

**THE MORPHOLOGY AND TRANSPORT OF MUCUS
IN MAMMALIAN AIRWAYS**

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ABSTRACT

The mucociliary clearance mechanisms in mammalian pulmonary airways have been re-examined. In this investigation Wistar rats, housed both under specific pathogen free (SPF) and normal animal house conditions, were examined. An intact airway system from the trachea down to the level of the terminal bronchioles was used. The airway preparation was rapidly isolated and examined under carefully controlled *in vitro* conditions. Specimens remained viable for at least 10 h. Mucociliary activity was observed through the intact bronchial wall with the aid of incident light. This function could be examined at all levels of the pulmonary tree in the same specimen. In contrast to the previously described presence of a continuous mucous blanket, the morphology of mucus in the airways of the rat has been shown to be discontinuous. Mucus is present as discrete particles of varying size. Under the light microscope these particles appeared to fall into three categories: droplets less than 4 μm in diameter; flakes 10–70 μm in diameter; and plaques which are conglomerations of droplets and flakes. Scanning electron microscopy reveals that droplets (i.e. single particles) may be as small as 0.5 μm and that composite particles made up of numerous aggregated droplets may be as small as 5 μm in diameter. Plaques are conglomerations of these particles. In the normal intrapulmonary airways only smaller particles are seen and are transported over the individual metachronal fields. In the larger extralobar airways these particles move together to be transported in well defined streams which may be up to 500 μm wide. These streams may follow a winding course up the trachea and more than one may be in operation at a time. Under conditions of hypersecretion such as occurs with chronic respiratory disease in rats (CRD) the number of particles increase peripherally and plaques may be found in small airways. The transport of mucus is however still intermittent and it never becomes confluent. Acute bronchitis results in wide-spread abnormalities of ciliary activity and mucus transport, which leads to total disorganisation of pulmonary clearance. "Chronic bronchitis" associated with CRD results in more organised abnormalities of mucociliary activity. Cilia may become inactive, reverse the direction of their effective stroke, beat retrogradely, and exhibit abnormal beat patterns which result in impaired mucus clearance. Squamous metaplastic areas further impede mucus transport. In general mucus transport rates were found to be faster in rats with "chronic bronchitis" than SPF rats, provided that the extent of the damage to the mucous membrane was

not too great in the "bronchitic" animal. This finding was confirmed by the examination of airway preparations approximately 19 h after the exposure to a charcoal aerosol. While significant amounts of charcoal were retained at the bifurcations of bronchi in SPF rats, most of the charcoal was cleared in "bronchitic rats". The only areas where particles were seen were on bronchitic patches or on whirlpools.

The findings of this study indicated that mucus was present in a discontinuous form, and that in both SPF and non-SPF animals no evidence for a mucous blanket was found.

PREFACE

I am indebted to my friend, Professor Joseph Iravani for introducing me to the study of pulmonary clearance mechanisms, and teaching me to master *in vitro* experimental techniques.

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CHAPTER 1

INTRODUCTION

The propulsion of mucus in the bronchi as a continuous sheet or blanket is a concept which has been proposed for many years and is to be found as a standard statement in many textbooks dealing with this subject. With the renewal of interest in tracheobronchial clearance mechanisms over the past twenty years, many factors influencing this function have been studied. These have included industrial and atmospheric pollutants, cigarette smoke and its various components as well as many other gases and dusts. A universal conclusion is that mucus serves an important function in lining the airways and protecting the underlying bronchial mucosa from harm. The term "mucous blanket" was conceived by Lucas and Douglas (1934) to describe their two-layer concept of the material lining the airways. This concept and term has become fixed in usage and in this thesis "mucous blanket" will be used when discussing the material lining the airways as envisaged by the above and subsequent authors. Particulate matter deposited on this mucous blanket is transported to the larynx and is thus eliminated from the lungs. This concept has governed the experimental design of most investigations concerning factors which alter mucus flow rate in the airways. Marker substances have usually been placed on the mucosa in the trachea and their rate of movement timed by direct observation. This rate of transport has been taken to indicate the flow rate of the mucous blanket.

However, recently several inconsistencies in the mucous blanket concept have been pointed out. Firstly, it has been suggested that the volume of mucus present in the very large area of the peripheral airways cannot be accommodated in the trachea, unless a very large increase in the gradient of transport velocity is present between these two areas (Kilburn 1968). Experimentally this transport velocity gradient has variously been shown to be fourfold (Barclay & Franklin 1937), and eight to fourteenfold (Asmundsson & Kilburn 1970). Neither of these gradients is great enough to accommodate a mucous blanket of uniform depth in the airways. Secondly, another aspect which needs clarification is the method whereby a continuous mucous blanket negotiates the natural obstructions occurring in the bronchi. These obstructions consist of the orifices of bronchial tributaries opening into the large airways, as well as naturally or pathologically occurring areas of squamous metaplasia. A significant portion of the bronchial diameter may therefore be unavailable for mucus transport in certain bronchi (Hilding 1957b). Thus the mucous blanket will have to divide to negotiate a path around these obstructions.

None of these inconsistencies have been satisfactorily explained and therefore the need has arisen to examine the secretion and transport of mucus in the pulmonary airways once again.

The development of a new *in vitro* system for the study of the mammalian pulmonary ciliated epithelium (Iravani 1969; Iravani and Van As 1972) overcame many of the difficulties experienced by earlier authors working in this field. A more precise observation of ciliary activity was made possible which resulted in the exact appreciation of the form of ciliary metachronism in pulmonary airways. Following on this a re-appraisal of the secretion and transport of mucus in the entire intact airway system could be made. The distribution and morphology of mucus was also examined by scanning electron microscopic techniques. The purpose of this study was to re-examine the morphology of mucus in the airways both *in vitro* and with scanning electron microscopic techniques. The purpose of this study was to re-examine the morphology of mucus in the airways both *in vitro* and with scanning electron microscopic techniques.

Some of the material presented in this communication is based on previously published work. (Iravani and van As, 1972; Van As and Webster, 1972; Van As and Hill, 1972; Van As and Wessels, 1972; Van As and Webster, 1974).

The nature of the method employed to gather the data for this thesis involved a sensitive and lengthy dissection technique, which, even under optimal conditions resulted in a failure rate of up to 60% of the animals prepared for observation. The preparation of a specimen took from 1–2,5 h depending on prevailing conditions. Similarly the preparation of tissues for scanning electron microscopy was time consuming as was the period spent inspecting the surface morphology. Therefore the total numbers of experiments described are small and do not lend themselves to statistical analyses or comparisons. More reliance was thus placed on an accurate description of the morphological observations made under different conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 CILIARY ACTIVITY

The first description of cilia is attributed to de Heide in 1684 by both Sleight (1962) and Rivera (1962), although Leeuwenhoek was probably the first person to observe them in 1677 (Sleight 1962) when he described "the incredibly thin feet or little legs by means of which a small . . . animalcule moved through the water" (in Sleight, 1962). The term cilia was coined by F.O. Muller in 1786 (Sleight 1962). Since that time much information has accumulated on the behaviour and structure of cilia in a wide range of animals and plants. Purkinje and Valentin (Sharpey 1836) and Sharpey (1830 and 1836) wrote the first reviews about cilia and described their presence in most of the main animal groups. Sharpey made a most notable contribution when he suggested that cilia "contain muscular substance throughout a greater or less part of their length by which they can be bent or extended". Gray (1928) summarised most of the early work on cilia in a monograph and, in addition, over a period of nearly 30 years made an important contribution to this field. His works have included observations on the mechanism of ciliary movement and the effect of various ions, oxygen and temperature on this mechanism. Two further reviews appeared almost simultaneously, one by Rivera (1962) and the other by Sleight (1962). These authors review in some detail the developments in the understanding of the structure, function, comparative anatomy, physiology, and to a limited extent, pharmacology of cilia and flagellae. It is of interest that these works indicate that the majority of investigations have been carried out on protozoa (*Opilana*, *Stentor*) Ctenophores, metazoa (*Mytilus*) to mention the most frequently studied species. However, attention has been paid to many phyla of the animal and plant kingdoms. In a very comprehensive review by Kinoshita and Murakami (1967) all known factors governing ciliary and flagellar movement were discussed. Two recent symposia have updated knowledge on ciliary activity. The first, in a symposium on structure, function and measurement of respiratory cilia (*Am. Rev. resp. Dis.*, 93: March 1966, part 2) the accent was placed on the evaluation of the present day knowledge of ciliated cells. Apart from reports of the effect of various chemical and physical factors or dusts on the ciliated epithelium, no new advances in the understanding of basic principle were reported in the symposium. The second symposium, a workshop for the evaluation of model systems to assess the

pulmonary responses to inhaled materials (Archs intern. Med., 126: September 1970) once again only reviewed existing methods up to that time for measuring mucociliary activity. The *in vivo* methods of Dalhamn (1970) reported previously (Dalhamn 1955, 1956, 1960) and the tracheal pouch model of Wardell *et al.* (1970) also reported earlier (Wardell and Chakrin 1970) were discussed.

The field of ciliary activity has been extensively discussed (in the reviews named above) and mention will thus be made of aspects which are conceptually recent developments.

Metachronal co-ordination of ciliary beat is a widely observed phenomenon and may take several forms. Knight-Jones (1954) described 4 types of metachronal co-ordination, namely symplectic (direction of the effective stroke of the ciliary beat and the direction of the wave of transmission coincide), diaplectic (direction of the effective stroke of the ciliary beat and the direction of the wave transmission in opposite directions), laeoplectic and dexioplectic (where the directions of the effective stroke of ciliary beat are to the left and to the right of the direction of the wave of transmission respectively). In its simplest form the metachronal wave resulting from each row of cilia being a fraction out of phase with its neighbours has the appearance of a wave travelling along the surface. Symplectic metachronal synchronisation results in the front of the wave forming at right angles to the direction of the effective stroke of the cilia. Symplectic metachronism described in *Opalina* (Okajima 1953) seems to be relatively rare in nature according to Sleigh (1962). Although not naming it as such, Hilding (1932a, 1932b, 1932c) described symplectic metachronal co-ordination in the nasal mucosa of man. The same phenomenon was probably also described by Lucas and Douglas (1934), in man, monkey and other mammals. No comment has been made about the type of metachronal organisation in mammalian pulmonary airways by Barnett and Miller (1966) or Dalhamn (1955, 1956, 1960), who has made an important contribution of the study of mucociliary activity in such airways. Barnett and Miller (1966) however, do describe waves with a definite wave length in cat trachea which may be interpreted as symplectic metachronism. They state that the cilia were not visible because of the presence of an optically dense medium above them but do not comment on the exact nature of this phenomenon. These waves then cannot be definitely said to represent symplectic metachronism. Ballanger (1949) discussing the ciliary activity of rabbit trachea *in vivo* does not describe symplectic metachronism. He makes the statement; "Analogy could

not be made to an ocean wave moving evenly across a large expanse of surface, but more to a field of clutching hands viewed from above in which all the hands were moving in the same general direction".

The most notable contribution to the understanding of metachronal synchronisation in mammalian pulmonary airways has been made by Iravani (1969). By viewing the cilia through walls of intact airways he was able to see that ciliated cells were grouped into metachronal "fields" of irregular dimension consisting of a few to several hundred cells per field. The organisation of the direction of ciliary beat and metachronal synchronisation of individual fields was entirely independent of neighbouring areas. Under normal conditions the direction of ciliary beat could be divergent in adjacent fields, but was never directed caudally (Fig. 3).

Barclay and Franklin (1937) and later Hilding (1957, 1963) labelled mucus with Indian ink and observed the transport of the material around openings of bronchial tributaries into the mainstem bronchus being studied. Hilding concluded that the mucous blanket divides and is thus transported around these orifices. Iravani (1967) was able to elucidate this phenomenon further by showing that the direction of ciliary beat became oblique to the axis of the bronchus where a branching site occurred. Mucus would therefore be moved away from the opening of the tributary.

2.2 MUCUS TRANSPORT

The main purpose of this review is to trace the development of the mucous blanket concept which proposes that the pulmonary airways are lined by a continuous layer of mucus from the small bronchioles up to the trachea.

2.2.1 Early work 1830 – 1932

Sharpey (1830 and 1836) investigated mucociliary activity in the respiratory system of birds and mammals. Using charcoal as an indicator he was able to study the movements of the indicator in the nasal mucous membrane of living rabbits. Gosselin in 1851 applied Sharpey's technique to human postmortem trachea and was able to detect movement 56 h after death (in Rivera 1962).

All the early literature subsequent to this indicates that the movement of indicators on excised membrane from various animals was studied. Lommel (1908) using freshly

excised dog trachea timed the rate of transport of lycopodium spores but commented on the unrepresentative nature of his findings. Von Gebhardt (1909) conducted a comprehensive investigation into the transport of particulate matter in intact animals (with large tracheostomes) as well as by excised dog trachea. He came to the conclusion that charcoal was the most suitable substance for this type of study, rejecting powder, sand and other similar substances. His results of mucus transport rates were also not reproducible in preparations which varied in their conditions of study. In the case of one experiment undertaken two minutes after the dog's death, the indicator was transported in the trachea at a rate of 5,3 cm per min. More frequently transport rates were 1 – 2,2 – 3,2 cm per min. Henderson and Taylor (1910) who studied the effect of expectorants on mucus flow during life in the trachea of anaesthetised cats, also noted wide variations in the rate of mucus transport. The fastest rate they observed was when indigo carmine was transported at a rate of 4 cm in 65 sec. The average rate was 1,5 – 2 cm per min. Henderson and Taylor make a most interesting statement in passing during the discussion of their technique namely, "we noted also that dust or stained mucus very frequently did not travel in a straight line but tended to curve to one side or the other. Streaks of stain were very commonly seen and Gebhardt (quoted above) seems correct in his conclusion that not all cilia in the trachea or on any ring are working at the same rate". This phenomenon which was then interpreted as ciliary currents was reported as early as 1830 by Sharpey when he found that charcoal was transported towards the pharynx when placed on extirpated nasal mucosa of rabbits *in vitro*. He was also the first person to prove that the propulsive effect of ciliary activity was towards the larynx in excised dog trachea bathed in tepid water (Sharpey 1836).

Further early contributions to the study of mucociliary activity and mucus transportation in pulmonary airways were made by Hill (1928), and Florey *et al.* (1932).

Hill (1928) used tracheal tissue harvested from a variety of animals (horse, sheep or ox) for *in vitro* study while immersed in a Ringer solution. For the transport of markers he found a fine suspension of lamp black to be the most suitable and recorded rates as fast as 3 cm per min. These transport rates varied and were of the same order in the horse, sheep, rabbit and hen trachea and frog gullet at 1 cm in 20 – 30 sec. under the best conditions. Transport rates in smaller animals, namely rats were slower at 1 cm in 2 – 2½ min. Florey *et al.* (1932) noted "a " "river" " of

secretion could be seen rapidly passing along the trachea towards the larynx". While observing the transport of a suspension of graphite ink in saline, they noted that the propulsion of the marker was not a smooth process and they state: "Some of the particles sink down and tend to remain, or at least are rather slowly moved forward until they are caught up on the strands of mucus". They also note after pilocarpine injection into the animal to produce an increase in the production of mucus that, "when a considerable quantity of mucus secretion was present, the mucus stream moved more quickly than when the normal " "resting" " secretion alone was present".

2.2.2 Mucus transport in the nose

During the 1930's a great deal of attention was paid by otorhinolaryngologists to ciliary activity and mucus transport in the nose and sinuses of man and a variety of animals. Yates (1924) studied the direction of flow of mucus in the nasopharynx of humans with markers (indigo carmine and lamp black). Anderson Hilding repeated this work in humans and dogs in more detail (1931, 1932a, 1932b, 1932c) and mapped out the direction of mucus transport in the nose. He showed that in the posterior two-thirds of the nose the mucus is transported towards the pharynx and towards the nares in the anterior third. In-between was an area where no transport occurred. He mapped out the course of mucus flow in the sinuses of a dog as well (1932c).

Subsequently several authors have confirmed and amplified the observations of Yates and Hilding. The most important contributions were made by Lucas and Douglas (1934), Proetz (1933, 1934, 1946), Lierle and Moore (1935), Ornston (1946), and Tremble (1947, 1948). Apart from mapping out the direction of ciliary currents by following the direction of the transport of markers in man and animals, the effects of drugs and chemical substances on cilia were observed by them. Lierle and Moore (1935), Ornston (1946) and Tremble (1947, 1948) who, in particular, studied the movement of mucus in normal persons and various disease states noted that the rate of transport is diminished in infective states but is normal in allergic persons. They also observed that localized eddy currents may form or that stasis may occur in pathological areas. All the abovementioned authors refer to the mucus in the nasal cavity and sinuses being present as a blanket, sheet or lining. Lucas and Douglas (1934) were the first to suggest that this mucous blanket had two components, a lower or sol layer in which the cilia beat, and a covering gel layer which was

propelled by the tips of the cilia during their effective stroke. This basic concept of a continuous mucous blanket born more than a hundred years ago, has been accepted widely and used as a basis for discussion and the interpretation of experimental results by authors such as Hilding (1961), Barnett and Miller (1966) Sadé (1970), and Asmundsson and Kilburn (1970).

The basic principles of the rates of transport and directions of mucus flow in the nose, as described by the earlier authors mentioned above, have been confirmed by more sophisticated modern techniques. Ewert (1965) using a Zeiss Diploscope was able to study the movement of non-irritant markers on the nasal mucosa in a large number of humans and concluded that the mean flow rate in the nose was 4,2 mm per min. The most recent investigations have been carried out following the course of radioactive tracers placed on the nasal mucous membrane. Proctor and Wagner (1965) first used this technique in humans utilizing macro-aggregated human albumen labelled with ^{131}I . A similar technique was used by Bang *et al.* (1967). While these techniques gave more information about mucus transport throughout the nasal mucosa, dispersion and streaming of the micro-droplets made accurate determination of the transit rate difficult. Quinlan *et al.* (1969) devised a greatly improved technique, using a non-dispersible radioactive particle. The particles were spherical beads of an anion exchange resin labelled with ^{99}Tc pertechnetate (TcO_4^-) ion. The course of the bead was followed on pictures obtained with a gamma camera. The transport rates of 9 mm per min measured by this technique were slightly higher than those reported previously by Ewert (1965) (4,2 mm per min) and Proctor and Wagner (1965) (6 mm per min). A marked degree of variation in transport rates in different subjects has been reported using radioactive markers on the nasal mucous membrane (Ewert, 1965; Bang *et al.*, 1967). Andersen *et al.* (1971) investigated the effect of varying conditions of relative humidity from 10% to 70% on transport rates in the nose and concluded that no relationship existed. Another interesting facet of ciliary activity in the nose which has not been widely commented on is that there is an increasing velocity gradient from the anterior to the posterior portion. Hilding (1931) first observed this and Quinlan (1969) confirmed the velocity gradient with his more precise radioactive techniques.

2.2.3 Mucus transport in the airways

During the past 40 years attention has been focused on ciliary activity and mucus transport in the pulmonary airways. Gordonoff and Mauderli (1936) observed the transport rates of charcoal and fragments of cork in a disconnected cranial portion of guinea-pig trachea. Their recorded rates of transport were 1 mm in 12 to 22 sec and they came to the conclusion that the power of ciliary beat is so small that the cilia laboured under the mucous blanket. At about this time Barclay *et al.* (1937) in a chance experiment noted that at the junction of bronchi the movement of cilia is such that mucus would be moved to either side of the airway, and also that in the trachea cilia move mucus spirally in a clockwise direction when viewed from the cranial aspect. Following this they showed the same phenomenon in cats' lungs (Barclay and Franklin, 1937) by exposing a bronchiole of 1 mm in diameter at the base of the lung and introducing Indian ink. They were thus also able to measure the rate of transport of the markers at various levels of the bronchial tree. The ink travelled at an average rate of 1 cm per 4 min in the lungs and 1 cm per min in the trachea. Indian ink injected subpleurally took 14 min to travel up to the tracheal cannula. These techniques were very similar to those employed by Hach (1925) who tracheostomised dogs and then fixed a bronchiole of 1 to 1½ mm in diameter to the pleura. A fuchsin stained suspension of lycopodium spores was introduced into the bronchiole the following day when the animal had recovered from the anaesthetic. Swabs of tracheal secretion were then obtained at 5 min intervals. As soon as a trace of the indicator was observed the animal was killed and the distance travelled by the indicator measured by opening the trachea and bronchi. The overall flow rate of mucus was estimated to be 4.6 mm per min by this technique.

Anderson Hilding who contributed significantly to the understanding of nasal clearance turned his attention to the airways and has made a number of widely accepted contributions to the principles governing mucus transport and ciliary activity, as well as re-inforcing previously held concepts. While investigating the death of a number of asthmatic patients (Hilding 1943) he came to the conclusion that the ciliary epithelium is altered in character and that failure of ciliary activity occurs together with an increase in the number of goblet cells and an attendant hypersecretion of tenacious mucus. These factors, he postulated (Hilding, 1949), combined to produce mucus plugging of the small airways. However other studies by Ornston (1946), Tremble

1947, 1948). and Ballenger (1949), failed to produce evidence that ciliary activity is altered in allergic states as measured by the ability of these epithelia to transport markers in the human nose or on the tracheal or nasal mucous membrane of rabbits. Hilding (1943, 1944, 1949) demonstrated in normal animals that cilia have the power to produce a negative pressure of -40 to -50 mm of water by moving a mucus plug occluding a tracheal lumen away from a manometer which was attached to the other end of the trachea. He demonstrated a similar situation in the frontal sinus of dogs (Hilding 1943). These observations formed the basis of his postulate that mucus hypersecretion in small airways result in "plug" formation in small airways causing atelectasis and death from asphyxiation in asthma.

The concept of ciliary currents or streaming described by Sharpey (1830; 1835), von Gebhardt (1909), Henderson and Taylor (1910), Barclay *et al.* (1937, 1938) and Barclay and Franklin (1937) was utilised by Hilding in the study of normal mucus transport as well as its pattern in disease states. By deciliating areas of trachea and bronchi obtained from freshly killed cows he showed (Hilding 1956) that the mucus, as indicated by the movement of Indian ink markers, accumulated on these deciliated areas. Similarly, cigarette tar accumulated on such areas. Hilding thought that this was suggestive evidence that noxious substances could therefore accumulate on similar areas occurring naturally where squamous metaplasia was present. In conjunction with the study of the effect of deciliated islands on mucus flow, Hilding also demonstrated with markers that the mucous blanket is diverted around bronchial openings. A striking phenomenon which he observed was the presence of whirlpool formation in bovine airways (Hilding, 1957a, 1957b). These areas were small and varied in size and number in different specimens. No conclusions were drawn from this observation.

In considering the overall transport of the mucous blanket from the terminal bronchioles to the trachea and larynx, Hilding (1957a, 1957b, 1961, 1963, 1965) points out that there is a rapid narrowing from the periphery of the airway system upwards. In the course of 8 branchings, the circumference of the airways diminishes tenfold from 344 mm to 34 mm. Von Hayek (1960) estimated that in the human there are more than 20 000 terminal bronchioles of approximately 0.5 mm in diameter. Thus Hilding points out (1965) that the width of the mucous blanket would be 30 metres at this level in the human. The airway system narrows down to a width of 50 mm in the trachea. Thus a large volume of mucus moves up into a

very confined area as it is transported cranially. Although no direct measurements in the airways have ever been made, Hilding (1961, 1965), pointed out that there was a velocity gradient in the nose and therefore probably in the lung. The other method in which this large volume of mucus could be accumulated in the upper airways was by an increase in the depth of the mucous blanket. The depth of the mucous blanket has however never been determined accurately (Hilding 1961, 1963, 1965). Other attempts at estimating the depth of the mucous blanket have also proved unsuccessful (Barnett and Miller, 1966).

In the majority of instances discussed thus far, the ciliated epithelium of the pulmonary airways has been examined *in vitro*. Dalhamn (1955, 1956, 1960, 1961) described a method of examining mucus transport and ciliary activity in the cranial portion of the trachea of experimental animals *in vivo* and *in vitro*. The *in vivo* method involved opening the trachea and observing the mucous membrane directly. Mucus transport rates were timed by observation of shed cellular elements or bubbles entrapped in mucus, and these were found to have a mean rate of 12,9 mm per min and 13,5 mm per min in two groups of rats. Artificial particles (i.e. charcoal, etc.) were not used as they had been shown to be propelled at varying speeds of flow (Dalhamn, 1956). Ciliary rates were timed with the aid of cinematographic techniques. Ciliary activity and mucus transport were compromised when the trachea was extirpated and studied *in vitro* under controlled conditions of humidity and temperature, suggesting that *in vivo* conditions were preferable (Dalhamn, 1956). In general the technique used by Dalhamn appears to be similar to that used by Proetz (1933) when observing and filming the activity of the nasal cilia in rabbits. Thus using this method, Dalhamn studied the effects of irritant gases (Dalhamn, 1956), cigarette smoke (Dalhamn, 1959; Dalhamn and Rylander, 1966; Dalhamn 1966), orthocresol and metacresol (Dalhamn, 1968), oxygen (Dalhamn and Rosengren, 1968, 1969, 1971a), tobacco smoke (Dalhamn and Rylander, 1969; Dalhamn, 1970b) and different aldehydes (Dalhamn and Rosengren, 1971b) on the function of the tracheal mucosa *in vivo* as well as *in vitro*. In all instances where the flow of mucus was measured it was thought to be a function of the movement of a mucous blanket.

The movement of the mucous blanket has been studied by other authors using similar techniques to those of Dalhamn. Barnett and Miller (1966) investigating the wave form of mucus transport consider that ciliary movement produces the waves in

the mucous blanket. They noted however that the transport of particulate matter showed no relationship to the wave properties observed. Lightowler and Williams (1969), using Dalhmn's technique showed a slowing of mucus flow after the exposure of rats to SO_2 , by observing the propulsion of markers placed on the mucous blanket in the trachea.

A simple method of measuring transport rates of the mucous blanket was devised by transilluminating the dissected trachea (usually of cats) *in vivo* and then observing the movement of particles injected between the carina and the observation point (Goldhamer *et al.* 1964). This method has been used to study the effect of gases, smokes and aerosols (Carson *et al.* 1966) and cigarette smoke (Weissbecker *et al.* 1971) on the velocity of particle transport in the trachea.

Yet other methods of observing mucociliary activity in the trachea of experimental animals have been devised. Sadé *et al.* (1970) observing the palates of frogs as well as rabbit, rat, cat and hen trachea *in vivo* and *in vitro*, concluded that a mucous blanket is a necessary intermediary in the transport system. Battista and Kensler (1970) developed a technique of exteriorising the intact trachea of Leghorn hens and observing the transport of markers under various experimental conditions. Normal transport rates were noted to be 15 mm per min. At the end of the experiment the trachea could be interiorised again. After exposure to irritant gas, they encountered difficulty in differentiating between ciliary inhibition, an increase in mucus viscosity, or an increase in the depth of the mucous blanket as being a cause of the alteration in transport activity. They also observed marked differences in depth of the mucous blanket under different conditions. Berke and Roslinsky (1971) were able to deposit an aqueous slurry of BaSO_4 at the level of the carina of rats without traumatising the trachea. The progress of the barium was then followed roentgenographically. A mean rate of 7,4 mm per min was observed.

An unusual method of observing the transport of standardised teflon discs from within the tracheal lumen of intact dogs by a fibre-optic bronchoscopic technique, has been described by Sackner *et al.* (1973). The transport rates were timed with the use of cinematographic techniques. The fastest rate of a single particle was 15 mm per min. Other particles were timed at 11,8 mm per min and mean velocity of all particles was 5,7 mm per min. Apart from indirect evidence obtained by Hach

(1925) and Barclay and Franklin (1937), no direct information was available on mucociliary function distal to the trachea *in vivo*. Kilburn (1967) devised an ingenious method of evaginating the bullfrog (*Rana catesbeiana*) lung through the glottis and studying the movement of labelled mucus along the various orders of ciliated ridges which separate the crypts (alveoli). Transport was timed with the aid of markers placed on the various ridges. Mean flow rates of 33 mm per min (longitudinal ridge), 12 mm per min (annular ridge), 5 mm per min (tertiary ridge) and 2 mm per min (quaternary ridge) were measured at an environmental temperature of 22°C. Particles deposited in crypts were generally not cleared in 4 to 6 h. Under conditions of excessive moisture these particles were retracted from the crypts towards ciliated tufts on the ridges. Filling of the crypts with enough fluid hastened the clearance of particles which would then be carried away on a continuous mucous blanket towards the pharynx. Transport rates in the pharynx of two animals were of the same order as that observed on the longitudinal ridges. Using these observations together with the fact that airways diminish from an aggregate circumference of 10 800 m at the 16th generation of the human lung (Kilburn, 1968) to 30 m at the 10th generation (Hilding, 1957b) and to 5 cm in the human trachea, he postulated that the mucous blanket must lose volume by the absorption of fluid to be accommodated in the respiratory tract (Kilburn, 1968; Asmundsson and Kilburn, 1970). Other factors supporting this hypothesis were that only a fourfold increase in mucus transport rates had been observed from the small bronchi to the trachea (Barclay and Franklin, 1937; and Kilburn 1967), and that secondly, natural obstructions such as bronchial orifices and squamous metaplasia impede mucus clearance as well as diminishing the effective surface area for mucus transport. It was suggested (Asmundsson and Kilburn, 1970) that even an 8 to 14 fold increase in the velocity of mucus transport from the level of the 8th to 12th bronchial generation (aggregate circumference 130 m) to the 50 mm circumference of the trachea, would not compensate for the magnitude of convergence of a mucous blanket uniformly 5 μ m deep (Dalhamn, 1956). Observations made on dog airways *in vitro* (Asmundsson and Kilburn, 1970) showed only an 8-fold increase in the velocity gradient of 1,6 mm per min in sub-segmental bronchi to 12,6 mm per min in the trachea. Therefore estimates of mucus viscosity were undertaken and they indicated that fluid from a 10th branch bronchiole would travel further up a 1 mm capillary tube than mucus from the trachea. This difference they suggested was due to an increase in mucus viscosity cranially. In addition mucociliary

activity was arrested by dropping 0,13 M NaCl on the mucosa but resumed as the excess fluid was apparently absorbed. These observations strengthened Kilburn's original hypothesis (Kilburn, 1968) that the large volume of mucus may be accommodated in the bronchial tree by the active resorption of fluid from the sol layer of the mucous blanket into the airway wall.

2.3 PULMONARY CLEARANCE OF RADIOACTIVE AEROSOLS

The external detection of radioactive particles deposited in the lung affords a method whereby pulmonary clearance can be studied (Albert and Arnett, 1955; Morrow *et al.* 1967). Provided that the aerosol is non-irritant or non-toxic, and the dose of the radioactive tracer is small, this procedure is deemed to be safe for use in human investigation (Booker *et al.* 1967; Thomson and Short, 1969). The dose delivered to the pulmonary tree is very low and has been reported to be 5 μC (Luchsinger *et al.* 1968), 200 μC (Sanchis *et al.* 1972), and 400 to 600 μC (Toigo *et al.* 1963), in human studies. In one study the lung dose was 0,5 mrad per day (only 3 times the dose rate from natural background) (Booker *et al.* 1967), and a total lung dose of 2.975 rad in another (Sanchis *et al.* 1972). A variety of radioactive tracers, namely ^{51}Cr , ^{131}I , $^{54}\text{MnO}_2$, ^{18}F , $^{99\text{m}}\text{Tc}$, ^{59}Fe , Th B and ^{198}Au have been tagged on to particles of carbon, polystyrene, Fe_2O_3 , albumen and gelatin. Particle size of the aerosol has varied from 0,8 μm to 70 μm . The majority of aerosols have been monodisperse, but in a few investigations have been didisperse or heterodisperse. (Albert, Lipmann, Spiegelman *et al.* 1967; Albert, Spiegelman *et al.* 1969; Albert, *et al.* 1969; Albert *et al.* 1971; Booker *et al.* 1967; Thomson and Short, 1969; Pavia *et al.* 1970; Luchsinger *et al.* 1968; Camner *et al.* 1971; Toigo *et al.* 1963; Morrow *et al.* 1967; Sanchis *et al.*, 1972). In many instances the delivered lung dose was only a small fraction of the original dose (Toigo *et al.* 1963; Luchsinger, 1968).

The removal of radioactive tracers appears to occur in two phases. The rates of transport in these two phases are very different from study to study, although in each report the initial phase is distinct from the second phase. In the different studies the clearance times of the initial and second phases overlap. No clear explanation for these widely differing results is found. The differing methods of aerosol administration, size of aerosol particles, duration of inhalations and total dose of particles may have played a part in causing the differences. Holma (1967) observed

a difference in clearance rates of 6 μm and 3 μm particles. The 6 μm particles were deposited higher up in the conducting airways and were transported out somewhat more rapidly than 3 μm particles (1,8 h versus 2,3 h) deposited lower down.

Camner *et al.* (1971) have noted a wide variation of transport rates from subject to subject, but individual reproducibility on repeated exposures was found. An initial rapid phase lasting from 30 min (Albert and Arnett, 1955; Morrow *et al.* 1967; Sanchis *et al.* 1972), 13 to 123 min (Luchsinger *et al.*, 1967), 2 to 3 h (Thomson and Short, 1969), and 10 to 20 h (Booker *et al.* 1967) has been observed. This initial phase occurs in association with radioactivity in the main conducting airways and is thought to represent the activity of the mucociliary escalator (Albert, Lipmann, Spiegelman, Strehlow *et al.* 1967; Camner *et al.* 1971; Luchsinger *et al.* 1968; Morrow *et al.* 1967; Sanchis *et al.* 1972). A second slower phase of removal of radioactivity may last 19 to 170 min (Luchsinger, 1968), 2 to 4 h (Albert and Arnett, 1955), 161 to 209 h (Holma, 1967), 70 days (Albert, Lipmann, Spiegelman *et al.* 1967) or as long as 150 to 300 days (Booker *et al.* 1967). This prolonged phase is thought to be a result of deposition of particles in the distal portion of the bronchial tree or at the alveolar level (Albert, Lipmann, Spiegelman, Strehlow *et al.* 1967; Morrow *et al.* 1967; Holma, 1967; Sanchis *et al.* 1972). The view has been held (Proctor, 1964; Green, 1970) that particles of 5 μm or larger do not penetrate the alveolar regions. Several studies indicate that these and larger particles may penetrate distal to the ciliated region (Booker *et al.* 1967; Thomson and Short, 1969). Several factors influence the distribution of deposition of particles in the respiratory tract. These are the size of the particle, depth of respiration and breath holding during exposure, calibre of the airway, and the degree of airway obstruction. Particles of 5 μm or larger do not diffuse but sediment by gravitational fall-out at a rate of 1 mm per sec. Thus in a three second breath holding period particles with unit density will sediment in all airways 3 mm or less in diameter. The depth of respiration and particle size may influence this site of deposition. Thus shallow breathing and the inhalation of larger particles favours deposition higher in the tracheo-bronchial region (Morrow *et al.* 1967). However Morrow *et al.* (1967) state that clearance kinetics indicate that physiological control is dominant in a given region and not particularly sensitive to particle size. Holma (1967) in contradistinction states that in both the initial and secondary phases of clearance the larger particles are eliminated more rapidly.

The application of this technique to the study of pulmonary clearance initially indicated that chronic lung disease (Thomson and Short, 1969) and smoking (Albert, Lipmann, Spiegelmann, Strehlow *et al.* 1967; Thomson and Short, 1969; Pavia *et al.* 1970) did not impair pulmonary clearance. Subsequent studies by these authors (Albert, Spiegelman, *et al.* 1969; and Albert *et al.* 1969; Pavia *et al.* 1971) indicated that pulmonary clearance was significantly slowed by cigarette smoking in man and experimental animals. However a long term study in the donkey showed that clearance function returns to normal rapidly in some instances after cessation of smoking (Albert *et al.*, 1971).

The clearance of labelled particles is presumably a function of the mucociliary escalator in the conducting airways. The clearance was noted to be discontinuous, apparently due to the intermittent discharge of particles from the bronchial tree into the trachea, (Albert, Lipmann, Spiegelman *et al.* 1967; Albert *et al.* 1969). Sanchis *et al.*, (1972) were unable to confirm this finding and emphasize the continuous nature of clearance. It appears that this type of investigation on lung clearance gives an overall picture of the method whereby the lung handles particles and gives no detailed or specific information on mucociliary activity.

2.4 SCANNING ELECTRON MICROSCOPY (SEM)

The characteristics of the SEM have been described in detail elsewhere (Black, 1970), and will not be considered here. The topographic observation of histological tissue by SEM has been used for a number of years. The development of preparative techniques which allow the study of non-conductive soft tissue with the SEM has provided additional information of the pulmonary cellular surface structure (Holma, 1969; Wang and Thurlbeck, 1970; Nowell and Tyler, 1971; Greenwood and Holland, 1972). This information cannot be obtained easily by the use of conventional histological techniques. The SEM has been used to study the topography of the respiratory epithelium by a number of workers but Nowell and Tyler (1971) are the only authors to allude to the presence of a mucous blanket. The mucus was seen in a terminal bronchiole and its presence inferred because the underlying respiratory epithelium was not visible.

2.5 SUMMARY

The literature review has shown that although the early workers referred to "ciliary streaming" when discussing the transport of particulate matter no uniform concept governing mucus transport was proposed. In 1934 Lucas and Douglas in a classic paper proposed the two layer sol and gel concept of the mucous blanket or sheet. This concept arose from observations of the nasal mucosa. Subsequently this concept has been utilised in investigations concerning tracheobronchial clearance mechanisms. It would appear that an assumption was made that conditions present in the nasal mucosa would also occur in the pulmonary airways.

CHAPTER 3

METHODS

3.1 INTRODUCTION

The preceding chapter reviewed the techniques available for studying tracheobronchial clearance mechanisms. Many of these techniques were unsatisfactory because of limited fields of view in *in vivo* preparations and the artificial environment created for *in vitro* preparations. Both these drawbacks precluded intensive study of pulmonary mucociliary activity. In this chapter an *in vitro* method for studying an intact airway system is described in detail.

3.1.1 Definition of terms

In this study animal models were used to simulate human diseases. It is understood that human disease patterns are very often only loosely imitated by animal models and therefore definitions and descriptions of the relevant animal models are necessary.

Acute bronchitis

This condition was precipitated by exposing SPF rats to freezing, moist conditions in a refrigerator for 0,5 h and room temperature for 0,5 h over a period of 12 h. Clinically the rats were ill on the following day, were sniffing, sneezing, had audible rhonchi and were in respiratory distress. An acute syndrome of this nature was never encountered in SPF animals not exposed to this treatment.

Chronic Respiratory Disease (CRD)

CRD, which occurs spontaneously in rodents, has been described in detail and is almost always due to an infection by *Mycoplasma pulmonis* (Lindsey *et al.* 1971). The morphology of *M. pulmonis* infection is described in detail by Lindsey *et al.* (1971) and presents in two main categories. The first is an epithelial hyperplasia, periluminal lymphocytic and plasma cell infiltration, and an accumulation of intra-luminal purulent exudate at all levels of the respiratory tract from the nasal mucosa to the respiratory bronchioles. The second category is confined to the lung and probably occurs as a result of the airway changes. These include atelectasis, in-

flux of macrophages and neutrophils into alveoli, and pulmonary abscesses. The airway also showed squamoid changes in the bronchial epithelium and Lindsey *et al.* (1971) refer to the airway changes as laryngitis, tracheitis and bronchitis.

As CRD occurred spontaneously in non-SPF rats, and resulted in bronchial abnormalities, it was convenient to use these rats as animal models of chronic bronchitis. Thus this bronchial syndrome will be designated "chronic bronchitis" for the purposes of this study. The histological features are illustrated in Fig. 6. The bronchial abnormalities were recognised easily under the dissecting microscope using magnifications up to X160. The changes consisted of bronchial thickening which resulted in a loss of translucency of the airway. Also seen often were patches of excessive thickening, brownish in colour, which could not easily be dissected off the bronchial wall. Fig. 6 illustrates the histological features of such an area. Abnormal ciliary activity is commonly associated with CRD (Iravani and van As, 1972) and is dealt with in detail in par 5.3.

Normal Rats

Wistar rats born and reared under SPF conditions were regarded as normal animals. Young rats weighing between 120–270 g were used as they were most likely to be normal. These rats had translucent, pinkish airways with no evidence of thickening or opacification when viewed under the dissecting microscope. If any of the topical features suggesting bronchial thickening were observed the rat was not used. Another important criterion of normality was the organisation of ciliary beat, which under normal conditions is always directed cranially and arranged in metachronal fields. These criteria have been discussed extensively elsewhere (Iravani 1969), and are also mentioned briefly in par. 4.3.

3.2 PHYSIOLOGICAL IN VITRO EXPERIMENTS

3.2.1 Animals

Wistar rats were used for all experiments. They were all fed a standard diet consisting of concentrated pellets. The rats fell into two groups, one housed under normal animal house conditions and the other born and raised under specific pathogen free (SPF) conditions. The atmospheric constituents to which the groups of rats were

exposed were identical except for the absence of bacteria in the SPF rooms. Animals removed from the SPF room and exposed to charcoal dust clouds had by necessity to be kept out of the SPF rooms to prevent unnecessary contamination of the colony. They were studied within a week of their removal. This period out of SPF conditions did not influence the pulmonary clearance mechanisms as no differences between the rates of ciliary beat or mucus transport could be observed when compared to rats not removed from SPF conditions.

Two groups of Wistar rats were used for the *in vitro* physiological experiments. The first group (28 rats) weighing from 120 – 270 g were kept in SPF conditions until the start of the investigation. Of these 28 experiments 11 were successful. The second group, although originally derived from SPF conditions, were removed at an early age and kept in normal animal house conditions for a period of 6 – 24 mth before study. This group was older, weighing from 180 – 400 g, and consisted of 36 rats. Of the 36 experiments 14 were successful. A further 4 SPF animals were exposed to freezing, moist conditions and room temperature alternately in an attempt to precipitate acute bronchial changes. This was an unpleasant experiment as the animals obviously experienced distress and pain. Therefore the experiment was terminated after the fourth animal.

3.2.2 Isolation and dissection of the airways

The technique used is similar to that described by Iravani and van As (1972). All animals were sacrificed by an intraperitoneal overdose of pentobarbital (250 mg/kg). The technique of sacrificing or rendering the rat unconscious by a sharp blow on the back of the head was not used. This method frequently results in pulmonary changes in the form of oedema or haemorrhage. In addition bleeding, resulting from the head injury, is frequently aspirated into the lungs, and whole blood has been observed to have a very strong depressant influence on ciliary activity (van As, unpublished observations). While the animal was in coma, and before respiration ceased, the trachea was exposed and cannulated with a snugly fitting polythene cannula. The cannula was securely tied in position with silk thread. The critical period of the dissection now commenced and had to be initiated before respiration ceased. The abdomen was opened and the lungs were inflated to the point of deep inspiration as judged from the movement of the diaphragms. The head, and trunk

below the diaphragms, were rapidly separated from the thorax and the pelt covering the thorax removed. The isolated thorax was immersed in a bath containing ice-cold Krebs Henseleit solution (Krebs and Henseleit, 1932) at a temperature of 5°C or less. From the time of tracheal cannulation till immersion of the thorax in cold Krebs Henseleit solution took 60 – 100 sec. Once the pulmonary tissues had commenced cooling the hypoxic period became less critical and the rest of the dissection could continue at a slower pace. The diaphragm was separated from the chest wall but not the basal portions of the lungs. The thoracic cage was removed by severing the ribs just lateral to the spine. The inferior vena cava and both ventricles were occluded with bulldog clamps. The pulmonary vein draining the left lung was exposed and the left atrium cannulated through this vessel. The cannula was firmly secured. A small incision was made in the arterial supply to the right inferior medial cardiac lobe (heart lobe) adjacent to the bronchus supplying this lobe. The heart lobe was then perfused with iced Krebs solution via the left atrium retrogradely through the pulmonary vascular bed to the opened pulmonary artery. The perfusion necessitated very gentle pressure and was continued until the heart lobe was rendered almost bloodless and no significant amount of blood was being flushed out of the artery. The volume necessary to perfuse the lobe adequately hardly ever exceeded 2 ml. When an excessive pressure was applied during the perfusion, or if a large volume was used, pulmonary oedema usually resulted. In these instances ciliary activity was compromised and as the airways were filled with fluid the specimen could not be studied.

After completion of perfusion the catheter was removed from the left pulmonary vein and the bronchovascular bundle was ligated. The lobe was removed by division distal to the ligature. All the other lobes, apart from the heart lobe, were ligated and removed in a similar fashion. The cardiovascular system, oesophagus and peri-bronchial connective tissues were dissected off the remaining extralobar airways, and the preparation removed from the vertebral column.

The specimen was mounted in an insulated bath (Fig. 1) and bathed in Krebs solution at a temperature below 5°C . The temperature was controlled in the bath with the aid of a thermostat (Hetotherm Ultrathermostat E-Type) and it never fluctuated by more than 0.2°C . The initial dissection was done under a dissecting microscope at a temperature below 5°C to arrest ciliary activity and diminish the

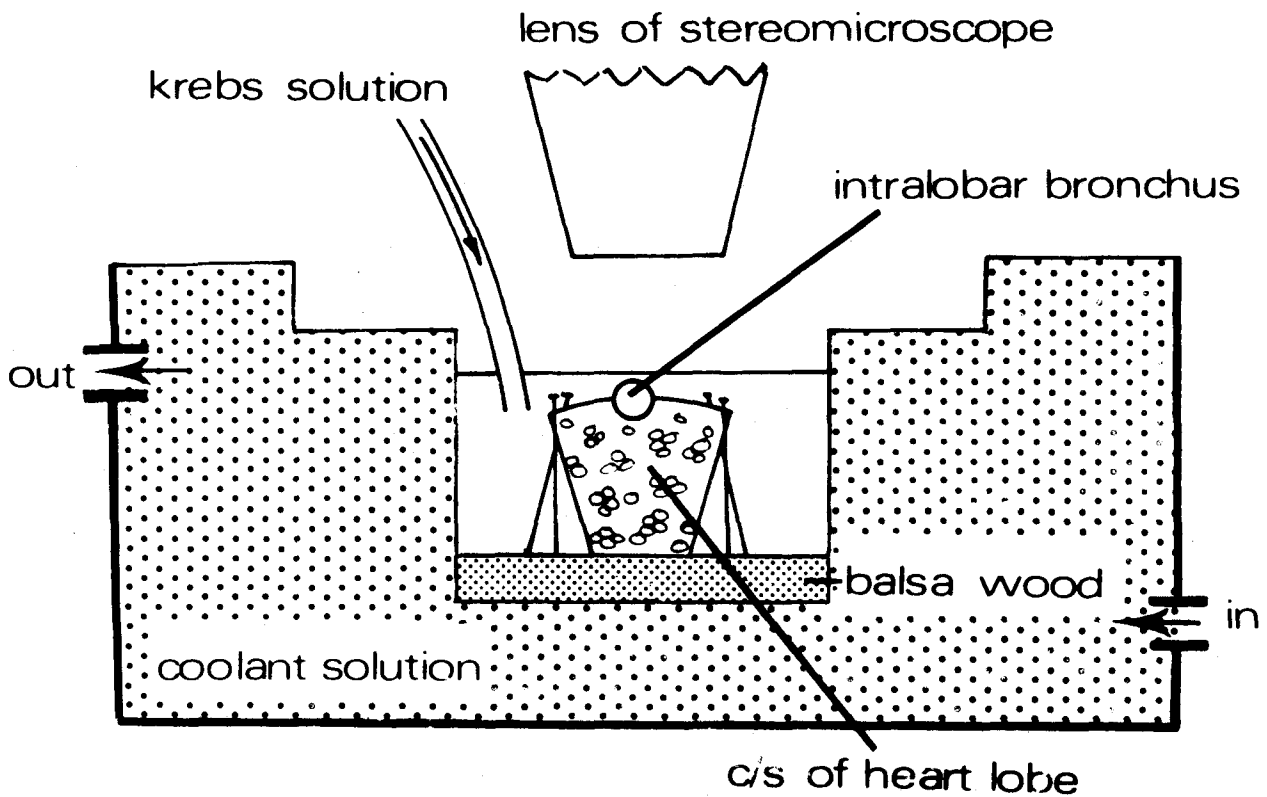


Figure 1 : Cross section of the insulated bath and airway preparation

The whole specimen is bathed in Krebs-Henseleit solution which acts as an extracellular fluid. The temperature of the Krebs solution is controlled by passage through an heat exchanger before entering the bath, where the coolant solution acts as an additional control.

metabolic requirements of the preparation. Observations of mucociliary activity were made at various temperatures between 17°C and 37°C depending upon the requirements of the experiment.

The volume of the bath in which the airway preparation was bathed was 40 ml with a turnover rate of 20 ml per min. The isotonic Krebs solution had a pH which was controlled by bubbling 5% CO₂ through it together with 20% O₂ prior to its passage through the bath. The mean pH observed during the experiments was 7.40 and the mean PO₂ was equivalent to 146 mm Hg at 20–22°C and 110 mm Hg at 35–37°C. 20% O₂ was bubbled through the reservoir continuously to saturate the Krebs-Henseleit solution at different temperatures. The differences in PO₂ are explained by the solubility coefficient of O₂ which is 0.003315 at 35°C and 0.004339 at 20°C. The effect of the salts in the Krebs solution can be ignored because this is a constant throughout, as can the ratio of the solubility co-efficients of O₂ to N₂ which varies from 2.20 at 35°C to 2.32 at 10°C, a change of less than 5%. The use of 95% O₂ in earlier work (Iravani 1967; Iravani and van As, 1972) was compared with 20% O₂ in this series. Using each animal as its own control, ciliary rates were counted at the level of the heart lobe bronchus in 4 rats at a temperature of 20–22°C first under conditions of 95% O₂ and then 20% O₂. No significant differences in rate between the two groups was noted, the 95% O₂ group showing a mean rate of 234 beats per min and the 20% O₂ group a rate of 242 beats per min.

The orientation of the specimen in the insulated bath was such that the membranous trachea was uppermost. The heart lobe was pinned in situ and the trachea and large airways were tethered by the tracheal cannula and pinning of the amputated lobes respectively. Once all the adherent peribronchial tissue had been cleared from the large airways, areas were selected for more precise dissection. These areas were usually in the middle and lower trachea, and the heart lobe bronchus just above its entry into the lobe. This dissection involved very careful removal of peribronchial stroma and musculature leaving in some instances only the mucous membrane intact. Perforation of the bronchus was a hazard at this stage of the dissection but not an irreparable complication. Bronchial tears could be easily sutured with a monofil thread (diameter 0.025 mm) attached to a 3 mm needle and resulted in no damage to ciliary activity in the surrounding areas.

The main intralobar bronchus of the heart lobe was exposed by gently teasing the alveolar tissues overlying it apart. Taking care not to rupture any bronchioles of significant size the entire main intralobar bronchus with its 12 side branches could be exposed. In all instances at least one side bronchus with its tributaries was exposed. One of the branches was exposed down to the terminal bronchiolar level.

From the time that the specimen was mounted in the insulated bath till the completion of study the airways were kept inflated at a constant pressure of 6 – 10 cm H₂O with air humidified by bubbling through water at room temperature (18 – 22°C). The pressure was constantly measured on a manometer and the pressure adjusted by means of alteration of the level of fluid in a water trap. The aim of keeping a constant pressure in the airways was to prevent atelectasis and airway closure. Keeping the larger airways at a constant diameter also facilitated the study of ciliary activity and mucus transport, as apposition of the airway walls caused disruption of ciliary function. Ciliary activity was seen to be inhibited by a constant intraluminal pressure greater than 10 cm H₂O (see par. 6.1).

Once the dissection was completed the temperature in the bath was raised slowly over a period of 30 min to the desired level which was either 20 – 22°C or 37 – 38°C. Although cilia were observed to commence activity at 5°C a further period of 1 h was allowed to elapse before mucus transport was studied. The reason for this was that at the time of the very rapid isolation of the thorax in ice-cold Krebs solution, ciliary activity was inhibited and the mucus present in the airways then, was still present when rewarming commenced. Specimens remained viable for more than 5 h after rewarming, as judged by the presence of ciliary activity.

Ciliary activity and mucus transport was studied using a stereo-microscope (Lietz) with magnification up to x320. The bronchial wall was illuminated with incident light using a fibre-optic system with a 500W halogen lamp as a light source (Volpi, A.G., Switzerland). This incident lighting which was easily adjustable, produced illuminated areas where desired and allowed the observation of the internal surface of the mucous membrane. Incident light reflected off the mucus-air and cilia-air interface and illuminated the individual mucus particles or the cilia on the cells. The pattern of mucus flow as well as its rate of transport could be

clearly assessed at all levels of the respiratory tract in the same specimen in a relatively short period. The rate of mucus transport was timed with a stopwatch using an eye piece with built-in graduated scale. When necessary more detailed study of restricted areas could be carried out using the opak or ultrapak system (Lietz Orthoplan microscope), with magnifications of up to x400.

3.2.2.1 Charcoal aerosol inhalation

In addition to the two groups of rats previously mentioned two further groups of 5 rats each were exposed to charcoal particles in a dust chamber. They were a normal young SPF group of rats (130 – 150 g) and an older one of non-SPF rats (270 – 300 g). These two groups are not comparable because of differences in their weights, but were chosen because the young SPF animals were clearly normal and the older non-SPF animals would almost certainly have been abnormal (see Definitions par 3.1.1). In our animal house clearly abnormal, young rats are difficult to find. The object of this investigation was to discover if patently abnormal rats handled a dust load differently to normal rats. The concentration of charcoal in the exposure chamber was 1 g/m^3 , and the diameter of the particles was $5 \text{ }\mu\text{m}$ or less. The animals were exposed for 5 h to deposit a significant dust load in the airways. They were examined 19 h later because firstly, this was practical and secondly, the time that elapsed after exposure would allow gross differences in clearance rates of the two groups of rats to express themselves.

A further three non-SPF rats weighing 230 – 270 g were exposed for 2 h to charcoal at a concentration of 200 mg/m^3 with a similar particle distribution as described above. As this was a more acute experiment a lower concentration of particles was administered for a shorter period of time than in the previous exposure. The purpose of this experiment was to demonstrate the patterns of the transport of mucus and record them photographically. Fig. 9 depicts the results in one of these rats and is representative of events occurring in all three specimens.

3.3 SCANNING ELECTRON MICROSCOPY (SEM)

Animals

All studies were performed on Wistar rats weighing between 200 and 300 g. Animals reared under both normal animal house conditions and a SPF environment were studied.

Preparation of Tissue

After the administration of a lethal dose of pentobarbital, intraperitoneally (250 mg per kg), a segment of trachea (5 rings) was removed from the rat before respiration ceased. Tissue from different experiments was prepared by one of two methods.

Method I. The tracheal rings were divided into fragments of $1 - 2 \text{ mm}^2$ and transferred rapidly into isopentane cooled in liquid nitrogen to approximately -180°C . After freezing for four minutes, the tissue was dried in a Cambridge freeze-dryer at -40°C for seven hours. When drying was complete the tissues were fixed in an atmosphere of osmium tetroxide (OsO_4) vapour in an evacuated dessicating jar. After four hours the tissues were transferred to a phosphate buffer for twelve hours. The specimen was then fixed in 5% gluteraldehyde in buffer at pH 7.4 for 4 h. The tissue was dehydrated through 50%, 70%, 90%, 95% and 3 changes in 100% alcohol, and 2 changes in propylene oxide and air dried.

Two SPF and 3 non-SPF rats were prepared by this method.

Method II. The tracheal segment was divided longitudinally, pinned out flat and rapidly immersed in gluteraldehyde at 5°C for 4 h. The tissue was washed in phosphate buffer and after being post-fixed in buffer containing 2% osmium tetroxide (OsO_4) was dehydrated in serial concentrations of ethanol. The alcohol was then replaced with clean amyl acetate, and the tissues dried by the critical point method (Anderson, 1951). The pieces of trachea were mounted on stubs and coated with gold in a vacuum evaporator and then examined with a Cambridge stereoscan electron microscope at a beam accelerating voltage of 20 kV., with the stubs titled at various angles to the electron beam.

Five SPF and 4 non-SPF rats were prepared by this technique.

3.4 SUMMARY

An *in vitro* method for the systematic study of tracheobronchial clearance mechanisms in intact airways has been described. It is possible to observe events from the level of the terminal bronchioles up to the trachea in the same specimen. The conditions created for this *in vitro* model approach those found *in vivo* very closely,

where the pH, PO_2 and temperature of the medium in which the preparation is bathed are rigidly controlled. In addition the airways are not opened and mucociliary function is observed through the bronchial walls by means of incident light. This, together with the fact that the airways are kept inflated with a small trans-bronchial pressure does not result in the mucosa being exposed to unnatural conditions.

CHAPTER 4

RESULTS – NORMAL RATS

4.1 INTRODUCTION

In the preceding chapter a technique for the systematic *in vitro* study of the pulmonary airways of the rat was described. In this chapter the results of the study of normal rats' (SPF) airways are described. SPF rats are defined as being normal and are used as control subjects, as non-SPF rats develop "chronic bronchitis" very readily.

4.2 PATTERN OF MUCUS SECRETION

Because of the nature of the preparation and the fact that extensive care had to be taken to avoid a significant period of hypoxia during the initial warm dissection before cooling, a proportion of experiments were not successful. A specimen was regarded as unsuitable for study of mucus transport if mucus was not seen at most levels of the respiratory tract. This lack of mucus transport occurred in approximately 60% of experiments. In most instances secretion of mucus occurred in the trachea and large airways. Ciliary activity was observed in all specimens. Under optimal conditions mucus transport could be studied for more than 5 h.

After opening the trachea before the insertion of the cannula, no mucus was seen with the naked eye. The low power microscopic view of the prepared airways showed pinkish translucent tracheal and bronchial walls.

On warming the preparation the cilia were reactivated at a temperature of approximately 5°C and their rate increased with further increases in temperature. Mucus transport commenced when a temperature of 17°C was reached. Residual mucus present in the tracheobronchial tree at the time of dissection was cleared first. Approximately 40 min after 38°C was reached, the secretion of mucus recommenced in successful experiments.

In normal rats the mucus was seen to be present at all levels of the airways except the terminal bronchioles. Mucus was observed in the airways in several different forms such as droplets, flakes or plaques. Small particle-like droplets less than

4 μm in diameter were observed throughout the tracheobronchial tree. These droplets were sparse in the small airways, but were more numerous in the trachea where goblet cells and mucous glands were more abundant. Droplets tended to aggregate together during their transport cranially, but were never seen to fuse or form the larger homogeneous flakes. Flakes which were 10 to 70 μm in diameter were observed with increasing frequency cranially. Frequently when several flakes and droplets came together during the course of their cranial transport, they were seen to aggregate together to form a larger body. These larger plaques varied in size and shape depending upon the number of flakes and droplets from which they were made. The process of aggregation of flakes to form plaques was readily observed and the flakes appeared to adhere as though bound by a visco-elastic bond. The bond between the flakes was not a tight one as the plaques frequently altered their shape when transport conditions changed. At times individual flakes appeared to separate from the parent plaque, often to be drawn rapidly towards it again as the plaque moved on. The observation made by Irwin (1969) that the path of successfully transported droplets was repetitive over the same order of metachronal fields in a particular airway was also observed. The overall pattern of distribution of droplets, flakes and plaques was more dense in the cranial portion of the specimen. Under normal conditions it was very unusual to encounter any mucus particle larger than droplets in the distal intralobar bronchus and its branches distal to the fourth or fifth branching site, i.e. in medium sized bronchi and peripheral airways (Fig. 2). Therefore in the small to medium sized intralobar airways there was a paucity of mucus.

Two of the 28 SPF rats exhibited mucus hypersecretion. The airways of these two rats were translucent, and none of the topical features normally associated with chronic respiratory disease were present when they were inspected with the dissecting microscope using magnifications of up to $\times 320$. In both specimens ciliary activity was normal and no areas associated with CRD (par 3.1.1) were seen (see par 5.3). In areas where mucus transport rates could be compared, e.g. the heart lobe bronchus or trachea, they were similar to rates in other SPF rats (rates in the heart lobe bronchus for these two rats are included in Table 1). The two rats with hypersecretion of mucus had numerous flakes and plaques in all the airways except the terminal bronchioles. In the terminal bronchioles a continuous layer of fluid-like secretion, showing the appearance of streaming, was seen, but it did not resemble morphologically mucus observed elsewhere in the airways. Therefore, apart from the

hypersecretion of mucus, these two rats satisfied all the criteria of normality (see Definitions – par 3.1.1). However these two rats' airways were not subjected to histological examination and therefore some doubt must remain as to their absolute normality.

4.3 MUCUS TRANSPORT

Under normal conditions all types of mucus particles were transported towards the trachea. The precise mode of transport varied according to the size of the particles. Droplets were moved from one contiguous metachronal field (Iravani 1969) to another. These metachronal fields of irregular dimensions varied in extent from a few cells to several hundred cells. The organisation of the direction of ciliary beat and metachronal synchronisation of individual fields was entirely independent of neighbouring areas, (Fig. 3). Frequently the direction of the effective stroke of ciliary beat was divergent in adjacent fields, but it was never directed caudally thus confirming the observation made by Iravani (1969). The path of succeeding transported droplets was repetitive over the same order of metachronal fields in a particular area studied. The larger flakes and plaques spread over a wider area and were propelled by the combined action of numerous metachronal fields.

With the progressive diminution of the airways circumference towards the trachea, the quantity of mucus observed there was much greater than in the smaller airways. In the peripheral airways, mucus was transported as discrete entities, but in major extralobar airways flakes and plaques tended to congregate and were frequently transported in well-defined streams along the ascending metachronal fields (Fig. 2). These streams were up to 500 μm wide and often took an indirect course upwards. Several streams were often in operation simultaneously and were always clearly separated from each other.

4.4 LOCATION DEPENDENCE OF THE VELOCITY OF MUCUS TRANSPORT

Observations on the location dependence of the velocity of mucus transport could only be made in the most extensively prepared specimens. In twelve preparations, (5 SPF and 7 non-SPF), a clear view was obtained in the airway system from the level of at least two terminal bronchioles up to the trachea. It was not possible to dissect and inspect the entire length of the bronchial tree in all the specimens studied as this was

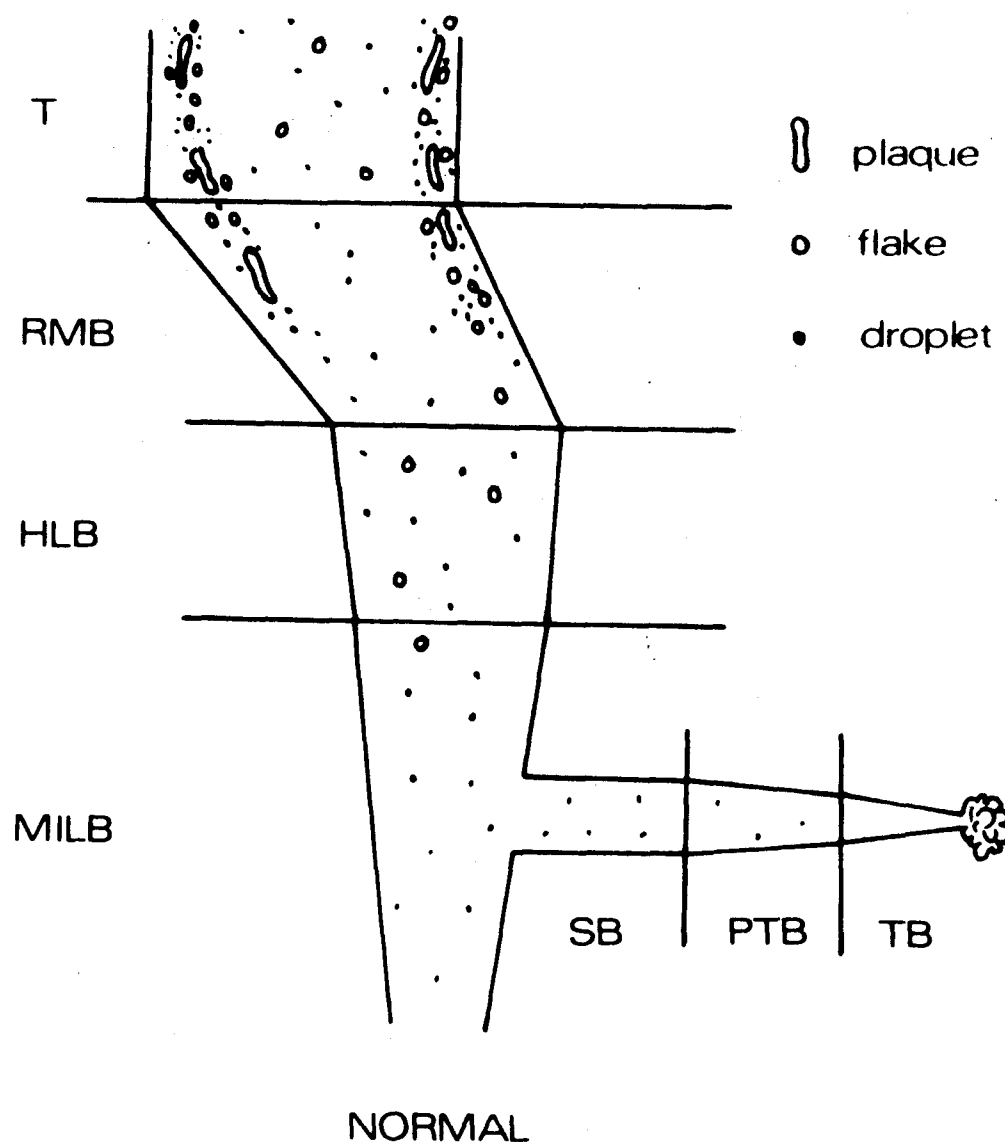


Figure 2 : Diagram of mucus in normal airways

A schematic representation of the distribution of the various particles of mucus i.e. droplets, flakes and plaques throughout the tracheobronchial tree in SPF rats.

TB = terminal bronchiole; PTB = preterminal bronchiole; SB = side bronchus;

MILB = main intralobar bronchus; HLB = heart lobe bronchus;

RMB = right main bronchus; T = trachea.

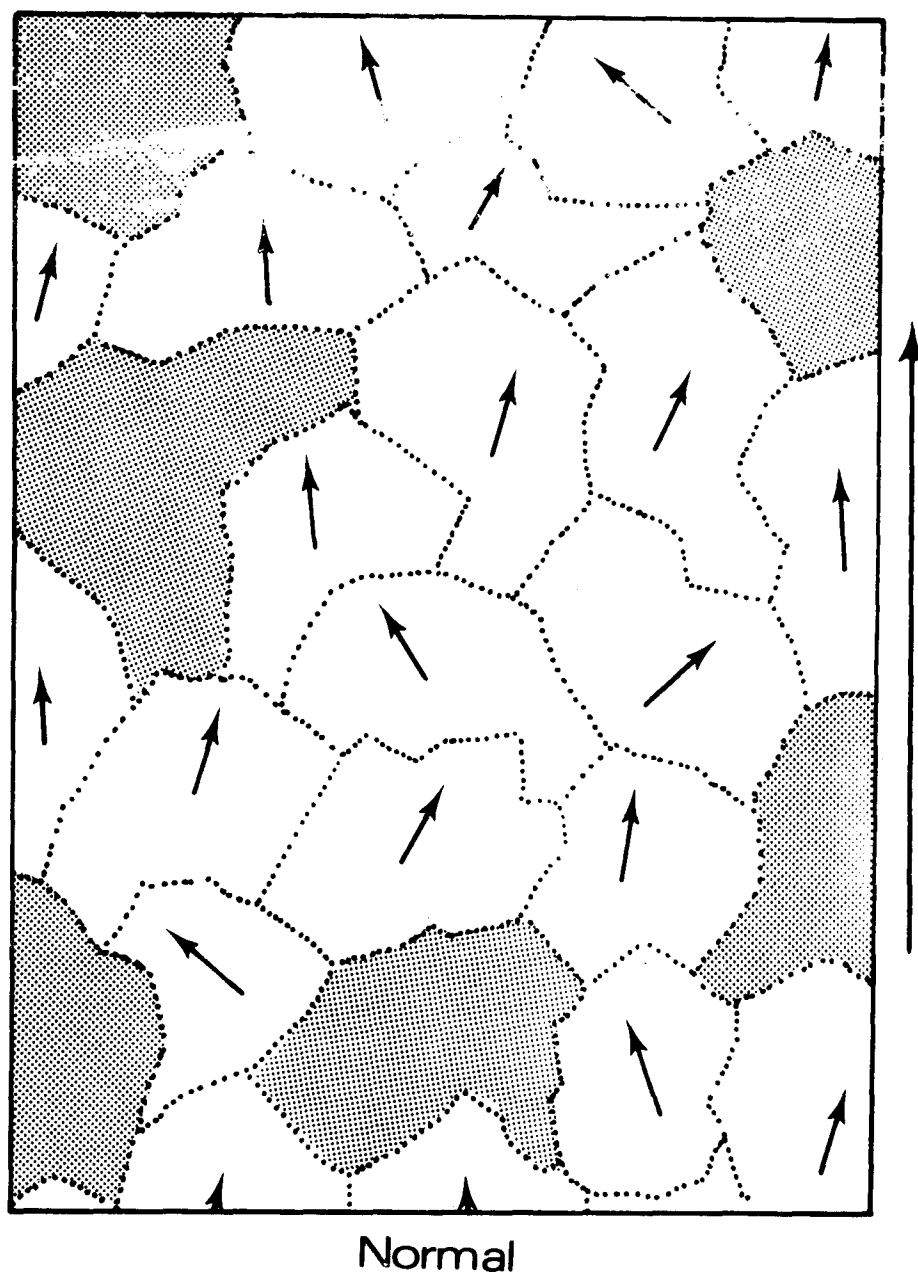


Figure 3 : Metachronal fields in a normal animal

Schematic representation of metachronal fields. The clear areas represent ciliated zones. The arrows within the stippled boundaries indicate the direction of the effective stroke of ciliary beat in those fields. These groups of cilia are metachronally synchronised in the individual fields. The grey zones are areas of goblet cells. The arrow on the right represents the overall direction of mucus transport cranially.

too time consuming. The velocity of the transport in the airways varied with the size of the mucus particles and with the location from which they were being transported. Most of the mucus in the more proximal airways system was transported as flakes and plaques. Small droplets which may have been freshly secreted from mucous cells could usually be followed for only a short distance before disappearing from the optical field.

Because of the many variables involved in the measurement of mucus transport velocities, it was decided to standardize this parameter. Therefore only the velocity of flakes between 10 to 20 μm in diameter was measured. Mucus transport rates at different levels of the bronchial tree could then be compared. In comparing different experiments the velocity of flakes in one specific region, i.e. the main lobar bronchus above its first branching site was measured. As the production of mucus distally in the pulmonary tree is normally sparse, the most comprehensive measurements were made in rats with mucus hypersecretion but with otherwise normal bronchi and ciliary activity (see par 4.2). The rats with hypersecretion yielded valuable information on the location dependence of transport as well as the absolute rates of transport in the very small airways. Flakes of mucus, which were timed to obtain transport rates, were seen very readily in the small airways of these two rats but not in the other SPF and non SPF animals whose airways were dissected completely.

Fig. 4 is illustrative of the location dependence of the velocity of mucus transport as measured in a rat with hypersecretion. It is apparent that the average velocity increased progressively cranially from a value of approximately 0,4 mm per min in the preterminal bronchioles to 11,5 mm per min in the upper portion of the lobar bronchus. This represents a 28 fold increase of velocity gradient in this relatively distal portion of the airway. On two separate occasions transport rates in the trachea of SPF and non SPF animals were timed at 15 mm per min. Thus assuming a rate of 0,4 mm per min in the preterminal bronchiole the velocity gradient would be nearly 40 fold.

Although overall there is an increase in velocity gradient cranially, it was observed that similar sized flakes in closely adjacent sites at the same level were transported at very different velocities. The variation of velocity was the consequence of the variation of the rate of ciliary beat in adjacent metachronal fields. Transport velocities

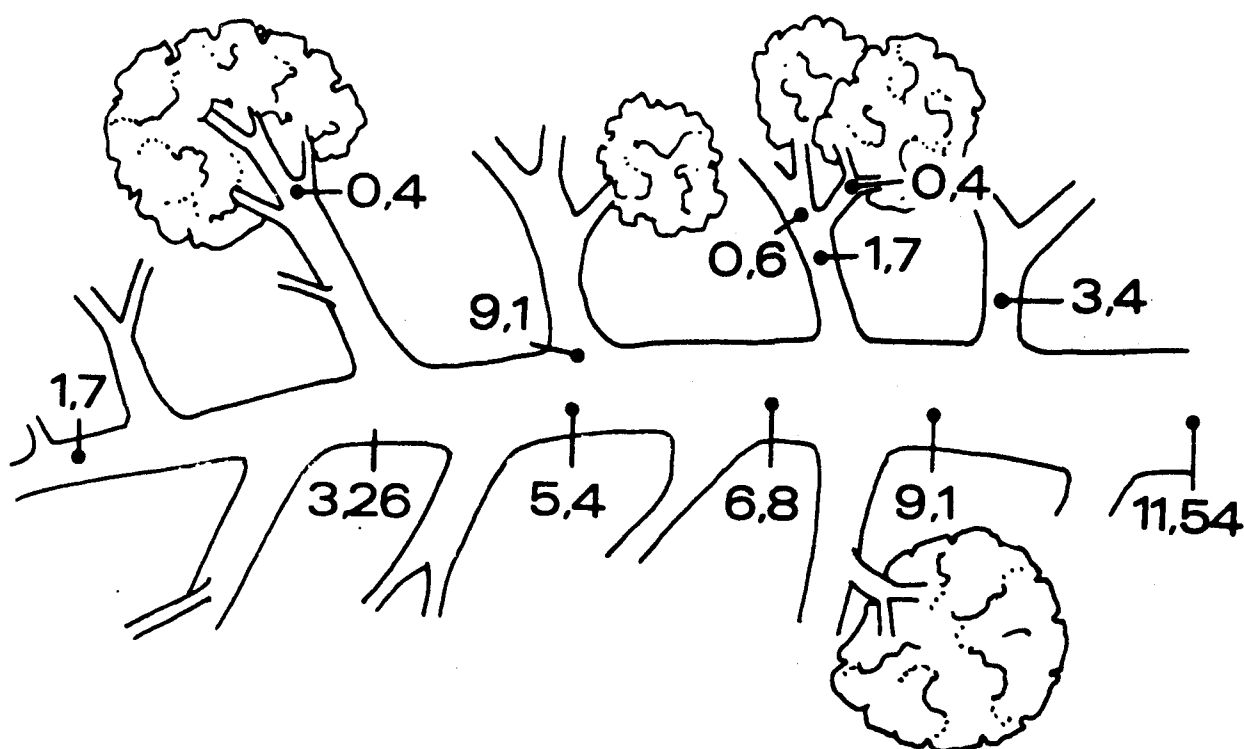


Figure 4 : Location dependence of mucus transport

Location dependence of velocity of mucus transport depicted in the airways of the right medial inferior lobe. Figures indicate the velocity of transport in mm per min at a particular site at 37°C. Diagram constructed through a drawing tube. X 10.

(After Iravani and van As, 1972.)

also varied considerably from rat to rat (Table I) and on this criterion alone no animal could be identified as normal or abnormal. For this reason (and reasons discussed in par 4.2), it was thought to be justifiable to use rats with hypersecretion to construct the location dependence of velocity, even though it may lead to a slight overestimate of transport velocities. The overestimation may result from the fact that conditions of hypersecretions ("chronic bronchitis") tended to result in higher transport rates than found in SPF animals (par 5.3 and Table I).

TABLE I – MUCUS TRANSPORT VELOCITIES^x

Normal (SPF) ⁺			"Chronic bronchitis" (CRD)*			Acute bronchitis**
m	n	SD	m	n	SD	m
++ 3,7	11	1,36	21,7	8	4,01	0,25
8,0	12	1,48	8,2	9	0,97	1,0
4,0	10	1,30	6,8	7	0,93	
2,1	10	0,85	4,1	10	0,96	
4,8	13	1,31	4,9	12	1,02	
4,8	11	1,28	13,4	15	3,15	
9,0	12	2,34	5,9	9	1,33	
3,6	9	1,32	6,9	11	1,44	
5,4	8	1,11	10,5	8	1,43	
3,8	9	0,73	4,9	10	1,03	
4,9	10	0,78	4,4	7	1,31	
			15,0	9	1,43	
			15,0	11	2,41	
			4,3	6	2,75	

x All transport velocities were measured in mm per min in the right inferior medial lobar bronchus just above the level of the first intralobar branching site.

++ Each value is the mean of the indicated number of observations (n) taken in as rapid sequence as transport conditions occurred.

+ The mean for the 11 experiments is 4,9 mm per min; range 1,2 and 12,0 mm per min.

* The mean for the 14 experiments is 9,0 mm per min; range 0,75 and 27,2 mm per min.

** Mucus transport occurred in only 2 of the 4 rats in which acute bronchitis was produced. No statistical evaluation has been included because of the small number of observations.

4.5 PULMONARY CLEARANCE OF INHALED PARTICLES

Fig. 5 demonstrates the distribution of charcoal particle aggregates seen 19 h after the inhalation of charcoal dust by a SPF rat. This pattern was representative of that seen in all five SPF rats exposed to the same conditions. It is apparent that the pulmonary clearance of charcoal in young animals living under SPF conditions is slow. Well demonstrated is the retention of aggregates of charcoal at the branching sites of main and side bronchi. Microscopically the particles appeared to be impacted on the epithelial surface as aggregates of charcoal. Normal ciliary activity could be observed in the area of the deposit as well as in adjacent regions. Histological examination of cross-sections of the areas where charcoal retention occurred, revealed a layer of particles lying on the ciliary epithelium. In contradistinction to the slow clearance of charcoal by SPF rats, the airways of the 5 non-SPF rats had almost completely been cleared of the particles. The only sites at which charcoal was seen were on "bronchitic" areas or on areas of whirlpool formation.

4.6 SUMMARY

Under SPF conditions the secretion of mucus in the pulmonary airways is sparse, most of the mucus being observed in the extralobar bronchi. No evidence for a continuous mucous blanket was found and a paucity of mucus was a striking feature. The transport of mucus was always in a cranial direction, the particles of mucus tending to congregate in the larger airways and be transported in well defined streams. The flow of mucus in these streams was intermittent but repetitive over the same order of metachronal fields. A velocity gradient of mucus transport was observed from the level of the terminal bronchioles up to the trachea. The order of this gradient was nearly 40 fold.

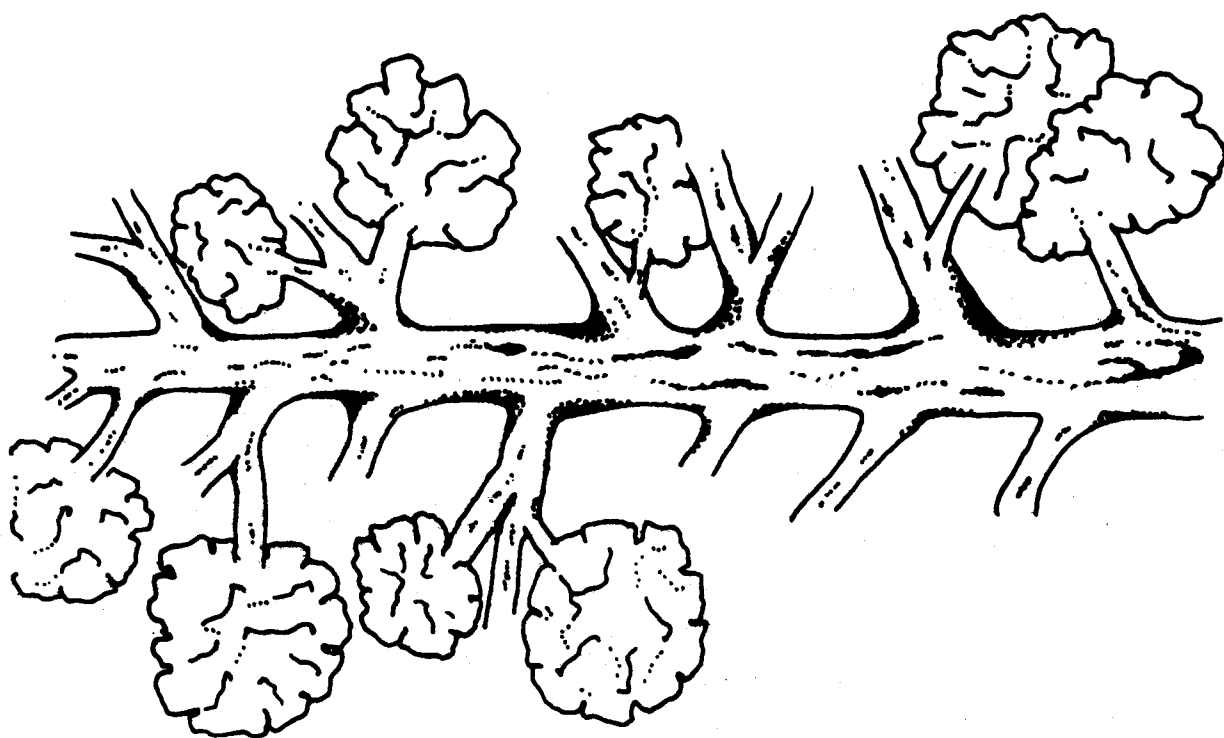


Figure 5 : Deposition of charcoal in airways

Intralobar airways of a SPF rat exposed to a charcoal dust cloud (1 g/m^3) for 5 h and examined 19 h later. Deposition of charcoal occurred mainly at bifurcations.

Diagram constructed through a drawing tube. x 12 (After Iravani and van As 1972.)

CHAPTER 5

RESULTS – “BRONCHITIC” RATS

5.1 INTRODUCTION

Once the pattern of mucus secretion in the airways had been recognised, as described in the previous chapter, the systematic study of tracheobronchial clearance under abnormal conditions could be undertaken. In the first instance non-SPF rats were used as they develop “chronic bronchitis” spontaneously. This abnormality has conveniently served as a bronchitic model. In this chapter the abnormalities observed in “bronchitic” rats are described. The morphology of the bronchial epithelium as seen with the scanning electron microscope in both normal and “bronchitic” rats is considered in this chapter.

“Bronchitis” developed spontaneously in the older and mature rats, or could be induced by exposing the animals to cold, moist conditions repeatedly. The presence of “bronchitis” was detected by easily palpable or audible rhonchi. During the course of the investigation two varieties of bronchitis were identified. They were acute bronchitis and “chronic bronchitis.”

5.2 ACUTE BRONCHITIS

This distinct syndrome was produced in only four rats. In order to precipitate the syndrome, rats were exposed alternately to freezing, moist conditions for 0,5 h and room temperature for 0,5 h over a period of 12 h. It was apparent that these animals became acutely ill, exhibiting sniffing and sneezing with audible wheezing, and were in respiratory distress. Their bronchial walls were always normally transparent but when the trachea was opened, mucoid secretion was found in 3 and in one it was mucopurulent. Examination of ciliary function in these 4 rats revealed many small patches (approximately 20 μm diameter) of ciliary inactivity, diffusely spread throughout the entire tracheobronchial tree. In addition to this, also diffusely distributed, there were small areas of abnormal ciliary activity showing ciliary inco-ordination and alteration of the direction of the effective stroke of ciliary beat. Thus mucus could be seen to be transported caudally in some areas or to follow a completely inco-ordinated course travelling at times caudally or laterally and then cranially. Although the velocity of

mucus flow over very short distances (20 to 30 μm) was regionally high, the overall cranial movement over significant distances was very sluggish. This overall slowing of transportation of mucus, despite the visual impression of very vigorous ciliary activity, probably resulted from the proportionately large area of mucous membrane with impaired pluricellular co-ordination. The rates of mucus transport are shown in Table I. Data on mucus transport in only two animals are available as the secretion of mucus did not develop in the other two (paragraph 4.2).

5.3 "CHRONIC BRONCHITIS"

"Chronic bronchitis" occurred spontaneously in many mature rats and consisted of the formation of multiple abscesses in one or several lobes very often involving entire lobes. An animal was accepted as suitable for the experiment if the heart lobe was not significantly involved by abscess formation. On opening the trachea, the mucus obtained was usually viscid and yellow-green in colour. On examining the dissected airways, many adherent discrete foci consisting of peribronchial inflammation were seen macroscopically and microscopically (Fig. 6). These foci occurred more frequently in the upper portion of the large bronchi and trachea. The tissues comprising the patches of peribronchitis could only be removed from the airway walls with some difficulty. Once these areas had been cleared, the underlying epithelial activity could be studied. It was found that most of the disturbances of mucociliary function occurred on the mucous membrane related to these areas of inflammatory change. All other areas with normally transparent walls were normal or only very rarely disturbed.

Under the areas of bronchial wall involved in the inflammatory process, two main abnormalities of mucociliary function were observed. They were —

- (i) Inactive zones in which no mucus transport or ciliary activity could be observed. These areas varied considerably in size, the largest zones being approximately 2 mm long and several hundred μm wide. However, in most instances these areas were smaller and only a few, or sometimes groups of metachronal fields were inactive. The small zones of ciliary inactivity did not impair mucus transport as it was moved by means of traction by adjacent cilia. Only on the larger zones of ciliary inactivity did stasis of mucus occur. Such areas of stasis are demonstrated in Fig. 9. In these areas ciliary movement was not observed and charcoal-labelled particles of mucus were

not transported for the duration of the experiment. In these areas the damage was extensive and transport was grossly impaired. In a few non-SPF animal numerous such areas were observed which resulted in an impaired overall clearance of mucus in the segment which was affected. In some zones a few cells could be stimulated to activity by rinsing their surfaces with physiological saline, via a thin catheter passed down the airway.

(ii) Active ciliated cells and cell groups with altered function.

The alterations of function consisted of:

- (a) Reversal of the effective stroke of ciliary beat in one or more cells. Thus in these cells the effective stroke of the cilia was mainly in a caudal direction, and consequently resulted in a reversal of mucus transport in that area. These areas extended from several cells up to several adjacent metachronal fields.
- (b) Disturbances of pluricellular metachronal co-ordination. The co-ordinated metachronism of ciliary beat in a cranial direction was absent and a disorganised individual activity of ciliated cells was apparent.
- (c) Altered beat form of the cilia. As opposed to the pendular effective and recovery stroke of the groups of cilia, it was noticed that a funnel-shaped type of activity was present. The effective stroke occurred in one semi-circular plane and the recovery stroke in the opposite plane giving the appearance of looking down on a funnel from above.

The abnormalities of the ciliated epithelium in "chronic bronchitis" had a profound effect on mucus transport, (Fig. 7). In larger zones exhibiting ciliary inactivity no mucus transport could be observed. In areas showing pluri-cellular metachronal inco-ordination and altered ciliary beat form, ineffective mucus transport was present. Reversal of the effective stroke of ciliary beat resulted in the caudal transport of mucus, and the consequent formation of whirlpools as the mucus was transported cranially and then caudally again on adjacent metachronal fields. Individual components of mucus arriving on these whirlpools could rotate without being transported cranially for several hours, or until by chance cranially directed metachronal fields picked them up. These whirlpools measured from 20 to 400 μm in diameter.

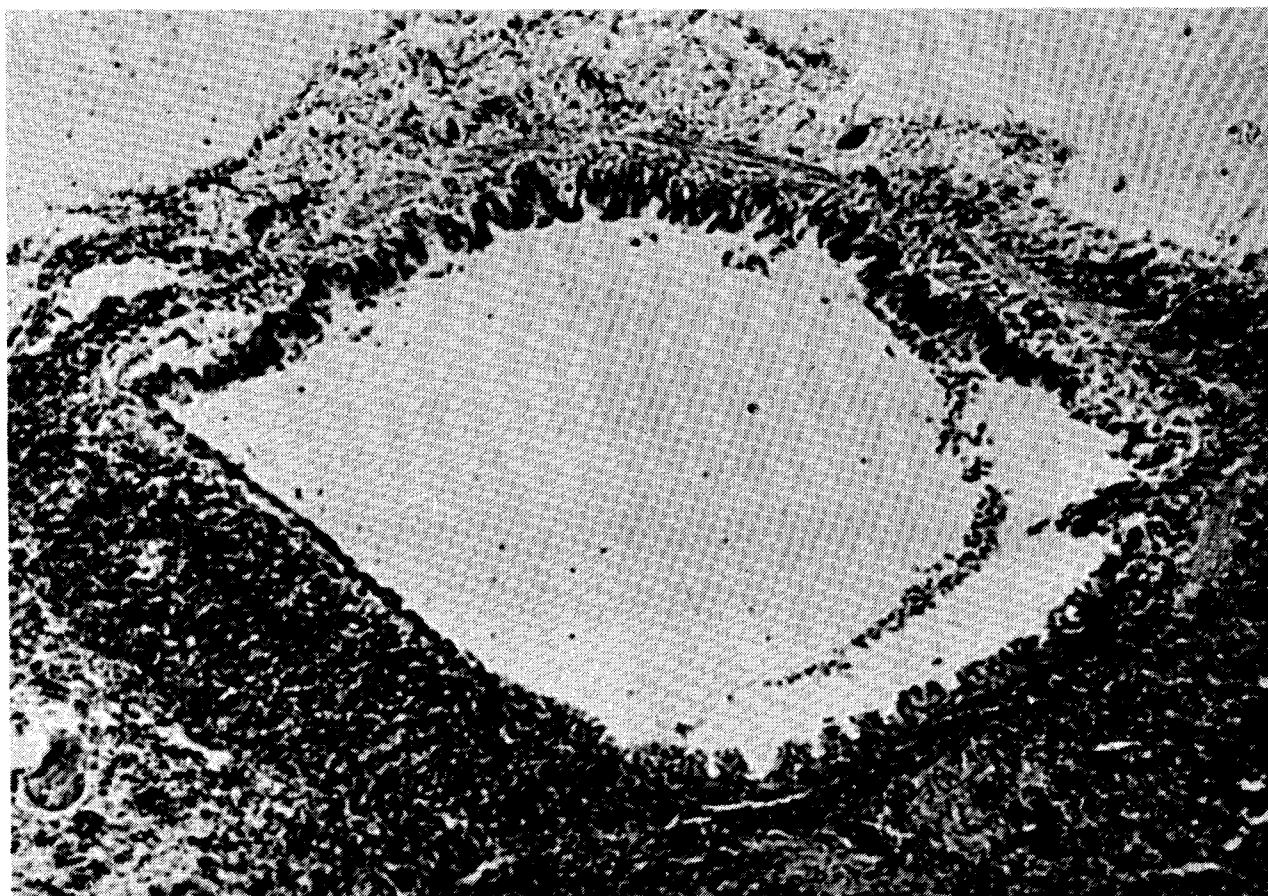
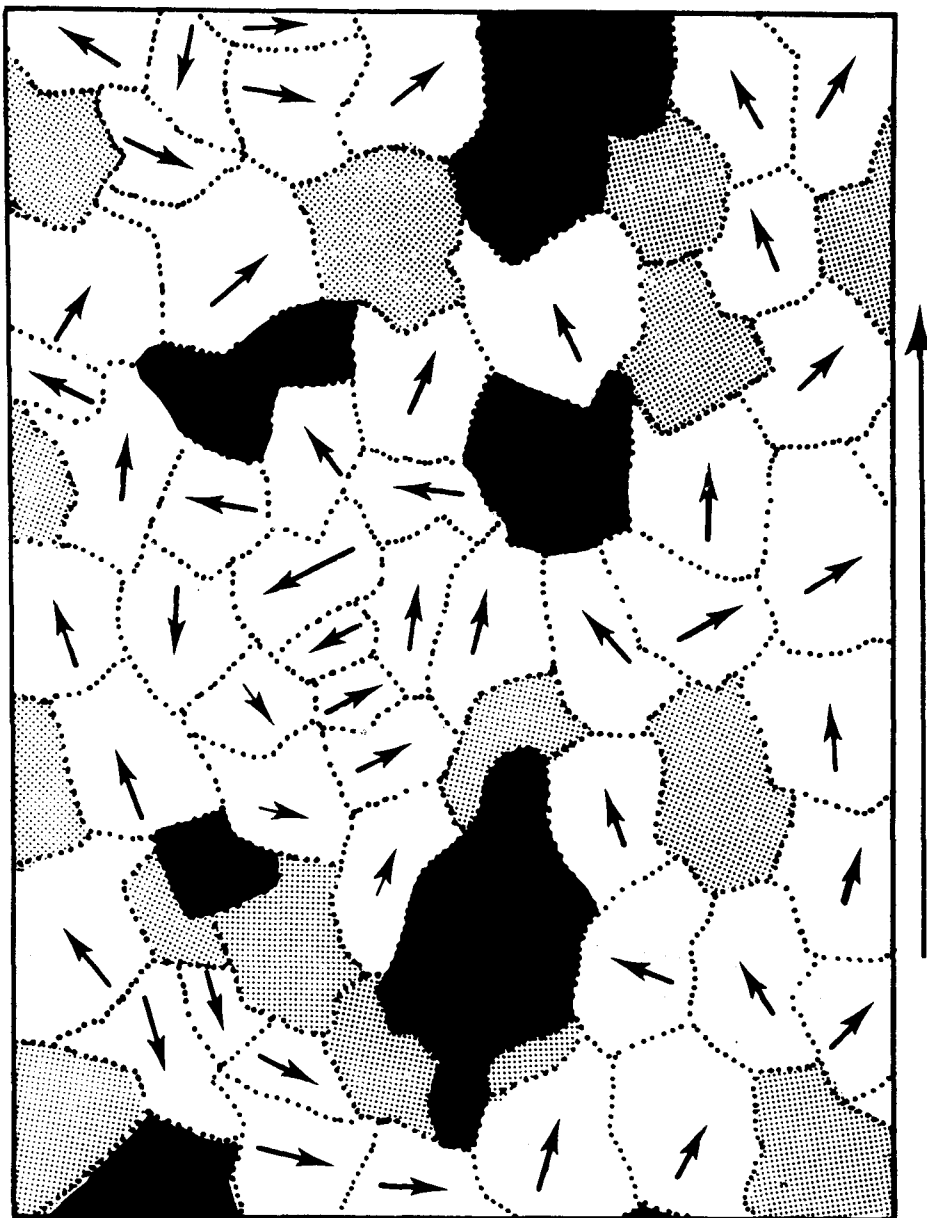


Figure 6 : Pathology of chronic respiratory disease

Cross section of the right main bronchus taken from a non-SPF rat. The area selected had bronchial wall thickening and exhibited abnormal ciliary activity and impaired mucus transport. A lymphocytic infiltrate is present, and the ciliary epithelium is abnormal in places. Haematoxylin and eosin. X 275.



Bronchitis

Figure 7 : Metachronal fields in a “bronchitic” animal

Diagrammatic representation of metachronal fields in a “bronchitic” area. The fields are separated by dotted lines. The grey areas are zones of goblet cells. The black areas represent zones of abnormal ciliary beat, ciliary inactivity and squamous metaplasia. The large arrow indicates the cranial direction. The small arrows show the direction of the effective stroke of ciliary beat in individual metachronal fields. In many areas this direction is retrograde and it can be seen how whirlpool formation occurs in mucus transport.

The pattern of mucus secretion was found to be basically similar to that seen in normal rats. The main difference was a quantitative one, much more being present both initially and when mucus secretion recommenced after rewarming the preparation. The larger flakes and plaques were seen more frequently lower down in the bronchial tree as in the case of rats with hypersecretion. In contradistinction to the normal appearance, a continuous stream of watery secretion was present in the proximal portion of the terminal bronchioles. However, the method of transport was an unco-ordinated streaming giving the appearance of turbulence (Fig. 8).

Average velocities of transport were usually higher than found in the normal animals (Table I). Here again, as in the normal rats, the velocity of transport varied with the size of the mucus particle, site of transport, and at adjacent sites for similar sized flakes. Particularly well demonstrated was the linear acceleration and deceleration of individual flakes and plaques within a distance of 100 μm or less. The rate of movement was determined by the degree of underlying ciliary activity. However, location dependence of velocity of transport was preserved, there being a general acceleration of velocity cranially.

5.4 PULMONARY CLEARANCE OF INHALED PARTICLES

Airways prepared after exposure to charcoal by inhalation depicted the transport of mucus in the large bronchi particularly well. For this purpose three non-SPF rats were exposed to a charcoal dust cloud with a 5 μm particle size at a concentration of 200 mg/m^3 . The passage of the streams could be recorded photographically. An example of this is shown in Fig. 9. This preparation illustrates the retention of charcoal on abnormal areas just above the bifurcation of the trachea; the transportation of mucus in a stream up the left aspect of the posterior membrane culminating in an area of retention; and the retrograde streaming and subsequent cranial transport of mucus in the upper portion of the trachea. This figure thus illustrates many aspects of both normal and abnormal transport. Of particular interest is the demonstration of areas where no movement of charcoal was seen at all. In these preparations it was clear that charcoal was transported only when attached to mucus particles. Mucus, on the other hand, moved only over areas where ciliary activity was visible. Charcoal, which had been deposited on areas where no ciliary activity was discernable, remained static. Similarly charcoal-labelled mucus which found its way onto an inactive area

would remain static until occasionally, by chance, a passing plaque came into contact with the static mucus, adhered to it, and pulled it off by traction. Adjacent to these bronchitic areas, ciliary activity was usually vigorous and mucus transport normal. However numerous areas of retrograde transport and whirlpool formation were observed.

5.5 SCANNING ELECTRON MICROSCOPY

Two SPF and 3 non-SPF rats were prepared using Method I and 5 SPF and 4 non-SPF rats using Method II (par 3.3). Photomicrographs of tracheal specimens are shown in Figs 10–18. Although mucus was clearly demonstrated by both techniques, it was observed that significant areas of the surface of all the specimens showed very little or no mucus. The results obtained indicate that it was difficult to distinguish between SPF and non-SPF rats using the topical ciliary and mucus morphology as indices. The main purpose, however, of this investigation was to attempt to document the surface morphology of mucus, and to confirm the presence or absence of a mucous blanket. No definite comparisons between SPF and non-SPF morphology were attempted. The results shown in Figs 10–18 are examples of the morphology observed in both SPF and non-SPF rats. No striking difference of the distribution of mucus between SPF or non-SPF animals could be demonstrated using Method I or Method II on either type of tissue. The photomicrographs (Fig. 10–18) were selected as they are representative of the surface morphology observed.

The results obtained with the preparation of a non-SPF rat trachea by Method I are shown in Figs 10–13. Fig. 10 illustrates the general configuration of mucus particles in an area of approximately 6 mm². The overall direction of mucus transport is towards the top. The width of the collection of mucus here is approximately 100 µm. Figs 11–12 illustrate the variability in size of mucus particles as well as the structure of the components. These figures show mucus particles on the ciliary surface of trachea varying from 0.5 to 10 µm diameter, lying either singly or grouped together. The smaller particles are homogeneous, but the larger ones appear to be made up of a number of particles. This is particularly evident when Fig 13 is examined. The larger particles of 5–10 µm have a laminar appearance and an uneven surface. It seems that these larger particles are an aggregation of the smaller ones as a result of adhesion and stratification. The smaller particles of 1–3 µm most probably represent the small-

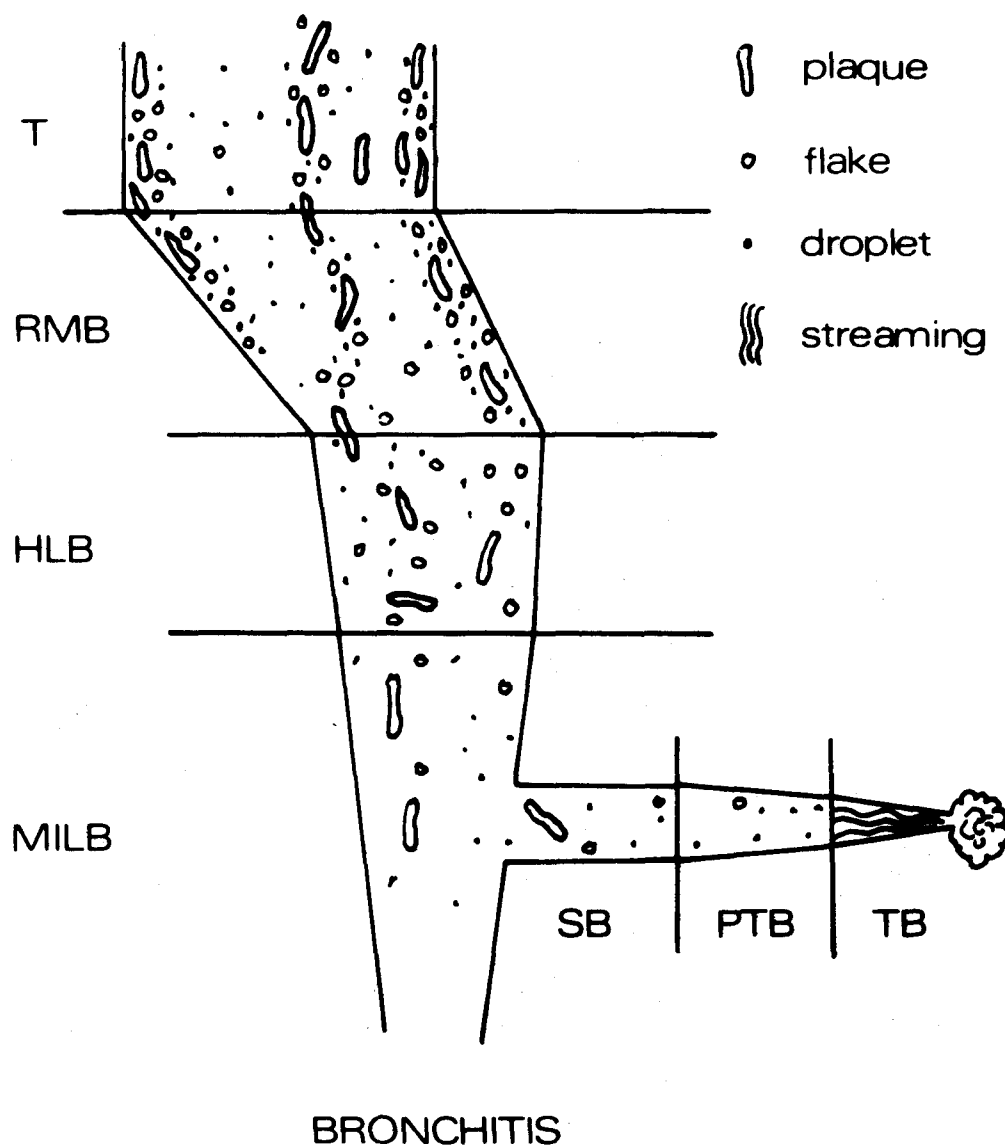


Figure 8 : Diagram of mucus in "bronchitic" airways

Schematic representation of the distribution of mucus particles in the tracheobronchial tree of non-SPF rats with "bronchitis". A similar distribution was observed in two normal animals with mucus hypersecretion. The principal difference between normal and "bronchitic" rats as regards mucus secretion is a quantitative one.

(See figure 2 for abbreviations)

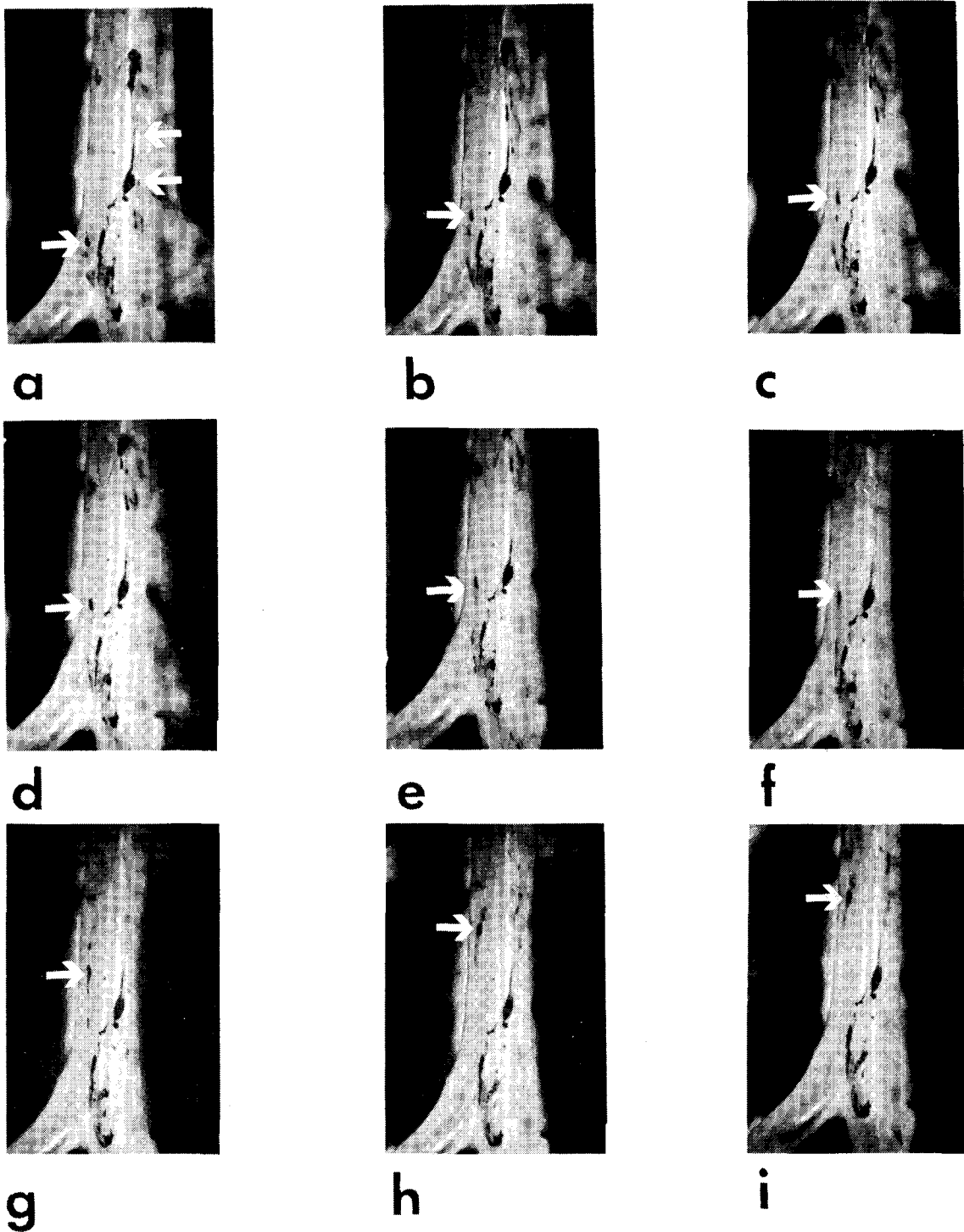


Figure 9 : Charcoal labelled mucus transport

Airway preparation after exposure of a non-SPF rat to charcoal dust particles for 2 h at a concentration of 200 mg/m^3 . The individual photographs were taken 5–8 sec apart while the temperature of the preparation was raised and mucus transport recommenced. The specimen is viewed through the membranous trachea. The right hand lower arrow in (a) shows a “bronchitic” area with retention of the marker throughout the duration of the experiment. The right hand upper arrow shows a labelled stream which is transported completely during the period of observation. Similarly the left hand arrows (a–i) show the formation of a stream which is transported normally. The other static patches of charcoal in the region of the tracheal bifurcation are also “bronchitic” areas.

est particles of mucus secreted and are presumably derived from goblet cells. The larger ones may be secreted as such from mucous glands or more probably are built up from a large number of smaller particles.

On the specimens of trachea examined, mucus particles were usually grouped together leaving large areas of intervening mucosa free of significant amounts of mucus. Mucus particles observed on these bare areas were usually smaller than 5 μm in diameter. On the entire surface of the segments of trachea scanned, only one other area had a significant collection of mucus. A portion of this collection of mucus is shown in Fig. 14. Many small particles of various diameters are present as well as one larger one, made up of numerous droplets.

The surface morphology illustrated in Figs 10 – 14 does not show cilia clearly. This is thought to be due to the fact that the freeze-drying technique used caused shrinkage of the tissues, and the surface tension forces during the drying process resulted in clumping or aggregation of the cilia. Thus, after the specimen had been studied, it was embedded in an epoxy resin and 60 sections each 2 μm thick were cut. The tissue was stained with haematoxylin and eosin. Inspection of the ciliary surface with a light microscope under oil immersion at a magnification of $\times 1000$ showed that the most distal portions of the cilia were folded and enmeshed in each other, giving the appearance of a solid layer, which could be mistaken for a mucous blanket. Above the cilia no evidence for a mucous blanket was found. Due to the fact that the mucus was so sparse, the plaques seen on the surface scanning views could not be found on the cross-sections.

Figs 15 – 18 illustrate the results obtained with Method II utilizing a SPF rat. The ciliary detail is clearly seen together with the surfaces of goblet cells and intercellular divisions. Collections of mucus and individual particles were found easily but not in as large a quantity as illustrated by preparation of the tissue by Method I. This may be due to the fact that SPF animals secrete less mucus than non-SPF animals.

5.6 SUMMARY

The principal difference between normal and "bronchitic" rats as regards mucus secretion is a quantitative one. In acute and "chronic bronchitis" there is an increase in the number of mucus particles in distal airways. However, even under conditions of mucus

hypersecretion associated with "chronic bronchitis", a continuous mucous blanket was never observed. In contradistinction to normal animals larger mucus particles, namely plaques and flakes were found lower down in the respiratory tract in "bronchitic" rats. Although mucus transport was totally disorganised under conditions of acute bronchitis, the overall rates of transport were somewhat greater in "chronic bronchitis" (mean 9,0 mm/min) than in normal animals (mean 4,9 mm/min) provided that the damage to the ciliary epithelium was not too great. The abnormalities of ciliary activity were most striking. In association with acute bronchitis there was a complete breakdown of the normal pattern of ciliary activity. More organised abnormalities were associated with "chronic bronchitis". In addition, the morphology of surface related structures as seen on the SEM are described. The morphology of mucus which is well demonstrated by this technique corresponds closely with the pattern which is recognised under physiological conditions in both normal, and "bronchitic" rats.

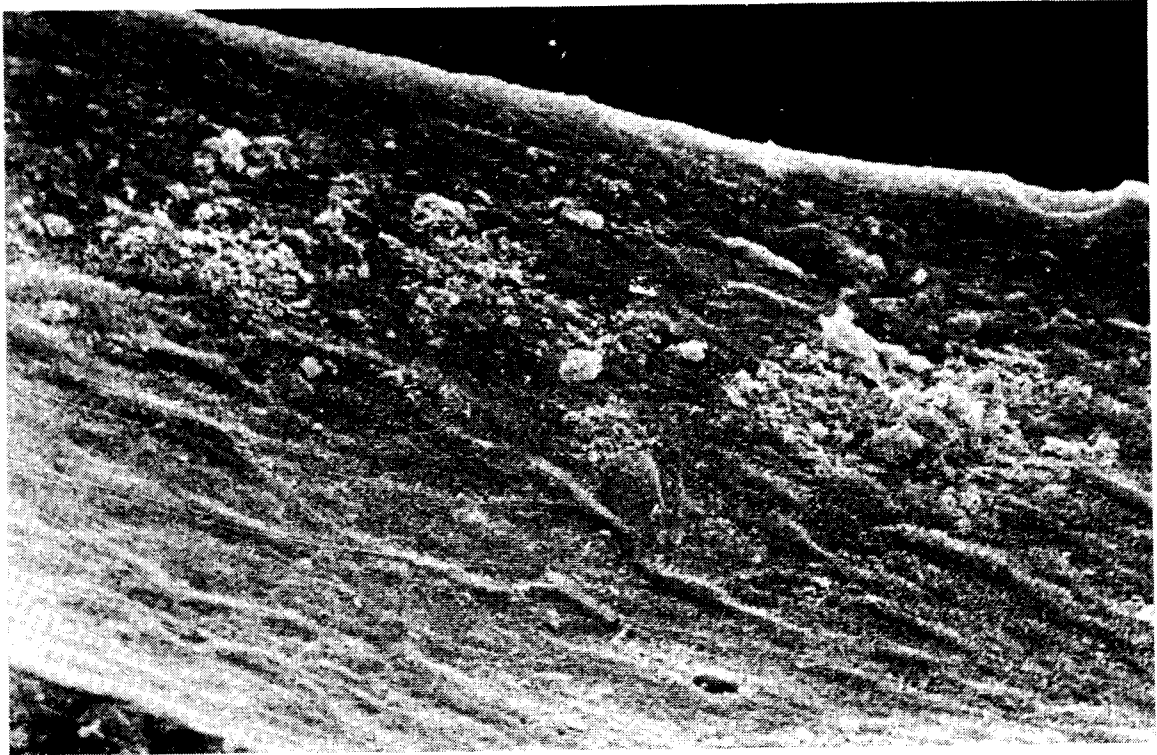


Figure 10 : SEM – overall view of mucus

Collections of mucus particles constitute several plaques. Particles of mucus are visible on an otherwise bare ciliary surface. Freeze-dried specimen. X 1500.

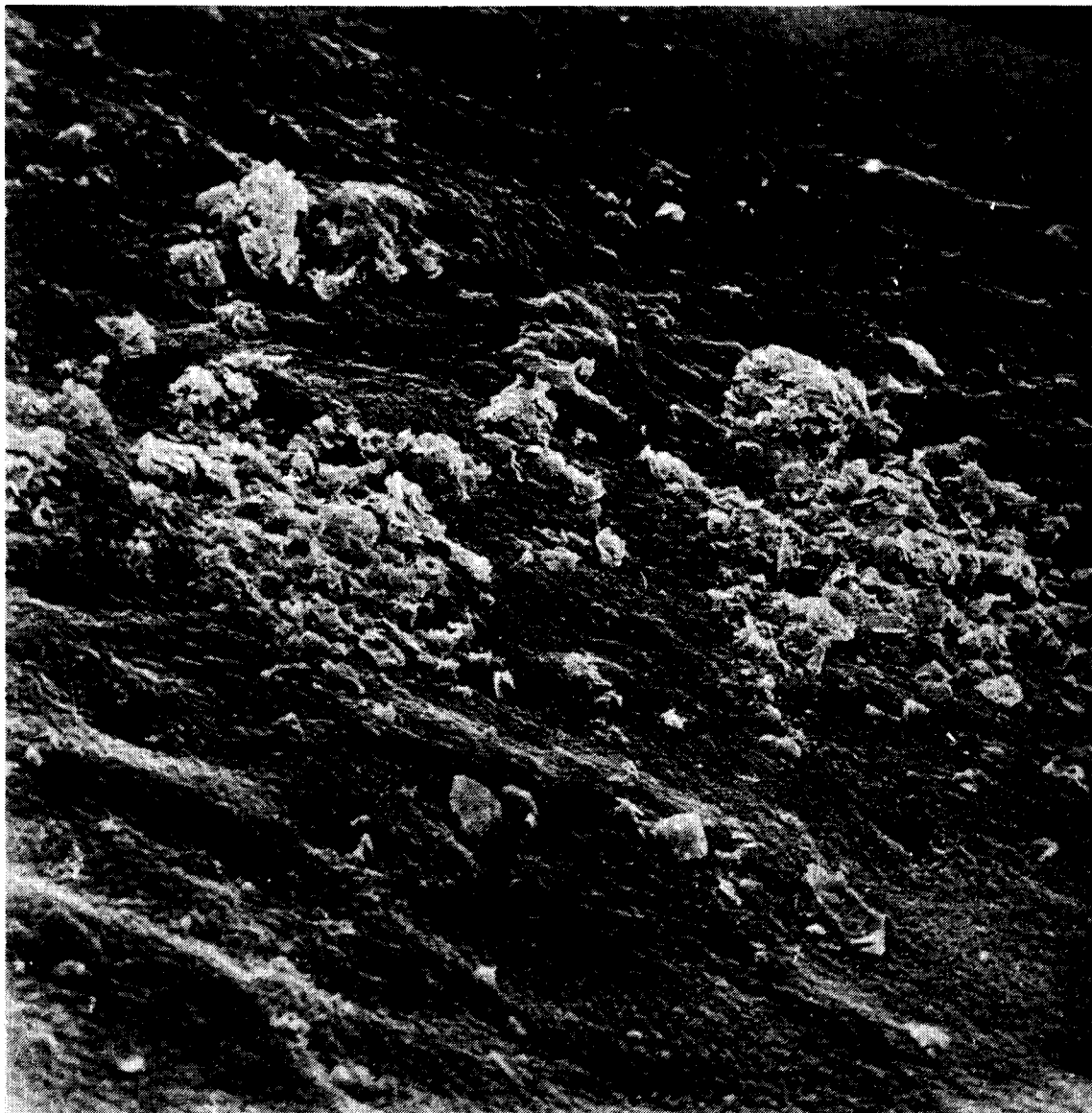


Figure 11 : SEM — view of two plaques

View of two plaques seen on the left side of figure 10. X 2 500

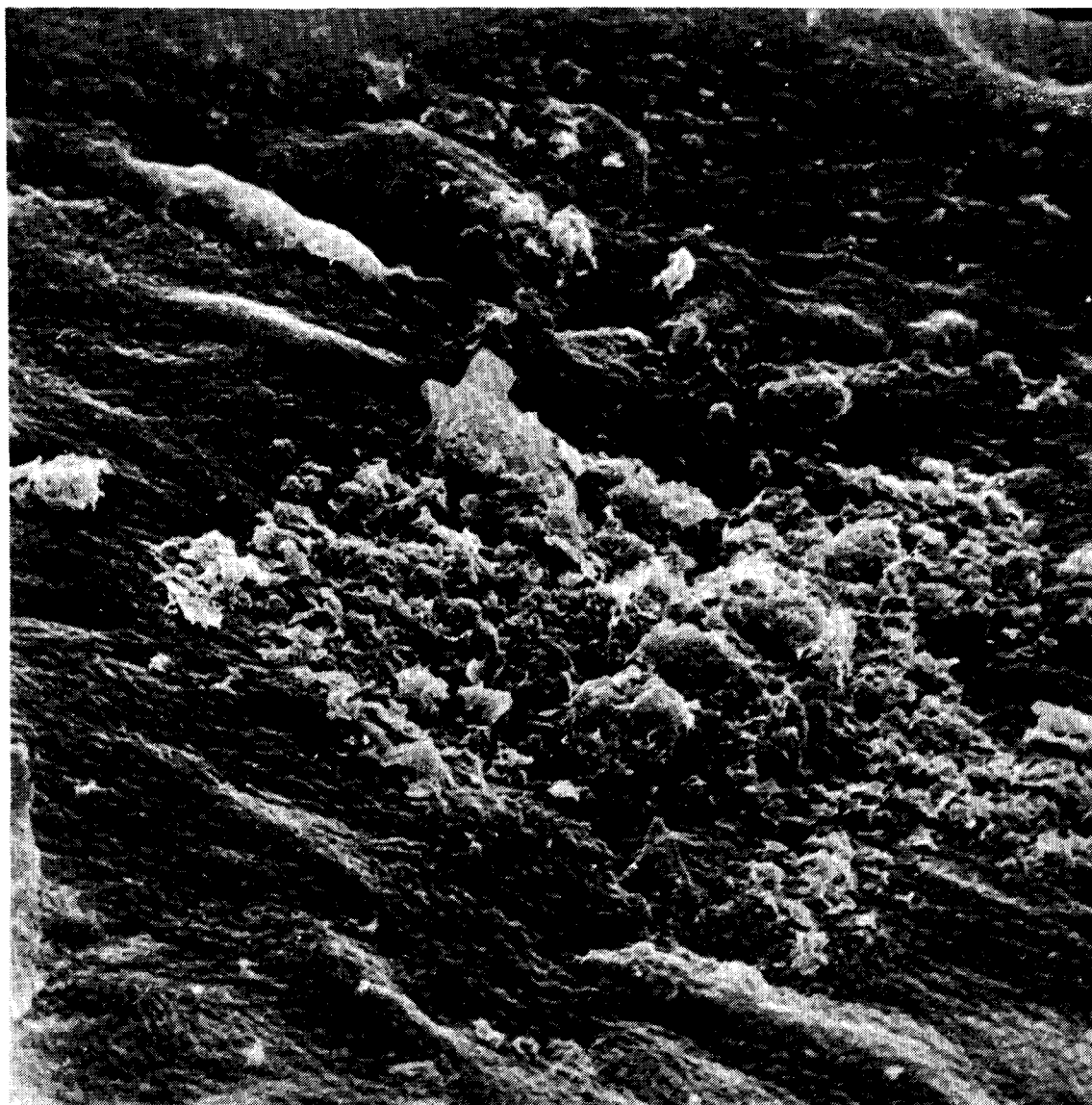


Figure 12 : SEM – view of one plaque

View of plaque on right hand side of figure 10. X 3,000.

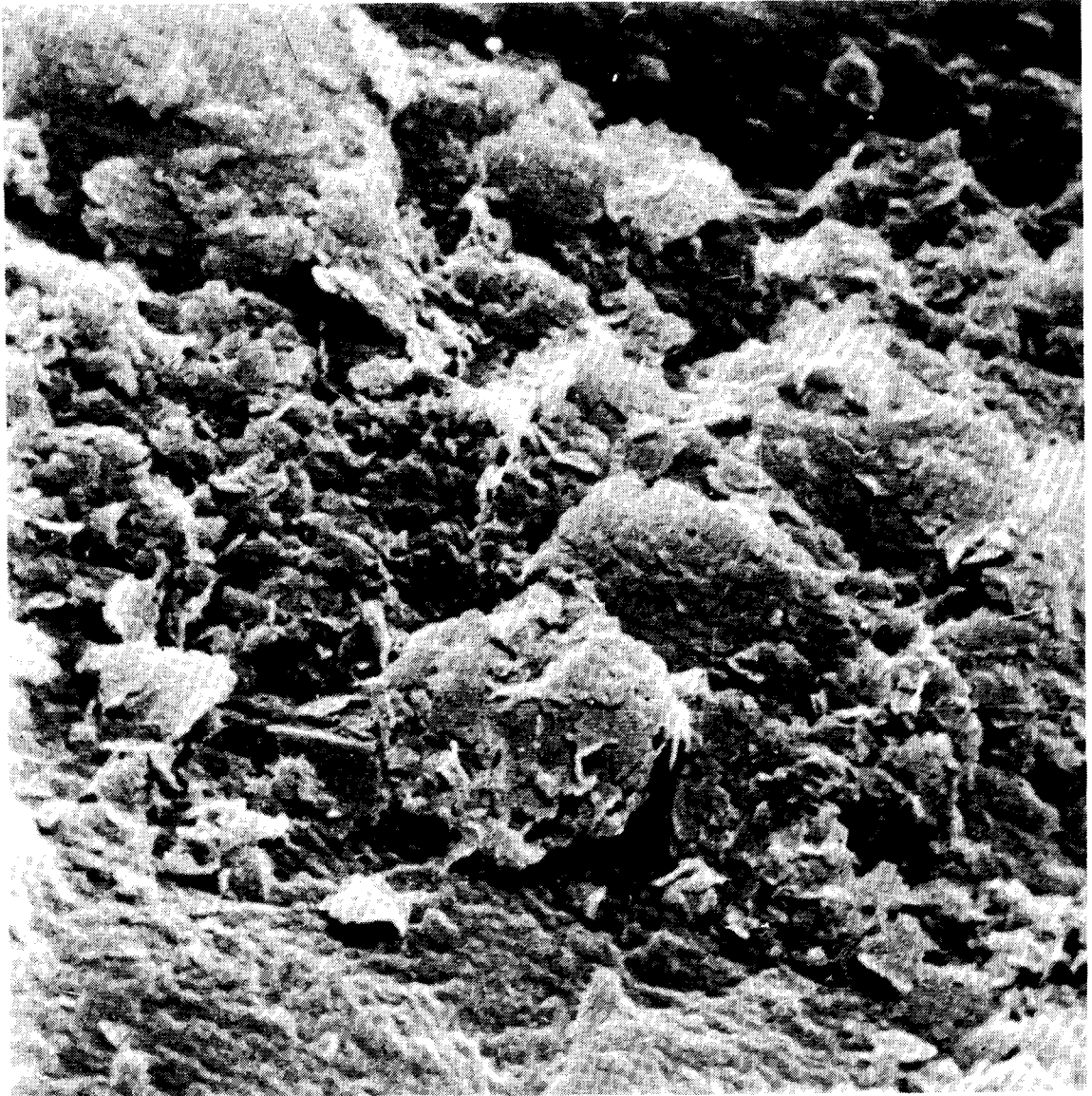


Figure 13 : SEM — composition of mucus particles

The composition of a plaque is shown. Droplets (well seen in the foreground) appear to be aggregated together to form the larger flakes. The plaque is then constituted by these larger and smaller mucus particles gathering together. X 8 500

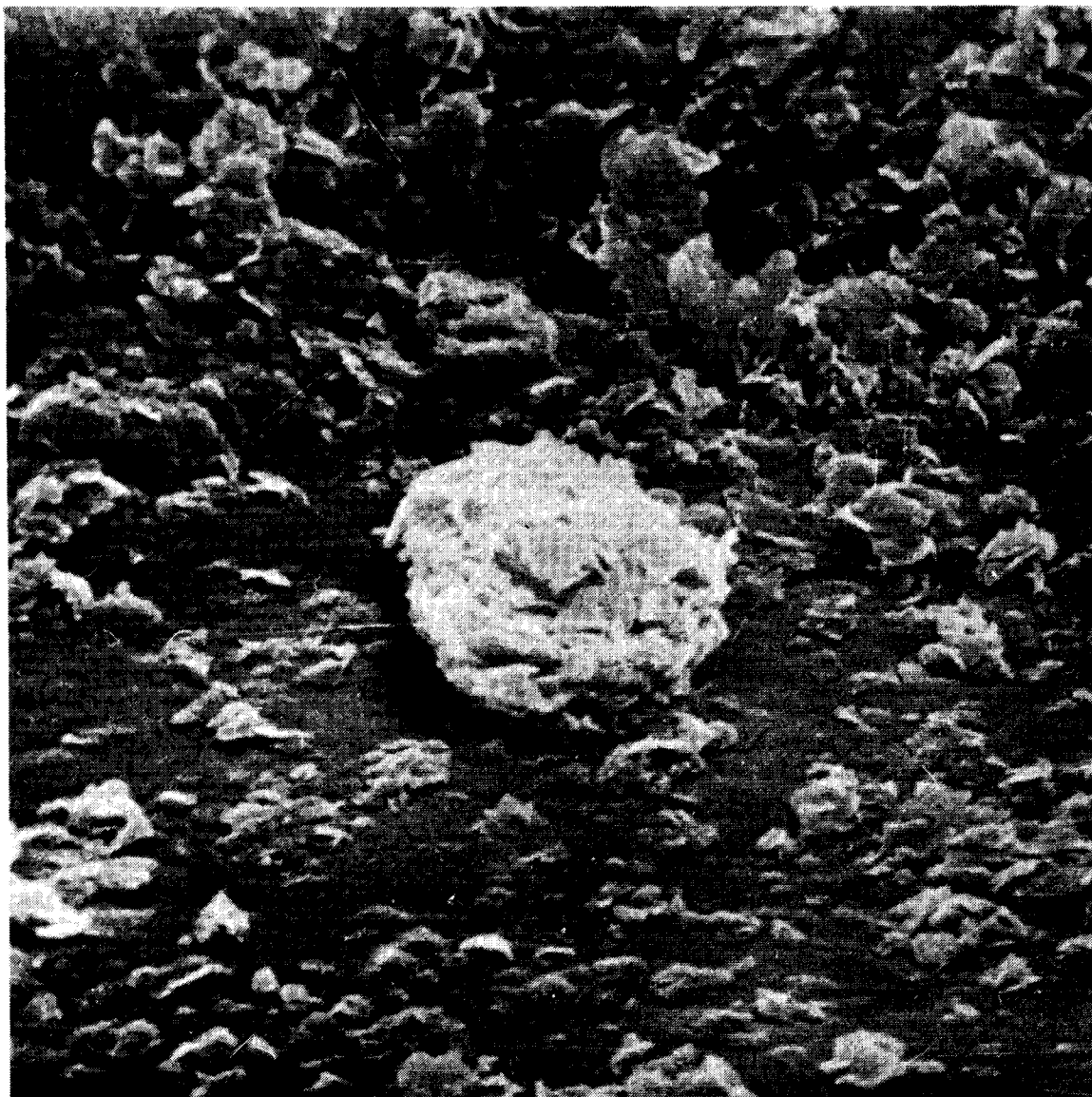


Figure. 14 : SEM – droplets of mucus and a flake

A portion of the only other area of trachea depicted in figures 10 – 13 showing mucus droplets and flakes. X 5,000



Figure 15 : The morphology of cilia and mucus

A particle of mucus on the ciliary epithelium. The grouping of ciliated cells and mucous cells is demonstrated. Critical point dried preparation. X 3 000



Figure 16 : SEM — Relationship of mucus to cilia

A small particle of mucus in an area adjacent to that in Figure 15. The relationship of the particle to the tips of the cilia is demonstrated. X 10,000



Figure 17 : SEM – Relationship of ciliated to non-ciliated cells

Mucus particle on tracheal epithelium. Cilia are relatively sparse in this area in comparison to the numbers of goblet cells. X 3,300

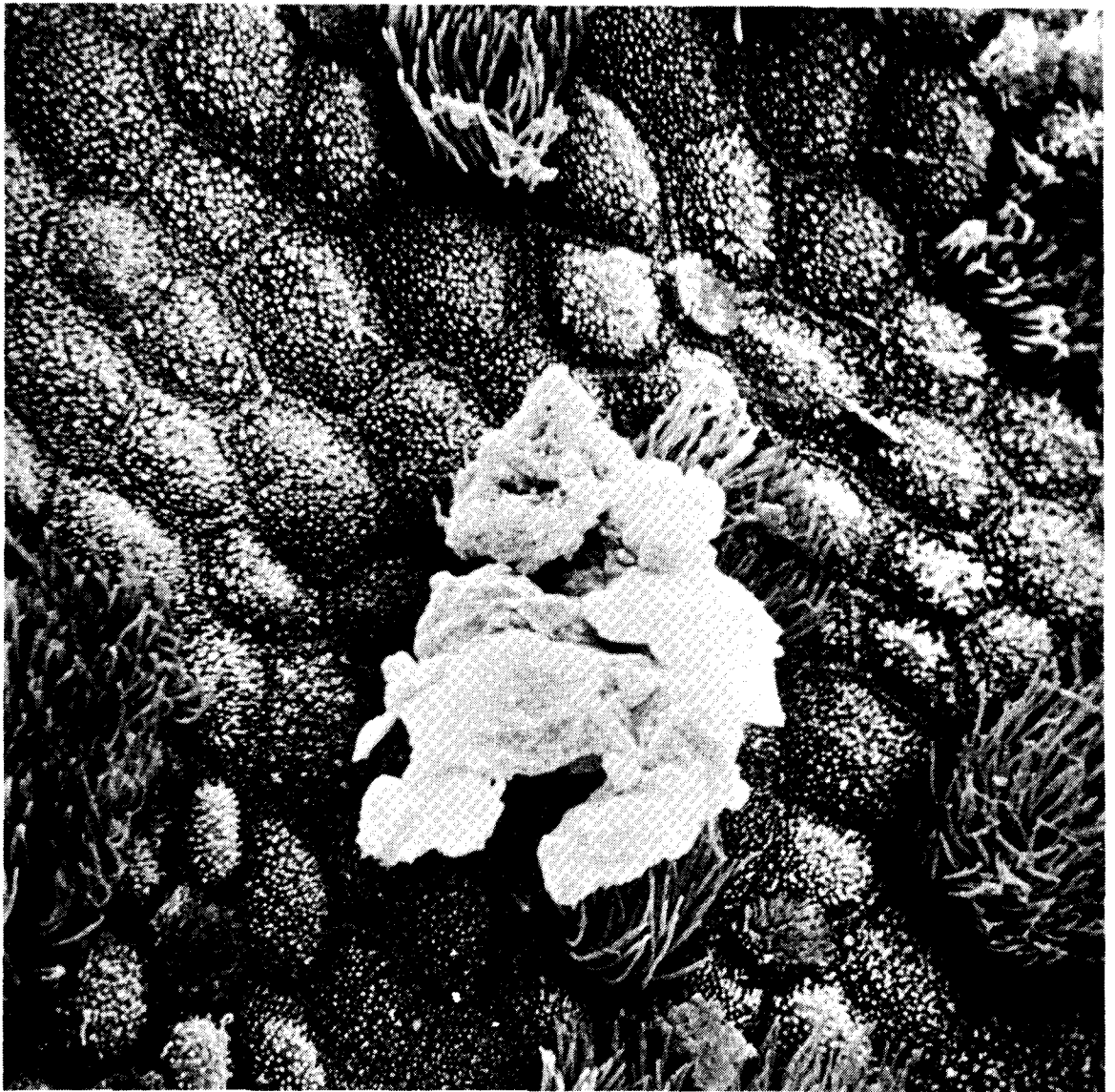


Figure 18 : SEM – Quartz particle attached to mucus

Mucus particles aggregated together on a tuft of cilia. Emmeshed in the mucus particles is a quartz particle. A large number of goblet cells are present in this area. X 3 000

CHAPTER 6

DISCUSSION

6.1 VALIDITY OF THE IN VITRO PHYSIOLOGICAL EXPERIMENTS

Satisfactory airway preparations were observed to function for as long as 10 h. The experiment was usually terminated before the preparation had ceased to function. Initially only approximately 40% of the experiments were entirely satisfactory, but this figure improved to nearly 80% as more experience was acquired. In all preparations ciliary activity was observed although the secretion and transport of mucus did not occur in all specimens. This latter function is probably a more sensitive one than that of ciliary activity which has been shown to continue under adverse conditions (Sharpey, 1835; Hilding, 1956). A further indication of the viability of the preparation was the demonstration of membrane potentials of cells of the mucous membrane which were comparable with those recorded in other ciliated epithelia *in vivo* (Iravani, 1969). A critical factor in the success of the method is to keep the warm ischaemia time as short as possible. Thus from the moment of tracheal intubation (animal still breathing), till the immersion of the isolated thorax in Krebs-Henseleit solution at 5°C, or less, normally did not exceed 90 sec. The cold ischaemia time was not critical and satisfactory results were obtained after storage of the isolated specimen in suitable conditions for several hours.

The method described yields ciliary beat rates and mucus transport rates which are similar to those previously reported (Barclay and Franklin, 1937; Dalhamn, 1956; Kilburn, 1967; Battista and Kensler, 1970; Sackner *et al.* 1973). Therefore although an *in vitro* method was used, ideal conditions of temperature control, pH and PO_2 were created in Krebs-Henseleit solution which simulates an extracellular fluid environment as closely as possible. In addition the lungs were kept inflated (the pressure not exceeding 10 cm H_2O) by passing humidified air through the intact airway system at very low flow rates. A constant pressure above 10 cm H_2O inhibited ciliary activity. This reaction which occurred very frequently, consisted of slowing of the ciliary rate, and ciliary beat would cease altogether if the pressure was not reduced. This response is different to that described by Sleight (1962) where the frequency of beat of the lateral cilia of *Mytilus* gill was increased by approximately 11% following an increase

of the hydrostatic pressure by 5 000 lb/in². Other ciliated protozoa however have shown a decrease in the rate of ciliary beat when a pressure of 1 000 lb/in² was applied (Sleigh 1962). Sleigh (1962) concludes that "it is impossible to determine at present the reason for the differences between results obtained using different material". The explanation for the inhibition of ciliary activity may be based on the observation that when cilia are compounded together their rate of beat may increase (Sleigh 1962). When the airway preparation is distended by a transmural pressure greater than 10 cm H₂O it is tempting to postulate that separation of the ciliated cells from each other by distension of the airway disrupts the tactile stimuli occurring between cells, and ciliary metachronism involving numerous cells is in some way disorganised leading to a slowing of ciliary rate. This phenomenon was however prevented by controlling the trans-bronchial pressure rigidly in the present study.

The automaticity of ciliary beat is a phenomenon which was first stressed by Verworn in 1889 (Sleigh 1962), who also noted that a cilium severed from its basal plate continued beating. The neurohumeral influences controlling ciliary beat have been discussed in detail by Rivera (1962), Sleigh (1962) and Kinoshita and Murakami (1967). The airway preparation which was used in this investigation was an *in vitro* model which had all its nervous connections severed. The specimen thus lost its potential to respond to neurohumeral stimuli, but ciliary rhythmicity was preserved. The ciliary rates observed with the present preparation (Iravani 1969) were very similar to those reported by Dalhamn (1956) who studied intact rats.

A particular advantage of this *in vitro* intact airway system is the fact that the bronchi were not opened and exposed to air. In addition the dissected airways were bathed in Krebs-Henseleit solution which acted as an extracellular fluid thus ensuring the adequate hydration of the tissues of the bronchial wall. The ciliary epithelium, being in contact with the humidified air used to keep the airways inflated, was allowed to function under conditions resembling the *in vivo* situation. Thus any surface fluid or mucus associated with the ciliary epithelium was preserved.

Rats were used exclusively in this study, but similar results have been obtained in the hamster (Van As unpublished data).

6.2 THE "MUCOUS BLANKET" CONCEPT

Many of the early workers in this field referred to ciliary streaming when studying transport in the trachea (Sharpey, 1830; Henderson and Taylor, 1910; Florey, *et al.* 1932). The term 'mucous blanket' arose out of reports by Hilding (1932a), Proetz (1933) and Lucas and Douglas (1934). All subsequent workers carrying out investigations on the tracheobronchial clearance mechanisms have used the term 'mucous blanket, sheet or layer' (Dalhamn, 1965; Hilding, 1965; Barnett and Miller, 1966; Kilburn 1968; Lightowler and Williams, 1969; Sadé *et al.* 1970; Nowell and Tyler, 1971; Wolfe *et al.*, 1972; Barton and Lourenco, 1973; Meyer *et al.*, 1973).

One of the problems of the mucous blanket concept has been the rationalisation of the fate of a supposedly large volume originating from the lower respiratory tract and being transported to a relatively narrow airway, the trachea. The width of the stream bed in the preterminal bronchioles has been calculated to be 30 m in the human (Hilding, 1960, 1965). This calculation was based on data obtained from von Hayek (1960). However, more recent evidence (Weibel, 1963) indicates that the aggregate circumference at the level of the preterminal bronchioles is 68 m (Table II). Hilding suggested that the elimination of mucus was achieved by an increase in the velocity of flow cranially as well as an increased depth of the mucous blanket to accommodate the additional volume. Available evidence (Barclay and Franklin, 1937; Kilburn 1967) suggests that there is only approximately a fourfold increase in the velocity gradient from the small airways to the trachea. Asmundsson and Kilburn (1970) thought that even an 8 to 14 fold increment in velocity of mucus flow would not be sufficient to compensate for the magnitude of mucus converging on a narrow stream bed of 50 mm. Their hypothesis is that an absorption of fluid occurs through the airway walls from the sol layer of the mucous blanket, thus reducing its volume. This hypothesis was supported by the observation that mucus in the trachea appeared to be stickier than in the smaller airways. The second problem is the method whereby the mucous blanket is transported past the openings of bronchial tributaries. Although Hilding (1965) showed that Indian ink markers were transported around these openings, he realised that a significant reduction of the effective bronchial circumference occurs at these sites. In one human specimen he found that 18 mm of a total circumference of 23 mm was occupied by the openings of these tributaries (Hilding, 1957b). Thus only 5 mm of intact bronchial surface at that

level was available for mucus transport. It is difficult to imagine a mucous blanket with its visco-elastic properties dividing easily to negotiate a path around these openings. This becomes more difficult to visualise if the mucus becomes increasingly viscid as it ascends in the airways.

The concept of a mucous blanket in the human lung may be examined theoretically by basing calculations on data derived from Weibel (1963), (Table II). The aggregate circumference and surface area of each bronchial generation is a straight-forward calculation. The volume of mucus in each generation is calculated assuming that the layer of mucus is uniformly 5 μm thick as has been suggested by Florey *et al.* (1932) and Dalhamn (1956). The 16th generation, (terminal bronchiole), represents the most distal zone of mucus production and therefore the mucous blanket. Velocities of mucus transport (column No. 1, Table II) are calculated by assuming that 0,4 mm per min as measured in a terminal bronchiole of rat's lung (Fig. 3) may be assumed to be a reasonable estimate of transport velocities in human lung at the same level. By maintaining a stable depth of mucous blanket (5 μm) it can be seen that transport rates in the trachea of 85 cm per min would have to be achieved. This velocity has never been recorded experimentally. At this flow rate the volume of mucus eliminated per day would be 355 ml (aggregate circumference x the velocity of transport x depth of mucous blanket x 1440). Conversely, by assuming a tracheal transport rate of 20 mm per min, which has been observed frequently in humans and experimental animals (column 2, Table II), transport rates of 0,001 mm per min would be present in the terminal bronchioles. This has not been observed in the experimental situation. At these flow rates only 8,2 ml of mucus would be eliminated per day. Both this and 355 ml per day are very different from the suggested figure of 0,75 ml/kg/24 h in adult human beings (Boyd 1972).

The above derived data, shown in Table II, indicates that a 2 000 fold velocity gradient would have to be present to allow a continuous mucous blanket, of uniform depth, to be transported from the terminal bronchioles to the trachea. This infers that either a mucous blanket is not present, or that previously reported transport rates are incorrect. Conversely Kilburn's hypothesis of fluid absorption may be correct and in this way an insufficient velocity gradient is overcome. If however, transport rates of both 0,4 mm per min in the terminal bronchioles and 20 mm per min in the trachea are correct then a fluid volume 347 ml (355 - 8 ml) would have to be absorbed each

day. The implication of this degree of dehydration of the mucus is that it is a very watery secretion in the small airways. Unfortunately no accurate data is available on the viscosity of mucus in the small airways. Asmundsson and Kilburn's (1970) assessment of mucus viscosity with capillary tubes did not exclude surface tension properties, and compared the mucus in the 10th bronchial generation with that in the trachea. It is unlikely that the 10th generation which is some distance from the terminal bronchiole would contain mucus which is significantly affected by secretions in the terminal bronchiole.

Thus to restate the problem. If a continuous mucous blanket is present from the terminal bronchioles up to the trachea, several mechanisms would have to operate to eliminate the mucus from the airways, as the trachea would not accommodate the mucus transported from the small airways. It has been suggested that the observed transport gradients of 4 fold (Barclay and Franklin 1937) and 14 fold (Asmundsson and Kilburn 1970) are not great enough to remove the mucus from the trachea, and maintain a uniform depth of the mucous blanket throughout the bronchial tree. The present study shows a measured gradient of nearly 40 fold over the whole length of the bronchial tree. Using available data from this and other papers (Table II) a 2 000 fold velocity gradient would have to be present to remove a mucous blanket of a uniform depth. Such a large gradient has never been observed. Basing calculations on an observed transport rate of 0,4 mm/min in the terminal bronchiole (Fig. 4), 355 ml of mucus would be produced per day. On the other hand a transport rate of approximately 20 mm/min has been observed frequently in the trachea, or similar airways (von Gebhardt 1909; Hill 1928; Kilburn 1967). This rate of transport in the trachea, if it reflected rates distally to it, would result in the production of only 8,2 ml of mucus per day. The difference in the two volumes transported, if 0,4 mm/min is the correct rate in the very small airways and 20 mm/min in the trachea, is 347 ml and this volume would have to be accounted for in some way. One way in which this volume could be accommodated is by an increase in the depth of the mucous blanket. Assuming a velocity gradient of 50 fold (0,4 mm/min to 20 mm/min) and that mucus secreted in the periphery only reached the trachea, the excess volume would result in an increase in the depth of the mucous blanket to approximately 200 μ m. This is a layer of considerable thickness and the electron microscopy studies (par 3.3 and 5.5) were specifically designed to detect such a layer. No such layer was observed in either SPF or non-SPF rats. The second way of accounting for the removal of the excess

volume would be the absorption of fluid into the bronchial wall. This has been suggested by Kilburn (1967) and Asmundsson and Kilburn (1970), and this factor together with an increase in the depth of the mucous blanket could conceivably compensate for the insufficient velocity gradient. It would also explain the increase in mucus viscosity which was observed between the 10th bronchial generation and the trachea (Asmundsson and Kilburn 1970).

However an important factor has been neglected in the foregoing theoretical calculations. This is the fact that mucous glands and goblet cells occur at all levels of bronchial tree (Dalhamn 1956). It is therefore reasonable to suggest that the volume of mucus delivered to the trachea is not only made up of that secreted in the periphery, but that a significant volume may be added to the postulated mucous blanket as it is transported cranially. Having established that the velocity gradient is 40 – 50 fold, this means that very much more fluid than the suggested 347 ml would have to be absorbed. This additional volume cannot be determined because it is difficult to estimate the rate at which mucus would be added to the bronchial tree at different levels as the mucous blanket ascended cranially.

More definite information has become available recently on the movement of fluid across the bronchial epithelium. Ion flux across canine tracheal epithelium has been shown to result in the active transport of Cl^- from the serosa to the mucosa (Olver *et al.*, 1974). The net flux of Cl^- is greater than the possible movement of Na^+ ions in the opposite direction, consequently an osmotic force may become available which results in the movement of water into the tracheal lumen (Marin *et al.* 1974). Therefore this preliminary evidence suggests that fluid is not absorbed but secreted by the bronchial mucosa thus casting some doubt on the fluid absorption theory. In the light of this information it seems that the compensatory mechanisms discussed above which are necessary to accommodate the mucous blanket in the airways, would be insufficient as further volume could be added to the mucus.

TABLE II — THEORETICAL CALCULATION OF MUCUS VOLUME AND TRANSPORT RATES IN THE HUMAN LUNG^x

Generation + n	Aggregate circumference cm	Mucus volume ml*	Transport rate (1) mm per min**	Transport rate (2) mm per min ++
0	5,7	0,033	858,6	20
1	7,7	0,017	635,6	14,8
2	10,4	0,0089	470,6	10,9
3	14,1	0,004	347,0	8,0
4	22,6	0,013	216,5	5,0
5	35,2	0,017	139,0	3,2
6	56,3	0,023	86,9	2,0
7	92,5	0,032	52,9	1,2
8	149,6	0,043	32,7	0,74
9	247,7	0,06	19,6	0,05
10	418,2	0,086	11,6	0,03
11	701,2	0,122	6,9	0,02
12	1222,5	0,179	3,9	0,01
13	2110,3	0,25	2,3	0,006
14	3808,9	0,38	1,3	0,003
15	6794,3	0,59	0,7	0,002
16	12353,2	0,87	0,4	0,001

x The basic morphometric data of the conducting airways was obtained from Weibel (1963) Table X1.2 p 139.

+ Generation 0 is the trachea and 16 is assumed to be the terminal bronchiole.

++ The values in this column were obtained by assuming a rate of 20 mm per min in the trachea.

* The total volume of mucus in the lung at any moment will be 2,72 ml if a uniform blanket of 5 μ m is present.

** The values in this column were obtained by assuming a rate of 0,4 mm per min in generation 16. Values similar to this have been observed in rat lung at that level. The calculation for mucus transport rates was made as follows:

$$\frac{12353,2}{6794,3} \times 0,4 = 0,7 \text{ mm per min}$$

The boxed figures in the columns Transport rate (1) and (2) are based on experimental observation.

6.3 DISCONTINUITY OF MUCUS IN THE AIRWAYS

In contrast to the studies reporting the presence of a mucous blanket in pulmonary airways, the most striking feature of this study was the relative paucity of mucus in the smaller airways of normal rats (Fig. 2). In these airways most of the mucus was in the form of small droplets less than 4 μm in diameter, a small number of flakes also being seen in the lobar bronchus. In the terminal bronchioles of normal rats no mucus was seen in any form. Above the level of the first branching site of the lobar bronchus components of mucus were more frequently seen. This distribution of mucus parallels the anatomical distribution of mucus-secreting cells and glands throughout the airways. In two animals, normal in all respects, hypersecretion of mucus was observed. In them the pattern and distribution of the components of mucus were similar to those seen in animals with "chronic bronchitis". Airway preparations derived from "bronchitic" animals showed all components of mucus present down to the preterminal bronchioles. The increase in peripheral distribution of mucus in these specimens reflected the increase in mucus-secreting cells throughout the lower airway system that is known to occur in association with chronic bronchial infections (Könn, 1966). An adequate explanation for the hypersecretion of mucus in two apparently normal animals is not known. Their bronchial epithelial morphology was not investigated by serial histological sectioning.

In conditions of hypersecretion ("bronchitis" and hypersecretion) a layer of secretion was present in the terminal bronchioles. The movement of this layer of secretion was apparently organised in respect of direction and velocity, giving the appearance of currents in a moving stream. The composition of this secretion is unknown, but it may be derived from the Clara cells present in this portion of the bronchioles (von Hayek, 1953; Kilburn, 1968). It is therefore unlikely to be mucoid. As this was the only site in the bronchial tree where a continuous layer of secretion was seen, it is possible that surfactant and other alveolar secretions played a part in its formation (Kilburn, 1968). Mucus secretions more proximal to the terminal bronchioles were always in the form of discrete droplets, flakes or plaques.

Barclay and Franklin (1937) described a spiralling mode of transport of Indian ink introduced into the peripheral airways of cat lungs. A tendency towards spiralling was also demonstrated in dog airways *in vitro* by Asmundsson and Kilburn (1970). The present study demonstrated that small droplets and larger flakes undergoing

cranial transport from the peripheral airways moved over individual contiguous metachronal fields with no tendency to systematic spiralling. At the level of the lobar bronchus the mucus particles tended to congregate and were transported en masse along well defined streams. These streams never followed a direct path and could deviate to either side of the trachea (Fig. 9). At the upper portion of the trachea these streams sometimes became confluent, but this could be attributed to the presence of a cannula in situ. Thus in none of the rats in this series or other small mammals studied so far has a mucous blanket or layer been observed lining any portion of the airways.

Most measurements of velocities of mucus transport in the intra-pulmonary airways have been derived either by indirect means or by placing indicators on the mucous membrane. However the fact that the components of mucus vary in size, mode and velocity of transport, complicates this measurement, especially when it is made in an indirect manner. In addition, similar components of mucus are transported at very different rates at closely adjacent sites. The transport velocity may also vary considerably within distances of approximately 20 – 100 μm when the passage of a single flake is timed. Thus if estimates of the velocity of mucus flow are to be meaningful standardisation of measurement is necessary. In this study flakes of 10 – 20 μm in diameter were chosen for the following reasons: they could be found in most regions of the airways; they were easily identifiable; their path of transport usually involved single metachronal fields; and their velocities of transport were only slightly slower than those of the small droplets (4 μm in diameter), which probably reflected the ciliary activity most closely. Because of the extreme variability in velocity of transport at a given site or for a given flake linearly, a mean value had to be taken of many readings. When investigating the location dependence of mucus transport, it was most convenient to use the normal rats with hypersecretion as suitable sized flakes were easily found throughout the pulmonary tree. When comparing transport velocities in different animals, measurements were always made at a specific anatomical site, namely the lobar bronchus just above its first branching site.

Although rapid velocities of mucus transport occurred with flakes, the majority of mucus is moved in the large airways in the form of plaques which are transported in streams of variable lengths and at variable intervals. The transport of individual plaques and those in streams is slower than that of the flakes. Thus the effective

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clearance time of the tracheobronchial tree is a function of the rate of movement of these plaques.

Estimates based on radioactive tracer elimination (Toigo *et al.*, 1963; Albert, Lipmann, Spiegelman, Strehlow *et al.*, 1967; Morrow *et al.*, 1967) has led Green (1970) to suggest that 90% of all materials physically deposited on the mucosa are cleared in less than 1 h. However, tracheobronchial clearance times of 2 h (Luchsinger, *et al.*, 1968), 2 to 3 h, (Thomson and Short, 1969), and 10 to 20 h (Booker *et al.*, 1967) have been observed. These prolonged clearance times are compatible with the findings of this study. The retention of charcoal particles for at least 19 h in SPF rat lung correlates with the slower rates of clearance of radioactive aerosols. Due to the discontinuity of mucus, a particle would not automatically be transported after deposition, but only after it came in contact with mucus. The physical characteristics of different substances may also determine their transport by the cilia. Charcoal, for example, is never transported unless attached to mucus. Apart from the widely differing clearance rates of radioactive aerosols discussed above, some authors have seen that particle movement may be sporadic and uneven. Florey *et al.* (1932) noted that particles of lamp black became stationary or moved slowly "until they are caught up in strands of mucus". Dalhamn (1956) never used charcoal particles as a marker for timing transport rates as their movement was unpredictable. Sade *et al.* (1970) also pointed out that although substances of widely differing surface properties and density were transported at a steady velocity, their movement ceased even when ciliary beat continued. Mucus placed on this epithelium moved immediately, and the blob of mucus could carry with it a large number of particles of any kind. This evidence, interpreted in the light of the findings in the present study, suggests that foreign particles will be transported only when they are in contact with particles of mucus. Intermittant transport may mean that particles become detached from the carrier or are deposited on an area of ciliary inactivity. This interpretation strengthens the concept of discontinuity of mucus in the airways.

The concept of a mucous blanket acting as a protective mechanical barrier initially conceived by Lucas and Douglas (1934), Hilding (1932a; 1932b; 1961) and Proetz (1934) has been used on numerous occasions as the basis for experimental design (e.g. Barnett and Miller 1966; Lightowler and Williams 1969; Weissbecker *et al.* 1971; and Wolfe *et al.* 1972) and discussion in reviews on the subject (Rivera 1962; and

Yeager 1971). It is pertinent perhaps at this point to speculate about the composition of the surface fluids related to the ciliary epithelium. It is generally accepted that cilia cannot function in a dry environment, e.g. air (Sleigh 1962). On the other hand the classical two-layer sol and gel concept of the mucous blanket is also not acceptable. The presence of the gel layer has been refuted by the findings of this study and mucus has been shown to be present in micro-particulate form as well as being sparsely distributed. The cilia themselves however have to exist in moist conditions of one form or another. Such a fluid layer was not observed under *in vitro* conditions in this study as the resolving power of the light microscope is limited to 4 μm . Histological techniques are all based on dehydration of the tissue and therefore any surface-related fluid would automatically be removed. The volume of the fluid, one could thus postulate, may vary from a mono- or polymolecular layer, which serves as a ciliary lubricant, to a layer of fluid in which the whole length of the cilium is bathed. In either event the particles of mucus would be transported at the level of the tips of the cilia. With the techniques presently available it is not possible to demonstrate the nature of this fluid layer. A mechanism whereby it can be constantly replenished has been demonstrated by Olver *et al.* (1974) and Marin *et al.* (1974). The osmotic gradient from the serosal surface to the mucosal surface resulting in a move of water in that direction could contribute toward the replenishment of water vapour lost during respiration. The respiratory epithelium is vulnerable as it is continually exposed to exogenous irritants, pollutants and infective agents (Green 1970). In view of the fact that mucus is sparse in the airways, other secretions such as secretory IgA, bronchial lactoferrin, bronchial lysozyme and some serum proteins (reviewed by Yeager 1971) become more important in their role of protecting the airways from primary invasion. These substances could well reside in the fluid layer related to the cilia.

6.4 ACUTE AND "CHRONIC BRONCHITIS"

The diffuse alteration in mucociliary function observed in the few animals that had contracted acute bronchitis probably represented a very early phase of bacterial or viral invasion after a non-specific injury or stimulus. The abnormalities were profound and resulted in gross alterations of the normal physiological process. Previous experience has shown that under normal conditions cilia are never stationary; ciliary inactivity therefore suggests either an irreparably damaged epithelium or moribund cells. In addition areas of altered ciliary beat result in inco-ordinated mucus transport,

often in a caudal direction. It is evident that the widespread changes cause a significant breakdown in the clearance function of the cilia which are further burdened by hypersecretion of mucus and the outpouring of inflammatory cells. These abnormalities are possibly due to toxic changes or perhaps are the effect of direct invasion of the epithelial cells by an infecting agent.

The chronicity of the bronchial abnormalities in animals with "chronic bronchitis" is indicated by the presence of peribronchial fibrosis, infiltration of the bronchial wall by chronic inflammatory cells, and areas of epithelial squamous metaplasia. It is not clear whether these changes are the sequelae of acute bronchitis or not. Abnormalities seen in the mucous membrane of these animals were much more localised than those found in acute bronchitis. A feature of zones of inactive cilia was that some ciliated cells could be stimulated to activity by rinsing their surfaces with saline. This suggests that the ciliary inactivity in "chronic bronchitis" is not always associated with moribund cells. It is possible that very viscous adherent mucus trapping the cilia was rinsed off by the saline allowing them free mobility once again.

Abnormalities of ciliary beat and mucus transport appeared to be more organised in "chronic bronchitis" than similar phenomena observed in acute bronchitis. A whirlpool type of movement was a regular feature of "chronic bronchitis". It is presumed that the rotary movement arose by chance in that mucus would be transported caudally for a certain distance, only to be returned cranially by adjacent fields and then picked up by the same caudally directed fields again. Whirlpools rotating for many hours were first observed in the bronchial tree by Hilding (1957a) in preparations from cows. He did not show an association between these whirlpools and bronchitis since the specimens were not reported to have been examined histologically.

Despite the hypersecretion of mucus, the presence of inflammatory cells and patchy abnormalities of ciliary activity, the overall rates of clearance were found to be greater in "chronic bronchitis" if the destruction of the ciliated epithelium was not too extensive (par 5.3(i) and Fig. 9). It would appear that the intervening normal mucosa is hyperactive probably as a result of the mechanical stimulus of excess mucus secreted at all levels of the respiratory tract.

Enhanced pulmonary clearance in old rats with "chronic bronchitis" was further demonstrated by the animals that were exposed to fine particulate charcoal. In all

instances the rats with "chronic bronchitis" had cleared almost all the charcoal from their respiratory tracts after 19 h, as compared with the large amount of charcoal retained in the lungs of young SPF rats. Charcoal aggregates were observed in "bronchitic" rats on large inactive zones or on whirlpools, demonstrating the length of time material can be retained in these areas. The delayed pulmonary clearance in SPF rats can not be adequately explained, as there was no visible ciliary abnormality and mucus transport was normal. It is possible that these young animals derived from SPF conditions, although possessing an adequate clearance mechanism had not been exposed to the stimuli that promote the full development of the mucociliary apparatus.

Toigo *et al.*, (1963) showed slow pulmonary clearance of radioactive aerosols in patients with chronic lung disease. Several other studies showed no difference between the clearance of radioactive aerosols in normals and smokers (Albert, Lipmann, Spiegelman, Strehlow *et al.*, 1967; Luchsinger *et al.*, 1968; Thomson and Short, 1969; Pavia *et al.*, 1970). There is however evidence for impaired clearance rates in smokers (Pavia *et al.*, 1971; Albert, Spiegelman, *et al.*, 1969; Albert *et al.*, 1969). Although these results are somewhat conflicting, Camner *et al.*, (1971) demonstrated that the technique is reproducible in individuals. Albert, *et al.*, (1969) showed that there is a wide range of variability in normality from subject to subject. The degree of impairment of clearance would depend on the amount of damage to the ciliary epithelium. This factor would be difficult to assess in the human when constructing an investigation, and this probably accounts for the variability in results.

The results of the experiments of this study suggest that the first abnormality in the development of bronchitis is most probably an impairment of ciliary function. Acute bronchitis induced by exposure of the animals to cold, moist conditions, led to abnormalities of ciliary function in numerous cells diffusely distributed throughout the entire tracheobronchial tree. Such a reaction would constitute a breach in the defence mechanism of the airway. In contradistinction rats with "chronic bronchitis" showed areas of mucociliary abnormality that were usually localised to regions with histological evidence of bronchitis and peribronchitis. This reaction is known to be the sequel to a mycoplasmal infection (Lindsey *et al.* 1971). Although no attempt was made to investigate the relationship between acute and "chronic bronchitis", as it was outside the scope of this study, it is tempting to postulate that a series of events may lead from one to the other. The entire respiratory tract is continually exposed

to foreign materials such as viruses, bacteria and atmospheric pollutants by inhalation. Any one of these agents may give rise acutely or after repeated exposure to an impairment of ciliary function and to damage of ciliated cells. Most of the damaged ciliated cells are replaced in the course of the normal regeneration of the mucous membrane. However, it is plausible to suggest that if the damage is severe or repeated, areas of squamous metaplasia may occur. These areas are inactive in terms of mucociliary clearance and are more vulnerable to invasion by inflammatory agents, leading to further damage and chronic inflammatory changes.

6.5 SCANNING ELECTRON MICROSCOPY (SEM)

The advantage of examining pulmonary surface morphology with a SEM lies not only in the large area which may be viewed and photographed rapidly (Nowell and Tyler, 1971), but also in the detailed inspection of surface related structures. These factors facilitate the study of the morphology of mucus and its relationship to the ciliated epithelium.

Attention has been drawn to the fact that different techniques of preparation of pulmonary tissue may either preserve mucus or remove mucus from the surface of the airways (Nowell and Tyler, 1971). The techniques of fixation used in this study are similar to those described by Nowell and Tyler (1971), viz. the use of a formaldehyde derivative, which they claim preserves mucus. The morphological evidence presented in this study supports the concepts which have arisen out of the direct observation of the *in vitro* preparations (namely the discontinuity of mucus). Thus it would seem that the exact method of fixation and drying is not critical, provided that the interval between the removal of the tissue and rapid fixation is short. Ciliary activity is vigorous under most circumstances and would result in the continued removal of mucus if this function were not arrested rapidly, by cold fixation with gluteraldehyde, or freezing in a solution at an extremely low temperature.

Allowing for the distortion of tissue during preparation, the mucus particles seen under the electron microscope resemble closely those observed *in vitro* with the light microscope. The greater resolution obtained with the SEM has revealed more precise information on the morphology of mucus. The concept that mucus is present as droplets, flakes and plaques is confirmed in principle. The plaques which were thought to be conglomerations of flakes and larger than 70 μm in diameter may be as small as 15 μm in diameter and are composed of both droplets and flakes (Fig. 11, 12 &

13). Flakes previously described as homogeneous bodies varying in size from 10 to 70 μm in diameter are seen to be as small as 5 μm in diameter. Although the formation of flakes has never been observed physiologically, it is now clear that they are constructed from a large number of aggregated droplets. These features are demonstrated in Figs 10 – 14. Droplets previously defined as mucus particles smaller than 4 μm are now seen to be as small as 0,5 μm in diameter. The relative paucity of mucus in the airways, even in conditions of "bronchitis" is well demonstrated. Significant collections of mucus were found in only two small areas of one of the tracheal segments studied. This segment of trachea was obtained from a mature animal which had grown up under normal animal house conditions. These animals all exhibit some features of "chronic bronchitis" associated with CRD and have a hypersecretion of mucus.

The two methods of preparation of tissue for SEM result in distinct differences in observed surface morphology (par 5.5). Mucus is more abundant in the freeze-dried specimen. This specimen is however from a non-SPF rat where a relative hypersecretion of mucus would be expected. Despite this, the mucus is present in well defined regions with large areas of intervening bare mucous membrane. The paucity of mucus in the specimen prepared by the critical point method may be due to its being washed off during the initial immersion in gluteraldehyde, although great care was taken to prevent this. Mucus is however, found quite easily when the specimen is viewed. The preparation was made from a SPF animal in which the paucity of mucus is expected even in the trachea. Ciliary detail, goblet cell morphology and other structures are clearly demonstrated by the critical point method. The surface of freeze-dried preparation appears to be composed of closely matted cilia with no visible goblet cells. When these preparations were sectioned and examined with the light microscope, the basal portions of the cilia were well separated but their tips were folded horizontally giving the appearance of an enmeshed layer (par 5.5). These findings may be attributed to shrinkage of the specimen during the preparation and to change in surface tension properties during drying. Thus of the two methods the critical point drying appears to be superior in the demonstration of mucous membrane morphology and mucus.

CHAPTER 7

CONCLUSIONS

An *in vitro* animal model to study the morphology and transport of mucus in mammalian airways was used. For the purpose of the study SPF Wistar rats were regarded as representative of normal conditions. Non-SPF Wistar rats are prone to develop chronic respiratory disease and were found to be a suitable model for the study of mucociliary function under conditions which resemble chronic bronchitis. Mucociliary activity was studied under controlled *in vitro* conditions by means of direct observation. Static conditions were examined by means of scanning electron microscopy as well as conventional histological techniques using the light microscope. From the findings of the study the following conclusions may be reached:

1. A discontinuity of mucus at all levels of the airway under both normal and abnormal conditions was observed. The classical gel layer proposed by Lucas and Douglas (1934) was not observed. Mucus was sparse in SPF animals and, although more plentiful in non-SPF animals, large areas of the epithelium were devoid of mucus particles.
2. In accordance with other reports (Iravani 1967 and 1969) where a location dependence of ciliary frequency was reported, the same phenomenon was documented with mucus transport. Velocities of 0.4 mm/min were observed in the terminal bronchiole and 15 mm/min in the trachea. This is a gradient of nearly 40 fold.
3. Under normal conditions ciliary activity was always present, and the effective stroke of ciliary beat directed cranially, resulting in unimpeded mucus transport. "Bronchitic" conditions resulted in widespread abnormalities of ciliary beat, in the form of reversal of the effective stroke of beat, abnormal beat form, and the cessation of ciliary activity. These abnormalities resulted in impaired mucus transport and whirlpool formation. However in adjacent normal areas the velocity of transport was normal or increased.
4. The inhalation of a charcoal marker demonstrated that these particles are transported only when adherent to mucus particles. Similar evidence has been reported previously (Florey *et al.* 1932; Dalhamn 1956).

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