456.6	594.5	716.8
LOWER TRACHEA	G1	G3	G2
	290.1	369.7	389.6
EXTRAPULMONARY BRONCHI	G1	G3	G2
	190.6	289.0	291.5
INTRAPULMONARY BRONCHI	G1	G2	G3
	131.2	224.0	279.1
SMALL BRONCHI*	G1	G2	G3
	75.5	118.4	186.1
BRONCHIOLI*	G1	G2	G3
	28.6	68.4	105.3

* = AT 1% AND 5% PROTECTION LEVEL

APPENDIX 4.13: PERCENTAGE INCREASE IN THICKNESS OF THE TRACHEOBRONCHIAL MUCOSAL LAYERS OF EARLY AND ADVANCED PNEUMONIC LESIONS FROM NORMAL SHEEP LUNGS

AIRWAY LEVEL	PERCENTAGE INCREASE IN THE THICKNESS					
	EPITHELIUM		SUBMUCOSA		MUCOSA	
	EARLY	ADVANCED	EARLY	ADVANCED	EARLY	ADVANCED
UPPER TRACHEA	81.6	31.5	24.6	59.3	30.1	56.9
LOWER TRACHEA	20.4	7.4	41.9	35.3	39.1	30.0
EP BRONCHI	27.9	00.0	57.3	60.5	53.0	51.7
IP BRONCHI	48.8	79.9	75.9	119.3	70.7	111.9
SMALL BRONCHI	46.2	121.0	62.2	157.7	56.8	146.5
BRONCHIOLI	93.0	235.6	118.6	298.7	104.2	268.2

APPENDIX 4.14: SUBMUCOSAL GLAND PARAMETERS AT DIFFERENT LEVELS OF NORMAL SHEEP TRACHEOBRONCHIAL AIRWAYS

PARAMETERS	TRACHEOBRONCHIAL LEVELS						
	L1	L2	L3	L4	SE	F-R	P<
NUMBER OF ACINI	10.2	7.9	5.2	4.5	0.7	11.98	0.001
TOTAL GLAND AREA (um^2)	43263	31028	20014	17340	4042	856	0.01
TOTAL ACINAR AREA (um^2)	25130	20522	12961	11753	2395	702	0.01
NON-ACINAR AREA (um^2)	18132	10555	7052	6392	1760	936	0.01
SINGLE ACINAR AREA (um^2)	2447	2558	2484	2554	1387	0.20	NS
GLAND AREA:ACINAR AREA	18:1	1.6:1	1.6:1	1.6:1	0.025	3.60	NS

APPENDIX 4.15: DUNCAN'S STATISTICAL ANALYSIS OF THE SUBMUCOSAL GLANDS OF NORMAL SHEEP LUNGS

AT 5% PROTECTION LEVEL

NUMBER OF ACINI	L1	L2	L3	L4
	10.2	7.9	5.2	4.5
TOTAL GLAND AREA	L1	L2	L3	L4
	43.3	31.0	20.0	17.3
TOTAL ACINAR AREA	L1	L2	L3	L4
	25.1	20.5	12.9	11.7
NON-ACINAR AREA	L1	L2	L3	L4
	18.1	10.6	7.0	6.4
SINGLE ACINUS AREA	L1	L2	L3	L4
	2.6	2.6	2.5	2.4
GLAND AREA:ACINAR AREA RATIO	L1	L2	L3	L4
	1.8	1.6	1.6	1.6

AT 1% PROTECTION LEVEL

NUMBER OF ACINI	L1	L2	L3	L4
	10.2	7.9	5.2	4.6
TOTAL GLAND AREA	L1	L2	L3	L4
	43.3	31.0	20.0	17.3
TOTAL ACINAR AREA	L1	L2	L3	L4
	25.1	20.5	12.9	11.7
NON-ACINAR AREA	L1	L2	L3	L4
	18.1	10.6	7.0	6.4
SINGLE ACINUS AREA	L1	L2	L3	L4
	2.6	2.6	2.5	2.4
GLAND AREA:ACINAR AREA RATIO	L1	L2	L3	L4
	1.8	1.6	1.6	1.6

APPENDIX 4.16: THE SUBMUCOSAL GLAND PARAMETERS AT DIFFERENT LEVELS OF EARLY PNEUMONIC LUNG LESIONS

PARAMETERS	TRACHEOBRONCHIAL LEVELS					
	L1	L2	L3	L4	SE	F-R
NUMBER OF ACINI	8.4	9.4	7.9	7.8	1.0	0.5
TOTAL GLAND AREA (um^2)	34625	36290	26191	32219	5809	06
TOTAL ACINAR AREA (um^2)	19201	23585	16405	18953	3469	07
NON-ACINAR AREA (um^2)	15424	12730	9786	13091	2869	06
SINGLE ACINAR AREA (um^2)	2252	2535	2027	2481	306	06
GLAND AREA:ACINAR AREA RATIO	18:1	1.5:1	1.7:1	1.7:1	0.16	1.1

APPENDIX 4.17: SUBMUCOSAL GLAND PARAMETERS AT DIFFERENT LEVELS OF ADVANCED PNEUMONIC LUNG LESIONS

PARAMETERS	TRACHEOBRONCHIAL LEVELS L1	L2	L3	L4	SE	F-R	P<
NUMBER OF ACINI	12.5	9.5	8.4	6.0	1.3	5.6	0.01
TOTAL GLAND AREA (um^2)	61579	44295	31812	48593	10566	13	NS
TOTAL ACINAR AREA (um^2)	35045	27851	17716	29717	6141	14	NS
NON-ACINAR AREA (um^2)	26533	16218	14292	18876	4627	13	NS
SINGLE ACINAR AREA (um^2)	2709	2997	2141	4893	689	30	NS*
GLAND AREA: ACINAR AREA RATIO	18:1	1.6:1	1.7:1	1.6:1	0.08	1.5	NS

* = P < 007

APPENDIX 4.18: DUNCAN'S STATISTICAL ANALYSIS OF THE SUBMUCOSAL GLAND PARAMETERS AT DIFFERENT LEVELS OF ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

NUMBER OF ACINI	L1	L2	L3	L4
	12.5	9.5	8.4	6.1
SINGLE ACINAR AREA	L4	L2	L1	L3
	4.9	3.0	2.7	2.1

AT 1% PROTECTION LEVEL

NUMBER OF ACINI	L1	L2	L3	L4
	12.5	9.5	8.4	6.1

APPENDIX 4.19: COMPARATIVE STATISTICAL ANALYSIS OF THE UPPER TRACHEAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND EARLY PNEUMONIC LESIONS

PARAMETERS	NORMAL	EARLY PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	9.7	7.9	0.8	2.2	NS
TOTAL GLAND AREA (um^2)	40611	34560	3625	14	NS
TOTAL ACINAR AREA (um^2)	23821	17944	2451	28	NS
NON-ACINAR AREA (um^2)	16789	15616	2184	03	NS
SINGLE ACINAR AREA (um^2)	2428	2209	88	31	NS
GLAND AREA: ACINAR AREA RATIO	17:1	1.9:1	0.2:1	2.1	NS

APPENDIX 4.20: COMPARATIVE STATISTICAL ANALYSIS OF THE LOWER TRACHEAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND EARLY PNEUMONIC LESIONS

PARAMETERS	NORMAL	EARLY PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	7.9	9.2	1.3	0.7	NS
TOTAL GLAND AREA (um^2)	31028	36290	7529	02	NS
TOTAL ACINAR AREA (um^2)	20522	23585	4098	02	NS
NON-ACINAR AREA (um^2)	10555	12730	3600	02	NS
SINGLE ACINAR AREA (um^2)	2558	2535	247	04	NS
GLAND AREA: ACINAR AREA RATIO	15:1	1.5:1	0.08	00	NS

APPENDIX 4.21: COMPARATIVE STATISTICAL ANALYSIS OF THE EXTRAPULMONARY BRONCHIAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND EARLY PNEUMONIC LESIONS

PARAMETERS	NORMAL	EARLY PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	5.2	7.9	0.6	10.5	0.01
TOTAL GLAND AREA (um^2)	20014	26191	3763	13	NS
TOTAL ACINAR AREA (um^2)	12961	16405	2632	08	NS
NON-ACINAR AREA (um^2)	7052	9786	1257	23	NS
SINGLE ACINAR AREA (um^2)	2485	2027	252	16	NS
GLAND AREA: ACINAR AREA RATIO	16:1	1.7:1	0.1	0.5	NS

APPENDIX 4.22: COMPARATIVE STATISTICAL ANALYSIS OF THE INTRAPULMONARY BRONCHI SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND EARLY PNEUMONIC LESIONS

PARAMETERS	NORMAL	EARLY PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	4.5	7.8	0.6	13.4	0.01
TOTAL GLAND AREA (um^2)	17340	32219	3645	83	0.5
TOTAL ACINAR AREA (um^2)	11753	18953	2131	57	0.05
NON-ACINAR AREA (um^2)	6392	13091	1691	79	0.05
SINGLE ACINAR AREA (um^2)	2554	2481	300	0.03	NS
GLAND AREA: ACINAR AREA RATIO	16:1	1.7:1	0.03	6.8	0.05

APPENDIX 4.23: COMPARATIVE STATISTICAL ANALYSIS OF THE UPPER TRACHEAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND ADVANCED PNEUMONIC LESIONS

PARAMETERS	NORMAL	ADVANCED PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	9.7	11.8	1.4	1.0	NS
TOTAL GLAND AREA (um^2)	40611	56801	9696	14	NS
TOTAL ACINAR AREA (um^2)	23821	31617	5799	09	NS
NON-ACINAR AREA (um^2)	16789	25183	4114	21	NS
SINGLE ACINAR AREA (um^2)	2428	2569	236	08	NS
GLAND AREA:ACINAR AREA RATIO	17:1	1.9:1	0.1	1.7	NS

APPENDIX 4.24: COMPARATIVE STATISTICAL ANALYSIS OF THE LOWER TRACHEAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND ADVANCED PNEUMONIC LESIONS

PARAMETERS	NORMAL	ADVANCED PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	7.9	9.5	0.9	1.6	NS
TOTAL GLAND AREA (um^2)	31028	44295	5022	35	NS
TOTAL ACINAR AREA (um^2)	20522	27851	2939	31	NS
NON-ACINAR AREA (um^2)	10555	16218	2345	29	NS
SINGLE ACINAR AREA (um^2)	2558	2997	325	09	NS
GLAND AREA:ACINAR AREA RATIO	15:1	1.6:1	0.06	0.3	NS

APPENDIX 4.25: COMPARATIVE STATISTICAL ANALYSIS OF THE EXTRAPULMONARY BRONCHIAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND ADVANCED PNEUMONIC LESIONS

PARAMETERS	NORMAL	ADVANCED, PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	5.2	8.4	0.3	64.7	0.001
TOTAL GLAND AREA (um^2)	20014	31912	3121	72	0.05
TOTAL ACINAR AREA (um^2)	12961	17716	1881	32	NS
NON-ACINAR AREA (um^2)	7052	14292	1487	118	0.01
SINGLE ACINAR AREA (um^2)	2485	2141	170	20	NS
GLAND AREA:ACINAR AREA RATIO	16:1	1.7:1	0.06	0.6	NS

APPENDIX 4.26: COMPARATIVE STATISTICAL ANALYSIS OF THE INTRAPULMONARY BRONCHIAL SUBMUCOSAL GLANDS BETWEEN NORMAL LUNGS AND ADVANCED PNEUMONIC LESIONS

PARAMETERS	NORMAL	ADVANCED PNEUMONIA	SE	F-R	P<
NUMBER OF ACINI	4.5	6.0	0.3	12.7	0.01
TOTAL GLAND AREA (um2)	17340	48593	9445	55	NS*
TOTAL ACINAR AREA (um2)	11753	29717	5467	54	NS*
NON-ACINAR AREA (um2)	6392	18876	4013	48	NS*
SINGLE ACINAR AREA (um2)	2554	4893	881	35	NS
GLAND AREA:ACINAR AREA RATIO	16:1	1.6:1	0.03	1.0	NS

*=0.07

APPENDIX 5.1

ALCIAN BLUE pH 2.6-PAS

SOLUTIONS

ALCIAN BLUE

Alcian blue	1 gm
Acetic acid	3 ml
Distilled water	97 ml

1% PERIODIC ACID

Periodic acid	1 gm
Distilled water	100 ml

SCHIFF'S REAGENT

Basic fuchsin	1 gm
N Hydrochloric acid	20 ml
Sodium metabisulphite	1 gm
Actived charcoal	2 gm
Distilled water	200 ml

METHOD

A) Place sections in xylol and then down to water.
B) Stain in Alcian blue solution for 5 min.
C) Wash well in tap water.
D) Treat with 1% periodic acid for 5 min.
E) Wash in distilled water.
F) Place in Schiff's reagent for 8 min.
G) Wash in running tap water for 10 min.
H) Dehydrate through graded alcohols to xylol.
I) Mount in DPX.

RESULTS

Acid mucopolysaccharids	Blue
Neutral mucins	Magenta
Mixtures of acid and neutral mucins	Purple

APPENDIX 5.2 DUNCAN'S STATISTICAL ANALYSIS OF THE PROPORTION OF DIFFERENT TYPES OF GLYCOPROTEIN AT VARIOUS TRACHEOBRONCHIAL LEVEL OF NORMAL SHEEP LUNGS

AT 5% PROTECTION LEVEL

L1	B	BR	R	P	RB
	0.1	2.8	6.3	32.1	58.7
L2	B	R	BR	P	RB
	1.1	4.2	4.7	27.4	62.7
L3	B	BR	R	P	RB
	0.3	0.7	3.9	22.4	72.8
L4	B	BR	R	P	RB
	0.05	0.6	3.4	19.3	76.7

AT 1% PROTECTION LEVEL

L1	B	BR	R	P	RB
	0.1	2.8	6.3	32.1	58.7
L2	B	R	BR	P	RB
	1.1	4.2	4.7	27.4	62.7
L3	B	BR	R	P	RB
	0.3	0.7	3.9	22.4	72.8
L4	B	BR	R	P	RB
	0.05	0.6	3.4	19.3	76.7

APPENDIX 5.3: HISTOCHEMICAL STAINING OF SUBMUCOSAL GLAND MUCOUS CELLS OF NORMAL SHEEP ASSESSED BY AREA

HISTO-CHEMICAL STAIN	PERCENTAGE OF TOTAL GLAND AREA STAINED							
	UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
BLUE	0.1	1.1	0.3	0.05	0.4	0.4	2.0	NS
BLUE-RED	2.8	4.7	0.7	0.6	2.2	1.4	2.1	NS
RED	6.3	4.2	3.9	3.4	4.5	1.3	0.9	NS
RED-BLUE	58.7	62.7	72.8	76.7	67.7	2.3	14.0	0.001
PURPLE	32.1	27.4	22.4	19.3	25.3	2.4	5.5	0.01

APPENDIX 5.4: DUNCAN'S STATISTICAL ANALYSIS OF THE PROPORTION OF EACH GLYCOPROTEIN AT DIFFERENT TRACHEOBRONCHIAL LEVELS IN NORMAL SHEEP LUNGS

AT 5% PROTECTION LEVEL

BLUE	L4	L1	L3	L2
	0.05	0.1	0.3	1.1
BLUE-RED	L4	L3	L1	L2
	0.6	0.7	2.8	4.7
RED	L4	L3	L2	L1
	3.4	3.9	4.2	6.3
RED-BLUE	L1	L2	L3	L4
	58.7	62.7	72.8	76.7
PURPLE	L4	L3	L2	L1
	19.3	22.4	27.4	32.1
ACID	L4	L3	L1	L2
	0.6	1.0	2.9	5.8
NEUTRAL	L1	L2	L3	L4
	65.1	66.8	76.6	80.1
MIXED	L4	L3	L2	L1
	19.3	22.4	27.4	32.1

APPENDIX 5.5: GLYCOPROTEIN TYPES OF SUBMUCOSAL GLAND MUCOUS CELLS OF NORMAL SHEEP LUNGS ASSESSED BY PERCENTAGE OF TOTAL COUNT

TYPES OF G.P	PERCENTAGE OF TOTAL GLAND AREA STAINED — UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
ACID	2.9	5.8	1.0	0.6	2.6	1.7	2.1	NS
NEUTRAL	65.1	66.8	76.6	80.1	72.2	2.4	9.3	0.01
MIXED	32.1	27.4	22.4	19.3	25.3	2.4	5.5	0.01

APPENDIX 5.6 DUNCAN'S STATISTICAL ANALYSES OF THE PROPORTION OF THE DIFFERENT TYPES OF GLYCOPROTEIN AT VARIOUS TRACHEOBRONCHIAL LEVELS IN EARLY PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

L1	B	BR	R	P	RB
	1.6	3.0	11.1	33.9	50.4
L2	B	BR	R	P	RB
	1.0	9.2	10.3	18.8	60.8
L3	B	BR	R	P	RB
	0.8	2.0	17.8	24.6	54.7
L4	B	BR	R	P	RB
	0.6	1.2	18.0	29.6	50.6

AT 1% PROTECTION LEVEL

L1	B	BR	R	P	RB
	1.6	3.0	11.1	33.9	50.4
L2	B	BR	R	P	RB
	1.0	9.2	10.3	18.4	60.8
L3	B	BR	R	P	RB
	0.8	2.0	17.8	24.6	54.7
L4	B	BR	R	P	RB
	0.6	1.2	18.0	29.6	50.6

APPENDIX 5.7: HISTOCHEMICAL STAINING OF SUBMUCOSAL GLAND MUCOUS CELLS ON EARLY PNEUMONIC LESIONS ASSESSED BY AREA

HISTOCHEMICAL STAIN	PERCENTAGE OF TOTAL GLAND AREA STAINED — UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
BLUE	1.6	1.0	0.8	0.6	1.0	0.9	0.2	NS
BLUE-RED	3.0	9.2	2.0	1.2	3.9	2.9	1.6	NS
RED	11.1	10.3	17.8	18.0	15.6	11.2	0.1	NS
RED-BLUE	50.4	60.8	54.7	50.6	54.1	14.0	0.1	NS
PURPLE	33.9	18.8	24.6	29.6	26.7	5.9	1.2	NS

APPENDIX 5.8: GLYCOPROTEIN TYPES OF SUBMUCOSAL GLAND MUCOUS CELLS ON EARLY PNEUMONIC LESIONS ASSESSED BY PERCENTAGE OF TOTAL COUNT

TYPES OF G.P	PERCENTAGE OF TOTAL GLAND AREA STAINED — UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
ACID	4.5	10.2	2.9	1.8	4.8	3.1	1.5	NS
NEUTRAL	61.6	71.0	72.5	68.6	69.7	6.3	1.0	NS
MIXED	33.9	18.8	24.6	29.6	26.7	5.9	1.2	NS

APPENDIX 5.9: DUNCAN'S STATISTICAL ANALYSES OF THE PROPORTION OF THE DIFFERENT TYPES OF GLYCOPROTEIN AT VARIOUS TRACHEOBRONCHIAL LEVEL IN ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

L1	R	BR	B	P	RB
	2.4	7.0	7.8	27.2	55.6
L2	R	BR	B	P	RB
	3.3	4.3	6.7	26.3	59.6
L3	B	R	BR	P	RB
	2.0	2.0	3.0	23.0	70.0
L3	R	B	BR	P	RB
	1.8	2.8	3.7	31.1	60.6

AT 1% PROTECTION LEVEL

L1	R	BR	B	P	RB
	2.4	7.0	7.8	27.2	55.6
L2	R	BR	B	P	RB
	3.3	4.3	6.7	26.3	59.6
L3	B	R	BR	P	RB
	2.0	2.0	3.0	23.0	70.3
L4	R	B	BR	P	RB
	1.8	2.8	3.7	31.1	60.6

APPENDIX 5.10: HISTOCHEMICAL STAINING OF SUBMUCOSAL GLAND MUCOUS CELLS IN ADVANCED PNEUMONIC LESIONS ASSESSED BY PERCENTAGE OF TOTAL COUNT

HISTO-CHEMICAL STAIN	PERCENTAGE OF TOTAL GLAND AREA STAINED: UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
BLUE	7.8	6.7	2.0	2.8	4.9	4.9	0.3	NS
BLUE-RED	7.0	4.3	3.0	3.7	4.5	2.0	0.8	NS
RED	2.4	3.3	2.0	1.8	2.5	0.6	1.1	NS
RED-BLUE	55.6	59.6	70.0	60.0	61.5	8.0	0.6	NS
PURPLE	27.2	26.3	23.0	31.1	27.0	4.2	0.6	NS

APPENDIX 5.11: GLYCOPROTEIN TYPES OF SUBMUCOSAL GLAND MUCOUS CELLS IN ADVANCED PNEUMONIC LESIONS ASSESSED BY PERCENTAGE OF TOTAL COUNT

TYPES OF G.P	PERCENTAGE OF TOTAL GLAND AREA STAINED: UPPER TRACHEA	LOWER TRACHEA	EP BRONCHI	IP BRONCHI	MEAN VALUE	S.E	F-R	P<
ACID	14.8	10.9	5.0	6.5	9.3	6.4	0.6	NS
NEUTRAL	58.0	62.8	72.0	62.4	64.0	8.0	0.5	NS
MIXED	27.2	26.3	23.0	31.1	27.0	4.2	0.6	NS

APPENDIX 5.12: COMPARATIVE STATISTICAL ANALYSIS OF THE HISTOCHEMICAL STAINING OF MUCOUS CELLS OF THE SUBMUCOSAL GLANDS FROM NORMAL LUNGS, EARLY AND ADVANCED PNEUMONIC LESIONS

AIRWAY LEVEL	HISTO CHEMICAL STAIN	PERCENTAGE OF TOTAL GLAND AREA STAINED: NORMAL	EARLY PNEUMONIA	LATE PNEUMONIA	S.E	F-R	P<
UPPER TRACHEA	BLUE	0.1	1.6	7.8	3.4	0.9	NS
	BLUE-RED	2.8	3.0	7.0	2.3	1.1	NS
	RED	6.3	11.1	2.4	2.4	3.2	NS
	RED-BLUE	58.7	50.4	55.6	8.0	0.3	NS
	PURPLE	32.1	33.9	27.2	5.1	0.5	NS
LOWER TRACHEA	BLUE	1.1	1.0	6.7	3.2	1.0	NS
	BLUE-RED	4.7	9.2	4.2	3.4	0.7	NS
	RED	4.2	15.6	3.3	5.1	1.8	NS
	RED-BLUE	62.7	60.8	59.6	10.4	0.02	NS
	PURPLE	27.4	18.8	26.3	4.8	1.0	NS
EXTRA-PULMONARY BRONCHI	BLUE	0.3	0.8	2.1	1.1	0.7	NS
	BLUE-RED	0.7	2.2	3.0	1.2	1.0	NS
	RED	3.9	17.8	2.4	9.0	0.9	NS
	RED-BLUE	72.8	54.7	70.3	10.2	0.9	NS
	PURPLE	22.4	24.7	23.0	4.2	0.1	NS
INTRA-PULMONARY BRONCHI	BLUE	0.05	0.6	2.8	1.4	1.2	NS
	BLUE-RED	0.6	1.2	3.7	0.8	4.8	0.05
	RED	3.5	18.0	1.8	7.5	1.4	NS
	RED-BLUE	76.7	50.6	60.5	8.9	2.2	NS
	PURPLE	19.3	29.6	31.1	3.4	3.5	NS

APPENDIX 5.13: COMPARATIVE STATISTICAL ANALYSIS OF THE PROPORTION OF GLYCOPROTEIN TYPES IN MUCOUS CELLS OF THE SUBMUCOSAL GLANDS FROM NORMAL LUNGS, EARLY AND ADVANCED PNEUMONIC LESIONS

AIRWAY LEVEL	TYPES OF G.P	PERCENTAGE OF TOTAL GLAND AREA STAINED: NORMAL	EARLY PNEUMONIA	LATE PNEUMONIA	S.E	F-R	P<
UPPER TRACHEA	ACID	2.9	4.5	14.8	6.5	1.0	NS
	NEUTRAL	65.0	61.5	58.0	7.4	0.2	NS
	MIXED	32.1	33.9	27.2	5.1	0.5	NS
LOWER TRACHEA	ACID	5.8	10.2	10.9	4.9	0.3	NS
	NEUTRAL	66.8	76.3	62.8	7.6	0.8	NS
	MIXED	27.4	18.8	26.5	4.8	1.0	NS
EXTRA-PULMONARY BRONCHI	ACID	1.0	3.0	5.1	1.5	2.0	NS
	NEUTRAL	76.6	72.5	72.7	4.2	0.3	NS
	MIXED	22.4	24.7	23.0	4.2	0.1	NS
INTRA-PULMONARY BRONCHI	ACID	0.6	1.8	6.5	1.5	4.5	0.05
	NEUTRAL	80.2	68.6	62.4	10.0	5.1	0.05
	MIXED	19.3	29.6	31.1	3.4	3.5	NS

APPENDIX 5.14 : DUNCAN'S STATISTICAL ANALYSIS OF THE COMPARISON OF THE TOTAL NUMBER OF ACTIVE CELLS PER LEVEL BETWEEN NORMAL LUNGS EARLY AND ADVANCED PNEUMONIC LESIONS

AT 5% PROTECTION LEVEL

MIXED	G1	G2	G3
	19.3	29.6	31.1
ACID	G1	G2	G3
	0.6	1.8	6.5
NEUTRAL	G3	G2	G1
	62.4	68.6	80.2

APPENDIX 6.1

LIQUID MODIFIED FM4 MEDIUM (Frey; et al. (1968)

NaCl	5.00 gm
KCl	0.40 gm
$MgSO_4 \cdot 7H_2O$	0.20 gm
$NaPHO_4$	4.03 gm
Glucose	10.00 gm
Phyto-Peptone	10.00 gm
NAD	0.10 gm
L-Cysteine HCP	0.10 gm
Eagle's vitamin soln x 100 (see below)	25.00 ml
0.4% phenol red	2.50 ml
Penicillin	10^6.00 units
1.0M NaOH to pH	7.80
Distilled water	1000.00 ml

NOTES ON PREPARATION

1) The medium was clarified by filtration through non sterile o.45 um and 0.22 um pore size filters and subsequently sterilized by filtration through a sterile 0.22 um filter.
2) The vitamin supplement used (Eagle's Vitamin Solution x 100) was as follows:

D-Biotin	20 mg
Calcium pantothenate	20 mg
Choline chloride	20 mg
Folic acid	20 mg
Riboflavin	2 mg
Myo-Iodositol	40 mg
Niacinamide	20 mg
Pyridoxine	20 mg
Thiamine	20 mg
Distilled water	200 ml

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

APPENDIX 6.2

GRAM-TWORT METHOD

SOLUTIONS

TWORT'S STAIN

0.2% absolute alcoholic neutral red	9 ml
0.2% absolute alcoholic fast green FCF	1 ml
Distilled water	30 ml

CRYSTAL VIOLET

Crytal violet	2 gm
95% alcohol	20 ml
Ammonium oxalate	1 gm
Distilled water	80 ml

GRAM'S IODINE

Iodine crystals	1 gm
Potassium iodide	2 gm
Distilled water	300 ml

METHOD

A) Place sections in xylol, then down to water.
B) Stain in crystal violet solution for 3 min.
C) Rinse in running tap water.
D) Treat with Gram's iodine for 3 min.
E) Rinse in tap water.
F) Decolourise in 2% acetic acid in absolute alcohol until no more colour comes away.
G) Rinse rapidly in distilled water.
H) Counter stain in modified Twort's stain for 5 min.
I) Differentiate in 2% acetic acid in absolute alcohol until no more stain comes from the section.
J) Clear in xylol and mount in DPX.