

GOBLET CELL INCREASE IN RAT BRONCHIAL EPITHELIUM  
ARISING FROM IRRITATION OR DRUG ADMINISTRATION -  
AN EXPERIMENTAL AND ELECTRON MICROSCOPIC STUDY

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PETER KEY JEFFERY, B.Sc.

Department of Experimental Pathology,  
Cardiothoracic Institute  
Brompton Hospital  
University of London

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### ABSTRACT

The development of new goblet cells in airway epithelium is one of the main features of mucus hypersecretory disease in man. The ultrastructural features of such development have been followed in the specific pathogen free rat, the increase being produced by the irritative effect of tobacco smoke and the effect of two drugs, isoprenaline and pilocarpine. The protection offered by phenylmethyloxadiazole, an anti-inflammatory drug, against the tobacco effect, has also been studied.

In preliminary normal anatomical studies the cell types present and their frequency at five airway levels was established.

Tobacco smoke caused cell changes only in the large airways, including an increase in goblet and ciliated cells, an increase in the number of cells in division and also in individual cell size and overall epithelial thickness.

Isoprenaline increased the number of goblet cells in the large and, more strikingly, in the distal airways while the proportion of ciliated cells was normal. The number of cells in division was increased and included goblet cells.

After pilocarpine the main feature was the reduction of cells with secretory granules presumably by their discharge.

It seems that in the large airway and after irritation the new goblet cell develops from the electron-dense nonciliated cell: electron-dense granules first are discharged then resynthesis involves an increase in number, size and electron-lucency of the granules. In the distal airways the electron dense nonciliated cell seems to develop from a more electron-lucent cell by loss of its abundant smooth endoplasmic reticulum.

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## CHAPTER I

### HISTORICAL INTRODUCTION

### Hypersecretion and Disease

Normally the volume of bronchial secretion is not sufficient to cause expectoration, any secretion being imperceptible and swallowed. In hypersecretory diseases, such as chronic bronchitis and cystic fibrosis, bronchial secretions are increased to a level where they are coughed up and expectorated as sputum (Reid, 1958). The observed increase in bronchial secretion is mirrored anatomically by an increase in the amount of tracheo-bronchial submucosal gland and by an increased number of surface epithelial goblet cells in the large airways, and by their appearance and subsequent increase in smaller bronchioli where they are normally sparse (Reid, 1954). The production of mucus by surface epithelial goblet cells is of the greatest functional significance and is the subject of this study.

### Anatomy of the Bronchial Lining Epithelium

In 1543 the Belgian, Vesalius, regarded as the founder of modern anatomy, described the lung as a "soft, spongy, thin, light, airy flesh". In 1602, Laurentius first described a membranous lining to the walls of the airways which in today's terms would have included both the mucosa and submucosa. Following this, the development of the light microscope and the observations of Malpighi,

von Leewenhoek and Bichat culminated in the theory that all physiological and pathological processes depended on changes within biological units called cells. This theory, postulated by Schleiden (1838) and Schwann (1839) and generally accepted after the publication of Virchows "cellularpathologie" in 1858 initiated much research into the microscopical anatomy of the lung and its airways.

#### LIGHT MICROSCOPE

##### Pre 1900

In 1834, and with the light microscope, Purkinje and Valentin published one of the first descriptions of the lining membrane of airways from a number of species of bird, reptile and mammal, including man. Discovering the cilia they proceeded to describe their movements in the trachea and bronchial tubes....."extending to their smallest divisions capable of examination" but could not ascertain their direction of beat.

In 1836, Sharpey published an extensive review of cilia in the animal kingdom and, in the mammalian respiratory system, traced the direction of beat, observing the movement of charcoal powder on a strip of dog airway in tepid water. He found the movement resulted in successive waves which he likened to wind in a cornfield and that it was clearly directed towards the trachea and larynx. He further

suggested that."whatever may be its other uses, it at least serves to convey the secretions along the membranes together with foreign matters if any are present".

In 1837, Henle and in 1847, Bowman reported that the mucous membrane was lined by a "pavement" (epithelium) resting on a basement membrane and composed of nucleated "particles" (cells) adhering together and of various size, form, and number. Henle described a secretion from this membrane the "principles" (precursors) of which Bowman regarded as being lodged in the epithelial cells. Even at this early stage, Bowman suggested that the secretion so formed within these cells may be....."thrown off" by virtue of minute chemical changes occurring in it, without the cell itself being altered in form. To these mucus-secreting cells and by virtue of their shape, the term goblet cell was later, in 1867, applied by Schulze. He showed that goblet cells have a theca....."filled with a mucous mass through which numerous highly refractile granules are distributed, and which projects from the upper rounded opening of the cell in the form of a small ball, that sometimes becomes altogether detached" (Schulze, 1872).

In 1853, Kolliker described more precisely, giving measurements of cell size and length of cilia, the histology of the epithelium. He found the trachea was laminated and of 3 layers: (i) a most superficial layer of ciliated cells (ii) a middle layer of vertically

elongated cells and (iii) the deepest layer of rounded cells. In the smaller bronchi and bronchioli the epithelial thickness gradually was reduced until it was of only a single layer. Schulze later (1871) disagreed, however, describing the lining of the trachea as a single layer with no evidence of stratification. Today we know that while the epithelium appears stratified it is, in fact, psuedostratified with all cells reaching the basement membrane (see Miller 1932).

In 1879 Frankenhaeuser studied the tracheal epithelium of a wide variety of mammals including rat and man and showed that while that of man, cattle and sheep was psuedostratified with goblet, intermediate and basal cells, that of rat was simple, columnar and lacking the intermediate layer. In 1881 Kolliker extended his study to the distal airways of man and found that the epithelium of the terminal bronchioli lacked goblet cells. In these small airways, ciliated cells were interspersed with nonciliated cells, later to be known as Clara cells.

#### Post 1900

In the latter part of the 19th century, improvements were made to microscopy, by the development of achromatic lenses and the use of improved embedding media, allowing thinner sections of tissue to be cut and stained by mono or polychromatic stains. Different cell types and organelles could now be visualised up to magnifications of the order of 1000 times.

The reviews of Miller (1932) and Lucas (1932) show that in the early part of the 20th century, little new information about the bronchial lining, came to light. Much attention was focused on the number and size of the cilia, on their relationship to underlying mitochondria and on their possible attachment to these and other cell organelles. A cuticle was shown to exist on the ciliated cells of man, rat and mouse. Some areas of epithelium were shown to be stratified due to friction and irritation. But essentially Miller's description of human epithelium is that of earlier workers with more emphasis on the transitional zone between the terminal bronchiolus and alveolus: a psuedostratified epithelium with ciliated, goblet, intermediate and basal cells, the thickness diminishing in bronchioli of 1 mm, goblet cells absent and basal cells rare in terminal bronchioli, and cilia absent (now known to be present) from the respiratory bronchiolus, where the nonciliated cuboidal epithelium gradually becomes flattened giving way to the simple squamous epithelial lining of the alveoli.

In 1934 Lucas and Douglas found that the frequency of ciliary motion was unaltered when the flow of overlying mucus was stopped and suggested that the mucous "blanket" must be of two layers, an upper highly viscous layer, resting on the tips of the cilia, and a lower, less viscous (periciliary) layer surrounding the cilia: only the tips of the cilia touched the mucus. It should be noted that the more recent

findings of Iravani and Van As (1972) show that the overlying mucus is, in fact, discontinuous, rather than as a blanket, the mucus consisting of droplets, or flakes, of about  $1\text{ }\mu\text{m}$  in size.

In 1937, Clara published the results of a histological study which concentrated on the terminal bronchioli of man and rabbit, describing in great detail the nonciliated cells which have since come to be known as "Clara" cells. The distinguishing features of this region were the absence of goblet cells and the presence of bulging nonciliated cells which secreted, in an apocrine manner, granules which did not stain for mucus. The goblet cells of the larger airways were also described, the ratio of goblet to ciliated cells varying in individuals and their numbers markedly increased in certain pathological diseases.

In the review by Policard and Galy (1945) no new cell type was described, but with reference to the intermediate cell they suggested that it was present even in the single layered epithelium of rats, mice and guinea pigs, between the ciliated cells, reaching the surface and bulging into the lumen, the apical surface covered by a cuticle similar to that of ciliated cells (He is probably referring to Clara-like cells).

In 1949, Macklin washed the living bronchiolar cells of mouse and hamster with an ammoniacal silver solution and defined two types of cell by microscopy. Ciliated cells were "dark" with a cuticle and nonciliated cells, which he considered to be Clara cells, were "light" with protruding

egg-shaped ends containing "small silverized particles" and, surmounting this, discoid areas with numerous granules.

In 1960 v.Hayek published a text on the human lung and reviewed the histology of the various airway levels adding new observations, mainly relating to the smallest bronchioli where goblet cells were absent. Describing cilia extending as far as and including the respiratory bronchioli, he found after Azan staining and within each ciliated cell, irregular large granules staining red and situated close to the nucleus, between it and the ciliated border. As the chromatin of the nucleus stained similarly he likened this to a nuclear secretion and to those granules found in ciliated cells after similar staining by Clara in 1937. In the latter case, these were thought to represent a precursor of secretion from nonciliated cells, thought by Clara to be derived by a metamorphosis of ciliated cells. v.Hayek also found similar granules between the cilia and occasionally within the mucus. Describing the nonciliated bronchiolar cells he found two forms which he regarded to be different functional states of the same cell type: (i) "club-cells" with protoplasmic processes projecting into the lumen and resembling those described by Clara and (ii) "nonciliated cells" which did not project beyond the ciliated cells. v Hayek, agreeing with Clara that the "club-cell" processes can be broken off and then "disolved" into the secretion, went on to describe in some of the nonciliated cells "paranuclear vacuoles"

which nearly filled the apex of the cell, causing them to resemble goblet cells the "secretion" finally bursting from the cell apex. Unlike Clara, v.Hayek (1962) does refer to the secretion in these small airways as mucus, eosinophilic and distinct from the basophilic mucus of the proximal airways. v.Hayek agreed with earlier workers in finding leucocytes (Miller, 1932 and Policard & Galy, 1945) and mast cells within normal epithelium (Taliaferro and Sarles, 1939; Fröhlich, 1949; Kent, Baker, Ingle and Li, 1954).

In 1968 Lamb recognised on morphological grounds, three forms of secretory cell: (i) medium sized cells, the maximum cell width being from half to one quarter the cell height: (ii) thin cells with only a narrow line of secretory product, lacking the flask-shaped expansion in the upper part of the cell and (iii) nearly spherical distended cells, the width being more than half the height. It would seem that these differences relate to the volume of secretion within the cells and not to the actual cell size.

#### Recent findings

In 1949 Fröhlich described a system of clear or light cells ("Helle Zelle") in the bronchial epithelium of man and other mammals. These cells, many of them argyrophilic, were present at all levels of airway and by using a silver technique, were shown in rabbit to have neural connections. This Fröhlich suggested, indicated they might be chemoreceptors.

In 1954, Feyrter also demonstrated that argyrophilic and clear cells were present in the bronchial tree of man, as well as in a wide variety of other body organs, regarding them as part of a "diffuse endocrine epithelial organ".

In 1963 Glorieux studied the bronchial epithelium of the cat and rabbit and showed the presence of argentaffin cells which, in the cat, received a very rich nerve supply probably from the vagus.

In 1965 and in the human, Bensch, Gordon and Miller found that cells similar to Kultschitsky cells (which they equate with enterochromaffin, argentaffin, argyrophil, basal clear or yellow cells) occurred at the base of tracheal and bronchial epithelium and particularly at their branching points. These cells were argyrophilic and in one case fluorescent and similar to those found in a nearby carcinoid tumour. In 1969, Lauweryns and Peuskins and in 1972 Rosan and Lauweryns described many such cells in the airways of young infants. In a more recent study in 1972, Lauweryns, Cokelaere and Theunynck described, in a variety of mammals, an arrangement of nonciliated cylindrical cells into so-called "neuroepithelial bodies". These groups of cells showed a marked argyrophilia, an intense yellow fluorescence and were supplied by nerves, thus resembling those found by Glorieux in 1963.

In 1972 Ericson, Hakanson, Larson, Owman and Sundler demonstrated similar cells within mouse tracheal epithelium but no similar report has been found for the rat.

## THE ELECTRON MICROSCOPE

### Early Studies

As early as 1873 Abbe showed that the smallest resolvable distance that could be obtained with the light microscope was half the wave length of light or 0.2  $\mu\text{m}$  and little could, therefore, be gained by magnifying beyond 1000 times. The discovery of the electron by T.T. Thomson in 1897 led to the development of the first electron microscope in 1931 and by 1945 resolution of the order of  $10 \text{ \AA}^{\circ}$  (INM) *sc* was possible (Hall, 1966).

The first electron microscopic studies of biological material were of micro-organisms, fixed in osmium vapour, dried on a formvar film and observed after a shadowing technique when metal was evaporated at a low angle across the specimen giving it a 3 dimensional appearance. In this way and in 1951 Engström was the first to observe tracheal cilia from a variety of species and in 1952, Engström and Wersall were the first to look at plastic-embedded sections of tracheal cilia. These workers described them in different mammals as 3 - 8  $\mu\text{m}$  long (6 - 7  $\mu\text{m}$  in man) with an axial core of 10 or 11 fibrils, surrounded by a delicate sheath, in number about 30 per square  $\mu\text{m}$  of cell surface and each with an associated basal body lying just below the cuticle of the cell. To each basal body there was a rootlet tapering towards its end, this longest in the "lower" animal forms.

In 1955 Policard, Collet and Giltaire showed the arrangement of internal fibrils in bronchiolar cilia as 9 single peripheral and 1 double central fibril, while in 1956 the studies of Rhodin and Dalhamn showed that it was of 11 filaments arranged as nine peripheral filaments (each of two sub-filaments) and two single central filaments. These latter workers found approximately 8 cilia per square  $\mu\text{m}$  of cell surface and estimated that an average sized cell would have about 270 such cilia projecting from its free apical edge. They also reported that at the extreme tip of the cilium the fibrils lost their typical arrangement and fused, one with the other. Small, 0.8 - 1.0  $\mu\text{m}$  long filiform (microvillus) projections were found between the cilia which Fawcett and Porter (1954) had previously suggested represented, at least in part, the cuticle seen and described with the light microscope.

LARGE AIRWAYS - In 1956, Rhodin and Dalhamn gave the most detailed description of the rat tracheal epithelium to date. Whilst describing the ultrastructure of ciliated, goblet and basal cells, they discovered a new cell type. Due to its regular arrangement of apical microvilli they referred to it as a "Brush" cell and suggested it might function in absorption or be a primitive, ciliated cell. This cell type has subsequently been reported at all levels of rat airway and rat alveolus (Meyrick and Reid, 1968) and in the airways of both the rabbit (Leeson, 1961 and Konradova, 1966) and pig (Baskerville, 1970a) as well as

extra respiratory sites such as the rat colon (Silva, 1966; Luciano, Reale and Ruska, 1968) and common bile duct (Riches, 1969). Although it has been reported at all levels of human airway (Spoendlin, 1959; Rhodin, 1959 and 1963; Watson and Brinkman, 1964 and Basset, Poirier, LeCrom and Turiaf, 1971) the illustrated photomicrographs do not resemble the original description.

Rhodin and Dalhamn (1956) also distinguished goblet from ciliated cells, goblet cells lacking cilia and having a more electron-dense cytoplasm with large numbers of secretory granules whose electron density was slight "...due to their content of polysaccharides which do not stain with osmium". They and Dalhamn in 1956, also made less detailed reference to narrow electron-dense cells which lacked the distal accumulation of electron-lucent secretory granules seen in goblet cells and probably representing an early stage in the elaboration of intracellular mucus. In a later study in 1959 Rhodin, compared the tracheal epithelium of rat with that of man and found that of rat was simple and columnar, whereas in man, it was pseudostratified. The ciliary rootlet was different, the cross striations found in man being absent in the rat. He also reported that in man both mucous and serous cells occurred in the epithelial layer. The "serous" cell was columnar but not of a goblet shape and its granules, in contrast to the mucous granules, stained with osmium. He likened this cell and its mode of secretion to that of the exocrine cells of the pancreas. Cells with similar granules were

also reported in the bronchus of the pig by Baskerville (1970b) although, in this case, the cytoplasm had the same electron density as did the adjacent ciliated cells.

In 1968, Frasca, Auerbach, Parks and Jamieson, describing the normal bronchial ultrastructure of dogs, reported the presence of a nonciliated "special type cell" with disc-like granules about 130 nm in diameter and containing finely fibrogranular material. This cell type appeared to be unique to the airways of the dog.

In 1969, Hansell and Moretti reported a "nonciliated cell" in the trachea of 3 week old mice which had not been previously described in any other mammalian trachea. This cell, 22  $\mu$ m high and 5  $\mu$ m wide was sometimes found as frequently as the ciliated cell. Smooth endoplasmic reticulum, present as short profiles, was the most characteristic feature of the cell. Mitochondria were similar to those of the nonciliated bronchiolar cells described by Karrer in 1956 and, with the light microscope, PAS positive material was found abundantly at the cell apex. As only one goblet cell was found, they believed these nonciliated cells secreted the bulk of the mucus found in mouse airways. In agreement with Osada (1963) who described the fine structure of human tracheal epithelium, they also found a small number of ciliated cells with a cytoplasmic density approaching that of the goblet cell.

With the electron microscope an intermediate cell layer (absent in rat and mouse) of vertically orientated spindle shaped cells resembling, in fine structure, the basal cells, has been described in the human (Rhodin, 1966), pig (Baskerville, 1970b) and rabbit trachea (Konradová, 1966).

In the large airways of all the species so far mentioned, a layer of basally situated cells has been found. They are described as polygonal or elongated cells with sparse electron-dense cytoplasm containing a few small ovoid mitochondria, a small Golgi apparatus and with tonofilaments which are often continuous with areas of cell attachment, (interalia Rhodin, 1966). Many of those which Rhodin and Dalhamn (1956) found in the rat were regarded, by them, as lymphocytes. The studies of human trachea by Marco, Sanchez-Fernandez and Rivera-Pomar (1966) have shown two types of basal cell: (i) a dark (i.e. electron-dense) cell with numerous mitochondria and abundant rough endoplasmic reticulum and (ii) a clear (i.e. electron-lucent) cell having these two cell components much reduced in number.

SMALL AIRWAYS - In 1955 Policard, Collet, and Giltaire-Ralyte first studied the bronchiolar epithelium of the rat after osmium fixation alone. Ciliated cells had shorter cilia than those of the larger airways, Clara cells had dome-shaped tops and were of two types, one with electron-dense cytoplasm and the other with lucent cytoplasm containing vacuoles 0.2 to

1  $\mu$ m in diameter. Neither type had mucous granules or showed any suggestion of secretion.

In 1956 Karrer published the first electron microscopic report of the epithelium of mouse bronchioli and recognised both ciliated and nonciliated cells. The cytoplasm of the nonciliated cells showed an abundance of mitochondria and endoplasmic reticulum. The majority of mitochondria were up to 1  $\mu$ m in diameter, spherical, and with a homogeneous matrix deficient in cristae but a second type, more elongated and with numerous cristae, was also found. The endoplasmic reticulum usually was of the smooth type, tubular or vesiculated and often concentric with the large mitochondria. These findings were substantiated by later studies on mouse airways (Rhodin, 1963; Niden and Yamada, 1966; Okada, 1969; Petrik and Collet, 1970a and b; Lauweryns, Cokelaere and Boussauw, 1971; Wang, Huang, Sheldon and Thurlbeck, 1971).

In 1958, Kisch described the smallest airways of the rabbit. Protruding from between the cilia of ciliated cells and from the apex of nonciliated cells he found small droplets less than 1  $\mu$ m in size. As well as these he described larger vesicles of several  $\mu$ m in diameter which protruded from nonciliated cells and contained fine granules, apparently the secretion. v.Hayek (1960) suggested this type of secretion might be of significance with regard to the copious bronchiolar secretion in asthma.

In 1960, Policard, Collet and Pregermain described the transition zone between the smallest bronchiolus and alveolus in the rat. The transition usually was by a cell type intermediate in structure, between the nonciliated bronchiolar cell and type II alveolar cell. This intermediate cell type bulged into the lumen and had cytosomes of  $0.5\ \mu\text{m}$  diameter which resembled those seen in the type II alveolar cell. They suggested these might represent the cells described by Clara in 1937. These observations have more recently received some support (see Balasova, 1968; Basset et al, 1971; Okada, 1969; Petrik and Collet, 1970).

In 1966, Niden and Yamada and in 1967, Niden, published observations on the mouse nonciliated bronchiolar or Clara cell. Their findings have led to the suggestion that these cells are responsible for the production of pulmonary surfactant, a substance rich in phospholipid and with a mucopolysaccharide component also (see page 27). Their description confirmed that of Karrer (1956) with the addition that electron-lucent secretory granules were found collected at the apex of the cell and which appeared to be extruded, one at a time, into the lumen. This suggested both a merocrine and an apocrine secretion by these cells.

In 1969 Sorokin described the bronchiolar epithelium of bats and found electron-dense membrane-bound droplets within the nonciliated cells. He suggested that these cells may be "classified among the serous cells".

Following this, in 1971, Cutz and Conen described the Clara cells of man and rabbit after glutaraldehyde followed by osmium fixation and in 1971 Askin and Kuhn made brief reference to those of rat. Both these groups of workers found apically situated electron-dense granules measuring about  $0.5 \mu\text{m}$  in diameter. In agreement with the findings of Freeman, Crane, Stephens and Furiosi, (1968) Askin and Kuhn (1971) also found electron-dense rod-shaped structures within the cells.

In 1971 and on the basis of cytoplasmic density, Lauweryns et al distinguished, in the mouse, 3 forms of Clara cell probably representing different functional states of the same cell type; a young form ("the clear type") and adult form ("the usual type") and an involutionary phase ("the dense type"). In the human, Basset et al (1971) also distinguished 3 forms: (i) cylindrical cells with an apical dome in which were numerous secretory granules (300-800 nm in diameter and with a granular content surrounded by a clear zone or halo; (ii) similar cells with fewer granules which lacked the halo and were homogeneously and only moderately electron-dense and (iii) cuboidal cells with a flattened apex, no secretory granules, numerous mitochondria and, occasionally, lamellated bodies. In 1972, and for the first time, Rosan and Lauweryns gave a detailed description of the bronchiolar cells of infants (600-1700g). They found the nonciliated cell to be the most frequent cell type but they differed from those of the mouse; they lacked the typical mitochondria of mouse nonciliated cells and had

less smooth endoplasmic reticulum. The nucleus was usually notched and often close to the cell apex. In the smallest airways ciliated cells had few cilia while cilia increased in number with the size of airway.

The pig is the only other species of those described that has the mitochondrial ultrastructure seen in the Clara cells of the mouse (Baskerville, 1970).

#### Recent findings

KULTSCHITSKY-LIKE CELLS - Since the first description of carcinoid tumours of the intestine in 1907 (see MacDonald, 1956; and Price's Textbook, 1966) and of the relationship between Kultschitsky-like cells and lung carcinoids (see Toker, 1966; and Bensch, Corrin, Pariente and Spencer, 1968) there has been interest as to whether or not Kultschitsky-like cells exist in the epithelium of the normal bronchial tree.

Bensch, Gordon and Miller (1965a and b) were the first to publish electron microscopic findings of a granulated cell in the bronchi of adult human airways which they regarded as the bronchial counterpart of the intestinal Kultschitsky or argentaffin cell. They found these cells wedged at the base of tracheal and bronchial epithelium and also in the submucosal glands which occurred particularly at the points of airway branching. In 1967 Gmelich, Bensch and Liebow,

extending these findings to the human bronchiolus, found identical cells both in the "normal" epithelium and in the adjacent carcinoid tumour. The main characteristic of these cells was an electron-lucent, or occasionally, electron-dense cytoplasm, containing an abundance of neurosecretory-like granules, about 140 nm in diameter and each with an electron-dense core surrounded by a clear zone between the core and the outer membrane. These cells did not reach the airway lumen and their processes were often found to extend for considerable distances between the other bronchial cells. These findings have been substantiated by Spencer (1968) and Basset et al (1971).

In 1970 Lauweryns, Peuskens and Cockelaere and in 1972, Rosan and Lauweryns, showed that these cells (their "AFG" cells) were abundant in the bronchial epithelium of infants; the smaller the infants the more were found and the more electron dense was the cytoplasm. Although they suggested that, on the basis of cell profile, cytoplasmic density and lipid inclusions their cells were distinct from those of Gmelich et al (1967), it would seem they are, at least, closely related. Similar cells have been described in the bronchial epithelium of chicken (Cook and King, 1969), tracheal epithelium of mouse (Ericson et al, 1972) and in extra and intrapulmonary airways of rat (Jeffery and Reid, 1973).

In the mouse, the light and electron microscopic report of Ericson et al (1972) showed that these cells were argyrophilic and took up tritiated dopamine and 5-hydroxytryptamine.

Similar cells, widely distributed throughout the body, have been grouped under the title of an Amine Precursor Uptake and Decarboxylation (APUD) endocrine system (see Caravalheira, Welsch and Pearse, 1968 and Pearse, 1969). Rosan and Lauweryns (1971) and Hage (1972) suggested the bronchial granulated cells should also be included as part of this system.

At the ultrastructural level attention has also been focused on the innervation of these cells. Observing the bronchial epithelium of the domestic chicken (1969), Cook and King showed axons associated with granulated cells and in 1970, Lauweryns, Peuskens and Cokelaere, found in the human infant, unmyelinated nerves with small, clear vesicles within 20 nm of these cells.

In 1972 and in the rabbit, Lauweryns, Cokelaere and Theunynck described "neuro-epithelial bodies" similar to granulated cells and with intracorpuseular nerve endings. For the first time in the rat and in 1973, Jeffery and Reid showed the association of what are probably both motor and sensory nerve endings within 15 nm of such cells.

GLOBULE LEUCOCYTES - In 1966 Kent, for the first time, described the fine structure of the "globule leucocyte" found at all levels of the respiratory tract of the laboratory rat. Most were found in the epithelium rather than the connective tissue and more in the larger than in the smaller airways. The cell, typically contained an eccentrically placed, lymphocyte-like nucleus, and its cytoplasm included membrane-bound ovoid "globules" usually homogenous and very electron-dense. These lacked the clear "halo" seen in mast cell granules and were not preserved when fixed with osmium alone. He believed these to be involved in an "immunological resistance", in particular to parasitic invasions. In 1968, Frasca et al described a similar cell in the bronchial epithelium of the dog after fixation with osmium alone ("special migratory cell-osmium") or acrolein ("special migratory cell-acrolein").

#### SCANNING MICROSCOPE

In 1969 and by scanning microscopy, Okada observed the mouse bronchiolus and confirmed that many of the bronchiolar cells protruded beyond the level of the ciliated cells. In 1971 Nowell and Tyler and in 1972, Nowell, Pangborn and Tyler, studied airway surface morphology in hamsters, horses and rats and occasionally found openings at the apex of the bulging bronchiolar nonciliated cells suggesting these might be apertures through which mucigen was discharged.

In 1972 VanAs and Wessels studied freeze-dried rat tracheal epithelium and found that the surface mucus was present as flakes or droplets as small as  $0.5\text{ }\mu\text{m}$  and not as a continuous "blanket" as previously thought.

Also in 1972 Greenwood and Holland observed a number of different levels of airway (nose through to segmental bronchi) and found ciliated cells were less numerous and cilia were shorter in the smallest airways. Numerous microvilli were clearly shown between the cilia, and cell junctions were demarcated by zones of microvilli which were slightly longer and present in greater density than over the rest of the cell surface.

In 1973 and in the rat, Parkinson and Stephens identified, by scanning microscopy, cells with a brush border. These were found infrequently in the normal terminal bronchiolar epithelium.

## SUMMARY

### Light Microscope

In summary, studies with the light microscope have shown the large airway epithelium of man to be psuedostratified with superficial, intermediate and basal layers and that of rat, to be simple. The superficial layer consists mainly of ciliated and mucus-secreting cells, the latter of several different types some of which do not have a typical "goblet" shape.

Extending distally and as airway diameter decreases, the epithelium becomes thinner and simpler, goblet cells are absent and are replaced by nonciliated cells, perhaps of more than one form, and whose function is still not understood.

More recent studies have shown a number of "clear" cells, many argyrophilic and fluorescent, present either singly or organised into "neuroepithelial bodies". The terms applied to these cells are confusing and it is obvious that there are many more "clear" cells than those accounted for by silver staining or fluorescence alone.

It has also been shown that leucocytes are a normal component of the surface epithelium and that one unusual granulated type, the "globule leucocyte" may be numerous.

#### Electron Microscope.

Electron microscopic studies have given more precise information about the cells identified by light microscopy and also revealed new types. The brush cell is a well documented example, while the existence of serous-like cells needs confirmation. The "special-type cell" found in dog (Frasca et al, 1968) and the "nonciliated cell" found in mouse trachea (Hansell and Moretti, 1969), have not been described in other species.

While the ultrastructure of the small airways of the pig is similar to that of the mouse, the Clara cell in these two species has an unusual ultrastructure when compared with most other species. In man and mouse, different forms of Clara cell have been described.

More recently basal granulated cells with "neurosecretory" granules have been shown to be present in relatively small numbers in a wide variety of species but, until the present study, not in the adult rat. Nerves have been shown to penetrate the surface epithelium, some of which lie adjacent to these granulated (Kultschitsky) cells.

The ultrastructure of the globule leucocyte has been detailed and this cell type distinguished from mast cells. Little is known of their origin or function.

#### Scanning Microscope

Finally, scanning microscopy has added little to our structural knowledge of the bronchial tree. Notably it has confirmed that the so-called mucous "blanket" is discontinuous and of small isolated droplets and flakes of mucus.

## Histochemistry of the Goblet Cell and

### Nonciliated Bronchiolar Cell

Although a wide variety of stains has been used to demonstrate mucus in goblet cells, those referred to in this study will be limited to the use of periodic-acid-Schiff, (henceforth referred to as P.A.S.) and Alcian blue (AB), singly or in combination.

The P.A.S. technique was first used to demonstrate epithelial mucus by McManus (1946) and Hotchkiss (1948) the resulting complex being magenta when viewed in the light microscope. Alcian blue was first introduced as a specific stain for the demonstration of acid glycoprotein by Steedman (1950) and, used at pH 2.6, stains both sialic acid and sulphate radicals bright blue. The combined AB/PAS stain was first introduced in 1956 by Mowry, the blue AB staining demonstrating the acidic component and the remaining magenta staining of the PAS reaction representing the neutral glycoprotein (McCarthy and Reid, 1964). Neuraminidase (i.e. sialidase) digestion was first introduced as a histochemical technique by Spicer and Warren (1960) and used in conjunction with the AB/PAS technique, demonstrated the presence of digestible (susceptible) sialic acid as the acidic component by the resulting loss of AB staining in the section. The presence of the sulphate radical as the acidic component in goblet cells was shown by uptake of radioactive sulphur in intestinal cells by Jennings and Florey (1956) and this has been correlated with AB staining by Curran

and Kennedy (1955).

#### GOBLET CELL

##### Mouse

In spite of relatively thorough histochemistry of many body organs there has been little histochemistry of the normal respiratory mucosa. Curran and Kennedy (1955) studied the distribution of sulphated mucopolysaccharides in the mouse and found AB staining in the respiratory epithelium which was evidently not due to sulphate since labelled sulphur was not incorporated into these cells. The results of Reid, McCarthy, Duvenci and Gibbons (1962), were in agreement with this and showed that the AB staining was due to the presence of a susceptible sialomucin present at all levels of mouse airway.

##### Rat

The rat showed, however, a regional distribution of acid glycoprotein: goblet cells of peripheral airways contained sialidase susceptible sialomucin, most (but not all) of the tracheal goblet cells took up labelled sulphate with no loss of AB staining after neuraminidase digestion, and the hilar bronchi showed a mixture of both types of goblet cell. Furthermore, the combined AB/PAS stain gave a range of colour; blue, purple (heliotrope) and magenta which indicated that a single cell could have a mixture of glycoprotein types. Those of the rat were predominantly purple staining with only a small number of pure magenta or blue staining cells to be seen (McCarthy and Reid, 1964).

On the basis of amount of stainable secretion and predominant colour, Sturgess and Reid (1973) distinguished four types of goblet cell; goblet cells with a small secretory mass staining either magenta or blue, full magenta and full blue goblet cells. From their photomicrographs it is clear that the small goblet cell variety does not conform to the typical goblet shape. Using these criteria in 1973, Jones, Bolduc and Reid, found that the majority of secretory cells of normal rat bronchi were predominantly magenta in colour.

#### Human

Using the combined AB/PAS technique, only a few goblet cells of human respiratory epithelium were found to stain red, most of them having at least some acid glycoprotein and staining either heliotrope or blue. Neuraminidase digestion showed most of these to contain at least some susceptible sialomucin although the amounts varied (McCarthy and Reid, 1964b).

#### THE NONCILIATED BRONCHIOLAR OR CLARA CELL

In 1937 Clara studied the histochemistry of the nonciliated cell in the human and rabbit terminal bronchiolus (see page 6 ). Recently, histochemical attention has been focused on the nonciliated cells of the mouse (e.g. Niden, 1967 and Azzopardi and Thurlbeck, 1969) with the suggestion that the secretion might be responsible, at least in part, for the production of surfactant (surface-tension reducing

substance). Little is known of the histochemistry of this cell in the rat (see Askin and Kuhn, 1971).

#### Human and Rabbit

Clara (1937) found the secretory granules of the nonciliated bronchiolar cell stained intensely with iron haematoxylin but found no staining of them by stains for mucus such as mucicarmine, mucihaematin, thionin and toluidine blue. On the basis of this, Clara presumed the apocrine secretion to be a non-mucus colloid. v.Hayek (1961) believed that the secretion from human nonciliated bronchiolar cells was of an eosinophilic "mucus" found in large amounts in some cases of asthma. Using both light and electron microscope Cutz and Conen (1971) showed that their apical membrane-bound granules were PAS positive, stained for bound lipids and with a variety of other stains including periodic acid-silver methenamine, Baker's acid haematin and Luxol fast blue: there was no reaction with Alcian blue or mucicarmine stains. The electron-density of the Clara cell granules was unaltered by Chloroform/methanol extraction but could be reduced after pepsin digestion. They concluded that these granules contained a phospholipid firmly bound to a nonlipid, most probably protein.

#### Mouse

In 1949, Macklin studied the nonciliated Clara cells of mouse, after supravital silver staining and found that while the cytoplasm did not stain with silver, the apical granules did. Niden (1967), found that the granules of these cells did not stain with PAS, AB and Sudan black.

(indicating an absence of mucopolysaccharide, acid mucopolysaccharide and free lipids) but did stain with Sudan black acetone, Baker's acid haematin, and silver hydroxylamine indicating the presence of a phospholipid. Intraperitoneal injections of tritiated palmitate or acetate resulted in the presence of autoradiographic grains over these cells within 5 minutes. In 1969 the histochemical studies of Azzopardi and Thurlbeck showed that the activity of the oxidative enzymes of the nonciliated cell surpassed that of both adjacent ciliated and nearby alveolar cells and that the nonciliated cell was rich in choline-containing phospholipids, probably present as lipoproteins. Other special mucus stains (e.g. mucicarmin and PAS) showed essentially, negative results although " a few positive granules were occasionally seen over some of the cells of the bronchiolar epithelium....."

#### Rat

In a study to find the origins of pulmonary surfactant, Askin and Kuhn (1971) showed that in the large alveolar (type II) cell, after chloroform-methanol extraction, there was almost complete loss of osmiophilic material from lamellated bodies; a less intense result was obtained with acetone extraction. Neither of these solvent extractions affected the nearby nonciliated cell granules. Tritiated palmitate localised by autoradiography, was consistently found to be

present in alveolar cells in greater amounts than in Clara cells.

these results, they suggested, were not in conflict with previous reports of phospholipid in Clara cell granules and suggested only that the phospholipid was not dipalmityl lecithin.

#### SUMMARY

In summary, goblet cells may contain neutral or acid glycoprotein, the latter due to either a sulphate radical or sialic acid, or a mixture of these. Clara cell granules would appear to contain a phospholipid of a kind probably bound to protein and there are suggestions that a neutral mucosubstance is also present.

#### Origin and Replacement of the Goblet Cell

##### in Bronchial Epithelium.

There is little known about the origin of the goblet cell in the respiratory tract. In 1881 Drasch and in 1885 Bockendahl described mitoses within respiratory epithelium. It is widely accepted that they develop from basal cells although, using the light microscope and as early as 1927, Schaffer suggested goblet cells could divide and more recently Condon 1942, Spencer and Shorter (1962) and Blenkinsopp (1967) have shown that many cells in the superficial layer may divide.

### ORIGIN FROM THE BASAL CELL

In 1881 in the trachea, Drasch described basal cells in division. These were thought to give rise to goblet cells via an intermediate cell ("Keilzellen") stage, the goblet cells finally giving rise to ciliated cells. Kolliker (1881) disagreed and suggested that the intermediate cell type (his "Erzatzzellen") gave rise separately to both ciliated and goblet cells.

The multipotential nature of basal cells was shown in 1953 by the experiments of Fell and Melanby on cultures of embryonic chick ectoderm. By giving an excess of vitamin A in the culture medium they induced the normal keratinizing squamous epithelium to change into a columnar type with ciliated and goblet cells. If the transformed epithelium was transferred back to a normal culture medium, basal cells ceased to form secretory cells reverting back to cells of the keratinizing type.

In 1962 Greenberg and Willms studied the regeneration of ciliated and mucus-secreting elements over a window of "Mersilene" cloth placed in the dog trachea and found that the parent cell was the reserve or basal cell.

In 1967, using tritiated thymidine to label D.N.A. Blenkinsopp investigated the mitoses of the tracheal epithelium of rat and found that each dividing basal cell gave rise to one basal and one "superficial"

cell; some of these "superficial" cells contained PAS positive material and were probably goblet cells.

Although there are electron microscopic reports of similarity in structure between basal and intermediate cells (e.g. Rhodin, 1966), there have been none showing stages of transformation from intermediate to goblet cells. The sparcity of basal cells in distal bronchioli suggests there is some other mechanism for the development of the goblet cells seen in such large numbers at this site in hypersecretory diseases.

#### ORIGIN FROM THE CILIATED CELL

In 1932 Hilding recorded the results of his experiments involving surgical closure of the nares of rabbits. He found an unexpected change in the epithelium: more or less complete metamorphosis of ciliated columnar cells to one of goblet cells devoid of cilia. This was thought to begin by the formation of a mucin droplet within the ciliated cell, followed by its enlargement, a lifting of the "ciliary plate" and its extrusion with complete loss of cilia. This was found to involve some 30 - 100% of cells and he believed this to be the chief pathological change in hypersecretory disease (Hilding, 1943).

Condon (1942) studied the rat epithelium after trauma and suggested that in the ciliated cell with vesicular cytoplasm, there was an

enlargement of vesicles and the formation of goblet cells. He also suggested that once having discharged, the goblet cell would return to a ciliated cell stage.

More recently, using the electron microscope and human tracheal epithelium, Osada (1963) described a small number of ciliated cells with cytoplasm of the same electron-density as goblet cells and with dense granules in the Golgi region. He suggested this as the first stage of transformation to a goblet cell.

#### ORIGIN FROM THE KULTSCHITSKY CELL.

The origin of goblet cells from argentaffin or Kultschitsky cells was suggested by Popoff in 1939. Using histochemical techniques and the intestinal tracts of rabbits and humans he found that goblet cells, when reaching a stage of functional exhaustion, did not perish but underwent a cytomorphosis into an "argentochrome" cell. This transformation had a rejuvenating effect and after a change in position was accompanied by dedifferentiation and a return to the normal secretory state.

Recently, using the electron microscope, Terzakis, Sommers and Andersson (1972) studied human segmental bronchi and found goblet cells containing small, dense, membrane-bound granules with a thin, low density peripheral halo, identical to the dense-cored granules of nearby "Kultschitsky" cells. These were not found in ciliated cells and they, therefore, felt that the neurosecretory appearing cells might

serve as precursors to goblet cells in diseased states.

#### ORIGIN FROM OTHER CELL TYPES

There are no descriptions in the literature of brush cells giving rise to goblet cells. No suggestion of the epithelial "serous" cell giving rise to goblet cells has been made (see Rhodin, 1959) and the role of the "goblet" cell depleted of its secretory granules is not clear (see Rhodin and Dalhamn, 1956 and Dalhamn, 1956).

That the nonciliated (Clara) cell could give rise to a mucus-secreting goblet cell has not seriously been considered.

In this present study, there has been a renewed attempt to pinpoint the origin or origins of the goblet cell at different levels of the respiratory tract.

#### Experimental Hypersecretion

Bronchial epithelium may be altered to produce increased mucus by a number of different agents including irritant gases, drugs or infection. The effects of infection will not be dealt with here (see interalia Sanford, 1968; Baskerville, 1972 and Gay, Maquire and Baskerville, 1972).

#### TOBACCO SMOKE AND OTHER IRRITANT GASES

A wide range of irritant gases (e.g. formalin, ammonia, chlorine, sulphur dioxide and nitrous oxide) have been used and their effects on the respiratory tract recorded. Haggard (1924) showed that all act by

inducing inflammation and the differing pathology of each is due, not so much to the difference in their chemical properties, as to differences in their physical properties, the more important of which is solubility. In the case of organic substances, such as those included in tobacco smoke, the action may be more complex and the systemic effect may be greater than their action as a pulmonary irritant. Depending on such factors as concentration, humidity and presence or absence of a particulate component, a range of effects from ciliostasis to cancerous invasions of the underlying tissue may be found.

The study of experimentally induced goblet cell hyperplasia in relation to respiratory irritation goes back to Lord Florey who, in the early 1930's produced goblet cell hyperplasia in cats and dogs with formol-saline (Florey, Carleton and Wells, 1932).

Subsequently, in 1958 and in the rat, Reid found a similar increase after exposure of rats to sulphur dioxide for 3 weeks (5 hours per day and 5 days per week). These animals had developed the stigmata of chronic bronchitis, hypersecretion of mucus; mucus was now found in the bronchi and also impacted in the alveoli. Histologically goblet cells increased in number in the large airways, appearing and increasing in the smallest airways in which they normally are absent. These changes were later (Reid, 1963) quantified, the excess of goblet cells persisting up to 3 months after exposure and in the absence of infection.

A similar increase, due to cigarette smoke was not described until 1958 when Mellors, using the light microscope, investigated the accumulation of smoke products in the airways of rats. In this latter case, the increase was not quantified as it was by Lamb and Reid, (1969).

### Tobacco Smoke

The American Indian herb, now known as Nicotiana tabacum, was first introduced into Europe in the early 16th century, when it was thought to have curative powers. Near the end of that century, smoking was condemned as responsible for all manner of ills but, in spite of this, it became an almost universal habit. Some of the first studies concerning the respiratory effects of cigarette smoke were to do with its ciliostatic effect, a phenomena now well established (Proetz, 1939; Hilding, 1956; Ballenger, 1960, and Dalhamn, 1964).

From statistical and post-mortem histological studies, a causal connection between smoking and cancer has been advanced (Doll and Hill, 1956; Chang, 1957; Auerbach, Gere, Forman, Petrick, Smolin, Muehsam, Kassouny and Stout 1957, and Auerbach, Stout, Hammond and Garfinkel, 1961), and it has been established as the major cause and predisposing factor in the development of chronic bronchitis (Royal College of Physicians' Report, 1962 and 1971).

Further work has concentrated on analysing the specific components of the smoke which bring about its effects (e.g. Kensler and Battista, 1963) and, more recently, with the development of a "safer" cigarette. The latter has involved studies on the value of filters (Dalhamn, 1964), on the sugar content of the tobacco and the pH and nicotine/tar content of the smoke (Elson, Betts and Passey, 1972). The publication of the Government's "Tobacco Tar Table", listing the tar and nicotine content of various brands of cigarettes, has intensified research into the development of synthetic tobaccos. Preliminary results, with the addition of anti-inflammatory agents to existing cigarettes, have given encouraging results (Jones, Bolduc and Reid, 1972).

The action of tobacco smoke on respiratory epithelium may be divided into its effects on goblet cell hyperplasia and ciliostasis ("bronchitic effect") and on the mitosis, and growth of epithelial cells ("cancer effect").

THE BRONCHITIC EFFECT - The most striking histological feature of chronic bronchitis is the hypertrophy of mucus-secreting elements: the surface goblet cells and the submucosal glands (Reid, 1954, 1958b and 1968). Few quantitative studies, similar to those made using sulphur dioxide (Reid, 1963) have been made after tobacco smoke.

Ultrastructural studies of the effects of tobacco smoke are even fewer in number and there is a need for more detailed analysis of the cellular changes.

Light microscopy - Early post mortem histological studies on smokers and non-smokers (Chang, 1957; and Ide, Suntzeff and Cowdry, 1959) showed that the tracheal and bronchial epithelium of smokers had significantly more surface goblet cells, fewer ciliated cells - those remaining had shorter cilia - and a thicker epithelium than non-smokers. The sub-mucosal gland was also increased in smokers (Ide et al 1959).

Passey and Blackmore (1967), Lamb (1967) and Lamb and Reid (1969), compared the effects of both cigar and cigarette tobacco given to rats. Both were found to increase the number of epithelial goblet cells, although significant quantitative results were only obtained from the lower dose ranges where metaplastic lesions were absent (Lamb and Reid, 1969). Furthermore, the increase was found to be dose-related and greatest in the largest airways.

In 1972 Jones, Bolduc and Reid, reaffirmed the increase<sup>d</sup> in goblet cells after tobacco smoke and showed this increase to be significant, especially in the largest airways. Associated with this there was also a histochemical change in the mucus (Jones, Bolduc and Reid, 1973).

Electron microscopy - Only one or two ultrastructural reports on the effects of whole tobacco smoke on respiratory epithelium have been found in the literature. In 1968b, Frasca et al reported the effects of tobacco smoke given for 44 - 420 days to dogs. In the early stages of exposure, there was an increased number of goblet cells and fewer cilia and by 420 days the number of cell layers had doubled with absence of both ciliated and goblet cells. A "special cell-type" with small (about 130 nm) disc-like inclusions and containing finely fibro-granular material was greatly increased in number while there was a decrease in the number of "special migratory cells" (i.e. globule leucocytes). No quantification of these changes was made.

More recently, Ailsby and Ghadiali (1973) have reported areas with atypical cilia in the airways of a 60 year old man who had smoke 25 cigarettes per day for 46 years. These, they suggested, would be useless in moving the mucus. These changes were not, however, shown to be directly related to the effects of cigarette smoke.

THE "CANCER EFFECT" - A correlation between smoking and lung cancer has long since been suggested and attempts to produce this experimentally have been made. Early in 1958 Leuchtenberger, Leuchtenberger and Doolin produced changes in the bronchial lining epithelium of mice exposed to whole tobacco smoke. Similar, but more extensive changes were produced

by "rubbing" cigarette smoke condensate onto the wall of the primary bronchus (Rockey, Spear, Ahn, Thompson and Hirose, 1962).

Passey and Blackmore, 1967, and Lamb, 1967, produced severe metaplastic lesions by giving 30/40 cigarettes (cigar or cigarette tobacco) per day to rats. They, and in 1969, Lamb and Reid, also showed that cigar smoke, from naturally cured tobacco, did not cause the massive increase in mitoses, seen with the equivalent amount of flue-cured cigarette tobacco.

A quantitative analysis of mitoses in the tracheal epithelium of rats, exposed to 25 cigarettes per day (Jones et al, 1972) has confirmed that cigarette smoke significantly increases the number of cells in mitosis and more recently, Leuchtenberger, Leuchtenberger and Ritter (1973) have shown cigarette smoke produces abnormalities in DNA synthesis chromosome complement, mitosis and cell growth.

#### Other Gases

With the light microscope, the respiratory effects of a wide range of gases including formol-saline (Florey et al, 1932) chlorine (Elms and Bell, 1963) nitrous-oxide (Freeman and Haydon, 1964; Freeman, Crane, Furiosi and Stephens, 1972) and sulphur dioxide (Dalhamn, 1956; Reid, 1963; Lamb and Reid, 1968; Mawdesley-Thomas, Healey and Barry, 1971) have been investigated and goblet cell hyperplasia

reported.

The ultrastructural changes have been reported after exposure to sulphur dioxide, nitrous oxide and acrolein inhalation.

In 1956 and in rats whose epithelium had been irritated by sulphur dioxide, Dalhamn showed a thickening of the epithelium which was now composed of tall slender, ciliated cells compressed laterally. No alteration in goblet cells was reported.

After nitrous oxide, Freeman and Haydon (1964) and Stephens, Freeman and Evans (1972) showed that due to ciliated cell hypertrophy, the cells of the distal airways of rat became more uniform in size though one third larger in area: few cilia remained. No ultrastructural observations on goblet cells were made.

The effects of one of the constituents of tobacco smoke, acrolein, was investigated by light, electron, and scanning microscopy, by Dahlgren and Dalen in 1972. They found that the cells of the less affected guinea pigs were deformed in shape, had cytoplasmic vacuoles and showed inter-cellular oedema, resulting in a narrowing of the epithelium. Goblet cells had discharged most of their mucus and cilia were degenerate. In the more severely affected animals, there was total exfoliation of both goblet and ciliated cells.

### The Anti-inflammatory Agent - Phenylmethyloxadiazole

The study of anti-inflammatory agents, which modify the tissue response to inflammatory agents, has been of special interest since the introduction of cortisone in the 1940's. Such agents include the steroid hormones, salicylates and quinine and antihistamine drugs.

In 1966 and 1969, Dalhamn and later, Dalhamn and Rylander (1971) showed that phenylmethyloxadiazole, (henceforth referred to as PMO,) a compound structurally related to the antitussive agent oxalamine, offered protection against the ciliostatic effect of tobacco smoke.

In 1972 Dahlgren and Dalhamn demonstrated a significant degree of protection against the destructive effect of acrolein exposure by intraperitoneal and oral dosage of PMO to guinea pigs, and more recently, Jones et al (1972) demonstrated that addition of PMO to tobacco (2% by weight) completely protected against the increase in goblet cells found in rats exposed to tobacco smoke without PMO. Less thickening of the epithelium and fewer mitoses were found in the animals whose cigarette included PMO.

No ultrastructural studies have yet been reported concerned with the affect of PMO on respiratory epithelium.

### ISOPRENALINE, PILOCARPINE AND OTHER DRUGS

The effect of drugs and in particular, isoprenaline sulphate and pilocarpine nitrate has been investigated recently with reference

to their effect on bronchial epithelium and secretion.

Isoprenaline (Isoproterenol, isopropylnoradrenalin, isopropylarterenol)

Isoprenaline (sulphate), henceforth referred to as IPN, first studied in 1940 by Konzett, is a sympathomimetic agent which acts, almost exclusively, on Beta-adrenergic receptors being a more powerful bronchodilator than adrenaline (Innes and Nickerson, 1965; Martindale, 1972). Particularly indicated as an inhalant in the treatment of acute broncho-constriction, IPN and its related bronchodilators have recently been less used due to an indicated correlation between the increased use of bronchodilator inhalants and a rise in the incidence of sudden death in asthmatics since their introduction. (Speizer, Doll and Heaf, 1968 and with Strang, 1968).

The effect of IPN on other body organs is better known; skeletal muscle vasculature, alimentary tract and notably the heart where necrosis and cell infiltration have been shown after only a single sub-cutaneous injection of 75mg/1,000g. body weight given to rats (Korb, 1966). At the cellular level and in both the kidney and salivary gland, it has been shown to alter the RNA/DNA ratio and induce mitosis and cell proliferation these effects being mediated via the intracellular messenger cyclic AMP (Barka, 1970; Malamud and Malt, 1971). Its effects on secretion are little known. There has been only one light microscopic report of its effect on bronchial epithelium (Sturgess and Reid, 1973), and although there have been ultrastructural studies of

its effects on other tissues, none concerning bronchial epithelium has been published.

LIGHT MICROSCOPE - In 1961, Selye, Veilleux and Cantin demonstrated a five-fold enlargement and weight increase in rat salivary gland after twice daily intraperitoneal injections of 50 mg. IPN for 17 days given to 135g. rats. There was an intense mitotic activity of serous, mucous and duct cells and an increase in their secretory activity. Although there was an increase in cell size, they concluded that most of the increase in gland size was due to cell proliferation. Later, in 1961, Brown-Grant produced a similar increase in mouse salivary gland but found mitoses only rarely and concluded that cell hypertrophy alone could account for the increase in size.

In 1962 Schneyer produced salivary gland increase up to 5 times the normal using lower doses; 6 to 12 mgs given to 215 g. rats for 12 days (higher doses were found to give high fatality, especially in males). Mitoses increased, especially during the early stages but he thought a progressive increase in cell size accounted for the increase in gland weight. As the water/dry weight ratio remained constant, this increase in size was not due to water imbibition. Alterations to the secretion of ions were found and the potassium levels in parotid and sub-maxillary secretion were very significantly increased. A marked reversal of these changes was found by 17 days

after withdrawal of the drug.

In 1968 and by uptake of labelled amino-acids, Barka reported a marked increase in protein synthesis in salivary glands as well as the peak in DNA synthesis at 28 hours.

In 1969 Mangos, McSherry, Benke and Spock, reported 10-fold enlargement of rat salivary glands after 5 mg. IPN given sub-cutaneously to 100g. rats for 32 days. The sodium content of the secretion was increased, there were alterations to the basic macro-molecules, and the sera of the IPN rats contained a factor which caused ciliary dyskinesia, thus resembling some aspects of the disease cystic fibrosis.

In 1973 Sturgess and Reid, for the first time, reported the effects of chronic administration of IPN on bronchial secretory cells. The number, staining characteristics, and size of the surface goblet cells was altered at all levels of airway and the changes quantified. There was a significant increase in the total number of goblet cells present at all levels. Using a combined Alcian blue and periodic acid Schiff (AB/PAS) technique, there was a four-fold increase in those staining with the AB component - i.e. those with predominantly acid-glycoprotein. No change in number was found in those staining only with PAS - i.e. those with neutral glycoprotein. After IPN, each goblet cell became more distinct, had more prominent secretory granules, and a larger volume of intracellular mucus.

ELECTRON MICROSCOPE - There have been very few ultrastructural reports of the effects of IPN on secretory cells and none concerned with bronchial epithelium.

In 1967 Takahama and Barka studied the acute effects of IPN in the 24 hour period following a single intraperitoneal injection of 16 mg/100g. BW given to female rats. By 1 hour there was a complete depletion of granules, at 4 hours the rough endoplasmic reticulum (RER) and the Golgi apparatus were hypertrophied, ribosomes increased in number and nuclei were swollen with prominent enlarged nucleoli. By 18 hours the secretory granules were restored.

In 1968 Kanda, Mayfield and Ghidoni also described the alteration in rat sub-maxillary gland following a single injection (16 mg/100 g. BW). They extended the study to 48 hours following the injection and found that by this time some granules contained concentric lamellae of dense material, resembling an "onion-skin" appearance. This they described as a new form of secretory granule which, they believed, represented an early stage in granule formation, with subsequent fragmentation of these lamellae giving rise to mature granules.

In 1969, Simpson described the response of the rat parotid gland to a single injection of 5 mg IPN given to 175 g. rats. Discharge began five minutes following the injection and was complete by 40 minutes. Accumulation of granules began at 6 hours and restitution of resting amounts was complete by 18 hours. Granules, similar to those described by Kanda et al (1968), were not found. Many lipo-fuscin granules

were, however, found in the cells of the IPN treated rats and in a later study these changes were attributed to cell damage which followed IPN (Simpson, 1972).

#### Pilocarpine nitrate

Pilocarpine nitrate (henceforth referred to as PCP) is a parasympathomimetic agent with the muscarinic effects of acetylcholine and affects innervated tissues in the same way as intense parasympathetic stimulation. Isolated independently by Gerrard and Hardy in 1875 it is an alkaloid obtained from the leaflets of the South American shrub Pilocarpus jaborandi and P. microphyllus which local indians chewed to invoke salivation. Early work showed it also caused marked sweating and in 1876 its actions on the pupil as well as the salivary glands were described by Weber (Koelle, 1965). Experimentally its actions have been described on the lacrimal, gastric, pancreatic and intestinal glands as well as the tracheal submucosal glands.

In 1932, Florey et al, gave repeated intravenous injections of pilocarpine to cats and studied the effects on the respiratory airways histologically. They found that while the submucosal gland was stimulated to secrete to a stage of exhaustion, the discharge from the surface goblet cells was not in excess of the normal and concluded that these surface structures lacked an innervation. The goblet cells were not, however, entirely unaffected, as they reported an increase in their intracellular content.

The work of Farber in 1942 showed that chronic administration of 10 mg. pilocarpine given subcutaneously 3 times daily for 2 - 8 weeks to kittens caused, in them, extreme loss of weight and severe nutritional disturbance. Morphologically lesions were seen in the pancreas with its acini and small ducts filled with thick, homogeneous osmiophilic material.

In 1973 Sturgess and Reid gave repeated subcutaneous injections of 10 mg. PCP to 250 g. rats and did find an increase in the total number of bronchial goblet cells. All histochemical types and particularly the small PAS goblet cell increased in number, the volume of secretion in each was greater and the secretory granules were larger and more prominent.

No ultrastructural reports of the effects of PCP on bronchial epithelium have been found in the literature.

#### Other drugs

The effects on bronchial epithelium of other drugs, in particular bromhexine hydrochloride ("Bisolvon"), have been investigated by light and electron microscopy.

The effects of Bisolvon, an expectorant, reported to change the structure of bronchial secretions and to reduce sputum viscosity have been recently investigated by electron microscopy.

In 1966 Merker gave intraperitoneal injections of 4 or 20 mg./kg.BW to 300 - 400 gram. rats for 6 days and examined the epithelium of medium and small bronchi. The control goblet cells had 300 nm. membrane-bound secretory granules with electron-dense finely granular contents. In the treated rats they increased in size and number with a "loosening-up" of the contents, loss of electron density, and often, fusion between the granules. Changes in some ciliated cells were suggestive of mucus transformation. These bronchial changes were also found by Spors (1970) who investigated the acid phosphatase activity in rat goblet cells after treatment with Bisolvon. In control goblet cells he found this activity was limited to the membrane of the secretory granule, while in those of the treated rats, it was spread over the matrix of the now "loosened" granule.

In 1967 Merker showed similar changes in the nasal glands of man, the loosening of the granules often beginning at the periphery, leaving a central dense-core.

In 1968, Geiseking and Baldamus investigated the human bronchial mucosa but failed to find the striking changes described by Merker (1966).

The effects of other drugs on bronchial epithelium have also been studied by light or electron microscopy. In 1960 v.Hayek reported changes in Clara and ciliated cells after injections of adrenalin and atropine respectively, Kent (1954) has found a decrease in the number of

globule leucocytes after cortisone and, using the electron microscope, Wang et al (1971) have caused alteration of Clara cells by injections of adrenaline.

#### Purpose of this Study

This present ultrastructural study is concerned with extending knowledge of the normal bronchial epithelium in the specific pathogen free rat and, in particular to establish the cell types present and their frequency at various airway levels. The morphological features of the various types of goblet cell, identifiable histochemically by light microscopy, have been correlated with their electron microscopic appearance.

Experiments with tobacco smoke have been designed to establish the cellular changes caused by mild irritation. In this way the structural changes associated with goblet cell increase can be followed, as well as their modification when protection against the tobacco effect is given by the anti-inflammatory drug phenylmethyl-oxadiazole.

The effects of two drugs, isoprenaline and pilocarpine on the bronchial epithelium, are also followed ultrastructurally since epithelial changes have already been reported by light microscopy.

## CHAPTER II

### MATERIAL AND METHODS

## MATERIAL AND METHODS

### Tissue

The adult laboratory rat (i.e. over 150g) was used in this study. Albino strains were obtained from either of two sources: specific pathogen free (henceforth referred to as SPF) rats from Carworth Europe, Alconbury, Huntingdon, or "Anticimex" rats from Anticimex Farm, Stockholm. The animals were free of respiratory infection (i.e. "clean") as judged by a low epithelial goblet cell count (1-2 goblet cells per oil field) and by the absence of, or minimal amounts (1-2 isolated sites) of lymphocytic cuffing around the airways (Reid, 1970).

From each animal, the trachea, left main bronchus, and single lobed left lung were taken. Fig. II-1 illustrates the levels 1-5 chosen from the single-lobed left lung of rat for examination by light and electron microscopy: (1) upper trachea (rings of cartilage) (1-6); (2) lower trachea (rings 15-20); (3) left extrapulmonary main bronchus; (4) left intrapulmonary bronchus; (5) distal bronchiolus of less than 0.4 mm in diameter.

Details of animal numbers are given with the appropriate results.

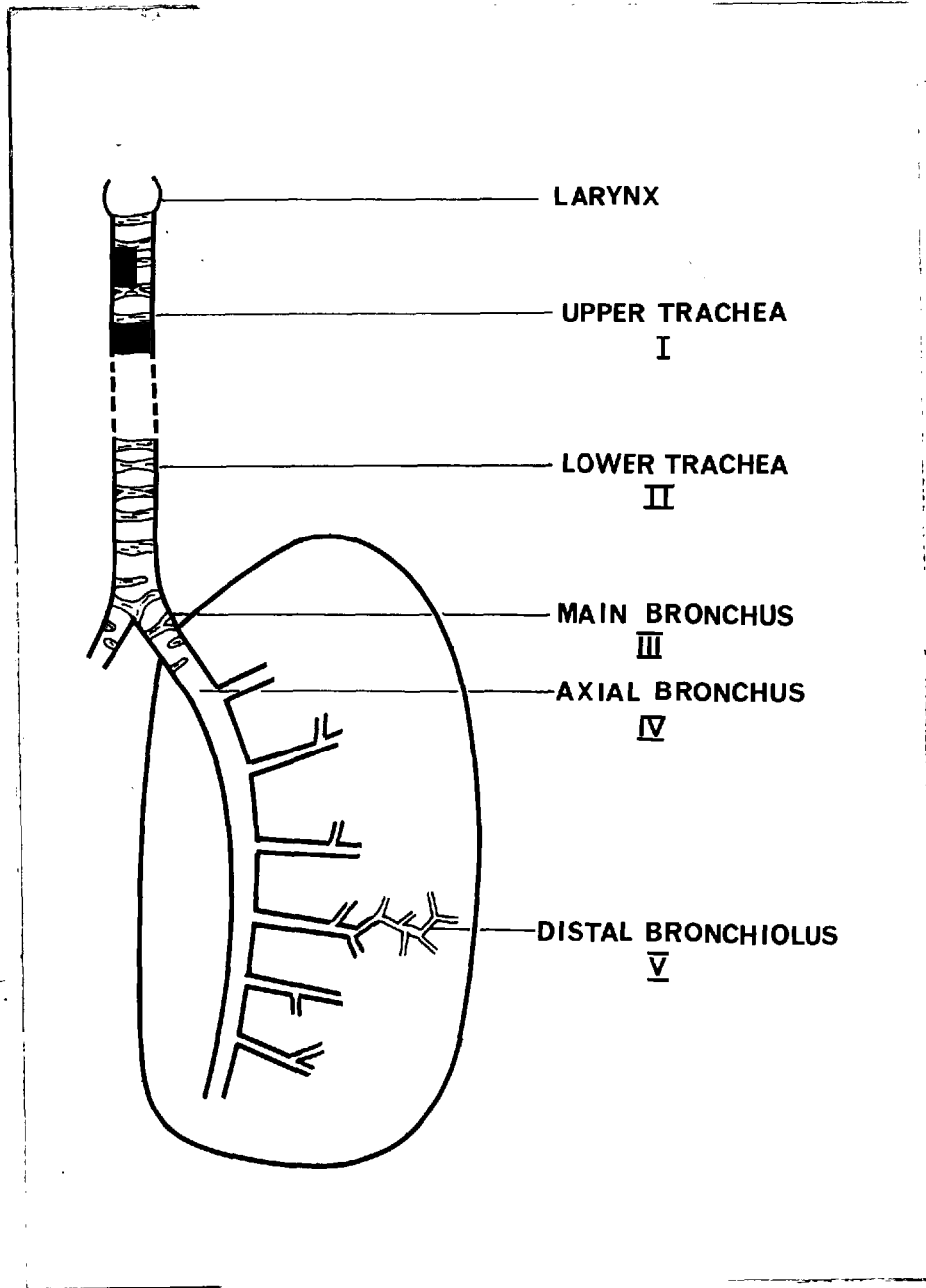


Fig. II-1 illustrates the levels 1 - 5 chosen from the single-lobed left lung of rat for examination by light and electron microscopy.

## Care of Animals

### USUAL CONDITIONS

The animals were kept on woodshavings in solid bottom polythene cages (North Kent Plastic Cages Limited, Dartford, Kent) and were given food and water ad libitum. The "Pasturised Breeding Diet" was obtained from Lillico & Company (Bexworth, Surrey). Water was changed daily and bedding twice weekly. All efforts were made to maintain the animal conditions as clean and hygienic as possible. Specific pathogen free rats (S.P.F.) were kept in an animal room used solely for S.P.F. rats and cared for by the person in charge of the experiment.

### EXPERIMENTAL CONDITIONS

During exposure to cigarette smoke, the animals were placed in thick wire mesh cages, suspended in the smoking cabinet. During the exposure, both exposed and control rats were deprived of food and water. Animals for drug studies were, at all times, kept under usual conditions and not deprived of either food or water.

## Experimental Procedure

### SMOKING EXPERIMENTS

Rats were exposed to cigarette smoke for approximately 4 hours each day for periods of up to six weeks.

#### The cigarettes

Two types of cigarette were used, manufactured and supplied by the research division of the Lorillard Corporation, Greensborg, North Carolina. All cigarettes were of the same size (80 cm long) and contained the same tobacco, but to the tobacco of one type was added 2% by weight of phenylmethyloxadiazole (henceforth referred to as P.M.O.). Both types of cigarette gave a smoke of similar pH - 4.8 and buffering capacity - 0.8 - (Elson et al, 1972).

#### Cabinets and smoking machines

Fig. II-2 shows the aluminium cabinet (A) used to house the animals during exposure to tobacco smoke. Air was delivered to the cabinet at a rate of about 10 litres per minute, via the Wright Auto-smoker machines (B) (Wright, 1972). These machines gave control for the length of cigarette burn. Each cigarette was "smoked" by a series of 4 "puffs" per minute, each puff lasting 4 seconds and drawing about 30 cc of air through the cigarette. The machines were

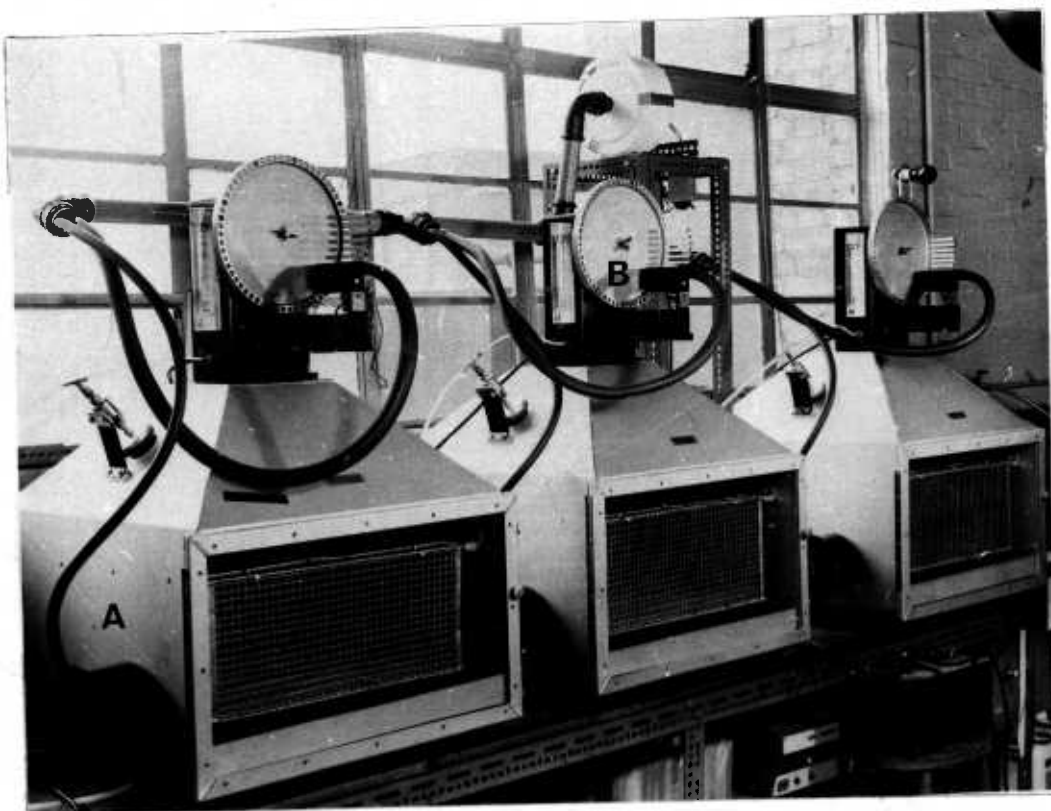


Fig. II-2 demonstrates the aluminium cabinets (A) used to house the animals during exposure to the cigarette smoke delivered from the Wright Autosmoker machines (B).

adjusted to start a new cigarette every 10 minutes and since a cigarette burned in 5 to 6 minutes, 4 minutes of room air only was delivered to the cabinets between cigarettes and clearing them of stale smoke.

#### DRUG EXPERIMENTS

The effect of two drugs, isoprenaline sulphate (isoproterenol, isopropylnoradrenalin), and pilocarpine nitrate (henceforth referred to as IPN and PCP respectively) was investigated. Animals were given a single daily injection of 10mg. PCP or 25 mg. IPN for up to 12 days. Drugs for injection were prepared sterile by the Hospital Pharmacy in 2 ml. vials at concentrations of 20 mg/ml (PCP) or 50 mg/ml (IPN) in distilled water with preservatives added. Animals were injected subcutaneously in the side with 0.5 cc of the drug, the sides being changed to avoid chronic irritation of the injection site. Subcutaneous injections allowed for high doses of the drug to be given which, by slow diffusion from the subcutaneous tissue, gave a low level of drug maintained over a long period of time. Control animals were given daily injection of 0.5cc sterile physiological saline (0.9%w/v).

#### Preparation of tissue for Microscopy

The following method of tissue preparation was used in all the experiments.

#### DISSECTION

Animals were injected intraperitoneally with an excess (about 1 ml)

of Nembutal (Sodium pentobarbitone) and dissected under deep anaesthesia from the ventral surface. The thorax was opened to expose the lungs, heart and thymus. A further cut was made between the coracoid processes and extended to part the sterno-hyoid muscle and salivary glands. The exposed trachea was then ligated immediately below the laryngeal cartilage and the fixative introduced by needle and syringe into the tracheal lumen. The lungs were slowly inflated with fixative until the pleura was tense and the lung margins sharply defined. A second ligature was usually tied immediately below the point where the needle was inserted to ensure inflation was maintained. Both the trachea and lungs were removed intact, care being taken not to stretch the trachea. The heart and thymus were then carefully dissected away (Fig. II-3) and the preparation immersed in the fixative. Tissue for light microscopy was mounted on a card in such a way as to preserve the normal laryngo-hilar length of the trachea.

Tissue for electron microscopy was removed from the primary fixative and, with the aid of a binocular microscope, further dissected for levels of airway (see Fig. II-1). The trachea and main extrapulmonary bronchi (levels I, II and III) were cut transversely as a series of rings, each containing a single cartilage plate. In the early experiments these were further cut into smaller pieces of about IX 3mm but it was later

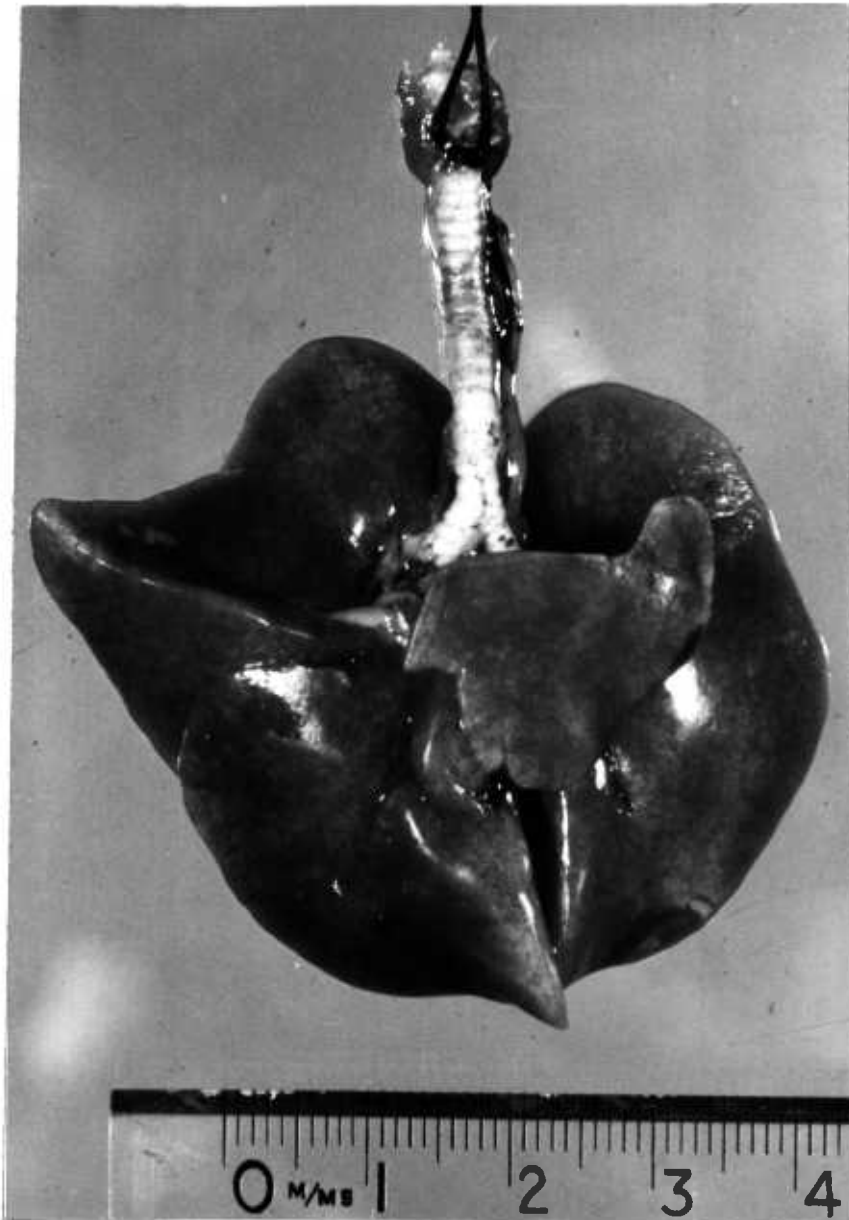


Fig. II-3 illustrates the trachea, bronchi and inflated lungs of a rat after their removal from the thoracic cage. The heart and thymus have been dissected and removed.

found better to embed as a complete ring for easier orientation of the block. The more proximal intrapulmonary airway (level IV) was taken from transverse cuts across the main axial pathway immediately distal to the hilum. Blocks of the distal intrapulmonary airways (level V) were taken from sections of the distal ends of the lateral airways and were 0.4mm or less in diameter, their position identified macroscopically by branches of the pulmonary artery which ran with them.

#### FIXATION.

Lungs were fixed for light microscopy by inflation and immersion in 10% neutral Formol-saline for at least 24 hours. The tissue was then processed through graded alcohols, chloroform used as a transitional medium, and embedded in paraffin wax. Unless otherwise stated, tissue for electron microscopy was subjected to double fixation.

Primary fixative - Glutaraldehyde (Taab Laboratories) was used as a 1-3% solution freshly prepared in 0.1M sodium cacodylate buffer adjusted to pH 7.4 with hydrochloric acid, the fixation time ranging between 2 and 4 hours (Sabatini, Bensch and Barnett, 1963). Before being transferred to the second fixative, the tissue was washed in several rinses of the buffer and allowed to stand overnight in the buffer at 4°C. (see appendix).

Post-fixation - Osmium tetroxide (Johnson Matthey Chemicals Limited)

was used as a 1% solution in the same buffer as used for glutaraldehyde, the tissue being immersed in the fixative for 90 minutes. The tissue was then quickly rinsed in the buffer or distilled water and transferred to 70% methanol.

DEHYDRATION -

Graded methanols were used for tissue dehydration, about 20 minutes time in each and three changes of absolute methanol and then transferred to 1,2 - epoxypropane for 30 minutes before embedding.

EMBEDDING.

Araldite (CIBA) with an added plasticiser was used as the embedding medium. This was made in bulk and stored in the freezer until required, (see Appendix).. Tissue was left for 2 hours' infiltration in a relatively large volume of the Araldite, which was occasionally stirred before being transferred to the final medium of Araldite held in silicone rubber moulds (Micron Limited). These were then placed in an oven at 60°C. for at least 48 hours' polymerization.

CUTTING AND VIEWING

Using glass knives, pale gold sections were cut on an L.K.B. Ultratome III, picked up on uncoated copper 200 mesh grids with

holes of 85  $\mu$ m square, stained and viewed in an A.E.I. EM6B.

### STAINING

Thick paraffin embedded sections were stained for light microscopy with either Mayers haematoxylin and aqueous eosin, or Alcian blue (pH 2.6) and periodic acid Schiff, referred to in future as H & E and AB/PAS respectively (see Appendix).

One  $\mu$ m Araldite embedded sections were stained with 1% Toluidine blue in a 1% Borax solution for examination by light microscopy (Mercer, 1963).

Ultrathin sections for electron microscopy were stained with methanolic uranyl acetate (Stempack and Ward, 1964) for 3 minutes, washed in methanol, dried and then post-stained in lead citrate at high pH (Reynolds, 1963) for 3 minutes, washed in dilute Sodium hydroxide and twice rinsed in previously boiled, distilled water.

### Quantification

The number of each cell type contributing to bronchial epithelium, the epithelial thickness, depth of the ciliary layer and the proportion of cell organelles was quantified.

#### EPITHELIAL CELL NUMBER AND PROPORTION

Using the light microscopic and 1  $\mu$ m Araldite embedded sections the number of each type of epithelial cell was counted. Sections were cut on an L.K.B. Ultratome III, floated onto distilled water, transferred to a clean glass slide, slowly dried in a 60°C. oven and stained with freshly prepared and filtered 1% toluidine blue in 1% borax solution kept at 60°C. in a water bath. A staining time of 2 - 3 minutes was found to give sufficient contrast, the sections then being washed in tap water, dried and mounted.

From each section 1.8mm of epithelium from the anterior wall was counted. Cells reaching airway lumen and with a nucleus in section were counted and distinguished from nucleated basal cells which did not reach the lumen. The number of certain migratory cells present in the epithelium was also recorded.

For the cell concentration (i.e. the number of cells occurring in 1.8 mm epithelium) the "error of repeat measurement of the mean" was calculated and found to be  $\pm 8$  cells.

The variance of the cell concentration found at airway level III within 3 sections of a single block (intrablock), 3 blocks of that level (interblock), 4 animals from the same batch (interanimal)

and of animals from different batches (interbatch) was calculated and the variances compared using the F test.

As expected, the interbatch variance was the largest of all and was significantly greater than the interanimal variance ( $P_F < 0.05$ ). Although larger, the interblock variance was not significantly greater than the interanimal variance but it was significantly more than the intrablock variance ( $P_F < 0.05$ ).

From this it was decided that the most satisfactory way to compare different experimental groups at any given airway level was by comparing from each the mean of 3 blocks from each of 2 or 3 animals.

Counts of epithelial cells were compared using a Chi squared test and individual means compared using a t test, values for P obtained from Fisher's and Yates' tables (1963), any value for P of less than 0.05 being accepted as statistically significant.

The above criteria required that the sections be cut as near to true transverse sections as possible and for this reason, the prior embedding of complete rings of airway was necessary.

#### EPITHELIAL THICKNESS AND DEPTH OF CILIARY LAYER

In each section stained with toluidine blue the thickness of the epithelium was measured from the basement membrane to the base of the cilia using a measuring eyepiece divided into 100 units where each unit = 1.3  $\mu$ m. The mean of three measurements, taken

from different parts of the anterior wall, was recorded. At the same time the mean depth of the ciliary layer was recorded, each measurement being taken from the luminal edge of the ciliated cell to the tips of the cilia from that cell.

#### EPITHELIAL CELLS AND THEIR ORGANELLES

The relative proportions of cell organelles of both goblet and ciliated cells was determined using a point counting technique on the electron micrographs. The fundamental principles of this technique and some of the more special problems relating to the application of it to electron microscopy are well reviewed by Weibel (1969). The method is based upon a principle first used by the geologist Delesse (1848) and later mathematically proved by Chayes (1954) and shows that in a complex mineral the areal proportions of a section of that mineral are equivalent to the volume proportions. The problem is then resolved into the determination of areas or the ratio of areas of the various components on a cut surface. Dunnill (1962) suggests that it is possible to estimate this area ratio in a manner which is both speedy and accurate, and also free from bias, by a point counting method. Chalkley (1943) applied this to histological sections and Wiebel (1969) to ultrathin sections.

In this present study and so as to make possible identification of the various cell types, only those epithelial cells reaching the airway

lumen and whose nucleus was included in the section were selected for point counting. It is recognised that this implies 'selection', but it was applied to both experimental and control groups so the comparisons are valid. Each cell selected was photographed and printed at a total magnification of either 15,000 or 18,000 times.

A simple lattice grid of parallel and equidistant lines, one inch apart was then superimposed on the photomicrograph, the intersections of the lines providing a system of points which were used as markers for point-counting volumetry (Weibel, 1969). The total number of points lying over the entire cell and the number of points over each organelle was recorded for each cell studied. The total 'hits' on each organelle was then expressed as a percentage of the total number of points over the whole cell and compared as between groups.

### CHAPTER III

#### NORMAL EPITHELIUM OF RAT AIRWAYS

As a preliminary to the study of experimentally induced changes in rat bronchial epithelium, the appearance of normal epithelium has been established, using both the light and electron microscope.

### Light Microscopy

Light microscopic studies were based on observations of formol-saline fixed, paraffin embedded, 4  $\mu$ m thick sections (henceforth referred to as "paraffin sections") and glutaraldehyde-osmium fixed, epoxy embedded, 1  $\mu$ m thick sections (referred to as "epoxy sections").

### PARAFFIN SECTIONS

Fifteen normal SPF rats of 4 different strains (CFE & CFY, Porton, Anticimex) were studied using either Haematoxylin and Eosin or Alcian blue/Periodic acid Schiff as histological stains.

With H & E the epithelium of the trachea and main bronchus (levels 1 - 3, see page.51) was pseudostratified, ciliated, low columnar and often cilia were patchy in distribution. Nonciliated cells sometimes bulged beyond the level of ciliated cells and into the airway lumen. Epithelium of level 4 was ciliated cuboidal, often with nuclei having their long axis aligned parallel to the

basement membrane and cilia were more numerous and more evenly distributed than in the extrapulmonary airways. The epithelium of the distal bronchiolus (level 5) was simple and cuboidal with cilia patchily distributed. Nonciliated cells often had apical processes which bulged into the lumen with a thinner "neck" region at its base. The nucleus was set high in the cell and partly included in the process. Tufts of cilia were seen in the lumen at all levels of airway and at the junction of the alveolar duct and respiratory bronchiolus.

The combined AB/PAS technique stained the glycoproteins within the epithelium (see page 25). Usually, the nonciliated secretory cells (goblet cells) contained a mixture of both red and blue secretory granules. Two types of goblet cell were distinguished on the basis of the predominant cell colour; (i) predominantly blue; (ii) predominantly red, each present in either a small or large form depending on the amount and distribution of intracellular secretion. In normal airways and at levels 1 - 4 the small goblet cell predominated and was predominantly red.

In a group of 10 normal animals the peripheral distribution of cells staining for glycoprotein was established. On each section of the left lung cross lines of reference were drawn, one line drawn transversely across the short axis of the lung from the hilum to the pleural edge and one drawn longitudinally bisecting, in the mid line,

TABLE III-1.STRUCTURES WITH AFFINITY FOR THE STAIN TOLUIDINE BLUE -1  $\mu$ m EPOXY SECTIONS

| <u>STRUCTURE</u>                                   | <u>AFFINITY</u>     |
|----------------------------------------------------|---------------------|
| Elastin - (of lamina propria<br>and blood vessels) |                     |
| Cartilage                                          |                     |
| Secretory granules of:                             |                     |
| Globule leucocytes                                 | Intensely positive  |
| Mast cells                                         |                     |
| Some nonciliated<br>epithelial cells               |                     |
| Certain cell constituents:                         |                     |
| Lysosomes                                          |                     |
| Nucleoli and<br>Peripheral heterochromatin         |                     |
| Red Blood Cells                                    |                     |
| Muscle                                             |                     |
| Collagen                                           |                     |
| Cytoplasm of nonciliated cells                     | Moderately positive |
| Mitochondria                                       |                     |
| Cytoplasm of ciliated cell                         |                     |
| Some nonciliated cell secretory<br>granules.       | Weakly positive     |

the long axis of the lung. The distal airways found between these two lines and the pleural edge were observed. On average two of the 5 lateral airways were cut in section and sometimes 3, all less than 0.4 mm in diameter at their most proximal ends. In none of these distal airways were cells staining for glycoprotein, found. These were the distal airways (level 5) taken for examination by electron microscopy.

#### 1 $\mu$ m EPOXY SECTIONS

Epoxy sections, 1  $\mu$ m thick and stained with toluidine blue were routinely taken from all blocks prior to preparation of ultra-thin sections. Table III-1 shows the various structures with affinity for stain. These proved not only necessary for block orientation, but also for quantification of the various cell types. The following results are taken from five levels of airway in a group of 20 normal SPF animals. Some of these had received injections of Normal saline as a control procedure in one of the experiments.

#### Anatomy

The upper trachea (level 1) was lined by tall, pseudostratified ciliated columnar epithelium with nuclei in two layers (Fig III-1 ). Two animals only had small lesions of stratified, polygonal cells, these areas being without cilia. Submucosal glands were plentiful and their duct openings onto the surface were seen. The lining epithelium of levels 2 and 3 was pseudostratified, ciliated and of low

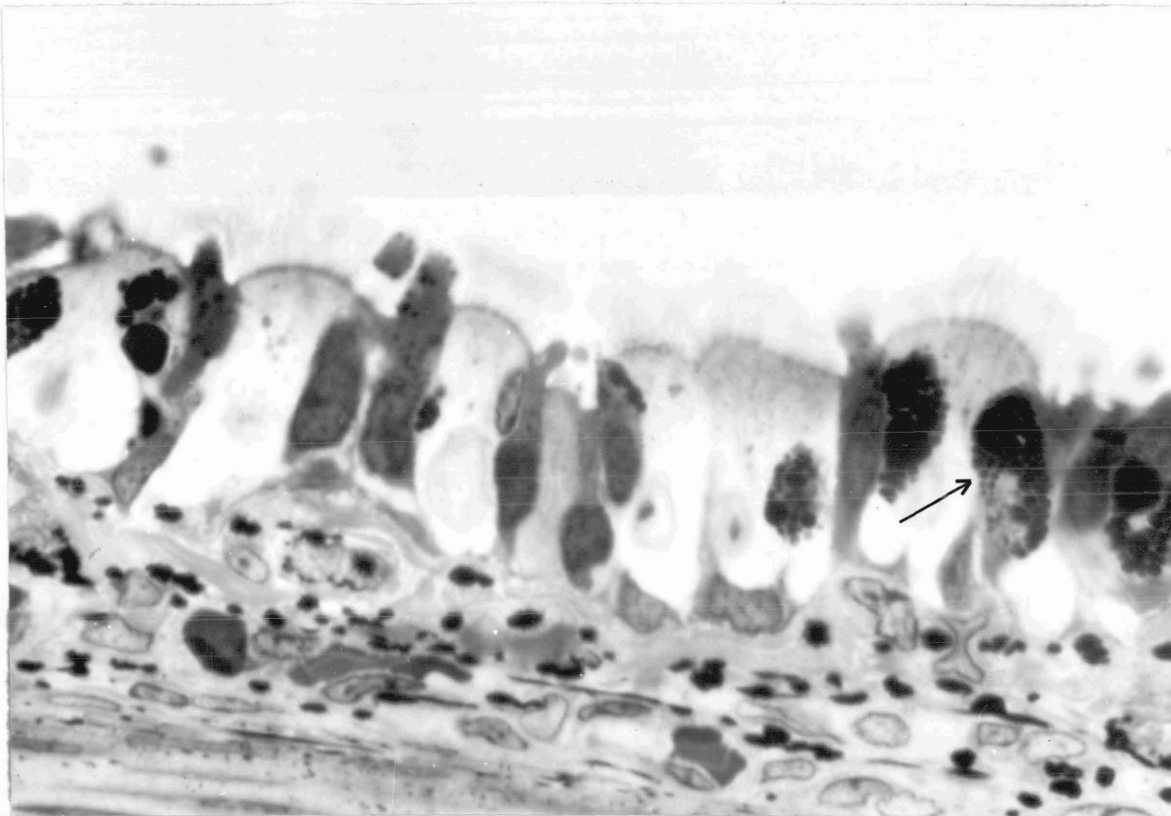


Fig. III-1. Photomicrograph of a transverse section of tracheal epithelium (level 1) as it appears in a 1  $\mu$ m epoxy embedded section stained with Toluidine blue; epithelial nuclei are at different levels. The nonciliated cells are those which bulge into the lumen and have cytoplasm with the strongest affinity for stain. Globule leucocytes are frequent (arrow) x 1500.

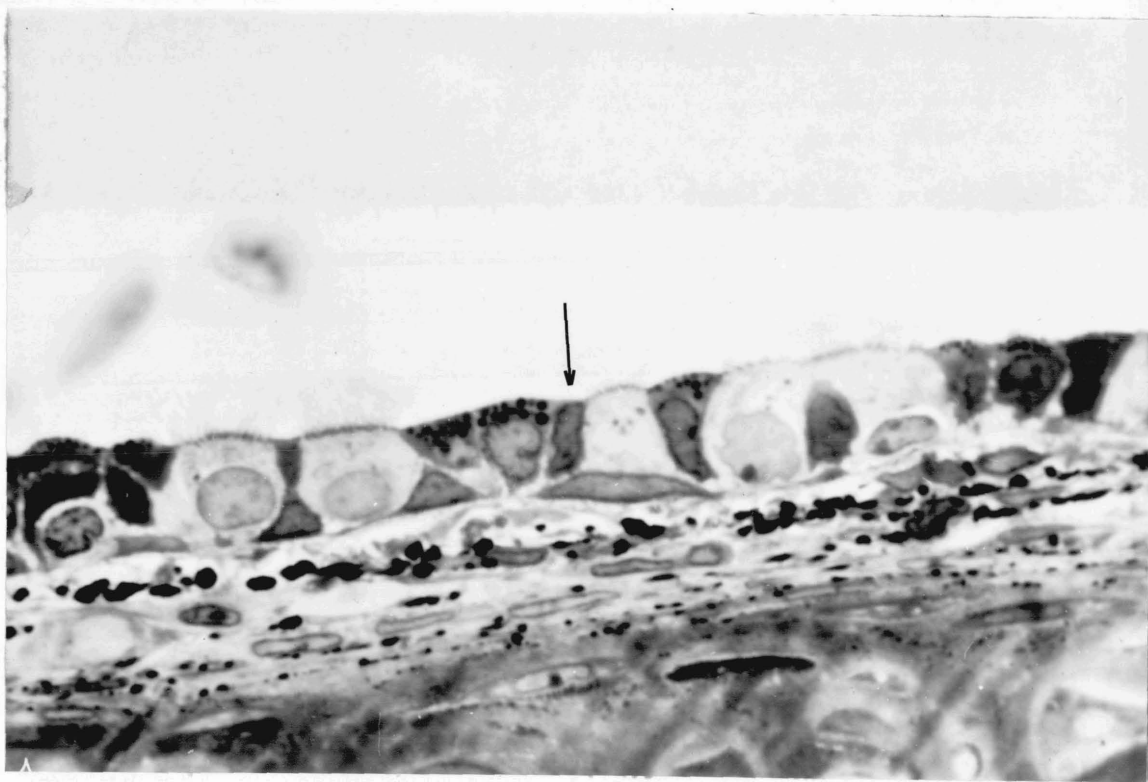


Fig. III-2. Photomicrograph of an epoxy-embedded section of epithelium from the main bronchus (level 3): epithelial nuclei are in two layers. Most of the nonciliated cells have intensely staining secretory granules: the occasional cell has none (arrow). Ciliated cells have a cytoplasm which stains weakly, while basal cells do not reach the airway lumen. x 1,500

columnar cells (Fig. III-2). Cilia were most numerous and most evenly distributed at level 4 (Fig. III-3). The epithelium of levels 4 and 5 was simple, low columnar or cuboidal and ciliated. (Fig. III-4).

In a good transverse section of an airway, all cells reached the basement membrane, but not all to the airway lumen. With these criteria, ciliated, nonciliated and basal cells were identified as the three main cell types (Fig. III-2) and see Table III-2.) Nonciliated cells, present at all levels, showed a tendency to bulge beyond the ciliated cells and into the airway lumen, this being most marked in the upper trachea. At other levels both flattened and bulging forms were present (Fig. III-3 & 4). On the basis of its secretory granules three types of nonciliated cell were distinguished (see Table III-2): (i) a cell with granules staining intensely; (ii) another with larger granules which stained only weakly and (iii) one without secretory granules and occasionally with a brush border.

Leucocytes, including lymphocytes and "globule leucocytes" (see page 21) were regularly found within the epithelium and this in the absence of subepithelial lymphocytic accumulation. They were present singly and appeared to be a normal epithelial component. Lymphocytes usually were basally situated, small cells with little

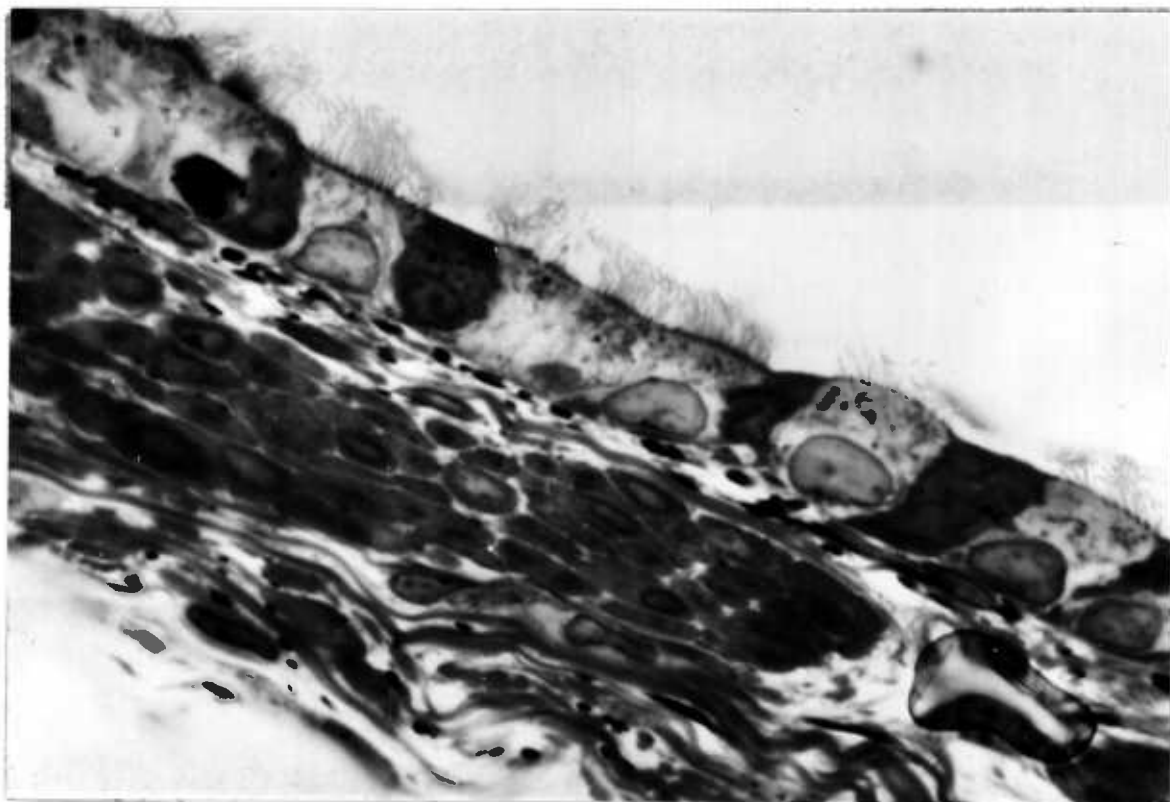


Fig. III-3. Photomicrograph of an epoxy-embedded section of epithelium from level 4: epithelial nuclei are at 1 level and ciliated cells are numerous. Nonciliated cells do not bulge into the lumen. x 1500.

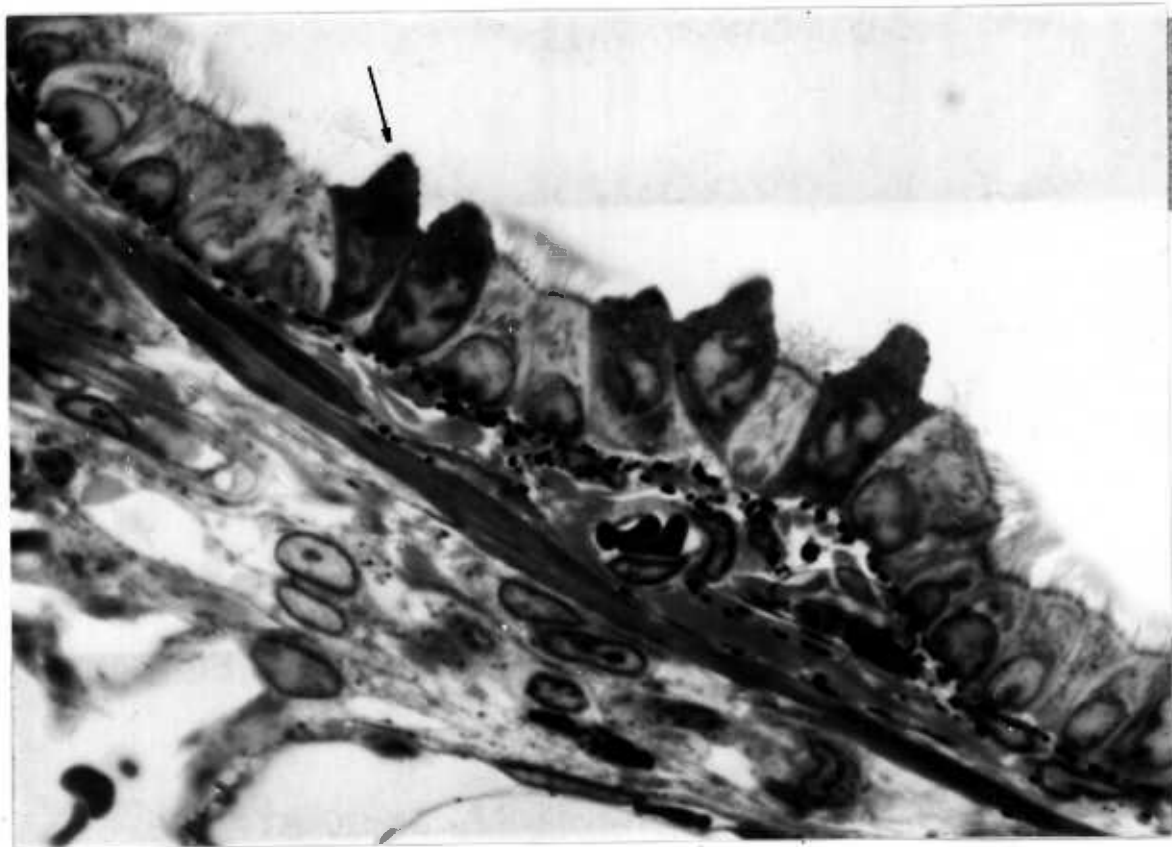


Fig. III-4. Photomicrograph of an epoxy-embedded section of epithelium from level 5: epithelial nuclei are at one level, ciliated cells are numerous and nonciliated cells have apices which bulge into the lumen and contain small intensely staining granules (arrow) x 1500.

TABLE III-2

MAIN CELL TYPES FOUND IN AIRWAY EPITHELIUM BY LIGHT AND ELECTRON MICROSCOPY

| <u>CELL</u> | <u>CYTOPLASM</u>  |                   | <u>GRANULES</u>    |        | <u>ENDOPLASMIC RETICULUM</u> | <u>NUCLEUS</u>         | <u>CELL PROJECTIONS</u>               |
|-------------|-------------------|-------------------|--------------------|--------|------------------------------|------------------------|---------------------------------------|
|             | L.M. <sup>1</sup> | E.M. <sup>2</sup> | L.M.               | E.M.   |                              |                        |                                       |
| CILIATED    | Weakly positive   | lucent            | none               |        | sparse                       | smooth in outline      | cilia and numerous filiform processes |
| NONCILIATED | positive          | dense             |                    |        | abundant                     | indented and irregular | few filiform processes                |
| (i)         | "                 | "                 | intensely positive | dense  | RER                          | "                      | "                                     |
| (ii)        | "                 | "                 | weakly positive    | lucent | RER                          | "                      | "                                     |
| (iii)       | "                 | "                 | none               |        | RER                          | "                      | few filiform or brush border          |
| (iv)        | intermediate      |                   | intensely positive | dense  | SER                          | "                      | blunt processes                       |
| BASAL       | positive          | dense             | none               |        | sparse                       | indented and irregular | few                                   |

1. 1 um epoxy-embedded section stained with toluidine blue.

2. Ultra-thin section - glutaraldehyde and osmium: uranyl acetate and lead citrate.

cytoplasm, negative to stain, and containing a small nucleus with heavily staining nuclear chromatin. Globule leucocytes were recognized by their eccentric nucleus and very strongly stained "globules" which filled the cell (Fig. III-1). Their shape varied from rounded to elongate and they were present at all levels within the epithelium. In the trachea, where they were most numerous they were often seen to extrude from the epithelium and lie free within the lumen. Occasionally, disrupted "globules" were seen with heterogeneous staining of their content.

#### Quantification

Epoxy sections were used to establish at each of the 5 airway levels the epithelial thickness, depth of the ciliary layer and the frequency of each cell type.

EPITHELIAL THICKNESS - Using a X8 measuring eyepiece and x 100 oil immersion objective, the thickness of the epithelium from the basement membrane to the luminal edge of the cell (i.e. to the base of the cilia) was measured at the five airway levels (Table III-3). From each section the mean of 3 readings from the anterior wall was taken.

The epithelium was thicker in the proximal airways than in the distal airways. That of level 1 was about twice the thickness of level 2 ( $P < 0.001$ ). That of level 2 was about  $\frac{1}{4}$  again as high

TABLES III-3 & 4EPITHELIAL THICKNESS AND DEPTH OF CILIARY LAYER OF NORMAL RATAIRWAY EPITHELIUMMEAN EPITHELIAL THICKNESS

| AIRWAY LEVEL | ANIMAL NUMBER | THICKNESS                  |               |
|--------------|---------------|----------------------------|---------------|
|              |               | Units*                     | $\pm$ $\mu$ m |
| I            | 5             | 23.3<br>(2.2) <sup>+</sup> | 30.2          |
| II           | 5             | 12.5<br>(0.7)              | 16.2          |
| III          | 6             | 9.8<br>(0.6)               | 12.7          |
| IV           | 9             | 9.4<br>(0.5)               | 12.0          |
| V            | 5             | 10.8<br>(0.3)              | 14.0          |

MEAN DEPTH OF CILIARY LAYER

| AIRWAY LEVEL | ANIMAL NUMBER | DEPTH        |               |
|--------------|---------------|--------------|---------------|
|              |               | Units        | $\pm$ $\mu$ m |
| I            | 5             | 4.3<br>(0.2) | 5.6           |
| II           | 5             | 4.6<br>(0.2) | 6.0           |
| III          | 6             | 3.8<br>(0.1) | 5.0           |
| IV           | 9             | 4.0<br>(0.1) | 5.2           |
| V            | 5             | 3.5<br>(0.1) | 4.6           |

\* 1 unit = 1.3  $\mu$ m

<sup>+</sup> SE of mean

as level 3 ( $P < 0.01$ ) and no significant difference in epithelial thickness was found between the remaining airway levels (3 - 5).

The greatest variation in thickness was found at level 1.

DEPTH OF CILIARY LAYER - The depth of the ciliary layer from the base to the tips of the cilia was measured and the mean of 3 readings taken at each level from the anterior wall in each animal (Table III-4). There was no significant difference in the depth of ciliary layer between levels 1 and 2, - in each case the mean depth was between 5.5 - 6  $\mu\text{m}$  - but they became shorter distally. The ciliary layer of levels 3 and 4 was similar and about 5  $\mu\text{m}$  deep, significantly less than either of levels 1 or 2 ( $P < 0.001$ ). At level 5 this layer was about 4.5  $\mu\text{m}$  deep and significantly less than that of levels 1 - 4 ( $P < 0.02$ ).

CELL TYPES - From the anterior wall ~~of~~ (one section from each level) the number of each cell type (see Table III-2) was counted in 10 high power oil fields (HPF), (i.e. cell concentration in 1.8mm epithelium). The mean for each airway level in several animals is shown in Table III-5, the percentage of each type in Table III-6.

In the upper trachea the greater cell height increased the likelihood of oblique sectioning and the appearance of more than two cell layers and for this reason consecutive fields were counted only where

TABLES III - 5 & 6

CELL CONCENTRATION AND PROPORTION IN 1.8mm LENGTH (10 HPF) OF NORMAL RAT AIRWAY EPITHELIUM

TABLE III-5 - MEAN CELL CONCENTRATION

| Airway level | Animal number | Ciliated          | Cell type         |                |                  | Basal            | Total             | Migratory       |
|--------------|---------------|-------------------|-------------------|----------------|------------------|------------------|-------------------|-----------------|
|              |               |                   | Nonciliated-dense | granule-lucent | none             |                  |                   |                 |
| I            | 4             | 49.25<br>(14.73)* | 57.75<br>(12.87)  | 0.5<br>(0.49)  | 78.75<br>(19.82) | 105.00<br>(4.72) | 291.25<br>(7.29)  | 41.50<br>(8.65) |
| II           | 5             | 85.20<br>(10.57)  | 68.20<br>(11.63)  | 1.20<br>(0.59) | 33.20<br>(4.48)  | 67.80<br>(4.70)  | 255.60<br>(8.59)  | 21.00<br>(3.73) |
| III          | 5             | 79.40<br>(12.72)  | 48.60<br>(10.03)  | 1.80<br>(1.57) | 36.80<br>(10.46) | 61.80<br>(4.26)  | 228.40<br>(15.17) | 9.20<br>(3.03)  |
| IV           | 6             | 111.66<br>(8.52)  | 42.16<br>(5.64)   | 0.83<br>(0.40) | 24.66<br>(7.33)  | 30.16<br>(7.13)  | 209.50<br>(15.68) | 7.50<br>(1.56)  |
| V            | 5             | 152.40<br>(1.96)  | 45.60<br>(10.75)  | 0.40<br>(0.40) | 34.60<br>(15.43) | 1.20<br>(0.74)   | 234.20<br>(4.81)  | 2.20<br>(0.86)  |

\* = se of mean.

TABLE III-6 - MEAN CELL PROPORTION

| Airway<br>level | Ciliated | Cell type                    |        |      | Basal | Migratory<br>% Total<br>Epithelial Cells |
|-----------------|----------|------------------------------|--------|------|-------|------------------------------------------|
|                 |          | Nonciliated-granule<br>dense | lucent | none |       |                                          |
| I               | 17*      | 20                           | 0.2    | 27   | 36    | 14                                       |
| II              | 33       | 27                           | 0.5    | 13   | 27    | 8                                        |
| III             | 35       | 21                           | 0.8    | 16   | 27    | 4                                        |
| IV              | 53       | 20                           | 0.4    | 12   | 14    | 4                                        |
| V               | 65       | 20                           | 0.2    | 15   | 0.5   | 1                                        |

\* to nearest 1%

there were 2 clearly defined cell layers, any areas of oblique section being omitted. Consecutive fields were counted at the remaining airway levels.

Apart from level 1 which showed a significantly higher cell concentration than at any other level ( $P < 0.02$ ), the remaining airway levels all had about 230 cells/10 HPF.

Ciliated Cells - There was a surprising sparsity of ciliated cells at level I and a progressive increase in their number more distally. Levels 2 and 3 resembled each other in having a mean of 82 ciliated cells/10 HPF about 34% of the population and this was significantly more than at level I ( $P < 0.05$ ). Level 4 showed a greater concentration of ciliated cells than at levels 2 or 3 ( $P < 0.05$ ) and level 5 had significantly more than at level 4 ( $P < 0.01$ ) there being about 152 ciliated cells/10 HPF - 65% of cells.

Nonciliated Cells - The most striking feature was the sparsity of cells distended with secretion and fulfilling the accepted description of a goblet cell. Furthermore, those which were distended usually had granules which stained only weakly (henceforth referred to as nonciliated cells with lucent granules). No significant difference in concentration was found at any level, those cells with lucent granules constituting

less than 1% of cells.

Those nonciliated cells with secretory granules staining intensely (henceforth referred to as nonciliated cells with dense granules), were the most common nonciliated cell type and numbered about 52/10 HPF, that is about 21% of cells. Their concentration did not differ significantly from level to level.

Those cells without secretion (henceforth referred to as nonciliated cells without granules) numbered about 32/10 HPF - 13% of cells - at levels 2 to 5 and were only significantly increased above this value at level 1 ( $P < 0.05$ ). Only a few cells with a distinct brush border could be detected these being more numerous (about 5/10 HPF) at level 1 than at levels 2 - 5 where they were also present.

Basal Cells - These showed a significant reduction in concentration more distally, being frequent in the large and rarely found in the smallest airways. Levels 2 and 3 resembled each other with about 65 basal cells/10 HPF - 27% of cells - this being significantly less than at level 1 ( $P < 0.001$ ) and more than found at level 4 ( $P < 0.01$ ). At level 5 only 1/10 HPF was found, significantly less than at level 4 ( $P < 0.01$ ).

Migratory Cells - This group included basally situated lymphocytic cells and "globule leucocytes" (see page 21). Those showing a nucleus in section were counted. Taken together there was a significant decrease in these cells with airway level. The highest concentration was found at level 1 with about 42/10 HPF, that is about 14% expressed as a percentage of total epithelial cell number. There were fewer at level 2 than at level 1 ( $P < 0.05$ ), levels 3 and 4 resembled each other in having fewer than at level 2 ( $P < 0.05$ ) and level 5 had only about 2/10 HPF, that is less than 1% which was significantly less than at level 4 ( $P < 0.05$ ).

#### Electron Microscopy

By electron microscopy, levels 1 - 5 of the respiratory tract have been examined. With double fixation, the degree of electron density of the various bronchial structures paralleled the "affinity for stain" seen in 1  $\mu$ m epoxy sections viewed by light microscopy (see Table III-2).

#### CILIATED CELL

Ciliated cells, at all levels, conformed to the classical structure as described by other workers: electron-lucent cytoplasm with many free ribosomes usually arranged as polysomes, short profiles of rough endoplasmic reticulum (RER), a few short profiles of smooth

endoplasmic reticulum (SER), often a well developed Golgi, and elongated mitochondria with well developed cristae. Mitochondria were seen throughout the cell, often in larger numbers in the supra-nuclear zone. It was quite characteristic to find 1 or 2 large electron-dense lysosomal structures close to and immediately above the nucleus. The nucleus was usually round, smooth in outline and without peripheral accumulation of chromatin (Fig. III-5). Cilia conformed to previous data with the typical arrangement of internal filaments but at high magnification the apical tip often possessed small projections, usually less than 10 and each about 20 nm in length (Fig. III-6). These were never seen on the lateral surface of the ciliary sheath. At the base and between the cilia, filiform projections were present in large numbers.

Very rarely a ciliated cell with a cytoplasm of electron density approaching that of goblet cells was seen but in these cases there were accompanying degenerative changes suggesting cell death; burst mitochondria, dilated RER, and many small vesicles in the cytoplasm.

#### NONCILATED CELLS

Nonciliated cells were recognized by their irregular indented nucleus with dense peripheral chromatin, and by their sparsity of surface filiform projections which, when present, were shorter than those of

ciliated cells. On the basis of cytoplasmic electron-density two types were found: (i) those with very dense cytoplasm contrasting strongly with that of neighbouring ciliated cells (Fig. III-7): (ii) those with lesser dense cytoplasm closely approaching that of ciliated cells (Fig. III-8). The former was by far the most frequent at levels 1 - 3 and the latter most frequent at level 5 with an overlap of each at level 4.

#### Nonciliated Cell - Dense cytoplasm

A group of nonciliated cells was found with a cytoplasmic density which contrasted strongly with that of ciliated cells. These had a cytoplasm rich in free ribosomes, RER throughout the cell and a supranuclear Golgi apparatus. This group could be subdivided on the basis of its secretory granules: (i) those cells with a predominance of electron-dense secretory granules (ii) those with predominantly electron-lucent granules and (iii) those without secretion.

NONCILATED CELL - DENSE CYTOPLASM - DENSE GRANULES - This was by far the most frequent type found. The dense granules were homogenously electron-dense with a very finely granular content lacking a peripheral clear zone (halo) and were well demarcated by a limiting membrane (Fig. III - 5 and 7). Cells of this type usually had few granules,

when they were at the apex of the cell and near the lumen, but the occasional cell was found with many such granules, filling the supranuclear zone.

Most of the granules were not osmiophilic and lost their electron-density when fixed with osmium alone, having only a peripheral rim of electron-dense material (Fig. III-9). Peripheral nuclear chromatin did not stain and occasional dense bodies, presumably lysosomes, were present in some cells. Both ciliated and nonciliated cells often had apical cytoplasmic blebs which was not a usual feature of cells pre-fixed with glutaraldehyde.

NONCILIATED - DENSE CYTOPLASM - LUCENT GRANULES - In the normal SPF animal, used for this study, these cells were very rarely found, it being usual to look at many sections from several animals before finding one. When present, it was easily recognised as a cell distended by an abundance of electron-lucent granules, usually larger than those of the previous group and each with an incomplete limiting membrane which often allowed the granules to fuse one with the other, (fig. III-10). The nucleus often was pushed to the base of the cell by the accumulating supranuclear secretory mass. In all respects these most closely resembled the goblet cells described by Rhodin and Dalhamn in 1956.

NONCILATED CELL - DENSE CYTOPLASM - WITHOUT GRANULES - Nonciliated

cells without granules were normally found with a low frequency of about 1 or 2 per section (about 1% of cells) and more often at levels 1 - 3 than at 4 and 5, when they usually showed a brush border of microvilli, 2  $\mu$ m high, and bundles of fibrils in the cytoplasm, in these respects resembling the classical description of a "brush cell" (Fig. III-11). The peripheral chromatin of the nucleus was not as well developed as in other nonciliated cells and usually lacked the indentation, being intermediate in structure between nonciliated and ciliated cells. Another feature regularly found and typical to this cell was the accumulation of membrane-bound vesicles numerous at the cell apex and diminishing in number towards the nucleus (Fig. III-12). The blunt microvilli, each distinct and with fine fibrils extending into their core after double fixation were distorted and lacked fibrils after osmium fixation alone (Fig. III-13).

Nonciliated Cells - Lucent Cytoplasm.

A group of nonciliated cells was found with its cytoplasm of about the equivalent electron density of ciliated cells and with an abundance of SER <sup>(in Clara cells)</sup>. These were infrequently found at levels 1 - 4 (Fig. III-8) but were the most frequent type of nonciliated cell at level 5 (Fig. III-14). The cytoplasm had few ribosomes, a moderate amount of RER, a small Golgi, and an abundance of SER, present in short profiles, especially at the cell apex (Fig. III-15). The apex of the cell usually lacked filiform processes and was characteristically

of short, blunt pseudopodia. These features were not found in any other nonciliated cell type. Mitochondria were of the conventional type with cristae and small electron-dense membrane-bound granules were found when they were usually at the cell apex and often within the pseudopodia. Electron-dense and membrane-bound rods often were found within the cytoplasm also (Fig. III-16).

After fixation by osmium alone the nonciliated cells of level 5 were altered in appearance. The short profiles of SER seen after prefixation by glutaraldehyde were absent, replaced by many small vesicles which filled the cell (Fig. III-17). Electron-dense granules were absent but a few incompletely membrane-bound bodies of similar size and with a smaller dense core were found (Fig. III-18). Each nonciliated cell also had a peripheral cytoplasmic zone lacking in organelles which was not seen after glutaraldehyde fixation (Figs. III-17 and 18).

After glutaraldehyde prefixation, on rare occasions, the apex of the cell produced a bleb-like process of more electron-lucent cytoplasm, often containing vesicles of SER (Fig. III-19). This feature was not, however, unique to the nonciliated cell and was as infrequently seen on ciliated cells and probably represented a fixation artifact (Fig. III-20).

Rarely a cell with an electron-lucent cytoplasm was seen which had a rounded nucleus lacking peripheral chromatin accumulation and protruding from the cell apex, short filiform processes, regularly

arranged. These usually had fibrogranular deposits identical to those seen by Sorokin (1968) in young developing ciliated cells (Fig. III-21).

#### BASAL CELL

This group comprised cells attached to the basement membrane, but not reaching the airway lumen (Fig. III-5). They were variable in shape often wedge-shaped or elongated with their long axis extending parallel to the basement membrane. Correspondingly the shape of the nucleus varied with that of the cell. Their cytoplasmic density and nuclear ultrastructure was identical with that of the nonciliated cell with dense cytoplasm. The cytoplasm was sparse, often with bundles of fibrils (tonofilaments) and with little in the way of other cell organelles. Mitochondria were round or ovoid and small.

#### NEURO-SECRETORY-LIKE GRANULATED CELL

On extremely rare occasions, a cell was found which did not conform to the criteria for a basal cell. Present singly and adjacent to the basement membrane, it differed from the basal cell in having an electron-lucent cytoplasm filled with membrane-bound granules, 130nm in diameter, each with an electron-dense core surrounded by a clear zone or halo (Fig. III-22). In all respects

this cell resembled that found in man by Bensch et al in 1965.

In the adult rat these were only found in the trachea.

#### MIGRATORY CELLS WITHIN THE EPITHELIUM.

Two types of migratory cell were found frequently within the epithelium in all sections of normal animals and without any evidence of inflammation, or sub-epithelial lymphocytic accumulation. These were most frequent at levels 1 - 3, found occasionally at level 4 and only rarely at level 5. No association with a particular type of epithelial cell was noted.

#### Globule Leucocyte

Globule leucocytes, in all respects resembling those described by Kent (1966), were found at basal, intermediate and superficial levels within the epithelium. Their most usual shape was round although elongated forms were found when their long axis was not in any particular plane. The most striking feature was the large number of spherical cytoplasmic granules, homogeneously electron-dense, apparently without internal structure, and with a closely adhering smooth limiting membrane (see Fig. III-11). The nucleus had an abundance of peripheral chromatin and the cell surface had many fine filiform processes extending into the intercellular space. After osmium fixation alone the cytoplasmic granules were altered with almost complete loss of electron-density, leaving a

finely granular matrix in which were embedded electron-dense splinter-like crystalloid structures (Fig. III-23).

#### Lymphocytes

Cells, in all respects resembling small lymphocytes, were found in normal epithelium, when they were invariably in the basal layer adjacent to the basement membrane. These were small rounded cells occasionally with large blunt pseudopodia, with a sparcity of cytoplasm which was electron-lucent. The cytoplasm contained few organelles: a few ribosomes, a rare profile of RER or perhaps a single mitochondrion. The nucleus, usually smaller than that of the epithelial cells had dense peripheral chromatin extending towards a central nucleolus (Fig. III-24).

#### EXTRAEPITHELIAL MIGRATORY CELLS

Macrophages were found within the airway lumen at all levels sometimes closely applied to the epithelium. These had a cytoplasm containing many dense bodies (Fig. III-25).

In the lamina propria fibroblasts were found frequently and cells (presumably mast cells) with granules of similar ultrastructure but smaller than those seen in the epithelial globule leucocyte were found infrequently.

### Intra-epithelial nerves

Nerves were found superficial to the basement membrane in all animals and adjacent to all cell types, but only in the extrapulmonary airways (levels 1 - 3). On no occasion were such nerves observed in any intrapulmonary airway. In structure, these resembled those previously described by Jeffery and Reid (1973) in the tracheal and bronchial epithelium of young 19 day old rats. Axonal varicosities with dense-cored vesicles and/or clear vesicles and some without vesicles, containing only neurotubules were found. The frequency of these nerves was not quantified but they appeared to be less frequent than those found in the younger animals. (Fig. III-26).

### SUMMARY OF RESULTS

Five levels of airway from the rat have been studied by light and electron microscopy: upper and lower trachea, main extrapulmonary bronchus, a large intrapulmonary bronchus near the hilum and a distal bronchiolus of less than 0.4mm. diameter

1. In the normal animal, cilia extend as far as and into the re<sup>s</sup>piratory bronchiolus.
2. In the adult rat (i.e. about 200g.), cells staining for glycoprotein are present in the epithelium of all but the distal airway - that is, lateral airways of less than 0.4mm diameter.
3. With the combined Alcian blue/periodic acid Schiff stains, the most frequently found cell staining for glycoprotein, is one with a small secretory mass which stains predominantly red.
4. Using epoxy embedded sections, stained with toluidine blue, ciliated, nonciliated, and basal cells are the main cell types found in all but the distal airways, where basal cells are usually absent.
5. On the basis of their secretory granules the nonciliated cell can be divided into three types: (a) those with intensely stained secretory granules, (b) those which have larger granules with little affinity for stain and (c) those which lack secretory granules or in which they are too small, or too few, to be observed by light microscopy.
6. A group of leucocytes, including both lymphocytes and "globule leucocytes", was regularly found in all but the distal airway

as a normal epithelial component: that is in the absence of inflammation, including sub-epithelial lymphocytic accumulation.

7. The epithelial thickness and depth of the ciliary layer was established at the five airway levels. Both are significantly decreased more distally.

8. Using epoxy sections it was possible to establish the frequency of each type of epithelial cell at the five airway levels:

(a) Ciliated cells progressively increased more distally. As a proportion of cells they were nearly four times as frequent in the distal bronchiolus as in the upper trachea. (b) Nonciliated cells with granules staining weakly (i.e. lucent) were present as less than 1% of cells and were never found in the distal bronchiolus.

They were the only cells which had a true goblet shape. Those nonciliated cells with granules staining intensely (i.e. dense) were the most frequent nonciliated cell and were present at all levels when they were 21% of cells. Nonciliated cells without granules were present also at all airway levels. (c) Basal cells decreased progressively more distally from about 27% of cells in the trachea to less than 1% in the distal bronchiolus. (d) Migratory cells were also decreased more distally, being rarely found in the bronchiolus. In the upper trachea they were most frequent when they were present as 14% when expressed as a percentage of the total epithelial cell number.

9. By electron microscopy and with the fixation and staining methods used, electron density was found to parallel the affinity for toluidine blue stain seen with the light microscope.

10. Ciliated cells were found as described previously in the literature, with the exception that at high magnification, the tips of their cilia had small projections, less than ten per cilium and each about 20 nm long.

11. As found by light microscopy, the three types of nonciliated cell distinguished by light microscopy, were also found. Most nonciliated cells without granules had a distinct "brush" border of microvilli. A fourth group of nonciliated cells, not distinguished by light microscopy, was found, infrequent proximally but as the most frequent nonciliated cell type in the distal bronchiolus. In contrast to the other nonciliated cells, this cell contained an electron-lucent cytoplasm with an abundance of smooth endoplasmic reticulum, present as short profiles especially at its apex (? Clara cell).

12. After glutaraldehyde, dense secretory granules were the most frequent type of secretory granule found when they were homogeneous and electron dense. After osmium fixation alone, these granules whether seen in nonciliated cells proximally or in Clara cells distally, did not stain.

13. Basally situated granulated cells (? Kultschitsky) were rarely found but, when present, were in tracheal epithelium and had electron-lucent cytoplasm filled with dense-cored vesicles.

14. Migratory cells were found normally at all but the most distal airway level. Globule leucocytes were found with granules which were homogeneously electron-dense after double fixation but which stained incompletely after osmium alone. Lymphocytes were found basally within airway epithelium as small rounded cells with blunt pseudopodia: its sparse and electron-lucent cytoplasm contained few organelles, and a nucleus with dense peripheral chromatin.

15. Nerves were found within the epithelium of all adult animals but only in extrapulmonary airways and in about the same numbers as found in young rats.

## CHAPTER IV

### THE EFFECT OF TOBACCO SMOKE ON RAT AIRWAY EPITHELIUM

The effect of tobacco smoke on rat airway epithelium was investigated by light and electron microscopy in two experiments. In all, a group of 112 male rats was used from which 30 were taken at random for the electron microscopic studies reported here. The detailed results of the histological studies on paraffin sections are reported elsewhere (Jones, Bolduc and Reid, 1972 and 1973).

Experiment A consisted of 24 animals of which 20 had been exposed daily (5 days each week) to tobacco smoke for up to 2 weeks, 4 animals being sacrificed on each of days 1, 2, 3, 7 and 14 of exposure. Experiment B consisted of 6 animals of which 4 had been exposed daily (4 days each week), to tobacco smoke for 6 weeks.

In each experiment, those animals to be exposed to tobacco smoke were equally divided into two groups: group I contained those exposed to tobacco smoke alone, while group II contained animals exposed to smoke from tobacco to which phenylmethyloxadiazole (PMO) had been previously added. For details see page 53.

## Effect on the Animals

### BEHAVIOUR AND APPEARANCE

During exposure to tobacco smoke, animals of both groups I and II huddled together in cage corners with their noses upturned. Their fur became wet, mainly around the face, and in a line along the spine. At the end of the day, many showed evidence of lacrimation and the eyes appeared congested. In each group, one or two animals showed evidence of similar nasal irritation. After removal from their cages they began to wash themselves and within half an hour were back to normal. During the early days of experiment B, it was noticed that animals of group II recovered more quickly than did those of group I. At this time the observer was ignorant of which cigarette was made of which tobacco. This difference was no longer apparent after further exposure, nor was it seen during experiment A.

### WEIGHT GAIN

At the beginning of experiment A, the range of body weight was 110-130g and in experiment B was 180-230g. There was, in all animals, a steady gain in weight.

### Experiment A

Animals in both groups I and II gained weight at a slower rate than did the controls, those of group II gaining less rapidly than group I (Fig. IV-1). At the end of the experiment, the mean weights of animals from both groups I and II were significantly less than that of the controls ( $P < 0.05$ ). Group II animals weighed significantly less than those from group I ( $P < 0.05$ ): this difference was not found in the older animals of experiment B.

### Experiment B

Overall, animals of both groups I and II gained weight at a slower rate than did the controls, failing to gain weight during the early exposure days but gaining at a faster rate over the days between exposure (Fig. IV-2). At the end of the experiment, the mean weights of animals from groups I and II were similar and both were significantly less than the controls ( $P < 0.001$ ).

### Effect on Airway Epithelium

The effect of tobacco smoke on the epithelium of two airway levels, 3 and 5, was studied by light microscopy, of 1  $\mu$ m epoxy sections, and electron microscopy.

MEAN BODY WEIGHT AFTER EXPOSURE TO TOBACCO SMOKE  
EXPERIMENT A

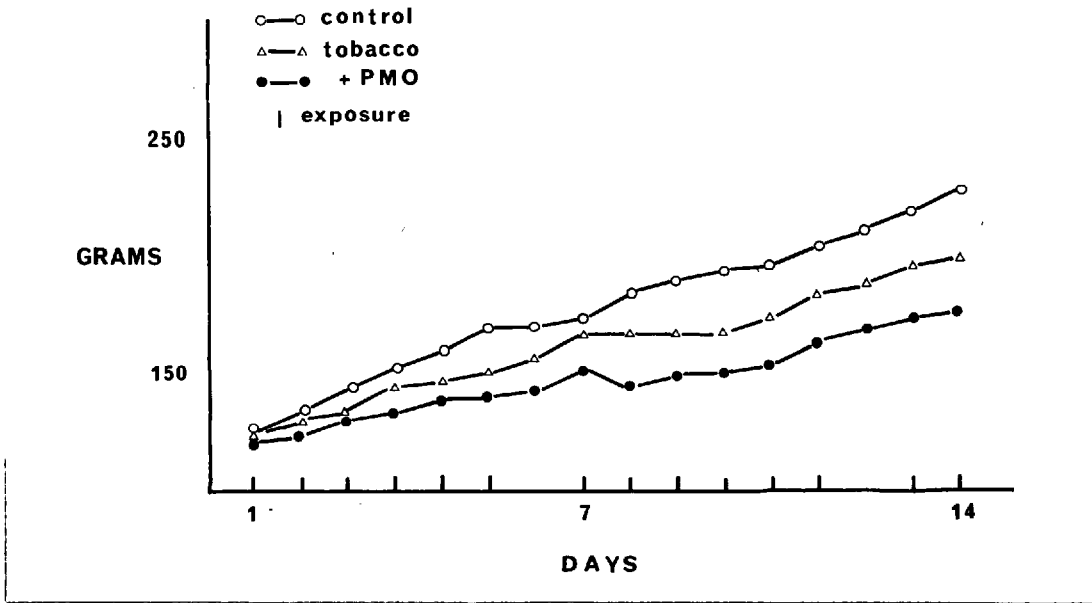


Fig. IV-1 shows the gain in body weight of those animals of experiment A.

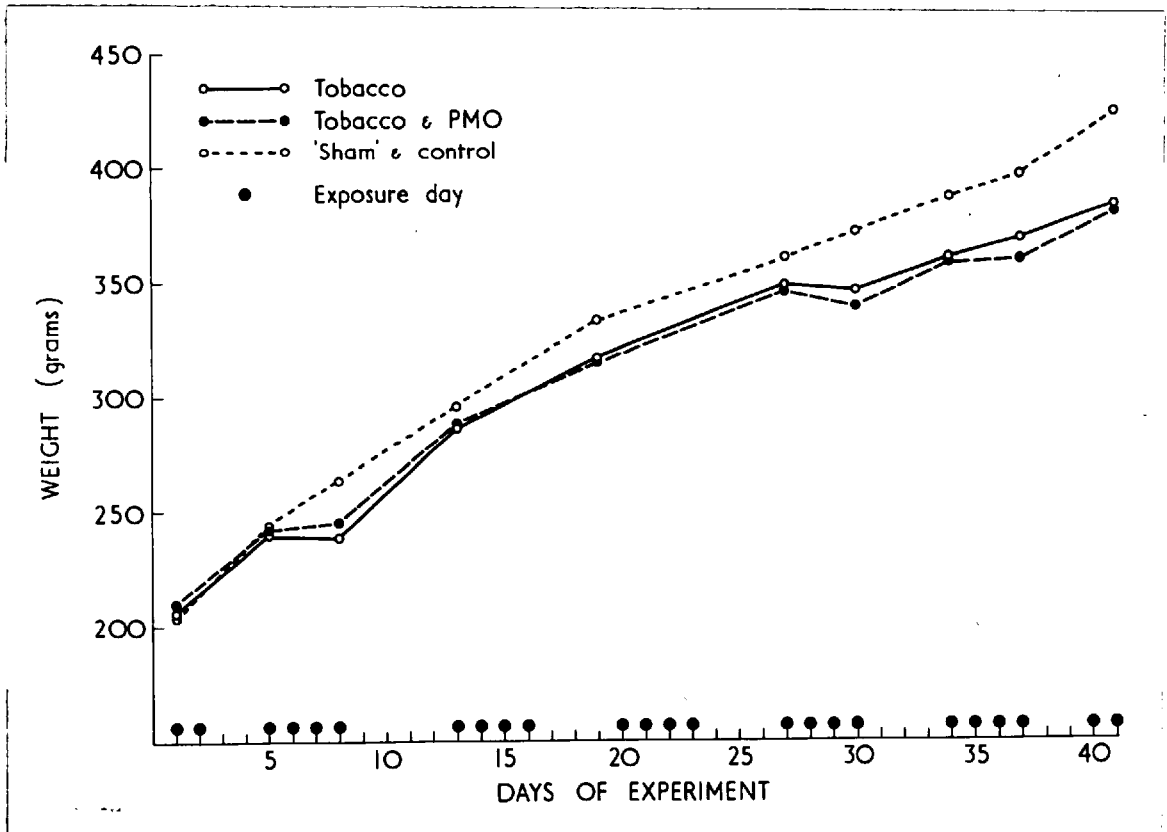


Fig. IV-2 shows the gain in body weight of those animals of experiment B.

#### MAIN BRONCHUS - LIGHT MICROSCOPY

From each of the 30 animals, 1  $\mu$ m thick epoxy sections of complete rings of airway, (main bronchus), were taken and stained with toluidine blue for examination by light microscopy (see page 58). Even during the early days of exposure, and especially by two weeks, a difference between control and exposed animals was easily recognised. The bronchial epithelium of the control animals was ciliated and cuboidal, while that of animals from groups I and II, while ciliated, was tall and columnar. Only very occasionally was an area devoid of cilia and of stratified basal-like polygonal cells found. At no time was there any evidence of epithelial ulceration.

All the types of epithelial cell found in the control animals were also found in the animals from groups I and II. No new cell type was found. In the control animals, nonciliated cells most commonly had secretory granules staining intensely with toluidine blue (i.e. dense granules). In contrast, many nonciliated cells of the animals from groups I and II contained a larger number of granules which had little affinity for stain, (i.e. lucent granules) (Fig. IV-3). Especially in the early stages of exposure, many of

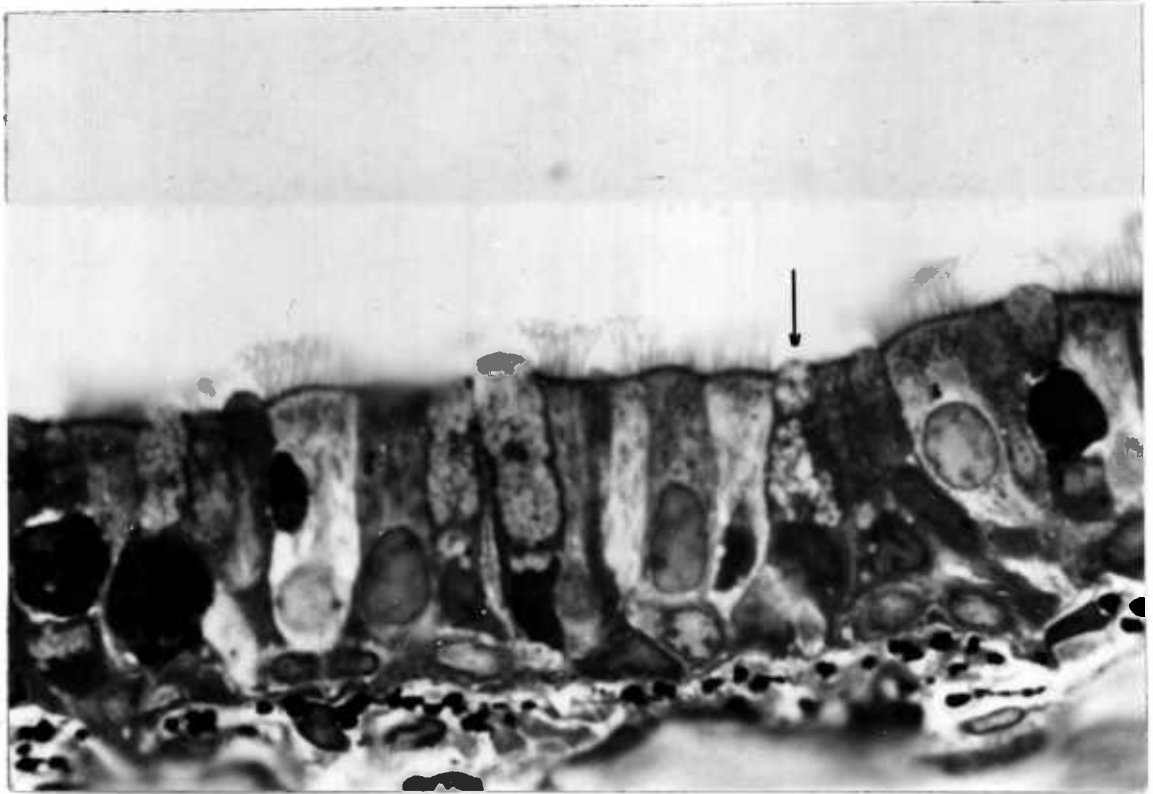


Fig. IV-3. Photomicrograph of (epoxy-embedded) section of thickened epithelium from level 3, taken from an animal exposed to cigarette smoke for two weeks. There are a number of cells filled with lucent granules: in one cell each granule has an intensely staining core (arrow).

these "goblet" cells had lucent granules containing a smaller but intensely staining core. Nonciliated cells without granules, basal cells and brush cells were found in both control and smoking animals, as were globule leucocytes and, occasionally, lymphocytes.

#### Epithelial Thickness

Using a x8 eyepiece with a measuring graticule divided into 100 units and an oil immersion objective so that 1 graticule unit =  $1.3\text{ }\mu\text{m}$ , the thickness of the epithelium was measured in the 24 animals of experiment A. Three different blocks of the main bronchus were taken from each animal and in a section from each the vertical distance from the basement membrane to the epithelial surface (i.e. base of the cilia) was measured at three positions over the anterior wall, and the mean of these three measurements calculated.

The mean epithelial thickness of the four control animals, two sacrificed at the beginning and two at the end of the experiment, was similar and equivalent to a thickness of  $12\text{ }\mu\text{m}$ .

After only one day of exposure, animals from both groups I and II had a thickened epithelium, but this was only significant in animals from group I ( $P < 0.05$ ). By day 2, animals from both groups I and II had a significantly thickened epithelium ( $P < 0.01$ ) and, but for day 3 which did not differ significantly from the controls,

the epithelial thickness increased up to about 27  $\mu\text{m}$  at two weeks, when the difference from the control value was highly significant ( $P < 0.001$ ) (Table IV-1). It was later found that the cell concentration and the number of cells in division was also less at day 3 than at the other days of exposure.

When the animals of group I were compared with those of group II, their epithelial thickness was found to be similar in the early days of exposure, but a tendency emerged by one week for animals of group I to have a thicker epithelium than those of group II ( $0.05 < P < 0.1$ ). At two weeks this tendency was more marked; the animals of group I now had a significantly thicker epithelium than those of group II ( $P < 0.05$ ).

#### Depth of ciliary layer

From each section used to measure epithelial thickness, the mean depth of the ciliary layer was calculated and recorded. The depth showed little variation: the mean of the controls was about 5  $\mu\text{m}$  and that of the groups I and II did not differ significantly from this value.

#### Concentration of cells - Experiment A

The number of cells occurring in 1.8 mm epithelium (i.e. 10 HPF) was counted in all the animals of experiment A. For each animal, a

TABLE IV-1

MEAN EPITHELIAL THICKNESS AFTER EXPOSURE TO TOBACCO SMOKEEXPERIMENT A

| CONTROL | GROUP | DAY     |        |        |         |         |
|---------|-------|---------|--------|--------|---------|---------|
|         |       | 1       | 2      | 3      | 7       | 14      |
|         | I     | 11.99   | 14.66  | 11.50  | 18.49   | 22.83   |
|         |       | (0.62)* | (1.01) | (1.00) | (0.70)  | (0.48)  |
|         |       | P<0.05  | P<0.01 |        | P<0.001 | P<0.001 |
| 9.49    |       |         |        |        |         |         |
| (0.47)  |       |         |        |        |         |         |
|         | II    | 11.83   | 14.00  | 12.50  | 15.83   | 19.66   |
|         |       | (1.14)  | (0.57) | (1.07) | (0.53)  | (0.50)  |
|         |       |         | P<0.01 |        | P<0.001 | P<0.001 |

\* SE of the mean. P values given where significantly  
different from controls.

section from each of 3 blocks of the main bronchus was counted and the mean for that level calculated: the mean and se for the group was calculated using the mean and se obtained from each animal in the group (see page 59).

CONTROL ANIMALS - Four unexposed animals were killed as controls, two on the first day and two on the last day of the experiment. The cell concentration did not vary significantly between these animals: the mean of the 4 animals was about 224 cells/10 HPF (i.e.  $223.9 - \text{se of mean} = 5.7$ ).

ANIMALS EXPOSED TO TOBACCO SMOKE - During the two weeks' exposure to tobacco smoke, the concentration of cells rose steadily to levels significantly higher than those of the controls ( $P < 0.01$ ). The cell concentration was increased after only one day's exposure. The greatest increase, seen in the animals from group I, was after 1 week while those of group II showed their greatest increase after the second week of smoking. At no time was there significant protection against this increase, by PMO: in both groups I and II there was an additional third as many cells present as in the control group (i.e. about 310/10 HPF).

#### Proportion of Cells

Since the cell concentration was found to increase with exposure to tobacco smoke, the proportion of each cell type was

calculated as a percentage of the total cell number (excluding migratory cells) and a comparison of animals made using these figures (Tables IV-2 and 3).

CONTROL ANIMALS - A comparison of the mean proportion of each cell type in those control animals sacrificed at the beginning of the experiment with those sacrificed at the end, showed that ciliated and basal cells varied by more than expected from the intra-animal variation ( $P < 0.05$ ). In view of this, the results from both groups of controls were pooled for comparison with the animals from groups I and II.

ANIMALS EXPOSED TO TOBACCO SMOKE - In both groups I and II and by the end of the first day of exposure, there was a significant shift from the control values in the proportion of each cell type ( $P_{\chi^2} < 0.001$ ). In both groups I and II the shift was similar (Fig. IV-4 and 5).

Ciliated Cell - Ciliated cells were not significantly altered during the early days of exposure, but between one and two weeks increased significantly ( $P < 0.05$ ).

Nonciliated cells - Nonciliated cells with dense granules showed a sharp decrease after day one of exposure ( $P < 0.01$ ) followed, in group I, by a recovery to near normal values at days two and three of exposure. Group II animals showed a slight recovery at this time, but this value was still significantly below the control value ( $P < 0.02$ ).

TABLE IV-2MEAN PROPORTION OF CELLS EXPRESSED AS A PERCENTAGE OF TOTAL CELLSAFTER EXPOSURE TO TOBACCO SMOKE ALONE - EXPERIMENT A

| CELL TYPE   | CONTROL          | EXPOSURE DAY              |                           |                          |                           |                            |
|-------------|------------------|---------------------------|---------------------------|--------------------------|---------------------------|----------------------------|
|             |                  | 1                         | 2                         | 3                        | 7                         | 14                         |
| CILIATED    | 26.24<br>(3.21)* | 27.50<br>(4.13)           | 22.83<br>(2.58)           | 22.16<br>(6.90)          | 25.33<br>(2.13)           | 35.83<br>(3.92)<br>P<0.05  |
| NONCILIATED |                  |                           |                           |                          |                           |                            |
| Granule     |                  |                           |                           |                          |                           |                            |
| Dense       | 25.83<br>(5.20)  | 5.16<br>(2.74)<br>P<0.01  | 18.50<br>(10.98)          | 17.16<br>(4.64)          | 1.36<br>(0.75)<br>P<0.01  | 1.83<br>(1.08)<br>P<0.01   |
| Lucent      | 2.09<br>(1.59)   | 2.19<br>(2.79)            | 10.33<br>(4.72)<br>P<0.05 | 6.66<br>(2.19)<br>P<0.05 | 5.50<br>(3.16)            | 17.50<br>(1.12)<br>P<0.001 |
| None        | 20.83<br>(6.67)  | 35.66<br>(3.34)<br>P<0.05 | 15.00<br>(6.33)           | 24.33<br>(4.80)          | 34.50<br>(6.08)           | 10.83<br>(3.35)            |
| BASAL       | 24.83<br>(2.63)  | 28.83<br>(3.71)           | 33.00<br>(4.93)<br>P<0.05 | 29.50<br>(3.00)          | 33.33<br>(2.62)<br>P<0.05 | 34.00<br>(2.19)<br>P<0.02  |

\*  $\pm$  SD. P values given where significantly different from controls.

TABLE IV-3

MEAN PROPORTION OF CELLS-EXPRESSED AS A PERCENTAGE OF TOTAL CELLS-AFTER EXPOSURE TO TOBACCO SMOKE + PMO - EXPERIMENT A

| CELL                   | CONTROL          | EXPOSURE DAY             |                           |                           |                           |                           |
|------------------------|------------------|--------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
|                        |                  | 1                        | 2                         | 3                         | 7                         | 14                        |
| CILIATED<br>CELL       | 26.24<br>(3.21)* | 33.66<br>(4.25)          | 26.16<br>(5.10)           | 21.83<br>(6.55)           | 24.83<br>(3.08)           | 34.00<br>(3.04)<br>P<0.05 |
| NONCILIATED<br>GRANULE |                  |                          |                           |                           |                           |                           |
| Dense                  | 25.83<br>(5.20)  | 3.49<br>(1.44)<br>P<0.01 | 6.49<br>(4.52)<br>P<0.02  | 6.83<br>(6.72)<br>P<0.02  | 0.53<br>(0.59)<br>P<0.01  | 1.44<br>(1.36)<br>P<0.01  |
| Lucent                 | 2.09<br>(1.59)   | 2.39<br>(2.18)           | 6.66<br>(1.08)<br>P<0.05  | 3.95<br>(1.06)            | 2.26<br>(0.75)            | 11.66<br>(3.05)<br>P<0.01 |
| None                   | 20.83<br>(6.67)  | 33.66<br>(1.95)          | 19.83<br>(4.84)           | 28.16<br>(10.27)          | 39.66<br>(2.85)<br>P<0.05 | 17.00<br>(2.99)           |
| BASAL                  | 24.83<br>(2.63)  | 27.33<br>(5.02)          | 41.00<br>(4.08)<br>P<0.01 | 39.49<br>(3.33)<br>P<0.01 | 32.83<br>(2.43)<br>P<0.05 | 35.83<br>(1.94)<br>P<0.01 |

\*±SD . P values given where significantly different from controls.

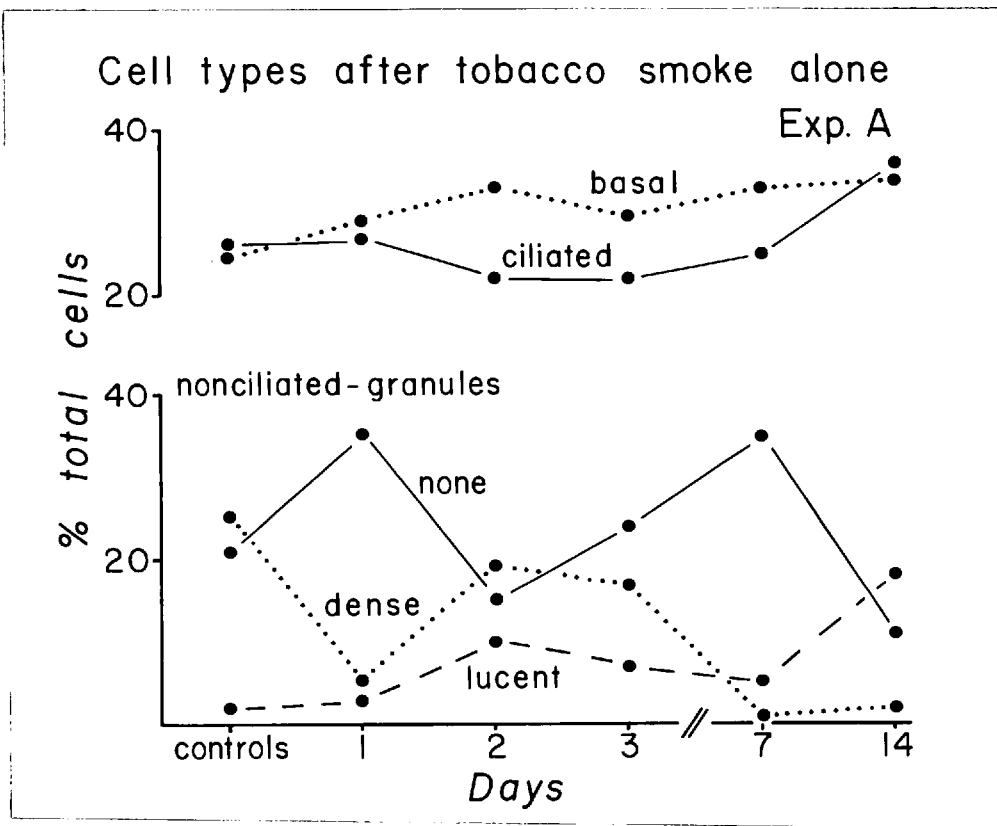


Fig. IV-4.

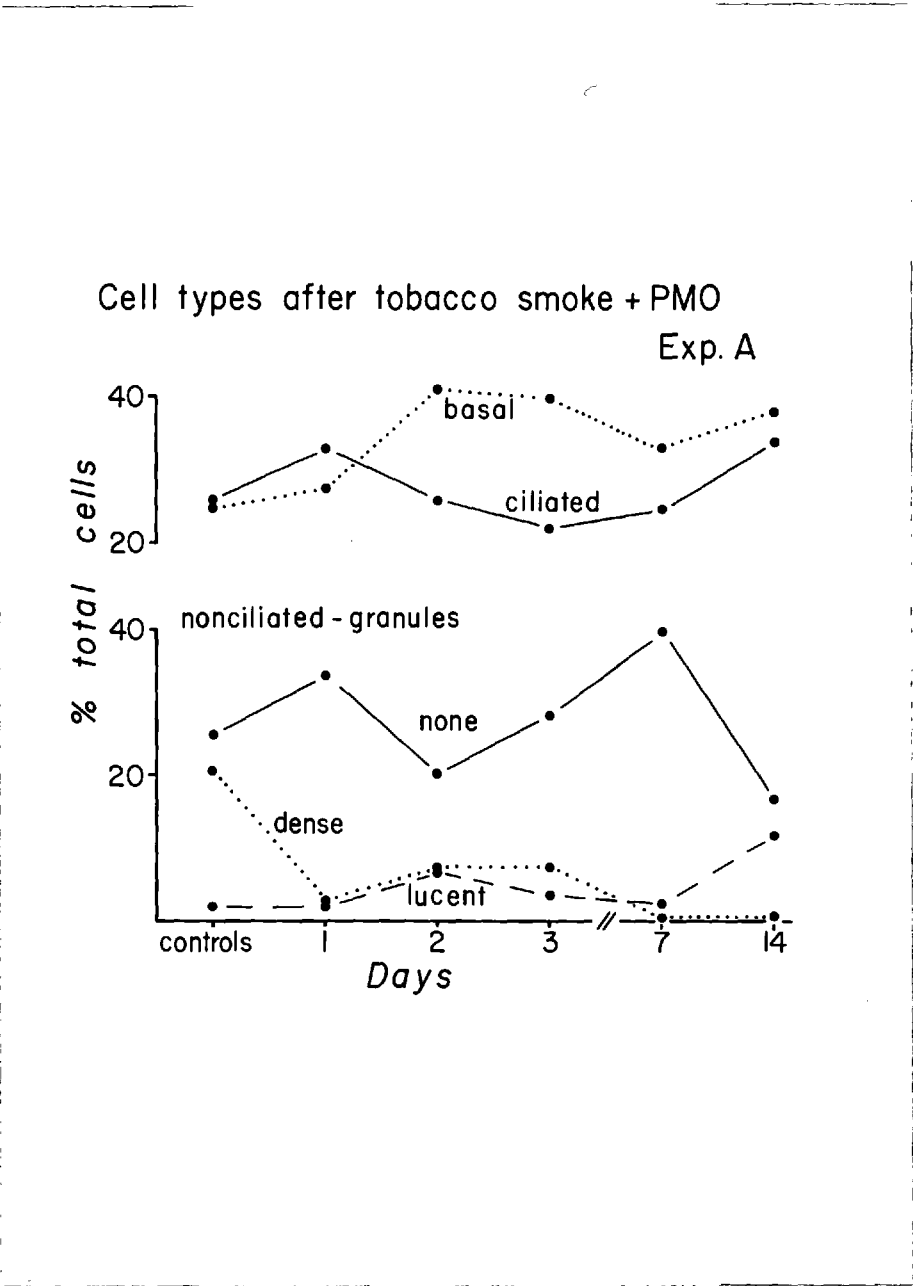


Fig. IV-5.

Nonciliated cells with lucent granules, normally present as 2% of cells, showed two significant peaks of increase at day two and at the end of the second week of exposure ( $P < 0.01$ ). When compared with the control value, the most marked increase occurred after two weeks and was seen in the animals from group I where this cell type increased to 18% of cells, nine times their normal proportion ( $P < 0.001$ ). PMO tended to protect against this increase ( $0.5^0 < P < 0.10$ ): animals of group II showed an increase of only five times when compared with the controls ( $P < 0.01$ ).

Nonciliated cells without granules showed two peaks of increase at day one and after one week ( $0.5^0 < P < 0.10$ ) returning to near control values on the other days of exposure. The increase seen in this cell type coincided with decreases in those nonciliated cells with secretory granules described above.

If the proportions of both types of secretory cell (i.e. dense and lucent) are considered as one, then it will be seen that throughout the 2 weeks of exposure to tobacco smoke there were always more secretory cells in the animals from group I than in those of group II.

Basal Cells - In both groups I and II there was a significant increase in basal cells after two days of exposure ( $P < 0.05$ ) and, but for day 3 in group I, this increase was significant and maintained at a similar

level throughout the exposure ( $P < 0.05$ ).

Migratory Cells - In number, this group of lymphocytes and globule leucocytes was increased, but when expressed as a percentage of the total epithelial nuclei, the normal figure of 4% was unaltered.

Concentration and proportion of cells - Experiment B

From each of the 6 animals of experiment B, one block of the main bronchus was taken and from each block 3 sections were counted for the cell concentration/10 HPF, the mean of the 3 sections being calculated. As only one block was taken from each animal the SD ( $\sigma$ ) for each group was estimated from the range ( $w$ ) by dividing the difference between the means of the two animals in each group by tabulated figures (see Paradine and Rivet, 1953) This method gave high figures for SD and while trends similar to those of experiment A were found, the differences between groups, even when large, were not found to be statistically significant.

As with experiment A, the cell concentration in both groups I and II was found to increase with exposure to cigarette smoke. The controls had a mean cell concentration of 222 cells/10 HPF (SD of mean = 51 ), similar to that of experiment A. With exposure to smoke for 6 weeks, the concentration increased to 291 cells/10 HPF in group I (SD of mean = 15 ) and 289 cells/10 HPF in group II (SD of mean = 57 ).

In the same way as experiment A, the percentage of each cell type was calculated from the total number of cells so that the animals in each group could be compared. Comparing the controls of experiments A with B, they were similar but for the proportion of ciliated cells which was greater in experiment B and for a few nonciliated cells with lucent granules found in experiment A but not in those animals of experiment B.

In both groups I and II the increase in cell concentration was accompanied by a shift, from the control values, in the proportion of cells: this was most significant in animals of group II ( $Px^2 < 0.001$ ).

When compared to the control value of 36%, ciliated cells increased in group I to 43% and reached their highest proportion in group II when they were 53% of cells (Fig. IV-6). Nonciliated cells with dense granules decreased, this being most marked in group II, while nonciliated cells with lucent granules (not present in the controls), appeared and increased to 5% in group I and to only 1% of cells in group II. Nonciliated cells without granules decreased in groups I and II.

Comparing the proportion of secretory cells (i.e. cells with dense granules and those with lucent granules) in groups I and II with the controls, there was an increase in secretory cells in the

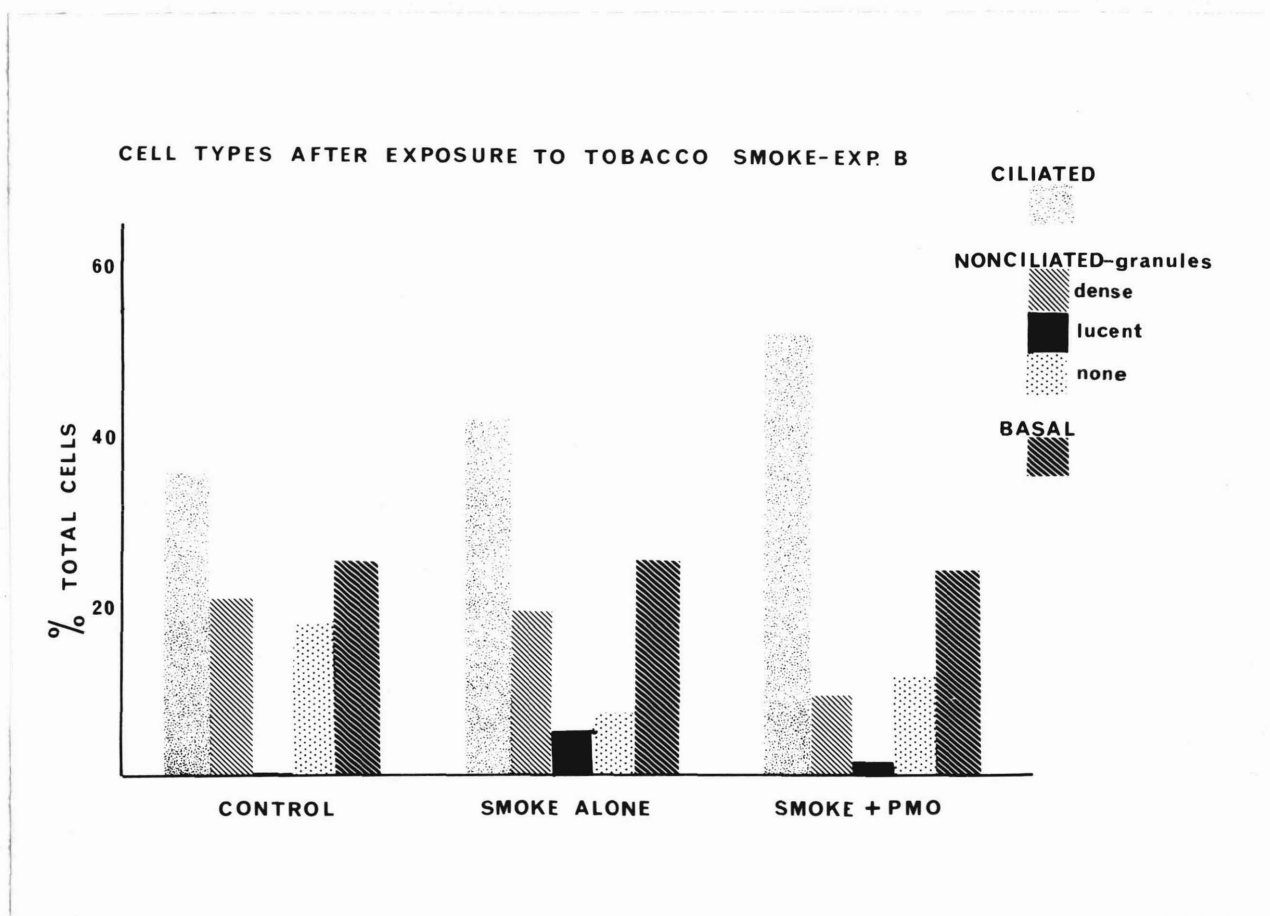


Fig. IV-6

animals of group I and a decrease to less than half the group I value, in the animals whose smoke included PMO (i.e. group II).

Basal cells increased in absolute number to maintain their normal proportion, and in contrast to experiment A, the number of migratory cells was slightly decreased in both groups I and II and thus their proportion as a percentage of epithelial nuclei, decreased from 3% to 1%.

#### Cells in Mitosis

Mitoses were found within the epithelium of all animals but especially in that of the animals from groups I and II. Their number was counted in experiment A. In the controls, a cell in division was rarely found: a maximum of 2 were found in 60 HPF (1 HPF = 0.18 mm epithelium). In the animals of groups I and II two peaks of increase after day one and after <sup>one</sup> ~~two~~ weeks of smoking, were found. The greatest increase was in those of group I-15/60 HPF at day one and 25/60 HPF at 1 week - as compared with 11 and 4/60 HPF on the same respective days in the animals from group II.

Of the 70 mitotic figures seen, nearly all were present in cells occupying a basal position within the epithelium. Only 3 such cells were seen in a mid or superficial position when they were present in animals from group I. On one occasion a cell in division also had secretory granules within the cytoplasm.

Cells in division measured 6-7  $\mu$ m in diameter and usually had the equatorial axis at right angles to the epithelium, although other orientations were seen.

#### Macrophages

Infrequently, macrophages about 20  $\mu$ m in diameter were seen in the airway lumen and close to the epithelium. In control animals each had a nucleus with a distinct nucleolus and, in the cytoplasm, toluidine blue positive inclusions. Those in both smoking groups were distinctive in that they also always had 1 or 2 larger inclusions up to 13  $\mu$ m in diameter, which appeared homogenous and yellowish and probably represented tar accumulation.

#### MAIN BRONCHUS - ELECTRON MICROSCOPY

The electron microscope was used to study the epithelium of the main bronchus of 12 animals, six animals from experiment A

(2 controls, 2 in group I and 2 in group II) killed after two weeks' exposure, and six animals from experiment B (2 in each group) killed after six weeks' exposure to tobacco smoke. Representative areas of the anterior wall were selected from 1  $\mu$ m sections stained with toluidine blue and these areas cut for electron microscopy. The results of both experiments A and B were similar and are here considered together.

#### Control Animals

The epithelium from control animals was ciliated, low columnar to cuboidal and in one case, flatter. Ciliated cells were regularly interspersed with nonciliated cells of the type with dense cytoplasm and dense granules (Fig. IV-7). On rare occasions, nonciliated cells with lucent granules were found when the granules usually also had an electron-dense core (see page 80). Brush cells were always present and of normal appearance (see page 81), and basal cells were frequent (Fig. IV-7). Lymphocytes and globule leucocytes were present and nerves were found with both ciliated and nonciliated cells. Macrophages were occasionally seen in the lumen.

#### Animals exposed to Tobacco Smoke

All the cell types found normally were also found in the smoking animals of both groups I and II. No new cell type was found.

Nerves were also found in these groups and although not quantified, were not obviously altered in number (see Fig. IV-11).

In both groups I and II and in both experiments A and B there were four significant deviations from the normal: (1) an increase in epithelial thickness (2) a change in the proportions of cell organelles; (3) a change in the type of secretion and the size and number of secretory granules produced by nonciliated cells and (4) a change in the size of certain cell organelles.

EPITHELIAL THICKNESS - CELL HYPERTROPHY - In both experiments A and B the increase in epithelial thickness was obvious by visual examination of electron micrographs (Figs. IV-8 and 9). The thickness was increased in both groups I and II when compared to the controls, the increase being most marked in group I. From experiment B measurements of a consecutive series of electron micrographs of bronchial epithelium of two controls and one animal from each of groups I and II were made. For each animal 10 measurements of the shortest vertical distance between the basement membrane and the luminal cell membrane of a ciliated cell were taken. The epithelium of the animal from group I was very much thicker (21  $\mu\text{m}$ ) than that of either group II (12  $\mu\text{m}$ ) or the controls (8  $\mu\text{m}$ ).

The increase in thickness was not due to an increase in cell number in a vertical direction, but to a cell hypertrophy accompanied by a change in cell shape: individual cells were taller and thinner (see Figs. IV - 10 to 12). A sample of 120 cells, 10 ciliated and 10 goblet cells from each of six animals (2 from each of groups I, II and the controls) showed that when compared to the controls there was a significant increase in total cell area as judged by a point-counting technique (see page 61) (Table IV-4). The increase was greatest in the animals of group I ( $P < 0.001$ ) but those cells of group II also showed a significant hypertrophy ( $P < 0.05$ ), if to a lesser extent than those of group I (see Figs. IV - 10 to 15).

The nonciliated cells (goblet cells) of group I showed significantly more hypertrophy than those of group II ( $P < 0.05$ ).

PROPORTION OF CELL ORGANELLES - Accompanying cell hypertrophy there was in both groups and both experiments, a significant shift in the proportion of cell constituents ( $Px^2 = 0.001$ ). The following results show the mean areal proportion of organelles in the mean total cell area as calculated from the results of point counting (see page 62).

TABLE IV-4MEAN CELL AREA - AS DETERMINED BY POINT COUNTING OF ELECTRONMICROGRAPHS - AFTER EXPOSURE TO TOBACCO SMOKE ALONE (I) OR WITHPMO (II)CILIATED CELL

| EXPERIMENT | CONTROL        | <u>GROUP</u>           |                        |
|------------|----------------|------------------------|------------------------|
|            |                | I                      | II                     |
| A          | 24.8<br>(1.4)* | 43.7<br>(1.7)<br>0.001 | 38.0<br>(2.8)<br>0.001 |
| B          | 26.6<br>(1.9)  | 39.7<br>(2.0)<br>0.001 | 35.8<br>(1.8)<br>0.01  |

GOBLET CELL

| EXPERIMENT | CONTROL       | <u>GROUP</u>           |                        |
|------------|---------------|------------------------|------------------------|
|            |               | I                      | II                     |
| A          | 25.7<br>(1.9) | 47.9<br>(2.3)<br>0.001 | 37.8<br>(1.9)<br>0.001 |
| B          | 33.7<br>(1.9) | 52.3<br>(2.6)<br>0.001 | 41.8<br>(3.2)<br>0.05  |

\*SE of the mean. P values given where significantly different from controls.

Magnification in Experiment A = x 15,000 and in Experiment B = x 18,000.

Ciliated Cell - There were two consistent intracellular changes in the ciliated cell: (i) The cytomatrix (i.e. polysomes and ground substance) normally 46% of the cell area was increased markedly in cells of both smoking groups, in group I to 60% and in group II to 57%, this contributing most to the increase in cell size. (ii) The areal proportion of the cell occupied by the nucleus fell from normally 34% to about 25% in groups I and II.

There was also, in experiment A, a slight fall in the proportion of cell organelles such as basal corpuscles, mitochondria, lysosomes, Golgi, vacuoles and endoplasmic reticulum, and in experiment B, an increase in their number to maintain the normal proportion.

Goblet Cell - There were three consistent changes seen within the nonciliated (goblet) cells: (i) In contrast to the ciliated cells the areal proportion of cytomatrix decreased with cell hypertrophy from normally 45% of the cell area to 35% in groups I and II: this decrease was most marked in experiment A. (ii) In group I the amount of intracellular secretion was increased from a normal 18% of the cell area to 38% (compare Fig. IV-13 with 14). In group II the areal proportion of secretion was increased, in experiment A, to 45% of the cell area while in experiment B it was 25% and less than that

seen in group I (compare Figs. IV-13, 14 and 15). (iii) With cell hypertrophy, the nucleus still showed the relative reduction seen with ciliated cells; there was a failure of the nucleus to increase its size with the increase in cell size and it remained of normal size.

SECRETION - NUMBER AND SIZE OF SECRETORY GRANULES - One of the most striking changes seen after exposure to cigarette smoke was the change in the type of secretion produced by nonciliated cells. In both experiments A and B there was a shift from cells containing secretory granules with a homogeneously electron-dense content and a distinct membrane (Fig. IV-13) to those with secretory granules with an electron-lucent content, without a clearly defined membrane and often confluent. (Figs. IV-14 and 15). The change was first indicated by the appearance of a thin, clear halo around the granule, which gave a fuzzy outline to it. It would seem that this change proceeded by a 'loosening' of the contents starting from the periphery and working inwards, the process presumably incomplete in some cells which contained lucent granules with an electron-dense core (Fig. IV-16).

In both experiments the number of secretory granules was greatest and their size largest in groups I and II. This change was quantified in experiment A by comparing the secretory granules from

10 cells of an animal in group I with 10 cells of an animal in the control group. In each case, cells with the largest amount of secretion were selected from five ultrathin sections of epithelium. Those cells from the control group were divided into three levels by three lines drawn across the width of the cell at right angles to the long vertical axis, one line drawn just touching the apical edge of the nucleus, one touching the apical cell membrane and a third bisecting these two in the supranuclear region (Fig. IV-17). Those cells of the smoking group were, owing to their larger size, divided into four levels by dividing the previously mentioned supranuclear zone into three equal parts.

In neither the control animal nor the animal from group I was there a significant difference in the size of secretory granules at different levels of the cell (Table IV-5). There was, however, a highly significant difference in granule size between the two groups: granules of group I were significantly larger than those of the control ( $P < 0.001$ ).

There was, in both control cells and cells of group I, a difference in the number of secretory granules at different levels of the cell. (Table IV-6). More were present in the most apical

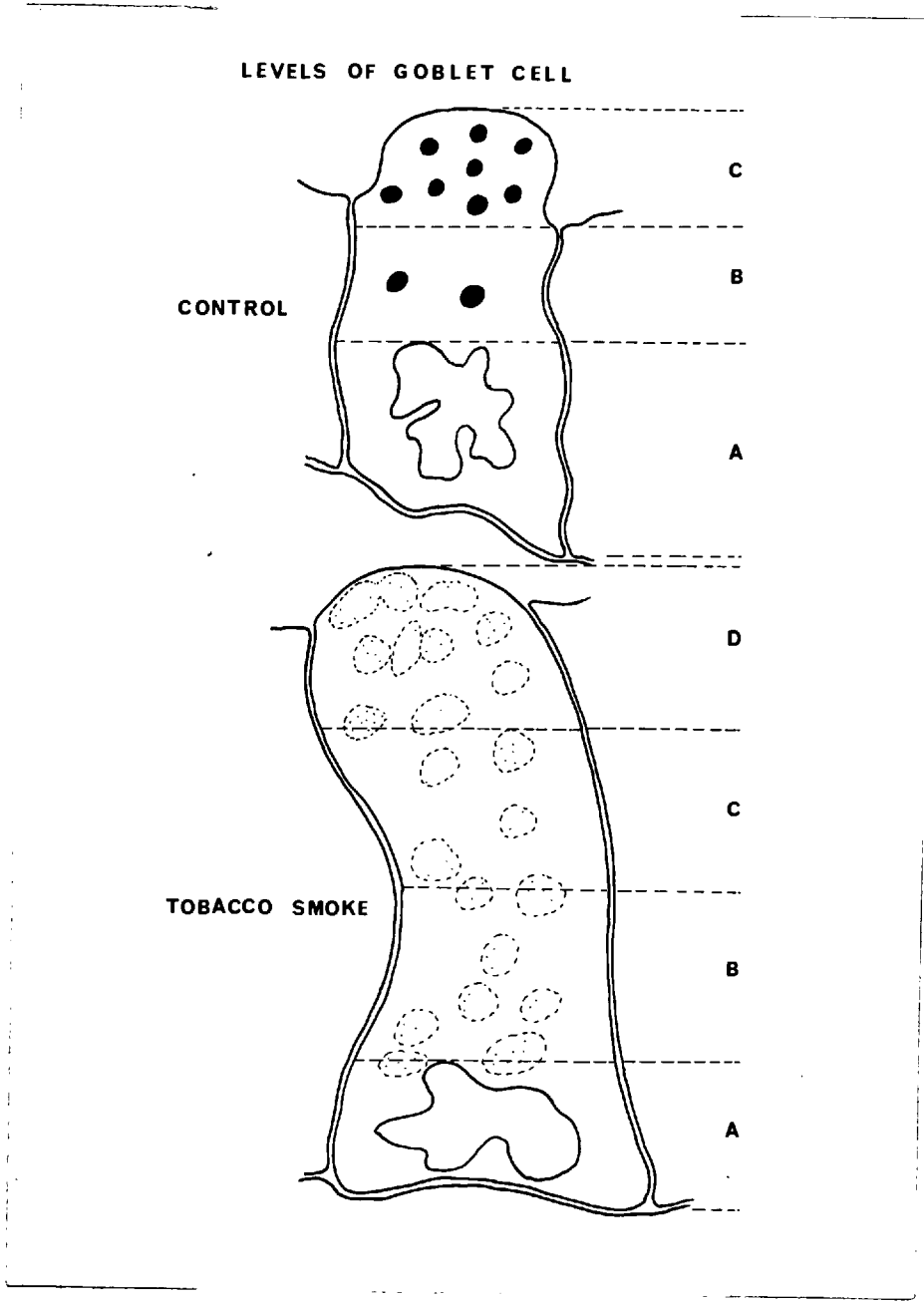


Fig. IV-17.

TABLE IV-5 & 6

GRANULE SIZE AND NUMBER IN 20 GOBLET CELLS - 10 FROM A CONTROL  
AND 10 FROM A RAT EXPOSED TO TOBACCO SMOKE ALONE - EXPERIMENT A

| <u>MEAN SIZE</u> |                 |              |                            |              |
|------------------|-----------------|--------------|----------------------------|--------------|
| LEVEL OF CELL    | CONTROL         | $\bar{x}$ nm | SMOKE ALONE                | $\bar{x}$ nm |
| A                | 8.96<br>(0.44)* | 597          | 12.15<br>(0.64)            | 809          |
| B                | 9.70<br>(0.26)  | 646          | 12.10<br>(0.27)            | 806          |
| C                | 8.78<br>(0.22)  | 585          | 11.14<br>(0.20)            | 742          |
| D                |                 |              | 11.11<br>(0.20)            | 740          |
| MEAN             | 9.15<br>(0.31)  | 609          | 11.63<br>(0.33)<br>P<0.001 | 775          |

| <u>MEAN NUMBER</u> |                 |                              |
|--------------------|-----------------|------------------------------|
| LEVEL              | CONTROL         | SMOKE ALONE                  |
| A                  | 2.90<br>(0.93)  | 5.81<br>(2.79)               |
| B                  | 11.80<br>(2.29) | 19.40<br>(1.57)              |
| C                  | 20.30<br>(2.48) | 27.60<br>(1.93)              |
| D                  |                 | 24.90<br>(2.37)              |
| MEAN               | 11.67<br>(1.90) | 19.43<br>(2.17)<br>* P< 0.02 |

\*SE of the mean

\* P value - control

than in the most basal level ( $P < 0.001$ ). At any level of the cell there were more secretory granules present in the smoking than in the control cells: the mean number in a cell was about 20 in group I and 12 in the control ( $P < 0.02$ ).

SIZE OF CELL ORGANELLES - Certain cell organelles were found to change in size after exposure to cigarette smoke. This change was quantified.

Cilia - In both smoking groups I and II, cilia were normal for size and structure. In both control and groups I and II, the cilia were similarly spaced: the distance between the mid point of adjacent cilia was in each case about  $0.36 \mu\text{m}$ .

Microvilli - Filiform processes (microvilli) were present on ciliated cells between the cilia and were numerous in both control and smoking animals. Microvilli were also present on nonciliated cells but were fewer in number and significantly shorter -  $0.3 \mu\text{m}$  - than those of normal ciliated cells -  $0.8 \mu\text{m}$ . ( $P < 0.001$ ). Unlike ciliated cells they showed no clear response to smoke exposure.

Compared to those of the control animals those of ciliated cells were considerably longer, but not wider, in the animals of groups I and II. These processes were measured from 15 ciliated cells of experiment B, 5 cells selected at random from each of groups

I, II and the controls. Where possible, 10 processes were measured from each cell. At a magnification of 18,000 times, those of the controls had a mean length of 13.6mm (se of mean = 0.9) equivalent to a length of 0.8  $\mu$ m. Those of group I were the longest, 1.2  $\mu$ m long (22.2mm: se of mean = 2.4) and significantly longer than those of the control ( $P < 0.002$ ) while those of group II measured 1  $\mu$ m in length (19.3mm: se of mean = 0.8) and also were significantly longer than those of the control ( $P < 0.01$ ).

Cilia/Microvilli Ratio - Using the same group of ciliated cells as above, the number of cilia and the number of microvilli in contact with and projecting from the cell surface was counted, and expressed as a ratio cilia/microvilli (i.e. C/M ratio). In the normal, the microvilli outnumber the cilia by about 2 to 1 (mean C/M ratio = 0.68 se of mean = 0.18). With smoking there was a trend to a relative increase in cilia and a decrease in microvilli (i.e. an increase in the C/M ratio). The greatest increase was seen in group I when the C/M ratio was 1.27 (se of mean = 0.17) (0.05  $P < 0.1$ ). The increase was not significant in group II when the C/M ratio was 0.99 (se of mean = 0.24).

Mitochondria - Mitochondria were never found swollen, burst or altered in internal structure. Compared to the controls, those of goblet cells

showed no structural response to cigarette smoke but those of ciliated cells were visably increased in length but not width.

From fifteen ciliated cells of experiment B, five selected at random from each of groups I and II and the control group, five of the largest mitochondria were chosen from each cell and their maximum lengths and widths recorded (mag = x18,000). Those of the control groups had a mean length of  $0.75 \mu\text{m}$  (13.4mm: se of mean = 1.1), while those of group I were significantly longer ( $P < 0.01$ ) with a mean length of  $1.6 \mu\text{m}$  (28.8mm: se of mean = 3.3). The maximum length recorded in group I was  $3 \mu\text{m}$ , about twice the control maximum. Those of group II were  $1.2 \mu\text{m}$  long (20.8mm: se of mean = 2.8), and also significantly longer than the control ( $P < 0.05$ ).

Some branching of mitochondria was normal but this feature did appear to be most common in those ciliated cells from group I.

Other Organelles - A few lysosomes were always present in both ciliated and goblet cells, no alteration in structure or number being apparent after smoking. In ciliated cells ribosomes were normally grouped as "rosettes" (i.e. polyribosomes), their density being the same in all groups. No "dilution" of ribosomes was found with cell hypertrophy, indicating some absolute increase in their number. Zones of cell contact, zonula occludens and macula adherens,

appeared in all groups without alteration from the normal.

Some ciliated cells in both control and groups I and II showed fibrogranular bodies - evidence of ciliogenesis (see Fig. IV-13).

OTHER CELLS - Nonciliated cells with only a few secretory granules were found in both the smoking and control animals and resembled those normally found (Fig. IV-17a).

Those nonciliated cells without granules were brush cells and were also found in both smoking and control animals. Those in animals exposed to smoke were somewhat taller and thinner than normal but were of normal structure (Fig. IV-18).

Basal cells were not altered in structure after exposure to cigarette smoke and showed all the normal characteristics already described (see page 83).

Kultschitsky cells were never found and migratory cells, globule leucocytes and occasionally lymphocytes were found in all groups without alteration (see Fig. IV-8).

CELLS IN MITOSIS - With the electron microscope, cells in mitosis were found but rarely. A single mitosis was found in an animal of experiment B exposed to tobacco smoke alone, when it was present in a basally situated cell (Fig. IV-19).

MACROPHAGES - As with the light microscope, macrophages identical to those seen in the alveoli were seen in the airway lumen of both control and smoking animals. Those of the controls had an abundance of electron dense inclusions of varied shapes (see page 85). Those of the smoking group differed in having larger, lipid-like inclusions (Fig. IV-20).

MYCOPLASMA - A small, isolated focus of sub-epithelial lymphocytic accumulation (limited to an 85  $\mu$ m length of epithelium) was found in one animal of group I in experiment B. In this case, there were numerous ovoid, or pear-shaped bodies, resembling Mycoplasma sp. on the apical cell membranes of the epithelial cells, particularly between the cilia and microvilli of ciliated cells (Figs. IV-21 and 22). Those which were pear-shaped were often seen to have an electron-dense rod-shaped core, projecting towards the epithelial cell (Fig. IV-22). At this localised site there was an infiltration of lymphocytes and plasma cells into the epithelium (Fig. IV-23): some of these infiltrating cells were in division (Fig. IV-24). The mycoplasmas measured about 0.46  $\mu$ m wide and had a maximum length of 0.73  $\mu$ m. They were seen in contact with the epithelial cell membranes, but were never seen within the cell or in the intercellular spaces.

#### DISTAL BRONCHIOLUS

##### Light Microscopy

Using 1  $\mu$ m epoxy sections, stained with toluidine blue, the distal airways of less than 0.4 mm in diameter (level 5) were

examined by light microscopy. Six rats, two in group I, two in group II and two from the control group of experiment A, were examined and the epithelial thickness, depth of ciliary layer and the number of each type of epithelial cell recorded. All the animals had a cuboidal or low columnar epithelium with ciliated cells interspersed with nonciliated cells with few or no dense granules. In most respects the epithelium was of normal appearance.

EPITHELIAL THICKNESS AND DEPTH OF CILIARY LAYER - Using a measuring eyepiece graticule, and in the same way as for the main bronchus, three measurements of the epithelial thickness, measured from the basement membrane to the base of the cilia, were recorded from each animal and the mean calculated. All animals had an epithelium of similar thickness, about 13  $\mu$ m.

Using the same sections as above, the mean of three measurements of the depth of the ciliary layer was recorded for each animal. All animals had a ciliary layer varying between 4 and 5  $\mu$ m in depth.

CONCENTRATION AND PROPORTION OF CELLS - In one section from each airway, the number of each cell type was recorded. The airways taken were somewhat smaller than usual (about 0.3 mm) and due to insufficient cells, only 5 to 8 high power fields (0.18 mm diameter) could be counted. Thus for comparison between groups, these figures were expressed as though for 10 HPF. There was no significant change in cell concentration with smoking: there was a mean of 232 cells/10 HPF

in the control group, 202 in group I and 236 in group II.

The percentage of each cell type was calculated from their respective totals and the mean of the groups compared. There was no significant change in the proportion of ciliated cells with smoking, each group showing a mean of about 72% of cells. Nonciliated cells with dense granules decreased somewhat from the control value of 11% to 5% in both groups I and II, and nonciliated cells without granules increased slightly from a control 16% to 22% in group I and 25% in group II. Basal cells were present as less than 1% of cells, an occasional one found in the controls and in group II. Migratory cells were found only in groups I and II when they comprised 1 - 2% of epithelial cells.

#### Electron Microscopy

The same distal airways examined by light microscopy were examined by electron microscopy in the six rats of experiment A.

CONTROL ANIMALS - In both animals from the control group, the distal airway epithelium appeared normal in every respect (see page 81). The epithelium was simple, cuboidal, or low columnar and of ciliated cells interspersed with nonciliated cells of the type with lucent cytoplasm, smooth endoplasmic reticulum and a few electron-dense

granules (Clara cells).

SMOKING ANIMALS - But for the presence of polymorphonuclear cells in one animal, both animals in group I had normal epithelium. No goblet cells were found and brush cells were present and of normal structure. In one animal several polymorphonuclear cells were found immediately beneath the epithelial basement membrane and occasionally within the surface epithelium (Fig. IV-25). The distal airways of both animals from group II lacked the polymorphonuclear cells seen in group I and in other respects had an epithelium of normal structure.

Macrophages were occasionally seen in the lumens of level 5: those of the smoking group had lipid-like inclusions like those seen in macrophages at level 2 (see page 125).

### SUMMARY OF RESULTS

1. The airway epithelium of rats exposed to tobacco smoke in two experiments, one of two weeks' duration (Experiment A) and one of six weeks' duration (Experiment B) was observed. To the animals of each experiment two types of tobacco smoke were given: tobacco smoke alone (Group I) and tobacco smoke to which the anti-inflammatory agent phenylmethyloxadiazole (PMO) had been added (Group II). The changes were similar in both experiments. Two airway levels were studied, the main extrapulmonary bronchus and the distal bronchiolus.
2. Those animals exposed to tobacco smoke gained weight less rapidly than their controls: the mean weight of the animals in groups I and II were similar in experiment B, but in the younger animals of experiment A, group II gained weight less rapidly than those animals of group I.
3. After two weeks the epithelium of the main bronchus exposed to tobacco smoke was increased in thickness about two-fold: animals of group I had the thickest epithelium and those of group II were partially protected from this increase. When compared to the controls there was no significant difference in the depth of the ciliary layer in either of the smoking groups.

4. The cell concentration (i.e. the number of cells in 1.8 mm epithelium - 10 HPF) was found to increase in animals of groups I and II when compared to the controls: the increase was proportional to the duration of exposure. There was no protection, by PMO, against this increase.

5. No new cell type was found in the epithelium of the animals of either smoking group I or II but, in both groups, and by day one, a significant shift in the proportion of each cell type occurred. With exposure, nonciliated cells with dense granules decreased sharply and nonciliated cells with lucent granules showed waves of increase, reaching a maximum at two weeks. Ciliated cells were increased by two weeks and by six weeks showed a most marked increase in the animals of group II. Basal cells were increased in experiment A but not in experiment B. Migratory cells were present in similar proportions to the controls. In the animals of group II, PMO partially protected against the increase in nonciliated cells with lucent granules: there were always far less secretory cells (i.e. dense and lucent granules considered as one) in those animals of group II than in those of group I.

6. The increase in epithelial thickness seen after exposure to smoke by light microscopy, was confirmed by electron microscopy,

the increase due to cell hypertrophy and a change in cell shape. With smoking there was a significant increase in the size of both ciliated and goblet cells: this was greatest in the animals of group I (i.e. there was partial, but significant, protection against this increase by the addition of PMO to tobacco smoke).

With cell hypertrophy, there was a significant shift in the normal proportion of cell organelles: in ciliated cells there was a marked increase in the cytomatrix (i.e. polysomes and ground substance) with a relative fall in the nucleus (it maintained normal size). In goblet cells there was an increase in secretory mass and a relative decrease in cytomatrix and size of the nucleus.

There was evidence of organelle hypertrophy, particularly of ciliated cell mitochondria, in those animals exposed to tobacco smoke: cilia were normal for size, density and structure.

7. In nonciliated cells with secretory granules (secretory cells) the type of secretion changed from electron-dense to electron-lucent and there were indications that the former had given rise to the latter. In both experiments the number of secretory granules within any secretory cell, was greater and their size larger in animals of groups I and II than in those of the control.

In neither control nor smoking groups was there a significant difference in the size of secretory granules when in any cell the mean of one level was compared with another.

8. Cells in division were found in animals from all groups, the greatest number found in animals from group I. Most were basal in position but some were superficial in cells with intracellular secretion. The increase occurred in waves with peaks at day 1 and after <sup>one</sup> ~~two~~ weeks of exposure. There was partial protection, by PMO, against this increase.

9. Macrophages were found in the airway lumen of all groups. Unlike those of the control animals the macrophages of animals from both groups I and II had distinctive, larger than normal inclusions which probably represented tar accumulation.

10. A Mycoplasma sp. was identified at a localised site in an animal from group I of experiment B: they were found in numbers between the cilia and microvilli. They were in contact with the cell but never seen within the cell or in the intercellular spaces.

11. But for a slight decrease in nonciliated cells with dense granules, smoking caused no alteration in the distal bronchiolus.

CHAPTER V

THE EFFECTS OF ISOPRENALINE AND PILOCARPINE

ON RAT AIRWAY EPITHELIUM.

The effects on rat airway epithelium of two drugs, isoprenaline sulphate and pilocarpine nitrate (henceforth referred to as IPN and PCP respectively), was investigated by light and electron microscopy, in two experiments A and B, each of 40 rats. In experiment B only IPN was used, the dose being higher and given for a longer period than in experiment A.

From experiment A, 12 male rats were selected for electron microscopy, 3 given 10 mg/100g body weight IPN for 6 days, 3 given 4 mg/100g body weight PCP for 12 days and a control group of 6 rats given injections of normal saline, 3 for 6 days and 3 for 12 days. The animals were taken randomly from each group. Airway levels 2, 3 and 5 were examined.

From experiment B, 4 male rats were selected for electron microscopy, 2 given 16 mg/100g body weight IPN for 12 days and 2 given injections of Normal saline. Airway levels 4 and 5 and a level intermediate to these (level 4a) were examined. Level 4a was a lateral airway taken close to the main axial pathway.

Before the experiments were started, the lungs were found to be "clean" from histological evidence: absence of lymphocytic cuffing and a low goblet cell count (Reid, 1970).

## Effect on Animals

### BEHAVIOUR

The behaviour, outward appearance, respiratory rates and rectal temperatures were recorded after injection of a drug on several days during experiment A. About one hour after injection the respiratory rate was observed, in 3 animals, chosen at random, each over a period of 15 seconds, the mean of the 3 being recorded. Control animals showed a mean of 30.5 breaths/15 sec (s.e. of mean = 1.8). In the same way, the mean rectal temperature was calculated from 3 animals using a 1 minute thermometer: the control mean was 36.2°C. (s.e. of mean = 0.2).

Normal saline injections had no obvious effect on animal behaviour.

### Pilocarpine

Within 10 minutes of a single subcutaneous injection of PCP there was lacrimal and salivary stimulation, apparent from the wet fur around the eyes and mouth. Defaecation was usual with characteristic watery, smelly stools. The hair arrector pili muscles were stimulated, the fur standing on end and giving the coat a spikey appearance. The mean rectal temperature was 36.6°C. (s.e. of mean = 0.2), the respiratory rate was 31/15 sec (s.e. of mean = 1.3), neither values differing significantly from the controls. Animals remained in an upright but hunched position, none died and all were fully recovered

and active by two hours following the injection.

### Isoprenaline

After a single subcutaneous injection of IPN, there was no lacrimal or salivary stimulation as seen in the PCP animals, but behaviour was more severely affected. Within minutes the animals toppled, flattened against the cage floor exhausted, and were unable to stand. When partially recovered they drank excessively, taking longer than two hours to return to normal. Among the rats given IPN, there was a 25% mortality in experiment A (1 rat died on day 2 and the other on day 3) and a 63% mortality in experiment B (8 female rats died on day 1 and 9 males and 2 females at the beginning of the second week). The two high mortality days of experiment B were unusually hot, 25°C. and 71% relative humidity and 27°C. and 66% relative humidity respectively. Autopsies showed there was lung congestion with superficially blackened areas of haemorrhage on the lung surface and some clear froth in the trachea.

The mean respiratory rate was significantly increased above the control and PCP animals to 36/15 sec. (se of mean = 1.5) ( $P < 0.05$ ). The mean rectal temperature for the group, although slightly depressed was 35.9°C. (se of the mean = 0.4) and not significantly lower than either the control or PCP group.

### WEIGHT GAIN

Body weights were recorded daily (morning) during experiment A and at injection days 1, 6 and 12 of experiment B.

#### Experiment A

Male rats from experiment A were received with a mean body weight of 200 g. and all steadily gained weight. At the beginning of injections they had a mean body weight of 250 g. (Fig. V-1).

After the start of injections the control group continued to steadily increase in weight until injection day 7 when the mean decreased due to half the group (presumably some of the heavier animals) being sacrificed. By 12 days, the control mean was 307.5g

The IPN animals failed to gain weight after the first injection with a decrease in mean body weight during the following two days. On the fourth and remaining days of the injection there was a recovery to the normal rate of weight gain, but no acceleration to catch up with the control group. The mean weight of the IPN group at the end of the 6th day was 260g. and significantly lower than both the control and FCP group ( $P < 0.001$ ).

After the start of injections, the FCP group continued to gain weight at the same rate as the controls. By the 12th day of injection the FCP group had a mean body weight which was significantly higher

than the controls due to their <sup>(controls)</sup> decrease in weight on the 7th day ( $P < 0.02$ ).

#### Experiment B.

Male and female rats from experiment B were received with a mean body weight of 100 g. being about 150 g. at the start of the injections (Fig. V-2).

The control male and females were of similar weight at the beginning of the experiment but by the twelfth day of Normal saline injections, the males had gained weight significantly more quickly than the females ( $P < 0.001$ ).

Male rats injected with IPN failed to gain weight as quickly as their male controls, by day 6 the mean weight being similar to that of the female control group.

Although the mean weight of the males, after 12 days of IPN injections, was significantly lower than that of their male controls ( $P < 0.01$ ), they had recovered slightly, having gained weight between days 6 and 12 at a faster rate than the female controls ( $P < 0.02$ ).

When compared to the female controls, the female rats injected with IPN did not show a failure to gain weight, the group mean always being similar to its matched control group (see Fig. V-2).

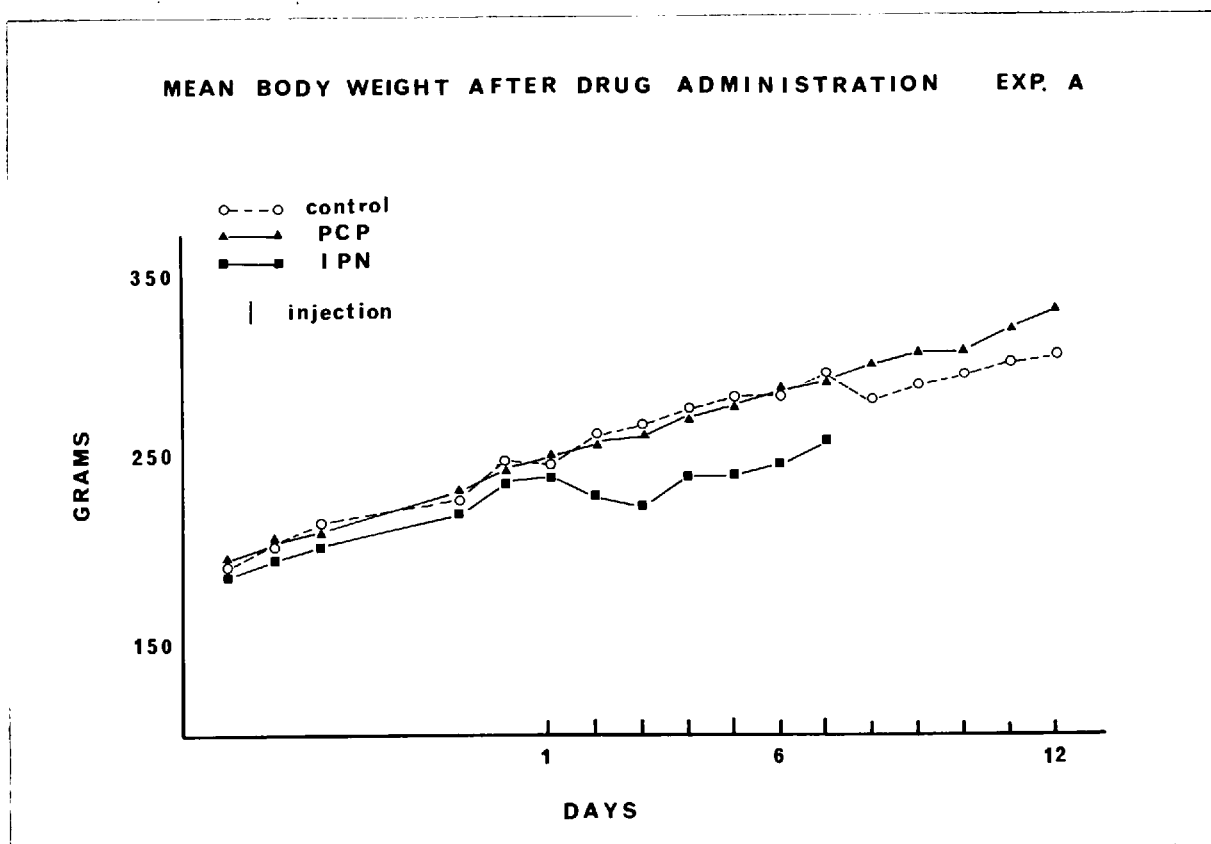


Fig. V-1

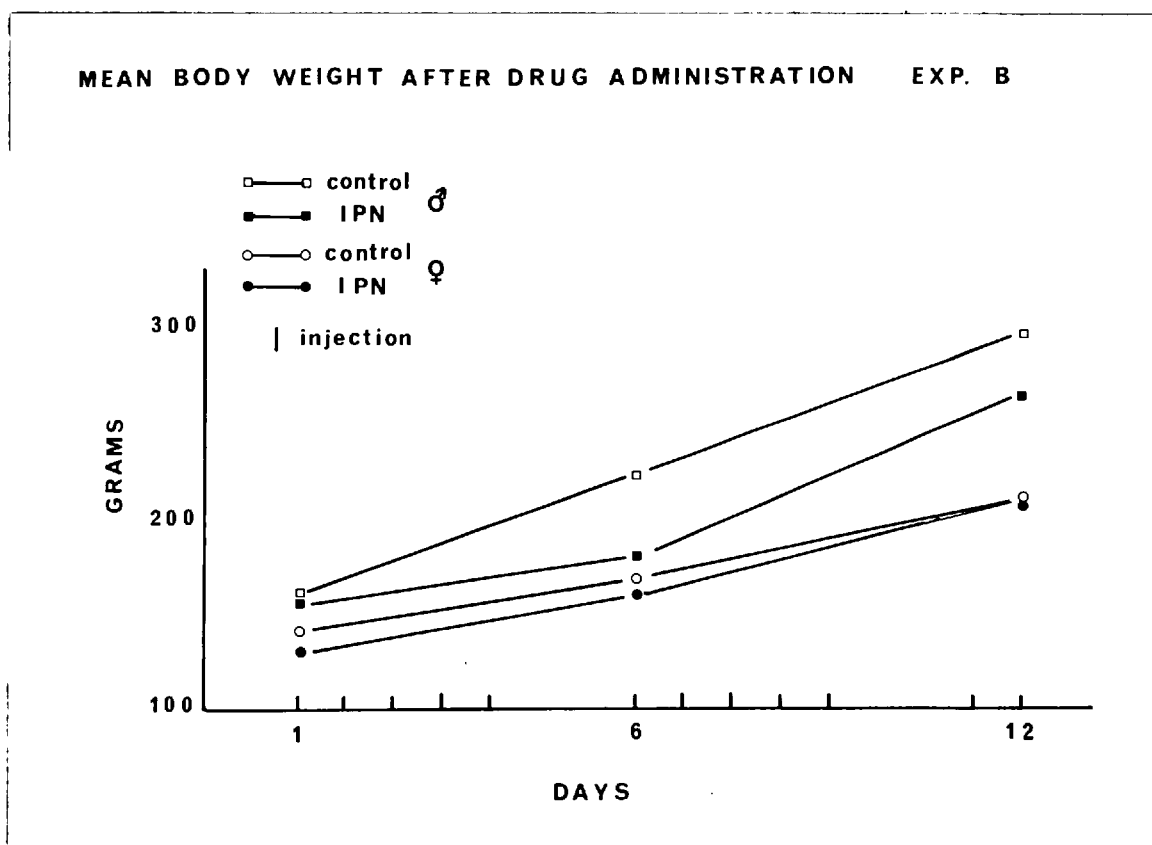


Fig. V-2

### Airway Epithelium

The epithelium from the animals selected for electron microscopy was observed also by light microscopic examination of 1  $\mu$ m epoxy sections stained with toluidine blue. The thickness of epithelium, depth of the ciliary layer and the number and type of epithelial cell was recorded.

### CONTROL ANIMALS

The six male control rats from experiment A and the two males from experiment B were examined by light and electron microscopy. In experiment A the three animals sacrificed at 6 days were compared with those three sacrificed at 12 days.

### Light Microscopy

The appearance of the epithelium was recorded at the 5 airway levels. But for one 12 day control of experiment A which had had its epithelium fixed in osmium alone and was excluded from these results, all the controls had an epithelium of normal thickness and a ciliary layer of normal depth for airway level (see page 71). In all but the 12 day controls of experiment A, the epithelial cells were of normal structure and frequency. The 12 day controls of experiment A did have more nonciliated cells with lucent granules than the 6 day controls: even at level 5, 2 such cells were found. Two cells in mitosis were also found at level 3, one in a basal cell and the other in a superficial cell with cytoplasm staining only weakly and in this respect resembling a ciliated cell (Fig.V-3).



Fig. V-3. Photomicrograph of bronchial epithelium (level 3) from a control rat showing a superficially located cell in division (arrow). The cell has cytoplasm with little affinity for stain.

The cell counts of both the 6 and 12 day controls were pooled for comparison with those of the IPN and PCP animals.

#### Electron Microscopy.

But for the more frequent than normal appearance of electron-lucent granules within the nonciliated cells, the fine structure of the epithelium at all levels resembled that of the normal animals already described (see page 77). The nonciliated cell with dense granules was the most frequently found nonciliated cell type and occasionally, in the 6 day but more especially in the 12 day controls, had granules in the process of changing from dense to lucent (Fig. V-4). At level 5 and especially in the 12 day controls, more cells than expected had electron-dense cytoplasm, many also having an electron-lucent granule developing in the Golgi region (Fig. V-5).

At no level was Mycoplasma sp. or any other sign of infection found.

### ISOPRENALINE ANIMALS

The airway levels of the three male rats of experiment A and two male rats from experiment B, given IPN, were examined by light and electron microscopy. The epithelial thickness, depth of the ciliary layer and the number and type of epithelial cell was observed at airway levels 2, 3, and 5 in the animals from experiment A, and measurements of cell size and secretory granule size and number from airway levels 4, 4a and 5 of experiment B.

### Light Microscopy

QUALITATIVE DESCRIPTION - The appearance of the epithelium was noted at the five airway levels of experiments A and B. The most characteristic change at all levels was the increase in nonciliated cells with lucent granules. There was also a tendency for isolated foci of sub-epithelial lymphocytic accumulation to occur in the IPN animals and the appearance of an occasional polymorphonuclear leucocyte within the epithelium. This was not found in the control or PCP animals. Otherwise, globule leucocytes and lymphocytes were found as they were in the controls.

In all three animals of experiment A, level 5 had the most altered epithelium with the cells appearing ciliated, tall and columnar and interspersed with nonciliated cells, many of which had a goblet shape and were filled with lucent granules (Fig. V-6).

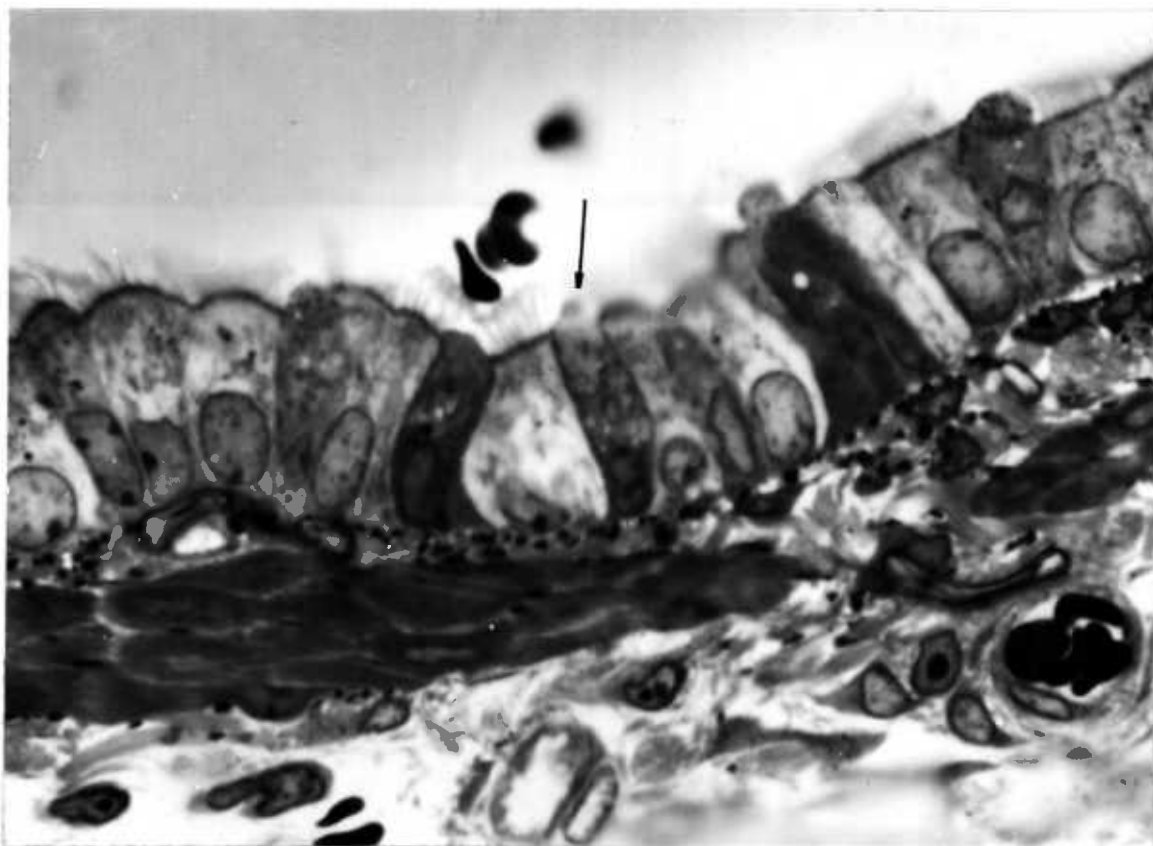


Fig. V-6. Photomicrograph of bronchiolar epithelium (level 5) of a rat given 25 mg IPN daily for 6 days. Compared to the normal (see page 69) the epithelium is thickened and goblet cells with lucent granules are present (arrow). x 1,500.

EPITHELIAL THICKNESS AND DEPTH OF CILIARY LAYER - As with the control animals, the epithelial thickness was measured at the three airway levels of the IPN treated rats from experiment A. Levels 2 and 3 each had a similar mean thickness to that of the corresponding levels of the controls, the mean values being about 14  $\mu\text{m}$  and 15  $\mu\text{m}$  respectively. (Table V-1). The epithelium of level 5 was, however, significantly thicker - 21  $\mu\text{m}$ -than that of the corresponding control mean - 14  $\mu\text{m}$  ( $P < 0.02$ ).

The depth of the ciliary layer was slightly reduced in the trachea but neither this nor that of the other levels, which were normal, were significantly different from the control mean.

CELL CONCENTRATION - As with the controls, the number of cells occurring in 1.8 mm length of epithelium (10 HPF) was counted in a section from each of the three airway levels of each IPN treated animal in experiment A (see Table V-2). But for a slight and insignificant decrease at level 5, the cell concentration was similar in the IPN and control animals.

PROPORTION OF EPITHELIAL CELLS - So as to compare the number of each cell type within a similar cell concentration, the numbers of each cell were converted to a percentage of their respective totals and the control and IPN groups compared in this way (Table V-3).

TABLE V-1

MEAN EPITHELIAL THICKNESS AFTER DRUG ADMINISTRATIONEXPERIMENT A

| Airway level | Control                    | Mean Number Units* |               |
|--------------|----------------------------|--------------------|---------------|
|              |                            | IPN                | PCP           |
| II           | 12.4<br>(0.9) <sup>+</sup> | 11.0<br>(1.5)      | 10.7<br>(0.4) |
| III          | 10.8<br>(0.9)              | 11.7<br>(2.0)      | 8.3<br>(0.9)  |
| V            | 10.8<br>(0.4)              | 16<br>(2.0)        | 11<br>(1.0)   |

\* 1 unit = 1.3  $\mu$ m.<sup>+</sup>SE of mean.

TABLE V-2

MEAN CELL CONCENTRATION AFTER DRUG ADMINISTRATIONEXPERIMENT A

| Airway level | Control                     | Mean Number Units* |                 |
|--------------|-----------------------------|--------------------|-----------------|
|              |                             | IPN                | PCP             |
| II           | 255.6<br>(8.6) <sup>+</sup> | 255.7<br>(9.9)     | 240.3<br>(12.7) |
| III          | 254.2<br>(12.6)             | 254.3<br>(16.8)    | 225.7<br>(8.1)  |
| V            | 239.0<br>(6.6)              | 229.7<br>(3.5)     | 240.0<br>(3.0)  |

<sup>+</sup>SE of mean.

TABLE V-3

MEAN PROPORTION OF CELLS AFTER DRUG ADMINISTRATIONEXPERIMENT B

| Level | Group   | Ciliated       | Cell type            |              |               | Basal         | Migratory<br>% Total<br>Epithelial Cells |
|-------|---------|----------------|----------------------|--------------|---------------|---------------|------------------------------------------|
|       |         |                | Nonciliated<br>dense | lucent       | none          |               |                                          |
| I     | Control | 33.6<br>(4.2)* | 26.6<br>(4.1)        | 0.4<br>(0.2) | 12.8<br>(1.5) | 26.2<br>(1.0) | 8.2<br>(1.6)                             |
|       | IPN     | 23.0<br>(4.7)  | 25.7<br>(3.7)        | 1.3<br>(0.4) | 21.3<br>(6.5) | 28.3<br>(4.8) | 7.3<br>(1.9)                             |
|       | PCP     | 29.7<br>(2.9)  | 12.0<br>(1.5)        | 0            | 31.3<br>(3.3) | 27.0<br>(3.8) | 5.7<br>(2.2)                             |
| II    | Control | 34.0<br>(2.31) | 21.6<br>(3.2)        | 0.9<br>(0.5) | 18.8<br>(5.9) | 24.6<br>(1.4) | 5.6<br>(0.2)                             |
|       | IPN     | 34.0<br>(2.9)  | 32.0<br>(3.6)        | 2.9<br>(2.6) | 9.3<br>(3.4)  | 22.0<br>(2.1) | 5.7<br>(0.9)                             |
|       | PCP     | 32.3<br>(1.2)  | 18.7<br>(4.4)        | 0.1<br>(0.1) | 26.3<br>(2.6) | 22.7<br>(2.6) | 2.7<br>(1.2)                             |
| III   | Control | 65.2<br>(1.8)  | 19.6<br>(4.7)        | 0.2<br>(0.2) | 14.6<br>(6.2) | 0.3<br>(0.2)  | 0.8<br>(0.3)                             |
|       | IPN     | 68.7<br>(2.0)  | 15.3<br>(1.2)        | 6.3<br>(3.0) | 9.3<br>(1.8)  | 0.4<br>(0.2)  | 2.5<br>(1.3)                             |
|       | PCP     | 62.0<br>(4.0)  | 15.0<br>(2.1)        | 0.3<br>(0.3) | 17.0<br>(1.5) | 5.6<br>(4.7)  | 2.7<br>(1.2)                             |

\*SE of mean.

The only significant change in the IPN animals was the increase in nonciliated cells with lucent secretory granules this being most significant at level 5 ( $P < 0.05$ ), with the trend at all three levels significant at the 1% level. As a proportion nonciliated cells with dense and those with lucent granules when taken together, (i.e. secretory cells) were increased at levels 3 and 5 and especially at level 3 where they were increased by 12%. At levels 3 and 5 there was a corresponding decrease in nonciliated cells without granules.

#### Electron Microscopy

The epithelium of the three animals of experiment A and the two of experiment B treated with IPN were examined by electron microscopy and their ultrastructure compared with that of the controls. The size of the nonciliated cells and their granule number and size were compared in experiment B.

EPITHELIAL APPEARANCE - Levels 2 and 3 - As with the controls, the most frequently found nonciliated cell in the IPN animal was that with dense granules. Many of these appeared, however, to have granules of larger size which were beginning to change to those of the electron-lucent type (Fig.V-7). Nonciliated cells with lucent granules, present in greater numbers than in the controls, had larger amounts of intracellular secretion, present as granules with or

without a dense core. (Figs. 8a and 8b).

Nerves, judged to be both motor and sensory, were found at levels 2 and 3 but not 5. Those in contact with ciliated and nonciliated cells often had intra-axonal dense-cored neurosecretory vesicles (Fig. V-9). They were not counted but no obvious change in their numbers was observed.

As with level 5, ciliated cells were normal in structure and there was no evidence of Mycoplasma or other infection.

Level 5 - In comparison with the 6 day controls, the change at level 5 was most striking. The epithelium had tall columnar ciliated cells frequently interspersed by nonciliated cells filled with large lucent and often confluent granules (Fig. V-10). These cells were usually large and distended by intracellular secretion. Intermediate stages between the nonciliated cell with lucent cytoplasm and dense granules and that with dense cytoplasm and lucent granules were found, the amount of smooth endoplasmic reticulum becoming less abundant and the amount of rough endoplasmic reticulum becoming more abundant in those cells with increasing numbers of lucent secretory granules. As well as a dense to lucent granule change, lucent granules often developed de novo from the Golgi zone. A suggested sequence in the production of a nonciliated cell with lucent granules from one with

dense granules is shown in Figs. V-11 to 13.

Evidence of ciliogenesis in cells with electron-lucent cytoplasm was seen. In one of these cells, electron-dense membrane-bound granules were also found which, in all respects, resembled those found in the nonciliated cells with dense granules (Fig. V-14). Brush cells were usually of normal structure, but for one cell found at level 5, which contained secretory granules (Fig. V-15). In this cell, the Golgi apparatus was somewhat larger than usual and the secretory granules were of intermediate electron density, some having a distinct membrane and others not. (Fig. V-16).

At level 5, mitoses were more frequently found in the IPN animals and then in both nonciliated cells with lucent granules (Fig. V-17) and in those with dense granules (see Fig. V-18).

A basally situated cell, judged to be migratory by the absence of desmosomes, and probably a lymphocyte, was also in division (Fig. V-19).

CELL SIZE, GRANULE SIZE AND NUMBER - At all the airway levels of the IPN animals of experiment A and B, the secretory granules within nonciliated cells appeared to be more numerous and larger than those of the controls. The changes, however, were not so pronounced in experiment B with no nonciliated cells with large numbers of lucent

granules being found at level 5. During examination of airway levels 4, 4a and 5 from an IPN rat and a control rat of experiment B, nonciliated cells, selected at random and with secretory granules, were photographed and the negatives of each projected onto a white page having a lattice-work of points spaced 19 mm apart. In this way, point counting volumetry was possible (see page 62).

Cell Area and Secretory Mass - Eleven nonciliated cells, all having a nucleus in section, were taken from the control and fourteen from the IPN rat and the mean results of cell size and secretory mass compared.

The mean cell areas in both the IPN and control animals were similar. As a percentage of this area, the proportion occupied by the nucleus was 26% in both cases, and that occupied by the cytomatrix was 67% in the control and 63% in the IPN treated animal. That occupied by secretion was 5% in the control when it was electron dense and 9% in the IPN treated animal, when 1% was electron-lucent, 2% electron-dense and 6% intermediate in density.

Number and Size of Secretory Granules - The number and size of secretory granules was recorded for each of 20 cells from the control and 25 cells from the IPN treated animals (Table V-4).

TABLE V-4

THE NUMBER AND SIZE OF NONCILIATED CELL SECRETORY GRANULES  
AFTER DRUG ADMINISTRATION - EXP. B.

MEAN NUMBER/CELL

| Airway level | Control       | IPN           |
|--------------|---------------|---------------|
| IV           | 16.8<br>(5.7) | 25.5<br>(3.4) |
| IVa          | 17.7<br>(4.4) | 25.3<br>(5.3) |
| V            | 14.7<br>(4.3) | 17.0<br>(4.8) |

MEAN SIZE/CELL-nm

| Airway Level | Control         | IPN             |
|--------------|-----------------|-----------------|
| IV           | 484.4<br>(73.4) | 783.0<br>(84.8) |
| IVa          | 470.0<br>(51.3) | 511.6<br>(65.8) |
| V            | 254.8<br>(39.6) | 397.5<br>(49.4) |

\*SE of mean

At each of the three airway levels the nonciliated secretory granules were more numerous and larger in nonciliated cells of the IPN animal.

The animals of experiment B, although not showing changes as severe as those seen in experiment A, did have at least a secretory response to the injections of IPN, this being of a similar type, if to a lesser extent.

#### PILOCARPINE ANIMALS

The airways of the three male rats of experiment A given PCP were examined by light and by electron microscopy of ultrathin sections. The epithelial thickness, depth of ciliary layer, number and type of epithelial cells was observed at levels 2, 3 and 5.

#### Light Microscopy

APPEARANCE OF EPITHELIUM AND CILIARY LAYER - But for level 5 in one animal which had a large number of basal cells, all three airway levels were similar to those of the control. The epithelium at levels 2 and 3 was slightly less thick than that of the controls but not significantly so, and the depth of the ciliary layer was not significantly altered.

CONCENTRATION OF CELLS - The number of cells occurring in 1.8 mm epithelium (10 HPF) was counted at the three airway levels, the means of the three animals, given PCP, being compared with those of the control

and IFN groups. Compared to the other two groups, the concentration of cells at levels 2, 3 and 5 was not significantly altered (see Table V-3).

PROPORTION OF CELLS - At each of airway levels 2, 3 and 5 there was a shift in the proportion of cells, this being most significant at level 2 ( $P_x^2 < 0.001$ ). At each of the three levels two nonciliated cell types, that with dense granules and that without granules, were altered with respect to the controls. Each trend was significant at the 2% level (see Table V-3). Nonciliated cells with dense granules decreased and this was most significant at level 2 ( $P < 0.05$ ). Those without granules increased, this also being most significant at level 2 ( $P < 0.01$ ). Nonciliated cells with lucent granules were present in similar, or even fewer numbers than in the controls so that the proportion of cells with secretory granules (i.e. dense and lucent) was always less than in the controls and about half that found in the IFN treated animals.

But for level 5, the proportion of ciliated and basal cells was similar to the controls. At level 5 and for no apparent reason, one animal showed a great number of basal cells - 15 per 1.8 mm epithelium - which pushed up the group mean for this cell type to some 6% of cells, but this was not significantly higher than the controls because of the larger standard error involved. There was no consistent change in the number of migratory cells.

Electron Microscopy

At all three airway levels the epithelium of the animals given PCP was similar, in ultrastructure, to the corresponding levels of their 12 day controls, with the exception that nonciliated cells without granules were much more frequently found (Fig. V-20). In contrast with the controls, more of the cells found in the PCP animals lacked a brush border (Fig. V-21). Nonciliated cells with dense granules often appeared in the process of discharging their secretion, suggesting that many of those without granules were the result of granule depletion. Small surface indentations were found indicating merocrine secretion (Fig. V-22) or cells with only a few large granules and concave apical surfaces were found suggesting expulsion of larger amounts of secretion (Fig. V-23). At level 3, a cell without cilia but with electron-lucent cytoplasm, long filiform apical processes and with evidence of ciliogenesis was also found to have electron-dense secretory granules (Fig. V-24). This cell was not found in any control and resembled that found in an IPN animal (see page 149). The epithelium of level 5 resembled that of the same level in the 12 day controls. While two animals had more nonciliated cells with lucent granules than normal, when compared to their 12 day controls, this was not significantly different (see page     for a comparison of 6 with 12 day controls). Other cell types were normal in structure.

### SUMMARY OF RESULTS

1. The airway epithelium of rats given injections of either pilocarpine nitrate (PCP) or isoprenaline sulphate (IPN) was studied in two experiments. In the first, the animals were divided into two groups, those given injections of PCP for 12 days and those given IPN for 6 days. In the second experiment, all the animals were given IPN at a higher dose for 12 days. Three airway levels were studied: lower trachea, main extrapulmonary bronchus and distal bronchiolus.
2. The behaviour of rats given either isoprenaline or pilocarpine was altered after their injections, but not that of the controls. Lacrimal and salivary flow increased after PCP and the animals had diarrhoea. After IPN the animals were unable to stand, drank excessively, had increased respiratory rates and several died.
3. In both experiments A and B, male rats failed to gain weight after injections of IPN but this was not found in the female rats of experiment B. The PCP animals continued to gain weight and were similar to their controls.
4. In experiment A the "12 day controls" had more nonciliated cells with lucent granules than did the "6 day controls".

5. At all airway levels, IPN caused a consistent rise in the proportion of nonciliated cells with lucent granules: the proportions of those with dense granules and without granules fluctuated, and there was no change in the proportion of ciliated or basal cells.

6. The distal bronchiolus of the IPN animal was the most severely affected airway level: the epithelium was thicker than normal and included more nonciliated cells filled with electron-lucent granules, these usually being of a typical goblet shape.

Secretory granules were larger and more numerous than in the controls and transitional stages between Clara and goblet cells were found.

7. After IPN, mitoses were numerous and found in both nonciliated cells with dense or lucent granules. A cell with evidence of ciliogenesis and also secretory granules was found, as was a brush cell with secretory granules.

8. The airway epithelium of the PCP animals resembled that of the controls and nonciliated cells with lucent granules were not increased in number. At all airway levels, the proportion of nonciliated cells with dense granules was significantly reduced while nonciliated cells without granules increased.

9. By electron microscopy more nonciliated cells without granules than is normal, were found: there was some evidence of cell discharge by merocrine secretion.

10. As with the IPN group an example of a cell in ciliogenesis and also with secretory granules, was found.

## CHAPTER VI

### DISCUSSION

### The Nonciliated Secretory Cell

In this present study, the first of its kind concerned with the respiratory tract of specific pathogen free (SPF) rats and with a comparison of extra with intrapulmonary airways, one of the most interesting new features is the frequent occurrence at all levels of airway of more than one morphological type of secretory cell. Secretory cells may be divided into those with electron dense cytoplasm and those with a more lucent cytoplasm.

DENSE CYTOPLASM - In the rat three types of cell with dense cytoplasm have been identified:

Type 1 - In the normal SPF rat, this type of nonciliated cell, a cell filled with electron-lucent granules and of a typical goblet shape, was rarely found but after irritation was frequently seen (up to <sup>18%</sup>~~9%~~ of cells). Rhodin and Dalhamn (1956) and Dalhamn (1956) described this cell as the most frequent nonciliated cell type usually 1 to every 4 ciliated cells. In the report by Dalhamn in 1956, bacteria were found between the cilia and it is likely that this was an "irritating" factor leading to goblet cell hyperplasia.

Type 2 - After glutaraldehyde fixation the most frequently found nonciliated cell was the type with electron-dense secretory granules.

After fixation by osmium alone few of these granules stained and in this respect these granules differ from those osmiophilic granules, presumably lysosomes, described in nonciliated cells by Rhodin and Dalhamn in 1956 and Rhodin in 1959. Those granules found in the following cell types, although dense after glutaraldehyde fixation, are also osmiophilic and thus differ from the granules found in the type 2 nonciliated cell: exocrine cells of the pancreas, surface epithelial cells of the fundic stomach, those of the uterine gland (Rhodin, 1963), and Paneth cells of the intestine (Halley, 1958; Rhodin, 1963). The serous cell granules of the tracheo-bronchial submucosal gland are not osmiophilic and thus resemble those found here (Meyrick and Reid, 1970).

Type 3 - Nonciliated cells (other than brush cells) without secretory granules and occasionally with lysosomes were described by Rhodin and Dalhamn (1956), Dalhamn (1956) and Rhodin (1957) and considered by them to represent a cell in an early stage of mucus synthesis. In the present study these cells were very rarely found *by electron microscopy*.

All the above three nonciliated cell types with dense cytoplasm with or without secretory granules, have in common with the basal cell, electron-dense cytoplasm, little or no smooth endoplasmic reticulum (SER), an irregular nucleus showing peripheral accumulation of chromatin, suggesting that they are all related.

After staining of 1  $\mu$ m epoxy sections with toluidine blue, the commonest cell has granules with strong affinity for stain. These probably represent the type 2 nonciliated cell. The most frequent nonciliated cell type in paraffin sections, stained by the AB/PAS technique, contained PAS positive secretory granules. (in rat). Thus although the evidence is circumstantial, it seems that the magenta staining granule, seen by light microscopy, represents the electron-dense granule seen by electron microscopy. Indeed such a correlation has already been made for the "two-toned" granules of the mouse Paneth cell (Selzman and Liebelt, 1962; Staley and Trier, 1965; Moe, 1968). With the electron microscope and in the mouse, granules of these latter cells were seen to have an electron-dense core and an outer less dense 'halo'. By cutting thinner than usual (2  $\mu$ m) paraffin sections and staining with AB or PAS or both in combination (AB/PAS), these workers showed that the outer 'pale' halo contained acid mucopolysaccharide while the inner dense core contained protein. All of the granule stained with PAS alone, but only the halo staining with AB and after AB/PAS the core stained magenta, the halo bluish-purple. Hally's (1958) earlier suggestion that this represented a fixation artifact is now not accepted (Staley and Trier, 1965).

Lucent cytoplasm - By electron microscopy and in the distal airway, the most frequently found nonciliated cell type had cytoplasm nearly as electron-lucent as the adjacent ciliated cell. This cell type was

not, however, exclusive to the distal airway being frequently found in the adult rat as proximally as the hilum and in the young 19 day old rat as far proximally as the trachea (unpublished results).

The characteristic abundance of SER found in this cell type in the rat, is also found in the nonciliated Clara cell (Karrer, 1956; Niden and Yamada, 1966) the tracheal nonciliated cell (Hansell and Moretti, 1969) and in the nonciliated cells of the nasal mucosa of the mouse (Matulionis and Parks, 1973). A survey of different types of cell found in the body has shown that only one other group of cells - the steroid producing cells - has this great abundance of SER (Enders and Lyons, 1964; Volk and Scarpelli, 1964; Christensen, 1965).

In particular the work of Christensen (1965) has shown this organelle to be most abundant in the testicular interstitial cells of the guinea pig, where it is thought to be the site of two enzymes, 17 hydroxylase and 17 desmolase, involved in the biosynthesis of androgens from cholesterol. The SER of this organ is very similar to that seen in the Clara cells of the rat after glutaraldehyde fixation and, in common with the latter, shows the same formation of dilated vesicles after osmium fixation alone. It is difficult to see what role steroid biosynthesis might play in the respiratory tract but cholesterol being a lipid might be a link, as lipids are thought to be a major component of surfactant, a surface tension reducing agent, possibly produced by

Clara cells (Niden and Yamada, 1966). These latter workers favour the role of SER in lipid metabolism and there is evidence for the cellular microsomal fraction, which includes membranes of SER, in lipid metabolism (Kennedy, 1961; Senior and Isselbacher, 1962).

The nature of Clara cell granules is not yet established. That some are PAS positive has been shown by some workers (Azzopardi and Thurlbeck, 1969; Cutz and Conen, 1971) and denied by others (Niden, 1967). In the rabbit, Cutz and Conen (1971), showed that the granules were digested by pepsin and suggested they were proteinaceous while in the mouse, Niden and Yamada, (1966) demonstrated the presence of bound lipids and phosphoglycerides within the granules. For those found in the rat Clara cells, Askin and Kuhn (1971) showed that they were not affected by chloroform/methanol extraction, as were the lamellated bodies of the type II alveolar cell, suggesting that the Clara cell granules were not composed of dipalmitol lecithin. Their lack of osmiophilia suggests that they are not of an unsaturated lipid.

Finally, the "special type cell" found in dog by Frasca et al (1968) and more recently in man (Miani, Pizzini and DeGasperis, 1971) has not been found in this study (see page 13). The short profiles of SER found in Clara cells do, however, bear some resemblance to the

'disc shaped granules' described by these workers and it may well be that these two cell types are related, the "special type cell" of the dog performing a similar function to the Clara cells of other species.

### The Response of Bronchial Epithelium to Irritation and

#### IPN Administration

At the dose levels used in this study, both the effects of tobacco smoke and IPN were in some ways similar, both for example increasing the numbers of nonciliated cells with electron lucent secretory granules; the former by direct irritation of the mucosa, the effect less dramatic more distally and the latter by a humoral route affecting the entire respiratory tract.

The significance of the shift from electron-dense to lucent secretion is not clear. Jones et al (1973) have shown an increase after tobacco smoke, in cells staining for acid glycoprotein (mainly sialidase resistant sialomucin). These cells are probably the counterpart of those with electron-lucent secretion (see page 160). The appearance of these cells certainly is a sensitive indicator of epithelial irritation.

Irritation by tobacco smoke also caused a cell hypertrophy, an increase in epithelial thickness, an increase of cells in division

(especially basal cells) and of cell concentration. The increase in epithelial thickness after tobacco smoke is not surprising as this is a common inflammatory response (see Haggard, 1924).

But this was not due to cell oedema (a dilution of cell organelles) since there was an increase to maintain, or even to increase, the proportion of cell volume normally occupied by organelles: - true cell hypertrophy.

Tobacco smoke alone and administration of IPN caused an increase in the number of cells in division, the former especially affecting the basal cells of the large airways and the latter causing differentiated goblet and nonciliated cells to divide. This study has clearly shown that the large airways of levels 1-3 of the rat, although psuedostratified, have two cell layers, a basal layer of polygonal or elongated cells and a more superficial layer of vertically orientated cells. The basal layer has been likened in structure to the germinal layer of the epidermis and like that epidermal layer, it has also been suggested to perform a germinal role, giving rise to the more superficial layer of maturing and differentiating goblet and ciliated cells (Rhodin, 1966; Blenkinsopp, 1967). In this way, the two compartment structure of the large airway may be compared with the dividing and maturation compartments of the intestinal epithelium and haemopoietic tissue of the body (Bolduc and Reid, 1973).

In the intestine the dividing compartment is at the base of the crypt and maturation occurs during migration to the villus tip while in myeloid tissue the dividing or mesenchymal compartment, is in the spleen and marrow of certain bones, with differentiation, in the marrow sinusoids and blood stream.

It is known that, in general, differentiation is associated with some loss of reproductive capacity, the prime example being the erythrocyte which loses its nucleus. Basically, three categories of cell are distinguished: 1. highly differentiated cells with no reproductive capacity and no provision for replacement as they are lost slowly (e.g. nerve cells); 2. specialised cells which are lost at a rapid rate (e.g. gut epithelium and skin) and replaced by cells of the same family type but which have not differentiated to the point where they have lost the ability to reproduce (i.e. stem or germinal cells) and 3. exceptions to the rule - specialised but still capable, under certain circumstances, of reproduction and found in cells with a long life span (e.g. liver and exocrine pancreas and many glands making hormones) (Ham and Leeson, 1957).

The features of bronchial epithelial cells would seem to place them, depending on their site, in categories 2 and 3. Those cells of the largest airways bear the brunt of irritation, have a relatively

short life span when compared to the distal airways (40 and 220 days respectively - Bolduc and Reid, 1973) and possess a germinal layer of undifferentiated basal cells, fulfilling the requirements of category 2 cells above. Those cells of distal airways are less exposed to irritation, have a longer life span and constitute a single compartment with both maturation and reproductive features - i.e. category 3 cells. In the latter case, IPN has been shown to stimulate both cell division and differentiation into mucus-secreting cells. The effect of IPN on DNA synthesis has already been mentioned (see page 45).

In the case of cigarette smoke, the stem cells of the large airways have been stimulated to divide either by direct stimulation by smoke products, or by axonal reflex via the intraepithelial nerves which are known to be particularly associated with this basal cell compartment (Jeffery and Reid, 1973). Indeed there is other evidence from the regenerating epidermis of salamander limbs to show the role of nerves in cell division and differentiation (Singer, 1952; Hay, 1960).

The absolute increase in cell concentration after tobacco smoke (not IPN) would indicate that cell replacement was somewhat faster than cell loss. Normally replacement and loss are in equilibrium. Goss (1960) has suggested that an increased functional

demand affects a tissue in broadly two ways: the tissue may respond by hyperplasia of its functional units with unlimited growth potential (e.g. liver) or it must rely on hypertrophy of its units (to this there is a physiological limit) because it has lost the capability of hyperplasia (e.g. heart, brain, kidney). In the case of airway epithelium, the functional demand has been the clearance of increased amounts of mucus (with entangled and dissolved tobacco products) produced by the increased numbers of goblet cells and the problem seems to have been resolved by a combination of hypertrophy of its functional units, perhaps to their limit, as well as <sup>by</sup> ~~of~~ their hyperplasia.

An increase in goblet cell size and in its secretory mass was seen both after irritation by smoke and after IPN administration. In the case of goblet cells of the intestine, it has been suggested that synthesis and discharge are continuous and that the resulting mucous mass present is the result of a balance between the two processes (see Moe, 1968). If this is so for the respiratory goblet cell, the increase could be due either to an increase in the rate of synthesis, a decrease in the rate of discharge or both.

The increase due to IPN is probably, in part, due to increased rate of synthesis, an effect it has been shown to have on protein synthesis acting via the intracellular messenger cyclic AMP (Barka, 1968).

The increase due to cigarette smoke was shown to be accompanied by an increase in granule number and size, the latter more likely as the result of granule retention due to an alteration in the cell membrane or poisoning, by tobacco products, of the active transport system involved in secretion. Such an imbalanced situation could lead to cell distension and eventual interruption of the apical membrane with discharge of content in an apocrine manner rather than the merocrine secretion normally seen.

PMO partially protected against the increase in mitoses, cell hypertrophy, and the increase in epithelial thickness seen after tobacco smoke alone. Fewer cells with lucent secretion and fewer secretory cells were found than after tobacco smoke alone: the mechanism of this protection is not clear. PMO may have acted in two ways: firstly by altering the tissue sensitivity to irritation (peroral administration of PMO has been shown to protect against tobacco smoke-induced ciliostasis - see Dalhamn, 1966) and secondly by its anticiliostatic effect (Dalhamn, 1969), keeping the overlying mucus with dissolved tobacco products moving and thereby lessening the exposure of the epithelium to the irritative effects of these tobacco products. The increase in ciliated cells after tobacco smoke, but particularly after tobacco smoke and PMO, would give some support to the latter suggestion.

### Origin of the Bronchial Goblet Cell

The nonciliated cell which increased most after irritation by smoke or after IPN administration was the type with electron-lucent secretory granules. This study shows that almost any cell type - e.g. ciliated or brush cell - is capable, under certain circumstances, of developing intracellular secretion and has also revealed the most usual route. This is slightly different in large than in small airways because of the absence of a dividing basal cell compartment in the latter.

Large Airways - Because of the similarity in cytoplasmic density and type of nucleus, the basal cell and nonciliated cell of the bronchial epithelium can be considered related. The latter are thought to arise from basal cells by their vertical polarization and the development of secretory elements: endoplasmic reticulum, and <sup>a apparatus</sup> Golgi (Miller, 1932; Rhodin and Dalhamn, 1956; Blenkinsopp, 1967; Policard and Galy, 1970). It is presumed that in the absence of irritation the overall structure of bronchial epithelium alters little since nonciliated cells with dense granules are continuously secreting in a merocrine way, with synthesis balancing discharge.

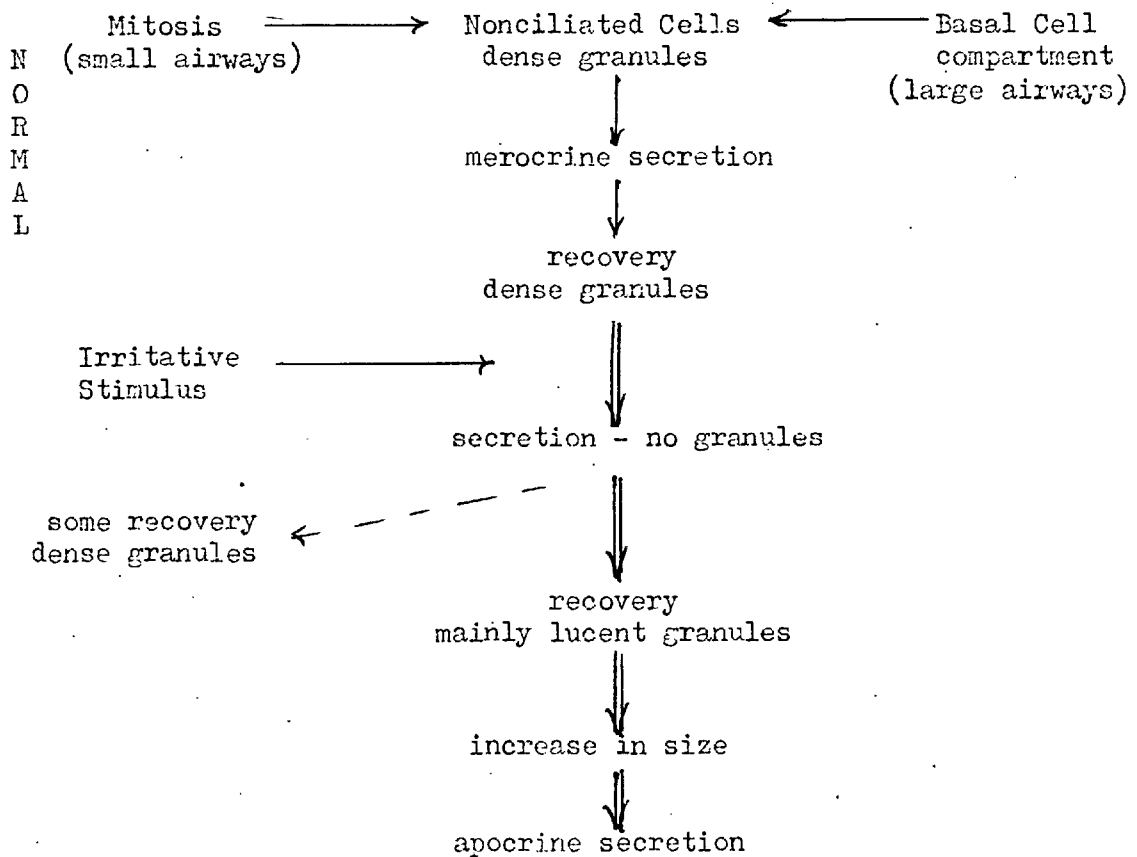
After tobacco smoke the first and immediate response is a sharp drop in the number of nonciliated cells with dense granules and a corresponding rise in those without granules. In the absence of

ulceration it must be presumed that this is due to discharge of secretion in excess of synthesis. Indeed there is evidence that these cells evacuate their contents (see page 154). By the second day of exposure, there is some recovery but not to normal levels and at the same time, nonciliated cells with lucent granules begin to appear (see page 104). Transition stages between these two cell types are normally present and it would seem that the synthesis of new secretion after discharge, following such a stimulus, is accompanied by a shift in the type of glycoprotein produced, fewer cells with dense granules (neutral glycoprotein) and more with lucent granules (acid glycoprotein) being produced. Transitional stages of dense to lucent granules have also been seen (see page 116). Evidence that both types of cell secrete is given by the fall in numbers of both. At the same time there is a corresponding rise in those without granules at day 3 of exposure. It is presumed that the next accumulation of secretion is accompanied by an even greater shift to cells with lucent granules, which by the second week of exposure, are present in relatively great numbers with almost complete suppression of nonciliated cells with dense granules.

The newly formed nonciliated cell with lucent granules then gradually increases in size, presumably due to increased synthesis

and decreased discharge to form goblet cells which are clearly visible by light microscopy.

At the same time, basal cells are feeding the system producing nonciliated cells with dense granules, making good any cell loss. A suggested series of events may be summarized in diagrammatic form below:



In this study, no evidence of Kultschitsky cells giving rise to goblet cells has been found (see Terzakis et al, 1972) and in view of the sparcity of this cell type it is felt that it would be an unlikely source of goblet cells.

Distal Airways - After isoprenaline goblet cells with lucent secretion developed in distal airways where they are normally absent. By light microscopy it was apparent that this increase was accompanied by a decrease in cells with dense granules and in those without granules. With the electron microscope the most characteristic feature of the cells normally present was the abundance of SER. Also in the apical region were small numbers of electron-dense granules. After IPN some of these cells had electron-lucent granules, particularly in the Golgi region and stages from few to many such granules were seen accompanied by a loss of SER, an increase in RER and in the electron-density of the cytoplasm, the last presumably due to an increased number of ribosomes.

That such Clara cells may produce mucoid secretions has received recent support. The nonciliated cell with SER which Hansell and Moretti (1969) described in mouse trachea was shown to have a PAS positive secretion. Similar cells have also been described in mouse nasal mucosa by Matulionis and Parks (1973) and were regarded by them as cells in an early stage of mucus secretion. In view of the absence of a basal cell compartment, it is suggested that the nonciliated cell with SER (Clara cells) form the most likely source of goblet cells seen at this distal site after isoprenaline and that in this situation both Clara and goblet cells maintain their numbers by cell division.

### The Effect of Pilocarpine on Secretion

After PCP and at all airway levels, there was an increase in nonciliated cells without granules and a decrease in cells with secretory granules. There was also evidence of cell discharge. These findings are in agreement with the effects of PCP on other secretory tissues but, although parasympathomimetic, whether it has the same effect as would be obtained by intense parasympathetic stimulation of the respiratory mucosa, is not clear.

Florey (1962) has shown that infusion of acetylcholine causes emptying of the mucous neck glands and to some extent of the goblet cells of the surface epithelium of the stomach. However, electrical stimulation of the vagi did not affect the surface goblet cells. In the colon PCP was found to empty the surface goblet cells and in this case faradic stimulation of the ends of the nerves supplying the colon caused considerable amounts of viscid mucus to be produced and histologically goblet cells were seen to be evacuated. Florey, (1962), believed this might have a bearing on the disease "mucous colitis" where there is no inflammatory lesion yet great amounts of mucus. By electron microscopy of the human small intestine (Trier, 1964) has shown that Paneth cells, and undifferentiated cells with dense granules but not distended goblet cells, are stimulated

to secrete after a single injection of either 5, 10 or 14 mg PCP.

The early light microscopic studies of cat respiratory epithelium by Florey et al (1932) failed to show goblet cell discharge following PCP and they concluded that these cells were not under parasympathetic control. The results of the study reported here are not in disagreement with Florey's findings since the cells affected in the rat by PCP and seen by electron microscopy would not have been easily visualised by light microscopy. That some of them have a nerve supply cannot be doubted after recent electron microscopic studies of the rat bronchial epithelium (Jeffery and Reid, 1973). In a recent light microscopic study, an increase of bronchial goblet cells following injections of PCP has recently been reported which is difficult to reconcile with the findings reported here (Sturgess and Reid, 1973).

#### Functional Aspects

The new structural findings reported here have a bearing on the normal function of the epithelium: removal of foreign material entangled in mucus, absorption, immunological protection as well as with the changes in hypersecretory disease, small airway blockage and increased viscosity of mucus.

CILIA - Cilia of the respiratory tract have been shown to beat in a fluid or periciliary layer (Lucas and Douglas, 1934) on which gobs or sheaths of mucus are seen (van As and Wessels, 1972). The mucous flow is regular and it is suggested that the primary function of ciliary metachronism is to convert the discontinuous activity of each cilium into overall continuous activity (Sleigh and Aiello, 1972). Sleigh (in press) assumes that when cilia propel mucus..... "only the tips of the effective stroke cilia penetrate the mucus and 'claw' it forward....." the elastic action of the mucus pulling more of the mucus forward. The finding of a structure not dissimilar to a "claw" in this study is intriguing and would support this particular method of moving mucus.

With irritation, cilia were of normal structure and density but the cells increased in number improving the escalator. This increase is interesting and has <sup>not</sup> been reported previously. ~~in a light microscopic study of human tracheal and bronchial epithelium from smokers and non-smokers. (Ide et al, 1959).~~ Their report ~~along~~ with the light microscopic study of Chang, 1959, and the electron microscopic report of Ailsby and Ghadialy (1973) indicated some ciliary damage, none of which was found in this present study.

Structural integrity does not, however, imply functional normality and there is good evidence for the potent and acute ciliostatic effect of tobacco smoke (Ballenger, 1960; Dalhamn, 1964 and see page 36).

#### Increase in Goblet Cells

An increase in bronchial goblet cells is one of the main features of hypersecretory disease in man (Reid, 1954) and this has been reproduced in this present study by both tobacco smoke and Isoprenaline. An extension of goblet cells to the periphery is also a feature of hypersecretory disease and in this present study was found in the animals treated with IPN, but not in those exposed to tobacco smoke *with this alone*.

Since goblet cells are only about  $\frac{1}{40}$  the volume of submucosal glands (Reid, 1960), the increase in goblet cells in the large airways is of less functional significance than their increase in the smaller airways where submucosal glands are absent. The appearance of goblet cells in the distal airways is, however, of great functional significance (Reid, 1954; Thurlbeck, 1973). The studies of Macklem, Proctor and Hogg (1970) have led them to the conclusion that, as with the alveolus, surfactant lines and

stabilizers the bronchiolus and Niden (1967), Azzopardi and Thurlbeck (1969) and Gil and Weibel (1971) have suggested that the bronchiolar Clara cells, at least in part, might be responsible for this secretion. If this is so, then the situation arises with IPN induced goblet cell increase that there is a decrease in cells producing surfactant, a replacement of these cells by mucus-secreting cells and a replacement of the surfactant lining by one of mucus. Macklem et al (1970) would regard such an airway, with loss of its surfactant, to have poor stability and to be more susceptible to narrowing with closure at high lung volumes.

In a recent study (1971) Karpick, Pratt, Asmundsson and Kilburn have correlated small airway goblet cell metaplasia with deaths due to acute respiratory failure and in the light of the findings after IPN, the excessive use of which is known to be associated with sudden death in asthmatics, it would seem appropriate to look more closely into the drug-induced appearance of goblet cells at this distal site.

#### Nervous control of bronchial epithelium

That nerves are present within the epithelium of rat extrapulmonary airways has been shown in the adult rat in this

present study and in young rats in an earlier study (Jeffery and Reid, 1973). The presence of neurosecretory vesicles implies that some, at least, are motor in function (Wolfe, Axelrod, Potter and Richardson, 1962; Richardson, 1966; Tranzer and Thoenen, 1967; De Robertis, 1967). They have been found adjacent to mucus-secreting, basal and ciliated cells. That they might be involved in the discharge of secretion and in basal cell division and cell differentiation has already been discussed (see pages 166 & 173) but their possible role with ciliated cells has not.

It is now believed that cilia beat spontaneously and that their metachronal co-ordination is hydrodynamic and not dependent on the electrical activity of cells. The regulation of their rate and direction of beat is probably under nervous and humoral control (Sleigh, in press). In this present study and in the previous one on young rats, nerves were found with some ciliated cells, but not with all, which would seem to cast doubt on their role in ciliary regulation. However, if metachronal co-ordination is under hydrodynamic control, as is suggested, then only the occasional cell need be regulated, the altered rate of cilia of that cell influencing the rate of those on neighbouring cells, the change

being smoothly passed over many cell boundaries. The control of ciliary rate would be seen to be most important in the large airways where mucus from goblet and submucosal gland cells is normally produced and where all the secretions of the more distal airways converge (see Kilburn, 1968). It is at this proximal site where intraepithelial nerves are present and numerous.

#### Passage of fluid through bronchial epithelium

Little is known about passage of fluids through bronchial epithelium. Richardson, Hogg, Bouchard, and Hall (in press) have shown that the tight junctions (zonula occludens) between bronchial cells do not normally allow molecules as large as those of horse radish peroxidase to pass through, but there is evidence to suggest that after exposure to tobacco smoke these may become permeable to such molecules (Hogg, personal communication). Sept 1969

With the discovery of the airway brush cell, Rhodin and Dalhamn (1956) suggested that this cell might function in absorption or be a primitive ciliated cell. Luciano, Reale and Ruska (1968) suggested it might be a chemoreceptor. In this present study, the brush cell comprising less than 1% of cells, has been shown to possess large

numbers of pinocytotic vesicles resembling those found in the absorptive cells of the kidney and intestine (Rhodin, 1963) suggesting an absorptive role by micropinocytosis.

The infrequency of these cells would suggest that they alone are not responsible for absorption. The numerous microvilli of ciliated cells, lengthened after cigarette smoke in this study, would suggest that they also might function in absorption and the recent studies of Hogg et al (in press), Erlandsen and Chase (1972) and Yardley and Brown (1973) have shown that goblet cells also may actively ingest particles as well as secrete mucus.

#### Immunological function of bronchial epithelium

The respiratory mucous membrane is a site ideally situated for the initiation of immune responses. In this present ultrastructural study, two cell types, which may be concerned with this, have been shown to be present normally. The globule leucocyte has been previously shown at this site by Kent (1966) while a small cell of the lymphocyte series has not previously been shown by electron microscopy.

The globule leucocyte, originally described by Weill in 1919 (Schollenleukocyten) and present in mucous membranes throughout the body in a wide variety of species (Kent, 1952) has been linked

with an immune function (Dawson, 1927; Dobson, 1966; Whur, 1966; Whur and Gracie, 1967). That their numbers may be controlled by tissue hormone levels has been suggested (Kent et al 1956; Kellas, 1961) and an increase in their number in magnesium deficient rats has been suggested to be due to histamine release by locally situated mast cells (Cantin et al, 1972). Kent (1966) reports, however, that their number decreases in inflammation. The present study gives no evidence either way since their numbers were little altered after either tobacco smoke or drug administration. Their normal distribution does indicate a function limited to the largest airways where much of the particulate matter is first trapped and an immunological role does seem likely. Murray, Miller and Jarrett (1968) believe this cell is involved in the transport of immunoglobulins through the epithelium and into the lumen. Most investigations agree that the cell originates from the lymphocytic series (Kent, 1952; Kellas, 1961; Toner, 1965) although some have suggested an origin from mast cells present in the subepithelial tissues (Murray et al, 1968; and Cantin et al, 1972). In the present study mast cells were seen normally in the subepithelial tissues with an ultrastructure similar to the globule leucocyte but they were never seen to migrate through the epithelial basement membrane. The normal presence of

a small lymphocyte within the epithelium would indicate a ready source of possibly pluripotential cells able to differentiate into immunologically active globule leucocytes.

As early as 1885, Flemming spoke of leucocytes with dark nuclei occurring in the tracheal epithelium "almost in every mammalian trachea" and since then, others have confirmed this observation (Miller, 1932; Andrew and Burns, 1947; v.Hayek, 1960). In the mouse, Andrew and Burns (1947) found them mostly at the base of the tracheal epithelium and believed they remained in this situation for long periods.

In 1966 and by autoradiography, Darlington and Rogers investigated the uptake of  $^3\text{H}$ -thymidine and  $^{35}\text{S}$  sulphate by similar lymphocytic cells found in the small intestinal epithelium of mouse. They comprised 9% of epithelial cells and had a low mitotic index. There was no evidence of migration of these cells through the epithelium and their results, taken with those of Gowans and Knight (1964), suggested that they might be a separate and self-perpetuating population of cells.

It is presumed that the lymphocytes found ultrastructurally in this present study represent those previously described by light microscopy. Their appearance and lack of organelles suggests they

are not actively secreting cells perhaps best being regarded as immunologically competent cells capable of division and of forming immunologically active cells should the appropriate antigen come along. It is tempting to speculate that, in the case of Mycoplasma infection with associated lymphocytic and plasma cell infiltration seen in this study, these cells formed the first line of defence by their division and differentiation into, on the one hand, plasma cells with dilated RER and probably forming antibody, and on the other, immunologically specific or "conditioned" lymphocytes (Yoffey, 1962).

More recently, a role of such lymphocytes (called trephocytes) in the control of growth has also been postulated (Loutit, 1962; Burch, 1968). The evidence, though circumstantial, is becoming more widely accepted. If this is so, the nutrients supplied could be non-specific (e.g. amino-acids) or preformed DNA or nucleoprotein (Loutit, 1962). That such cells are in a resting state and capable of a renewed cycle of development with pluripotential possibilities, is recognised (Yoffey, 1962).

#### Future Study

The present work has established, in detail, the transformation of nonciliated cells into goblet cells and while supporting the role

of basal cell as the precursor of a nonciliated cell, it has not established the structural changes involved in the latter transition. While cell profiles are found which could represent cells intermediate between basal and nonciliated cells, it is difficult to be sure that these are not oblique sections of already differentiated nonciliated cells. Transitional stages between basal and ciliated cells were never seen and it would be of interest to follow the growth and differentiation of basal cells more precisely at the ultrastructural level, using tritiated thymidine to label the dividing basal cell compartment as did Blenkinsopp (1967) in the lung and Merzel and Leblond (1969) in the small intestine using light microscopy.

The administration of the drugs isoprenaline and pilocarpine for periods of up to 12 days has given new information about the chronic effects of high doses of these drugs. It is felt that additional information would be gained by studying the effect of these drugs after a single injection, at doses nearer to physiological levels and also during recovery. The results could be compared with similar, but less detailed ultrastructural studies on salivary glands (see page 44). It would also be of interest to see if other sympathomimetic drugs such as salbutamol have similar effects to those seen after IPN administration. The anti-inflammatory drug, PMO,

has partially protected against the changes seen after tobacco smoke alone and the question is raised as to whether, given intraperitoneally or perorally, it will protect against similar changes caused by other agents such as Isoprenaline or exposure to sulphur dioxide.

It would be of advantage to be able to study the histochemical features of the different secretory cells demonstrated by electron microscopy, as has been done with histological techniques prepared for light microscopy (see page 25). Although, to date, staining of 1  $\mu$ m thick sections of bronchial epithelium by Alcian blue or periodic acid Schiff has proved unsuccessful in this laboratory, it may be that new staining techniques will be found more satisfactory (see Pool, 1973). If sufficiently dense staining of secretory cells by conventional mucin stains can be achieved, then there is no reason why such stain could not be detected at the electron microscopic level (see Glauert, 1965).

The electron microscope has shown differences of structure thought to be associated with neutral and acid glycoprotein, but has not distinguished between the acidic mucins - sialomucin and sulphomucin. Studies of the incorporation of  $^{35}\text{S}$  into surface goblet cells, visualized at the ultrastructural level, would help to elucidate which type of cell contains mainly a sulphomucin and where in the cell this type of mucus is found.

It is hoped that by understanding the structural changes associated with bronchial hypersecretion in an animal model such as the rat, advances may be made in the quest for controlling such changes in man: the use of an anti-inflammatory drug such as PMO is an encouraging first step in this direction.

### GENERAL SUMMARY

The detailed results have been summarised at the end of each chapter. The following points are particularly relevant to the increase in goblet cell number found in hypersecretory diseases such as chronic bronchitis and in an animal model of hypersecretion such as that described here in the specific pathogen free (SPF) rat.

The frequency of the various epithelial cell types has been determined in the normal so that after tobacco smoke and isoprenaline, an increase in goblet cells and ciliated cells also, could be identified. These increases were at the expense of the nonciliated cell with dense granules.

In the normal, various types of nonciliated cell can be distinguished and only few are typical goblet cells. Their secretory granules are so small and sparse that it is unlikely they are identifiable by conventional light microscopy of paraffin sections. In 1.µm epoxy sections, toluidine blue distinguishes two types of secretory granule, one staining intensely and the other only weakly. Affinity of tissue structure for stain correlates well with electron density and it is reasonable to assume that these granules represent respectively the electron-dense and electron-lucent granules seen by electron microscopy. In the

normal SPF rat the cell with predominantly lucent granules is rare.

Tobacco smoke causes an increase in epithelial thickness, cell hypertrophy, increase in cell concentration and a significant increase in nonciliated cells with lucent granules. This alteration occurs without epithelial ulceration and as no new cell type is seen, suggests a shift in the existing cell population. Within the population of nonciliated cells there is a shift from cells with dense granules to those with lucent granules and granules with transitional features are seen.

In the nonciliated cell, tobacco smoke also gives rise to an increase in number and size of secretory granules, a larger part of the cell than normal being occupied by secretion: these cells now look like typical goblet cells. The increase in goblet cell number does not represent so much an increase in secretory cells as in a build-up of their intracellular secretion.

The concentration of other epithelial cells is also increased, in particular ciliated cells, their cilia being normal in size, density and structure.

Isoprenaline sulphate also causes an increase in goblet cells with lucent granules: tobacco smoke causes an increase only in

the large airways, isoprenaline an increase at all levels, particularly in distal bronchioli where these cells are not normally found. In the case of the isoprenaline-induced goblet cell increase, there is no increase in cell concentration, nor epithelial ulceration. At this distal site, goblet cells are seen to develop from Clara cells normally present and numerous.

The metaplasia of the normal nonciliated cell to a goblet cell with lucent secretion is the most sensitive indicator of epithelial irritation. It seems that this change is accompanied by a histochemical shift from a neutral to an acid secretion, but the significance of this to its viscosity is not known.

Finally, an anti-inflammatory agent phenylmethyloxadiazole has protected against many of the changes seen after tobacco smoke and this is an encouraging first step in the control of bronchial epithelial hypersecretion.

## APPENDIX

The following procedures were used in the preparation of fixatives and embedding medium for electron microscopy and glycoprotein stains for light microscopy.

#### Fixative

Both glutaraldehyde and osmium were made up in sodium cacodylate buffer. All stock solutions were kept at 4°C.

#### BUFFER

The buffer was freshly prepared immediately before fixation:

Stock solutions ... (A) 0.1M sodium cacodylate

(B) 0.1N hydrochloric acid

8.3ml of B was made up to 100ml with A giving a buffer of pH 7.2.

#### GLUTARALDEHYDE

The fixative was freshly prepared before fixation.

Stock solution... 25% glutaraldehyde in aqueous solution (Taab Laboratories).

12ml of the stock solution was made up to 100ml with buffer, giving an approximately 3% solution.

OSMIUM

A solution of osmium was made up at regular intervals, kept at 4°C. and used as required: 0.5g osmium tetroxide (Johnson Matthey Chemicals Limited) was dissolved in 50ml of buffer to give a 1% solution.

Both fixatives were used at room temperature.

Embedding Medium

Araldite epoxy resin (CIBA) was used as the embedding medium made up in bulk (2 lbs), poured into smaller bottles and frozen, to be thawed and used as required. The following ingredients were used:

Epoxy resin (CY212) 1 lb.  
Hardener (HY960) 1 lb.  
Accelerator (DY064) 13.5ml.  
Di-n-Butyl Phthalate - approximately 20 ml.

Staining for Glycoprotein

The following schedule was used for the staining of neutral and acid glycoproteins in paraffin-embedded sections.

ALCIAN BLUE STAIN AND P.A.S.Solution required:

- A) 1% Alcian blue in 3% Acetic acid
- B) Periodic acid.....1% aqu.
- C) Schiff reagent.....supplied by G.T. Gurr
- D) Sulphurous Acid rinse:
  - Distilled water.....90 ml.
  - N.HCl..... 5 ml.

10% Potassium Metabisulphate..... 5 ml.

Method:

- 1) Bring sections to distilled water.
- 2) Place in freshly filtered solution of Alcian blue, 30 mins.
- 3) Wash in running tap water (roughly  $\frac{1}{2}$  hour). Treat with 0.3% sodium carbonate for 30 minutes.
- 4) Rinse in running tap water for 20 minutes.
- 5) Place in Periodic acid.....5 minutes.
- 6) Wash in running tap water.....5 minutes.
- 7) Rinse well in distilled water.
- 8) Stain in Schiff reagent.....3 minutes at 18-22°C.
- 9) Transfer directly to 1st Sulphurous acid rinse...2 minutes.
- 10) Transfer directly to 2nd Sulphurous acid rinse...2 minutes.
- 11) Wash well in running water.....5 - 10 minutes.
- 12) Dehydrate clear and mount.

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### ELECTRON MICROGRAPHS

The following are the electron micrographs referred to in Chapters III - V. Photomicrographs, graphs and tables are included in the text, where appropriate, and are not found here.

Unless stated otherwise, all electron micrographs are of tissue fixed in glutaraldehyde and osmium and stained with uranyl acetate and lead citrate.

KEY

The following abbreviations have been used:

|     |                              |     |                                 |
|-----|------------------------------|-----|---------------------------------|
| CC  | Ciliated cell                | fi  | fibrils                         |
| NCC | Nonciliated cell             | co  | collagen                        |
| BaC | Basal cell                   | Bv  | blood vessel                    |
| BRC | Brush Cell                   | nu  | nucleus                         |
| GL  | Globule leucocyte            | Go  | Golgi apparatus                 |
| L   | Lymphocyte                   | ly  | lysosomes                       |
| bm  | epithelial basement membrane | SER | smooth endoplasmic<br>reticulum |
| lu  | airway lumen                 | RER | rough endoplasmic<br>reticulum. |

ILLUSTRATIONS

CHAPTER III

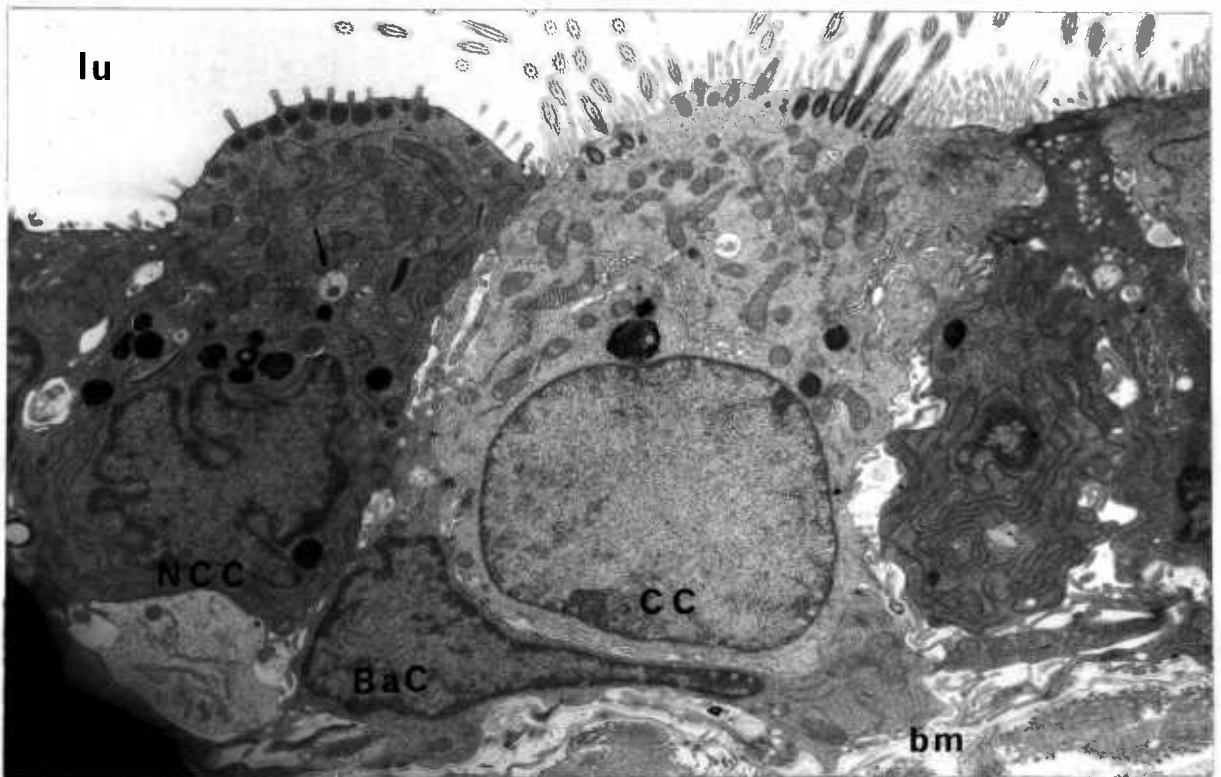


Fig. III-5 illustrates a ciliated cell, a nonciliated cell with dense-secretory granules at its apex, and a basal cell in a normal extrapulmonary bronchus (level 3). The nonciliated cell also has a single electron-lucent granule (arrow) x 7,500.

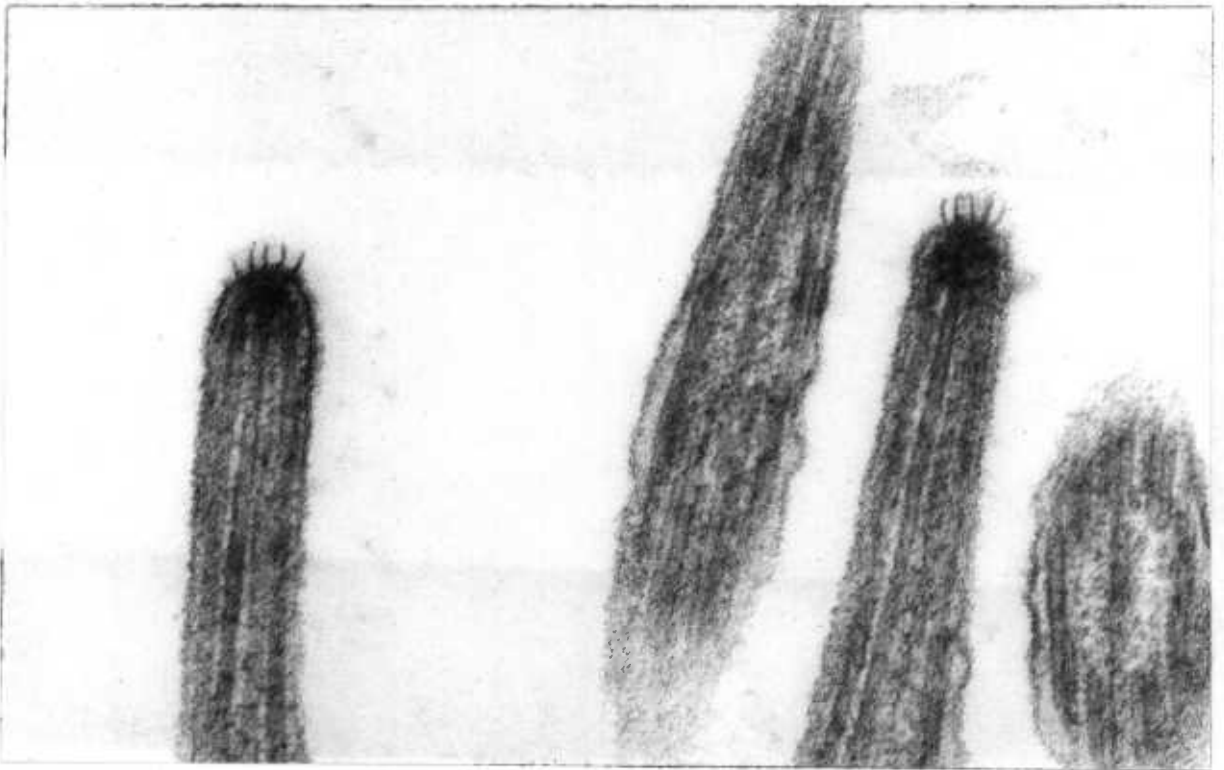


Fig. III-6, shows the tips of two normal tracheal cilia each with small projections. x 120,000.

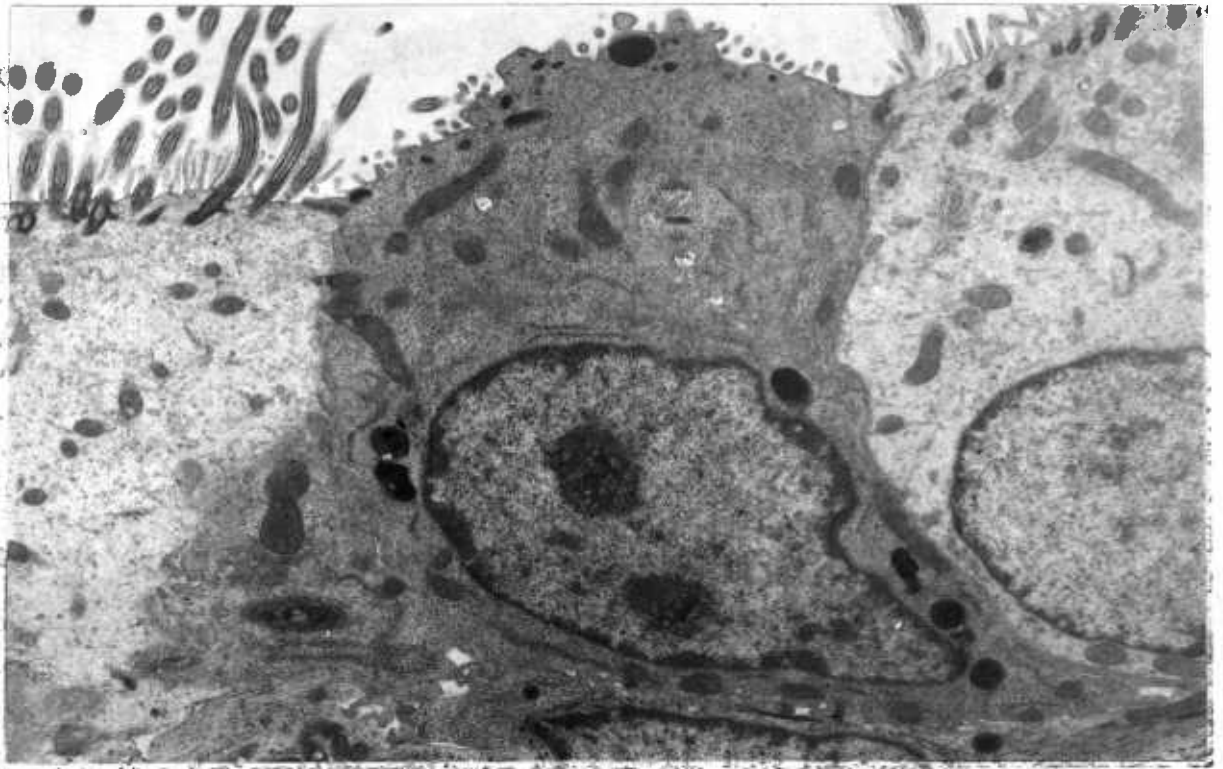


Fig. III-7 illustrates a nonciliated cell at level 4 with electron-dense cytoplasm which strongly contrasts with that of its two neighbouring ciliated cells. x 7,500.

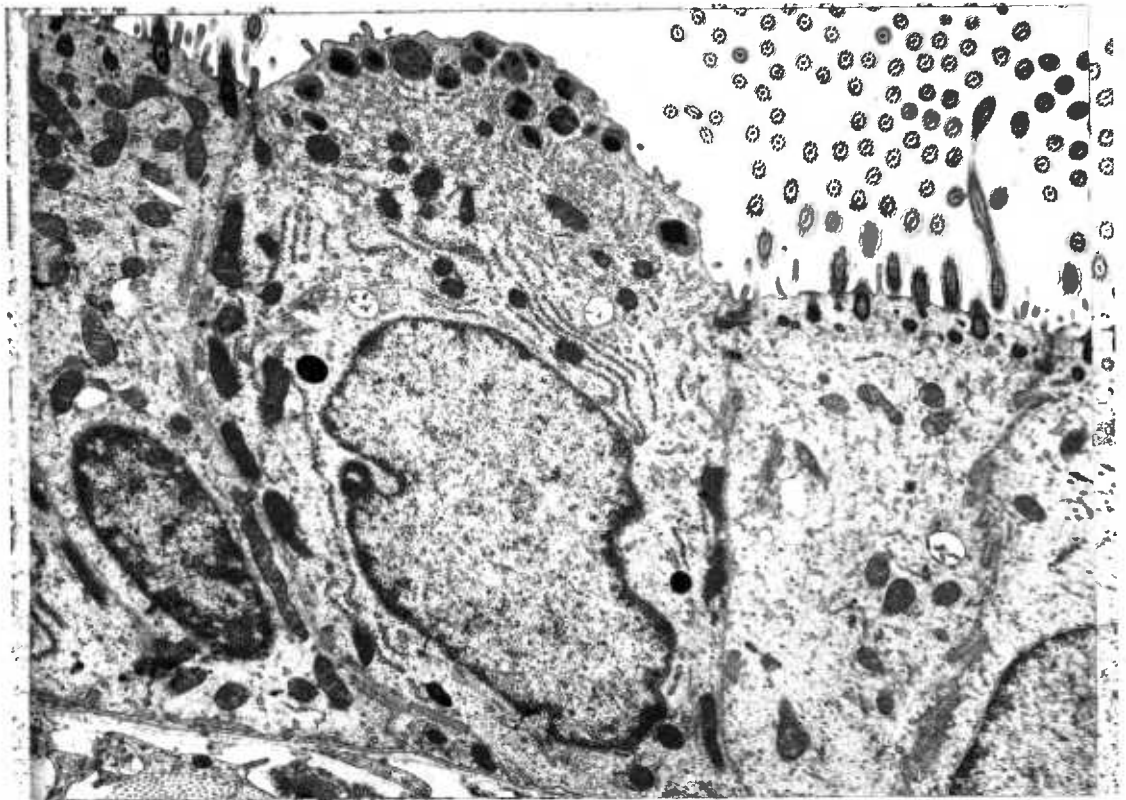


Fig. III-8 illustrates a nonciliated cell at level 4 with electron-lucent cytoplasm of similar density to its neighbouring ciliated cells. Unlike the cell depicted in the previous figure, this cell has an abundance of smooth endoplasmic reticulum. x 7,500.

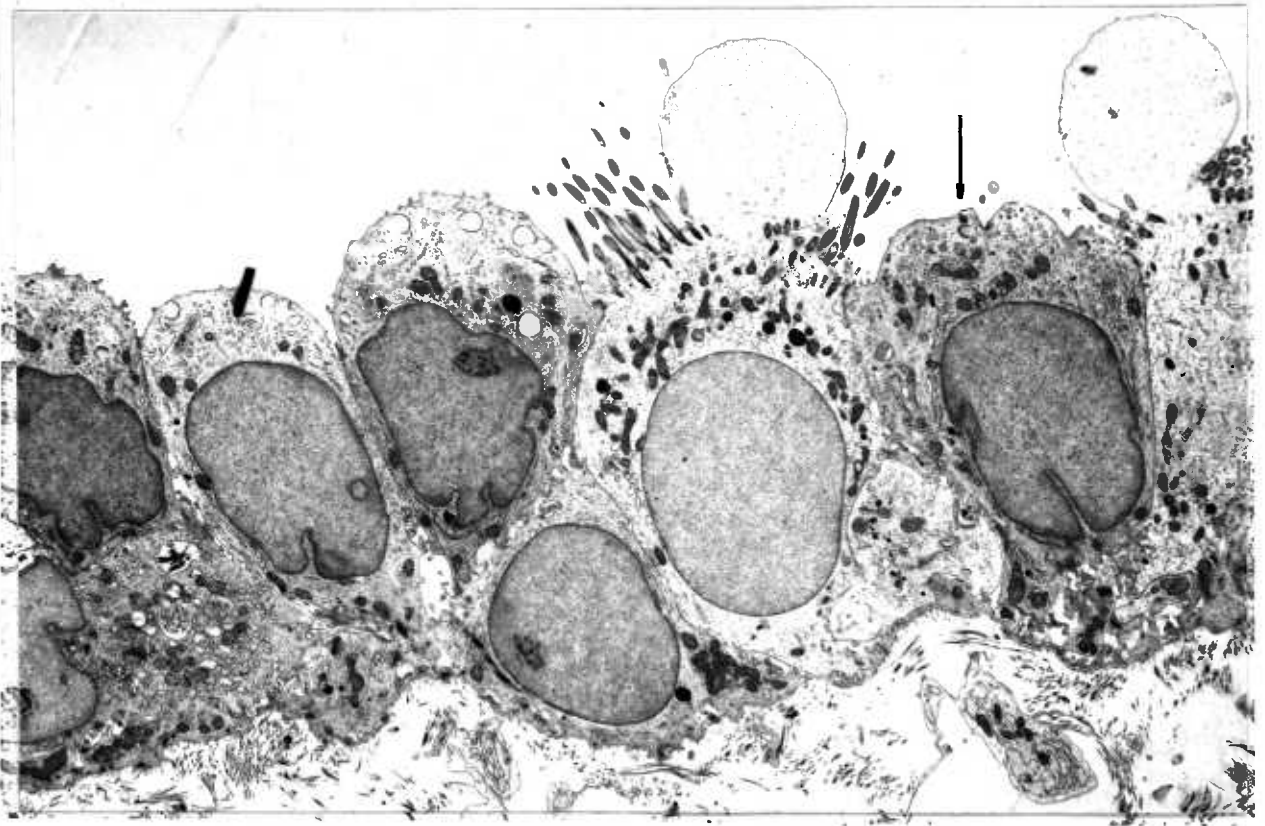


Fig. III-9 shows the appearance of bronchial epithelium (level 3) after fixation in osmium alone. Most nonciliated cell granules are not stained although there is some staining of the peripheral edge: smaller granules are stained (arrow) as are lysosomes. Cytoplasmic blebs from both ciliated and nonciliated cells are frequent. x 4,500.



Fig. III-10 illustrates a nonciliated cell with dense cytoplasm in normal bronchial epithelium (level 3): the cell has large electron-lucent secretory granules which lack a distinct membrane allowing some granules to fuse (arrow) x 10,500.

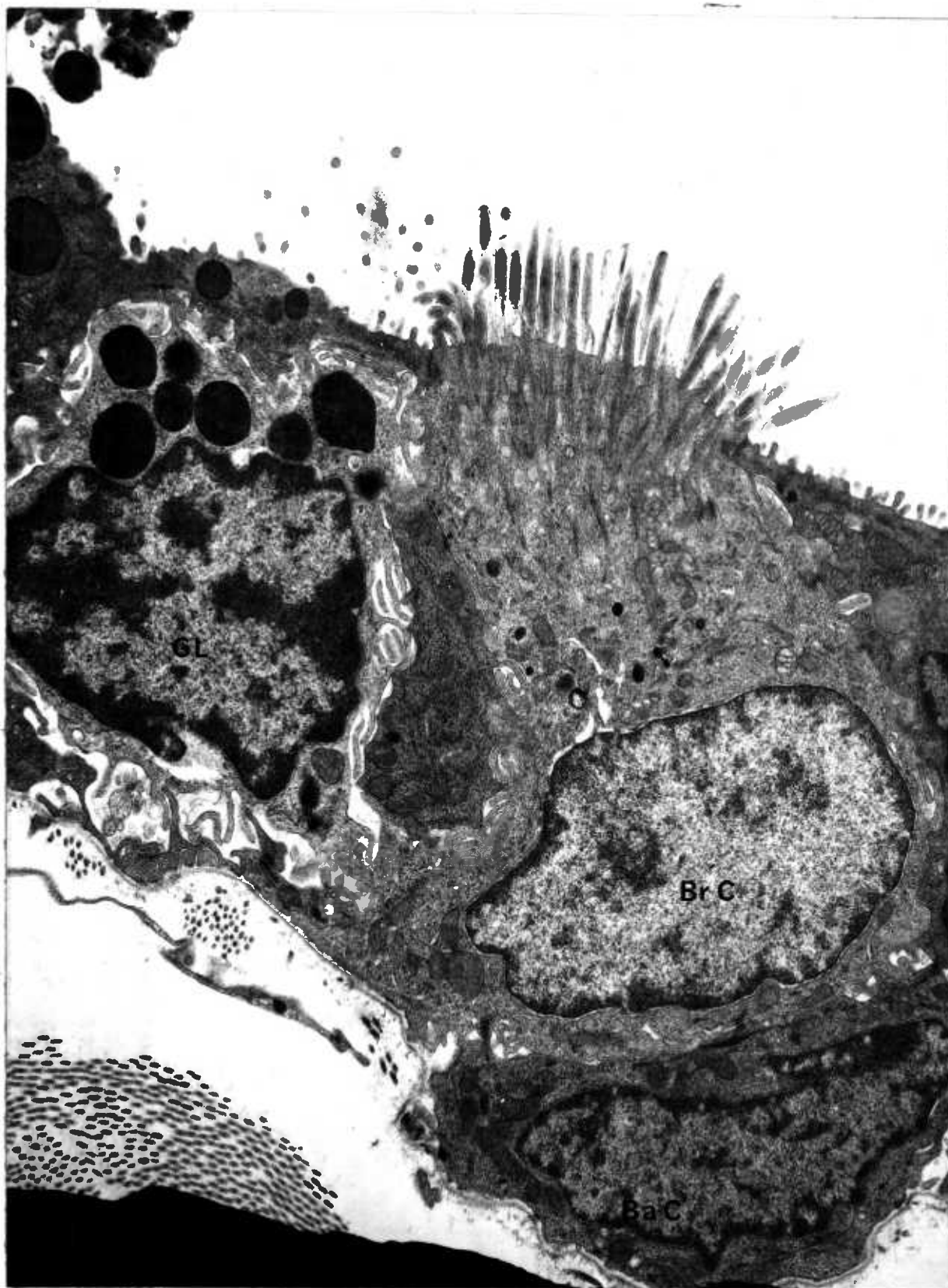


Fig. III-11 illustrates a nonciliated cell (brush cell) without secretory granules and with a brush border of microvilli: the cytoplasm also contains many bundles of fibrils. Beneath lies a basal cell and, to the left, a globule leucocyte with dense globular inclusions and many filiform processes projecting into the intercellular space. x 10,500.

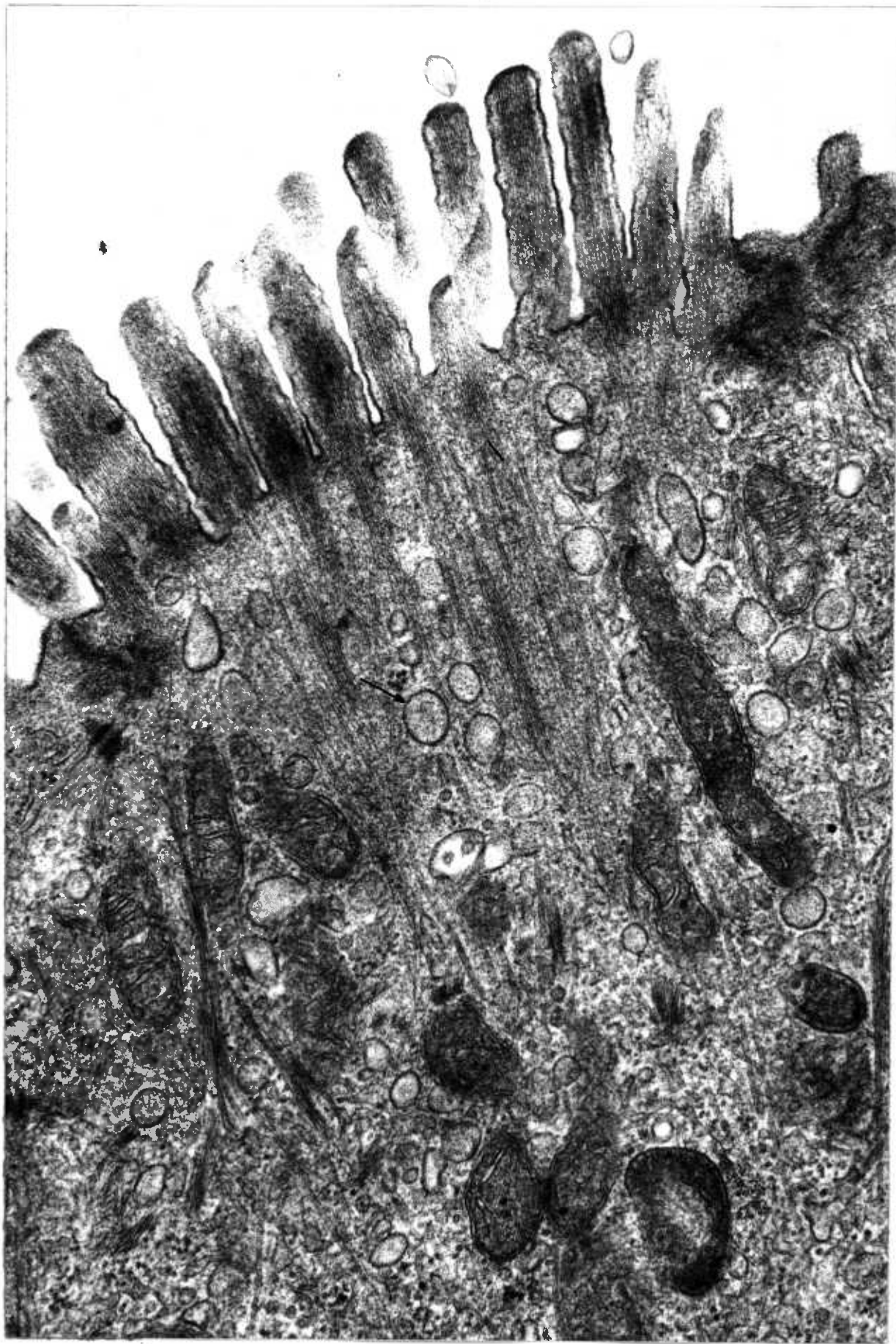


Fig. III-12 shows the apex of a "brush" cell with many pinocytotic vesicles (arrow). Each blunt microvillus has fine tubules which extend into the cell. x 52,500.

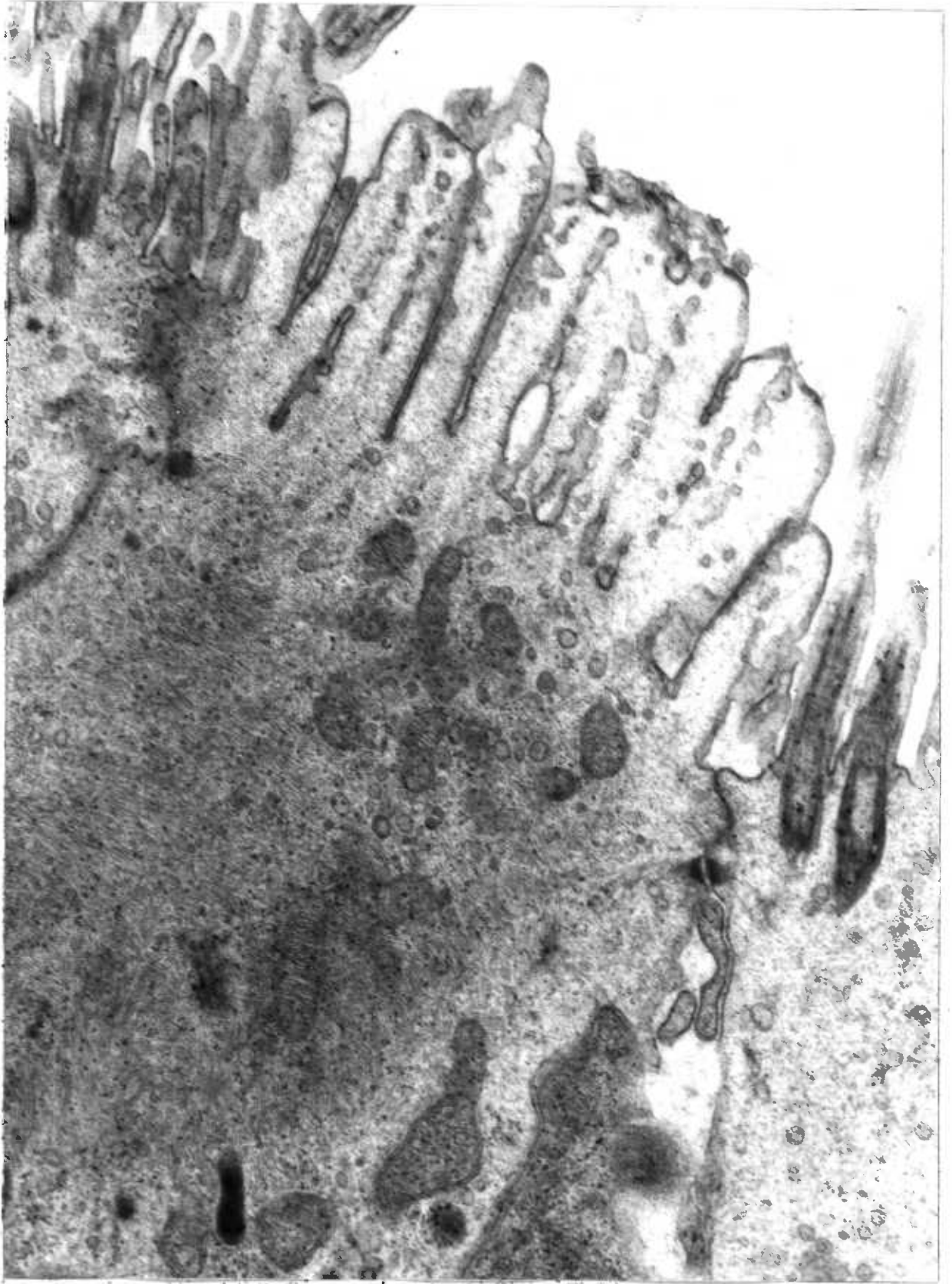


Fig. III-13 illustrates the apex of a brush cell after fixation by osmium alone: the microvilli are distorted, indistinct and lack internal structure. Adjacent cilia are not affected. x 45,000.

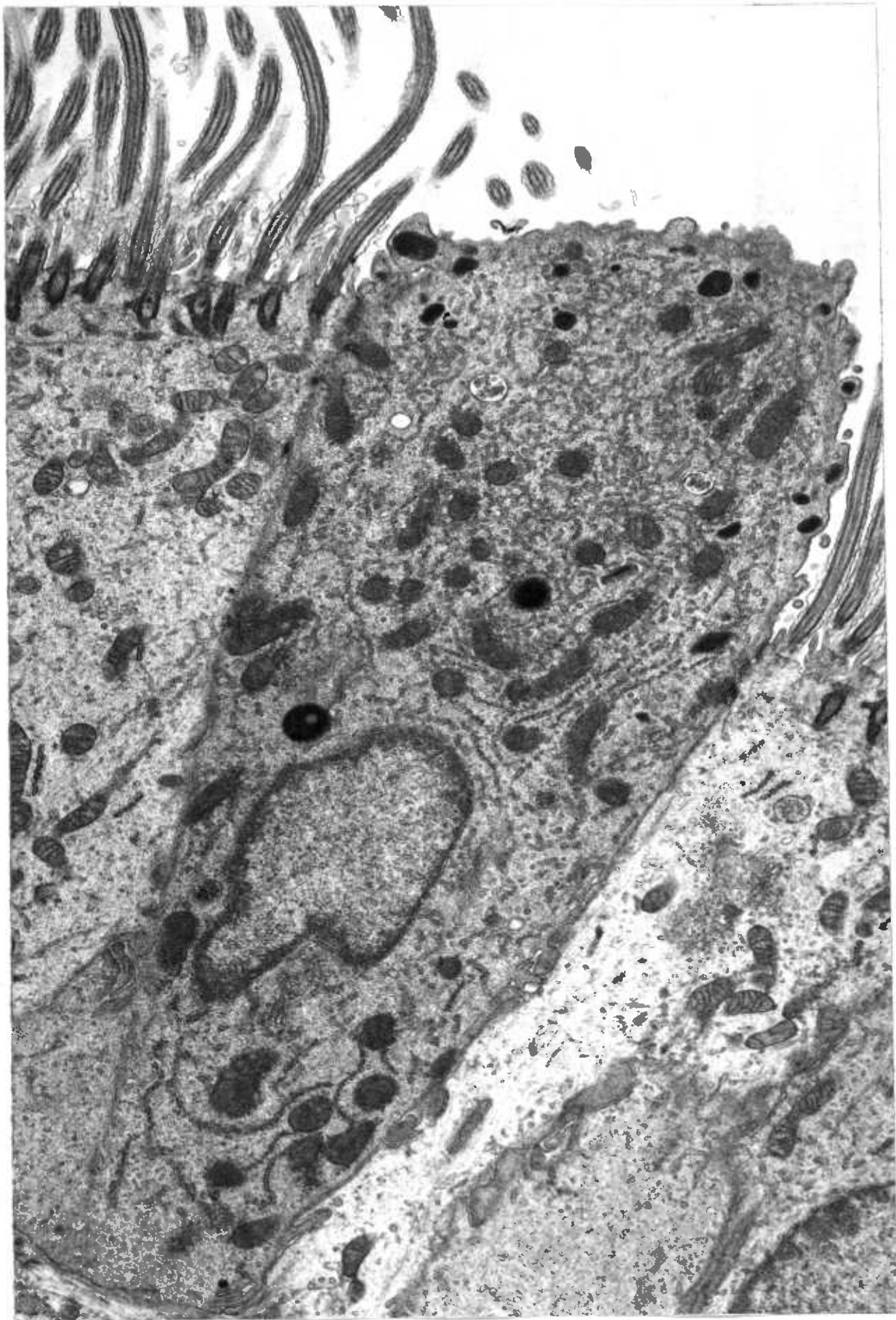


Fig. III-14 illustrates a nonciliated cell at level 5 with cytoplasm of density approaching that seen in the adjacent ciliated cells. The apex of the cell has an abundance of smooth endoplasmic reticulum. x 13,500.

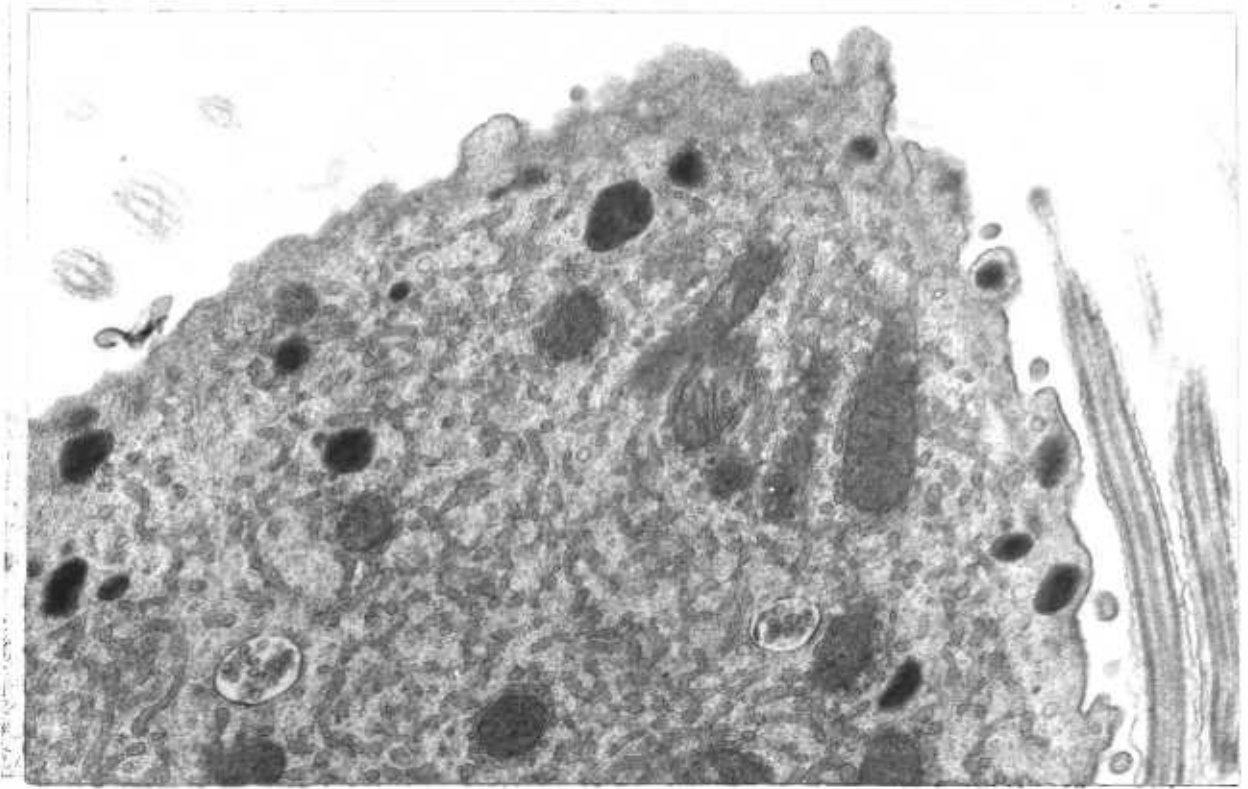


Fig. III-15 illustrates the apex of the nonciliated cell depicted in the previous figure: dense granules and short profiles of smooth endoplasmic reticulum are present and the cell membrane lacks filiform processes. x 22,500.

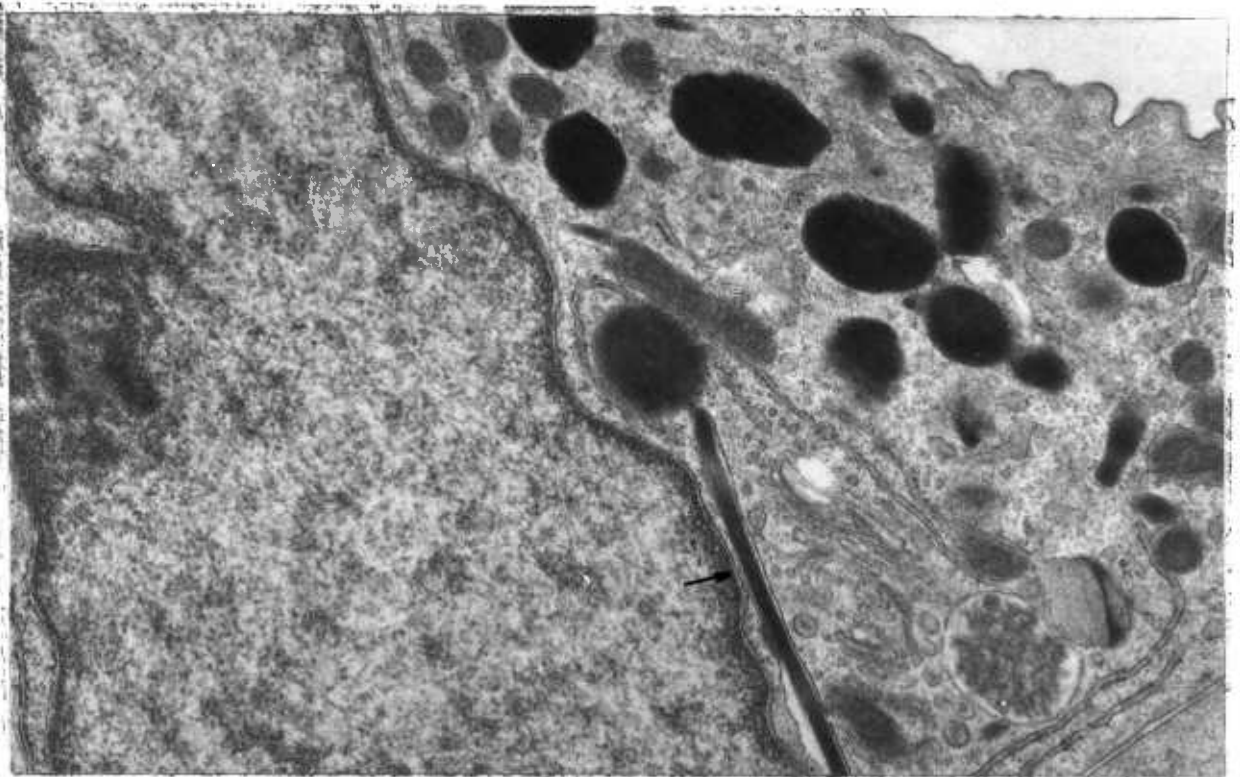


Fig. III-16 depicts a membrane-bound rod (arrow) often found within the nonciliated cell with electron-lucent cytoplasm. x 22,500.



Fig. III-17 illustrates a nonciliated cell at level 5 after fixation by osmium alone: the cell is filled with small vesicles and lacks short profiles of SER. There is also an outer zone of cytoplasm free of organelles (arrow).x 12,000.

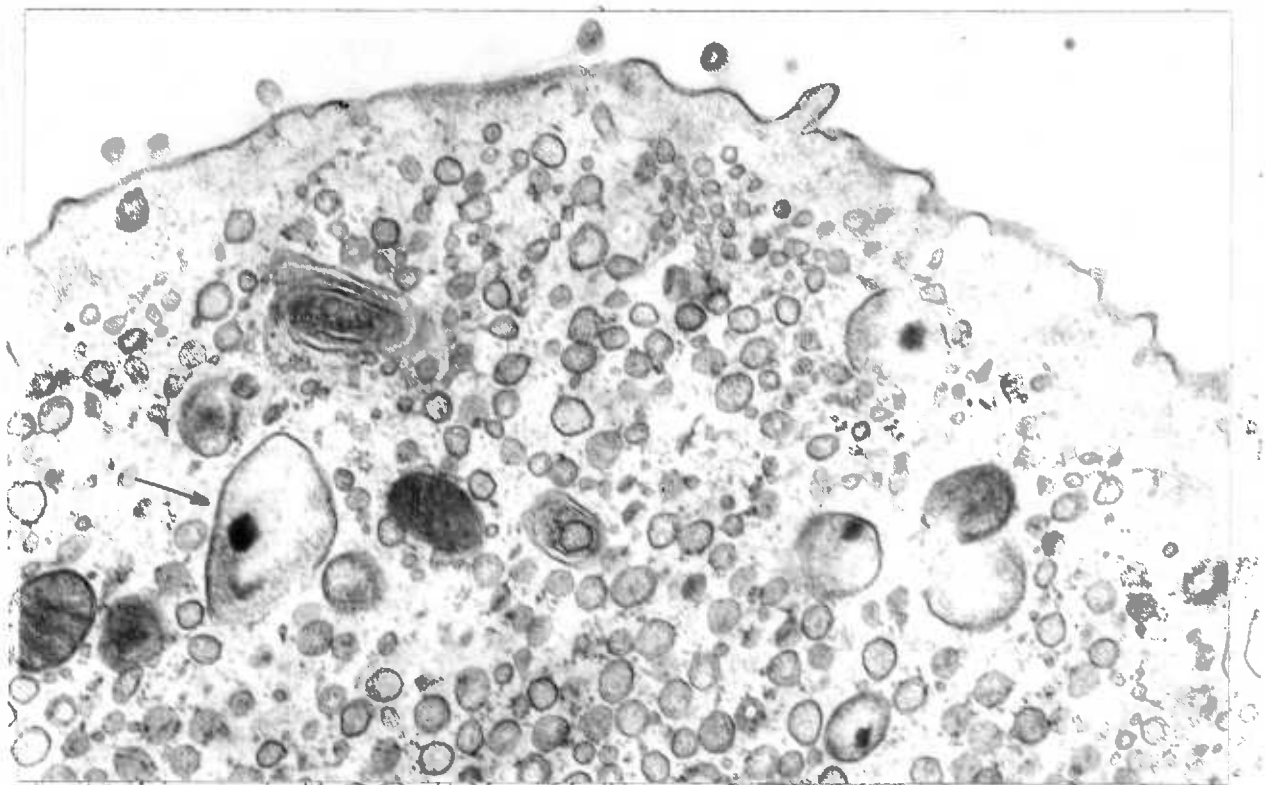


Fig. III-18 shows the apex of a nonciliated cell of level 5 fixed in osmium alone: structures with incomplete membranes and with an electron-dense core are found (arrow). x 9,000.

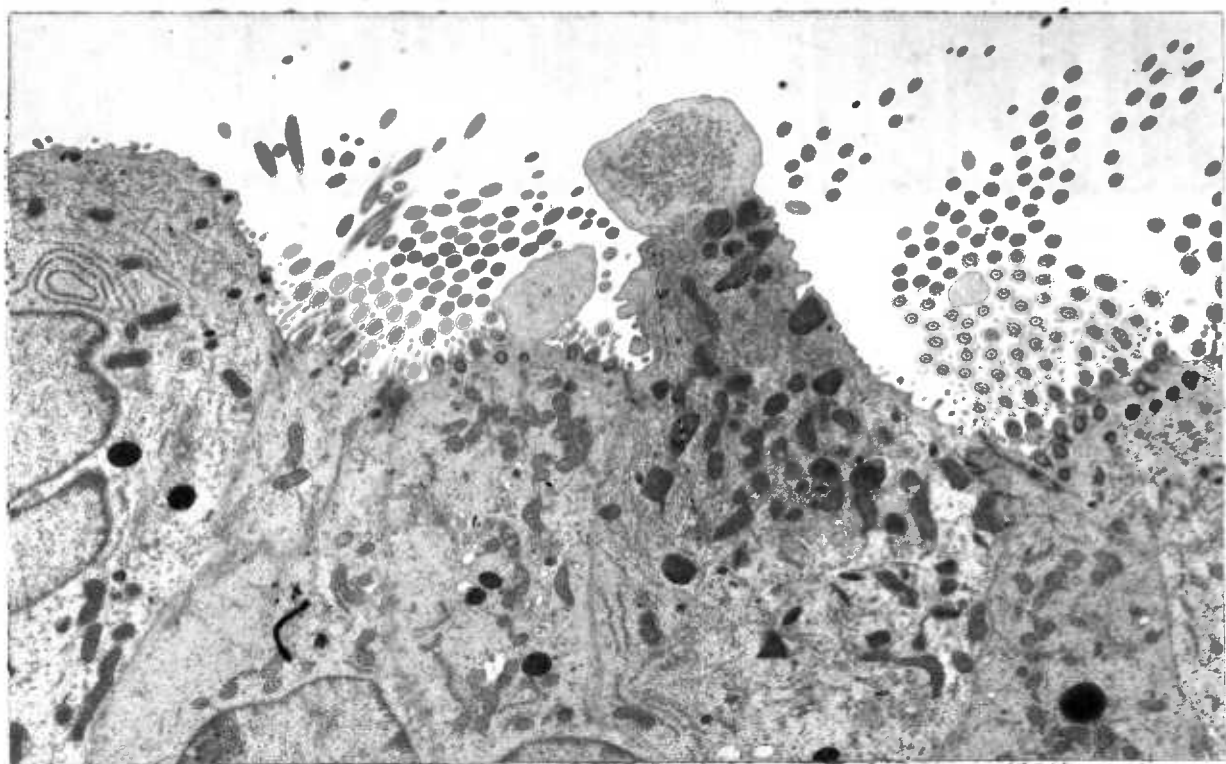


Fig. III-19 illustrates a nonciliated cell at level 5: from the apex there is a bleb-like process containing profiles of SER. x 6,000.



Fig. III-20 depicts a bulging cytoplasmic process from a ciliated cell at level 5: this also contains a few profiles of SER. x 12,000.

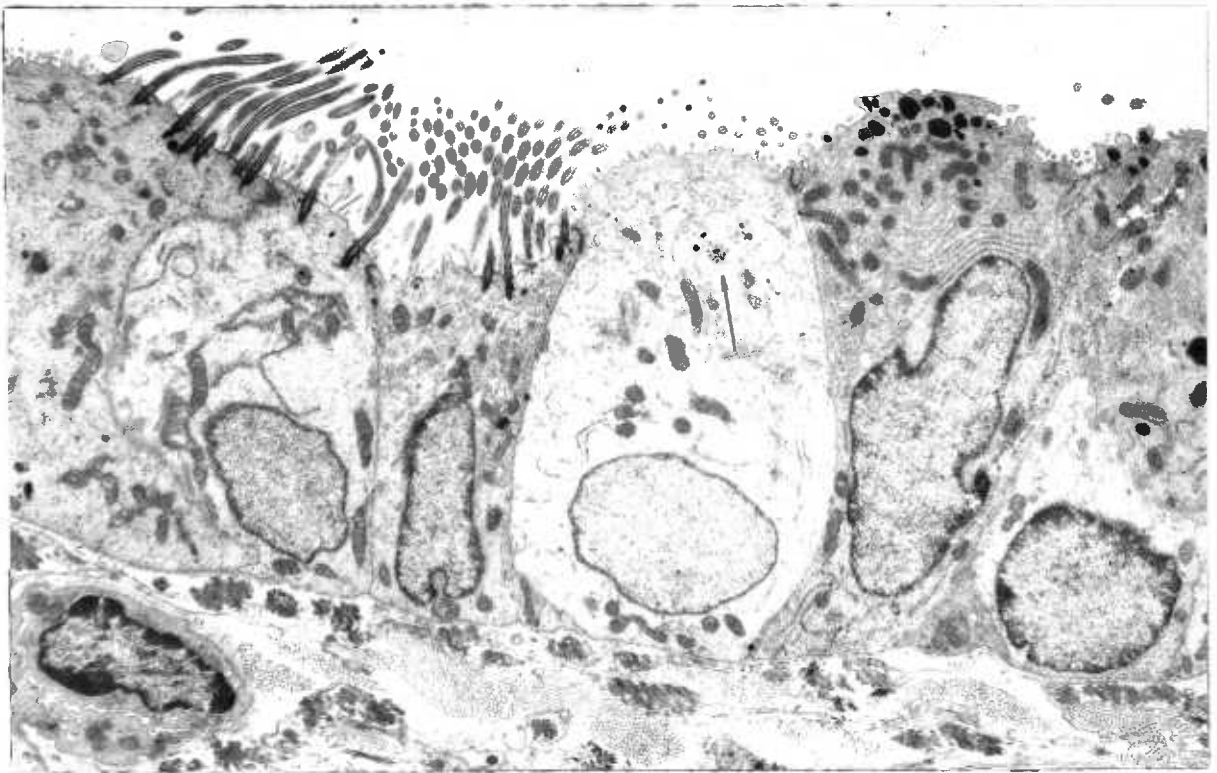


Fig. III-21 illustrates a cell without cilia and with an accumulation of fibrogranular material (arrow): this and the lucency of its cytoplasm suggests it is a developing ciliated cell. x 4,500.



Fig. III-22, illustrates a granulated(?Kultschitsky) cell at the base of tracheal epithelium: the cytoplasm is electron-lucent and contains many granules each with a smaller electron-dense core. x 17,500.



Fig. III-23 illustrates a globule leucocyte after fixation by osmium alone: the granules are incompletely stained showing electron-dense splinter-like fragments in a lucent matrix (arrow). Compare with Fig. III-11. x 7,500.

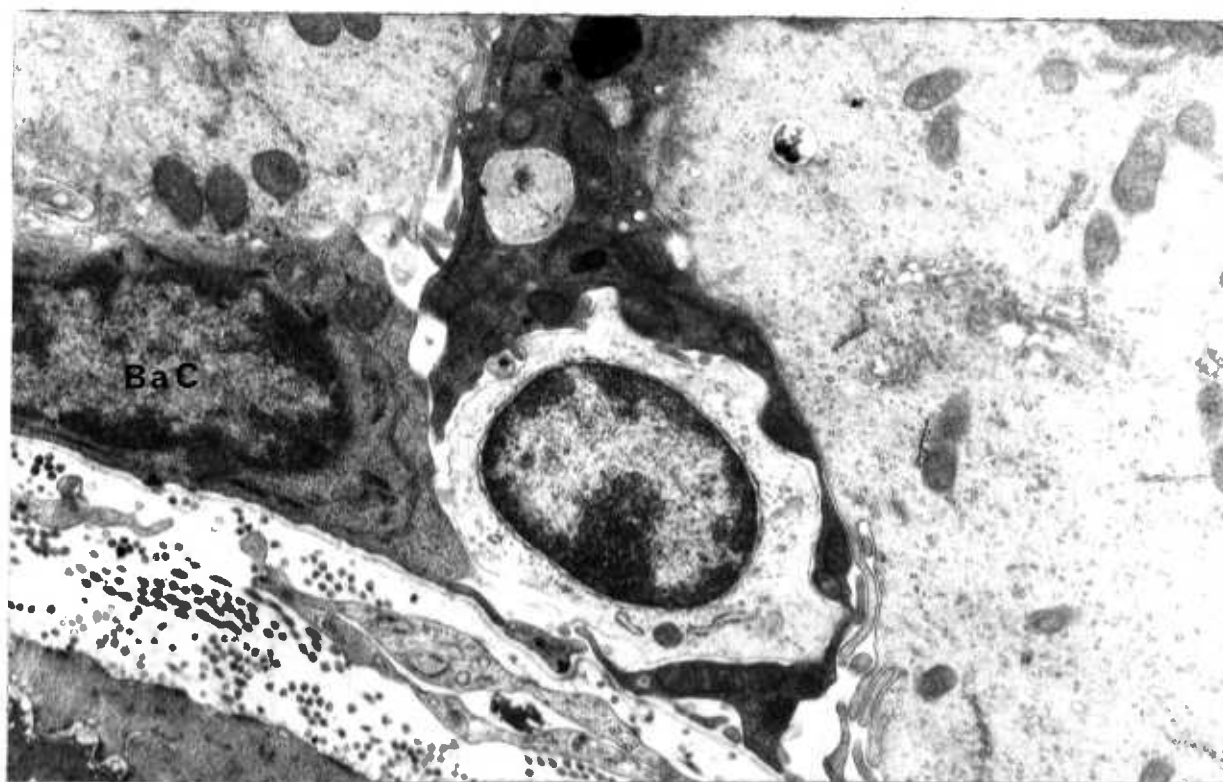


Fig. III-24 illustrates a small lymphocyte at the base of the epithelium and adjacent to a basal cell. The cytoplasm of the lymphocyte is electron-lucent and contains few organelles. x 15,000.

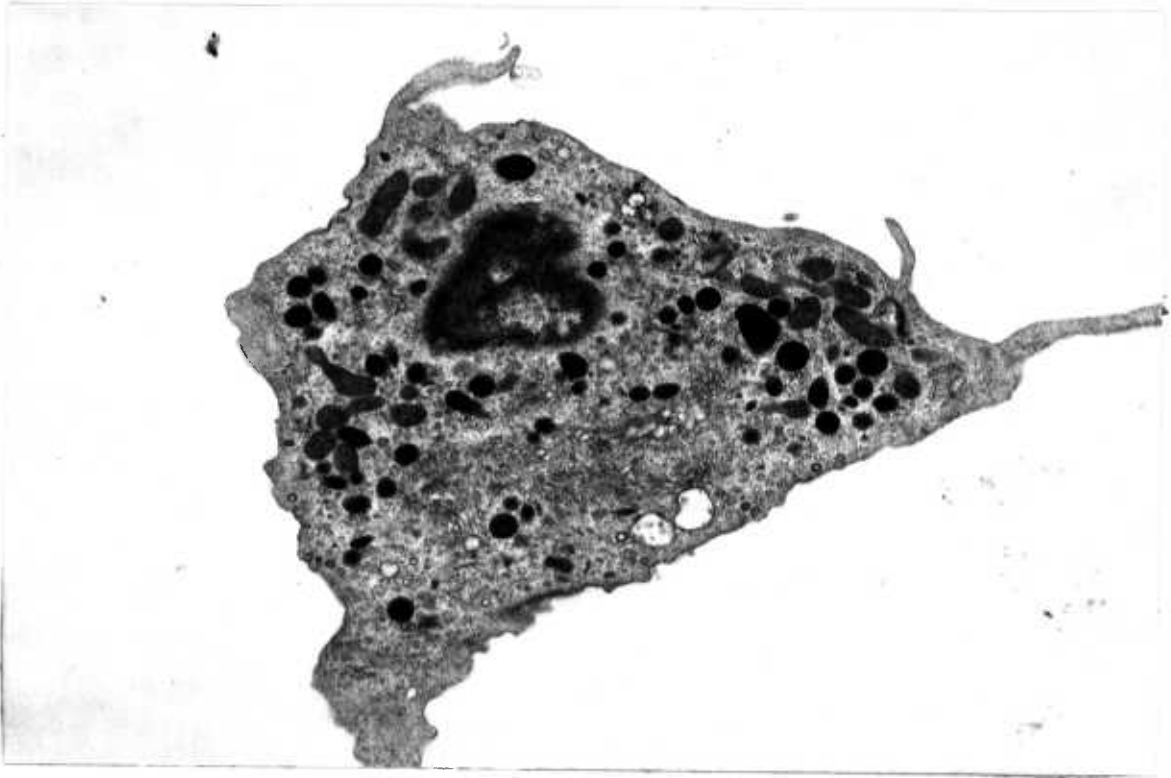


Fig. III-25 illustrates a macrophage in a control animal: the cytoplasm has an abundance of electron-dense inclusions. x 10,500.

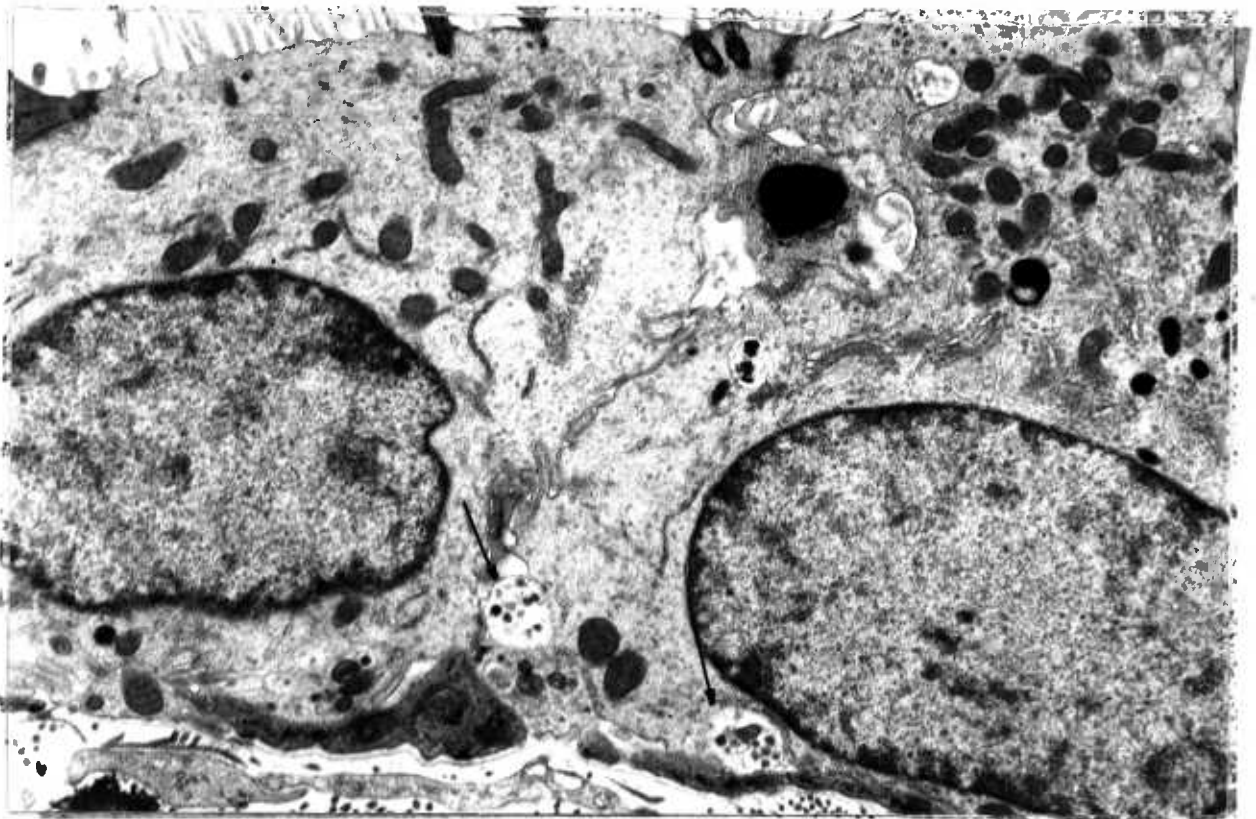


Fig. III-26 depicts two intra-epithelial nerves (arrows) in normal bronchial epithelium (level 3): each has dense-cored neurosecretory vesicles. x 12,000.

## ILLUSTRATIONS

### CHAPTER IV

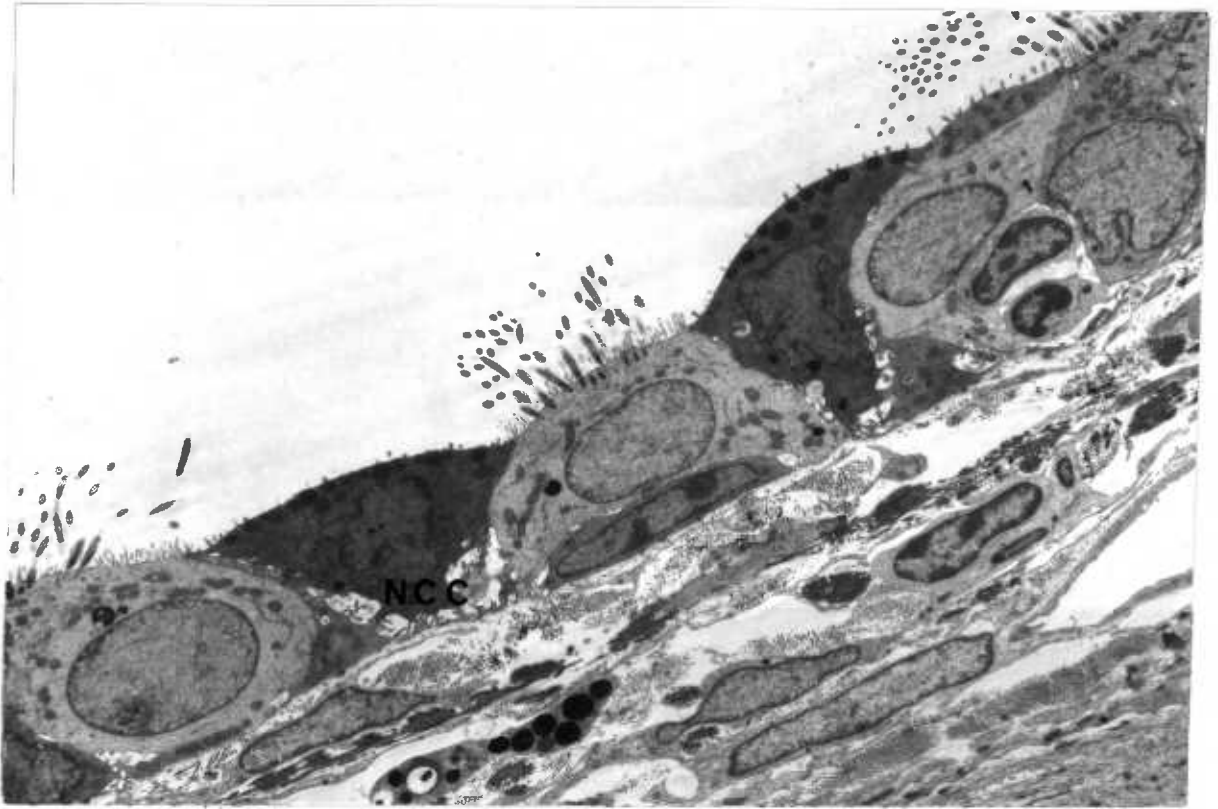


Fig. IV-7 illustrates the epithelium (level 3) of a control rat: nonciliated cells have a few electron-dense granules x 3,750.

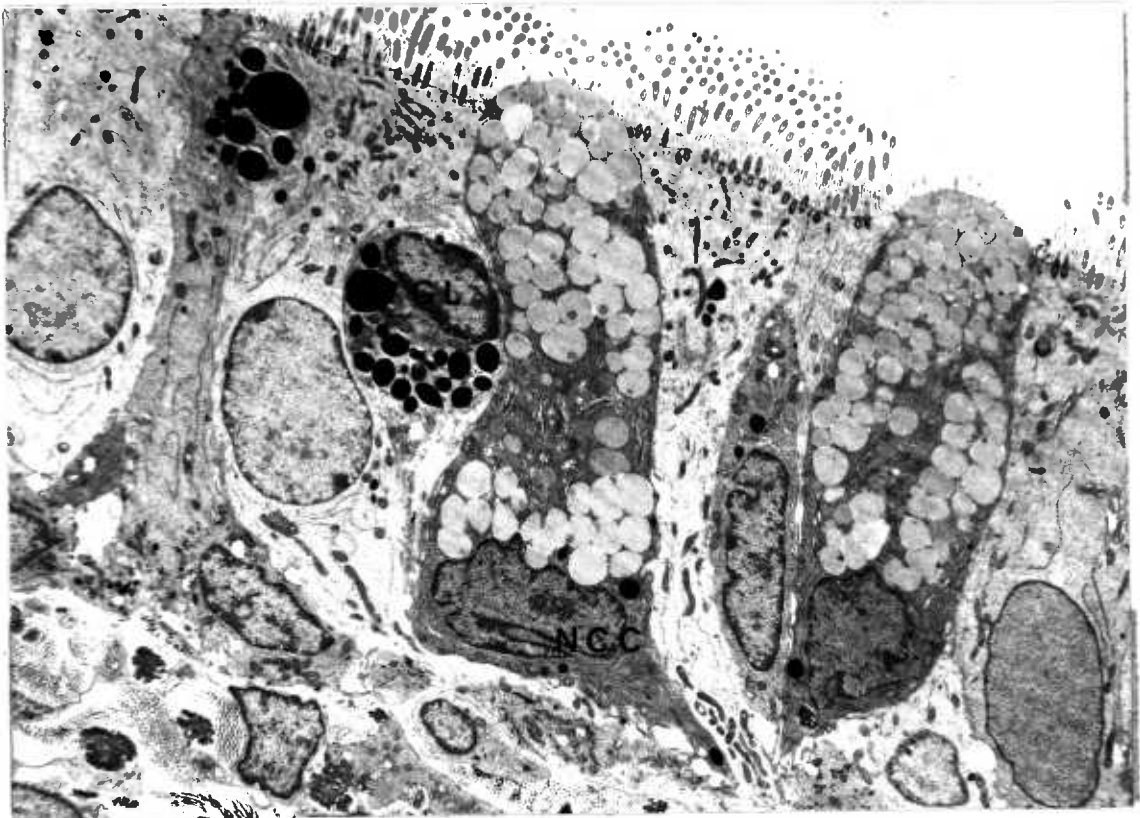


Fig. IV-8 illustrates the epithelium (level 3) of a rat exposed to tobacco smoke for 2 weeks: nonciliated cells now have a great number of electron-lucent secretory granules. The rounded cell to the left of centre is a globule leucocyte. x 3,750.



Fig. IV-9 illustrates the epithelium (level 3) of a rat exposed to tobacco smoke for 2 weeks but whose smoke included PMO: some nonciliated cells have dense and others lucent secretion. x 3,750.

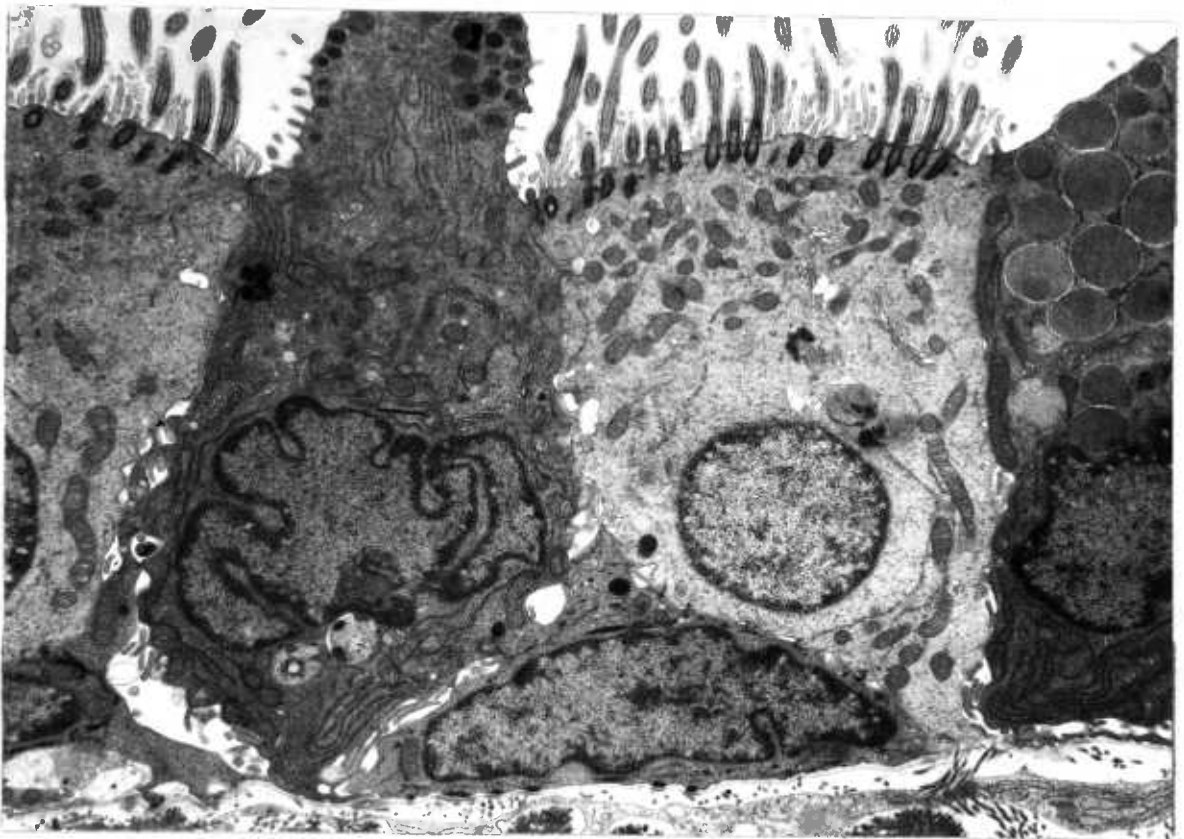


Fig. IV-10 illustrates a ciliated cell<sup>in</sup> epithelium (level 3) taken from a control rat: the cell cuboidal in shape. x 7,500.



Fig. IV-II illustrates a ciliated cell after exposure to tobacco smoke for 2 weeks: the cell is taller and thinner than normal and the cytoplasmic ground substance is increased. A group of intraepithelial nerves is present (arrow) x 7,500.

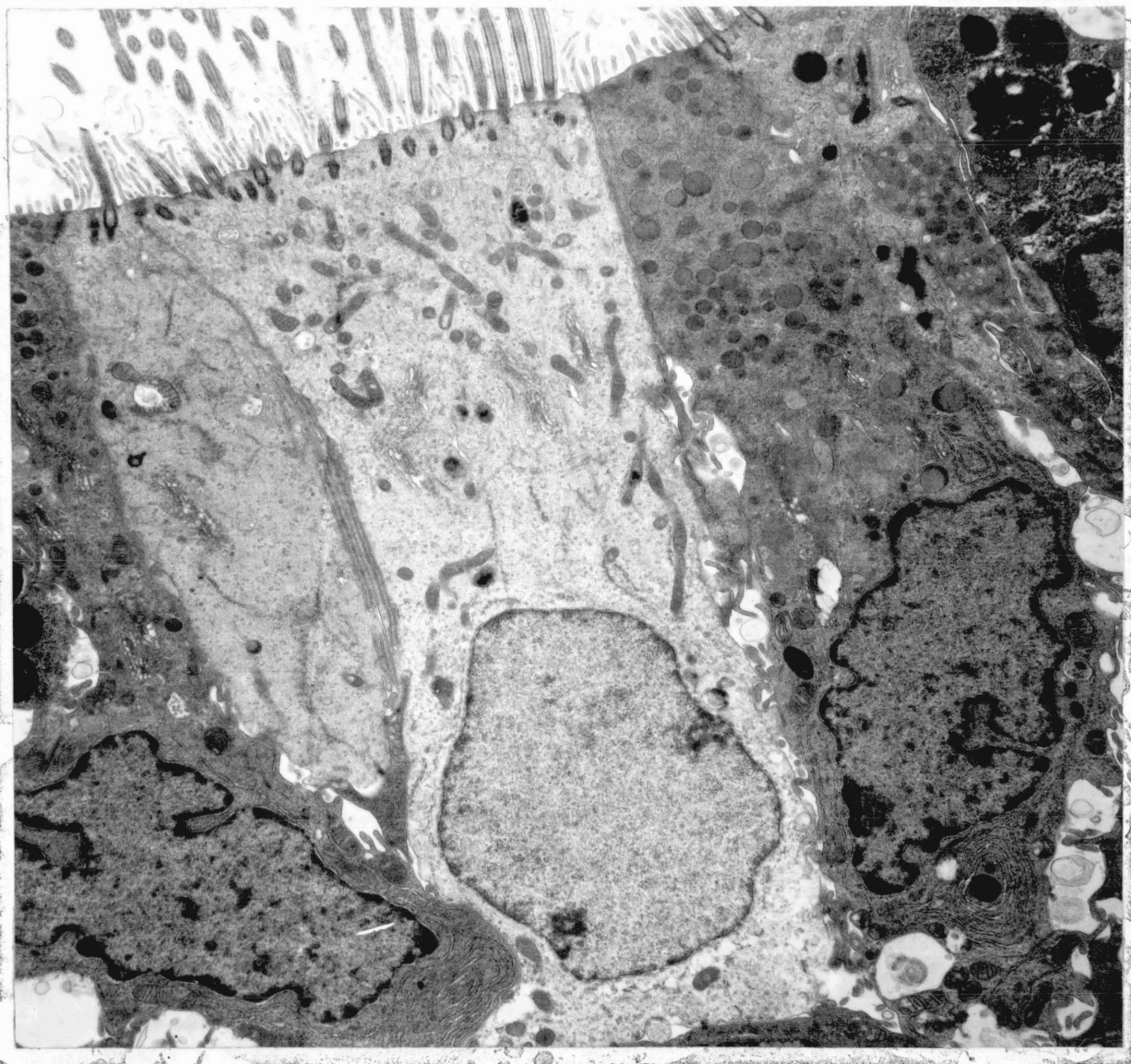


Fig. IV-12 illustrates a ciliated cell after exposure to tobacco smoke to which PMO had been added: the changes are not as severe as those shown in the previous figure. x 7,500.



Fig. IV-13 illustrates a nonciliated cell in the epithelium (level 3) of a control: each secretory granule is distinct and homogeneously electron-dense. x 9,000.



Fig. IV-14 illustrates a nonciliated cell in epithelium (level 3) exposed to tobacco smoke for two weeks. Compared to the cell in the previous figure there is more intracellular secretion, each granule is larger, electron-lucent and there is a tendency for the granules to fuse with each other (arrow) x 9,000.

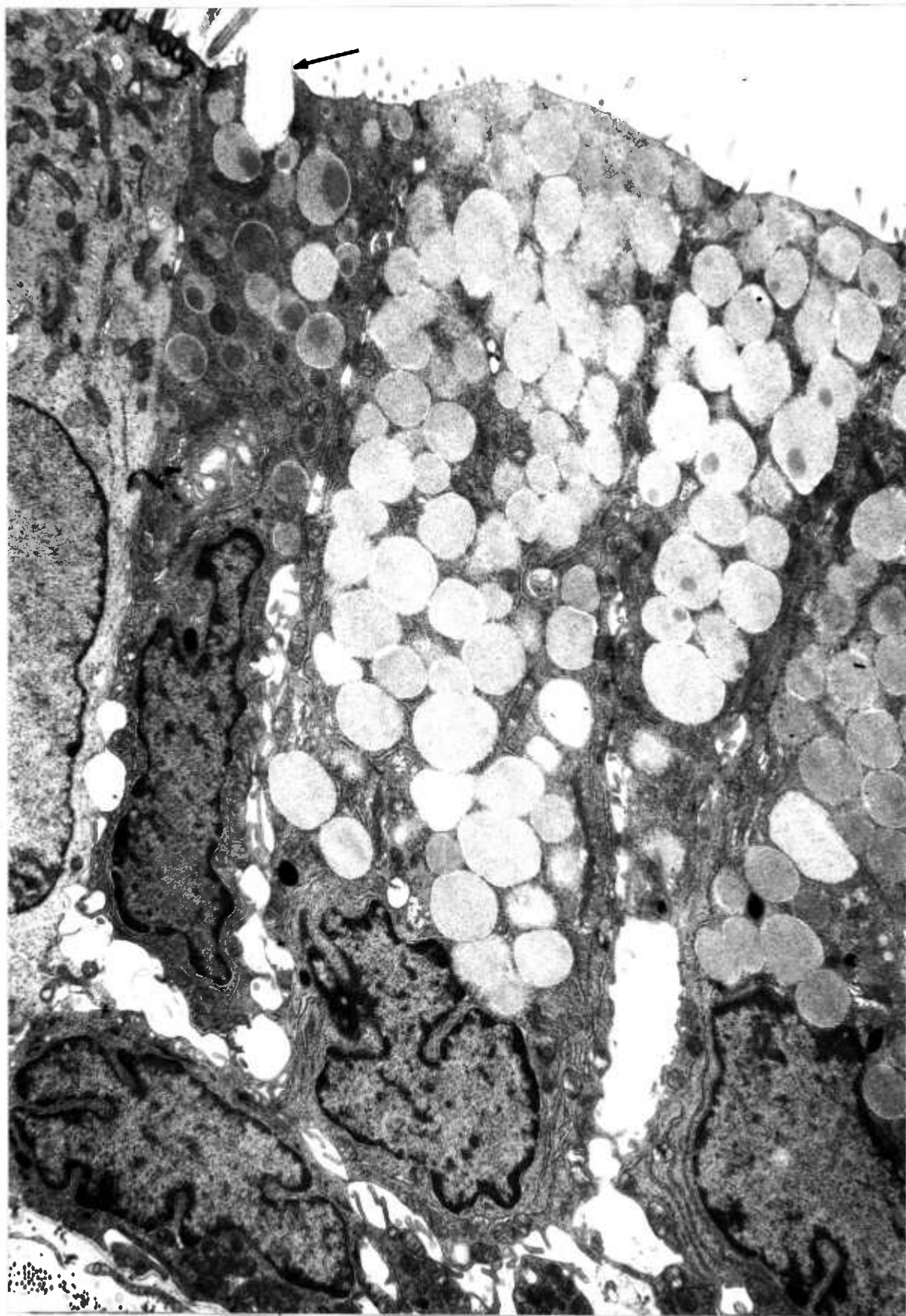


Fig. IV-15 illustrates a nonciliated cell in bronchial epithelium (level 3) of a rat exposed to tobacco smoke + PMO for two weeks: the cell is filled with large lucent secretory granules, some of them confluent. A granule is being discharged from an adjacent cell (arrow). x 9,000.

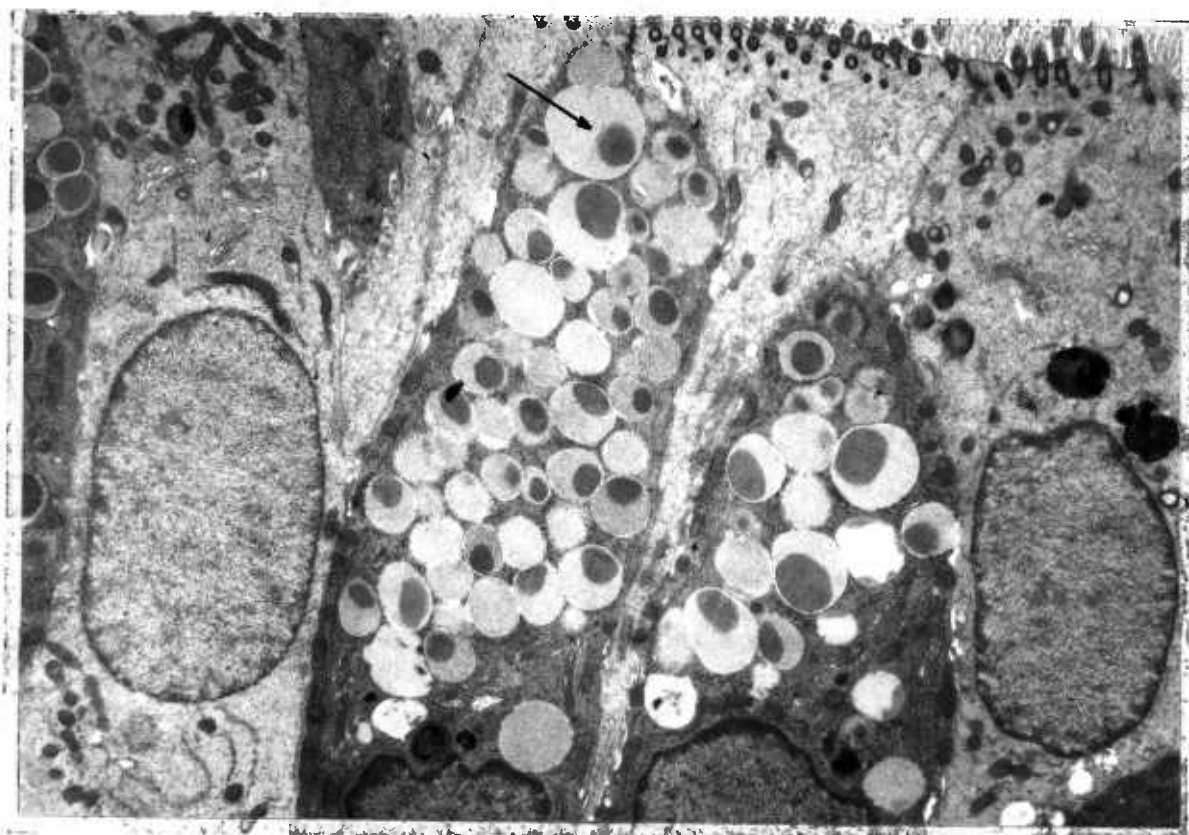


Fig. IV-16 shows lucent secretory granules within a nonciliated cell exposed to tobacco smoke for six weeks: each granule has a smaller electron-dense core (arrow). x 6,000.

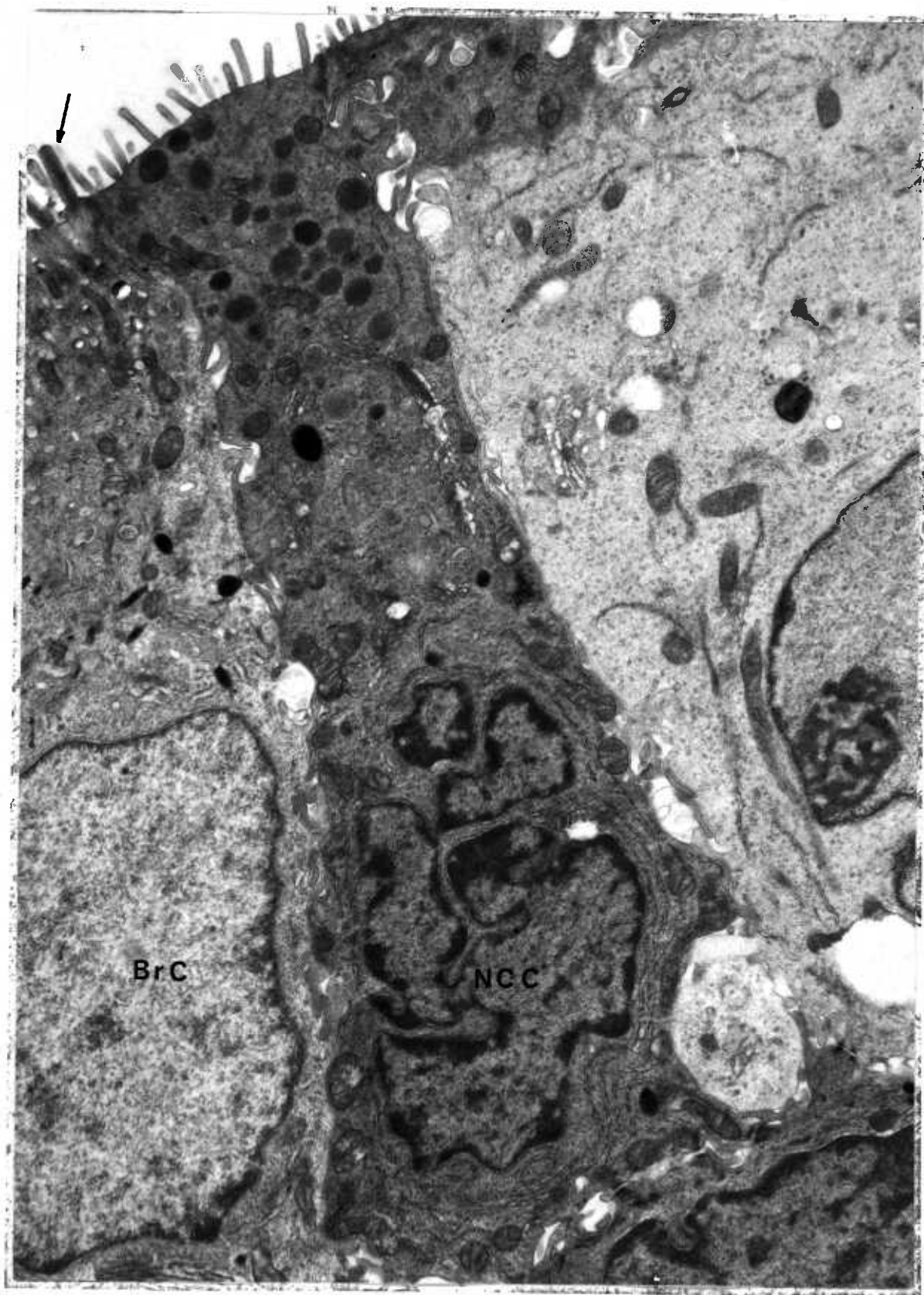


Fig. IV-17A shows a nonciliated cell with dense granules in bronchial epithelium (level 3) exposed to tobacco smoke for six weeks: a few microvilli project from the luminal edge and are thinner than those of the adjacent brush cell (arrow). x 12,000.

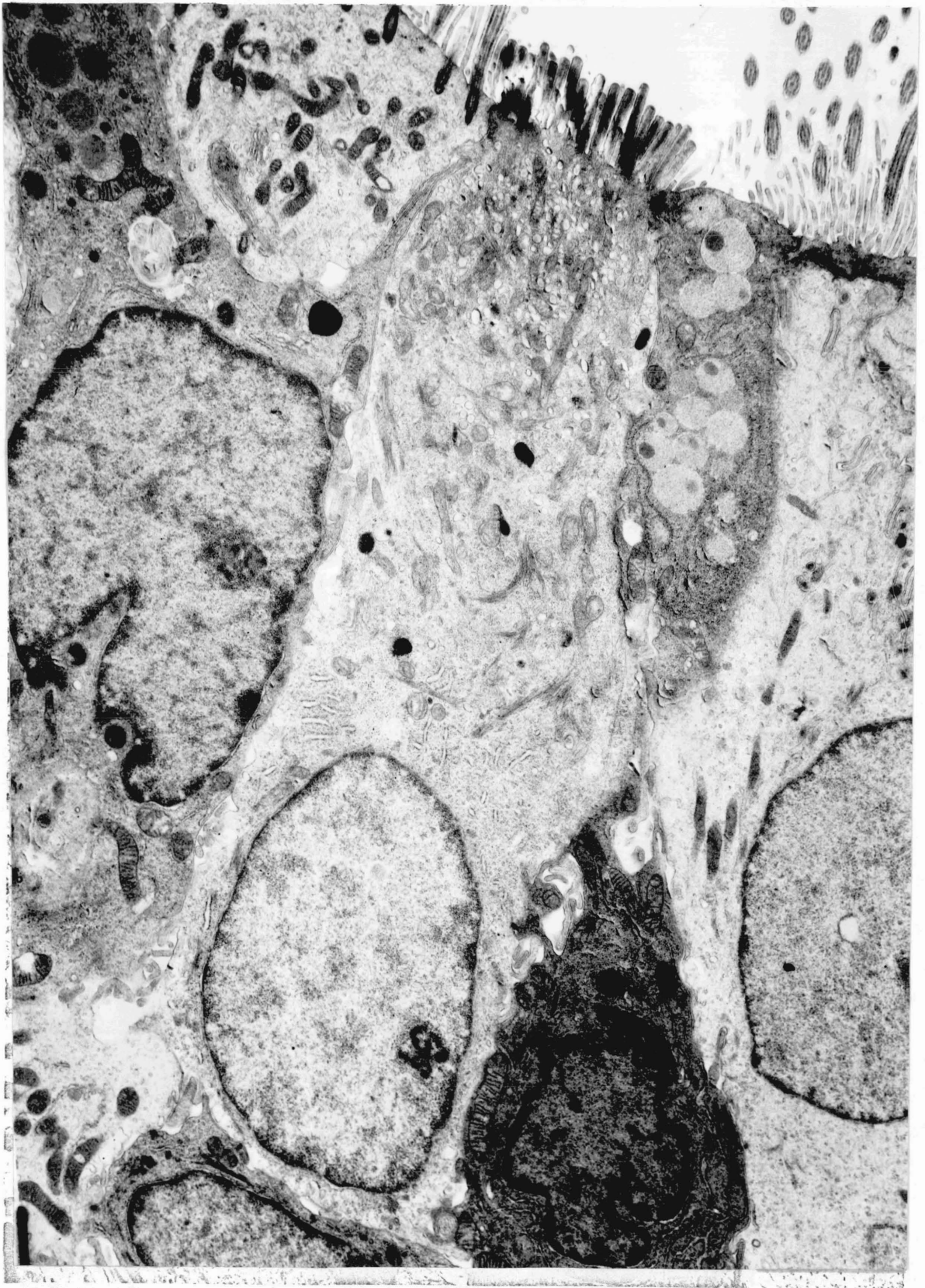


Fig. IV-18 illustrates a brush cell in bronchial epithelium (level 3) exposed to tobacco smoke for 6 weeks: the cell is taller than usual but of normal structure. x 9,000

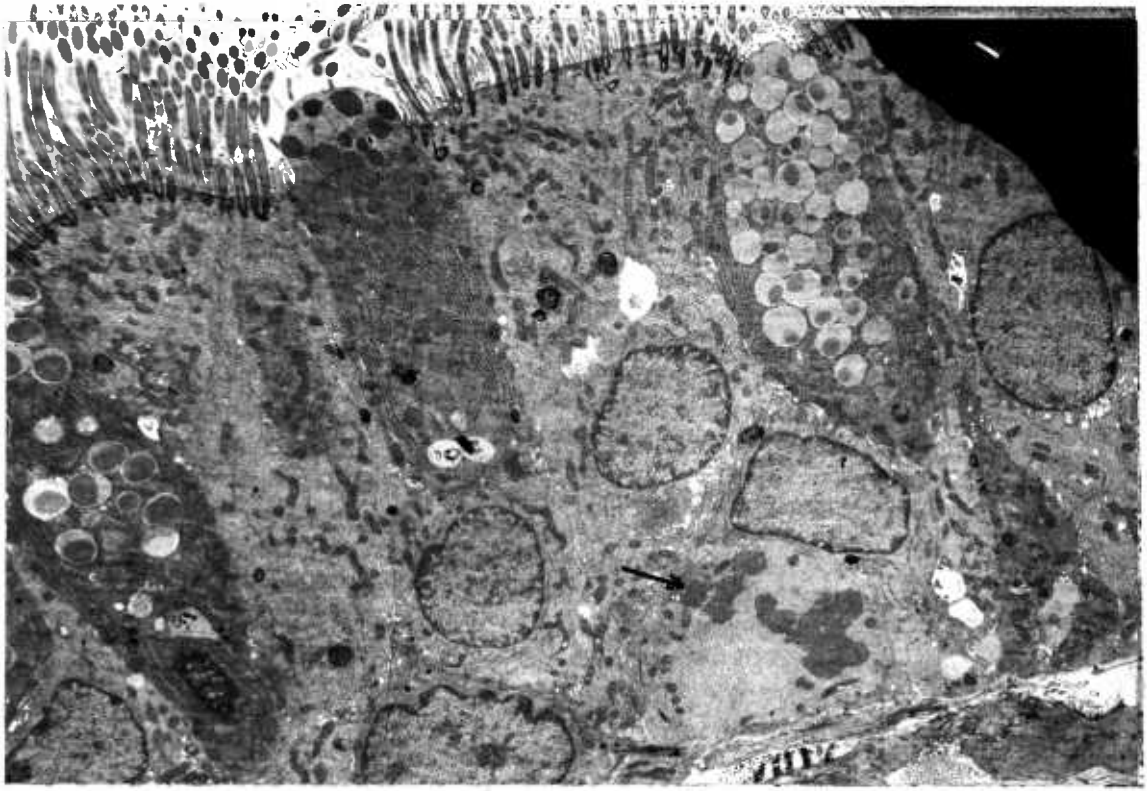


Fig. IV-19 illustrates a basal cell in division in bronchial epithelium (level 3) exposed to tobacco smoke alone for 6 weeks: chromosomes are cut in section (arrow) x 3,750.

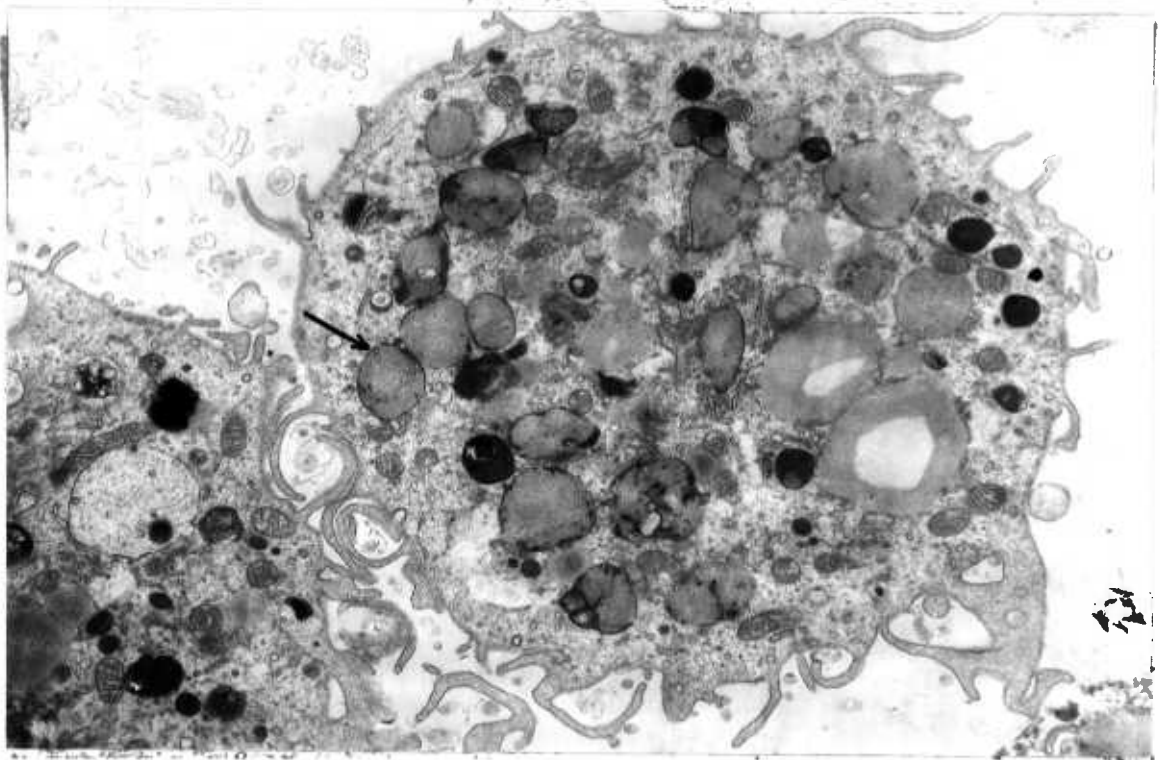


Fig. IV-20 illustrates an airway macrophage in an animal exposed to tobacco smoke and PMO for 6 weeks. As well as electron-dense inclusions, all had larger less dense membrane-bound inclusions which probably represent tar accumulation (arrow) x 9,000.

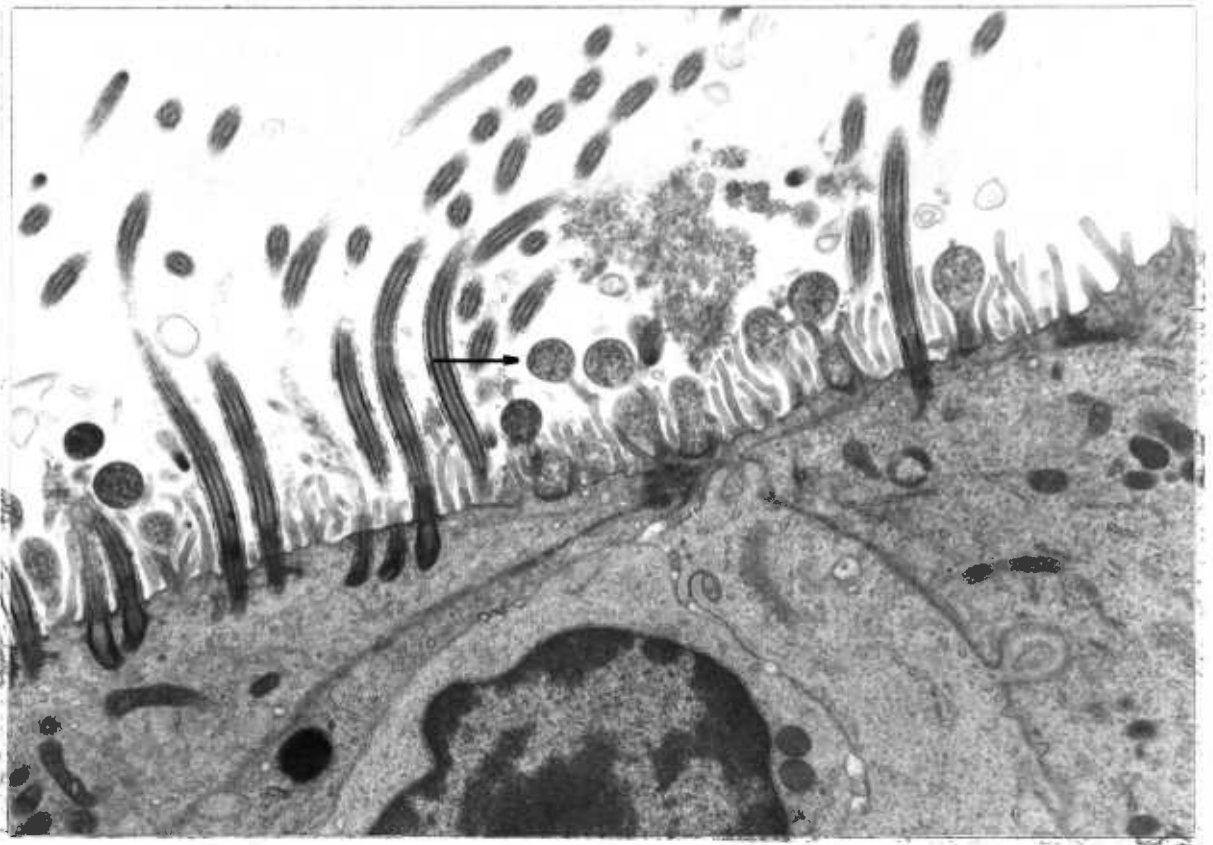


Fig. IV-21 shows numerous ovoid and pear-shaped bodies of Mycoplasma sp. between the cilia of bronchial epithelium (level 3) exposed to tobacco smoke for 6 weeks (arrow). A lymphocyte is migrating through the epithelium between the ciliated cells. x 12,500.



Fig. IV-22 shows the above Mycoplasmas at higher magnification: some have a process with a dense core projecting towards the epithelium (arrow). x 30,000

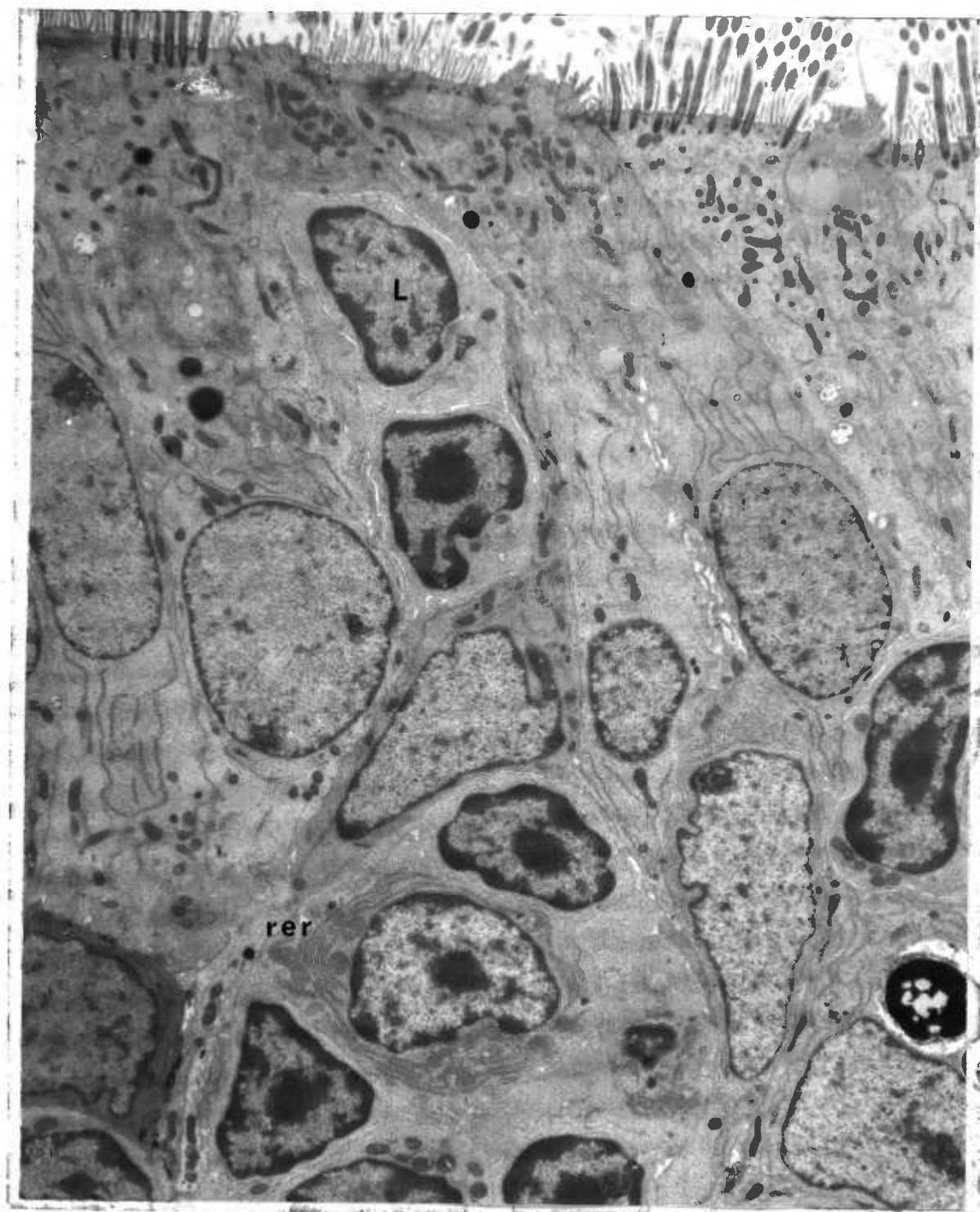


Fig. IV-23 illustrates infiltration of lymphocytic cells into bronchial epithelium at a site where mycoplasmas were in evidence. A cell with much RER, presumably developing into a plasma cell, is also present. x 6,000.

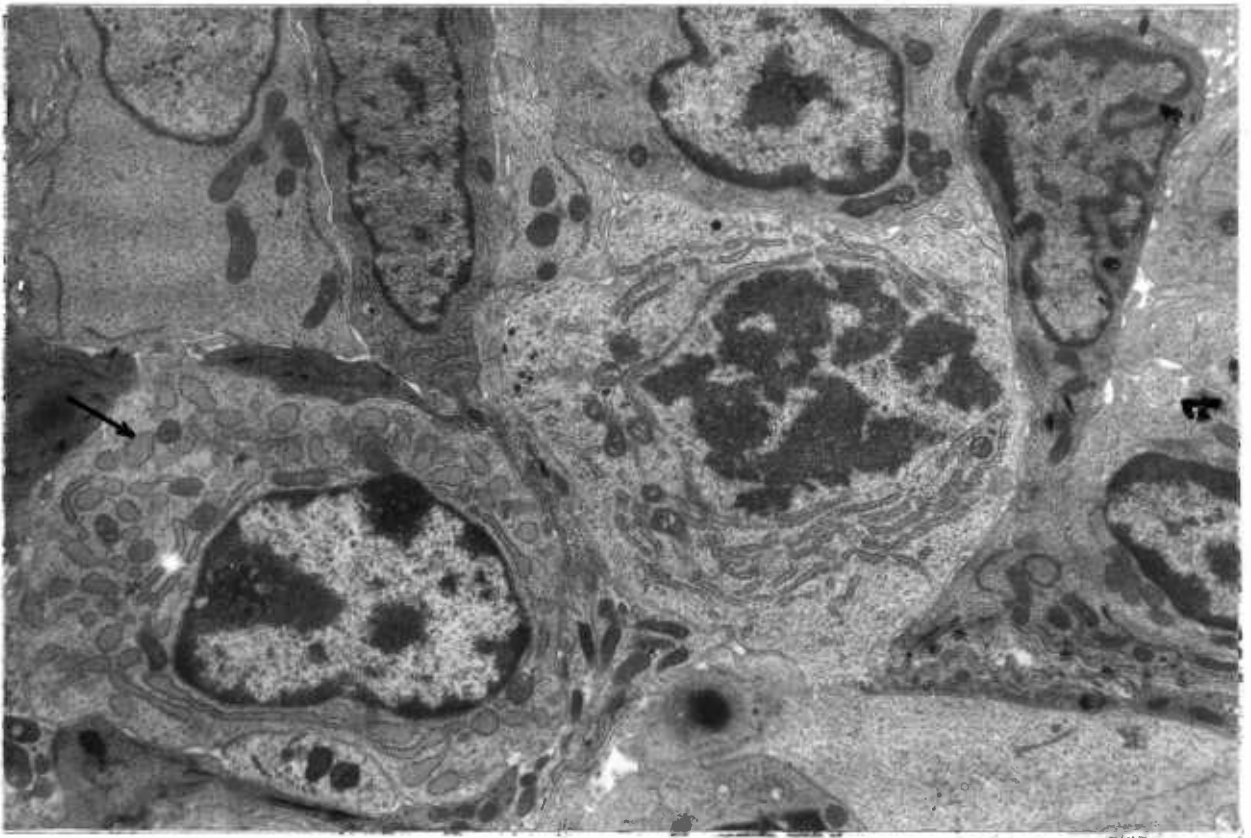


Fig. IV-24 shows, at a site where there is Mycoplasma infection, a cell with much RER (presumably to become a plasma cell) within the epithelium and in division. There is an adjacent plasma cell with distended RER, (arrow). x 7,500.

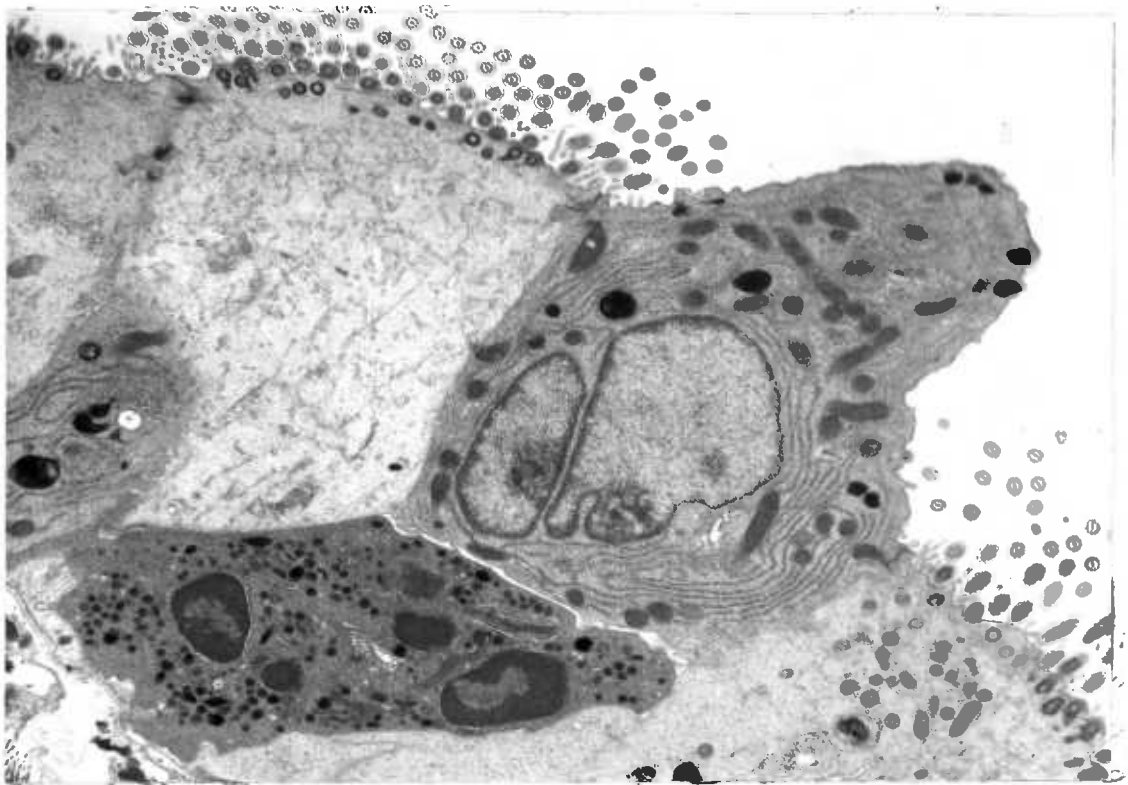


Fig. IV-25 illustrates bronchiolar epithelium (level 5) from a rat exposed to tobacco smoke for two weeks: a polymorphonuclear leucocyte lies within the epithelium between a nonciliated cell and the basement membrane. x 7,500.

## ILLUSTRATIONS

### CHAPTER V

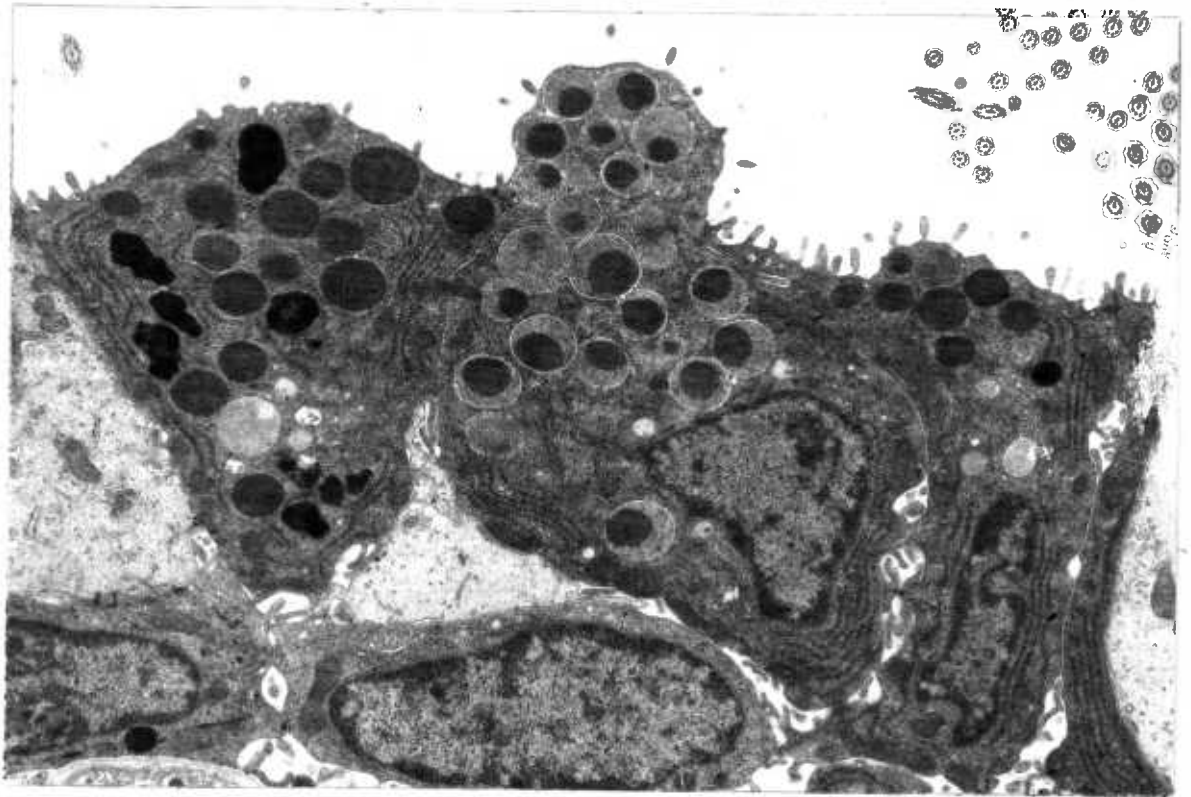


Fig. V-4 illustrates a nonciliated cell in bronchial epithelium (level 3) of a 12 day control rat: many of the secretory granules have an electron-lucent halo x 9,000.

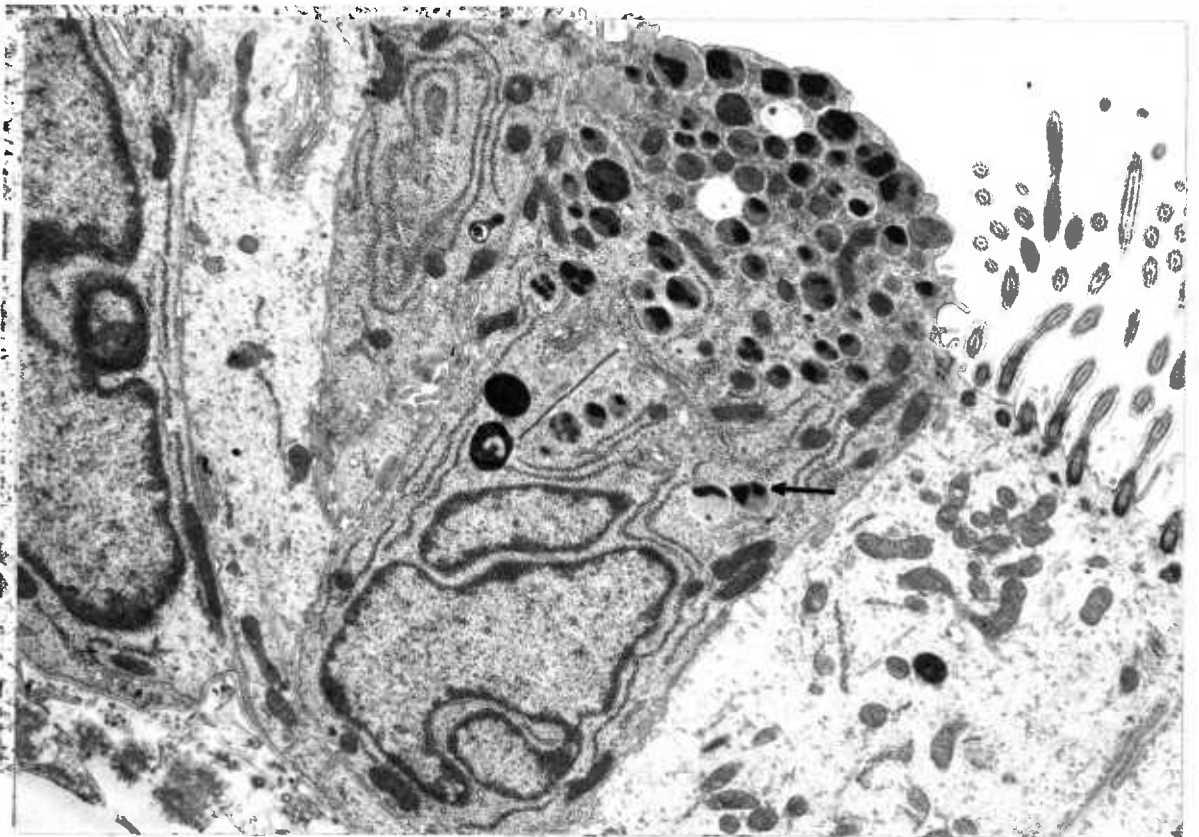


Fig. V-5 illustrates a nonciliated cell in bronchiolar epithelium (level 5) of a control rat: there is less SER than usual and lucent granules are developing in the Golgi region as well as from apical dense granules (arrow) x 9,000.

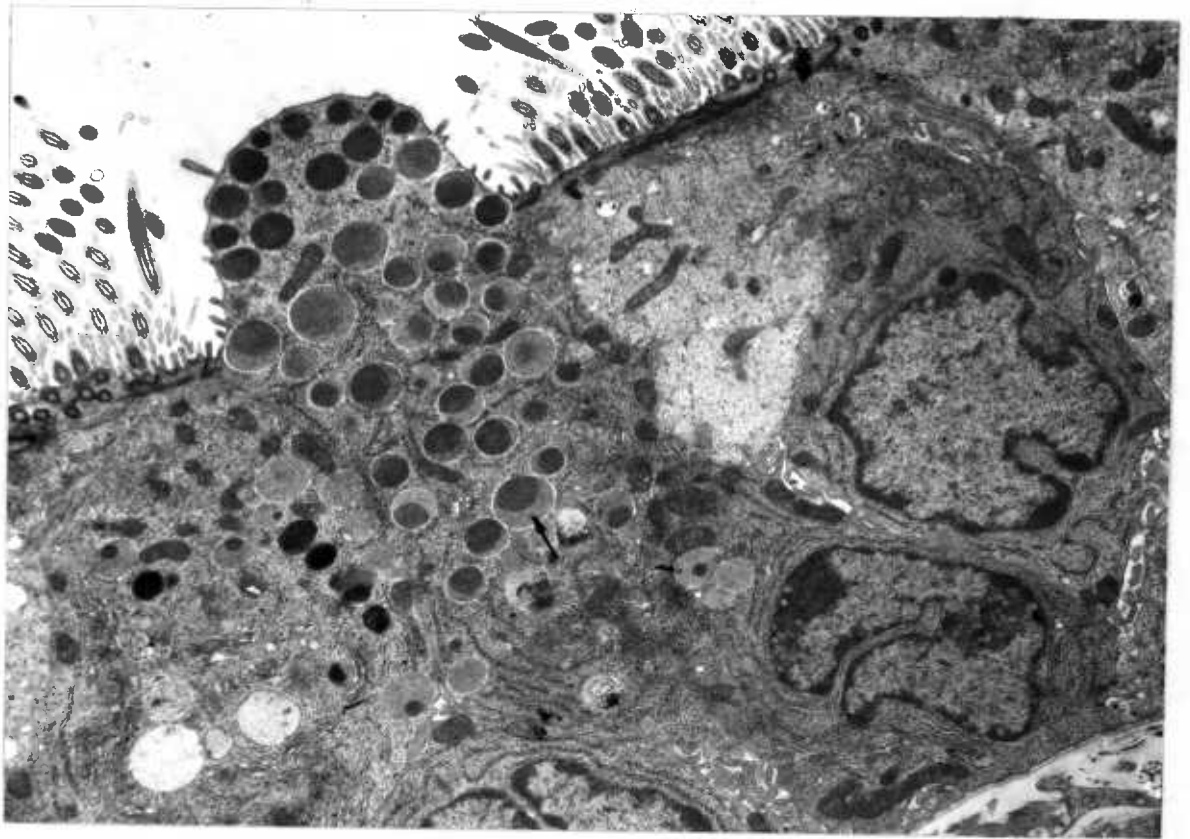


Fig. V-7 illustrates a nonciliated cell in the tracheal epithelium (level 2) of a rat injected with IPN: many of the secretory granules have an electron-lucent halo (arrow). x 7,500.

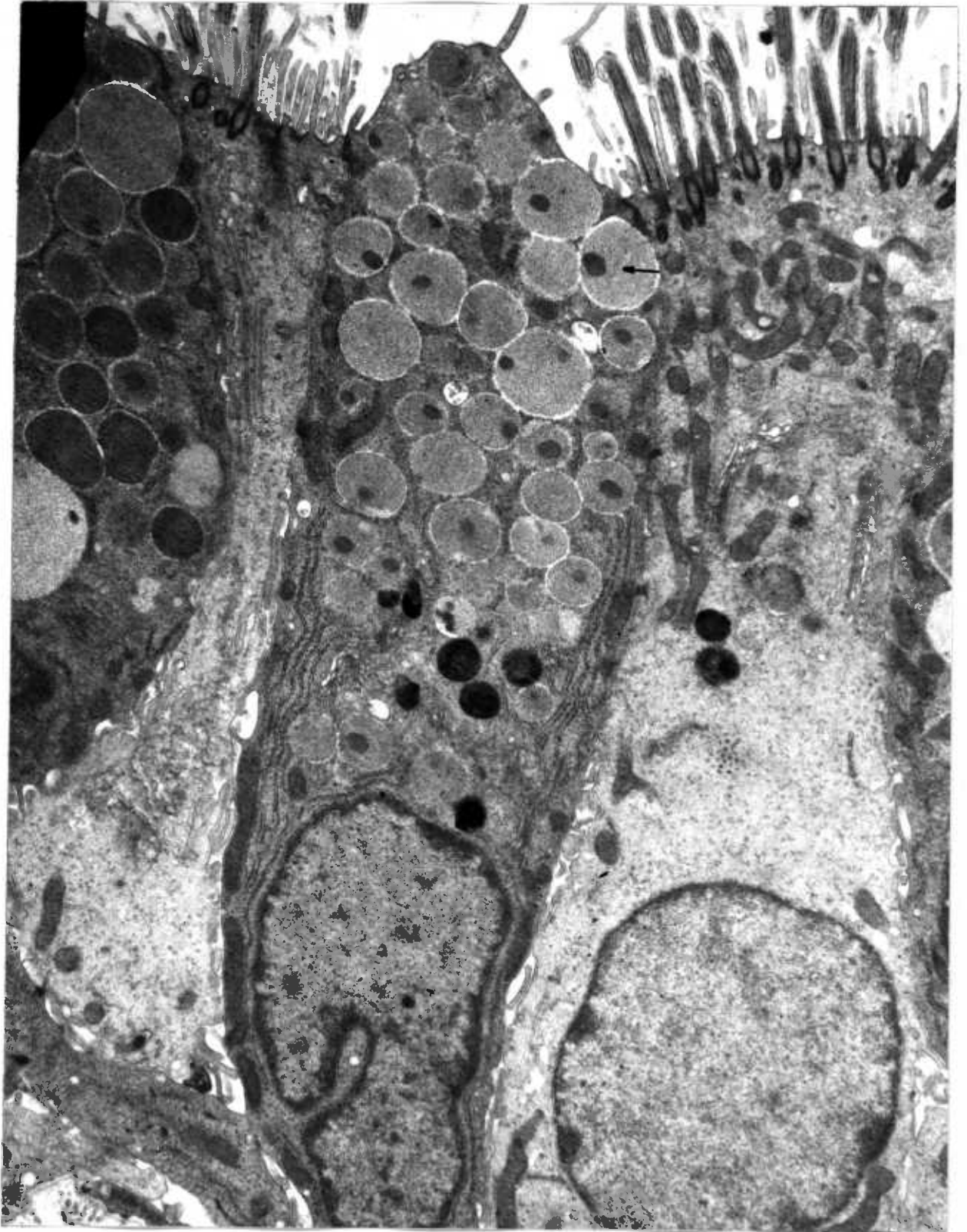


Fig. V-8a illustrates a nonciliated cell in the epithelium (level 3) of a rat treated with IPN: the electron-lucent granules each have a dense core (arrow) x 12,000.

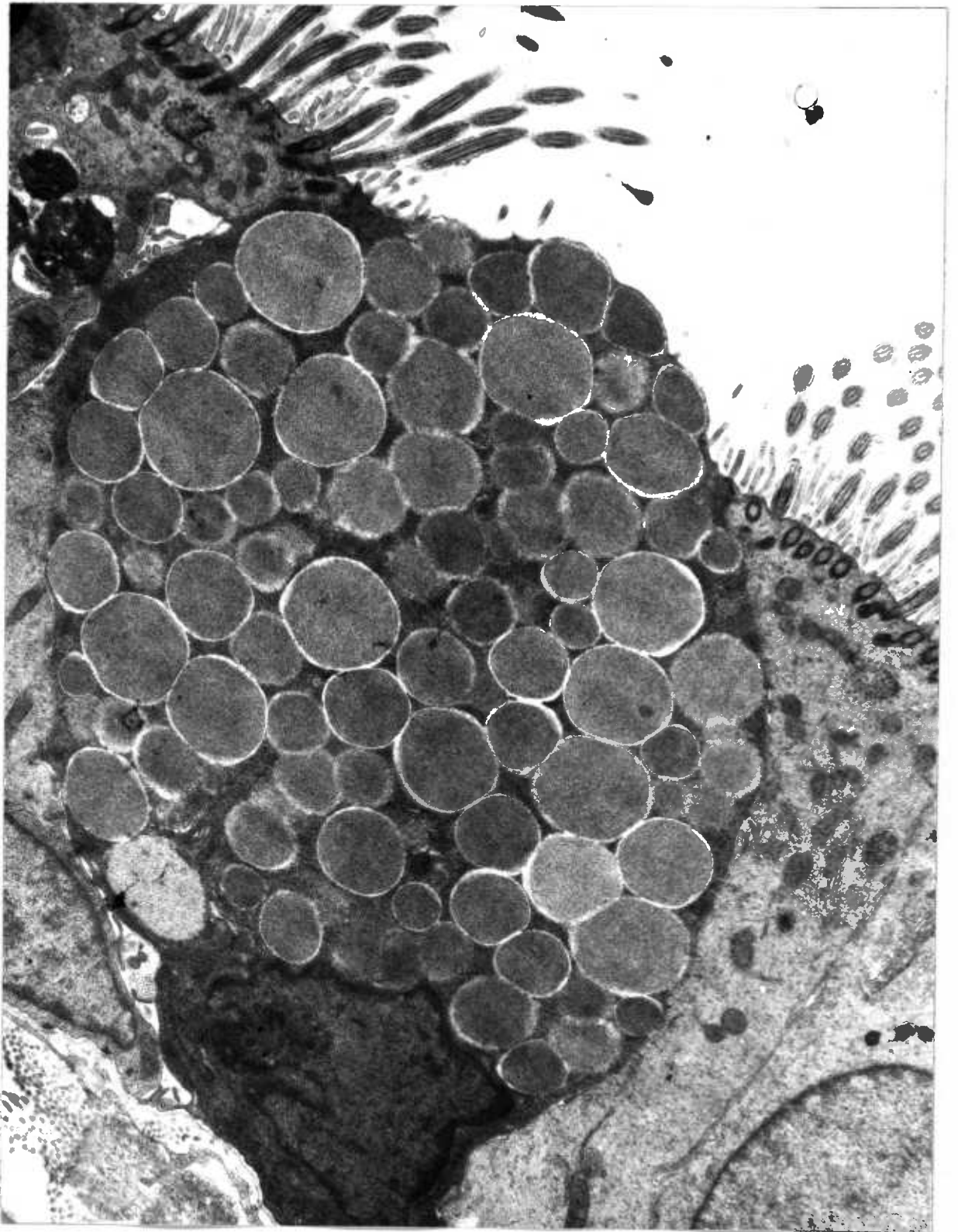


Fig. V-8b illustrates a nonciliated cell in the epithelium (level 3) of an IPN treated rat: the electron-lucent granules are large and numerous and the cell, goblet shaped x 10,500.

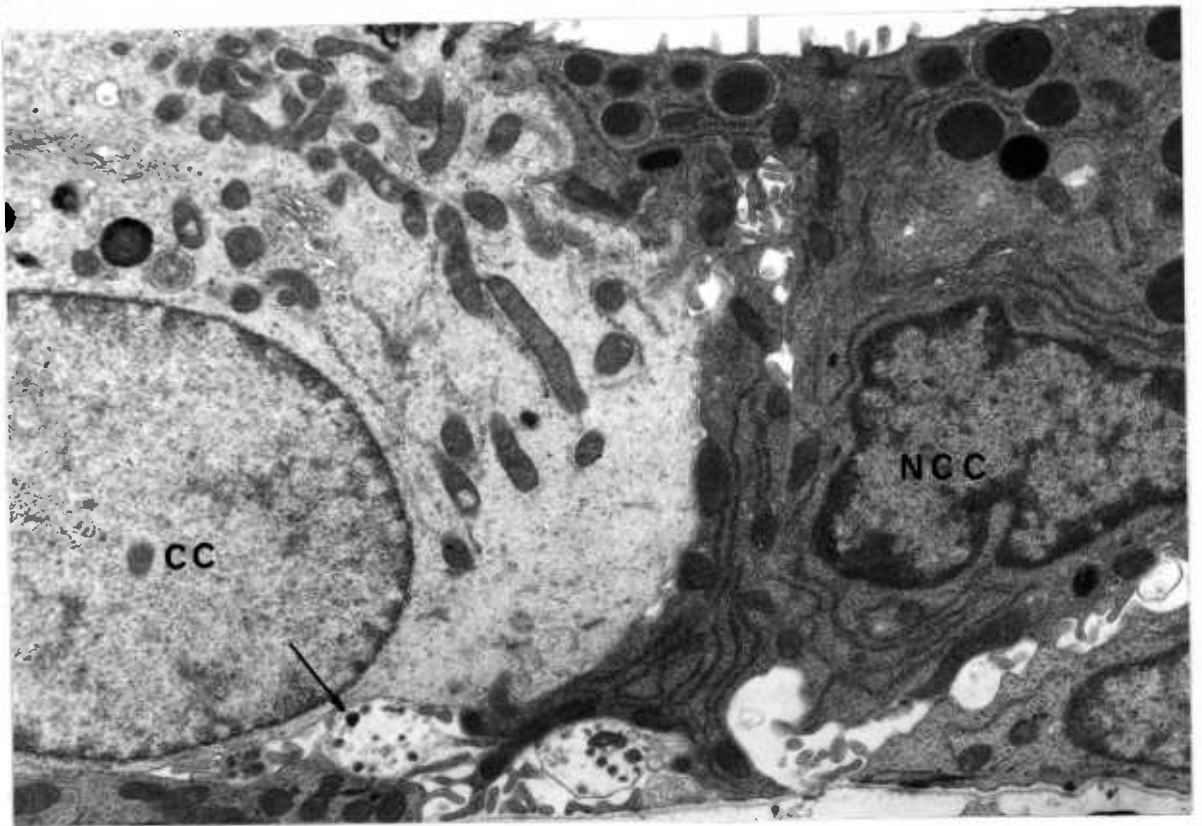


Fig. V-9 illustrates two intraepithelial motor nerves present in a rat injected with IPN, one at the base of a ciliated cell and the other beneath a nonciliated secretory cell. Each axon has dense-cored vesicles (arrow). x 12,000.

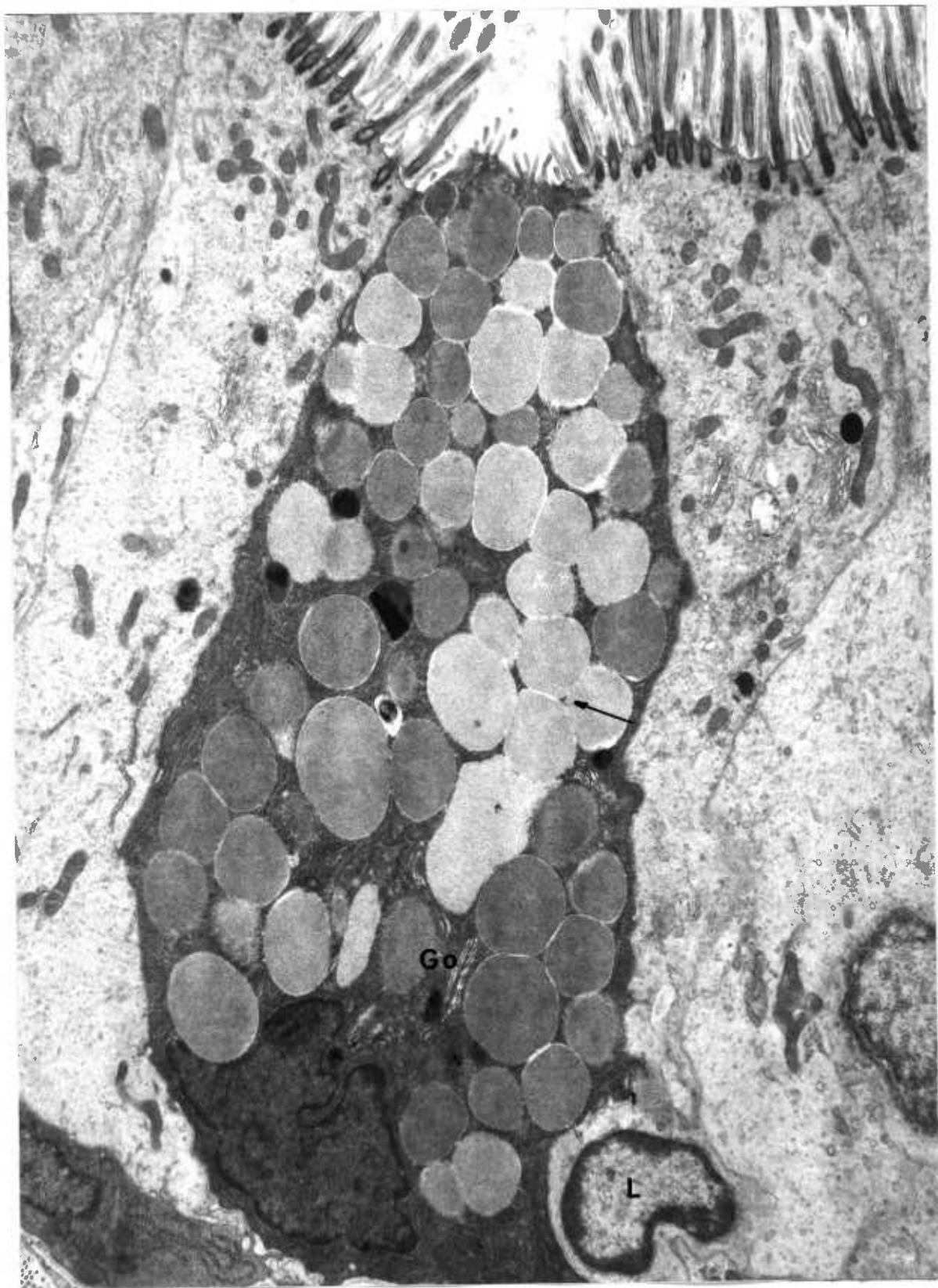


Fig. V-10 illustrates a nonciliated goblet cell in the bronchiolar epithelium (level 5) of a rat injected with IPN: the cell is full of large, lucent and often confluent secretory granules (arrow). These cells were frequently found. x 9,000.

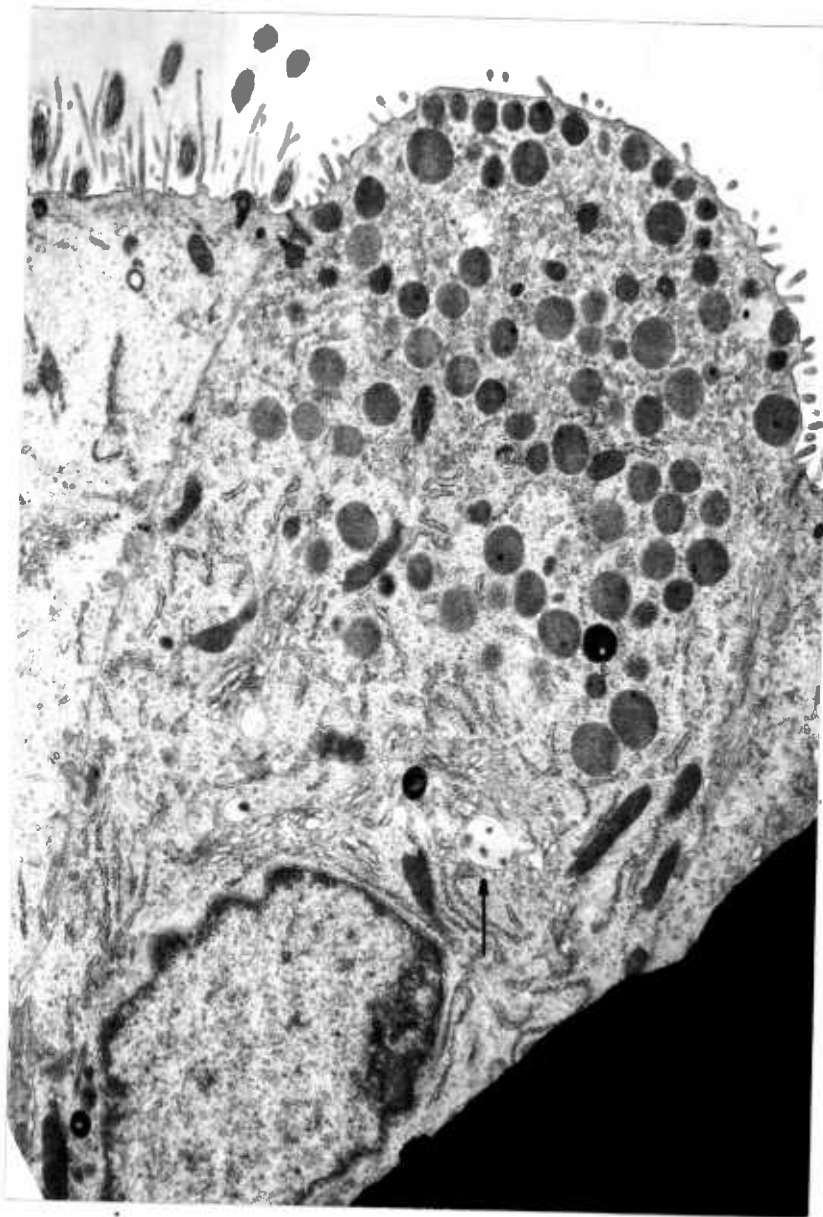


Fig. V-11 illustrates a bronchiolar (level 5) Clara cell with dense granules and much SER: the granules are, however, more numerous than normal (see Fig. III-14) and there are a few lucent granules in the Golgi region (arrow) x 9,000.

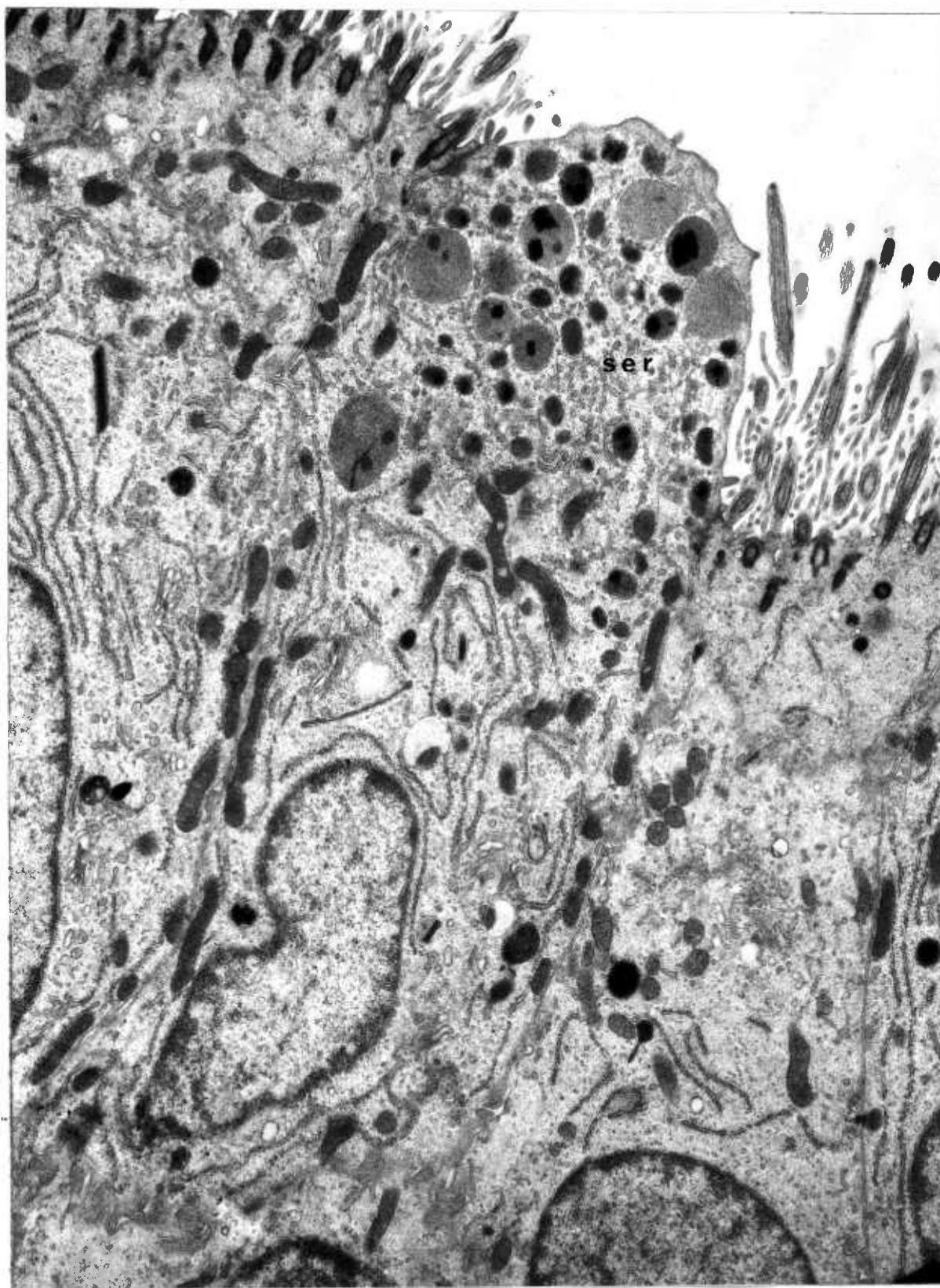


Fig. V-12 depicts a nonciliated bronchiolar cell (level 5) in a more advanced stage of transformation than in the previous figure; the granules are more lucent and there is less SER. x 12,000.

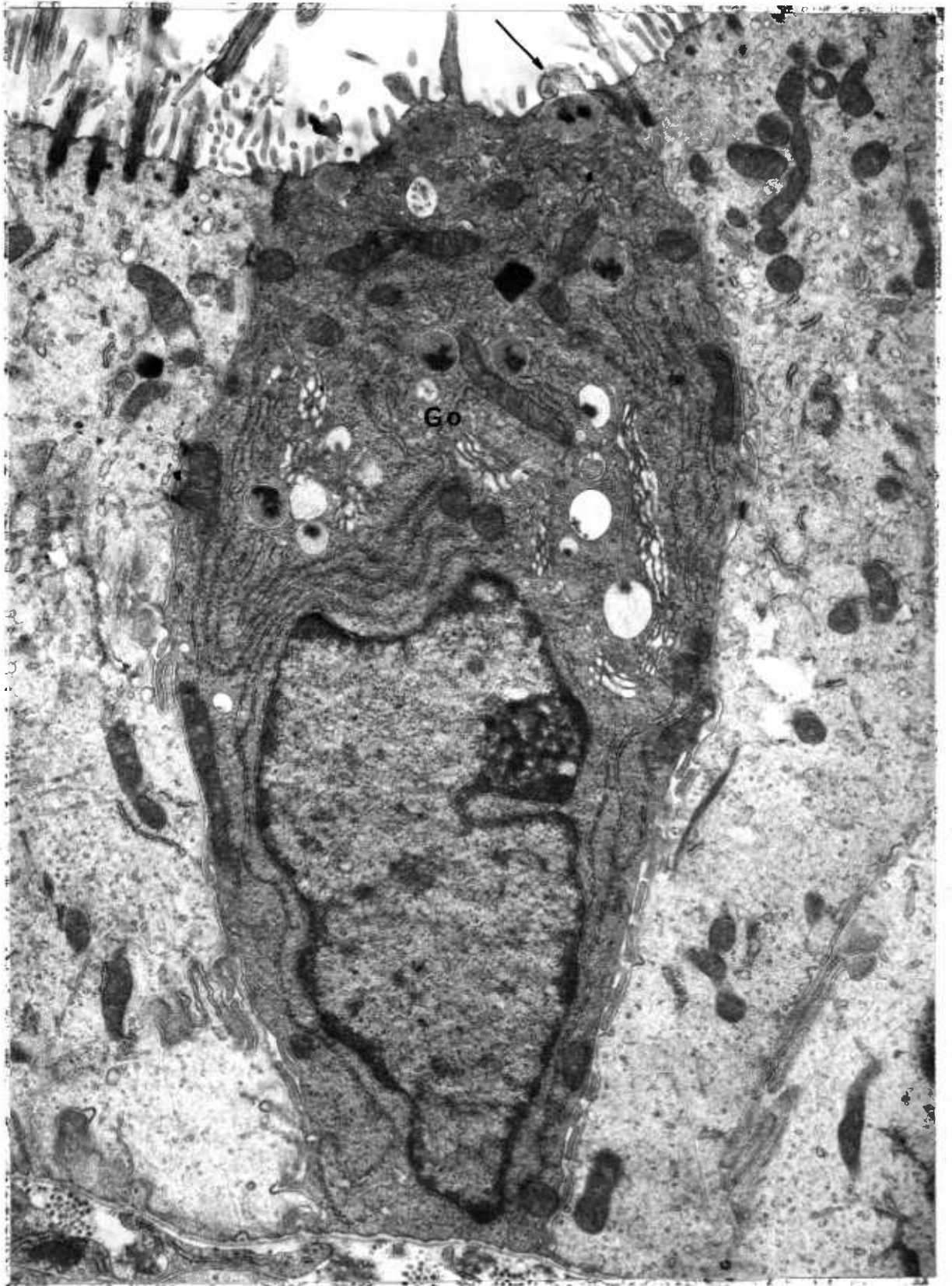


Fig. V-13 depicts a nonciliated bronchiolar cell (level 5) in a later stage of transformation than that seen in the previous figure: the now dense cytoplasm has little SER as short profiles, a well developed Golgi, abundant RER and many lucent granules, some of which are being secreted (arrow). From such a cell and by increased synthesis, a goblet cell might develop. x 13,500.

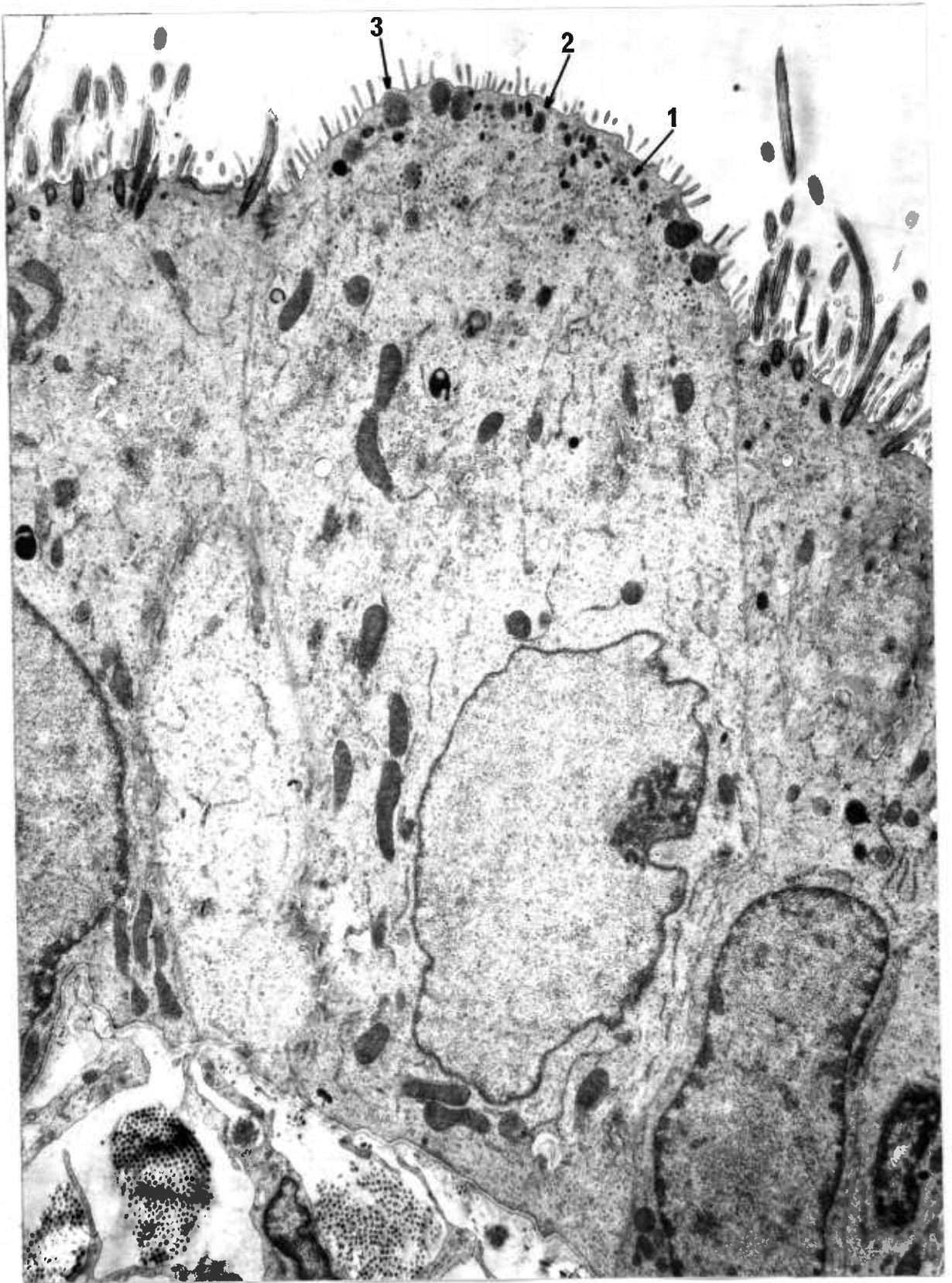


Fig. V-14 illustrates a cell without cilia (level 5) but resembling a ciliated cell in having lucent cytoplasm with fibrogranular bodies - evidence of ciliogenesis - and apical filiform projections. The apex has many dense granules similar to those of Clara cells in varying stages of formation (arrows 1-3). IPN treated rat. x 9,000.

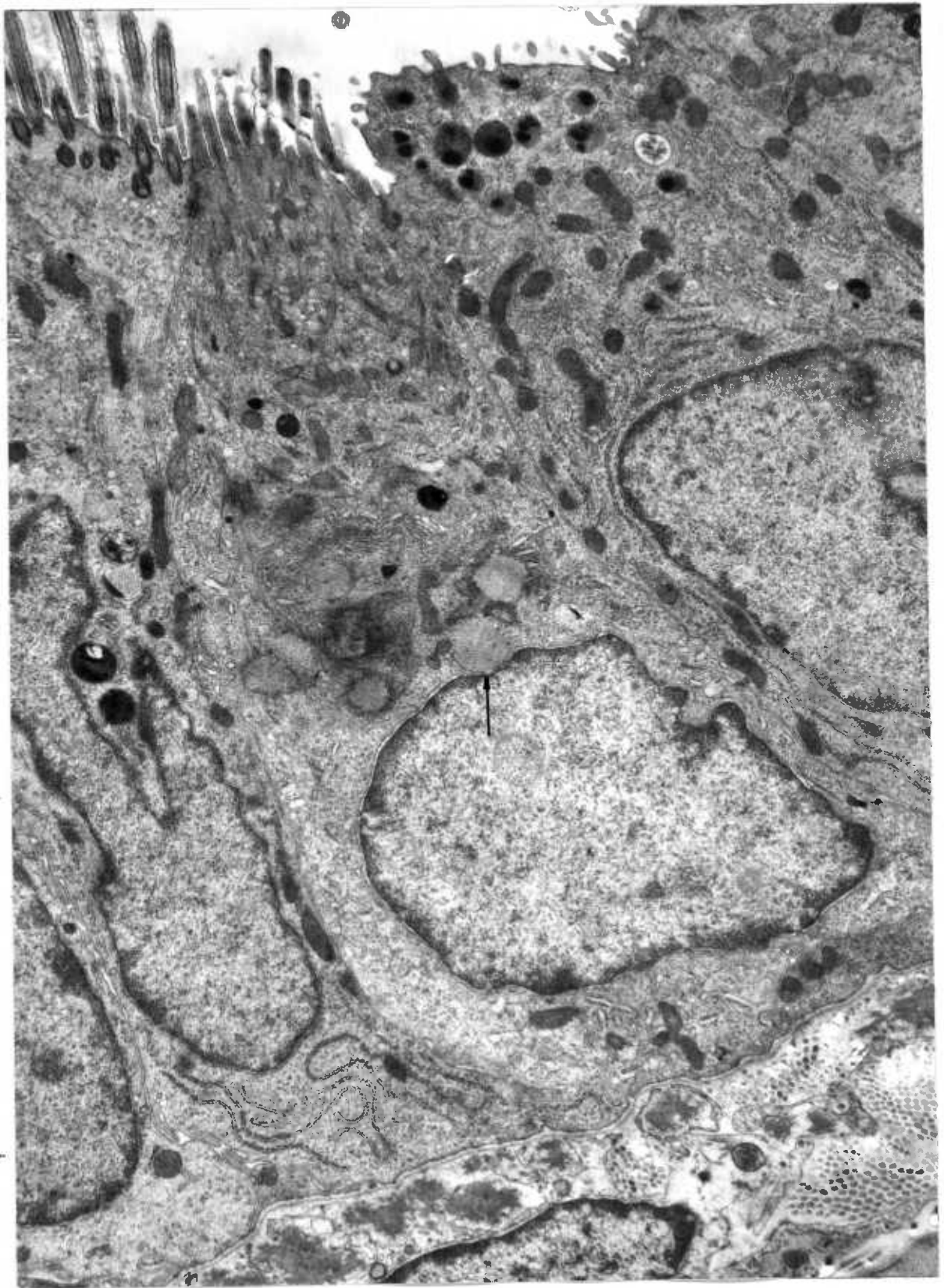


Fig. V-15 illustrates a brush cell in bronchiolar epithelium (level 5) of a rat injected with IPN: the cell contains secretory granules in the Golgi region (arrow) x 12,000.



Fig. V-16 shows the Golgi region of the brush cell, depicted in the previous figure, at higher magnification: the Golgi is well developed and some of the granules have a distinct membrane (arrow). x 30,000.

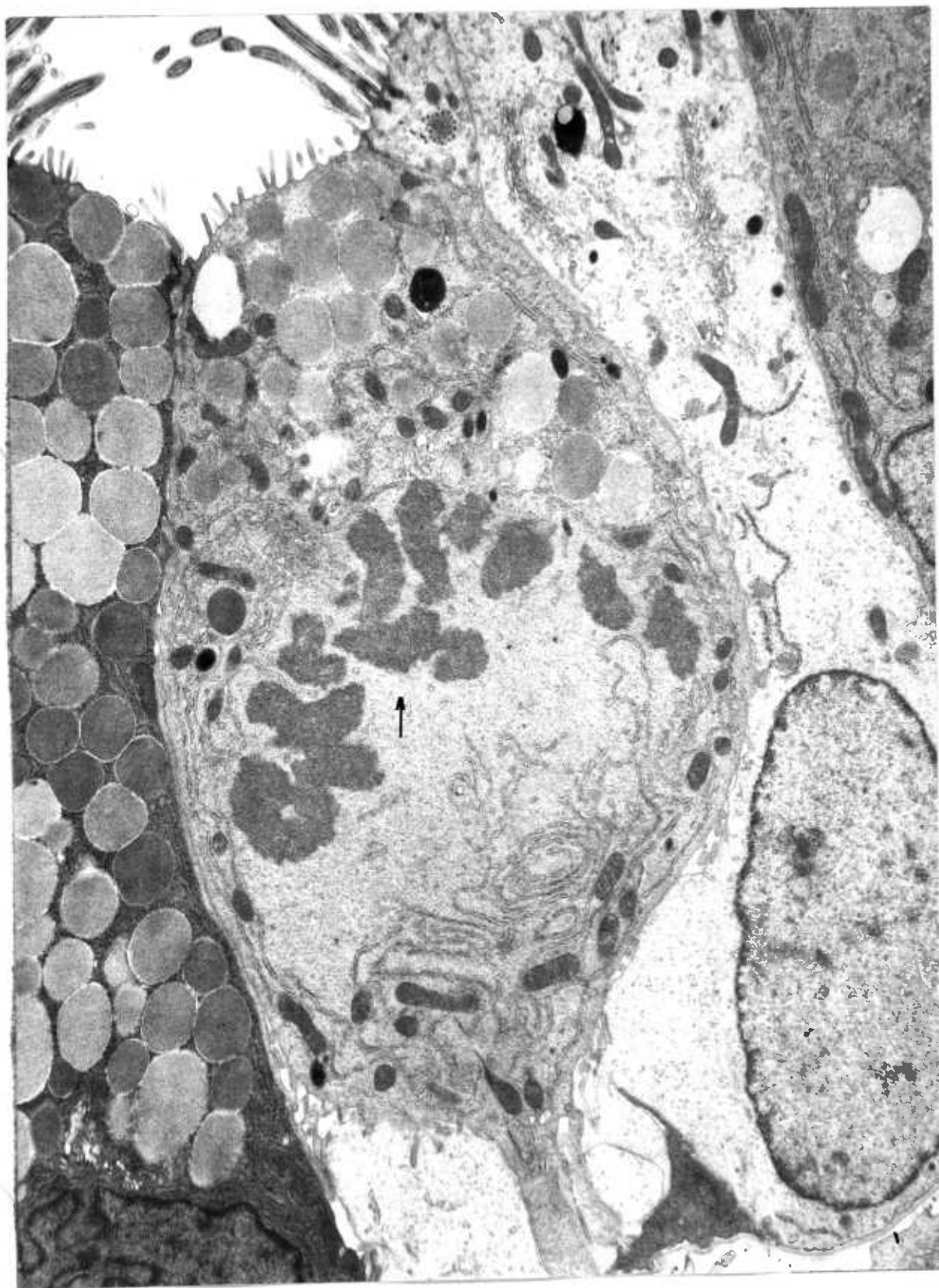


Fig. V-17 shows a nonciliated bronchiolar cell in division (level 5): chromosomes are cut in section (arrow) and the nuclear membrane is indistinct. The cell has many lucent secretory granules similar to those found in the adjacent goblet cell. IPN treated rat. x 9,000.



Fig. V-18 illustrates a nonciliated bronchiolar cell (level 5) in division: there are a few dense granules in the apical region (arrow). IPN-treated rat. x 13,500.

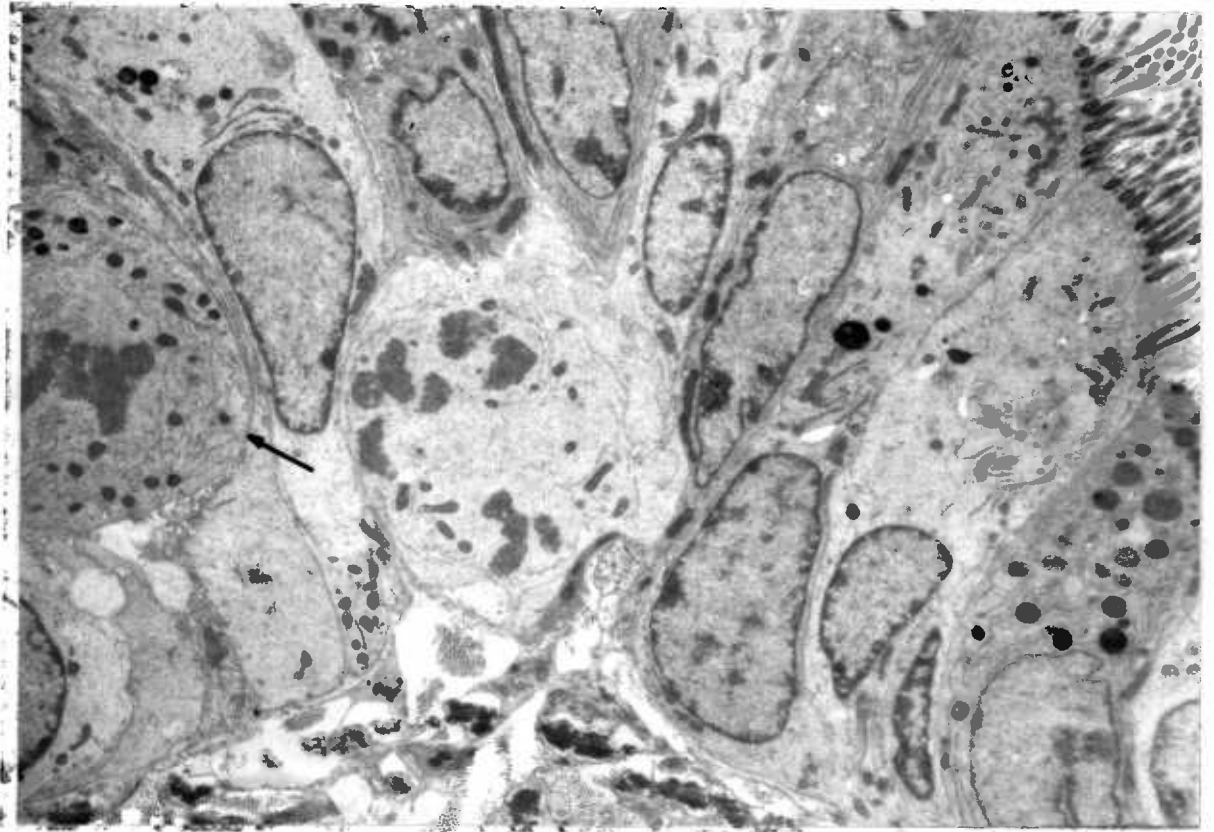


Fig. V-19 shows a migratory cell (probably a lymphocyte) in division (level 5): the cytoplasm is electron-lucent and contains few organelles. Part of a nonciliated cell in division is seen on the left (arrow). IPN-treated rat. x 5,250.

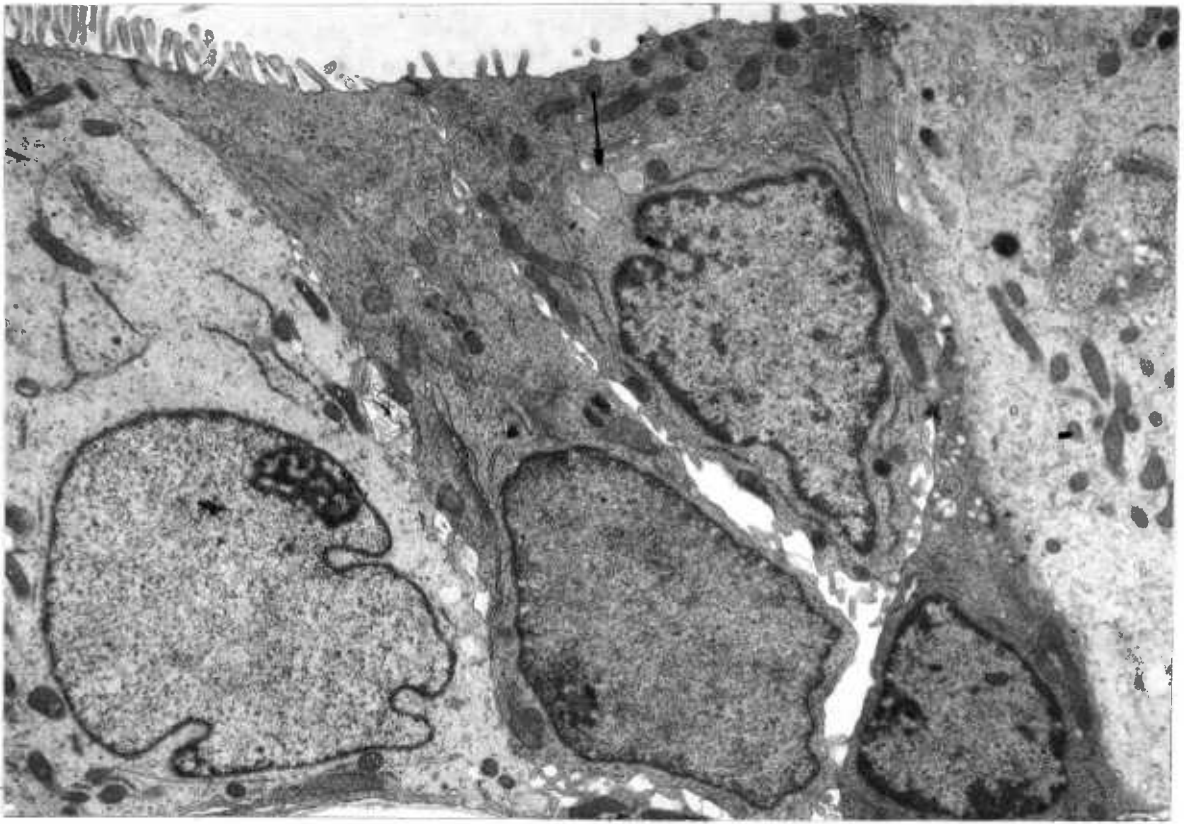


Fig. V-20 illustrates a nonciliated cell without granules found frequently in the bronchial epithelium (level 3) of rats injected with PCP. The cell on the right also has a reduced number of secretory granules (arrow). x 9,000.

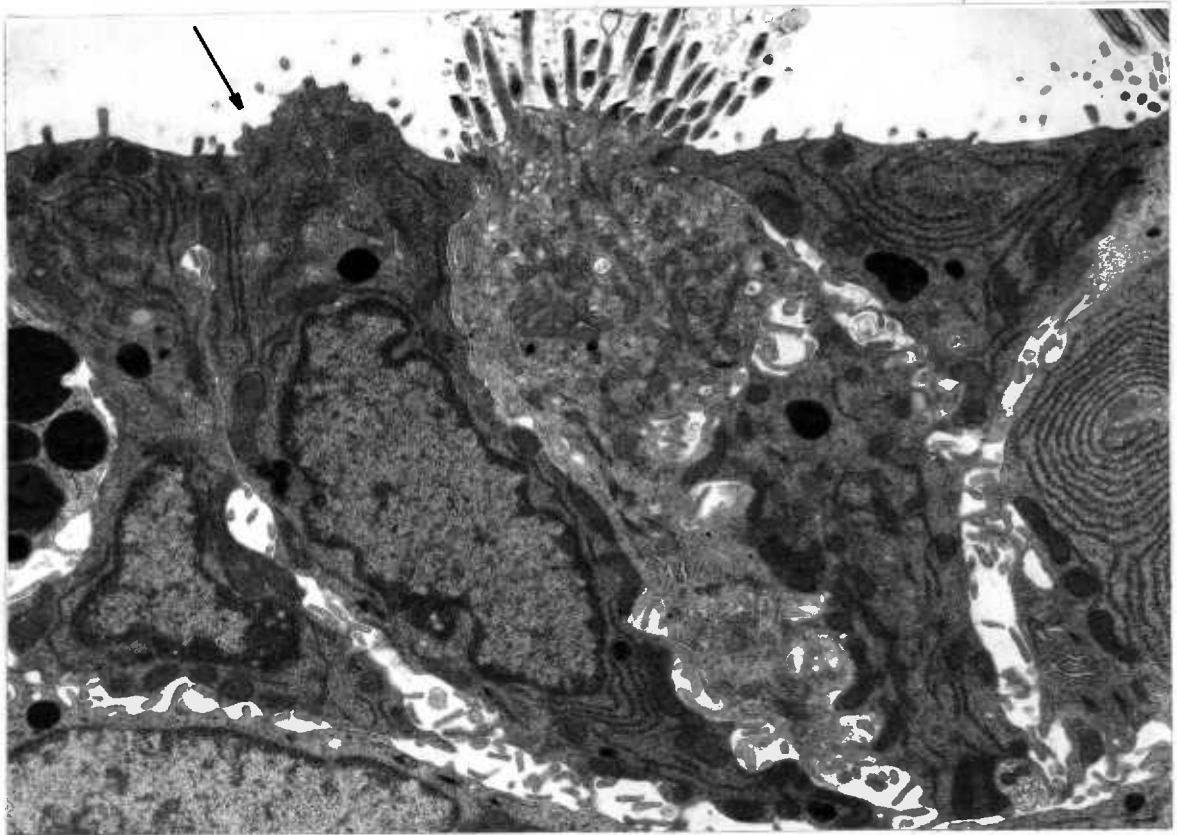


Fig. V-21 compares the nonciliated cell without secretory granules, found frequently in the epithelium of animals given PCP (arrow), with a brush cell, the type of cell without granules usually found in the controls. Level 2. x 9,000.

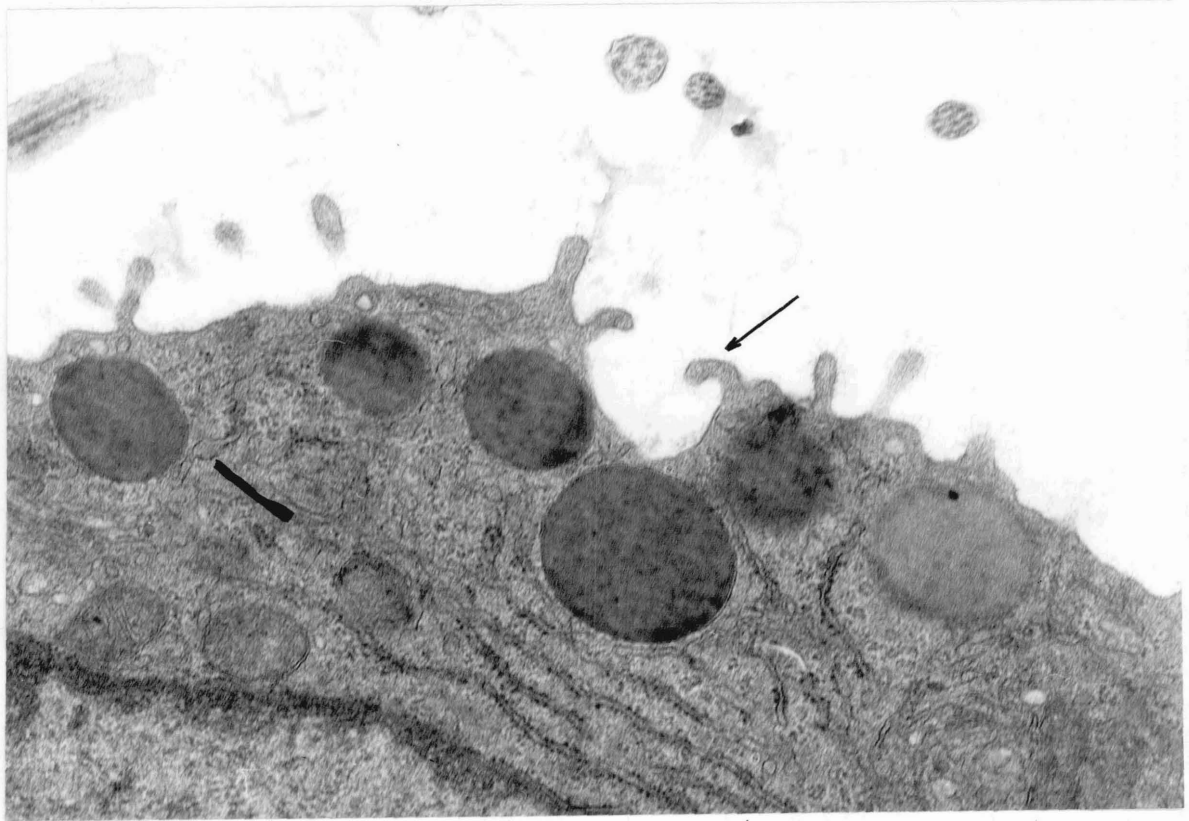


Fig. V-22 depicts the apex of a nonciliated cell in the epithelium (level 2) of an animal given PCP. The cell contains few dense granules: one appears to have been recently discharged leaving a small indentation at the cell surface (arrow). x 30,000.

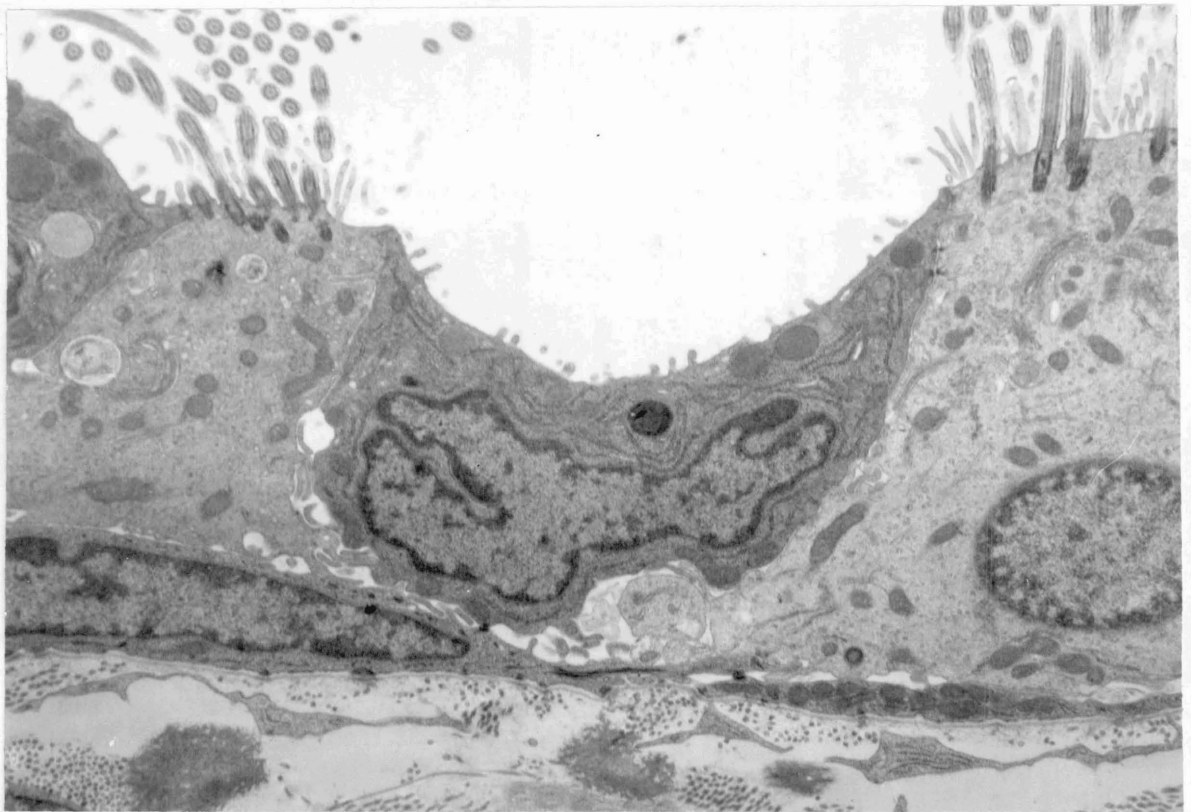


Fig. V-23 illustrates a nonciliated cell in the epithelium (level 3) of an animal given PCP: the cell contains a few dense granules and has a concave apex which might suggest recent exhaustion of its secretion. x 9,000.

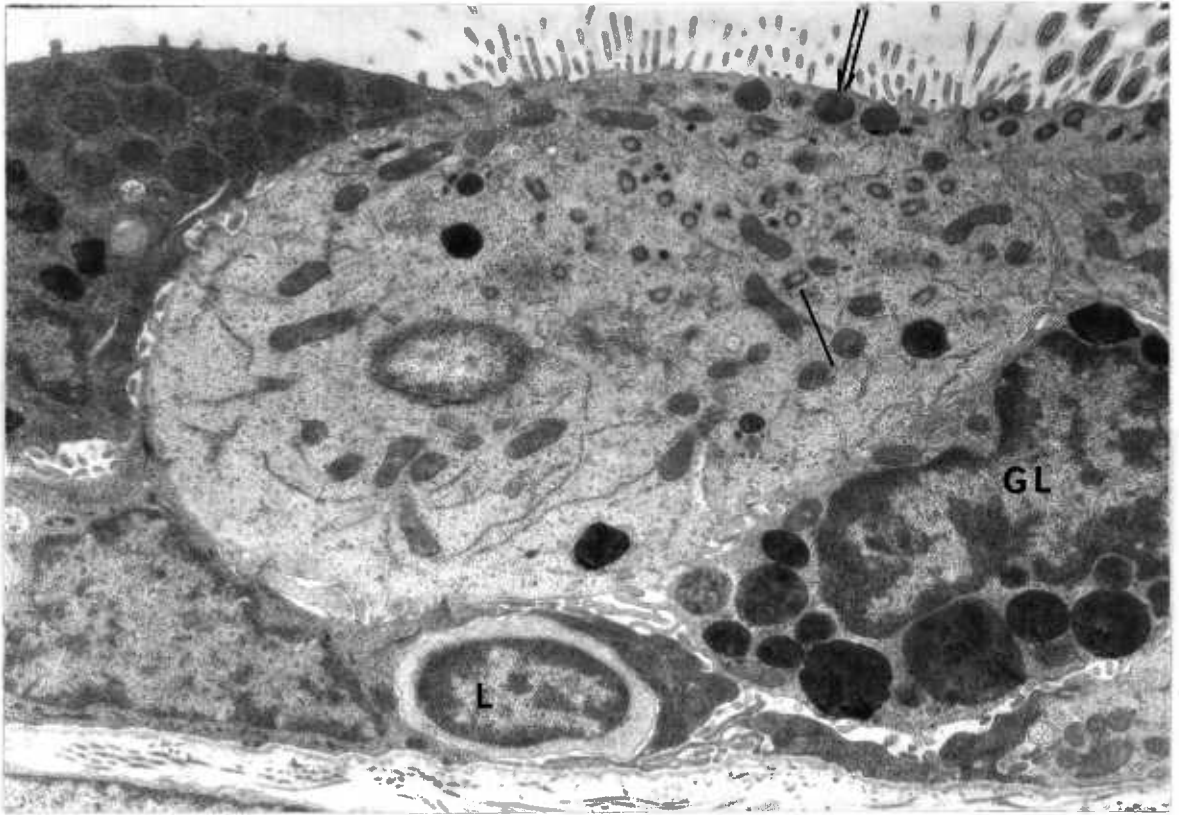


Fig. V-24 illustrates a cell without cilia but with lucent cytoplasm, long filiform processes and evidence of ciliogenesis. The cytoplasm contains many centrioles (single arrow) as well as three dense secretory-like granules (double arrow). Level 3 - PCP-treated rat. x 9,000.