A QUANTITATIVE ANALYSIS OF THE EFFECT OF TOBACCO SMOKE AND PHENYLMETHYLOXADIAZOLE ON RAT AIRWAY EPITHELIUM

Thesis submitted for the Degree of Master of Philosophy in the University of London

by

ROSEMARY CRISTIAN JONES, FIMLS.

Department of Experimental Pathology
Cardiothoracic Institute
Brompton Hospital
University of London

August 1976

ABSTRACT

Irritation of airway epithelium by tobacco smoke increases goblet cell number, modifies the type of glycoprotein produced and leads to hypertrophy of the surface epithelium and submucoeal glands. Each of these are features of human airway disease and have been produced experimentally.

A combination of Alcian Blue-periodic acid Schiff techniques and quantitative methods of analysis have demonstrated, in the specific pathogen-free rat, the way the glycoprotein changes within a goblet cell and within the cell population after inhalation of tobacco smoke - with or without the addition of the anti-inflammatory agent phenylmethyloxadiazole (PMO).

Increase in goblet cell number occurs earlier than previously shown. Change in glycoprotein synthesis, however, precedes increase in goblet cell number and thus is the most sensitive and earliest index of response of this cell population to irritation.

The range of intracellular combinations of glycoprotein within rat airway goblet cells has been established. In the normal, the extrapulmonary airway goblet cells produce mainly neutral glycoprotein alone or neutral with acid while, in the intrapulmonary airway, most cells produce only acid or a mixture.

Quantitative analysis has shown that conversion of one goblet

cell type to another, when caused by irritation, follows certain pathways. Initially, throughout the airways, cells produce both acid and neutral or acid glycoprotein alone. Ultimately, cells produce only acid glycoprotein - sialidase resistant sialomucin - and the amount of intracellular secretion is increaeed. Thus, in some airway regions, there is finally a shift from production of neutral glycoprotein while, in others, normal production is maintained. Modification of glycoprotein occurs in both existing and newly formed cells.

In the extrapulmonary airways, PMO protects against increase in goblet cell number. It does not protect against change in glycoprotein; it partially protects against epithelial hypertrophy but, paradoxically, produces greater increase in intracellular glycoprotein within goblet cells and gland hypertrophy.

CONTENTS

Page

LIST OF TABLES

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

LIST OF ILLUSTRATIVE MATERIAL

CHAPTER IV

CHAPTER V

CHAPTER I

HISTORICAL INTRODUCTION

Since the respiratory surface is continuous with the outer surface of the body, and thus with Man's environment, a system of protection is necessary for the delicate airway mucosa and alveolar surface of the lung against airborne and noxious agents - such as viruses, bacteria or atmospheric pollutants. Foreign particles, which either escape or by-pass the efficient filtering system of the upper respiratory tract, enter the lower airways. Here, mucus plays an important role in providing a mechanical barrier between airborne particles or gaseous products and cilia, as well as the apical cell membrane, and by trapping foreign particles.

Mucus does not form a continuous blanket across the epithelial surface (Iravani and van As, 1972), rather there is a two-phase system in which the epithelial surface is covered by a fluid layer, of low viscosity, with streaks of mucus present on its surface (Lucas and Douglas, 1934). Mucus and particulate matter are propelled along the fluid layer by the whip-like action of the cilia (Sleigh, 1974) and perhaps even "clawed" forward by tiny projections at the cilia tips (Jeffery and Reid, 1975). Usually, after passing through the larynx, such secretions are swallowed and any foreign material is effectively removed from the respiratory system. Mucus is essential to the transport system within the airways since the clearance of particles is no longer effective in its absence, even though the cilia still beat (Sade, Eliezer, Silberberg and Nevo, 1970). Other roles for the

function of mucus within the airways have been postulated, including the regulation of heat and water exchange and control of serum transudate through the airway mucosa (Negus, 1963).

In the normal airway only the amount of secretion necessary for the transport system to function efficiently is produced - by mucus-secreting cells of the surface epithelium of the airways (goblet cells) and of the submucusal gland (mucous and serous cells), and from a small amount of serum transudate (Lopez-Vidriero, Das, Picot, Smith and Reid, 1976).

Hypersecretion of mucus is characteristic of certain airway disease - in human chronic bronchitis increase in the volume of secretion is the basis of the clinical definition of the disease (Medical Research Council Report, 1965) and results directly from structural (Reid, 1965; de Haller and Reid, 1965) and metabolic changes (Sturgess and Reid, 1972; Coles and Reid, 1976) in mucus-secreting cells,and from increase in serum transudate (Bonomo and D'Addebbo, 1964).

Increase in the number of goblet cells of the surface epithelium, and their appearance in distal airway regions, are characteristic changes in human hypersecretory states. It is this cell and the nature of its secretory product that are the subject of this study, which attempts to trace their pattern of change within the airways of an experimental animal model. The animal used

is the rat, in which changes within the airway epithelium are induced by inhalation of tobacco smoke.

The introduction to this study includes five main sections:

Secretory cells of airway epithelium

Epithelial glycoproteins and their histochemical identification

Histochemistry of the goblet cell of airway epithelium using AB-PAS techniques

Animal models of airway disease
Part I - Epithelial changes induced by tobacco

Animal models of airway disease
Part II - Epithelial changes induced by irritant gases and other agents

SECRETORY CELLS OF AIRWAY EPITHELIUM

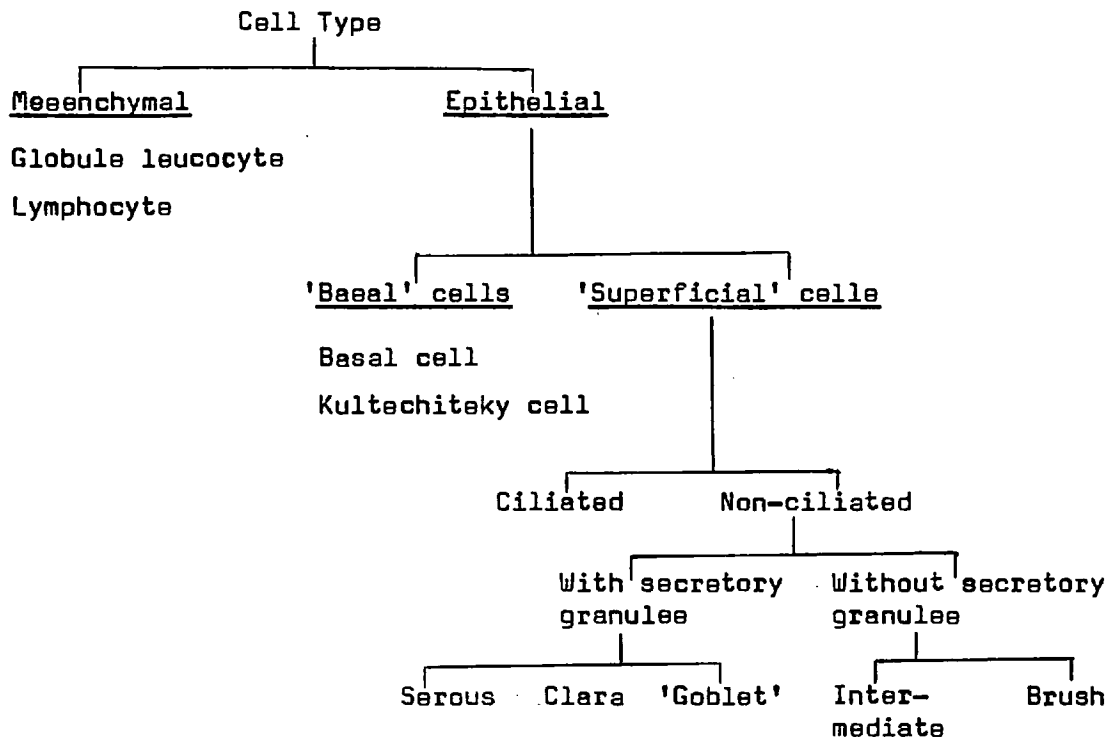
Airway Epithelial Cell Types

As early as 1602, Laurentius described a membrane lining the wall of the trachea and bronchus. In 1712, Morgagni described the membrane in more detail, including the tracheal gland and its duct but not until the 19th Century were cell types within the membrane identified. Ciliated cells were first described by Henle (1837) and goblet cells - chalice or beaker cells - by Knauff (1867), Schulze (1871) and Frey (1874). The studies of Frankenhaeuser (1879) and Waller and Björkman (1882) additionally identified basal cells and spindle-shaped cells (now considered to be the intermediate cells) between cells in the basal layer and the ciliated and goblet cells.

The presence of at least four cell types, including a single secretory cell, has been recognised within the airway epithelium since the 1870s. Not until recently, with the greater resolution of the electron microscope, were further cell types recognised.

Dalhamn (1956) and Rhodin and Dalhamn (1956) first described the ultrastructure of the cells and cilia of rat airway epithelium. Currently, 10 cell types - 8 epithelial and 2 mesenchymal - are recognised within rat airway epithelium, although not all are found in every species (see Jeffery and Reid, 1975).

Theee 10 cell typee are illustrated below:



Three secretory cell typee are now recognised within rat airways by their ultrastructural characteristics.

The airway epithelium is thus a complex structure of many cell types, each with a different function. Both the structure and function of the airway epithelial celle in eeveral mammalian epeciee have been the eubject of a recent review (Breeze, Wheeldon and Pirie, 1976). It is the ultraetructure of the secretory cell population that is of particular relevant to the present study. Other cell typee - such ae the baeal and intermediate cell - aleo are referred to in the

Discussion of the present findings, in Chapter VI, and it is for this reason that the following sections are included on cell frequency and secretory cell ultrastructure.

Frequency of Cell Types in Rat Airway Epithelium

Using 1 μm toluidine blue stained sections, Jeffery and Reid (1975) established the frequency of the various cell types in rat airway epithelium. This was a necessary preliminary to their study of the cell of origin and the pattern of development of new secretory cells within airway epithelium after irritation. From these findings of cell frequency the epithelium of the extrapulmonary airways was described as "pseudo-stratified, ciliated and columnar" and that of the intrapulmonary airways as "simple, ciliated and cuboidal".

In sections 1 μm thick and stained with toluidine blue, 5 cell categories are distinguished: (i) basally situated cells, (ii) ciliated cells, and then non-ciliated cells (iii) without secretory granules, (iv) with densely stained granules and (v) with unstained granules. Categories (i), (iii) and (iv) thus include more than one cell type (vide infra).

The frequency of the basally situated cells (basal and Kultschitsky cells) decreases from 37% of the total epithelial cell population in the trachea, to less than 1% in the peripheral airways.

Ciliated cells are present throughout the airway, increasing in number between the proximal and peripheral airway region: they comprise between 17 - 35% in the extrapulmonary airways and increase to 65% in the bronchioli.

Within the extrapulmonary airway epithelium the number of non-ciliated cells without secretory granules (intermediate and brush cells) decrease from between 27 - 15% but, within the lung, they then maintain roughly their proportion of the total cell population (about 15%).

Cells with densely stained granules (serous or Clara cells) are frequently found at each airway level (about 22%), while those with unstained but visible granules ('goblet' cells) are rare ($<1\%$), although present at each level of the airway.

From ultrastructural studies of epithelial cell types it is known that those cells with granules that stain with toluidine blue in the extrapulmonary airways are serous cells, in the proximal regions of the intrapulmonary airways either the serous or Clara cell, and in the peripheral airways the Clara cell alone.

Ultrastructure of Secretory Cells in Rat Airway Epithelium

The following features of the secretory cells within rat airway epithelium led Jeffery and Reid (1975) to class them separately:

Serous cell

The serous cell of the epithelial surface was characterised by electron-dense cytoplasm with much endoplasmic reticulum. The nucleus was irregular in shape, found toward the base of the cell and, as in most secretory cells, the Golgi apparatus was just superficial to it. The apical region of the cell contained secretory granules. Each granule was round, limited by a membrane and, after fixation with glutaraldehyde and osmium tetroxide, electron-dense. Each measured about 600 nm in diameter and varied in number between 1 and 35 per cell. Even when only a few granules were present, these also were found in the apical region of the cell.

'Goblet' cell

The structure of their 'goblet' cell resembled that of the serous cell in the density of its cytoplasm, the position and outline of its nucleus and the presence of a well-developed Golgi apparatus. In contrast, the granules of this cell were electron-lucent, each had an incomplete membrane and often the granules were confluent. The granules were larger than those of the serous cell.

Clara cell

The structure of the Clara cell resembled that of the serous in the electron-density of its secretory granules and the irregularity of its nuclear outline, but differed in that its

cytoplasm was of low electron-density and contained much smooth endoplasmic reticulum. The secretory granules were smaller than those of the serous cell and although they were bound by a discrete membrane their outline was irregular.

Correlation between Secretory Cell Types identified by Electron and Light Microscopy in Rat Airway Epithelium

From ultrastructural studies, the type of granule distinguishes the 'goblet' from the 2 other secretory cell types present within rat airway epithelium. While the serous and Clara cells may be distinguished from each other by some difference in granule number, it is more by difference in their cytoplasm.

Serous and 'goblet' cells

Jeffery and Reid (1975) suggested both from their findings and from evidence from other studies (Staley and Trier, 1965; Moe, 1968), that the serous cell (with electron-dense granules) corresponded to the cell staining with periodic acid Schiff (PAS) in light microscopy sections and the 'goblet' cell (with electron-lucent granules) to that staining with Alcian Blue (AB). These are the 2 essential stains used in the present study. The difference in the density of the granules in tissue prepared for electron microscopy, that was at one time considered to be an artefact of fixation (Hally, 1958), is now no longer accepted (Staley and Trier, 1965).

Clara cell

In some studies the granules of the Clara cell have been reported to stain with PAS (Cutz and Conen, 1971) but not in others (Niden, 1967). Certainly in the present study, in the most peripheral region of the intrapulmonary airway epithelium in the rat, cells with very small apical caps of intracellular granules staining with PAS frequently have been seen; and, indeed, in the present study have been found to comprise - at this level of the airways - a specific secretory cell population. That the Clara cell does not contain acidic groups has been verified by its failure to stain either with mucicarmine or with AB (Cutz and Conen, 1971).

Comment on the nature of the secretory granules of the Clara cell

Although it is accepted that the serous and 'goblet' cells secrete glycoproteins, the former mainly neutral and the latter acid, the nature of the secretory product of the Clara cell has not been clearly established. For this reason the following brief description of current opinion is included. From the most recent study it would seem that the granules of the Clara cell may incorporate at least both protein and a sugar (Petrick and Collet, 1974).

Digestion by papain has indicated the presence of a protein within the granules of the Clara cell (Cutz and Conen, 1971). Bound lipids and phosphoglycerides also have been demonstrated (Niden

and Yamada, 1966). Azzopardi and Thurlbeck (1969) and Cutz and Conen (1971) considered that the cell might contain a lipoprotein while other studies suggested the cell to be the source of pulmonary surfactant (Niden, 1967; Etherton and Conning, 1971). A more recent study by Petrik and Collet (1974) failed to show uptake of tritiated choline by Clara cell granules in the mouse. Since choline is a specific precursor of lecithin (and therefore of dipalmitoyl lecithin - the main component of surfactant), they considered that surfactant was not synthesised by the Clara cell. Other tritiated compounds - leucine and galactose - were incorporated by the Clara cell granules, suggesting the synthesis of a secretion containing protein and, since acetate also was incorporated by the cell, possibly cholesterol. Petrik and Collet suggested that in the bronchioli the Clara cell secretion contributed to the formation of the basal layer (the "hypophase" of the alveolar lining - see Scarpelli, 1968) and that the Type II pneumonocyte, in addition to contributing to the basal layer of fluid, produced a surface film one molecule thick with tension-reducing properties. While the nature of the granule of the Clara cell requires further study, the possible presence of protein and sugar would explain the staining of the cell, in light microscope studies, with PAS.

EPITHELIAL GLYCOPROTEINS AND THEIR HISTOCHEMICAL IDENTIFICATION

Epithelial Glycoproteins

The important constituents of epithelial mucus in establishing its rheological properties are the glycoproteins. They are characterised by a single polypeptide chain with branched oligosaccharide side chains and differ in structure from the other major group of polypeptide-sugar compounds - the glycosaminoglycans. The latter are present in connective tissue, and are characterised by linear side chains of alternating sequences of sugar residues containing uronic acid (primarily hexosamine and hexuronic acid residues). Of the amino-acids, large amounts of threonine and serine are present and the following carbohydrates are present in glycoproteins from respiratory airway epithelium: N-acetylneuraminic acid, fucose, galactose, N-acetylglucosamine and N-acetylgalactosamine.

Epithelial glycoproteins may or may not contain acid radicles, those without being termed neutral. The basic structure of the neutral and acid glycoprotein is otherwise similar. Of those glycoproteins containing acid groups, N-acetylneuraminic acid (synonym sialic acid) and sulphate are the only acid radicles identified in man.

Spicer, Leppi and Stoward (1965), in their classiciation of glycoproteins, suggested that those containing sialic acid and sulphate esters be described respectively as "sialomucins" or "sulphomucins" and these terms of reference are now commonly adopted.

Sialomucins may be further subdivided into those in which the sialic acid is either sensitive or resistant to removal from the glycoprotein structure by a sialidase (Warren and Spicer, 1961). The basis, however, for the resistance of some sialomucin to the enzyme is not clear. An increase in resistance has been attributed to the presence of O-acetyl groups on the sialic acid molecule (Faillard, 1959), but bovine cervical mucus is not O-acetylated and yet is sialidase resistant. It is considered that steric arrangement and perhaps protein binding may contribute to resistance to the enzyme. Two forms of sialic acid have been identified in bovine submaxillary gland mucin - N-acetyl O-diacetylneuraminic acid and a N,O-diacetyl form (Blix and Lindberg, 1960) and histochemical studies have demonstrated the presence of both sensitive and resistant forms (Quintarelli, Tsuiki, Hashimoto and Pigman, 1961; Jones and Reid, 1973a). Neuberger and Marshall (1966) comment that both types of O-acetylated neuraminic acid are "slowly susceptible" to sialidase but that some are more speedily cleaved. It may be that the sialic acid of bovine submaxillary gland with the extra acetyl group is the more resistant to digestion. Biochemical and

histochemical studies both have shown that de-acetylation with an alkali can change a 'resistant' to a 'sensitive' form (Gibbons, 1963; Ravetto, 1968; Jones, 1971).

In summary, at least 4 glycoproteins may be identified histochemically - (i) neutral, (ii) sialomucin sensitive to the enzyme sialidase, (iii) sialomucin resistant to sialidase, and (iv) sulphomucin. Each of these groups are identified in the present study.

Histochemical Identification of Epithelial Glycoproteins

A wide range of techniques may be used to identify epithelial glycoproteins histochemically, including basic dyes, enzyme digestion, autoradiography, fluorescent antibody methods and chemical modification of the tissue such as methylation and saponification procedures (see Spicer and Henson, 1967). An earlier study within the Department (Lamb, 1969) considered many of the methods available for the demonstration of glycoproteins and selected a small group of histochemical methods that allowed the identification of each variety of glycoprotein in airway epithelial secretory cells. In a later study (Jones, 1971; Jones and Reid, 1973 a & b) concerned with the effect of pH on AB staining, it emerged that, by the combined use of variations of the Alcian Blue-periodic acid Schiff (AB-PAS) techniques, it was possible to identify the 4 types of glycoprotein present (vide supra). The basis of the staining of tissue groups by these techniques and the

background to their introduction as histochemical methods are given with the details of the methods in Chapter II (p. 83).

HISTOCHEMISTRY OF THE GOBLET CELL OF AIRWAY EPITHELIUM USING AB-PAS TECHNIQUES

Normal Airway Epithelium

Rat

In 1950, Leblond applied the PAS stain to a wide range of tissues in the rat and reported that the granules in goblet cells of both the tracheal and bronchial epithelium stained intensely.

In a study which employed the use of AB-PAS staining, sialidase digestion and autoradiography McCarthy and Reid (1964a) further reported, in rat airway epithelium, the staining of the goblet cells and identified their glycoproteins. Throughout the airways, some goblet cells stained blue after AB-PAS staining (acid glycoprotein), while others stained purple (acid and neutral glycoprotein) or red (neutral glycoprotein). In the tracheal epithelium stained with AB-PAS, most goblet cells stained purple and a few blue, or red. Of 1,000 counted cells, sialidase had no effect on the percentage staining purple, and many cells took up ^{35}S-sodium sulphate. It was concluded that in this cell population the acid groups produced, included sialidase resistant sialomucin and sulphomucin. In the intrapulmonary airways,

the goblet cells found grouped at the bifurcations of the axial and lateral pathways mostly stained blue although other cells staining purple, or red, were present. Here, as in the trachea, the staining of most goblet cells was unaffected by sialidase, while others changed from blue or purple staining, to red. Goblet cells staining blue, and not affected by sialidase, also took up labelled sulphate. Thus the acid glycoproteins of this goblet cell population were identified as mainly sialidase resistant sialomucin and some sulphomucin. In the bronchioli the few goblet cells present stained blue or purple and, after sialidase, became red - a sialidase sensitive sialomucin.

Sturgess (1970) and Sturgess and Reid (1973) reported 2 histochemical types of goblet cell in rat airway epithelium (cells containing acid or neutral glycoprotein) and further characterised the cells by their amount of glycoprotein. Goblet cells were described that were either full of secretion or contained only a small amount (in the apical region of the cell). This identification of goblet cell types was a necessary preliminary to the study of changes in the airway epithelium after drug administration.

Mouse

In the same study, McCarthy and Reid (1964a) reported the staining,and identified the glycoproteins of goblet cells in the airway epithelium of the mouse. After AB-PAS, goblet cells in

both the trachea and bronchial epithelium stained red more often in the mouse than in the rat, and those that were blue or purple became red after sialidase. There was no uptake of ^{35}S-sodium sulphate and it was concluded that only sialomucin sensitive to sialidase was produced.

Man

In a study of the goblet cells of human airway epithelium, McCarthy and Reid (1964b) reported that few cells stained red with AB-PAS; most stained blue or heliotrope (a blue and red mixture that corresponded to the purple-staining goblet cells of the rat and mouse). After sialidase, some of the cells staining blue or heliotrope became red, others changed from blue to heliotrope, while yet others were unaffected. Thus the goblet cells staining blue or heliotrope in sections stained with AB-PAS, could contain sialidase sensitive sialomucin. While both sialidase sensitive and resistant sialomucin were identified, the presence of sulphomucin was not established. Lamb (1969), with the use of staining techniques and uptake of ^{35}S-sodium sulphate, later identified sulphomucin in goblet cells throughout human airways.

Fetal Airway Epithelium

Rat

In their study, Jeffery and Reid (1976) described mainly the ultrastructure of the airway epithelium of the developing fetal rat.

After 21 days' gestation, they reported that goblet cells in both the trachea and proximal regions contained but little secretion but that, within some cells, both acid and neutral glycoprotein could be identified.

Man

In the developing airway epithelium in man, the goblet cell glycoproteins also have been described (de Haller, 1969). Secretory cells were present in the epithelium by the 13th week of intra-uterine life. At this time, both acid and neutral glycoprotein were present within goblet cells, and some cells containing neutral glycoprotein also were reported to have an apical cap of intracellular acid glycoprotein. The number of goblet cells producing neutral or acid glycoprotein was roughly equal. The pattern of secretion by the goblet cells of the human airways then changed during childhood-when the pattern more closely resembled that of the adult - with cells producing mainly acid glycoprotein.

ANIMAL MODELS OF AIRWAY DISEASE

Part I - EPITHELIAL CHANGES INDUCED BY TOBACCO

The habit of tobacco smoking has been established as a major contributing factor in the development of chronic bronchitis (Royal College of Physicians, 1972). Histological studies of human post-mortem material have shown that the thickened tracheal and bronchial epithelium of smokers contain significantly more goblet cells distended with secretion and fewer ciliated cells, as compared with the epithelium of non-smokers (Chang, 1957; Ide, Suntzeff and Cowdrey, 1959). The submucosal gland also has been shown to be increased in smokers (Ide, Suntzeff and Cowdrey, 1959; Thurlbeck, Angus and Pare, 1963; Field, Davey, Reid and Roe, 1966).

The Effect of Tobacco Smoke on Airway Epithelium - Goblet Cell Hyperplasia and Gland Hypertrophy

Rat

Mellors (1958) reported an increase in goblet cell number in rat airway epithelium after exposure to 200 cigarettes (given between 1-4 daily) although his findings were not quantified. Fluorescent staining of frozen tissue sections also showed an accumulation of smoke products in the mucus overlying the epithelium, with penetration into the apical region of the cells.

Lamb and Reid (1969a) exposed rats to smoke either from standard cigarettes (flue-cured tobacco) or from specially prepared cigarettes made from cigar shredded tobacco (air-cured

tobacco). Animals were exposed daily to 5, 10 or 20 cigarettes over a period of weeks and quantitative analysis showed that each tobacco produced a dose-related increase in goblet cells. The greatest increase in their number was in the trachea and in the proximal regions of the lung. They found little difference between the effect on the goblet cell number of either type of tobacco but mentioned that the number of mitoses was greater in animals exposed to the flue-cured cigarettes as compared with the cigar tobacco. In the rats exposed to only 10 cigarettes a day, and not at the other dose levels, they reported that there was a change from sialomucin to sulphomucin but no quantitative analysis of goblet cell glycoproteins was included in the study.

Lamb

In lambs exposed to tobacco smoke administered via a tracheostomy tube, Mawdesley-Thomas and Healey (1973) measured the amount of mucus collected and quantitatively assessed change in goblet cell number and gland hypertrophy by image analysis. Results for the goblet cells were inconclusive with increase in cell number in some animals but the gland showed a significant and dose-related increase in size. The results of a similar and earlier experiment, while not statistically significant for the goblet cell population, showed a trend towards an increase in their number with more cells producing acid glycoprotein (Mawdesley-Thomas, Healey and Barry, 1971).

Other studies

Other studies have been concerned with the effect of tobacco smoke on mucus transport within airways. Experimental studies have established that tobacco smoke inhibits ciliary activity, slowing the speed of the beat and its direction and thus inhibiting mucus transport within airways (Falk, Tremer and Kotin, 1959; Dalhamn, 1964; Kaminski, Frencher and Calandra, 1968). Hilding (1956, 1961) has suggested that a reduction in mucus clearance would lead to accumulation of inhaled cigarette products which would prolong their effect on the cells of the airway epithelium leading to an increase in their potential carcinogenic effect.

The Effect of Tobacco Smoke on Airway Epithelium - Proliferation of Other Airway Cells

In addition to its effect on the mucus-secreting cells of airway epithelium, tobacco smoke produces cell proliferation. Many epidemiological studies have shown a correlation between tobacco smoking and development of lung cancer. Some of the studies based on statistical evaluation of data include those of Kennaway and Kennaway (1947), Sadowsky, Gilliam and Cornfield (1953) and Doll and Hill (1956). Histological studies also have shown that tobacco smoking produces cell proliferation in human airway epithelium. Auerbach and his colleagues reported an increase in the occurrance of basal cell hyperplasia in the bronchial epithelium of smokers

as compared with non-smokers; there was a loss of cilia and also stratification of the epithelium, the presence of atypical cells and squamous metaplasia (Auerbach, Petrick, Stout, Statsinger, Muesham, Forman and Gere, 1956; Auerbach, Gere, Forman, Petrick, Smolin, Muesham, Kassouny and Stout, 1957; Auerbach, Stout, Hammond and Garfinkel, 1961). Spain, Bradess, Tarter and Matero (1970) also have shown that the incidence of squamous cell metaplasia is increased at a younger age in smokers than in non-smokers.

Mouse

Proliferative changes in the airway epithelium of mice exposed to tobacco smoke have been reported by Leuchtenberger, Leuchtenberger and Doolin (1958). Together with atypical basal cell hyperplasia and squamous metaplasia there was an increase in deoxyribonucleic acid (DNA) and nuclear volume. An early increase in protein also has been reported to be followed by a gradual increase in DNA, both changes being reversed by stopping of exposure (Leuchtenberger, Leuchtenberger, Zebrun and Shaffer, 1960). Later studies reported an additive effect of viral infection with exposure to smoke (Leuchtenberger, Leuchtenberger, Ruch, Tanaka and Tanaka, 1963).

Rabbit

Similarly, in the rabbit, Holland, Kozlowski and Booker (1963) reported generalised basal cell hyperplasia with and without atypical cells after exposure to cigarette smoke over long periods - 2-5 years.

Dog

Auerbach and his colleagues have reported changes produced experimentally in the bronchial epithelium of beagle dogs that were similar to the changes reported in their earlier studies of post-mortem material of human smokers. Tobacco smoke (also given via a tracheostomy tube) produced proliferation of the bronchial epithelium and atypical cells (Auerbach, Hammond, Kirman, Garfinkel and Stout, 1967). In a further study, the presence of invasive bronchiolar-alveolar tumours (12 dogs) and early invasive squamous cell carcinoma (2 dogs) were reported after exposure to cigarette smoke without filters: cigarettes with filters produced non-invasive bronchiolar-alveolar tumours (Auerbach, Hammond, Kirman and Garfinkel, 1970).

Other studies

Proliferation of the bronchial mucosa has been produced in dogs by the repeated application of cigarette smoke condensate to the primary bronchus (Rockey, Spear, Ahn, Thompson and Hirose, 1962)

Other studies have reported similar histological findings in the rat, guinea-pig and rabbit (Blacklock, 1961) and, in the rat, (Stanton, Miller, Wrench and Blackwell, 1972) the appearance of metastases in other organs, including hilar and distant lymph nodes, kidney and bone.

Comment In the present study, the effect of tobacco on weight gain in rats is reported. This is routinely recorded in experimental

studies within the Department and is used as an index of the general health and response of the animals during exposure to an irritant. Haag, Larson and Weatherby (1960) and Elson and Passey (1963) have reported a reduction in weight gain in rats similarly exposed. The study of Elson and Passey (1963) confirmed that this was the result of a metabolic mediated effect and not of decreased food intake, since the control animals given the same amount of food gained relatively more weight than exposed animals.

...../Part II

ANIMAL MODELS OF AIRWAY DISEASE

Part II - EPITHELIAL CHANGES INDUCED BY IRRITANT GASES AND OTHER AGENTS

In 1932 Florey, Carlton and Wells published the findings of experiments carried out to determine the conditions of mucus discharge from airway goblet cells and submucosal gland, together with the histological changes. Histological examination of material showed that both vagal stimulation and administration of pilocarpine depleted the cells of the gland of their secretion, which was seen lying free in the lumen of the acini and in the gland ducts, but that the goblet cells retained their secretion. Attempts to study the goblet cells further, following the application of an irritant (mustard oil), gave inconclusive results, since there was desquamation of the epithelium. In discussion of these findings, further experiments were described which illustrated the effect of irritation upon the airway mucosa. Daily injections (between 5–14) of formalin (each of 3 cc at a concentration between $\frac{1}{4}$ - $\frac{1}{8}$%, injected into the airway via a glass tube passed through the glottis) produced changes in the epithelium of treated cats. Ciliated, columnar epithelial cells were replaced by cuboidal shaped cells resembling the transitional epithelium of the bladder, goblet cells were greatly increased in number and the gland showed evidence of exhaustion of secretion. From these studies and other observations of goblet cell increase in both airway epithelium in human bronchitis, and in a 'patch' of ileum inserted into the abdominal wall of a dog,

they concluded that chronic irritation of mucous membranes containing goblet cells resulted in an increase in their number.

The Effect of Sulphur Dioxide on Airway Epithelium

Rat

In 1963 Reid reported the results of exposure of rats to sulphur dioxide. Her aim was to produce in the laboratory animal, by repeated and prolonged exposure to an irritant, the changes seen in human airway epithelium in chronic bronchitis. After exposure to between 200 - 300 ppm for 5 hours a day over a period of 6 weeks, the number of goblet cells in the main bronchi was increased and the cells extended into distal lung airways. The importance of the study was the production of goblet cell metaplasia within the airways, in the absence of infection.

A further study of changes in rat airway epithelium after sulphur dioxide was made by Lamb and Reid (1968) in which histopathological changes in the surface epithelium and submucosal gland were measured. The main purpose of the study of the surface epithelium was to establish (i) whether destruction and desquamation of the epithelium necessarily preceded goblet cell increase, and (ii) the type of glycoprotein produced by the newly developing goblet cells. Both the change in number and histochemical changes within the goblet cell population were quantified.

Within the trachea and proximal airways, the first 4 days' exposure to the irritant produced a loss of the normal epithelial structure and its replacement with two layers of flattened cells, with a consequent reduction in goblet cell number. After 3 weeks this epithelium was replaced by one which was hypertrophied although occasional patches of the flat regenerating type were still present; but at no time was the epithelium denuded nor was there evidence of severe inflammation.

After 3 weeks of exposure to sulphur dioxide the goblet cell number throughout the airways was increased and the cells extended into peripheral airways. There was then a further increase, after 6 weeks' exposure, at each level of the airways save in the main bronchi, where the 3-week level was maintained. The increase in goblet cell number was accompanied by a relative increase in the number of cells producing an acid glycoprotein. By 3 weeks, almost all goblet cells in the proximal airways - which, in control animals, contained a sensitive form - now contained sialomucin resistant to sialidase. The newly appearing goblet cells in peripheral airways first produced sialomucin sensitive to sialidase and then (after 3 weeks' exposure) produced the resistant form. Increase in synthesis of sulphated glycoprotein sought by uptake of ^{35}S-sodium sulphate and autoradiography was reported for both the goblet cells and gland.

The number of cells in mitosis also was counted and within the surface epithelium their number was greatest after the first

4 days of exposure. Within the airway epithelium their number remained increased except within that of the distal airways. The number of cells in mitosis in the distal airways was reduced after 3 weeks but the number of goblet cells continued to increase through the 6 weeks of exposure to the irritant.

The results of the study confirmed that mitosis need not directly precede goblet cell increase and that newly formed cells changed their pattern of glycoprotein production. In addition, it was reported that the gland, in contrast to the epithelial surface, showed no evidence of damage during the period of exposure to the irritant. The duct cells even retained their cilia, but after 4 days - in both the ducts and acini - there was an increase in the number of mitoses which still was increased after 6 weeks of exposure, when the gland was hypertrophied.

The reversal of the changes produced within airway epithelium by exposure to sulphur dioxide has been followed. In her study, Reid (1963) found that increase in goblet cell number persisted for up to 3 months after the cessation of exposure and that, during the recovery period, the goblet cells became distended with secretion. In their later study Lamb and Reid (1968) confirmed that 35 days after the last exposure period goblet cells within the proximal airways were still increased in number and that a relative increase in production of sialidase resistant sialomucin was maintained.

Subsequently, Mawdesley-Thomas and Healey (1969) and Mawdesley-Thomas, Healey and Barry (1971), by the use of an image analyser, quantitatively assessed goblet cell hyperplasia in rat airway epithelium after exposure to sulphur dioxide. In both the tracheal and bronchiolar epithelium they reported a dose-related response to the irritant.

Quevauviller, Huyen and Garcet (1970) have reported the results of exposures of rats to sulphur dioxide (300 ppm) for periods between 80-195 hours. They found an 8-fold increase in the goblet cell number in the bronchial epithelium and that the concurrent administration of S-carboxy-methyl-cysteine prevented the appearance of some of the new goblet cells. Huyen (1973) briefly reported that its use also prevented change to sialidase resistant sialomucin and sulphomucin in goblet cells of the airway epithelium of rats similarly exposed.

Lamb

The effect of sulphur dioxide on airway epithelial secretory cells in the lamb also was reported in the study of Mawdesley-Thomas, Healey and Barry (1971). They found an increase in the relative number of goblet cells containing acid glycoprotein. The change in the nature of the mucus of the goblet cells, however, was thought to be due to the incorporation of sulphur dioxide directly into the mucus of these cells rather than a chemical alteration in the nature of the mucus produced by the cell.

Dog

A further species was investigated by Chakrin and Saunders (1974) who exposed beagle dogs to sulphur dioxide and reported histopathological changes within the airway epithelium. These findings were related to histochemical changes in a later study. Exposure produced a regional response in that, together with reduction in the number of goblet cells and an increase in ciliated cells at the proximal end of the segmental bronchi, there was an increase in the number of goblet cells at its distal end and in the bronchioli. In the trachea, the epithelium - this was squamous in type - showed a loss of cilia and of goblet cells. This type of epithelium extended to the lobar bronchus, with squamous hyperplasia in some places and thickening of the epithelium from 3 to 5 or 6 cell layers, and an increase in mitotic activity. None of these epithelial changes were quantified.

A further study also showed evidence of mucus hypersecretion, with mucus impacted in alveoli and in the lumen of the airways of dogs exposed to sulphur dioxide (Spicer, Chakrin and Wardell, 1974). The mucus within the goblet cells was found to stain only weakly, (staining black with the high iron diamine stain in control animals and grey in those exposed) but still produced sulphomucin. The goblet cells of the small airways contained more secretion than those of the large, but that their glycoprotein also stained less well than in control dogs. They concluded that exposure to the irritant had produced a decrease in the number of acidic groups within the goblet cells of the airway surface.

Other studies

In two studies reported, there appeared to be no increase in goblet cell number produced as the result of exposure to sulphur dioxide. In 1967, Goldring, Cooper, Ratner and Greenberg exposed Syrian hamsters to high doses (650 ppm) for periods of 12-212 days. The gas was given with and without the additional challenge of influenza virus, but they gave no report of goblet cell increase and in only 2 of the 78 animals exposed were there proliferative changes within the epithelium. Additionally, Lightowler and Williams (1969) reported a decrease in mucus flow rates, and histological changes within the lung, but no difference in the number of goblet cells in the epithelium of the trachea or main bronchi of control rats and those exposed to 200 ppm sulphur dioxide for 16 weeks. In the bronchiolar epithelium, development of bronchiectasis was associated with an increase in the number of goblet cells (with an increase in cells containing an acid glycoprotein). It would seem that, in the animals in which spontaneous infection had developed in small airways, high numbers of goblet cells in control animals equalled the effect of the irritant and that in the exposed animals there was no synergistic effect between infection and the effect of irritation.

The Effect of Other Gases on Rat Airway Epithelium

Chlorine

Exposure of rats to 40 ppm chlorine gas also has produced goblet cell hyperplasia and an extension of these cells into

peripheral airways (Elmes and Bell, 1963). Young rats (those below 20 weeks in age) showed the greatest difference from their corresponding controls; older animals showed only marginal differences from their control group because of goblet cell proliferation with increased age and the onset of spontaneous lung disease.

Nitrogen dioxide

In 1964, Freeman and Haydon reported goblet cell increase with no evidence of inflammatory cell infiltration in rats exposed to 25 ppm nitrogen dioxide for 40 days. Goblet cells were demonstrated by PAS staining of tissue sections but no further analysis of their glycoprotein was made. Similar changes in the epithelium of trachea, bronchi and bronchioli were reported after long-term exposures to a lower dose of nitrogen dioxide (15 ppm) by Freeman, Crane, Furiosi, Stephens, Evans and Moore (1972) but, again, no analysis of the glycoproteins was made.

The Effect of Other Agents on Airway Epithelium - Different Species

Isoprenaline

Rat Sturgess and Reid (1973) first reported the effect on the mucus-secreting cells of airway epithelium of the β-adrenergic drug isoprenaline. Other studies had already established that the drug produced increase in both the size and number of the secretory cells in salivary glands. Subcutaneous administration of the drug (x6 daily injections of 25 mg/100 g body weight) produced an increase

in the number of goblet cells throughout the airways. In the trachea, where the changes were quantified, there was a 4-fold increase in cells containing acid glycoprotein with no change in the number containing the neutral form. The number of cells containing both large and small amounts of acid secretion was increased but the type of acid glycoprotein was not analysed. The tracheal submucosal gland was also increased in size.

Pig Baskerville (1976) later adopted the use of isoprenaline to produce goblet cell hyperplasia and gland hypertrophy in the pig. Intramuscular injection (x6 daily injections of 75 mg to pigs weighing between 32-40 lbs) produced a significant increase in the total number of goblet cells in bronchial epithelium ($p < 0.001$). The cells contained acid glycoprotein that was a sialomucin. Many goblet cells also were full of acid secretion. In the recovery period after administration of the drug, the number of the full cells containing acid glycoprotein persisted up to 16 days, their number was then reduced but still somewhat above the control value 56 days after the last injection; and, by 84 days, they had regained the control level.

Methacholine

Cat Daily administration of methacholine to cats (3 mg/Kg in 3 doses), over a period of 90 days, recently has been reported to produce both goblet cell metaplasia and bronchial gland hypertrophy (Kleinerman, Sorensen and Rynbrandt, 1976). Changes were only

briefly described but it was reported that, while the glycoprotein of the gland was unchanged and mainly neutral, the goblet cells showed a loss of acid groups.

While many studies have described animal models of chronic bronchitis in which the airway changes characteristic of the disease have been produced, few have used the 'model' to study the pattern of development of the changes in mucus-secreting cells of the surface epithelium. In as much as the goblet cells of the airways have been studied, only limited quantitative analyses have been made of the change in glycoprotein accompanying goblet cell hyperplasia. Details of the pattern of change within the goblet cell population after irritation have not been established.

PROTECTIVE EFFECT OF ANTI-INFLAMMATORY AGENTS AGAINST AIRWAY CHANGES INDUCED BY IRRITANTS

Since 1966, Dalhamn and his colleagues have described the protective effect on airway epithelium exposed to irritants of certain anti-inflammatory agents. Dalhamn (1966, 1969) first reported that the anti-tussive drug oxalamine citrate reduced the degree of ciliostasis produced in cat trachea by tobacco smoke. The drug was found to be effective when given orally either before or after the exposure to the smoke, or when added to the tobacco.

Intraperitoneal administration also of oxalamine citrate to guinea-pigs was reported to 'protect' against acrolein-induced inflammatory changes within the airways and lung parenchyma (Dahlgren and Dalhamn, 1967) and against coughing induced by ammonia aerosol (Dahlgren and Dalhamn, 1967; Dalhamn and Raud, 1968).

Further study of the three components of oxalamine citrate - citric acid, diethylamine citrate and phenylvinyloxadiazole (PVO) - identified the latter as the component responsible for its protective effect against ciliostasis (Dalhamn and Rylander, 1971). Because PVO proved unstable and had a strong tendency to polymerise, a derivative - phenylmethyloxadiazole (PMO) - was prepared and tested at various concentrations and it was reported that the addition of 2% (by weight) of PMO to tobacco was effective against ciliostasis.

In 1972, Dahlgren and Dalhamn reported the results of further studies in which the protective effect of intraperitoneal or oral dosage of PMO was assessed in guinea-pigs exposed to acrolein. They found that PMO provided a significant amount of protection against the destructive effect of the aerosol on the respiratory system. In the same series of experiments a further anti-inflammatory agent - phenylbutazone - was also found to have a significant action when administered orally but not when given by intraperitoneal injection.

THE PURPOSE OF THE PRESENT STUDY

Some of the studies described earlier in this chapter have reported a change in the neture of the glycoprotein produced by goblet cells of the airway epithelium of the ret by exposure to an irritant (Lemb end Reid, 1968, 1969e). The purpose of this study is to enelyse further the neture, the pattern and the timing of changes in glycoprotein produced within eirway goblet cells in response to irritetion end to relate them to any change in goblet cell number et verious airway levels. The specific pethogen-free ret is used in these studies. It provides a suiteble animal model in which goblet cells are present throughout the airways, save the most peripherel bronchioli, but in animals with 'clean' lungs are present only in low numbers.

Assessment of glycoproteins within a populetion of goblet cells is based on deteiled quentitetive analysis of veriations in the staining with combined Alcian Blue-periodic acid Schiff techniques. In this way, acid glycoprotein cen be distinguished from neutral glycoprotein, and the former is further characterised by the acid radicle it conteins - as sielomucin sensitive or resistant to the enzyme sielidase, or sulphomucin.

The irritant used is tobacco smoke. This is preferred to other egents such as sulphur dioxide because, at a relatively low

'dose', it leaves intact the structure of the epithelium, while producing the appropriate changes. It was also possible to include in the study an analysis of the protective effect of an anti-inflammatory agent - phenylmethyloxadiazole (PMO). Previous studies have shown that the addition of PMO to tobacco smoke protects against ciliostasis. While this effect had been studied in short-term experiments (Dalhamn and Rylander, 1971) no study has traced the pattern of the effect of PMO on the structure of the airway epithelium irritated by tobacco smoke.

A comparison is made of exposures as short as hours with those up to several weeks.

CHAPTER II

MATERIAL AND METHODS

TISSUE

The adult male laboratory rat was used in this study (i.e. over 200 g). Specific pathogen-free (henceforth referred to as SPF) Sprague-Dawley rats were obtained from Anglia Laboratory Animals (formerly 'Carworth Europe', Alconbury, Huntingdon - CFY strain) or from Anticimex (Evelund, Sollentuna, Sweden). These animals corresponded to Grade 4 of the Medical Research Council classification for SPF rats (Laboratory Animals Centre Manual, 1974).

For pulmonary studies using an animal 'model', it is important that animals free of respiratory infection be used, since only then may any changes found be related to the conditions of the experiment without the complication of superadded infection. The laboratory rat is particularly prone to naturally occurring respiratory disease arising from bacteria, virus or mycoplasma infection. Since commercial colonies of SPF rats appear to succumb to spontaneous outbreaks of respiratory disease from time to time, it has proved necessary to screen even SPF animal stock in the laboratory before the start of an experiment (Lamb, 1975; personal observation).

The animals were judged to be free of respiratory infection by low epithelial goblet cell counts (1-2 goblet cells per oil immersion field - 0.18 diameter) and by minimal amounts of lymphocytic cuffing around the airways. It has been found that even in germ-free rats some lymphocytic cuffing is present around the airways (Giddens and Whitehair, 1969) but the degree of cuffing remains a good marker for screening rats for respiratory studies (Reid, 1970a). In well maintained

SPF rats 1-2 small isolated sites of lymphocytic cuffing in the trachea and at bifurcations of the lateral wall of the axial pathway with the lateral pathway branches are common. This degree of lymphocytic cuffing is designated Grade I. Occasionally, in some animals 3-4 sites of lymphocytic cuffing may be present at the main intrapulmonary airway bifurcations (Grade II) while in others thin continuous (Grade III) or thick continuous (Grade IV) cuffing of the airways may be found. Before each experiment 3-4 animals from each batch of animals were sacrificed and only if lymphocytic cuffing was minimal (Grade I) were animals from the same batch accepted for experimental studies. It has been found (personal observation) that animals designated Grade I usually maintained their status throughout the experimental period: at the end of the experiment the control animals sometimes showed slightly less cuffing and the exposed animals sometimes slightly more (up to Grade II).

From each animal, the trachea, main bronchi and the single lobed left lung were taken. The details of animal numbers are given with the appropriate results.

CARE OF ANIMALS

Usual Conditions

The animals were kept on wood-shavings in solid bottom polythene cages which were held in open racks (both obtained from North Kent Plastic Cages Ltd., Dartford, Kent). The open racks were preferred to laminar air-flow racks which have been recommended for use with SPF stock,

but which more recently have been shown to encourage passage of dust and organisms from cage to cage (Clough, Hill and Blackmore, 1973).

The animals received food (Plowco Pasteurised Breeding Diet, Lillico & Co., Betchworth, Surrey) and water ad libitum. The drinking water was changed daily and did not contain any antibiotic. The water containers were changed weekly, being then disinfected in a solution of Cetavlon and washed thoroughly. The bedding was changed twice a week and the cage washed and disinfected in a solution of Lysol once a week. Environmental factors have been shown to influence the number of respiratory infections (Giddens, Whitehair and Carter, 1971) and every effort was made to maintain the animals in conditions that were 'socially' hygenic. Animals were kept in a room used solely for the experiment (this room at any time housing only SPF rats) and were cared for by the author.

Experimental Conditions

During exposure to tobacco smoke, the animals were placed in heavy gauge wire mesh cages, which were suspended over trays containing sawdust in the smoking cabinets.

During the exposure period all rats were not given food or water.

EXPERIMENTAL PROCEDURE

Rats were exposed to tobacco smoke for approximately 4 hours a day. The details of the exposure, and the time interval following the last

exposure to tobacco smoke before the animals were sacrificed, are given with the appropriate results. In these experiments no animal died as the direct result of exposure to tobacco smoke.

Type of Cigarette

The cigarettes were manufactured and supplied by the Research Division, Lorillard Corporation (Greenborg, North Carolina, USA). Two types of cigarette were supplied for the experiments reported in this study. All cigarettes were of the same size (85 mm long) and contained the same type of tobacco (see below), which was a mixture of flue-cured and air-cured tobacco in approximately equal proportions. To the tobacco of one type was added 2% by weight of 3-Phenyl-5-Methyl-1,2,4-Oxadiazole (PMO): since each cigarette contained 1000 mg of tobacco, it contained about 20 mg of PMO.

It was important to establish that the addition of PMO did not appreciably alter the characteristics of the tobacco. For example, a reduction in pH of the smoke could have led to more ready absorption of nicotine by the animals (Elson, Betts and Passey, 1971). The result of analysis of each of the two types of tobacco is given in Table II.1 (Betts, personal communication). The two types of cigarette burnt at the same rate and there was no significance in any difference in the analysis of the two types of tobacco.

Exposure Cabinets and Smoking Machines

Figure II.1 shows the aluminium cabinet used to house the animals during exposure to tobacco smoke. The delivery of air and tobacco smoke

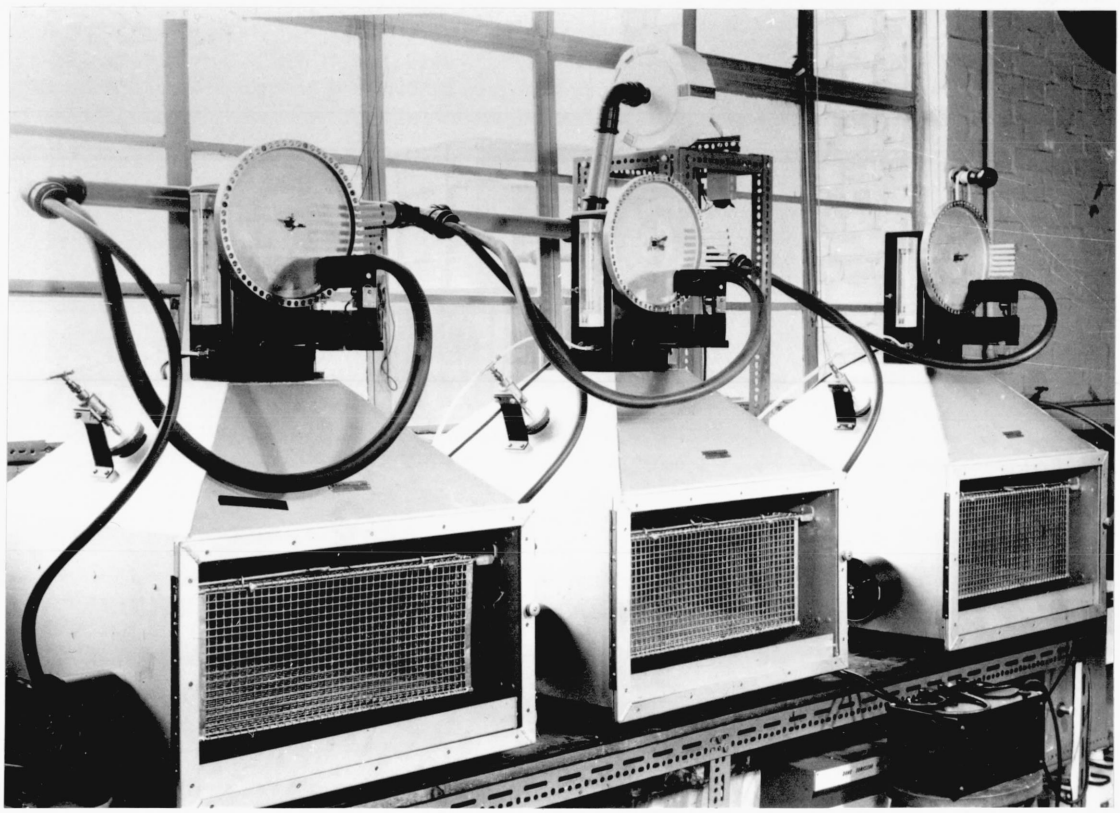
Table II.1 Analysis of tobacco with and without the addition of PMO from cigarettes smoked to a 10 mm butt length (2 sec puff every 58 secs).

		Untreated cigarettes	PMO treated cigarettes
(a)	Sugar content (as % weight of tobacco)	6.00	5.90
(b)	Total particulate matter (mg/cigarette)	17.00	20.85
(c)	Nicotine content (mg/cigarette)	1.98	1.94
(d)	pH	4.85	4.83
(e)	Buffering capacity	0.77	0.82

Estimation (a) is made by analysis of the tobacco, (b) and (c) by analysis of the smoke condensate, and (d) and (e) by analysis of the smoke.

Fig. II.1 Cabinets (A) and Wright Autosmoker (B) used to expose animals to tobacco smoke.

Fig. II.1



to the cabinet was operated by a series of valves controlled by appropriate timing mechanisms and a Wright (1972) Autosmoker (B). The system was designed by Research Engineers Ltd., (Shoreditch, London). Once in operation the machine could be left to continue the exposure schedule. A simple safety valve was incorporated into the system allowing air to enter the cabinet should air fail to enter by the usual route, but at no time did this valve operate.

A constant stream of room air was delivered into each cabinet by an oil-free diaphragm pump (Charles Austen Pumps Ltd., Byfleet, Surrey) at a rate of 10 litres per minute via the Wright Autosmoker machine. This machine controlled the 'smoking' of the cigarettes, the tobacco smoke being added intermittently to the air entering the cabinet. Each cigarette was 'smoked' at a rate of 4 puffs per minute, each puff lasting 2 seconds and drawing 30 ccs of air through the cigarette. These smoking parameters are within the range of those obtained for human subjects - puff volume (ml) 22-56, puff duration (secs) 0.97-2.32, puff frequency (puffs/min) 1-4 (Schur and Rickards, 1957; Keith, 1962).

The machine was adjusted to start a new cigarette every 10 minutes. Since each cigarette took 6 minutes to burn, air alone entered the cabinets during the 4 minutes between cigarettes. The air and smoke were removed continuously from the bottom of the exposure cabinet to the outside of the building by an exhaust system.

Most of the work reported in this study was carried out using the system of exposure described above. The initial experiment (reported in

Chapter III) was carried out using a similar system which differed only in having a less efficient exhaust system. This led to an increase in temperature and humidity within the cabinets during exposure, although an earlier study (Jones, Baetjer and Reid, 1971) found that these factors did not influence goblet cell number in rat airway epithelium. Because of the less efficient exhaust system, control animals were removed from the room during the exposure period.

At the end of each exposure period for each experiment, the delivery tube through which air and tobacco smoke had entered the cabinet was washed free of tar condensate with acetone.

PREPARATION OF TISSUE FOR MICROSCOPY

All animals were injected intraperitoneally with an excess (1-1.5 ml) of sodium pentobarbitone (Veterinary Nembutal, Abbott Laboratories, Queensborough, Kent).

Dissection

The ventral surface of the thorax was opened to expose the lungs, heart and thymus. The trachea and oesophagus were then exposed and a ligature tied immediately below the larynx. The trachea (cut above the larynx) and oesophagus and thoracic block were carefully removed intact.

Macroscopic Appearance of Tissue

On removal from the body, all lungs appeared pink and healthy. There was no increase in the size of the lungs relative to body weight of

the animals exposed to tobacco smoke (due to oedema).

Fixation

The lungs were slowly inflated (until the lung margins were sharply defined) with fixative introduced, via a blunt-ended needle and a syringe, into the tracheal lumen immediately below the ligature. The heart and thymus were then carefully dissected away (see Fig. II.2) and the lungs and trachea mounted onto a card in such a way as to preserve the normal laryngo-hilar length of the trachea. The card and tissue were then immersed in fixative.

All tissue was fixed in 10% neutral phosphate buffered formol saline at pH 7.0 (Carlton, 1973) for at least 24, and up to 48, hours. Lamb (1969) has shown that this fixative provides good preservation and has no adverse effect on subsequent AB-PAS staining of glycoproteins in bronchial epithelial tissue, which is the main staining technique used throughout this study.

Selection of Tissue Blocks

From the tissue taken from each animal, four tissue blocks were selected for processing. The larynx and oesophagus were removed and discarded and the trachea divided into three equal parts (these were usually each about 10 mm long). The tissue blocks were thus (1) upper trachea, (2) mid-trachea, (3) lower trachea and main bronchi, and (4) the left lung.

Fig. II.2 Trachea, main bronchi and inflated lungs (ventral surface) of rat after removal from the thoracic cage. The heart and thymus have been removed.

Fig. II.2

Processing of Tissue

All tissue was processed for paraffin wax embedding by a Histokinette (E.7326) processing machine (British American Optical Co. Ltd., Slough, Berks.) using the schedule given in Table II.2.

The tissue was given a final change in wax under partial vacuum (500 mm Hg) and embedded in fresh wax (Fibrowax, melting point 57-58^{o}C, R.A. Lamb, London).

The upper three rings of trachea (judged by the number of cartilage plates) from block (1) were embedded transversely while the remaining part was embedded in a longitudinal plane. The other tracheal blocks (2) and (3) were also embedded longitudinally. Tissue embedded in a longitudinal plane was orientated such that the posterior wall of the trachea was sectioned, and sections of the lower trachea thus included the carina and each of the walls of the main bronchi.

The left lung was embedded so that the ventral edge was sectioned. Ideal sections of the left lung then exposed the axial pathway from the lung hilum to the periphery, as well as the lateral pathways which run in a ventral direction in the lung (see Fig. II.3, p. 82). In all sections prepared for this study both the second and third lateral pathways were always included; the fourth and fifth lateral pathways were only occasionally present, as well as only a small part of the first lateral pathway (close to the last plate of cartilage present at the lung hilum).

Table II.2 Tissue processing schedule

10%	Neutral buffered formol saline		-	2 hours
50%	Alcohol		-	2 "
70%	"		-	2 "
90%	"		-	2 "
100%	"	I	-	2 "
"	"	II	-	2 "
"	"	III	-	3 "
Chloroform		I	-	2 "
"		II	-	3 "
Wax		I	-	2 "
"		II	-	2 "

In the results reported in Chapter III, the epithelium of the trachea and main bronchi, the axial pathway and the second and third lateral pathways were examined without any further subdivision, while in Chapters IV and V the epithelium of the airways was examined at the seven levels as listed below and illustrated in Fig. II.3:

(I) Upper trachea
(II) Mid-trachea
(III) Lower trachea and main bronchi
(IV) Upper axial pathway (medial wall)
(V) Lower axial pathway (" ")
(VI) Second lateral pathway
(VII) Third " "

The medial wall of the axial pathway was chosen because, unlike its lateral wall, the epithelium was free of lymphocytic cuffing in virtually all animals. The second and third lateral pathways were included from this region of the airways for the reason given above.

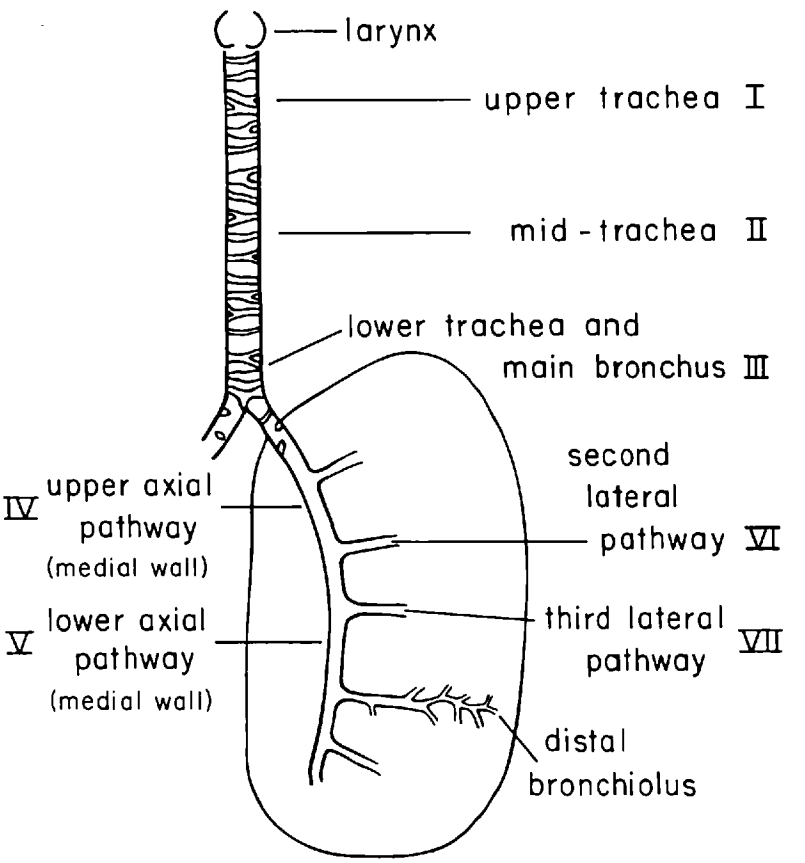
Since in this study no changes were observed in the smallest airways - the distal bronchioli (0.4 mm or less in diameter) - no further reference is made to this region.

Preparation of Serially Cut and Mounted Tissue Sections

Serial paraffin sections were cut at 4 μm with an MSE Base Sledge Microtome (Measuring and Scientific Equipment Ltd., Crawley, Sussex). All sections were mounted in serial order, being floated out on water thermostatically controlled at 56°C, and picked up on to clean glycerin albuminised slides. The slides were drained of excess water, dried initially on a hotplate at 54-56°C and dried for a further 16-24 hours in

Fig. II.3 Illustration of airway levels chosen for examination from the trachea and main bronchus, and left lung of rat.

Fig. II.3


larynx
upper trachea I
mid-trachea II
lower trachea and
main bronchus III
second
lateral
pathway VI
third lateral
pathway VII
distal
bronchiolus
IV upper axial
pathway
(medial wall)
V lower axial
pathway
(medial wall)

an incubator at 37°C. Spare sections were mounted and, in certain cases, these were used to repeat results when there was section damage.

BACKGROUND TO TECHNIQUES USED IN THIS STUDY

Sialidase Digestion

This technique was introduced as a histochemical method by Spicer and Warren (1960) being derived from earlier chemical techniques (Gottschalk and Lind, 1949). The terminal position of sialic acid on the carbohydrate chain of the glycoprotein allows its removal by the enzyme sialidase (syn. neuraminidase, Receptor Destroying Enzyme - see Blix, 1957). Filtrates with this activity have been obtained from cultures of Vibrio cholerae (Burnet, McCrea and Stone, 1946) as well as from Diplococcus pneumoniae, Clostridium perfringens and certain other bacteria (see Madoff, Annenberg and Weinstein, 1961). The purification and properties of sialidase from Vibrio cholerae (which is used in this study) have been described by Ada, French and Lind (1961). To obtain full enzymatic activity calcium ions are added to the enzyme (Drzeniek, 1973). The application of this technique allows the identification of two forms of sialic acid - one is sensitive and the other resistant to the action of the enzymes.

Periodic Acid Schiff

Periodic acid Schiff was one of the first, and perhaps the most important, techniques applied to tissue sections for the demonstration of carbohydrates (McManus, 1946; Hotchkiss, 1948). This technique was the basis on which later methods were developed for the demonstration of glycoproteins and subsequently allowed their characterisation at the intracellular level.

Periodic acid forms aldehydes from 1, 2 glycol groups (Malprade, 1934). This oxidizing agent produces the aldehydes required by breaking the carbon bonds (C-C) of various structures present when they are present in the form of adjacent 1,2 glycol groups (CHOH-CHOH) converting them to aldehydes (CHO) but does not hydrolyse nucleic acids. Similarly, sialic acid may be demonstrated by PAS (Montreuil and Biserte, 1959). Released aldehydes restore the quinoid structure of the Schiff reagent forming a coloured product in the tissue. There have been several modifications to the method of preparation of Schiff reagent (see Pearse, 1960) whose basis is the dye basic fuchsin. The most common method of preparation of Schiff is that described by de Tomasi (1936), which was used for the preparation of the reagent used throughout this study (R.A.Lamb, personal communication).

Alcian Blue

The background of the preparation that led to the production of the Phthalocyanine dye Alcian Blue (AB) has been described by Haddock (1948). While its copper nucleus provides the colour property of this dye, copper phthalocyanine itself is an insoluble pigment which must be made soluble to be used as a dye. Abrahart (1968) later described the process by which AB 8GS was prepared and the chemical and histochemical properties of AB 8GX (the variant currently on the market) have been described by Scott and his colleagues (Scott, Quintarelli and Dellovo, 1964; Quintarelli, Scott and Dellovo, 1964a & b). Alcian Blue 8GX is made soluble by the addition of isothiouronium groups which confer up to four positively charged groups on each dye molecule. It is this property which allows it to stain polyanions in tissue sections.

The mechanism by which AB stains acid glycoproteins is not fully understood, although it is thought most likely a form of electrostatic charge attraction (Scott et al, 1964). Other possible aspects of its mechanism have been described by Spicer (1960), Dorling (1969), Stoward (1963), Goldstein (1961, 1962) and by Yamada (1964) but attraction between polyanions such as acid glycoproteins and positively charged dye molecules (Coulombic attraction) leading to the formation of a salt-link is probably one of the most important factors responsible for staining tissue groups by AB; with other factors such as London-van der Waals forces, dipoles, hydrogen bonds or "hydrophobic" bonds playing some part.

The effect of pH

The staining of acid glycoproteins by AB is modified by pH. This effect is linked to the dissociation of the acid groups in glycoproteins. The pK_a value (dissociation constant) of the carboxyl radicle of sialic acid (pK_a 2.6 - Svennerholm, 1956) differs from the sulphate radicle of ester sulphate. The pK_a value of sulphate esters in epithelial glycoproteins is not known. Sulphuric acid is known to have two pK_a values: dissociation of the first hydrogen is at so acid a level of pH as to be almost immediate, while the second hydrogen atom has a pK_a value of 1.94. The pH of the AB staining solution thus determines ionisation of the acid groups of glycoproteins present in the tissue which, although present in fixed tissue, behave as if in solution.

Although dissociation occurs over a wide range of pH, it is maximum over a narrow range, taking place at pH values near the level of dissociation. Staining thus takes place over a range of pH and varies over this range according to levels of dissociation. At an unfavourable

pH level acid radicles no longer dissociate, bind and thus stain with AB.

Alcian Blue-Periodic Acid Schiff

The original method of Steedman (1950) which introduced AB as a stain for acid glycoproteins was improved by Lison (1954) and Mowry (1956). The later modifications improved the selectivity of the stain by lowering the pH of the staining solution to between 2.4 and 2.6 and allowed sequential staining of the carbohydrate component by PAS. The combined AB-PAS method for the demonstration of both the neutral and acid components of acid glycoproteins was introduced by Mowry and Winkler (1956) and Mowry and Morard (1957) and was based on the earlier technique of Ritter and Oleson (1950) which used a combination of Hale's dialysed iron technique with PAS. Although AB-PAS techniques now in use stained acid glycoproteins well, they did not distinguish the various acid radicles identified in glycoproteins by biochemical techniques, i.e. sialic acid and sulphate.

The use of ^{35}S labelled sulphate techniques established that AB stained sulphomucin (Curran and Kennedy, 1955) while sialidase treatment established its staining of sialomucin (Spicer and Warren, 1960). Later modifications to the preparation of the AB solution gave selective staining of sulphated glycoprotein; staining at pH 1.0 (Spicer, 1960), the addition of a salt (Scott and Dorling, 1965) and AB prepared in aluminium sulphate (Heath, 1962) are examples of methods used in this way. Other stains claimed to be specific for the demonstration of sulphomucin include Aldehyde Fuchsin (Spicer and Meyer, 1960) and High iron diamine (Spicer, 1965). The technique of acid hydrolysis and AB-PAS staining has also been used to identify acid radicles in tissue sections: sialic acid is

removed and sulphate unaffected by this treatment (Lamb and Reid, 1969b).

Each of the techniques mentioned above was used in a quantitative study which identified the glycoproteins in the mucous and serous cells of the human bronchial submucosal glands (Lamb and Reid, 1969b, 1970). The stains claimed to be specific for sulphate radicles, however, failed to stain all sulphomucin present and the technique of acid hydrolysis gave rise to the technical difficulty of occasional lose of sections by the technique in use. A more recent study in the Department has exploited the use of AB solutions prepared at various pH levels to identify the acid radicles of epithelial glycoproteins (Jones and Reid, 1973a & b). This study established the range of pH between 2.6 and 0.5 over which sialomucin and sulphomucin selectively bind with AB (see Fig. II.4); sialomucin resistant to the enzyme sialidase was found to retain Alcian blue staining to a lower level of pH (1.5) than the sensitive form (pH 1.7) but both failed to stain at pH 1.0, where sulpomucin alone retained its affinity for the dye. This pattern of staining occurred when only single types of acid glycoprotein were present within the tissue and even when present in combination.

The combined AB-PAS techniques have been used throughout this study in preference to other histochemical techniques for the identification of epithelial glycoproteins. They provide a relatively simple and reliable method of analysing both neutral and acid glycoproteins, and the type of acid glycoprotein within cells.

STAINING METHODS

Prior to staining, or to treatment with sialidase, sections were dewaxed with xylol, washed with 100% and then 70% alcohol and washed in running tap water.

Fig. II.4 Dissociation of acidic groups in epithelial glycoproteins and range of pH over which each acid glycoprotein stains with AB.

Fig. II.4

SIALOMUCIN - Sialic Acid ($-CO_2H$)

$$-COOH \underset{}{\overset{pK_a\ 2.6}{\rightleftharpoons}} -COO^- + H^+$$

ABpH 2.6 - 1.5

SULPHOMUCIN - Sulphate Ester ($-SO_3H$)

$$CH_2-O-SO_3H \underset{}{\overset{pK_a\ \text{unknown}}{\rightleftharpoons}} CH_2-O-SO_3^- + H^+$$

ABpH 2.6 - 1.0

Sialidase Digestion

The method was according to Spicer and Warren (1960) save that a 1:3 dilution of the enzyme with distilled water was used instead of the full strength enzyme. Lamb (1969) has shown that up to 1:6 dilution of the enzyme with distilled water gives the same histochemical result as the full strength enzyme and is less costly for routine use. The sialidase used was Receptor Destroying Enzyme (RDE) from Vibrio cholorae (Wellcome Research Laboratories, Beckenham, Kent).

Sections were brought to distilled water and incubated overnight (16 hours at 37°C) in the following solution of RDE (pH 5.55): RDE (20 ml), distilled water (60 ml) and 4% calcium chloride (10 ml). The sections were then washed well in running tap water and stained with ABpH 2.6-PAS by the technique described below.

Alcian Blue-Periodic Acid Schiff Techniques

One batch of Alcian Blue (AB) 8GX was used throughout this study (RevectoR stain, batch no. 67146, C.I.* 74240 supplied by Hopkins and Williams Ltd., Chadwell Heath). In an early study a detailed investigation was made of each stage of the AB-PAS technique (Jones, 1971) which resulted in the standardized method currently used in the Department. This includes checking of dye batch, method of preparation of staining solution and storage, and in the case of AB, prevention of dye diffusion within sections stained at low pH (see below).

* Colour Index number of classification accorded to each dye standardized by the Society of Dyers and Colourists (see Conn, 1961)

Two preparations of AB were used: either 0.1% AB in 3% acetic acid - pH 2.6 (Mowry, 1956) or 1.0% AB in 1N hydrochloric acid - pH 1.0 (Spicer, 1960). Details of the AB-PAS techniques are given in Table II.3.

Treatment with sodium carbonate insolubilises AB 8GX in the tissue converting it into a fast or stable compound (Mowry, 1960). Lev and Spicer (1964) reported that after staining with the variant AB 8GS at pH 1.0 dye diffuses from stained to previously unstained areas of epithelial glycoproteins in sections washed in tap water, because of pH change. This effect has been confirmed for the variant AB 8GX by a quantified analysis of diffusion (Jones, 1971). Thus, sections stained with AB at pH 1.0 were simply blotted to remove excess stain and transferred directly to the sodium carbonate rinse. Sections were then stained with periodic acid Schiff (PAS).

A modification of the PAS method of McManus (1946) was used with a shorter timing for periodic acid oxidation (5 instead of 10 minutes) and treatment with the Schiff reagent (3 instead of 15 minutes). This is the standard technique in use in the Department. The reduction in oxidation and treatment with Schiff reagent gives a pale magenta colour, but this is preferred since there is less opportunity of masking small amounts of acid glycoprotein stained with AB. The Schiff reagent (R.A. Lamb, London) was stored at $4^{o}C$, brought to room temperature before use and used only on the one day.

Haematoxylin and Eosin

A standard method of Erhlich's haematoxylin and eosin (H & E) was

Table II.3 AB-PAS staining techniques

1. Sections were washed in distilled water and stained in AB either at pH 2.6 or at pH 1.0 for 30 minutes*
2. Treated with 0.3% sodium carbonate for 30 minutes
3. Washed in running tap water and rinsed in distilled water

The method of PAS staining was as follows:

4.	1% aqueous period acid	-	5 minutes
5.	Running tap water	-	10 "
6.	Distilled water	-	5 "
7.	Schiff reagent (18-22°C)	-	3 "
8.	Sodium sulphite rinse** I	-	5 "
9.	" " " II	-	5 "
10.	Running tap water	-	10 "

11. Sections were dehydrated in 70% and then 100% alcohol, cleared in xylol and mounted under clean glass coverslips in Dammar Xylol

* Sections stained with AB at pH 2.6 were washed in tap water, rinsed in distilled water and placed in the sodium carbonate rinse, while those stained with AB at pH 1.0 were simply removed from the stain and blotted and transferred directly to the rinse (see text for further details).

** Sodium sulphite rinse: 1N hydrochloric acid (5 ccs), 10% potassium metabisulphite (5 ccs) and distilled water (90 ccs).

used (Carlton, 1973): the haematoxylin powder C.I. 75290 and eosin Y C.I. 45380 were supplied by R.A. Lamb (London).

The routine procedure was to stain each of four consecutive tissue sections in one of the following ways: (i) ABpH 2.6-PAS, (ii) sialidase ABpH 2.6-PAS, (iii) ABpH 1.0-PAS and (iv) H & E. The same sequence was used for staining sections from each tissue block. The first three sections were used to assess goblet cell number, staining and amount of goblet cell glycoprotein, and the latter to assess the degree of lymphocytic cuffing at the end of the experiment, measurement of epithelial height and histological details. Measurements of tracheal gland were made from sections stained either with (i) or (iv).

STAINING CHARACTERISTICS OF EPITHELIAL GLYCOPROTEINS IN RESPONSE TO AB-PAS TECHNIQUES

While previous studies have established the staining response of goblet cell glycoproteins to AB-PAS techniques at epithelial sites (Jones and Reid, 1973a; Fletcher, Jones and Reid, 1976), it has been reported that the goblet cell glycoprotein stained one of a range of colours, i.e. blue, blue-red, red-blue or red. The goblet cell glycoprotein of goblet cells of rat airway epithelium differs in that it stains either with AB (blue) or with PAS (red) in response to these techniques (see p. 117)

In this study, the glycoproteins of rat airway epithelium, however, have not been found to differ from other epithelial glycoproteins in

response to the three AB-PAS techniques used to identify the nature of the glycoprotein. Figure II.5 illustrates the staining characteristics of epithelial glycoproteins in response to these techniques.

Method of Analysis

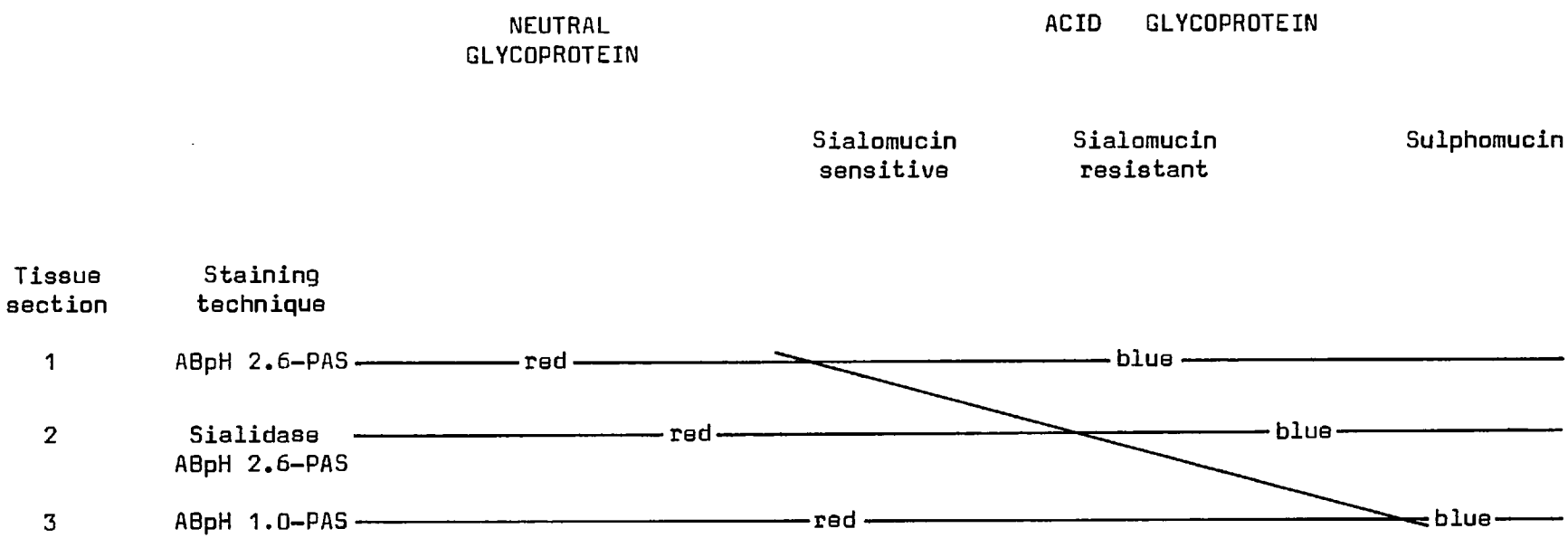
In a section stained with ABpH 2.6-PAS, neutral glycoprotein stains red and each type of acid glycoprotein blue (see Fig. II.5). Pre-treatment of the section with sialidase removes acid radicles sensitive to its action and subsequent staining with ABpH 2.6-PAS reveals neutral glycoprotein and sialidase sensitive sialomucin as red, while sialidase resistant sialomucin and sulphomucin stain blue. In a section stained with ABpH 1.0-PAS each type of glycoprotein stains red save sulphomucin which still stains blue. This approach to the histochemical analysis of intracellular glycoproteins has been applied successfully in studies of goblet cells of fish epithelia (Fletcher, Jones and Reid, 1976) and mucous and serous cells of the pig bronchial gland (Jones, Baskerville and Reid, 1975).

QUANTITATION

Tissue sections were examined by light microscopy using x8 eyepiece and x100 objective lens (field size - 0.18 mm diameter). All measurements were made on airway epithelium which showed no evidence of lymphocytic cuffing. This included virtually all the epithelium of the trachea and main bronchi and that of the medial wall of the axial pathway. In the lateral pathways, the epithelium at the bifurcation with the lateral wall

Fig. II.5 Staining characteristics of epithelial glycoproteins in tissue sections stained by AB-PAS techniques.

Fig. II.5


NEUTRAL GLYCOPROTEIN
ACID GLYCOPROTEIN
Sialomucin sensitive
Sialomucin resistant
Sulphomucin
Tissue section
Staining technique
1
ABpH 2.6-PAS
red
blue
2
Sialidase ABpH 2.6-PAS
red
blue
3
ABpH 1.0-PAS
red
blue

of the axial pathway was excluded because of cuffing: approximately the first 2 mm (10-12 HPF) along the distal wall of each lateral pathway was avoided, while measurements were recorded for the next 10 HPF (usually all that are available before the next branch of these bronchi). Sequential measurements were recorded from the same region of the proximal wall of the same lateral pathway.

Goblet Cells

In tissue sections stained with AB-PAS techniques, the glycoprotein within a goblet cell is demonstrated but not the cell outline. Throughout this study results are expressed for goblet cells rather than for 'area of glycoprotein within a goblet cell'. In an airway only those goblet cells in which the glycoprotein was in contact with the surface edge of the epithelium were included in a count made along the epithelium; only an occasional cell was excluded from the count for this reason. Where so little glycoprotein was present that positive identification of a goblet cell within the epithelium could not be made without question, it was also excluded from the count. This occurred infrequently, perhaps on one or two occasions at each airway level.

Examination of the airway epithelium of control animals showed variation in the distribution of goblet cells. At any airway level, in some animals the goblet cells were fairly evenly distributed while in others the distribution was irregular. To overcome this variation, goblet cells were counted along the total length of epithelium available at each airway level (which was of similar length in each animal). For example, for the seven airway levels previous described (p.80) -

Level I - included both walls of the upper trachea (approx. 20 mm)
" II - " " " " " mid-trachea (" ")
" III - " " " " " lower trachea (" 40 mm)
and four walls of the main bronchi
" IV - upper medial wall of axial pathway (30 HPF = 5.4 mm)
" V - lower " " " " " (" ")
" VI - second lateral pathway (20 HPF = 2.6 mm)
" VII - third " " (" ")

The goblet cell count was then expressed per unit length of epithelium - a unit of 3 mm was chosen since this was the maximum length of epithelium available at levels VI and VII: **low number of goblet cells identified as containing different types of glycoproteins precluded a length of 1 mm.**

Repeatability of cell counts

To establish the repeatability of cell counts made by the observer, goblet cells (in sections stained with ABpH 2.6-PAS) were assessed along the same length of epithelium on two separate occasions (each made one week apart). The results are given in Table II.4. At each airway level, the total number of cells counted on each occasion was very similar, as was the proportion of the total cell number occupied by each cell type.

Intra-animal and inter-animal variation

Goblet cell counts were made on each of five consecutive tissue sections of trachea (stained with ABpH 2.6-PAS) from one control and one exposed animal. In each case the standard deviation (s.d.) for intra-animal readings was low as compared with its mean (control - mean 30.81, s.d. 4.69; tobacco - mean 87.63, s.d. 4.94).

Similar counts were made for five control and five animals exposed

Table II.4 Assessment of repeatability of goblet cell counts: two counts (a & b) were made (each one week apart), along the same length of epithelium, at three airway levels.

		Total no. cells in 3 mm epithelium	No. Goblet Cells (%) of each type*			
			L.PAS	S.PAS	L.AB	S.AB
AIRWAY LEVEL						
Trachea	a	26	2	73	12	13
	b	32	4	69	9	18
Axial pathway	a	54	4	61	5	30
	b	61	7	60	6	27
Lateral pathway	a	47	3	36	19	42
	b	40	4	34	18	44

* L.PAS Goblet cells containing a large number of secretory granules staining with PAS
S.PAS " " " " small " " " " " " "
L.AB " " " " large " " " " " " AB
S.AB " " " " small " " " " " " "

Further details are given on p.

to tobacco. The standard deviations of the inter-animal groups were greater than those of the intra-animal groups (control - 21.35, s.d. 7.85; tobacco - 78.59, s.d. 11.64). The inter-animal variance, however, was not significantly greater than the intra-animal variance. Because of the low levels of variance, goblet cell counts were made only on three consecutive tissue sections from each animal (each section stained using a different AB-PAS technique) and a low number of animals within any group was acceptable, especially so because of the wide inter-group variation between control and exposed animals.

Area of secretory mass

In a goblet cell the width and height of the secretory mass of acid glycoprotein, judged as the area staining with AB, was measured if either the width or height of the secretory mass was 5 units or more of the eyepiece graticule (5 units = 6.5 μm). Maximum measurements were recorded for each cell included. The product of the measurements of width and height was taken as an 'index' of the area of secretory granules occupying a cell.

Epithelial Height

In tissue sections stained with H & E the height of the epithelium was measured from the basement membrane to the apical membrane of an epithelial cell using an eyepiece graticule. The graticule was divided into 100 units, each unit being equal to 1.30 μm.

Tracheal Gland

Since in the rat trachea most of the gland is present immediately

below the larynx, the upper 3 mm was used for the measurement of the tracheal gland.

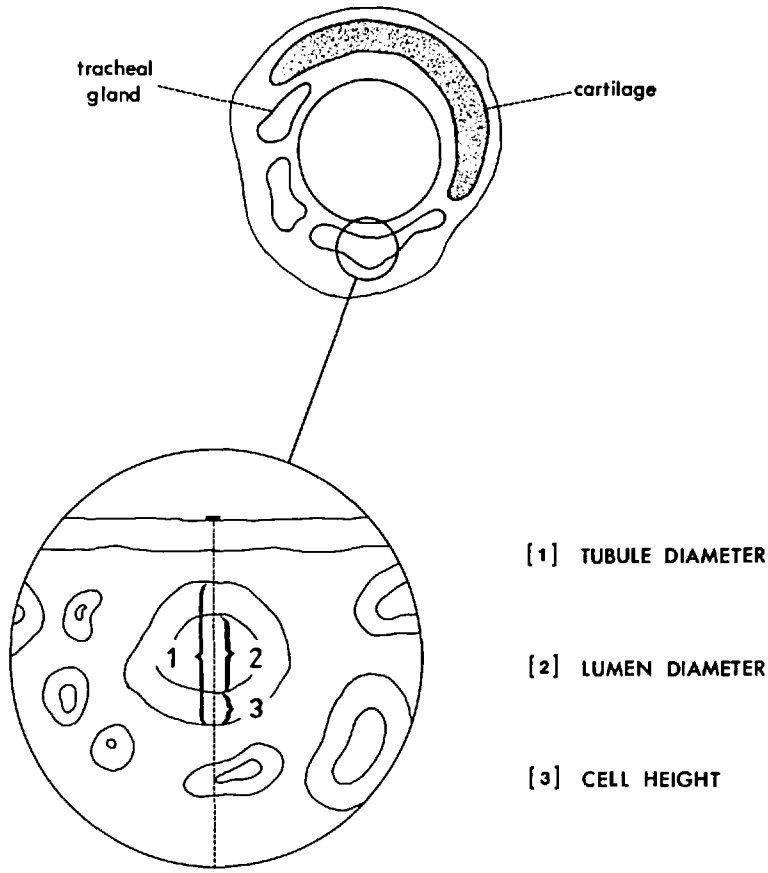
Serial transverse sections were prepared and gland measurements were made on sections 160 μm apart. Using the same eyepiece graticule used for the measurement of epithelial height, in each section examined three measurements were recorded for each mucous gland tubule - along the maximum diameter, perpendicular to the lumen of the trachea, the lumen diameter and cell height of each wall (x2). From these measurements the tobule diameter, from basement membrane to basement membrane, was calculated (see Fig. II.6 where 1 = tubule diameter, 2 = lumen diameter and 3 = cell height).

Evaluation of Results

Standard methods were used for the variance ratio test (f-test), calculation of Student's t-test and the chi-squared test (Paradine and Rivett, 1953) and values of significance read from standard probability tables (Fisher and Yates, 1963).

Fig. II.6 Illustration of measurements made of rat tracheal gland.

Fig. II.6


tracheal gland
cartilage
1
2
3
[1] TUBULE DIAMETER
[2] LUMEN DIAMETER
[3] CELL HEIGHT

CHAPTER III

THE EFFECT ON RAT AIRWAY EPITHELIUM OF SIX WEEKS' EXPOSURE TO TOBACCO SMOKE WITH AND WITHOUT PHENYLMETHYLOXADIAZOLE

DETAILS OF THE EXPERIMENT

The effect of six weeks of exposure to tobacco on rat airway epithelium was investigated. In total the experiment included 44 male rats from which 24 were taken at random for the studies reported here. The results of a study of cell turnover are reported elsewhere (Bolduc and Reid, 1976b) as are the electron microscopy findings for this experiment (Jeffery, 1973).

Animals were exposed either to tobacco or to tobacco to which PMO was added (see p. 69), or were untreated (controls). The number of animals in each group and the details of the exposure schedule are given in Table III.1.

The exposed animals did not receive consecutive days of exposure to smoke, the pattern of exposures being as illustrated in Fig. III.1. All animals were sacrificed approximately 20 hours after the last exposure period finished.

EFFECT ON THE ANIMALS

Behaviour and Appearance

During exposure both animals exposed to tobacco and to tobacco + PMO huddled together in the cage corners. Their fur became wet and at the end of a day's exposure to smoke many animals were lacrimating and had eye congestion. During the first 8 exposures it was noticed that animals in the group exposed to tobacco + PMO recovered more quickly than those exposed to tobacco. At this time the groups were coded and the observer was ignorant of the type of smoke

Table III.1 Exposure schedule and number of animals in each group.

No. days' exposure per week	No. cigarettes per day	No. animals/group		
		Tobacco	Tobacco + PMO	Controls
		9	9	6
2	25			
4	"			
4	"			
4	"			
4	"			
4	"			
2	"			
TOTAL 24 exposure days over a period of 40 days	600		24	

received by each group. Within a few minutes of removal from the exposure cabinet the animals exposed to tobacco + PMO recovered quickly, burrowed amongst the wood-shavings in their cages and started cleaning themselves and moving about. The animals exposed to tobacco took about 15 minutes to recover before starting this activity. After further exposures the difference was no longer apparent. The 'cleaning' activity took place before the animals chose to eat or drink.

Weight Gain

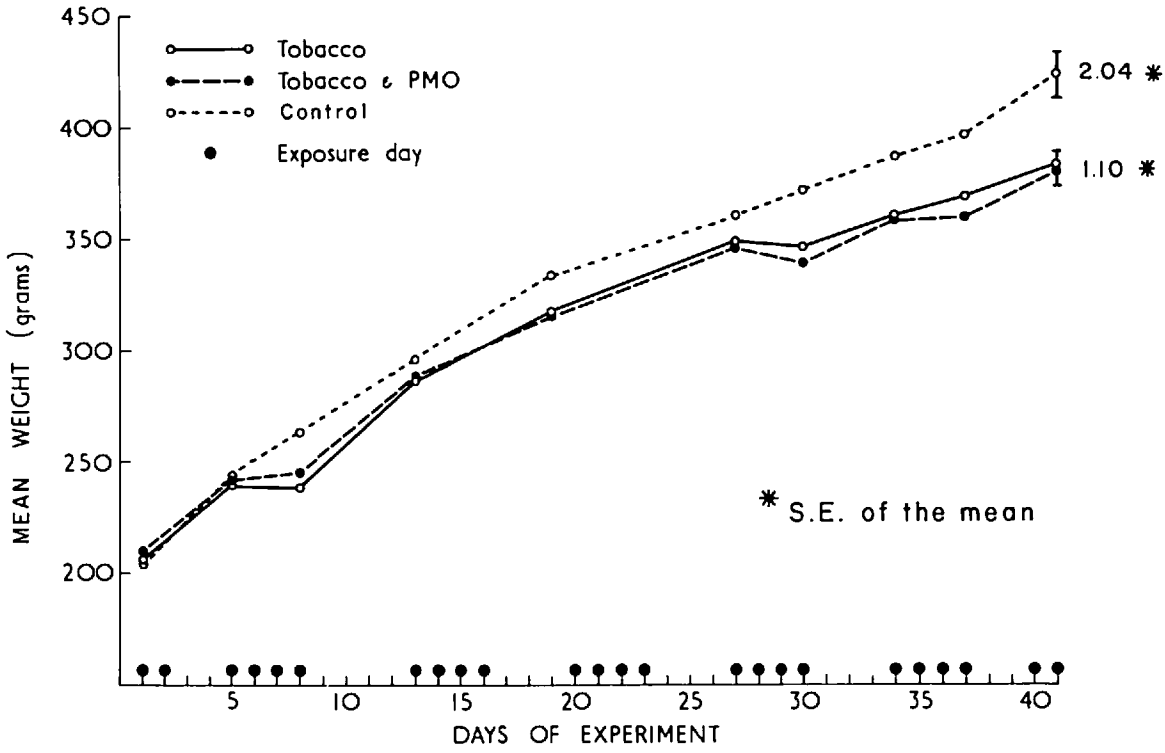
At the beginning of the experiment the range of body weight for the animals was 180-235 g. There was a steady gain in weight in all animals. Overall, animals in both groups exposed to tobacco smoke gained weight at a slower rate than did the controls, failing to gain as much weight on the days of exposure but gaining at a relatively faster rate on the days between (Fig. III.1). At the end of the experiment the mean body weights of the animals exposed either to tobacco or to tobacco + PMO were similar and both were significantly less than the controls ($p < 0.001$ for each).

EFFECT ON AIRWAY EPITHELIUM

In this experiment the effect of tobacco smoke on the epithelium of the trachea, axial and lateral pathways was studied: (the results from the second and third lateral pathways are combined to give a mean value for this airway level). At each airway level measurements were made of:

Fig. III.1 Gain in body weight of control rats and rats exposed to tobacco or to tobacco + PMO. Days of exposures are as illustrated, the days between representing days of rest.

Fig. III.1


Tobacco
Tobacco & PMO
Control
Exposure day
2.04 *
1.10 *
* S.E. of the mean
MEAN WEIGHT (grams)
450
400
350
300
250
200
5
10
15
20
25
30
35
40
DAYS OF EXPERIMENT

(i) the height of the epithelium

(ii) the total number of goblet cells

(iii) the number of goblet cells staining in a given way after ABpH 2.6-PAS, sialidase ABpH 2.6-PAS or ABpH 1.0-PAS

(iv) the distribution of goblet cells containing acid or neutral glycoprotein

(v) the distribution of goblet cells containing sialomucin sensitive or resistant to sialidase or sulphomucin

(vi) the intracellular distribution of granules containing each type of acid glycoprotein

(vii) the area occupied by secretory granules within goblet cells containing acid glycoprotein

(viii) the size of the tracheal submucosal gland.

Epithelial Height

At each airway level, in each of the 24 animals, the height of the epithelium was measured with an eyepiece graticule as the vertical distance between the basement membrane and the epithelial surface. The results of measurements of epithelial height are given in Table III.2.

In the trachea of each animal, 10 measurements were made over the plates of cartilage and 10 between. The mean value for each group of animals was calculated from the mean values for individual animals within a group. The epithelial height over and between the cartilage plates was assessed separately because early inspection had suggested an increased thickness of the epithelium between the plates, but in no group was there a significant difference between

Table III.2 Mean epithelial thickness (μm), at three airway levels in control rats and rats exposed to tobacco or to tobacco + PMO

	CONTROLS	TOBACCO	TOBACCO + PMO
Trachea			
Over cartilage plates	11.12 (0.47)*	19.05 (1.64)	14.28 (1.37)
Between cartilage plates	12.81 (0.75)	20.89 (1.15)	15.62 (1.91)
Axial pathway	9.21 (0.87)	11.20 (1.21)	10.84) (1.48)
Lateral pathway	8.02 (0.26)	8.10 (0.94)	8.48 (0.60)

* S.E. of the mean

the values for the two sites. Similarly, the mean value of 10 measurements from the axial and from the lateral pathways of each animal was used to calculate the group mean value at these airway levels.

In the animals exposed to tobacco the height of the tracheal epithelium was increased both over and between the cartilage plates as compared with both the animals exposed to tobacco + PMO ($p < 0.02$ and < 0.05 respectively) and the controls ($p < 0.001$ for each). While there was some increase in the height of the tracheal epithelium of the animals expoeed to tobacco + PMO as compared with the control animals, the difference was not significant.

In the intrapulmonary airways only in the axial pathway was there some increase in the height of the epithelium in the exposed animals as compared with the controls, but there was no significant difference between any of the groups.

Evidence was sought whether the increase in the epithelial thickness in the trachea was due to either an increase in cell number or size. Along the intrapulmonary airways cell counts were not made since virtually only a single epithelial cell layer was present. At each point where measurements of tracheal height were made along an airway, the number of nuclei between the basement membrane and the surface edge of the epithelium was counted. The mean cell number and standard error of the mean of measurements

for animals exposed to tobacco or to tobacco + PMO and control animals was 1.90 (0.05), 2.80 (0.16) and 2.10 (0.02) respectively over the plates of cartilage, and 3.10 (0.14), 3.20 (0.16) and 2.65 (0.18) between. At either site there was no significant difference between any of the groups. Thus the increase in epithelial height was due to hypertrophy rather than hyperplasia of cells.

Goblet Cell Number

The goblet cell number was assessed in tissue sections of rat airway stained with ABpH 2.6-PAS. For each group of animals the mean value for the absolute number of goblet cells at each airway level is expressed as the number per 3 mm epithelium. These results are given in Table III.3 and illustrated in Fig. III.2.

In the tracheal epithelium of the animals exposed to tobacco, the goblet cell number was significantly above that of animals exposed to tobacco + PMO and control animals ($p < 0.001$ for both). There was no significant difference in the number of goblet cells in the tracheal epithelium of animals exposed to tobacco + PMO and the controls. In the axial pathway, while there was an increase in the goblet cell number per unit length of epithelium in both animals exposed to tobacco and tobacco + PMO, the increase was not significantly different from the control value; neither was the increase in the lateral pathway of those exposed to tobacco, while in those exposed to tobacco + PMO there was no difference from control animals.

Fig. III.2 Mean number of goblet cells in 3 mm of airway epithelium of exposed and control animals.

Fig. III.2

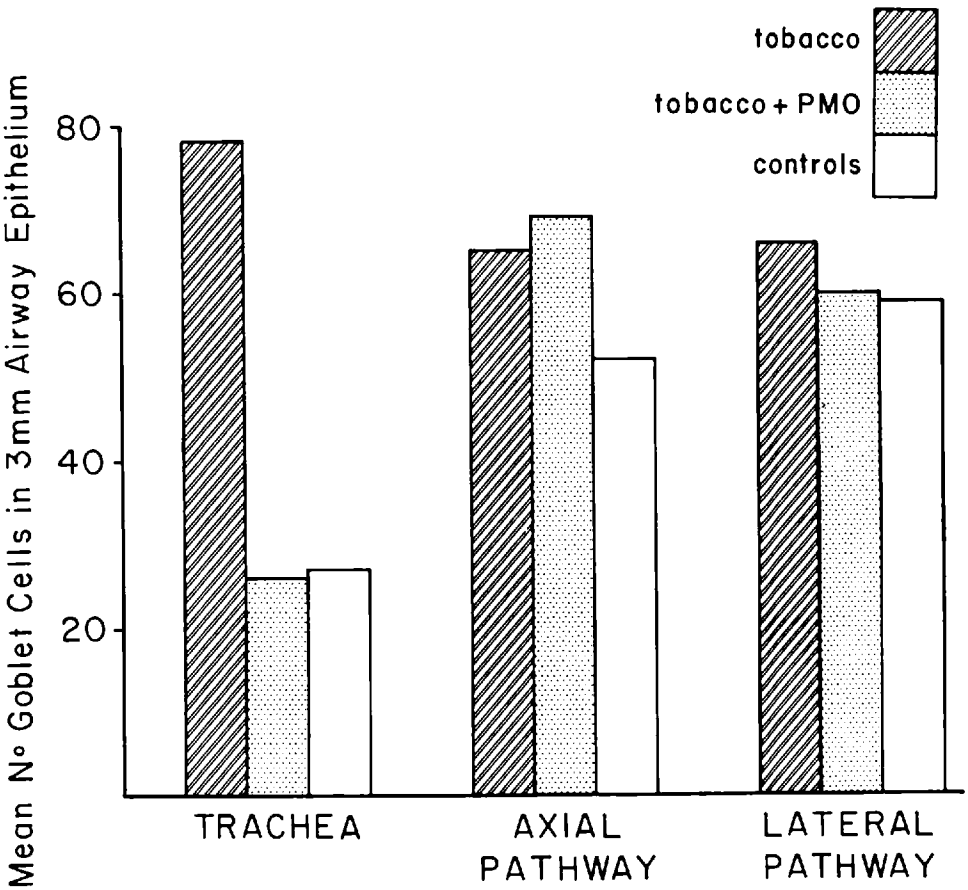

tobacco
tobacco + PMO
controls
Mean N° Goblet Cells in 3mm Airway Epithelium
80
60
40
20
TRACHEA
AXIAL PATHWAY
LATERAL PATHWAY

Table III.3 No. goblet cells in 3 mm airway epithelium in rats exposed to tobacco or to tobacco + PMO and controls.

	Trachea	Axial Pathway	Lateral Pathway
TOBACCO	78.2 (5.30)*	63.56 (6.75)	66.26 (10.64)
TOBACCO + PMO	25.48 (2.46)	69.99 (8.92)	59.62 (6.93)
CONTROL	27.04 (1.34)	52.33 (7.79)	59.15 (6.41)

* S.E. of the mean

Distribution and Type of Glycoprotein within Goblet Cells of Rat Airway Epithelium

The intracellular glycoproteins of goblet cells of rat airway epithelium are present as granules which are visible by light microscopy (x8 eyepiece and x100 objective - field size 0.18 mm). Goblet cells were assessed both by their amount of glycoprotein and by its staining characteristics.

For the purpose of quantitation where glycoprotein within a goblet cell was present only in the upper half it was classified as having a 'small' number of secretory granules, and if the glycoprotein extended into the basal half as having a 'large' number.

Some goblet cells were found to contain only granules of neutral glycoprotein, some only grenules of acid glycoprotein and some a mixture of both types. In those goblet cells containing both neutral and acid forms the granules of acid glycoprotein were found to occupy the apical region.

In the experiment reported here goblet cells containing neutral glycoprotein alone (PAS +ve)were counted separately from those containing acid glycoprotein (AB +ve): the latter group thus included cells containing acid glycoprotein alone or acid glycoprotein together with some neutral glycoprotein, the presence of acid radicles being the important feature. Goblet cells were classified in one of four ways:

(i) Large PAS +ve (L.PAS)
(ii) Small PAS +ve (S.PAS)
(iii) Large AB +ve (L.AB)
(iv) Small AB +ve (S.AB)

Whereas S.AB cells were often of the mixed variety, with acid glycoprotein in the apical region of the cell and neutral glycoprotein below, in this experiment the L.AB cells more often contained only acid glycoprotein, occasionally containing only a few granules of the neutral form.

The degree of exposure to tobacco or to tobacco + PMO was such that there was no overt epithelial damage - the epithelial structure remained intact, there was no evidence of epithelial shedding, and the goblet cells were well stained by the techniques used and readily identified. Nor was there any evidence of squamous cell metaplasia or cell proliferation or of oedema or mucus impaction within alveoli.

Distribution of Goblet Cells of Each Type after AB-PAS Techniques in Airway Epithelium of Control Rats and Rats exposed to Tobacco or to Tobacco + PMO

The number of goblet cells staining with AB or with PAS, expressed as the absolute number of cells in 3 mm of airway epithelium, in tissue sections stained with ABpH 2.6-PAS, sialidase ABpH 2.6-PAS and ABpH 1.0-PAS, is given in Tables III.4-12 for control rats and rats exposed to tobacco or to tobacco + PMO for 6 weeks. The number of goblet cells of each type in airway epithelium stained with ABpH 2.6-PAS was assessed in all rats in each group, and in 6 rats in each group after sialidase ABpH 2.6-PAS or ABpH 1.0-PAS.

...../Text continues p. 137

Table III.4 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 2.6–PAS – TRACHEA

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	3.72	10.76	27.94	47.61
2	2.37	9.78	31.74	50.67
3	0	7.11	10.00	36.66
4	0.45	9.45	17.55	52.38
5	2.34	5.88	25.08	59.40
6	1.74	8.25	12.69	47.13
7	0.75	8.70	29.55	50.43
8	0.15	6.18	19.96	27.30
9	1.17	7.98	12.69	58.32
Total	12.69	74.09	187.20	429.90
Mean	1.41	8.23	20.80	47.77

Tobacco + PMO					
(a)	1	0.70	1.02	10.28	18.18
	2	0.42	2.34	10.51	25.89
	3	0.90	3.69	9.62	16.03
	4	2.55	8.60	5.12	14.45
	Total	4.57	15.65	35.53	74.55
	Mean	1.14	3.91	8.88	18.63
(b)	5	2.31	13.87	1.92	3.92
	6	0.63	14.25	1.21	2.11
	7	4.32	12.28	0.32	2.32
	8	0.24	13.50	0.24	3.75
	9	0.51	17.01	0.84	3.44
	Total	8.01	70.91	4.53	15.54
	Mean	1.60	14.18	0.90	3.10
Control					
	1	1.44	10.14	0.54	12.37
	2	1.11	19.62	1.22	10.37
	3	0.18	17.02	1.60	10.41
	4	1.80	13.61	1.62	10.50
	5	1.08	10.49	1.20	11.69
	6	0	12.00	0	12.22
	Total	5.61	82.88	6.18	67.56
	Mean	0.94	13.81	1.03	11.26

Table III.5 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 2.6–PAS – AXIAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	13.83	38.48	15.90	32.19
2	6.36	31.37	17.20	27.69
3	1.23	23.61	8.43	35.82
4	1.41	23.47	8.02	13.41
5	2.85	31.41	5.70	22.38
6	1.17	27.12	5.94	18.74
7	3.00	19.83	6.49	42.15
8	0.33	17.62	3.12	11.28
9	3.78	32.13	2.16	16.41
Total	33.96	245.04	72.96	220.01
Mean	3.77	27.23	8.11	24.45

Tobacco + PMO				
1	7.92	55.80	14.79	36.24
2	3.03	29.79	10.72	72.98
3	5.85	35.38	8.91	45.75
4	2.85	13.09	8.31	16.65
5	3.15	18.66	14.34	43.38
6	4.02	20.70	8.79	20.46
7	3.78	17.95	4.20	13.41
8	0.75	12.03	17.67	49.22
9	1.83	13.89	3.24	26.65
Total	33.18	217.29	90.97	324.74
Mean	3.68	24.14	10.10	36.08
Control				
1	5.07	25.91	5.93	10.08
2	4.02	22.38	4.89	11.64
3	3.81	29.49	5.89	27.32
4	1.41	21.66	4.50	11.19
5	0.21	13.33	7.66	13.54
6	8.61	41.76	4.21	29.49
Total	23.13	154.53	33.08	103.26
Mean	3.86	25.76	5.51	17.21

Table III.6 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 2.6-PAS - LATERAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	11.64	47.49	25.83	57.90
2	2.49	25.83	5.82	41.64
3	2.91	26.25	7.08	29.58
4	0	17.47	5.82	20.40
5	4.14	22.89	4.98	19.98
6	2.49	34.98	22.50	13.74
7	4.56	31.65	7.08	20.39
8	0.39	15.81	2.91	19.98
9	1.23	17.07	4.14	17.91
Total	29.85	239.44	86.16	241.52
Mean	3.32	26.60	9.57	26.84

Tobacco + PMO				
1	4.14	22.07	17.49	36.61
2	1.65	15.15	16.64	65.41
3	0.81	19.98	5.81	17.50
4	0.81	17.07	5.81	30.00
5	4.14	32.49	6.66	17.49
6	4.14	25.41	9.15	33.75
7	0	9.50	14.98	26.64
8	0	9.50	6.66	18.73
9	1.65	18.74	7.49	12.48
Total	17.34	169.91	90.69	258.61
Mean	1.92	18.87	10.07	28.73
Control				
1	4.14	20.82	2.07	13.33
2	7.89	41.25	4.14	19.57
3	4.98	29.16	9.14	24.98
4	2.91	31.14	19.56	23.33
5	1.65	20.82	10.83	25.41
6	2.07	12.90	12.91	12.90
Total	23.64	156.09	58.65	119.52
Mean	3.94	26.02	9.78	19.92

Table III.7 Number of goblet cells in 3 mm airway epithelium after staining with sialidase ABpH 2.6–PAS – TRACHEA

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	0.24	10.29	17.91	72.68
2	0	2.85	2.58	78.53
3	0	4.14	0.75	48.54
4	0	3.15	7.14	80.45
5	0	0	7.17	51.12
6	0.33	9.18	16.32	67.32
Total	0.57	29.61	51.87	398.64
Mean	0.10	4.94	8.65	66.44

Tobacco + PMO					
*(a)	1	0	1.02	0.15	19.35
	2	0	2.22	2.22	24.59
	3	0	2.98	0.36	16.42
	Total	0	6.22	2.73	60.36
	Mean		2.07	0.91	20.12
(b)	5	0	7.77	0	3.75
	6	0	8.39	1.36	2.26
	7	0	7.11	0	3.53
	Total	0	23.27	1.36	9.54
	Mean		7.75	0.45	3.18
Control					
	1	0	4.98	1.17	20.17
	2	0.78	13.76	0.66	10.38
	3	0.11	18.72	0.91	14.50
	4	3.72	18.06	0.30	16.22
	5	0.77	23.77	1.66	14.50
	6	0	10.00	0	0.66
	Total	5.38	89.29	4.60	76.97
	Mean	0.90	14.88	0.77	12.83

* Results from animals 1 - 3 and 5 - 7 represent 3 animals chosen from each group designated (a) & (b) - see p.

Table III.8 Number of goblet cells in 3 mm airway epithelium after staining with sialidase ABpH 2.6-PAS - AXIAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	5.55	22.21	7.32	54.44
2	0.81	23.41	2.82	53.00
3	0	19.24	0	58.75
4	1.20	39.99	1.80	34.24
5	6.51	34.45	0	22.60
6	0.63	31.35	0	31.54
Total	14.70	170.65	11.94	254.57
Mean	2.45	28.44	1.99	42.43

Tobacco + PMO				
1	0.33	31.95	1.68	36.39
2	3.33	32.22	2.76	49.72
3	0.27	37.20	0	39.15
4	0.87	17.10	1.29	32.88
5	0	17.60	0	35.15
6	5.31	22.98	3.0	24.33
Total	10.11	159.05	8.73	217.62
Mean	1.68	26.50	1.45	36.27
Control				
1	0	11.28	0	13.10
2	0	14.86	0	14.68
3	1.47	18.50	0	19.61
4	0	16.08	0	12.89
5	1.92	18.56	0	16.16
6	0.90	15.07	0	11.38
Total	4.29	94.35	0	87.82
Mean	0.72	15.73		14.64

Table III.9 Number of goblet cells in 3 mm airway epithelium after staining with sialidase ABpH 2.6-PAS - LATERAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	1.65	57.90	1.65	46.65
2	0	20.41	0	24.15
3	7.08	55.58	3.75	37.08
4	9.52	24.92	1.58	23.49
5	5.82	43.32	4.14	18.13
6	2.49	32.91	2.91	32.50
Total	26.56	235.04	14.03	182.00
Mean	4.43	39.17	2.34	30.33

Tobacco + PMO				
1	2.91	26.69	3.33	37.90
2	0.39	34.53	0	19.14
3	0	16.66	0	22.49
4	13.31	34.98	13.32	54.25
5	15.82	42.90	1.65	11.25
6	0.81	27.48	4.14	34.56
Total	33.24	183.24	22.44	229.09
Mean	5.54	30.54	3.74	38.18
Control				
1	0.39	14.90	0	11.25
2	0.39	12.90	0.39	11.66
3	0.39	23.32	2.07	14.06
4	1.23	22.90	0	14.89
5	1.23	20.40	1.23	11.24
6	0.39	28.56	0	8.82
Total	4.02	122.98	3.69	71.92
Mean	0.67	20.50	0.62	11.99

Table III.10 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 1.0–PAS – TRACHEA

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	14.69	68.31	2.67	5.55
2	12.21	69.00	0.78	7.02
3	6.44	17.01	0	0
4	3.00	38.88	0.48	0.48
5	5.45	31.17	0.45	11.19
6	17.97	47.37	0.36	7.38
Total	59.76	271.74	4.74	31.62
Mean	9.96	45.29	0.79	5.27

	Tobacco + PMO				
*(a)	1	2.55	9.80	0	0.99
	2	6.48	8.49	2.22	7.38
	3	3.12	18.60	0	0
	Total	24.50	36.89	2.22	8.37
	Mean	8.16	12.29	0.74	2.79
(b)	5	0.60	6.00	0	0
	6	0.54	7.32	0	0
	7	0	18.44	0	0
	Total	1.14	31.76	0	0
	Mean	0.38	10.58		
	Control				
	1	1.38	12.98	0	2.28
	2	1.02	12.08	0	0.39
	3	2.40	19.91	0	0
	4	3.15	19.90	0	0
	5	1.05	13.65	0	1.05
	6	0	10.75	0	0
	Total	9.00	89.27	0	3.72
	Mean	1.50	14.88		0.62

* Results from animals 1 - 3 and 5 - 7 represent 3 animals chosen from each group designated (a) & (b) - see p.

Table III.11 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 1.0-PAS - AXIAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	12.24	76.67	0	1.14
2	6.42	45.21	0	2.37
3	4.62	76.83	0	0
4	5.55	55.36	0	2.61
5	5.31	32.97	0	0
6	4.86	47.67	0	0
Total	39.00	334.71	0	6.12
Mean	6.50	55.79		1.02

Tobacco + PMO				
1	17.31	43.98	0.33	2.64
2	6.72	68.64	1.56	3.96
3	12.88	66.39	0	3.69
4	5.94	65.49	0	10.71
5	3.78	43.97	0	4.77
6	3.57	39.59	0	3.27
Total	50.20	328.04	1.89	28.77
Mean	8.36	54.67	0.31	4.79
Control				
1	0	31.70	0	0
2	0.63	18.79	0	5.43
3	10.41	40.76	0	0.75
4	0	25.32	0	0
5	16.65	53.73	0	0.57
6	2.10	14.52	0	0
Total	29.79	184.82	0	6.75
Mean	4.97	30.80		1.13

Table III.12 Number of goblet cells in 3 mm airway epithelium after staining with ABpH 1.0–PAS – LATERAL PATHWAY

Animal Group and No.	GOBLET CELLS			
	L.PAS	S.PAS	L.AB	S.AB
Tobacco				
1	12.48	93.33	0	2.01
2	2.49	83.73	0	0.81
3	3.15	57.12	0	1.98
4	10.83	38.81	0	0
5	19.56	52.89	0	0
6	10.41	73.74	0	0.39
Total	58.92	399.62	0	5.19
Mean	9.82	66.60		0.87

Tobacco + PMO				
1	6.66	49.14	0	0
2	5.82	98.73	0	3.75
3	0	47.50	0	0
4	23.31	89.19	0	2.49
5	10.65	40.39	0	0
6	6.66	42.48	0	0
Total	53.10	367.40	0	6.24
Mean	8.85	61.23		1.04
Control				
1	0.81	18.73	0	0
2	2.07	48.75	0	1.23
3	5.40	49.14	0	3.33
4	1.44	19.42	0	0.36
5	6.66	51.66	0	0
6	0	17.50	0	0
Total	16.38	205.20	0	4.92
Mean	2.72	34.20		0.82

The staining pattern of epithelial glycoproteins in response to AB-PAS techniques has been described previously (p. 94). Briefly, after ABpH 2.6-PAS cells staining with AB contain acid glycoprotein while those staining with PAS contain the neutral form. After sialidase ABpH 2.6-PAS cells staining with AB may contain sialomucin resistant to sialidase or sulphomucin and cells containing sulphomucin are identified by their staining with AB after ABpH 1.0-PAS.

Analysis of the pattern of staining in the way which is illustrated in Table III.13 identifies further the types of glycoproteins within the goblet cell population. Thus acid glycoprotein is identified as either one, or a combination, of three types - sialomucin sensitive or resistant to sialidase or sulphomucin occurring alone or in combination. In the following results the description in the text is by reference to the type of glycoprotein within a goblet cell rather than to its staining properties.

The Effect of Tobacco on the Distribuiton of Goblet Cells containing Different Types of Glycoprotein

(i) Cells containing Acid or Neutral Glycoprotein

At each airway level in rats exposed to tobacco there was an increase in the number of goblet cells containing acid glycoprotein compared with the number in the control animals (L.AB + S.AB cells - Tables III.4-6). Both cells containing a large and small amount of acid glycoprotein were increased. The greatest increase in the absolute number of acid glycoprotein containing cells was in the

Table III.13 Identification of glycoproteins within goblet cells of rat airway epithelium

In tissue sections stained with either (a) ABpH 2.6-PAS
(b) sialidase ABpH 2.6-PAS or
(c) ABpH 1.0-PAS

No. goblet cells staining with AB after (a)
= no. goblet cells containing <u>acid glycoprotein</u>

No. goblet cells staining with PAS after (a)
= no. goblet cells containing <u>neutral glycoprotein</u>

Difference between no. goblet cells staining with AB after (a) and (b)
= no. goblet cells containing <u>sialomucin sensitive to sialidase</u>

Difference between no. goblet cells staining with AB after (b) and (c)
= no. goblet cells containing <u>sialomucin resistant to sialidase</u>

No. goblet cells staining with AB after (c)
= no. goblet cells containing <u>sulphomucin</u>

trachea, the increeses being similar but less in the axial end lateral pathwaye. In addition, the relative number of cells containing acid glycoprotein, as compered with those containing neutral glycoprotein, was increased from 46% of the total goblet cell populetion in control animals, to 88% in the trachee of enimels exposed to tobacco. There was e small increase in these cells in the intrapulmonary airways; in the exial pethway from 44 to 51% end in the leteral pethway from 49 to 54%.

Analysis of results by the chi-squared test showed a significant difference in the distribution of the verious types of goblet cell at each airway level, in enimals exposed to tobacco as compared with the control distribution (Teble III.14). Within the goblet cell population of exposed animels there wes a shift to cells producing acid glycoprotein et the expense of cells producing neutral glycoprotein, in the trachea ($p < 0.001$) and in the axial and lateral pathweys ($p < 0.01$ for both). In the trachea and in the axial pathway the shift was to goblet cells containing lerge and small amounts of glycoprotein while in the lateral pathwey it was to cells containing only a small amount.

Table III.14 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. ABpH 2.6-PAS

TOBACCO/CONTROLS		Actual value	Expected value	A-E
Trachea	L.PAS	13	26	- 13
	S.PAS	74	358	- 284
	L.AB	187	26	+ 161
	S.AB	429	293	+ 136
$Px^2 <$				0.001
Axial pathway	L.PAS	34	42	- 8
	S.PAS	245	282	- 37
	L.AB	73	60	+ 13
	S.AB	220	188	+ 32
$Px^2 <$				0.01
Lateral pathway	L.PAS	30	40	- 10
	S.PAS	239	259	- 20
	L.AB	86	98	- 12
	S.AB	242	200	+ 42
$Px^2 <$				0.01

(ii) Cells containing Different Types of Acid Glycoprotein

After sialidase ABpH 2.6-PAS staining at each airway level in animals exposed to tobacco, there was an increase in the absolute number of goblet cells containing acid glycoprotein unaffected by sialidase digestion - that is, an increase in cells containing sialomucin resistant to sialidase or sulphomucin - as compared with the number in control animals (L.AB + S.AB cells - Tables III.7-9). The relative number of goblet cells containing these glycoproteins was also increased from 46% in control animals to 94% in the trachea of animals exposed to tobacco, from 47 to 58% in the axial pathway and from 37 to 43% in the lateral pathway.

There was a significant difference in the distribution of the various types of goblet cell at each airway level in animals exposed to tobacco as compared with the distribution in control animals (Table III.15). Within the goblet cell population of exposed animals there was a shift to cells producing sialidase resistant sialomucin and sulphomucin ($Px^2 < 0.001$ at each airway level). In Table III.15 and in Tables that follow (Tables III.16-19), in some instances large and small cells staining with PAS were grouped together, as in some instances were large and small cells staining with AB. The data was grouped in this way to give a sufficient number of cells within an 'Expected' group (i.e. $\geqslant 5$) an insufficient number precluding analysis of results by the chi-squared test. At each airway level the shift was to cells containing these acid glycoproteins (from Tables III.7-9 it was apparent that this was due mainly to cells containing only a small amount), and in the axial and lateral pathways there was also a shift to cells now containing a large amount of the neutral form (vide infra).

Table III.15 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. Sialidase ABpH 2.6-PAS

TOBACCO/CONTROLS		Actual value	Expected value	A-E
	L.PAS	1	14	- 13
	S.PAS	30	244	- 214
Trachea				
	L.AB	52	14	+ 38
	S.AB	399	211	+ 188
$Px^2<$				0.001
	L.PAS	15	10	+ 5
	S.PAS	171	229	- 58
Axial pathway				
	L.AB+S.AB	267	214	+ 53
$Px^2<$				0.001
	L.PAS	27	9	+ 18
	S.PAS	235	278	- 43
Lateral pathway				
	L.AB	14	9	+ 5
	S.AB	182	162	+ 20
$Px^2<$				0.001

After ABpH 1.0-PAS staining, in rats exposed to tobacco, only in the trachea was there a small increase in the absolute number of goblet cells containing sulphomucin above that in control animals (L.AB + S.AB cells - Tables III.10-12); the relative number of these cells was increased only from 3 to 9%. There was no increase in the number of these goblet cells in the axial and lateral pathways.

In the trachea and in the lateral pathway there was a significant difference in the distribution of the various types of goblet cell in the animals exposed to tobacco ($Px^2 < 0.001$ for both) but, as compared with the control distribution, the shift was to cells containing a large amount of glycoprotein staining with PAS - which with this staining technique may be either a neutral glycoprotein or sialomucin. In the trachea there was also some shift to cells containing sulphomucin (Table III.16); from the absolute values in Tables III.10-12 it was apparent that this was due mainly to cells containing only a small amount of this glycoprotein.

From these results it can be concluded that in the tracheal epithelium of the animals exposed to tobacco there was an increase in the number of cells containing acid glycoprotein in either large or small amounts, and that this increase was due mainly to cells containing sialomucin resistant to sialidase and to a small extent to those containing sulphomucin. In the intrapulmonary airways, as in the extrapulmonary, the main increase in cells containing acid

Table III.16 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. ABpH 1.0-PAS

TOBACCO/CONTROLS		Actual value	Expected value	A-E
Trachea	L.PAS	60	33	+ 27
	S.PAS	272	322	- 50
	L.AB+S.AB	37	14	+ 23
	$Px^2 <$			0.001
Axial pathway	L.PAS	39	51	- 12
	S.PAS	335	317	+ 18
	L.AB+S.AB	6	12	- 6
	$Px^2 <$			NS
Lateral pathway	L.PAS	59	33	+ 26
	S.PAS	400	421	- 21
	L.AB+S.AB	5	10	- 5
	$Px^2 <$			0.001

glycoprotein was in cells containing sialomucin resistant to sialidase. In the intrapulmonary airways there was some evidence that some cells contained sialomucin sensitive to sialidase since there was a shift to L.PAS cells after sialidase ABpH 2.6-PAS. The description of a shift from one goblet cell to another demonstrated by sialidase ABpH 2.6-PAS and ABpH 1.0-PAS is included in the section of results given later in this chapter which describes the combination and location of granules of different types of acid glycoprotein within goblet cells.

The Effect of Tobacco + PMO on the Distribution of Goblet Cells containing Different Types of Glycoprotein

(i) Cells containing Acid or Neutral Glycoprotein

In the tracheal epithelium of the animals exposed to tobacco + PMO it was apparent that there was a difference in response between animals within the group. It was striking that in 4 animals exposed to tobacco + PMO (Nos. 1-4, see Table III.4), there were more goblet cells containing acid glycoprotein (L.AB + S.AB cells) than in each of the remaining 5 animals within the group (Nos. 5-9). Comparison of the mean number of cells containing large and small amounts of acid glycoprotein showed a significant difference in these cell types between the first group of animals and the second ($p < 0.001$ and < 0.01 respectively). The findings for these two groups, which are referred to as tobacco + PMO (a) and (b), are analysed separately.

Comment The absolute number of goblet cells was greater in those animals in tobacco + PMO (a) than in (b). As compared with the control animals, however, there was no significant difference in the total goblet cell number in the group tobacco + PMO (a) (mean 32.58, S.E. 2.19) while that in the group tobacco + PMO (b) (mean 19.80, S.E. 0.89) was significantly reduced ($p < 0.01$).

In the trachea of animals in the group tobacco + PMO (a) there was an increase in the number of goblet cells containing acid glycoprotein as compared with the number of these cells in control animals (L.AB + S.AB cells - Table III.4). There was an increase in both the mean absolute number and in the relative number of these cells from 46% in control animals to 84% in those in the group tobacco + PMO (a). Analysis of results by the chi-squared test showed a significant difference in the distribution of the various goblet cell types as compared with the control distribution (Table III.17). Within the goblet cell population of the trachea of the exposed animals there was s shift to cells producing acid glycoprotein at the expense of cells producing neutral glycoprotein ($p < 0.001$). The shift was to cells containing both large and small amounts of glycoprotein.

In the trachea of those animals in the group tobacco + PMO (b) there was no increase in the number of goblet cells containing acid glycoprotein above that in control animals (L.AB + S.AB cells - Table III.4). There wae a significant difference in the distribution

Table III.17 Goblet cell types in rat airway epithelium. Actual values - TOBACCO + PMO - compared with those Expected on the basis of the control distribution. ABpH 2.6-PAS

TOBACCO+PMO/CONTROL		Actual value	Expected value	A-E
Trachea (a)	L.PAS	6	5	+ 1
	S.PAS	16	67	- 51
	L.AB	36	5	+ 31
	S.AB	74	55	+ 19
P_{x^2}				0.001
Trachea (b)	L.PAS+S.PAS	79	55	+ 24
	L.AB+S.AB	21	46	- 25
P_{x^2}				0.001
Axial pathway	L.PAS	33	49	+ 21
	S.PAS	217	327	- 110
	L.AB	91	70	+ 21
	S.AB	323	219	+ 104
P_{x^2}				0.001
Lateral pathway	L.PAS	17	36	+ 3
	S.PAS	170	233	- 63
	L.AB	91	88	+ 3
	S.AB	259	180	+ 79
P_{x^2}				0.001

of goblet cells of each group (PAS or AB) as compared with the distribution in control animals ($P_{x^2} < 0.001$) but there was a shift away from cells containing acid glycoprotein to cells containing the neutral form (Table III.17); from Table III.4 it was apparent that this was due mainly to cells containing a small amount of neutral glycoprotein.

In the intrapulmonary airways there was no apparent difference in the response between animals exposed to tobacco + PMO which, for analysis of the change at this site, were thus treated as a single group.

In the axial and lateral pathways of the animals exposed to tobacco + PMO, there was an increase in both the absolute and relative number of goblet cells containing acid glycoprotein compared with the number in control animals. In the axial pathway the increase was from 46% of the total goblet population in control animals to 62% in those exposed; and the increase in the lateral pathway was from 49 to 65%. Within the goblet cell population of the exposed animals there was a shift to cells producing acid glycoprotein - to cells containing both large and small amounts - and some shift to those containing a large amount of neutral glycoprotein; these shifts were at the expense of the cells containing a small amount of the neutral form.

(ii) Cells containing Different Types of Acid Glycoprotein

Since cells containing acid glycoprotein were present in the tracheal epithelium of animals in the group tobacco + PMO (a) and not in the group tobacco + PMO (b), the findings after staining with sialidase ABpH 2.6-PAS and ABpH 1.0-PAS are described only for the former group.

After sialidase ABpH 2.6-PAS staining, there was an increase in the absolute number of goblet cells containing sialomucin resistant to sialidase and sulphomucin in animals exposed to tobacco + PMO as compared with control animals (L.AB + S.AB cells - Tables III.7-9). The relative number of these cells was increased from 46% in the trachea of control animals to 91% in the animals in the group tobacco + PMO (a). In the axial and lateral pathways the increase was from 47 to 57% and from 37 to 54% respectively.

There was a significant difference in the distribution of the various types of goblet cells in the trachea of animals in the group tobacco + PMO (a) and in the intrapulmonary airways of the tobacco + PMO group as a whole as compared with the control distribution (Table III.18).

Table III.18 Goblet cell types in rat airway epithelium. Actual value - TOBACCO + PMO - compared with that Expected on the basis of the control distribution. Sialidase ABpH 2.6-PAS

TOBACCO+PMO/CONTROLS		Actual value	Expected value	A-E
Trachea (a)	L.PAS+S.PAS	6	37	- 31
	L.AB+S.AB	63	32	+ 31
$Px^2 <$				0.001
Axial pathway	L.PAS	10	9	+ 1
	S.PAS	159	200	- 41
	L.AB+S.AB	227	187	+ 40
$Px^2 <$				0.001
Lateral pathway	L.PAS	33	9	+ 24
	S.PAS	183	283	- 100
	L.AB	22	9	+ 13
	S.AB	229	166	+ 63
$Px^2 <$				0.001

There was a shift to cells producing sialomucin resistant to sialidase at each airway level ($Px^2 < 0.001$) and in the lateral pathway there was a shift also to cells now containing a large amount of the neutral form; from Tables III.7-9 it was again apparent that the shift was mainly to cells containing only a small amount of sialomucin resistant to sialidase.

...../Text continues p. 152

After AB pH 1.0–PAS staining, only in the trachea and in the axial pathway of rats exposed to tobacco + PMO was there a small increase in the absolute number of goblet cells containing sulphomucin above the number present in control animals (L.AB + S.AB cells – Tables III.10–12). There was an increase in the relative number of these cells from 3% in the trachea of control animals to 15% in the group tobacco + PMO (a), and from 3 to 8% in the axial pathway of the group as a whole.

At each airway level there was a significant difference in the distribution of the various types of goblet cell in animals exposed to tobacco + PMO as compared with the distribution in control animals ($Px^2 < 0.001$ for each airway level), while in the trachea and axial pathway there was a shift to cells producing sulphomucin (Table III.19), in the lateral pathway and in the trachea also there was a shift to cells containing a large amount of glycoprotein staining with PAS, that may be either neutral glycoprotein or sialomucin.

It can be concluded that only in the trachea of animals in the group tobacco + PMO (a) (and not in those in tobacco + PMO (b)) was there an increase in the number of goblet cells containing either large or small amounts of acid glycoprotein, and that this increase was due mainly to cells containing sialomucin resistant to sialidase and to a few containing sulphomucin. In the intrapulmonary airways of the group as a whole, there was a similar increase save that there were fewer cells containing sulphomucin and these were present only in the axial pathway. In the lateral pathway there was some evidence also that some cells

Table III.19 Goblet cell types in rat airway epithelium. Actual value - TOBACCO + PMO - compared with that Expected on the basis of the control distribution. ABpH 1.0-PAS

TOBACCO+PMO/CONTROLS		Actual value	Expected value	A-E
Trachea (a)	L.PAS	25	5	+ 20
	S.PAS	37	63	- 26
	L.AB+S.AB	10	5	+ 5
	p_x^2			0.001
Axial pathway	L.PAS	50	55	- 5
	S.PAS	328	341	- 13
	L.AB+S.AB	31	13	+ 18
	p_x^2			0.001
Lateral pathway	L.PAS	53	30	+ 23
	S.PAS	367	386	- 19
	L.AB+S.AB	6	9	- 3
	p_x^2			0.001

contained sialomucin sensitive to sialidase since there wes e shift to L.PAS cells efter sialidase ABpH 2.6-PAS, a shift similar to thet seen in animals exposed to tobacco.

Relationship between Goblet Cell Number and Intracellular Glycoprotein in Rat Airway Epithelium efter Exposures to Tobacco or to Tobacco + PMO

Extrapulmonary airways

(i) The effect of tobecco

An increase in the absolute number of goblet cells in the tracheal epithelium of enimals exposed to tobacco was entirely due to an increese in the absolute number of goblet cells containing acid glycoprotein. This was accompanied by an increase in the proportion of these cells in the total. Most of the cells contained sielomucin resistant to sialidase.

(ii) The effect of tobacco + PMO

In the tracheal epithelium in 4 of the 9 animals exposed to tobacco + PMO there was no increase in goblet cell number elthough there was a change in the type of glycoprotein produced by the goblet celle. As in the animals exposed to tobacco alone, there was an increase in the relative number of cells containing acid glycoprotein - due mainly to an increase in cells conteining sialomucin resistant to sialidase. In the remaining animals within the group there was a reduction in goblet cell number end most of the goblet cells contained neutral glycoprotein.

Intrapulmonary airways

(i) The effect of tobacco and tobacco + PMO

In both the axial and lateral pathways of animals exposed either to tobacco or to tobacco + PMO there was virtually no increase in goblet cell number but an increase in the relative number of cells containing acid glycoprotein that was also sialomucin resistant to sialidase. The relative increase in such cells was greater in animals exposed to tobacco + PMO than in those exposed to tobacco alone.

Intracellular Distribution and Location within Goblet Cells of Secretory Granules containing Different Types of Glycoprotein

There were particular patterns of response to the staining techniques used in this study that identified the type of glycoprotein within a class of goblet cells and its location within a cell.

Neutral Glycoprotein

From ABpH 2.6-PAS staining of the epithelium it was apparent that, at each airway level, in each group of animals there were cells containing only neutral glycoprotein (i.e. L.PAS and S.PAS cells). In all, the glycoprotein occupied at least the apical region of the cell, but extended further into the basal half in some cells (L.PAS) than in others (S.PAS).

As described previously on p.117 other goblet cells contained some neutral glycoprotein in combination with the acid form. In these cells the neutral glycoprotein occupied the area below that

containing the acid. (Since sialidase had almost no effect on the population of cells containing small amounts of any acid glycoprotein, it seemed likely that, in these cells at least, neutral glycoprotein was combined with a sialidase resistant sialomucin).

Acid Glycoprotein

Within the airway epithelium there was a reduction in the relative number of L.AB cells in tissue sections treated with sialidase and stained with ABpH 2.6–PAS, as compared with the number in sections similarly stained but untreated with the enzyme (see Table III.20): the relative number of the S.AB cell type, however, was increased.

...../Text continues p. 158

Table III.20 Percentage of L.AB cells in airway epithelium stained with (a) ABpH 2.6-PAS or (b) sialidase ABpH 2.6-PAS in rats exposed to tobacco or to tobacco + PMO and in control rats.

		Trachea	Axial pathway	Lateral pathway
TOBACCO	(a)	27	13	14
	(b)	11	3	3
TOBACCO + PMO	(a)	19*	14	17
	(b)	4	2	5
CONTROLS	(a)	4	11	16
	(b)	2	0	2

* There was a reduction from 27 to 4% of L.AB cells in the trachea of those animals in the group tobacco + PMO (a) in which there was a striking shift to cells containing acid glycoprotein and virtually no change in the number of L.AB cells in the animals exposed to tobacco + PMO (b) in which the shift did not occur.

At each airway level in animals exposed to tobacco or to tobacco + PMO, and in the intrapulmonary airways of control animals, there was a significant difference in the distribution of goblet cells of each type after sialidase ABpH 2.6-PAS as compared with the distribution of goblet cells of each type in sections stained with ABpH 2.6-PAS alone ($Px^2 < 0.001$ for each - Tables III.21-23).

In the epithelium of the trachea and axial pathway in animals exposed to tobacco, and in the intrapulmonary airways of control animals and those exposed to tobacco + PMO, the difference was due to a shift away from cells containing a large amount of acid glycoprotein (L.AB) toward cells containing a small amount of glycoprotein which was not affected by sialidase digestion - that is, toward S.AB cells containing sialomucin resistant to sialidase or sulphomucin. The action of the enzyme was thus to convert L.AB cells in sections stained with ABpH 2.6-PAS to S.AB cells in sections stained with sialidase ABpH 2.6-PAS.

(i) Sialomucin sensitive and resistant to sialidase

In general, since more goblet cells contained an acid glycoprotein unaffected by sialidase treatment (and excluding here the number of cells which shifted from the L.AB category and which could thus contain a sialidase sensitive sialomucin) than the eventual number of goblet cells found to contain sulphomucin, it follows that many cells contained only sialomucin resistant to sialidase.

In addition, where an increase in the absolute number of S.AB cells

Table III.21 Distribution of goblet cells types in rat airway epithelium after Sialidase ABpH 2.6-PAS (Actual value) compared with the distribution based on values after ABpH 2.6-PAS (Expected value). CONTROL

SIALIDASE ABpH 2.6-PAS/ ABpH 2.6-PAS		Actual value	Expected value	A-E
Trachea	L.PAS	5	6	- 1
	S.PAS	89	90	- 1
	L.AB	5	6	- 1
	S.AB	77	73	+ 4
$Px^2 <$				NS
Axial pathway	L.PAS	4	14	- 10
	S.PAS	94	92	+ 2
	L.AB	0	20	- 20
	S.AB	88	61	+ 27
$Px^2 <$				0.001
Lateral pathway	L.PAS	4	14	- 10
	S.PAS	123	88	+ 35
	L.AB	4	33	- 29
	S.AB	72	68	+ 4
$Px^2 <$				0.001

Table III.22 Distribution of goblet cell types in rat airway epithelium after Sialidase ABpH 2.6-PAS (Actual value) compared with the distribution based on values after ABpH 2.6-PAS (Expected value). TOBACCO

SIALIDASE ABpH 2.6-PAS/ABpH 2.6-PAS		Actual value	Expected value	A-E
Trachea	L.PAS	1	9	- 8
	S.PAS	30	51	- 21
	L.AB	52	128	- 76
	S.AB	399	294	+ 105
	$Px^2 <$			0.001
Axial pathway	L.PAS	15	27	- 12
	S.PAS	171	194	- 23
	L.AB	12	58	- 46
	S.AB	255	174	+ 81
	$Px^2 <$			0.001
Lateral pathway	L.PAS	27	23	+ 4
	S.PAS	235	183	+ 52
	L.AB	14	66	- 52
	S.AB	182	186	- 4
	$Px^2 <$			0.001

Table III.23 Distribution of goblet cell types in rat airway epithelium after Sialidase ABpH 2.6–PAS (Actual value) compared with the distribution based on values after ABpH 2.6–PAS (Expected value). TOBACCO + PMO

SIALIDASE ABpH 2.6–PAS/ABpH 2.6–PAS		Actual value	Expected value	A–E
Trachea (a)	L.PAS	5	7	– 2
	S.PAS	16	50	– 34
	L.AB	36	23	+ 13
	S.AB	75	52	+ 23
$Px^2 <$				0.001
Axial pathway	L.PAS	10	20	– 45
	S.PAS	159	129	+ 39
	L.AB	8	53	– 45
	S.AB	218	193	+ 25
$Px^2 <$				0.001
Lateral pathway	L.PAS	33	15	+ 18
	S.PAS	183	148	+ 35
	L.AB	22	79	– 57
	S.AB	229	225	+ 4
$Px^2 <$				0.001

was accompanied by a corresponding decrease in the number of L.AB cells by sialidase, many of the L.AB cells here contained only sialomucin sensitive and resistant to sialidase. (The small number of goblet cells containing sulphomucin also precludes this type of acid glycoprotein within many cells in the above population).

In the lateral pathway of animals exposed to tobacco, the shift was away from cells containing acid glycoprotein unaffected by sialidase toward cells staining with PAS. This staining response indicated cells containing either large or small amounts of a glycoprotein that was only sialomucin sensitive to sialidase, and as the greater shift was away from cells containing a large amount it was the majority of these cells that contained such a glycoprotein.

(ii) Sulphomucin

Goblet cells containing glycoprotein staining with AB after ABpH 1.0-PAS - that is, cells containing sulphomucin - were rarely seen in rat airway epithelium. No cells containing a large amount of this glycoprotein were seen in control animals and only the occasional cell in those exposed to tobacco or to tobacco + PMO. In each animal group it was the cell containing only a small amount of sulphomucin that was more frequently seen, and these cells contained fewer granules of this glycoprotein stained with AB, than the number of granules staining with AB in the small cell population after sialidase ABpH 2.6-PAS. Here, some granules of acid glycoprotein in some of the S.AB cells which stained with AB at

pH 2.6 after sialidase, did not stain with AB at pH 1.0. This suggested that in these cells sialomucin resistant to sialidase was combined with sulphomucin.

Since, on occasion, a cell containing a large amount of sulphomucin did not contain any glycoprotein staining with PAS, it could be taken that such a cell contained only sulphomucin.

These findings thus show that within rat airway epithelium a goblet cell may produce each of the following types of glycoprotein:

(i) neutral glycoprotein;
(ii) sialomucin sensitive to sialidase;
(iii) sialomucin sensitive to sialidase and resistant;
(iv) sialomucin resistant to sialidase;
(v) sialomucin resistant to sialidase and sulphomucin;
(vi) sulphomucin.

From ABpH 2.6–PAS staining of the epithelium, it was apparent that any acid glycoprotein occupied at least the apical region of a cell, and (as in cells containing neutral glycoprotein) that this extended further into the basal half in some cells (L.AB) than in others (S.AB). The pattern of conversion of L.AB to S.AB cells by sialidase suggested that, when combined within a cell, sialomucin sensitive to sialidase occupied the lower part of the intracellular secretory mass, with either sialomucin resistant to sialidase or sulphomucin, or both, at the cell apex. And, where sialomucin resistant

to sialidase and sulphomucin were present together, the latter occupied the apical region. Each single type of acid glycoprotein could occupy only the apical or extend into the basal half of a goblet cell.

Area occupied by Secretory Granules within Goblet Cells containing Acid Glycoprotein

In the tracheal epithelium of animals exposed either to tobacco or to tobacco + PMO, the relative increase in goblet cells containing acid glycoprotein was accompanied by more cells containing an increase in its amount. In tissue sections stained with ABpH 2.6-PAS the relative number of L.AB cells was increased from 4% in control animals to 27% in those exposed to tobacco and to 19% in those exposed to tobacco + PMO. Thus, at this airway level in exposed animals, proportionately more goblet cells contained more secretory product that was acid glycoprotein.

At each airway level in all animals exposed to tobacco + PMO, however, the large AB staining goblet cells appeared more distended than this cell type in either animals exposed to tobacco or in control animals. This was due to an increase in the cell area occupied by secretory granules because of an increase in the number of granules rather than size.

At each airway level, cells were measured if either the width or height of the secretory mass was 5 units or more of an eyepiece graticule

and the product of these two measurements taken as an 'index' of the area of granules occupying a cell (see p.100). Cells were measured along the full length of the epithelium at each airway level. In control animals, as compared with animals exposed to smoke, there were fewer cells at each airway level with enough secretory granules staining with AB to be included in the count.

One animal was chosen at random from the group exposed to tobacco and from the control group while two animals were chosen from the group exposed to tobacco + PMO. Of the animals exposed to tobacco + PMO, one had shown a marked and one a lesser shift to cells secreting acid glycoprotein but both gave a high area index with little difference between their area size. These results for animals in each group are given in Table III.24.

At each airway level the mean cell area occupied by secretory granules staining with AB was greater in the animal exposed to tobacco + PMO than in either of the other animals. In both the axial and lateral pathways of the animal exposed to tobacco + PMO the value for the secretory mass was significantly increased above that for the animals in each of the other groups (tobacco + PMO/tobacco and tobacco + PMO/controls $p < 0.001$ respectively). In the trachea, the value for the animal exposed to tobacco + PMO was significantly ($p < 0.02$) above that exposed to tobacco (since taller but thinner cells gave a lower index of secretory mass in the tobacco exposed animal) but was not significantly increased above the control.

Table III.24 Width and height (μm) of the secretory mass staining with AB, and their product as an index of area (μm_2), in goblet cells of rat airway epithelium.

	TRACHEA			AXIAL PATHWAY			LATERAL PATHWAY		
Animal group	Width	Height	Area	Width	Height	Area	Width	Height	Area
Tobacco	5.20 (0.69) *	10.53 (1.43)	47.99 (3.92)	6.31 (0.38)	6.50 (0.40)	49.92 (3.53)	6.12 (0.25)	5.01 (0.50)	30.66 (3.22)
Tobacco + PMO	8.71 (0.47)	7.15 (0.39)	61.35 (3.26)	8.70 (0.43)	7.80 (0.23)	63.01 (3.92)	7.61 (0.28)	6.96 (0.35)	53.91 (3.81)
Controls	7.51 (0.80)	8.23 (0.75)	56.44 (3.98)	6.31 (0.53)	5.75 (0.48)	36.21 (4.27)	5.59 (0.93)	4.47 (0.44)	28.16 (4.87)

* S.E. of the mean

Tracheal Submucosal Gland

The structure of the rat tracheal gland has been described by Sturgess (1970) and has been found to resemble that of the human bronchial gland (Meyrick, Sturgess and Reid, 1969) in consisting of a duct system leading from the mucous and serous secretory tubules of the gland to the surface lumen of the airway.

Tracheal gland hypertrophy was assessed by analysis of the mucous tubule population in the way previously described on p.101. In both the normal gland, and in the gland of exposed animals, adequate measurement of the serous tubules was difficult and unsatisfactory because of their small size, their structure and their scanty distribution and which prevented their use in the assessment of gland hypertrophy. In addition, in the glands of the exposed animals it was obviously the size and number of the mucous tubules that was greatly increased while there was little change in the serous tubules.

The mean value of measurement of the mucous tubules in the glands of animals in each group are given in Table III.25.

Mucous tubule size

In both animals exposed to tobacco and to tobacco + PMO, the mean tubule diameter was significantly increased above that in control animals (tobacco/control $p < 0.05$; tobacco + PMO/control $p < 0.02$). The distribution by size of tubules in control and exposed animals is illustrated in Fig. III.3. In both the animals

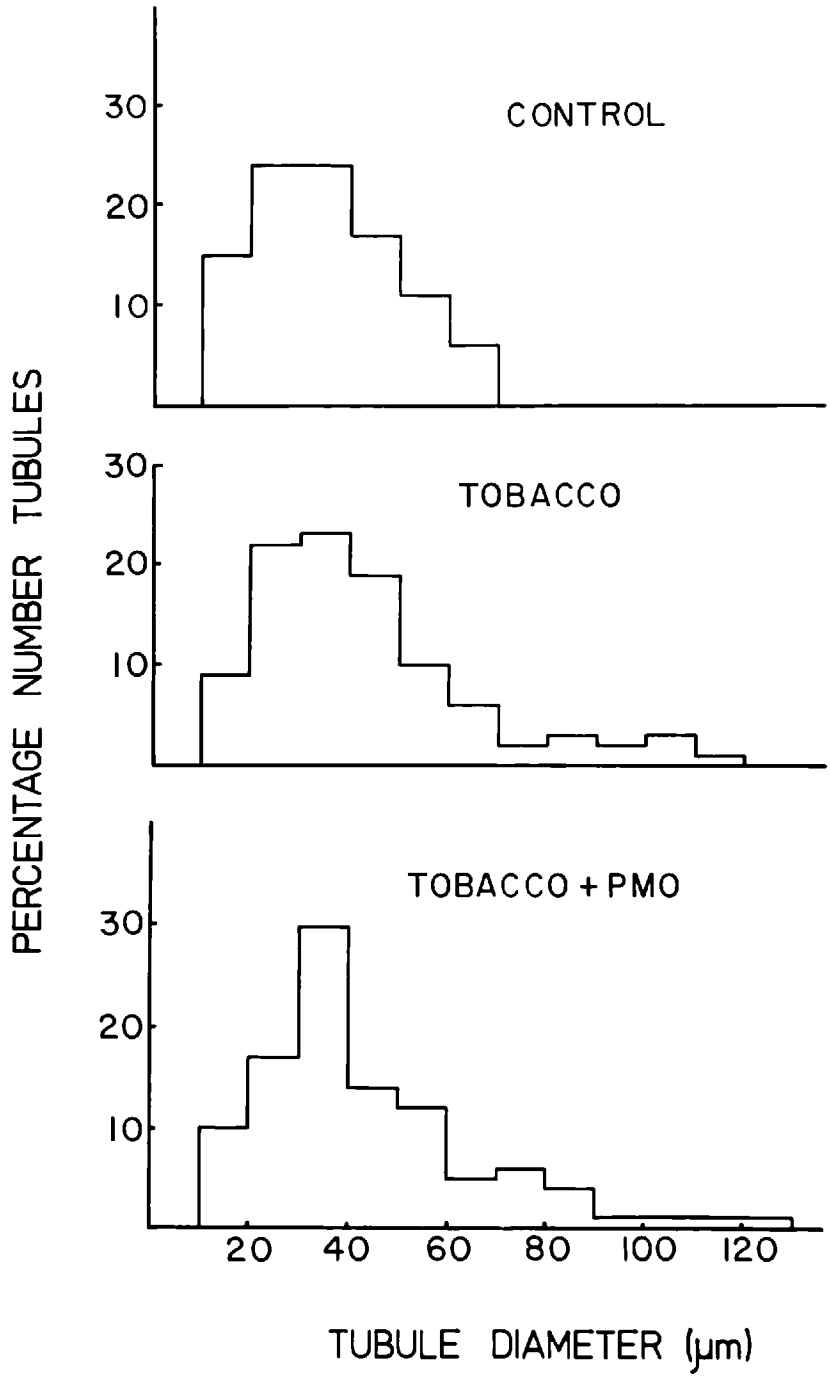
Table III.25 Tracheal gland measurments in rats exposed to tobacco or tobacco + PMO and controls.

	Mean tubule diameter (μm)	Mean lumen diameter (μm)	Mean cell size (μm)
TOBACCO	42.79 (1.83)*	27.92 (1.73)	7.50 (0.18)
TOBACCO + PMO	43.66 (1.67)	29.16 (1.67)	7.23 (0.13)
CONTROL	36.55 (2.32)	24.09 (2.54)	6.26 (0.19)

* S.E. of the mean

Fig. III.3 Distribution of tracheal gland tubules by size in control rats and in rats exposed to tobacco or to tobacco + PMO.

Fig. III.3


CONTROL
TOBACCO
TOBACCO + PMO
30
20
10
PERCENTAGE NUMBER TUBULES
20
40
60
80
100
120
TUBULE DIAMETER (μm)

exposed to tobacco or to tobacco + PMO, there were larger gland tubules than found in control animals - that is, tubules over 70 μm in diameter. In the animals exposed to tobacco, tubules up to a maximum diameter of 120 μm appeared and in those exposed to tobacco + PMO tubules up to a maximum diameter of 130 μm.

The distribution of the absolute number of mucous tubules by size is given in Table III.26. Analysis of these results by the chi-squared test (Table III.27) shows a significant difference in the population of tubules in both exposed groups of animals as compared with that of control animals (both tobacco/controls and tobacco + PMO/controls <0.001). In both exposed groups the significant difference in the distribution of tubules is caused by a shift toward tubules of a larger size; above 60 μm in those exposed to tobacco and an even greater shift to tubules above 50 μm in those exposed to tobacco + PMO. In addition, there is also some shift toward tubules of a smaller size - that is, between 40-49 μm in animals exposed to tobacco and to between 30-39 μm in animals exposed to tobacco + PMO.

Table III.26 Distribution of mucous tubules by size (μm) in rat tracheal gland.

Tubule size (μm)	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	90–99	100–109	110–119	120–129	Total No. tubules
TOBACCO	14	35	36	30	16	10	3	4	3	4	1	0	156
TOBACCO + PMO	19	33	57	27	23	9	11	8	2	1	1	2	193
CONTROLS	14	22	22	15	10	6	0	0	0	0	0	0	89

Table III.27 Size of mucous tubules in rat tracheal gland. Actual values – (i) Tobacco and (ii) Tobacco + PMO compared with those Expected on the basis of the control distribution.

	Tubule size (μm)	Actual value	Expected value	A–E
TOBACCO/CONTROLS				
	10 – 20	14	25	– 11
	20 – 39	35	39	– 4
	30 – 39	36	39	– 3
	40 – 49	30	26	+ 4
	50 – 59	16	18	– 2
	60 –119	25	11	+ 14
$Px^2 <$				0.001
TOBACCO + PMO/CONTROLS				
	10 – 20	19	30	– 11
	20 – 29	33	48	– 15
	30 – 39	57	48	+ 9
	40 – 49	27	33	– 6
	50 – 59	23	22	+ 1
	60 –129	34	13	+ 21
$Px^2 <$				0.001

Mucous tubule cell size

In each exposed group the increase in tubule size was accompanied by a significant increase in the mean cell height (tobacco or tobacco + PMO/controls $p < 0.001$). The distribution of mucous cells by size is given in Table III.28. Analysis showed a significant difference in the cell population of the exposed animals as compared with the controls (tobacco or tobacco + PMO/controls $Px^2 < 0.001$ - see Table III.29). In both exposed groups of animals the significant difference in the distribution of cells is caused by a shift to cells of a larger size, that is in cells between 7 - 16.9 µm in height.

...../Text continues p. 177

Table III.28 Distribution of mucous cells by size (μm) in rat tracheal gland.

Cell size (μm)	1 - 2.9	-4.9	-6.9	-8.9	-10.9	-12.9	-14.9	-16.9	Total number of cells
TOBACCO	5	27	110	98	25	35	6	6	312
TOBACCO + PMO	9	37	150	114	28	41	2	1	382
CONTROLS	11	28	89	29	9	8	1	3	178

Table III.29 Size of mucous cells in rat tracheal gland. Actual values - (i) Tobacco and (ii) Tobacco + PMO as compared with those Expected on the basis of the control distribution.

	Cell size (μm)	Actual value	Expected value	A-E
TOBACCO/CONTROLS				
	1 - 2.9	5	19	- 14
	3 - 4.9	27	49	- 22
	5 - 6.9	110	156	- 46
	7 - 8.9	98	51	+ 47
	9 -10.9	25	16	+ 9
	11 -16.9	47	21	+ 26
$Px^2<$				0.001
TOBACCO + PMO/CONTROLS				
	1 - 2.9	9	24	- 15
	3 - 4.9	37	60	- 23
	5 - 6.9	150	191	- 41
	7 - 8.9	114	62	+ 52
	9 -10.9	28	19	+ 9
	10 -16.9	44	26	+ 18
$Px^2<$				0.001

Mucous tubule lumen size

While there was also an increase in the mean lumen diameter in the exposed animals, the increase was not significantly different from the controls. There was a significant difference, however, between the distribution by size of the mucous tubule lumen in each exposed group as compared with the controls (tobacco or tobacco + PMO/controls $Px^2 < 0.001$ - see Tables III.30 & 31). The difference in distribution is caused not by a shift towards an increase in the size of tubule lumens, but by a distribution where the actual values for lumen size are more sharply peaked than the expected values in the range above 10 and below 20 μm and above 50 and below 110 μm in both the animals exposed to tobacco and in those exposed to tobacco + PMO.

Comparison of the mean mucous tubule lumen with mean tubule size showed no significant difference in this ratio between the control and exposed values (control 0.66; tobacco 0.65; tobacco + PMO 0.67).

Mucous tubule number

Because of the structure of the rat tracheal gland, and its position away from the plates of cartilage, it was not possible to assess gland hypertrophy by the gland to wall ratio in the way described for the human bronchial submucosal gland by Reid (1960). From serial sections of the upper 3 mm of trachea, the mean number of tubules per tracheal ring was calculated for animals in each group,

Table III.30 Distribution of mucous tubule lumens by size (μm) in rat tracheal gland.

Tubule lumen size (μm)	1-9.9	10-19.9	20-29.9	30-39.9	40-49.9	50-59.9	60-69.9	70-79.9	80-89.9	90-99.9	100-109.9	Total No. Tubules
TOBACCO	18	60	28	20	11	7	3	4	3	2	0	156
TOBACCO + PMO	26	61	35	25	17	9	9	7	0	2	2	193
CONTROLS	21	19	19	20	6	4	0	0	0	0	0	89

Table III.31 Size of mucous tubule lumens in rat tracheal gland. Actual values - (i) tobacco and (ii) tobacco + PMO as compared with those Expected on the basis of the control distribution.

	Tubule lumen size (μm)	Actual value	Expected value	A-E
TOBACCO/CONTROLS				
	1 - 9.9	18	37	- 19
	10 - 19.9	60	33	+ 27
	20 - 29.9	28	33	- 5
	30 - 39.9	20	35	- 15
	40 - 49.9	11	11	0
	50 -109.9	19	7	+ 12
$Px^2 <$				0.001
TOBACCO + PMO/CONTROLS				
	1 - 9.9	18	37	- 19
	10 - 19.9	60	33	+ 27
	20 - 29.9	28	33	- 5
	30 - 39.9	20	35	- 15
	40 - 49.9	11	11	0
	50 -109.9	19	7	+ 12
$Px^2 <$				0.001

as was the number of tracheal sections that included gland.

For each animal group, the mean number of tubules per tracheal ring is illustrated in Fig. III.4. In both exposed groups, the number of tubules was increased above the number in the control group, although the increase was significant only in the animals exposed to tobacco + PMO (tobacco + PMO/controls $p < 0.01$). The number of tubules was also significantly increased in the animals exposed to tobacco + PMO as compared with those exposed to tobacco (tobacco + PMO/tobacco $p < 0.05$).

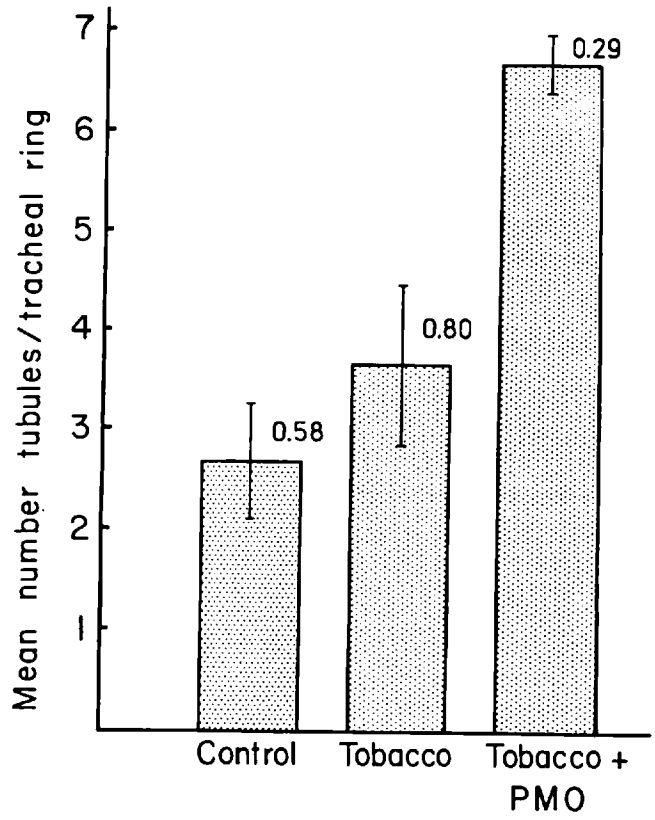
From the number of tissue sections including gland, it was apparent that the depth of the gland was increased in the animals exposed to tobacco (mean 69.04, S.E. 3.64/100 μm trachea) as compared with control animals (mean 57.57, S.E. 3.07/100 μm trachea), although the increase was not significant. In animals exposed to tobacco + PMO, however, there was a significant increase in the depth of the gland (mean 75.86, S.E. 3.36/100 μm trachea) above that in control animals ($p < 0.05$).

Mucous and serous cell glycoproteins

There was no change in the histochemistry of the glycoprotein produced by mucous cells of the rat tracheal gland in response to exposure to tobacco or to tobacco + PMO. After exposure to smoke virtually all of the mucous cells, as in the normal rat gland also, contained glycoprotein which stained with AB at pH 2.6, both with and

Fig. III.4 Mean number of tubules per tracheal ring in control rats and rats exposed to tobacco or to tobacco + PMO.

Fig. III.4


Mean number tubules/tracheal ring
7
6
5
4
3
2
1
0.58
0.80
0.29
Control
Tobacco
Tobacco + PMO

without previous sialidase treatment, and with AB at pH 1.0 with the AB-PAS technique. That is, in both the normal and hypertrophied rat tracheal gland the mucous cells contain sulphomucin.

Similarly, there was no change in the histochemistry of the glycoproteins produced by the serous cells. These cells contain a glycoprotein which stains with PAS using the AB-PAS technique, with AB at pH 2.6, but occasionally they stain with AB at pH 1.0. This pattern of staining suggests that most of the serous cells produce glycoprotein without acid groups (that is, a neutral glycoprotein) but that some acid groups - particularly sulphate - are present within certain cells.

Modification by PMO of the Effect of Tobacco on Airway Epithelium

Exposure of rats to tobacco with and without the addition of PMO produced some airway epithelial changes which were different and some which were similar. Some of the differences in response showed that PMO offered a 'protective' effect against the changes produced by tobacco.

Comparison of change in epithelial height

The 'protective'effect:

The increase in epithelial height in the tracheal epithelium produced by exposure to tobacco was partly prevented by the addition of PMO to the tobacco.

Comparison of change in absolute goblet cell number

The 'protective' effect:

In the tracheal epithelium, increase in the absolute number of goblet cells produced by exposures to tobacco was completely prevented by the addition of PMO.

Absence of 'protective' effect:

In the intrapulmonary airways, there was no difference between the response in animals receiving tobacco with or without PMO, there being a small increase in the goblet cell number above that in control animals.

Comparison of change in goblet cell glycoprotein

Absence of 'protective' effect:

At each airway level, the addition of PMO did not prevent the change produced by tobacco in the nature of the secretory granules of goblet cells from neutral to acid glycoprotein, that was mainly a sialidase resistant sialomucin.

There was at each airway level an increase in the area of secretory mass of acid glycoprotein in goblet cells after exposure to tobacco which was not prevented by the addition of PMO, but was even increased.

Comparison of change in tracheal submucosal gland

Absence of 'protective' effect:

In the tracheal gland, tobacco produced an increase in mucous tubule size and cell size which was not prevented by the addition of PMO. Nor did PMO prevent an increase in mucous tubule number or in the depth of gland per unit length of trachea where it was associated with an even greater increase than that produced by tobacco alone.

SUMMARY AND COMMENT

The following summarises the effect of exposure of rats to tobacco smoke for 6 weeks:

1. Failure to gain weight as rapidly as control animals, the weight gain being less over the days of exposure to smoke than on the days of rest between.

2. Extrapulmonary airway changes

   (i) There is hypertrophy of tracheal epithelium without any obvious epithelial damage (such as desquamation, squamous metaplasia or hyperplasia).

   (ii) The number of goblet cells is increased.

   (iii) There is an increase in both the absolute and relative number of goblet cells containing acid glycoprotein at the expense of those containing neutral glycoprotein.

   (iv) The increase in cells containing acid glycoprotein is mainly from cells containing sialomucin resistant to sialidase, with the appearance of few extra cells containing sulphomucin.

   (v) There is an increase in the number of goblet cells containing a large amount of acid secretory product.

   (vi) There is an increase in the tracheal submucosal gland due to an increase in the mean mucous tubule diameter with an increase in mucous cell size, but no change in the glycoproteins of the mucous and serous cells.

3. Intrapulmonary airway changes

(i) There is no epithelial hypertrophy or evidence of damage.

(ii) There is only a small (and insignificant) increase in the number of goblet cells.

(iii) There is an increase in the relative number of goblet cells containing acid glycoprotein which, as in the extrapulmonary airway, is due mainly to an increase in cells containing sialomucin resistant to sialidase.

(iv) The amount of acid glycoprotein is also increased within many goblet cells.

4. The addition of PMO protects against only some of the effects produced by tobacco smoke. The action of PMO is summarised in Table III.32. Its greatest protective effect is against an increase in goblet cell number in the tracheal epithelium.

It seems that the effective action of PMO on suppression of the total goblet cell number in response to continued exposures to tobacco is more efficient in some animals than in others. It gives partial protection against the increase in epithelial height. It gives no protection against a change in the type of glycoprotein from neutral to acid produced by the goblet cells. It

Table III.32 The effect of tobacco and 'protective' effect of PMO.

EFFECT OF TOBACCO	EFFECT OF PMO (Protection or Enhancement)
Failure to gain weight	NO protection
Extrapulmonary Airways	
Increase in epithelial height	PARTIAL "
Increase in goblet cell number	COMPLETE "
Change in goblet cell glycoprotein	NO "
Increase in amount of secretory product containing acid glycoprotein	ENHANCEMENT
Increase in tracheal submucosal gland size	"
Intrapulmonary Airways	
Change in goblet cell glycoprotein	NO protection
Increase in amount of secretory product containing acid glycoprotein	ENHANCEMENT

causes, within cells, a greater increase than tobacco alone in the amount of acid secretory product. It did not protect against increase in the tracheal submucosal gland size; neither did it produce any change in mucous and serous cell glycoproteins within the gland.

5. Goblet cells of rat airway epithelium have been found to produce a wide range of glycoproteins. They may contain neutral or acid glycoprotein alone or in combination and of the acid glycoprotein both sialidase sensitive and resistant sialomucin and sulphomucin are found. They may be present singly or combined but only certain intracellular combinations occur. Six intracellular distributions of glycoprotein have been established and where only single forms of acid glycoprotein are found within goblet cells they may be present in either large or small intracellular amounts. Where acid and neutral glycoprotein occupy a cell, the acid form is found in the apical region, where sialomucin resistant and sensitive sialomucin are found together it is the resistant that is found in the cell apex, and where sialomucin and sulphomucin are found the latter occupies the apical zone.

CHAPTER IV

THE EFFECT ON GOBLET CELL NUMBER OF RAT AIRWAY EPITHELIUM OF SHORT PERIODS OF EXPOSURE TO TOBACCO SMOKE WITH AND WITHOUT PHENYLMETHYLOXADIAZOLE

DETAILS OF THE EXPERIMENT

In this experiment the response of the goblet cells in rat airway epithelium was studied for periods of exposure to tobacco smoke up to two weeks in length. Animals were sacrificed so that the early response during this period could be analysed - that is, after 1, 2, 3 or 7, or after 14 days.

In total a group of 68 male rats was included from which 40, selected at random, were used for the studies described here. As in the experiment reported previously in this study, animals were exposed either to tobacco or to tobacco + PMO or were untreated (control animals). The number of animals sacrificed from each group on each day of the exposure period is given in Table IV.1.

Animals exposed for 7 or 14 days did not receive consecutive days of exposure. Those animals exposed for 7 days received 6 continuous days of exposure, followed by a 1-day break and a further single day of exposure to smoke. A similar schedule was adopted over the appropriate number of days for animals exposed to smoke for 14 days; that is, 6 continuous days of exposure, 1 of rest, 6 of exposure, 1 of rest and 2 of exposure.

All animals exposed either to tobacco or to tobacco + PMO were sacrificed approximately 20 hours after their last exposure finished. Three control animals were sacrificed at the beginning

Table IV.1 Exposure schedule and number of animals sacrificed from each group on each day of the exposure period.

No. days' exposure	No. cig. (total)	No. Animals/Group		
		Tobacco	Tobacco+PMO	Controls
1	25	3	3	3
2	50	4	4	
3	75	4	4	
7	175	3	3	
14	350	3	3	3
Total no. animals		17	17	6 = 40

of the experiment and 3 at the end. Although there were rather fewer goblet cells per unit length of epithelium in the animals secrificed at the end of the exposure period, since there was no statistically significant difference in cell number nor was there a difference between the two groups in the distribution of cells containing various cell types of glycoproteins, the results for all control animals were taken together.

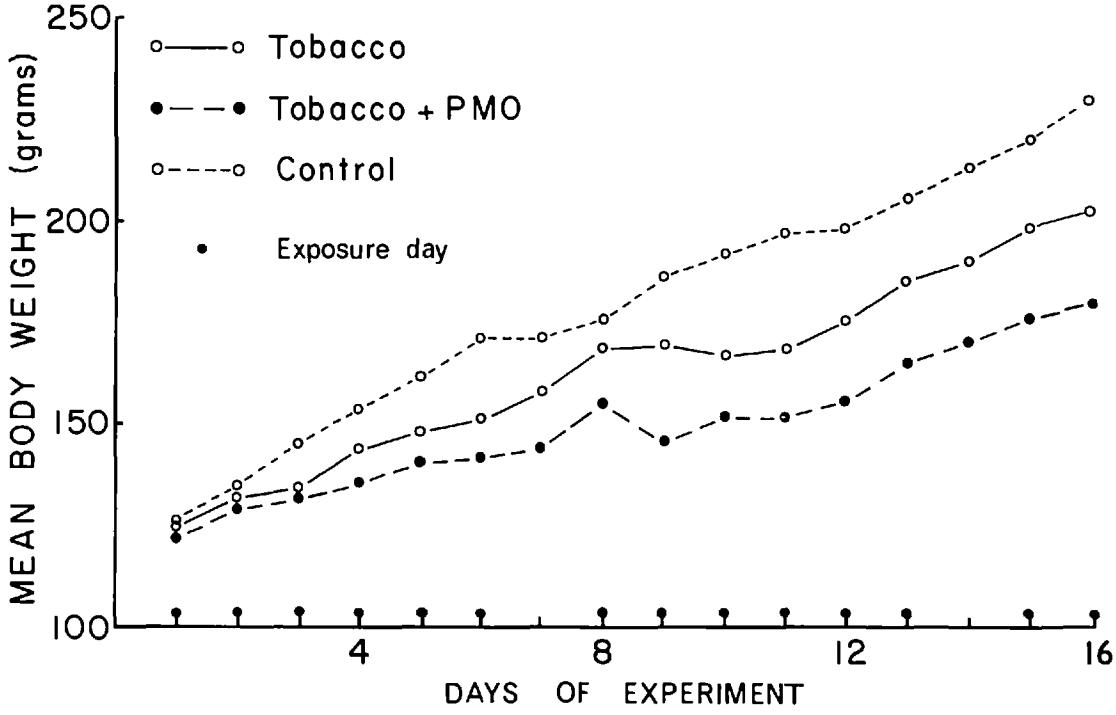
EFFECT ON ANIMALS

Weight Gain

In all animals there was a steady gain in weight over the days of the experiment. The mean daily weights for each animal group are shown in Fig. IV.1. The values for the exposed groups were consistently below those for the control animals, with the animals exposed to tobacco + PMO having the lowest mean weight on each day. After 6 days of exposure there was a significant difference between each of the groups: the control group was significantly increased above the groups exposed to tobacco or to tobacco + PMO ($p < 0.001$ for both) and the group exposed to tobacco significantly increased above that exposed to tobacco + PMO ($p < 0.02$). As the result of the first day's rest from exposure there was then less difference between the groups on day 8, but there was then a significant difference between the mean weights of the three animal groups by the end of the exposure period: the control group was significantly increased above each of the exposed groups ($p < 0.05$ for both) and the group exposed to tobacco significantly increased above that

Fig. IV.1 Gain in body weight of control rats and rats exposed to tobacco or to tobacco + PMO (see text).

Fig. IV.1



	Day 6		Day 16	
	Mean	S.E.	Mean	S.E.
Controls	171.3	4.29	230.0	10.0
Tobacco	151.3	2.39	202.5	6.57
Tobacco + PMO	141.7	2.77	179.2	5.99

exposed to tobacco + PMO ($p < 0.01$). By the end of the exposure period the second day of rest did not seem to allow a similar recovery in the mean weight of each exposed group of animals. (The relevant mean body weight and S.E. of the mean for each group of animals on days 6 and 16 are given with Fig. IV.1).

EFFECT ON AIRWAY EPITHELIUM

Goblet cells were assessed in rat airway epithelium at the seven airway levels previously described in Chapter II: the upper, mid and lower trachea + main bronchi - levels I, II and III, the upper and lower axial pathway - levels IV and V, and the second and third lateral pathways - levels VI and VII.

Before assessing the effect of exposures to tobacco smoke, the number of goblet cells at the airway levels selected for this study was assessed in the epithelium of control animals. The findings for the control animals are described at the beginning of the appropriate results section that follows.

In both control and exposed animals all results for goblet cell number are based on cells counted in tissue sections stained with ABpH 2.6-PAS.

Difference in Goblet Cell Number between Airway Levels in Control Animals

The number of goblet cells in 3 mm epithelium at each airway level

Fig. IV.2 Number of goblet cells per unit length of airway epithelium in control rats.

Fig. IV.2

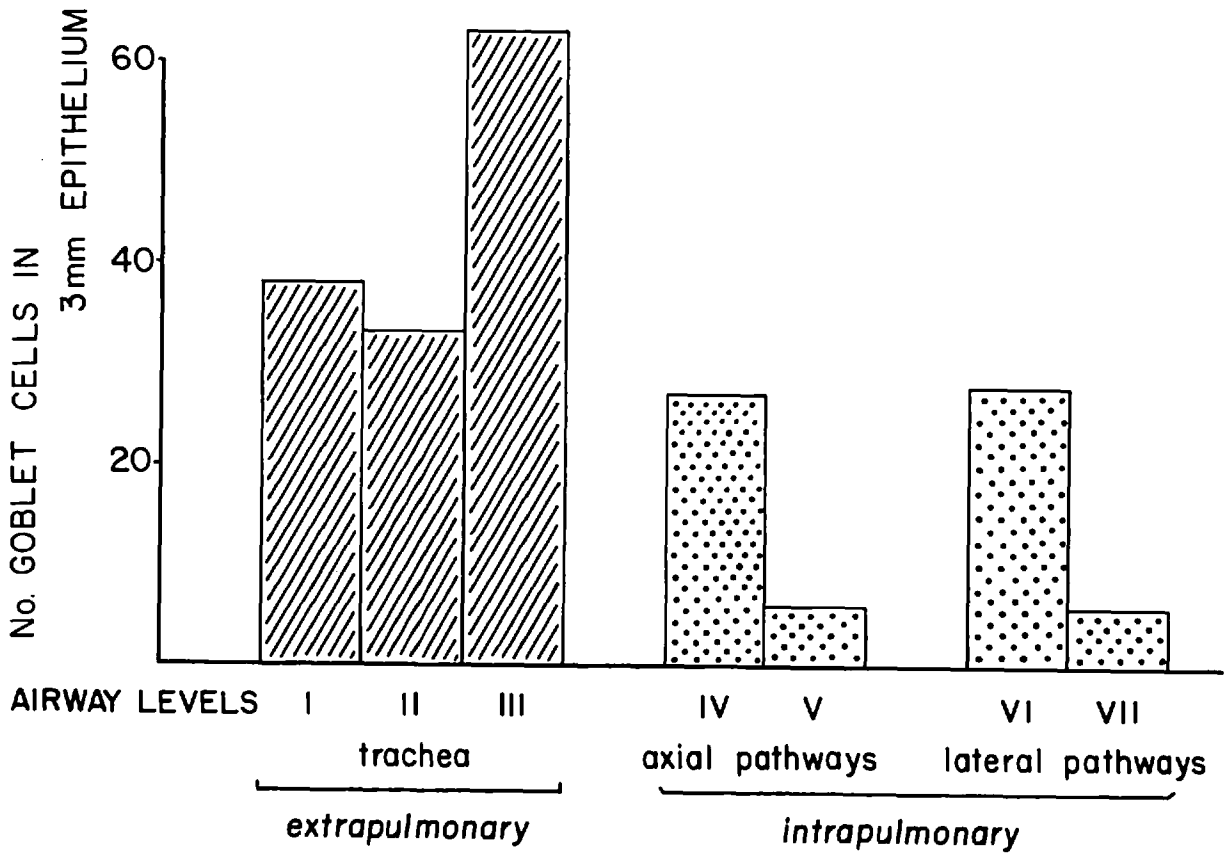

No. GOBLET CELLS IN 3mm EPITHELIUM
60
40
20
AIRWAY LEVELS
I
II
III
IV
V
VI
VII
trachea
axial pathways
lateral pathways
extrapulmonary
intrapulmonary

Table IV.2 Number of goblet cells in 3 mm epithelium of control rats

AIRWAY LEVELS						
I	II	III	IV	V	VI	VII
34.16	41.06	64.78	27.30	5.96	27.69	5.99
(8.29)*	(8.10)	(6.47)	(6.46)	(2.47)	(6.40)	(2.04)

* S.E. of the mean

P values are given where there is a significant difference in goblet cell number between airway levels:

Levels III v I	$p < 0.02$	Levels IV v V	$p < 0.02$
" " v II	$p < 0.05$	" VI v VII	$p < 0.02$
" " v IV and VI	$p < 0.01$		
" " v V and VII	$p < 0.001$		

in control animals is illustrated in Fig. IV.2 and is based on those values given in Table IV.2.

The concentration of goblet cells per unit length of epithelium varied according to the airway level. The greatest concentration of goblet cells was at Level III, where the goblet cell number was significantly above that of each of the other airway levels (see Table IV.2 for p values). Similar numbers of goblet cells were found at Levels I, II, IV and VI and significantly more cells found at these levels than at Levels V and VII, where similar and the least numbers of goblet cells were found.

Change in Absolute Number of Goblet Cells in Response to Tobacco or to Tobacco + PMO for Various Periods up to 14 Days

In these results the patterns of change in goblet cell number in the airway epithelium of animals exposed to tobacco or to tobacco + PMO are described by comparison of the absolute cell number after each exposure time with that of the corresponding airway level in control rats.

Different patterns of change in goblet cell number were seen in the extrapulmonary and intrapulmonary airways. In animals exposed either to tobacco or tobacco + PMO, the pattern in the extrapulmonary airways showed a 'wave' effect (see Fig. IV.3) while in the intrapulmonary airways the pattern was one of steady increase over the exposure period (see Fig. IV.4). In addition,

for animals exposed either to tobacco or to tobacco + PMO the pattern of change was similar at these levels .

The changes in the extrapulmonary airways (Levels I-III) are described separately from those for the intrapulmonary airways (Levels IV-VII) first for the animals exposed to tobacco and then for animals exposed to tobacco + PMO.

Exposures to tobacco

The effect of exposures to tobacco on the goblet cell number in airway epithelium of exposed animals are illustrated in Figs.IV.3 and 4. Values for significant differences in goblet cell number in response to exposures to tobacco are calculated from figures given in Tables IV.3 and 4 and from those for control animals given previously in Table IV.2.

Extrapulmonary airways (Airway levels I-III, see Fig. IV.3)

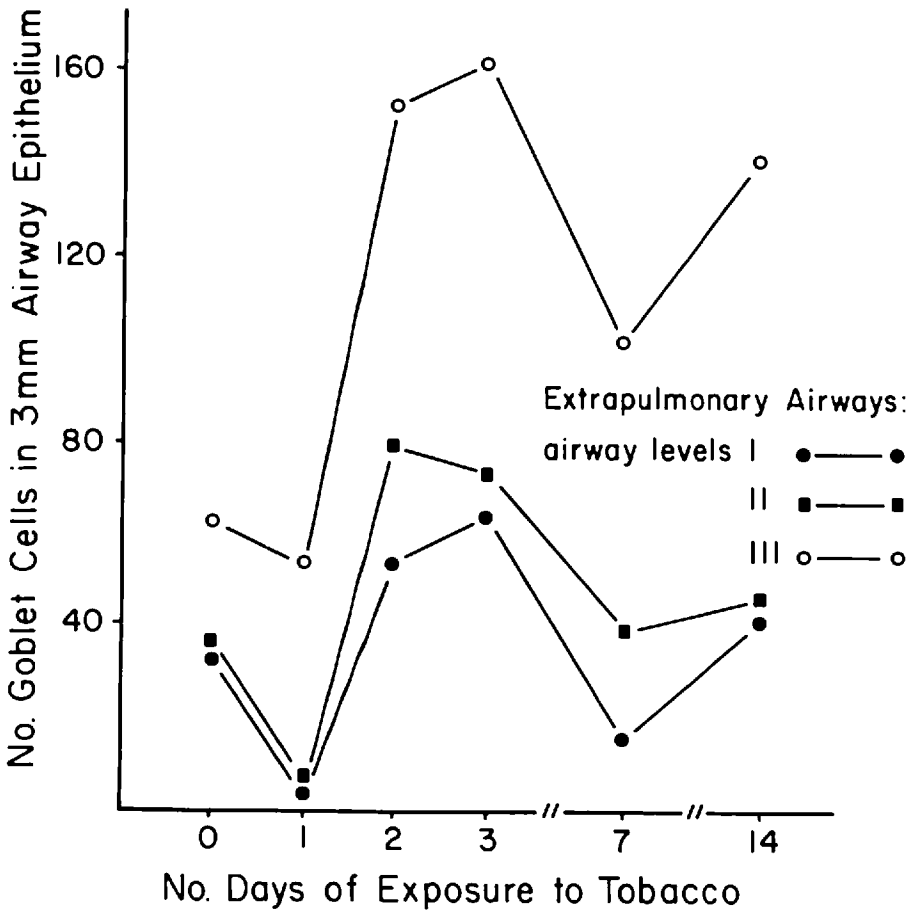
Day 1 After the first day of exposure the number of goblet cells in the extrapulmonary airways of animals exposed to tobacco was reduced below control values, the decrease being significant at Levels I and II but not at Level III.

Days 2-3 After 2 or 3 days of exposure there was a striking increase in the number of goblet cells above control values. At Levels I and II the increase seen after 2 days' exposure was not significant because of wide variation at these airway levels between

Fig. IV.3 Change in goblet cell number in extrapulmonary airways of rats exposed to tobacco for periods up to 14 days.

Day 0 = Control animals

Fig. IV.3


No. Goblet Cells in 3mm Airway Epithelium
160
120
80
40
0
1
2
3
7
14
No. Days of Exposure to Tobacco
Extrapulmonary Airways:
airway levels I
II
III

animals in the exposed group, but at Level III there was a larger increase in cell number which was significantly increased above the controls ($p < 0.05$). After 3 days' exposure, however, the goblet cell number was significantly increased at each airway level ($p < 0.05$, < 0.02, < 0.001 for Levels I–III respectively).

Day 7 Again after 7 days' exposure the goblet cell number was reduced at each airway level, although only at Level I was the reduction below that of the control value ($p < 0.05$); at Level II the goblet cell number was not significantly different from the controls while at Level III it was still increased ($p < 0.02$).

Day 14 After 14 days' exposure the goblet cell number was increased at each airway level, although only at Level III was it significantly increased above the control ($p < 0.001$).

Intrapulmonary airways (Airway levels IV–VII, see Fig. IV.4)

Day 1 After 1 day of exposure the goblet cell number was increased in the animals exposed to tobacco at airway levels IV and V but significantly so only at Level V ($p < 0.01$). At Levels VI and VII it was similar to the control values.

Day 2 After 2 days' exposure the goblet cell number was increased above the control values at Levels IV and V ($p < 0.02$ and < 0.001 respectively) but still not significantly increased at Levels VI and VII.

Days 3, 7 and 14 After 3, 7 and 14 days' exposure the goblet cell number was significantly increased above the control value at each

Fig. IV.4 Change in goblet cell number in intrapulmonary airways of rats exposed to tobacco for periods up to 14 days.

Day 0 = Control animals

Fig. IV.4

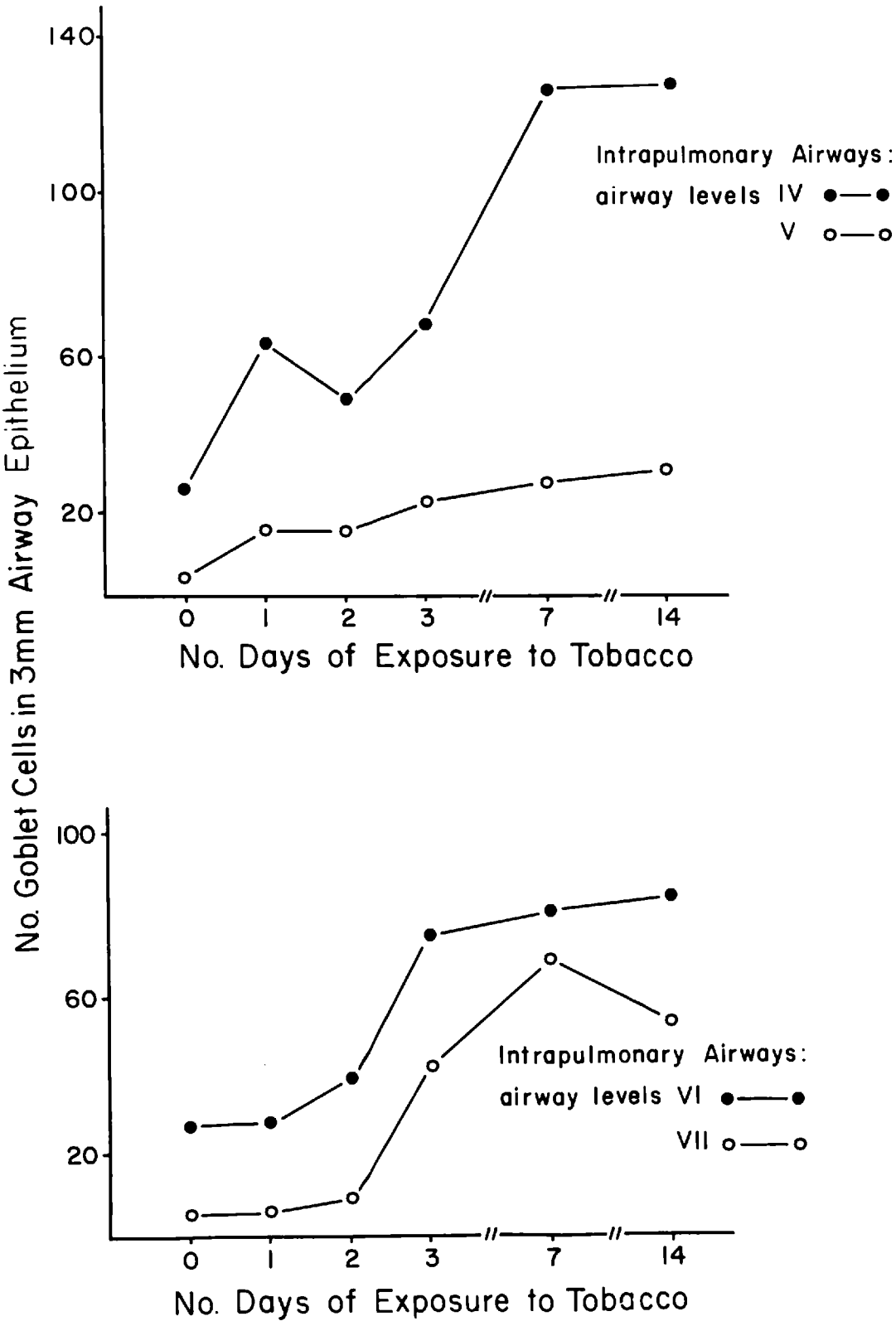

No. Goblet Cells in 3mm Airway Epithelium
140
100
60
20
Intrapulmonary Airways:
airway levels IV
V
0
1
2
3
7
14
No. Days of Exposure to Tobacco
100
60
20
Intrapulmonary Airways:
airway levels VI
VII
0
1
2
3
7
14
No. Days of Exposure to Tobacco

Table IV.3 Number of goblet cells in 3 mm epithelium in extrapulmonary airways of rats exposed to tobacco.

	No. Days' Exposure to Tobacco				
	1	2	3	7	14
AIRWAY LEVELS					
I	4.85 (1.89)*	53.96 (16.35)	64.28 (9.91)	16.99 (2.80)	40.80 (13.49)
II	6.76 (0.91)	78.57 (25.63)	74.67 (9.87)	47.94 (16.23)	51.25 (12.10)
III	53.09 (4.63)	152.92 (33.33)	162.12 (14.90)	101.85 (8.33)	140.70 (4.45)

* S.E. of the mean

Table IV.4 Number of goblet cells in 3 mm epithelium in intrapulmonary airways or rats exposed to tobacco.

AIRWAY LEVELS	No. Days' Exposure to Tobacco 1	2	3	7	14
IV	63.54	50.89	69.94	127.80	131.83
	(7.43)*	(3.54)	(11.25)	(8.27)	(24.27)
			p<0.01	p<0.001	p<0.05
V	16.78	17.69	24.79	29.20	32.55
	(5.26)	(0.29)	(0.03)	(4.84)	(9.70)
			p<0.02	p<0.02	p<0.05
VI	28.80	40.17	76.63	82.30	85.35
	(9.70)	(14.57)	(15.10)	(10.36)	(6.26)
			p<0.02	p<0.01	p<0.001
VII	6.69	10.44	43.10	70.53	54.89
	(0.19)	(5.63)	(12.12)	(5.39)	(18.13)
			p<0.01	p<0.001	p<0.05

* S.E. of the mean

P values given for increases in goblet cell number after 3, 7 and 14 days of exposure above control value: p values for days 1 and 2 are given in the text.

airway level (see Table IV.4 for these values of significance).

Exposures to Tobacco + PMO

The effect of exposures to tobacco + PMO on the number of goblet cells of rat airway epithelium are illustrated in Figs. IV.5 and 6. Values for significant differences in the goblet cell number in response to exposures to tobacco + PMO are calculated from values given in Table IV.5 and 6 and the values for control rats given previouely in Table IV.2.

Extrapulmonary airways (Airway levels I-III, see Fig. IV.5)

Days 1-14 After 1 day's exposure the goblet cell number in the extrapulmonary airways of animals exposed to tobacco + PMO was reduced below the control value ($p < 0.01$, < 0.01 and < 0.001 for airways levels I-III respectively).

At airway levels I and II the goblet cell number did not regain the control value at any time during the exposure period: after an increase in number after 2 days' exposure the number was reduced after 3 days ($p < 0.02$ and < 0.01 respectively) and after 7 days of exposure ($p < 0.01$ for both) followed by an increase in cell number after 14 daye when the number was not significantly different from control values.

At airway level III the goblet cell number was successively increased after 2 and then further after 3 days of exposure, but

Fig. IV.5 Change in goblet cell number in extrapulmonary airways of rats exposed to tobacco + PMO over periods up to 14 days.

Day 0 = Control animals

Fig. IV.5

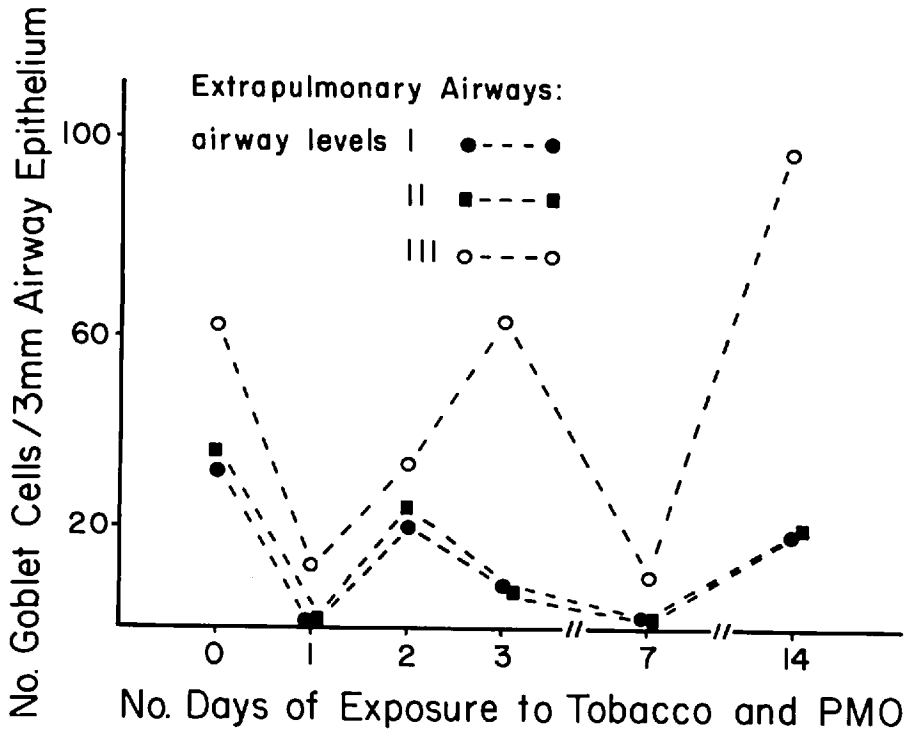

No. Goblet Cells/3mm Airway Epithelium
100
60
20
Extrapulmonary Airways:
airway levels I
II
III
0
1
2
3
7
14
No. Days of Exposure to Tobacco and PMO

Table IV.5 Number of goblet cells in 3 mm epithelium in extrapulmonary airways of rats exposed to tobacco + PMO

	No. Days' Exposure to Tobacco + PMO				
	1	2	3	7	14
AIRWAY LEVELS					
I	1.62 (0.27)*	20.91 (12.85)	8.72 (2.42)	2.15 (0.75)	18.53 (4.19)
II	0.92 (0.52)	24.72 (14.07)	7.87 (1.32)	1.02 (0.17)	19.76 (5.30)
III	14.68 (4.80)	33.59 (15.44)	62.53 (22.68)	12.50 (0.67)	98.27 (17.58)

* S.E. of the mean

after 3 days the number only equalled the control value. After 7 days of exposure the goblet cell number was again reduced below that of the control value ($p < 0.001$). After 14 days the cell number was further increased but was not significantly different from the controls.

Intrapulmonary airways (Airway levels IV-VII, see Fig. IV.6)

Days 1-14 In the intrapulmonary airways of the animals exposed to tobacco + PMO the goblet cell number increased steadily in response to exposures: by 7 and 14 days the goblet cell number was significantly increased at each airway level ($p < 0.001$ for each airway level on each of these days).

Comparison of the Increase after 14 Days of Exposures

Comparison of the increase in goblet cell number after 14 days (the longest period of exposures) revealed different patterns of response by the epithelium at different airway levels.

The number of goblet cells in 3 mm airway epithelium of animals exposed to tobacco or to tobacco + PMO for 14 days and control animals is given in Table IV.7, where the goblet cell number for the exposed animals is also expressed as a percentage of the control value which equals 100%.

The effect of tobacco

In the intrapulmonary airways, where the goblet cell number was

Figs. IV.6 Change in goblet cell number in intrapulmonary airways of rats exposed to tobacco + PMO over periods up to 14 days.

Day 0 = Control animals

Fig. IV.6

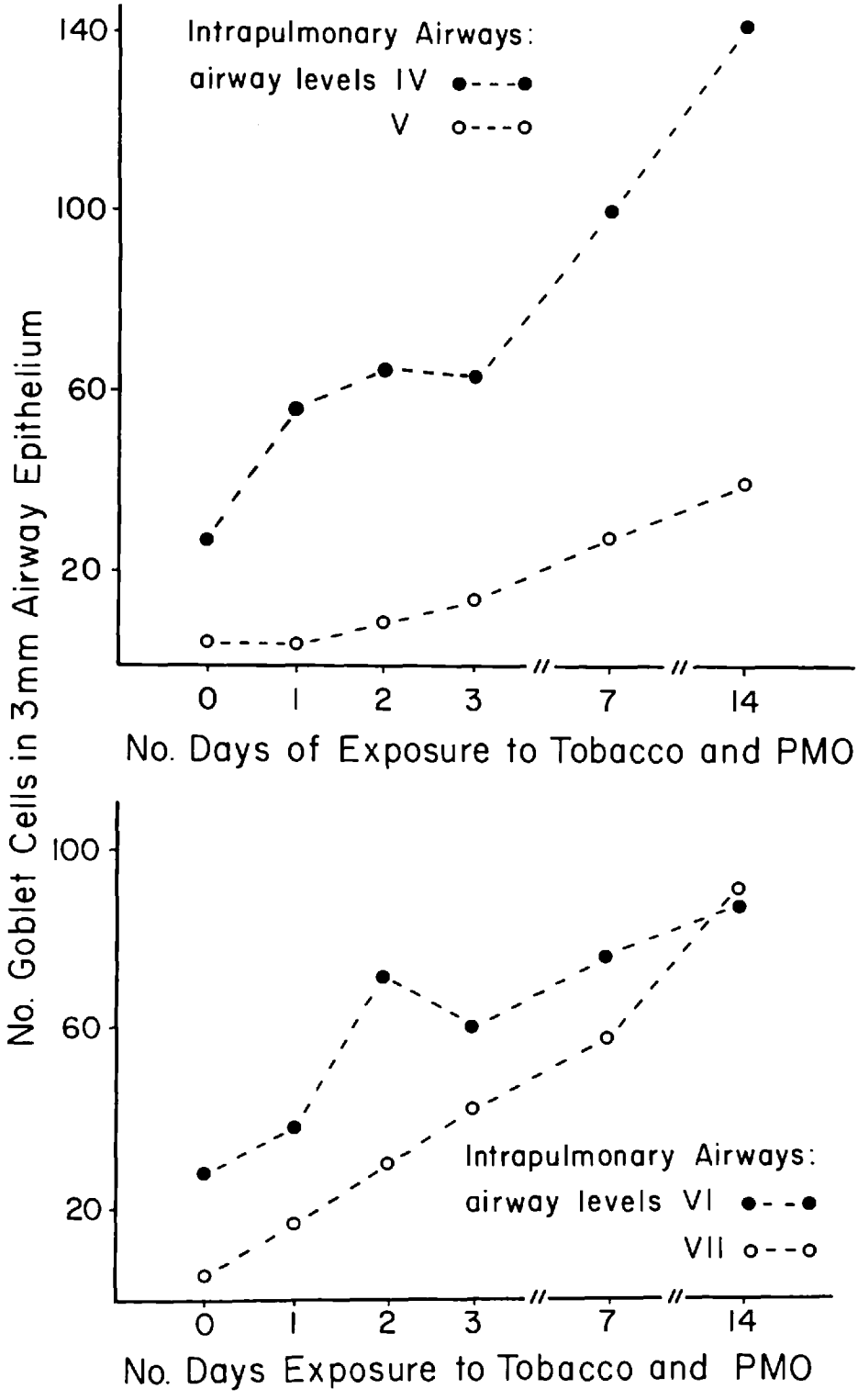

140
100
60
20
Intrapulmonary Airways:
airway levels IV
V
No. Goblet Cells in 3mm Airway Epithelium
0
1
2
3
7
14
No. Days of Exposure to Tobacco and PMO
100
60
20
Intrapulmonary Airways:
airway levels VI
VII
0
1
2
3
7
14
No. Days Exposure to Tobacco and PMO

Table IV.6 Number of goblet cells in 3 mm epithelium in intrapulmonary airways of rats exposed to tobacco + PMO.

	No. Days' Exposure to Tobacco + PMO				
	1	2	3	7	14
AIRWAY LEVELS					
IV	56.34 (15.97)*	65.27 (0.92)	63.53 (10.25)	100.90 (7.53)	143.09 (6.31)
V	4.81 (1.19)	9.91 (1.03)	12.46 (1.08)	26.52 (2.45)	43.13 (5.93)
VI	38.32 (23.96)	72.03 (19.01)	59.53 (14.27)	76.03 (4.73)	87.87 (7.29)
VII	17.50 (4.88)	30.30 (7.35)	42.09 (10.39)	58.15 (10.61)	91.05 (8.97)

* S.E. of the mean

significantly increased above the controls the following features emerged:

(i) Those regions of the airways with the greatest number of goblet cells per unit length of epithelium in control animals also contained the greatest number of goblet cells in response to tobacco. For example, compare the number of goblet cells at airway levels IV and V with that at level V and VII in control and exposed animals (Table IV.7).

(ii) The response of airways containing similar numbers of goblet cells in control animals was not the same after exposures to tobacco: in control animals there were similar numbers of goblet cells at airway levels IV and VI, and similar numbers at levels V and VII, but after exposure to tobacco the increase was greater at airway level IV than at VI and greater at level VII than at V (see Table IV.7).

(iii) Airway regions with the least number of goblet cells in control animals, showed the greatest percentage increase in goblet cell number in response to tobacco, e.g. levels V and VII (Table IV.7).

Table IV.7 Comparison of the number of goblet cells in airway epithelium of control rats and rats exposed to tobacco or tobacco + PMO for 14 days: (a) absolute cell number, (b) absolute value for exposed animals expressed as a percentage of the control value which equals 100%.

	CONTROL	TOBACCO		TOBACCO + PMO	
	(a)	(a)	(b)	(a)	(b)
AIRWAY LEVELS					
I	34.1	40.0	119.6	18.5	54.2
II	41.0	51.2	124.8	19.7	57.7
III	64.7	140.7	217.4	98.2	151.7
IV	27.3	131.8	482.7	143.0	523.8
V	5.9	32.5	550.8	43.1	730.5
VI	27.6	85.3	309.0	87.8	318.1
VII	5.9	54.8	928.8	91.0	1542.3

It is difficult to assess similar aspects of the response in the extrapulmonary airways. Even after 14 days of exposure to tobacco there was only a small increase in the goblet cell number above the control value at Levels I and II although there was a significant increase at Level III.

The effect of tobacco + PMO

In the intrapulmonary airway epithelium of animals exposed to tobacco + PMO the response after 14 days of exposure was similar to that described above (i - iii) for animals exposed to tobacco, save that at airway level VII the goblet cell number exceeded that at Level VI. Nor was it possible to assess similar aspects in the extrapulmonary airways of animals exposed to tobacco + PMO since there was a decrease in goblet cell number below control values at Levels I and II, and the increase at Level III was not significantly different from the controls; even at this airway level, however, there was a trend apparent with regard to (i).

Change in Goblet Cell Number in Response to Tobacco or to Tobacco + PMO relative to Previous Exposure Period

In these results the effect of exposures to tobacco or to tobacco + PMO on the number of goblet cells in airway epithelium is compared between successive days of the exposure period - both the change in absolute cell number and rates of change.

Change in absolute goblet cell number

The effect of tobacco In the extrapulmonary airways of animals exposed to tobacco the absolute number of goblet cells after 2 days of exposure was significantly increased above the number after only 1 day of exposure ($p < 0.05$ for each level). For the following period of exposure to tobacco, only after 7 days was the goblet cell number at airways Levels I and II significantly different and, in this instance, below the number present in response to the previous period of exposure ($p < 0.01$ and < 0.05 respectively).

Although the absolute goblet cell number after 2 and 3 days exceeded the number after 14 days of exposure to tobacco, there was no significant difference between any of these values, i.e. more effect achieved.

In the intrapulmonary airways the goblet cell number was significantly increased only at Levels V and VII between days 3 and 7 of exposures ($p < 0.001$ and < 0.05 respectively) and at Level IV between days 3 and 7 ($p < 0.01$).

The effect of tobacco + PMO In the extrapulmonary airways of the animals exposed to tobacco + PMO, the absolute number of goblet cells was significantly increased at airways Levels I and II between days 3 and 7 ($p < 0.05$ and < 0.02) and between days 7 and 14 ($p < 0.02$ for both), but at airway Level III was only significantly increased between days 7 and 14 ($p < 0.01$).

In the intrapulmonary airways, only at Levels IV and V was the goblet cell number significantly increased between successive periods of exposure and this was between days 3 and 7 ($p < 0.05$ and < 0.01) and between days 7 and 14 ($p < 0.05$ for both).

Rate of change in goblet cell number

The rates of change in goblet cell number between various periods of exposure for animals exposed to tobacco or to tobacco + PMO are shown in Figs. IV.7 - 10: for each period of exposure the goblet cell number is expressed as a percentage of the value for the previous exposure period.

The effect of tobacco In the extrapulmonary airways of animals exposed to tobacco the greatest rate of decrease in the goblet cell number occurred after the first day of exposure at airway Levels I and II and between days 3 and 7 at Level II (see Fig. IV.7).

At airway levels I and II the rate of increase in the goblet cell number was greatest between days 1 and 2 of the exposure period: at airway level III, the rates of increase were similar between days 1 and 2, and 2 and 3, of the exposure period and were greatest at these times.

In the intrapulmonary airways exposed to tobacco, the greatest rate of increase in the goblet cell number occurred at Levels IV and V after the first day of exposure: at Levels VI and VII the greatest increase occurred between days 2 and 3 and there was a progressive

Fig. IV.7 Extrapulmonary airways (Levels I - III) Change in the percentage goblet cell number between periods of exposure to tobacco: the goblet cell number after each period of exposure is expressed as a percentage of the value of the previous exposure period which = 100%.

Fig. IV.7

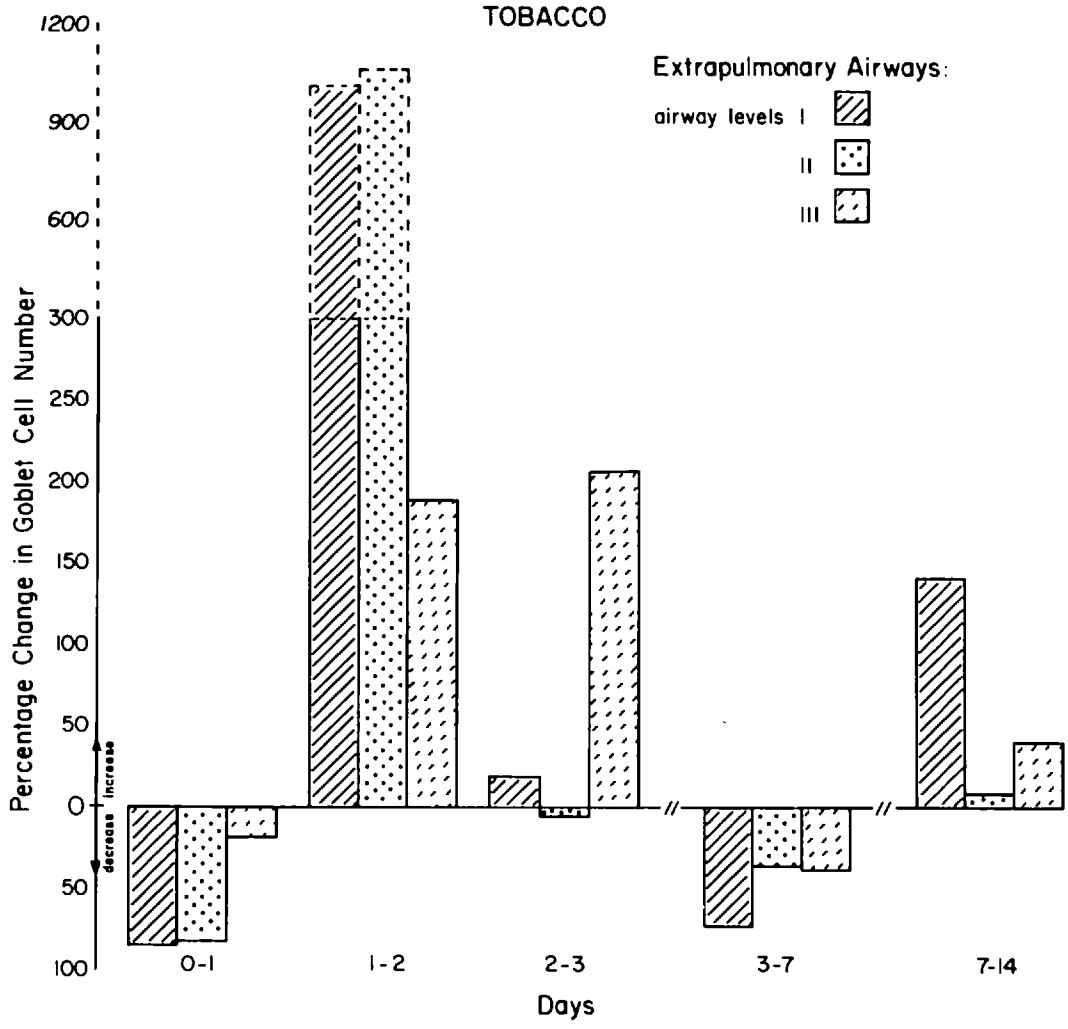
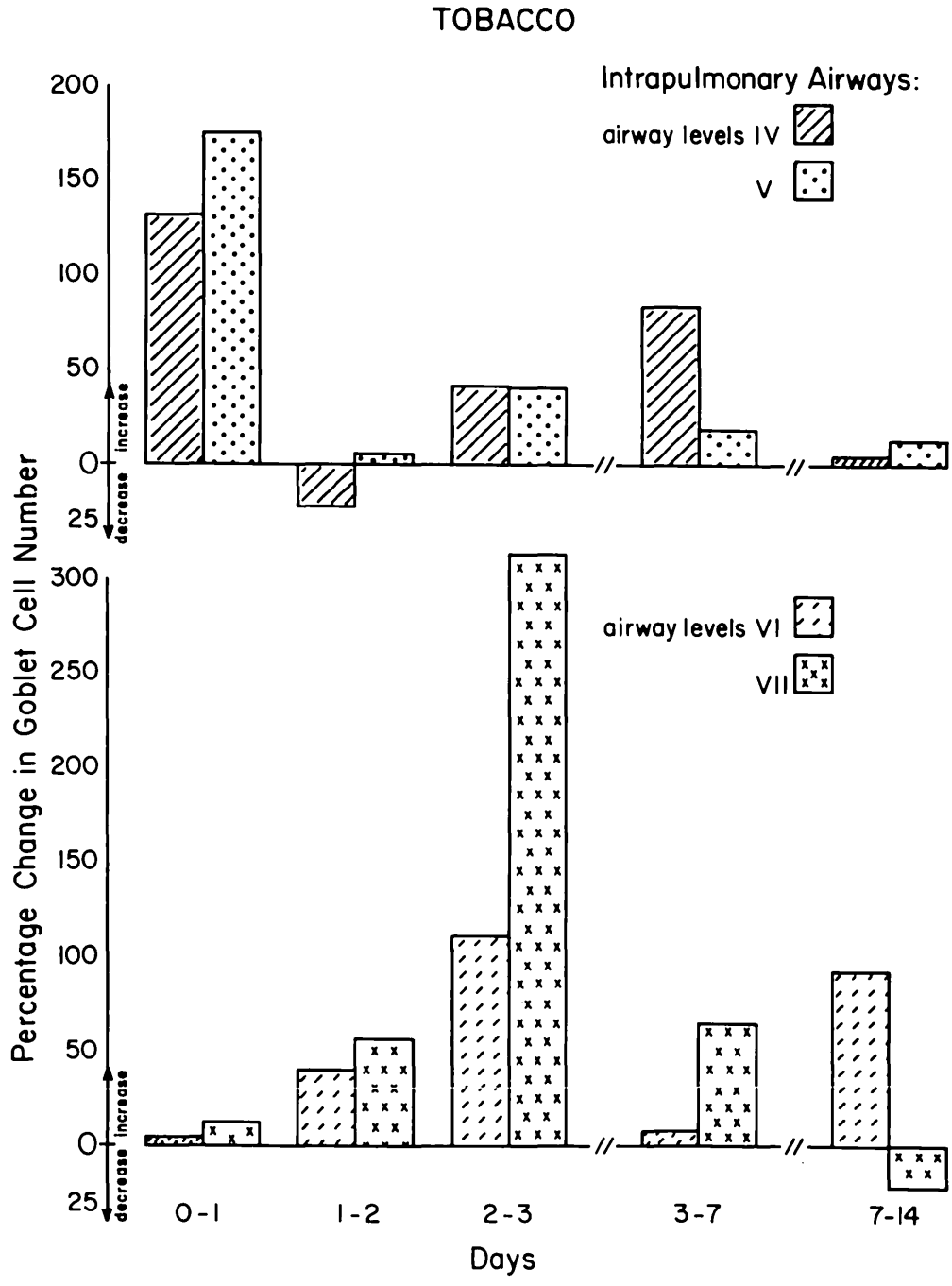

TOBACCO
Extrapulmonary Airways:
airway levels I
II
III
Percentage Change in Goblet Cell Number
1200
900
600
300
250
200
150
100
50
0
50
100
decrease
increase
0-1
1-2
2-3
3-7
7-14
Days

Fig. IV.8 Intrapulmonary airways (Levels IV - VII)
Change in the percentage goblet cell number between periods of exposure to tobacco:
the goblet cell number after each period of exposure is expressed as a percentage of the value of the previous period which = 100%.

Fig. IV.8


TOBACCO
Intrapulmonary Airways:
airway levels IV
V
airway levels VI
VII
Percentage Change in Goblet Cell Number
200
150
100
50
0
25
increase
decrease
300
250
200
150
100
50
0
25
increase
decrease
0-1
1-2
2-3
3-7
7-14
Days

increase in the rate between the early periods of exposure up to this time (see Fig. IV.8).

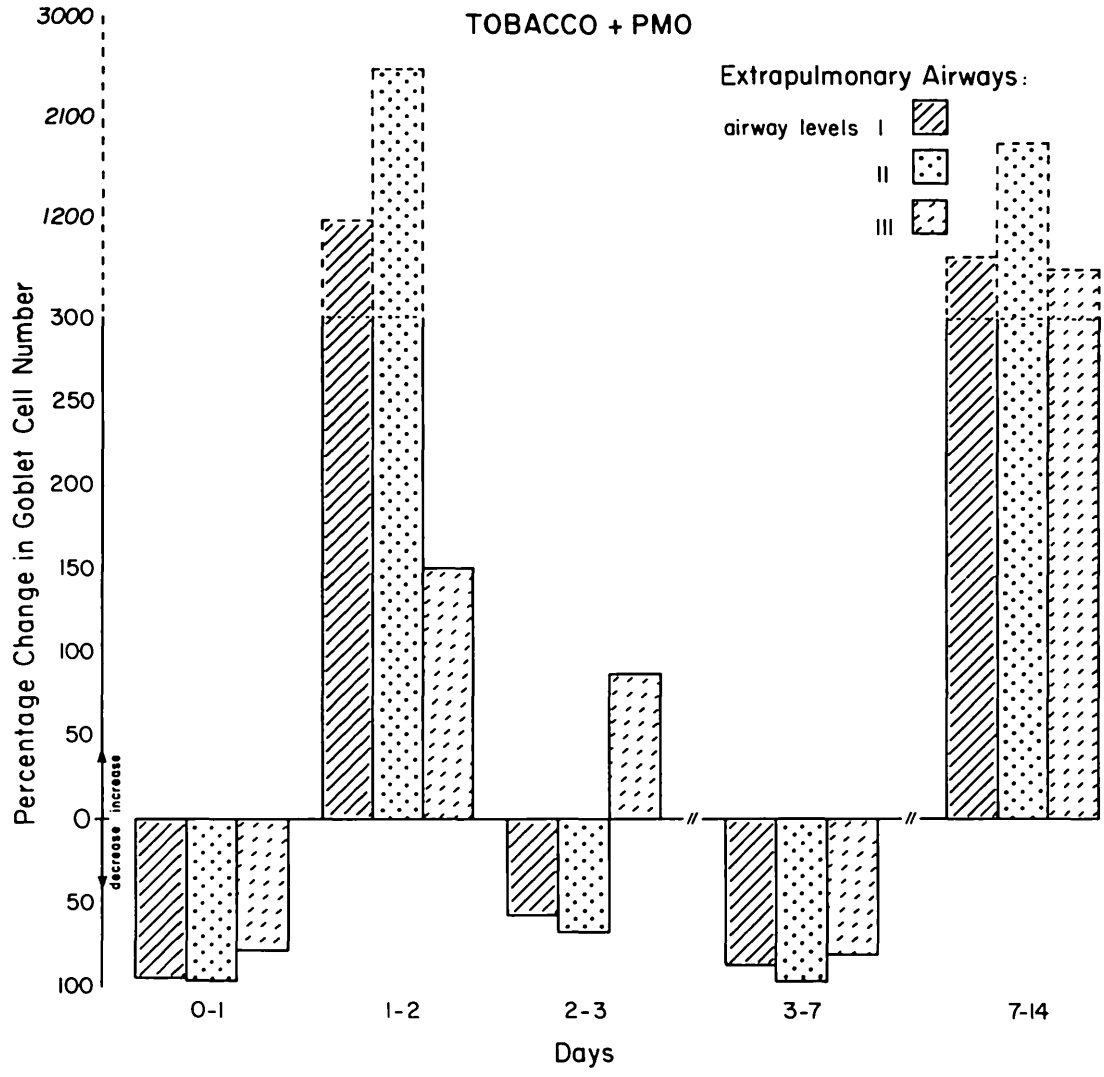
The effect of tobacco + PMO In the extrapulmonary airways of the animals exposed to tobacco + PMO the rates of decrease in the goblet cell number were similar after the first period of exposure and between days 3 and 7. At airway levels I and II alone the decrease that occurred between days 2 and 3 was less than that seen at the other times during the exposure period (see Fig. IV.9).

At airway levels I and II, the rate of increase was greatest between days 1 and 2 of the exposure period, although the increase in rate between days 7 and 14 was almost as great: at airway level III, the greatest rate of increase occurred at the end of the exposure period, between days 7 and 14.

In the intrapulmonary airways of the animals exposed to tobacco + PMO, at airway level IV the greatest rate of increase occurred after the first day of exposure while at Level V the rates of increase were similar between days 1 and 2 and between days 3 and 7: at airway level VI the greatest increase occurred between days 1 and 2 and at Level VII it occurred after the first day of exposure (see Fig. IV.10).

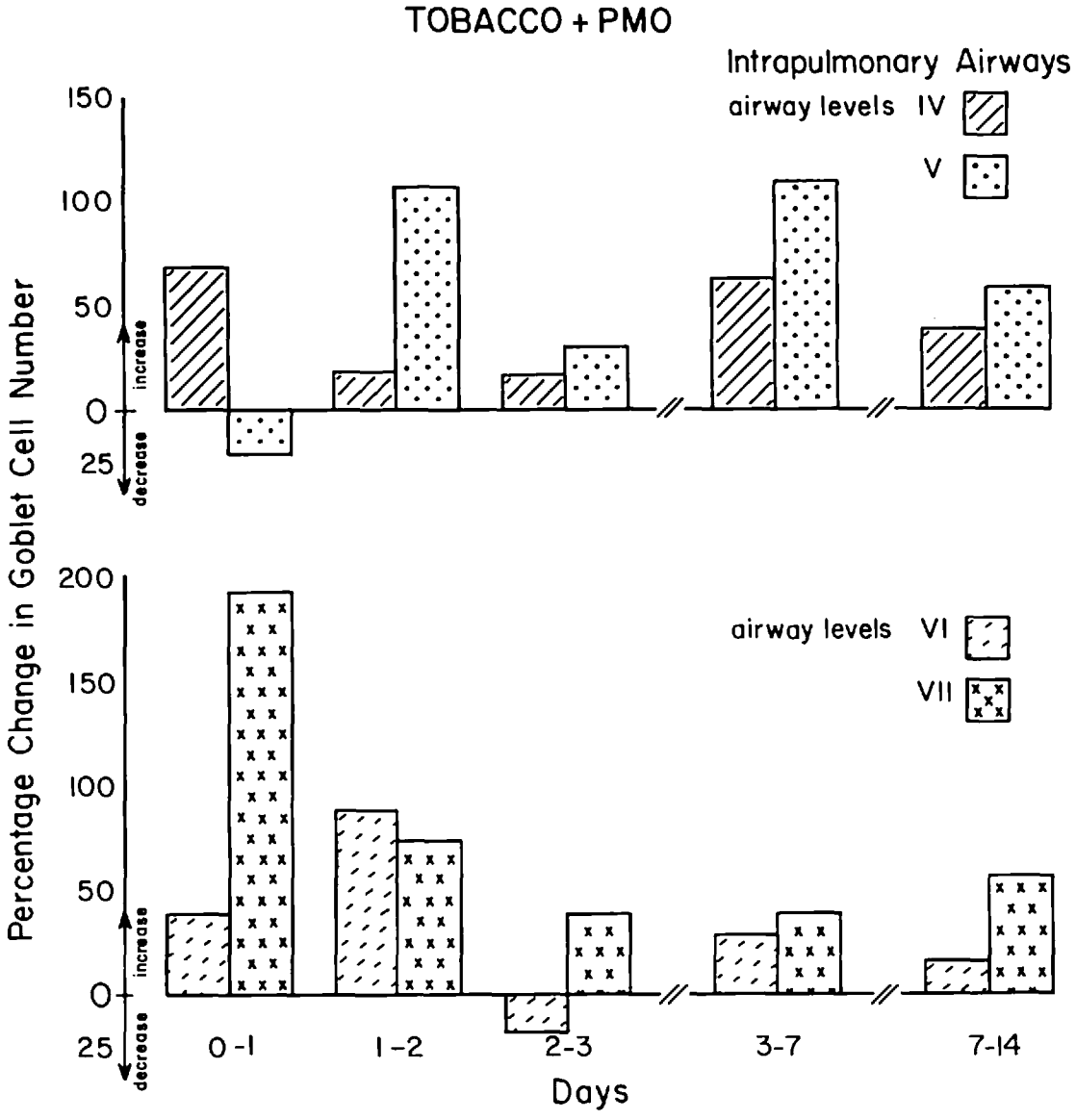
Fig. IV.9 Extrapulmonary airways (Levels I - III)
Change in the percentage goblet cell number between periods of exposure to tobacco + PMO: the goblet cell number is expressed as a percentage of the value of the previous period which = 100%.

Fig. IV.9


TOBACCO + PMO
Extrapulmonary Airways:
airway levels I
II
III
Percentage Change in Goblet Cell Number
3000
2100
1200
300
250
200
150
100
50
0
50
100
increase
decrease
0-1
1-2
2-3
3-7
7-14
Days

Figs. IV.10 Intrapulmonary airways (Levels VI - VII)
Change in percentage goblet cell number between periods of exposure to tobacco + PMO: the goblet cell number after each period of exposure is expressed as a percentage of the value of the previous period which = 100%.

Fig. IV.10


TOBACCO + PMO
Intrapulmonary Airways:
airway levels IV
V
airway levels VI
VII
Percentage Change in Goblet Cell Number
150
100
50
0
25
increase
decrease
200
150
100
50
0
25
increase
decrease
0-1
1-2
2-3
3-7
7-14
Days

Modification by PMO of the Changes in Goblet Cell Number in Response to Tobacco

As in the experiment reported in Chapter III, the addition of PMO 'protected' against some of the airway changes produced by tobacco.

Comparison of the change in absolute goblet cell number

The 'protective' effect:

(i) In the extrapulmonary airways increase in the absolute number of goblet cells produced by exposure to tobacco at Levels I and II was completely prevented, and at Level III greatly reduced, by the addition of PMO to the tobacco.

(ii) In the intrapulmonary airways, at Level V, the increase in goblet cell number produced by early exposures to tobacco - that is, after 1, 2 and 3 days of exposures - was greatly reduced by the addition of PMO to the tobacco (while the difference did not achieve significence on day 1 the difference was highly significant on each of the other days - tobacco/tobacco + PMO $p < 0.001$ for both days 2 and 3).

Absence of 'protective' effect:

(i) In the epithelium of the extrapulmonary airways the pattern of change in response to tobacco with and without PMO was

similar: both produced a reduction in goblet cell number after 1 and after 7 days of exposure, with a relative increase on the days between, and a further increase in goblet cell number toward the end of the exposure period (although there was such an increase after 14 days of exposures only at Level III in response to tobacco with PMO).

(ii) In the intrapulmonary airways again the pattern of change was similar in response to tobacco with and without PMO: both produced an overall increase in goblet cell number with the highest count at the end of the exposure period. In terms of absolute goblet cell number, however, more goblet cells were present after 14 days of exposures to tobacco + PMO than without, although the difference between the two groups was never significant.

(iii) The particuler patterns of response by the intrapulmonary epithelium for both absolute and percentage increase in goblet cell number, apparent after 14 days of exposures, were similar in response to tobacco with and without PMO.

(iv) At one intrapulmonary airway level, Level VII, there were slightly more goblet cells after most exposures to tobacco with PMO than without but again the differences were never significant and it seemed that PMO had no effect.

Comparison of the rates of change in goblet cell number

The 'protective' effect:

(i) In the extrapulmonary airways the rate of decrease in goblet

cell number after 1 and after 7 days of exposures was greater in response to tobacco with PMO than without. In the upper levels of the extrapulmonary airways (Levels I and II) there was also a decrease after 2 and 3 days of exposure to tobacco with PMO.

Absence of 'protective' effect:

(i) In the extrapulmonary airways exposures to tobacco with and without PMO produced similar effects in that the greatest rate of increase in goblet cell number occurred at Levels I and II, between 2 and 3 days of exposuree. The rates of increase, however, were greater in response to tobacco with PMO and there was a second and striking increase in rate of goblet cell change between days 7 and 14 in response to this type of smoke.

(ii) In the intrapulmonary airways there was little similarity in the pattern of response with regard to rate of change in the goblet cell number after exposures to tobacco with or without PMO. While at Levels IV and V the addition of PMO reduced the rate of increase in goblet cell number in response to the first exposure to tobacco, it produced subsequently a comparatively greater increase after longer periods of exposure. And, at Levels VI and VII, the greatest rate of increase in goblet cell number occurred in response to earlier exposures to tobacco with PMO (after day 1 and between days 1 and 2) than without (where the greatest increase occurred between days 2 and 3).

SUMMARY AND COMMENT

1. Rats exposed to tobacco smoke for 14 days, with and without PMO, failed to gain weight as rapidly as control animals. The addition of PMO thus gives no protection against the failure to gain weight induced by tobacco smoke and is associated with an even greater impairment of weight gain.

2. There is a regional variation in the number of goblet cells per unit length of epithelium in the airways of control rats. The greatest number of goblet cells is present in the lower trachea and main bronchi: the upper and mid-region of the trachea and proximal airways contain similar numbers of goblet cells while the least number of goblet cells is present in the peripheral airways.

3. Exposure of rats to tobacco smoke for 14 days produces an increase in goblet cell number at each airway level investigated in this study. The pattern of change leading to such an increase differs in the extrapulmonary airways from the intrapulmonary airways

Extrapulmonary airways Broadly, there is both decrease and increase in the goblet cell number over 14 days of exposure to tobacco smoke, with a similar pattern of response in each of the three extrapulmonary airways levels. Decreases in the goblet cell number occur after 1 and after 7 days of exposures. The first decrease represents the initial response of the epithelium

to tobacco smoke, where the goblet cells empty their secretion and thus are no longer identifiable in tissue sections stained with AB-PAS. The second decrease, after 7 days of exposures, appears to be a similar response to that seen on day 1 since the rats had not been exposed on the previous day.

During the 14 days of exposure to tobacco smoke the greatest rate of increase in the goblet cell number occurs between days 1 and 2 in the upper and mid-region of the trachea. In the lower trachea there is evidence of a lag period in this response since it occurs, to a lesser extent and equally, between days 1 and 2 and between days 2 and 3. At the end of 14 days' exposure both the greatest increase in absolute goblet cell number and the greatest percentage increase occur in the lower region of the trachea

Intrapulmonary airways Unlike the extrapulmonary response, there is a progressive increase in the goblet cell number in the intrapulmonary airways over the period of exposure to tobacco. The greatest rate of increase in goblet cell number occurs after 1 day's exposure in the axial pathway, and between 2 and 3 days of exposure in the lateral pathways. This suggests a lag period in the response of the lateral pathways as compared with that of the axial pathway.

In response to 14 days of exposures to tobacco smoke, the greatest increase in absolute goblet cell number occurs in the airway regions containing the highest number of goblet cells in control rats - that is, the upper axial and second lateral pathways. The greatest response - that is, the greatest percentage

increase in goblet cell number, occurs in regions with the least number of goblet cells in control animals - the lower axial and third lateral pathways.

4. Exposures to tobacco smoke with PMO for 14 days produces an increase in the goblet cell number only in the lower region of the trachea and in the intrapulmonary airways. The patterns of change in goblet cell number are similar to those seen in response to tobacco smoke alone both in the extrapulmonary and in the intrapulmonary airways.

Extrapulmonary airways The addition of PMO gives no protection in the extrapulmonary airways against decrease in goblet cell number in response to exposure to tobacco smoke, due to emptying by the goblet cells of their secretion. It completely prevents increase in the goblet cell number, however, in the upper and mid-region of the trachea, and greatly reduces the increase in the lower trachea.

Intrapulmonary airways The addition of PMO gives no protection against the increase in goblet cell number in the intrapulmonary airways in response to exposure to tobacco; in fact, it is associated with an even greater increase in the goblet cell number.

CHAPTER V

THE EFFECT ON GOBLET CELL GLYCOPROTEINS OF RAT AIRWAY EPITHELIUM OF SHORT PERIODS OF EXPOSURE TO TOBACCO SMOKE WITH AND WITHOUT PHENYLMETHYLOXADIAZOLE

GOBLET CELL GLYCOPROTEINS

This chapter reports the histochemical findings in the experiment for which the results of goblet cell number were described in Chapter IV.

Because of the possible change in glycoproteins after short periods of exposure to tobacco smoke, with or without phenylmethyloxadiazole (PMO), goblet cells were characterised by the full range of intrscellular types of glycoproteins that could be identified within the individual cell. Goblet cells were identified in the way previously described (Chapter III, p.118) save that those cells containing a mixture of glycoprotein staining with either AB or with PAS were quantified separately from those staining with AB alone. The varieties of goblet cell described here thus include L.PAS, S.PAS, LAB-PAS, S.AB-PAS, L.AB and S.AB. Where both neutral and acid glycoprotein were present within a cell the acid glycoprotein occupied the apical region (see Chapter III, p.117).

Distribution of Goblet Cells containing Neutral and Acid Glycoprotein in Control Animals

The number of goblet cells of each type in the airway epithelium of control animals is given in Tables V.1 and 2. The distribution of goblet cells containing neutral glycoprotein alone (PAS), acid and neutral glycoprotein (AB-PAS) or acid glycoprotein alone (AB) at each airway level is illustrated in Fig. V.1. As in

Fig. V.1 Percentage of goblet cells staining with PAS, AB-PAS or with AB in airway epithelium of control rats.

Fig. V.1

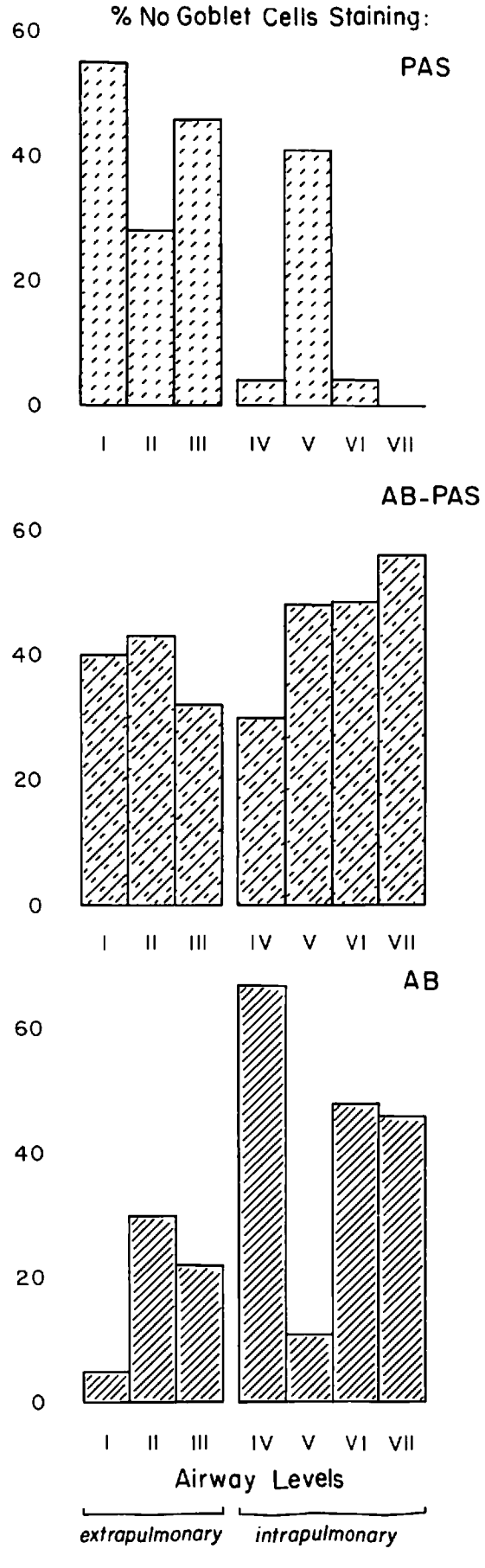

% No Goblet Cells Staining:
PAS
60
40
20
0
I II III IV V VI VII
AB-PAS
60
40
20
0
I II III IV V VI VII
AB
60
40
20
0
I II III IV V VI VII
Airway Levels
extrapulmonary
intrapulmonary

Table V.1 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB in the extrapulmonary airways of control rats. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
Airway Levels						
I	1.70 (1.17)*	17.02 (5.83)	1.18 (0.52)	12.40 (5.20)	0	1.76 (0.39)
II	0.27 (0.17)	10.88 (5.49)	1.94 (0.88)	15.51 (5.44)	0	8.16 (2.29)
III	3.67 (1.42)	26.13 (7.71)	8.04 (3.97)	12.76 (2.74)	1.11 (0.57)	13.07 (4.80)

* S.E. of the mean

Table V.2 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB in the intrapulmonary airways of control rats. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
Airway Levels						
IV	0	1.00 (0.44)*	0.19 (0.02)	7.98 (1.88)	0.27 (0.14)	17.87 (8.50)
V	0.55 (0.34)	1.97 (0.87)	0.36 (0.31)	2.58 (0.98)	0	0.69 (0.28)
VI	0	1.14 (0.40)	0	13.23 (4.47)	0	13.23 (7.92)
VII	0	0	0	3.34 (2.24)	0	2.81 (1.96)

* S.E. of the mean

Chapter III, results are described by reference to the type rather than the staining property of glycoprotein within goblet cells.

At each airway level in the epithelium of control animals there were goblet cells containing acid or neutral glycoprotein alone or in combination. The percentage number of goblet cells containing both types of glycoprotein was the more uniform throughout the airways, while those containing only neutral glycoprotein were found predominantly in the extrapulmonary airways (Levels I-III) and in a single intrapulmonary airway (Level V), and those containing only acid glycoprotein predominantly in the intrapulmonary airways (Levels IV, VI and VII).

From the values in Tables V.2 and 2, it is apparent that the majority of goblet cells in the airway epithelium of control animals are 'small' - that is, they contained only small amounts of any type of glycoproteins.

Change in the Number of Goblet Cells containing Neutral and Acid Glycoprotein after Exposure to Tobacco or Tobacco + PMO

Two airway levels were chosen for detailed analysis of the effect of exposure to tobacco or tobacco + PMO on the glycoproteins of the goblet cell populations. Because of the different patterns of change in the goblet cell number found in the extrapulmonary from the intrapulmonary airways, one level of each was chosen - in each case the level with the greatest number of goblet cells in the control animals, i.e. Level III (extrapulmonary) and Level IV (intrapulmonary). Values for airway levels I-II and V-VII are included in Tables V.7-16 at the end of the following section of results and the findings for these airway levels briefly described.

The Effect of Tobacco

The effect of exposure to tobacco on the number of goblet cells staining with PAS, with AB-PAS or with AB in airway epithelium at Levels III and IV are illustrated in Figs. V.2 and 3. These results are based on values given in Tables V.3 and 4, and are compared with the values for the control animals given previously in Tables V.1 and 2.

Airway Level III (Fig. V.2 - extrapulmonary)

Cells containing only neutral glycoprotein

Days 1-14 There was a fall in the number of goblet cells containing only neutral glycoprotein in the epithelium at airway level III after periods of exposure to tobacco. Cells containing a large amount of

Fig. V.2 Changes in the number of goblet cells staining with PAS, AB-PAS or AB in 3 mm epithelium at airway level III in rats exposed to tobacco.

Day 0 = Control value

For clarity PAS-staining cells are shown separately.

Fig. V.2

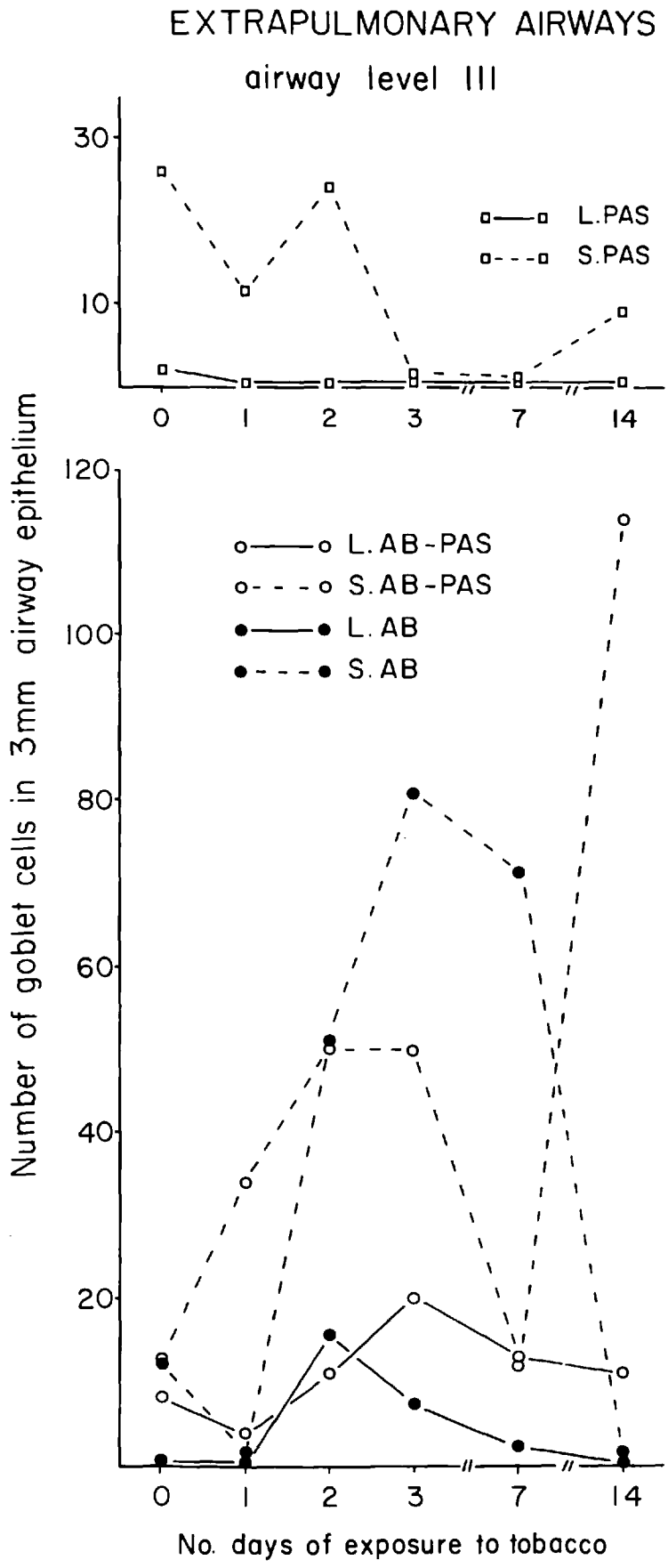

EXTRAPULMONARY AIRWAYS
airway level III
30
10
L.PAS
S.PAS
0
1
2
3
7
14
120
100
80
60
40
20
L.AB-PAS
S.AB-PAS
L.AB
S.AB
Number of goblet cells in 3mm airway epithelium
No. days of exposure to tobacco

Table V.3 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level III in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0.70 (0.85)*	12.36 (2.56)	3.69 (1.51)	33.57 (3.24)	0	2.77 (0.43)
2	0.23 (0.12)	24.83 (15.77)	10.66 (5.89)	49.76 (22.31)	15.52 (9.61)	51.92 (12.23)
3	0.67 (0.40)	2.64 (1.78)	19.95 (12.87)	50.49 (12.85)	7.25 (5.11)	81.33 (14.96)
7	0	0.47 (0.28)	13.30 (2.19)	12.43 (1.46)	2.53 (0.39)	72.52 (7.33)
14	0.86 (0.64)	9.24 (6.54)	11.19 (6.89)	116.72 (14.12)	0	2.32 (0.70)

* S.E. of the mean

neutral glycoprotein (L.PAS) were rarely seen. The number of cells containing a small amount of neutral glycoprotein (S.PAS) fell below the control value after day of exposure, regained the control value after 2 days but was again less than half the control value by the end of the 14-day exposure period.

Cells containing acid glycoprotein

The greatest change in goblet cell number after exposure to tobacco occurred in those cells containing small amounts of glycoprotein that was either a mixture of acid and neutral glycoprotein or acid glycoprotein alone. When cells containing a small amount of acid and neutral glycoprotein increased, cells containing acid glycoprotein alone decreased and vice versa. There was little change in the number of goblet cells containing large amounts of acid glycoprotein alone or in combination and no rise and fall in these cell populations as in those containing small amounts of these glycoproteins.

Days 1-14 Cells containing a small amount of acid and neutral glycoprotein (S.AB-PAS) were increased at airway level III after 1 day and increased further after 2 and 3 days of exposure when the increase was 4-fold (after Day 3 $p < 0.02$). The number of this cell type then fell and was at only the control value after 7 days. After 14 days, however, it had increased strikingly with a 10-fold increase above the control value ($p < 0.001$). It was this cell that predominated in the increased goblet cell population after 14 days.

In contrast, cells containing a small amount of acid glycoprotein (S.AB) were reduced after 1 day of exposure but increased after 2, 3 and 7 days of exposure when the increase above the control was 6-fold ($p < 0.001$). After 14 days of exposure the number of these cells had fallen dramatically so that this cell type was only occasionally seen.

The number of goblet cells containing a large amount of acid and neutral glycoprotein (L.AB-PAS) was reduced below the control value after 1 day of exposure. The greatest number of these cells was found after 3 days' exposure, although their number was not significantly increased above the control value. The number of these cells then reduced after 7 and after 14 days of exposure with the number of cells at the end of the exposure period being similar to the control value.

Cells containing a large amount of acid glycoprotein alone (L.AB) were first seen after 2 days of exposure when their greatest number was found ($p < 0.001$ as compared with the control value). Subsequently, after each exposure period, their number fell so that by 14 days none were seen.

Airway Level IV (Fig. V.3 - Intrapulmonary)

Cells containing only neutral glycoprotein

Days 1-14 At airway level IV (as at Level III), there was little change in the number of goblet cells containing only neutral glycoprotein. No cells containing a large amount of neutral glycoprotein (L.PAS) were seen and there were only a few cells containing a small amount of neutral glycoprotein (S.PAS) after any exposure period, there being a small increase in their number above that of the controls at 7 days.

...../Text continues p. 253

Fig. V.3 Changes in the number of goblet cells staining with PAS, AB-PAS or AB in 3 mm epithelium at airway level IV in rats exposed to tobacco.

Day 0 = Control value

Fig. V.3

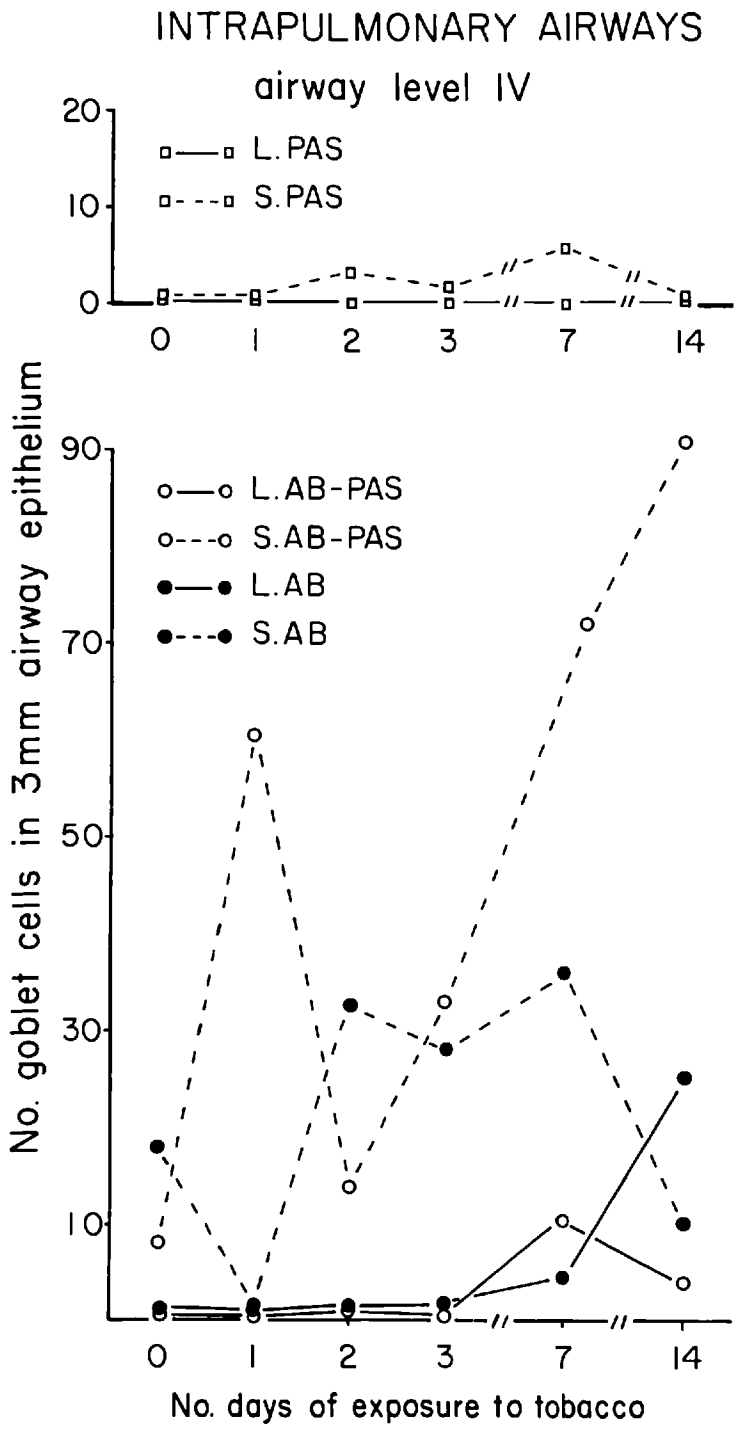

INTRAPULMONARY AIRWAYS
airway level IV
20
10
0
L.PAS
S.PAS
0 1 2 3 7 14
No. goblet cells in 3mm airway epithelium
90
70
50
30
10
L.AB-PAS
S.AB-PAS
L.AB
S.AB
0 1 2 3 7 14
No. days of exposure to tobacco

Table V.4 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level IV in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	0.55 (0.43)*	0	60.28 (10.38)	1.32 (0.87)	1.55 (0.05)
2	0	3.23 (0.54)	1.00 (0.54)	14.42 (5.94)	1.93 (0.55)	30.31 (4.19)
3	0	1.72 (0.78)	0.34 (0.21)	34.16 (10.60)	1.53 (0.98)	32.19 (15.40)
7	0	5.88 (4.01)	10.36 (7.37)	72.27 (19.46)	3.47 (1.04)	35.83 (18.71)
14	0	1.12 (0.71)	3.29 (2.80)	91.95 (4.97)	25.28 (15.78)	10.18 (5.24)

* S.E. of the mean

Cells containing acid glycoprotein

Days 1-14 The pattern of change in cell populations containing acid and neutral glycoprotein or acid glycoprotein alone was similar at this airway level to that seen at Level III. That is, after 1 day of exposure the number of goblet cells containing a small amount of acid and neutral glycoprotein (S.AB-PAS) showed an 8-fold increase above the control value ($p < 0.001$) but after 2 days of exposure the cell number was reduced to just above the control value. (This fall was earlier than at Level III). There was then a subsequent increase in the number of these cells after each of the following number of days of exposure with the maximum increase occurring after 14 days, when it was almost 12-fold ($p < 0.001$).

After 1 day of exposure the number of cells containing a small amount of acid glycoprotein alone (S.AB) was reduced below the control value. Their number then increased above that of the control after 2, 3 or 7 days of exposure before being reduced again below the control value after 14 days of exposure.

Cells containing large amounts of acid glycoprotein, either alone or in combination with neutral glycoprotein, were rarely seen save after 7 and 14 days of exposure. The greatest number of goblet cells containing a large amount of acid and neutral glycoprotein (L.AB-PAS) were found after 7 days of exposure and those containing a large amount of acid glycoprotein alone (L.AB)

after 14 days, although neither value was significantly increased above that of the corresponding control value.

The Effect of Tobacco + PMO

The results for the changes in the number of each type of goblet cell in the airway epithelium of rats exposed to tobacco + PMO are presented in Figs. V.4 and 5 and in Tables V.5 and 6.

Airway Level III (Fig. V.4 - extrapulmonary)

Cells containing only neutral glycoprotein

Days 1-14 After 1 day of exposure the number of goblet cells containing large and small amounts of neutral glycoprotein (L.PAS and S.PAS) was reduced at airway level III: subsequently either cell variety was rarely seen.

Cells containing acid glycoprotein

Days 1-14 After 1 day of exposure the number of goblet cells containing a small amount of acid and neutral glycoprotein (S.AB-PAS) was reduced in the epithelium at airway level III. After 2 and 3 days of exposure their number was then increased but was not significantly above the control value: it was then reduced to just above the control value after 7 and after 14 days of exposure.

Cells containing a small amount of only acid glycoprotein (S.AB) decreased after 1 day of exposure. After 2 and 3 days their

Fig. V.4 Changes in the number of goblet cells staining with PAS, AB-PAS or AB in 3 mm epithelium at airway level III in rats exposed to tobacco + PMO.

Day 0 = Control value

Fig. V.4

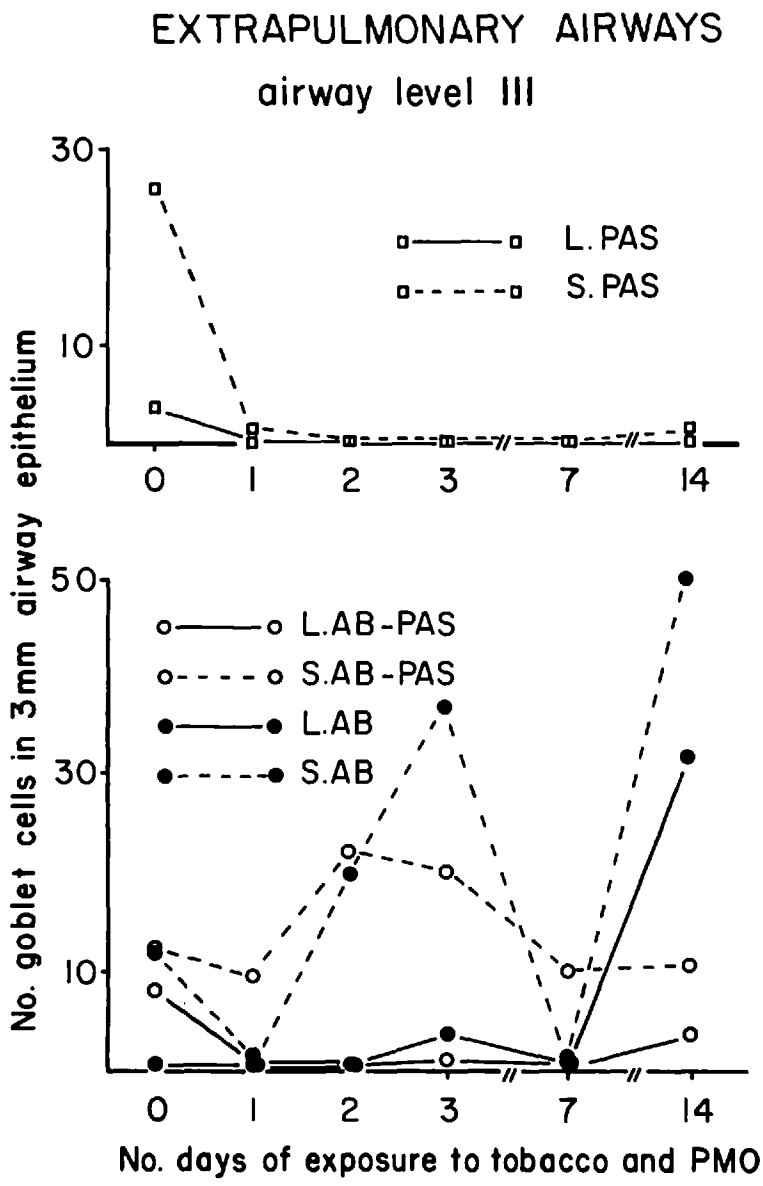

EXTRAPULMONARY AIRWAYS
airway level III
30
10
L. PAS
S. PAS
0
1
2
3
7
14
50
30
10
L.AB-PAS
S.AB-PAS
L.AB
S.AB
No. goblet cells in 3mm airway epithelium
No. days of exposure to tobacco and PMO

Table V.5 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level III in rats exposed to tobacco + PMO. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0.81 (0.12)*	1.93 (1.48)	0.57 (0.17)	9.96 (3.74)	0.76 (0.04)	0.65 (0.12)
2	0	0.23 (0.20)	0.11 (0.60)	21.69 (10.14)	1.13 (0.74)	10.44 (5.31)
3	0	0	1.30 (0.84)	19.73 (6.36)	3.71 (3.59)	37.67 (15.25)
7	0	0.36 (0.26)	0.05 (0.02)	11.33 (0.38)	0	0.63 (0.37)
14	0	1.67 (0.17)	3.61 (0.64)	10.62 (4.70)	31.77 (12.48)	50.60 (8.29)

* S.E. of the mean

number was increased and it then reduced, so that after 7 days of exposure this cell type was only occasionally seen. After 14 days of exposure their number was strikingly greater, a 4-fold increase above the control value being seen ($p < 0.01$).

Cells containing large amounts of acid and neutral glycoprotein, or acid alone (L.AB-PAS and L.AB) were rarely seen after any days of exposure save at the end of the exposure period when there was a large increase in the number of cells containing a large amount of acid glycoprotein alone ($p < 0.05$ when compared with the control value).

Airway Level IV (Fig. V.5 - intrapulmonary)

Cells containing only neutral glycoprotein

Days 1-14 As at airway level III there was little change in the number of cells containing large or small amounts of neutral glycoprotein (L.PAS and S.PAS) in animals exposed to tobacco + PMO: these cells were rarely seen.

Cells containing acid glycoprotein

Days 1-14 The pattern of increase and decrease in cell populations containing small amounts of acid and neutral glycoprotein or acid glycoprotein alone is most clearly seen here at airway level IV. After 1 day of exposure the number of cells containing a small amount of acid and neutral glycoprotein (S.AB-PAS) was increased above the control value ($p < 0.05$). After 2 days their number was then decreased to just above the control value and then increased after 3, 7 and 14 days of exposure, the greatest increase occurring after 14 days when the increase was 8-fold ($p < 0.001$ when compared with the control value).

Fig. V.5 Changes in the number of goblet cells staining with PAS, AB–PAS or AB in 3 mm epithelium at airway level IV in rats exposed to tobacco + PMO.

Day 0 = Control value

Fig. V.5

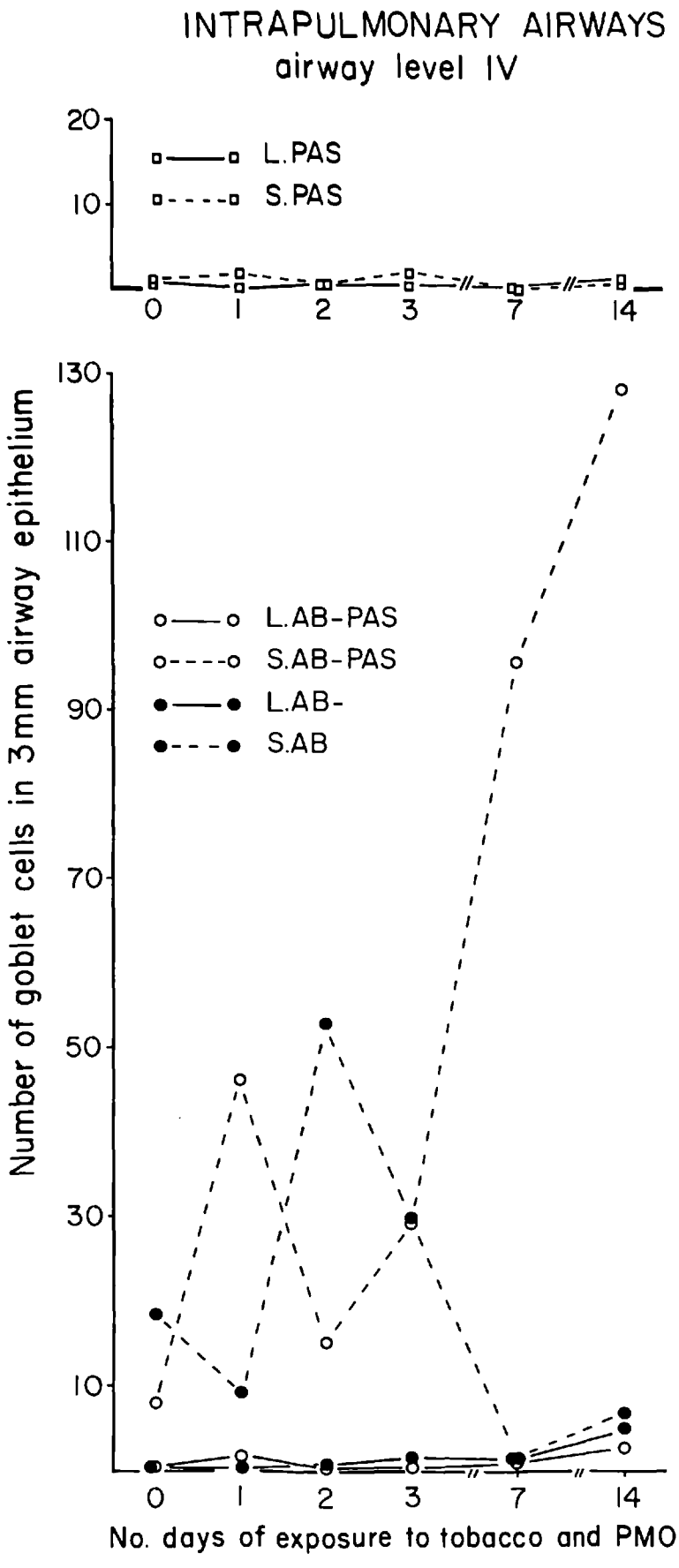

INTRAPULMONARY AIRWAYS
airway level IV
20
10
L.PAS
S.PAS
0
1
2
3
7
14
130
110
90
70
50
30
10
L.AB-PAS
S.AB-PAS
L.AB-
S.AB
Number of goblet cells in 3mm airway epithelium
No. days of exposure to tobacco and PMO

Table V.6 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level IV in rats exposed to tobacco + PMO. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	1.47 (0.50)*	1.21 (0.32)	45.46 (13.08)	0	8.20 (5.81)
2	0	0.78 (0.60)	0	12.49 (8.96)	1.00 (0.50)	51.00 (12.96)
3	0	2.45 (1.32)	0.16 (0.09)	29.31 (10.90)	1.79 (0.70)	29.89 (8.46)
7	0	0	1.04 (0.87)	95.72 (7.09)	2.13 (1.70)	2.05 (1.04)
14	0	1.62 (0.94)	2.72 (0.79)	128.03 (8.29)	4.68 (1.27)	6.70 (1.34)

* S.E. of the mean

After 1 day's exposure the number of cells containing a small amount of acid glycoprotein alone (S.AB) was decreased below the control value. This number was then increased after 2 days of exposure when the greatest number of these cells was seen, there being a 4-fold increase above the control value ($p < 0.05$). After 7 and 14 days of exposure the number of these cells then fell, their number being above the control value after 7 days but below the control value at the end of the exposure period.

Cells containing large amounts of either acid and neutral glycoprotein or acid glycoprotein alone (L.AB-PAS and L.AB) were rarely seen after any period of exposure.

The effect of tobacco and tobacco + PMO

Airway levels I, II and V-VII (Tables V.7 - 16)

In animals exposed either to tobacco or to tobacco + PMO the response was, in general, similar at airway levels I, II and V-VII to that already described for Levels III and IV. After exposure to either type of smoke for periods up to 14 days, the main changes were in the number of cells containing small amounts of acid glycoprotein (S.AB-PAS and S.AB cells). The response of the absolute number of goblet cells of each type at Levels I, II and V-VII was similar in each of the following ways:

(i) the number of cells containing a large amount of neutral glycoprotein (L.PAS) was unchanged and this cell type rarely seen;

(ii) the number of cells containing a small amount of neutral glycoprotein (S.PAS) was reduced in the extrapulmonary airways (Levels I and II) and little changed or only slightly increased in the intra-pulmonary airways (Levels V-VII);

(iii) the number of cells containing a large amount of either acid and neutral or only acid glycoprotein (L.AB-PAS and L.AB) was unchanged or only slightly increased, save at Level VI in animals exposed to tobacco and at Level VII in those exposed to tobacco + PMO, where cells containing only the pure form were particularly increased after Day 14;

(iv) the number of cells containing a small amount of either acid and neutral or only acid glycoprotein (S.AB-PAS and S.AB) was usually increased, save after 1 and 7 days at airway levels I and II in animals exposed to tobacco and at these airway levels after each period of exposure to tobacco + PMO; the increase was mainly in cells containing a mixture of acid and neutral glycoprotein.

...../Text continues p. 274

Table V.7 Number of goblet cells in 3 mm epithelium staining with PAS, with AB-PAS or AB at airway level I in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	2.20 (0.69)*	0	2.70 (0.81)	0	0
2	0	0.25 (0.12)	1.25 (0.10)	28.70 (5.14)	1.53 (0.22)	22.23 (6.33)
3	0	3.92 (1.06)	2.80 (1.12)	37.57 (4.97)	0.28 (0.14)	19.71 (1.85)
7	0	0.46 (0.26)	0.37 (0.18)	16.19 (2.13)	0	0
14	0.77 (0.21)	4.99 (1.92)	3.85 (1.77)	26.57 (7.32)	0	4.62 (0.64)

* S.E. of the mean

Table V.8 Number of goblet cells in 3 mm epithelium staining with PAS, A8-PAS or A8 at airway level II in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		A8-PAS		A8	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	1.35 (0.58)	0	3.50 (0.54)	0	0
2	0	10.63 (4.23)	3.69 (1.42)	42.69 (8.59)	1.22 (0.86)	20.33 (2.62)
3	0	0	0.56 (0.43)	68.08 (8.79)	0.37 (0.91)	5.66 (2.19)
7	0	0	0.30 (0.23)	45.31 (10.11)	0	2.34 (1.91)
14	0	5.66 (0.62)	2.60 (0.45)	38.83 (0.86)	0	4.10 (0.73)

* S.E. of the mean

Table V.9 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level V in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	6.45 (2.00)*	0	7.59 (2.15)	0.58 (0.20)	2.11 (0.97)
2	0	2.65 (0.61)	0	8.12 (1.80)	0.67 (0.54)	6.25 (1.33)
3	0	2.21 (0.77)	0	11.33 (1.27)	0.63 (0.43)	10.62 (1.00)
7	0	0	0	20.87 (2.16)	0	8.33 (2.24)
14	0	5.30 (0.59)	0	24.81 (6.23)	0.32 (0.20)	2.13 (1.00)

* S.E. of the mean

Table V.10 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level VI in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	3.88 (0.44)	0	24.93 (2.89)	0	0
2	0	1.04 (0.54)	0	32.13 (8.62)	0	7.02 (2.43)
3	0	1.25 (0.18)	0	4.56 (1.76)	0.63 (0.54)	70.19 (13.99)
7	0	0	0	42.72 (15.97)	0	39.58 (8.82)
14	0	4.59 (1.90)	0	46.99 (8.34)	15.89 (8.69)	17.88 (6.92)

* S.E. of the mean

Table V.11 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level VII in rats exposed to tobacco. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	3.66 (0.59)	0	3.03 (0.81)	0	0
2	0	0	0	8.95 (3.87)	0	1.95 (0.05)
3	0	2.85 (0.10)	0	10.45 (3.15)	2.65 (0.89)	27.15 (3.59)
7	0	0.56 (0.40)	4.45 (2.16)	26.29 (3.04)	7.04 (3.56)	32.12 (4.81)
14	0	1.87 (0.17)	3.32 (1.95)	27.45 (2.49)	6.19 (1.95)	16.10 (3.60)

* S.E. of the mean

Table V.12 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level I in rats exposed to tobacco + PMO. ABpH 2.6-PAS

GOBLET CELLS

	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	0	0	1.62 (0.10)	0	0
2	0	0.09 (0.07)	0	2.64 (0.04)	0.09 (0.07)	18.08 (6.00)
3	0	0	0.44 (0.30)	7.12 (1.73)	0	1.16 (0.28)
7	0	0	0	2.15 (0.62)	0	0
14	0	1.43 (0.26)	0.37 (0.25)	2.61 (1.26)	0.37 (0.15)	13.88 (4.97)

* S.E. of the mean

Table V.13 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level II in rats exposed to tobacco + PMO. ABpH 2.6-PAS

GOBLET CELLS

	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	0	0	0.92 (0.43)	0	0
2	0	0	0	4.66 (0.41)	0	19.83 (6.83)
3	0	0.13 (0.10)	0	3.14 (1.48)	0	4.61 (1.34)
7	0	0	0	1.02 (0.36)	0	0
14	0	1.20 (0.23)	0.91 (0.37)	10.31 (0.71)	2.02 (0.90)	5.32 (0.95)

* S.E. of the mean

Table V.14 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level V in rats exposed to tobacco + PMO. ABpH 2.6-PAS

	GOBLET CELLS					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	0	0	4.81 (0.61)	0	0
2	0	1.80 (0.42)	0	6.10 (0.40)	0	2.00 (0.01)
3	0	2.10 (1.00)	0	8.20 (2.14)	0.20 (0.20)	2.06 (1.40)
7	0	0	0	26.52 (6.07)	0	0
14	0.14 (0.10)	7.72 (1.64)	2.23 (0.73)	29.69 (2.61)	1.57 (0.65)	1.78 (0.34)

* S.E. of the mean

Table V.15 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level VI in rats exposed to tobacco + PMO. ABpH 2.6-PAS

GOBLET CELLS

	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	5.74 (2.97)	0	31.39 (6.32)	0	1.21 (0.49)
2	0.21 (0.17)	2.71 (1.70)	1.65 (0.60)	25.21 (4.68)	4.17 (1.47)	38.12 (6.72)
3	0.21 (0.17)	2.08 (1.03)	0	17.79 (8.86)	7.07 (2.37)	32.38 (10.40)
7	0	11.01 (4.30)	0	40.03 (2.40)	0	25.00 (8.46)
14	0	0	0	55.12 (2.10)	9.07 (3.35)	23.68 (3.70)

* S.E. of the mean

Table V.16 Number of goblet cells in 3 mm epithelium staining with PAS, AB-PAS or AB at airway level VII in rats exposed to tobacco + PMO. ABpH 2.6-PAS

GOBLET CELLS

	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
No. days exposure						
1	0	2.52 (0.79)	0	14.98 (3.38)	0	0
2	0.21 (0.17)	2.71 (1.70)	1.65 (0.60)	25.21 (4.68)	4.17 (1.47)	38.05 (6.72)
3	0	2.51 (0.16)	0	20.62 (6.05)	3.76 (0.25)	15.20 (3.17)
7	0	8.88 (1.44)	5.68 (0.35)	21.37 (3.27)	2.09 (1.10)	20.20 (0.14)
14	0	12.61 (1.01)	4.48 (2.14)	45.32 (1.99)	12.40 (5.90)	16.24 (2.42)

* S.E. of the mean

Change in Percentage Number of Goblet Cells containing 'Small' Amounts of Acid and Neutral or only Acid Glycoprotein in response to Exposure to Tobacco or to Tobacco + PMO

The percentage of goblet cells containing acid or neutral glycoprotein in airway epithelium of control rats and those exposed to tobacco or to tobacco + PMO is illustrated in Figs. V.6 and 7. The number of cells containing 'small' amounts of glycoprotein is illustrated as separate groups (S.PAS, S.AB-PAS and S.AB cells) while those containing 'large' amounts of glycoprotein are grouped together. The latter is taken as a single group since there was comparatively little change in the proportion of these cells or in the proportions within the group of cells containing different types of glycoproteins. Since the effect of exposure to tobacco or tobacco + PMO was greatest on cells containing small amounts of acid glycoprotein (S.AB-PAS + S.AB) the findings for these particular cell types are described in relation to the change in the total goblet cell number.

The Effect of Tobacco

Airway level III (Fig. V.6 - extrapulmonary)

As compared with control animals, it was apparent that exposure of rats to tobacco for periods up to 14 days produced an increase in the proportion of the total goblet cell number containing small amounts of acid glycoprotein (S.AB-PAS+S.AB cells), whether or not the total goblet cell number was increased (cf. the response after 1 day with that after each of the subsequent exposure

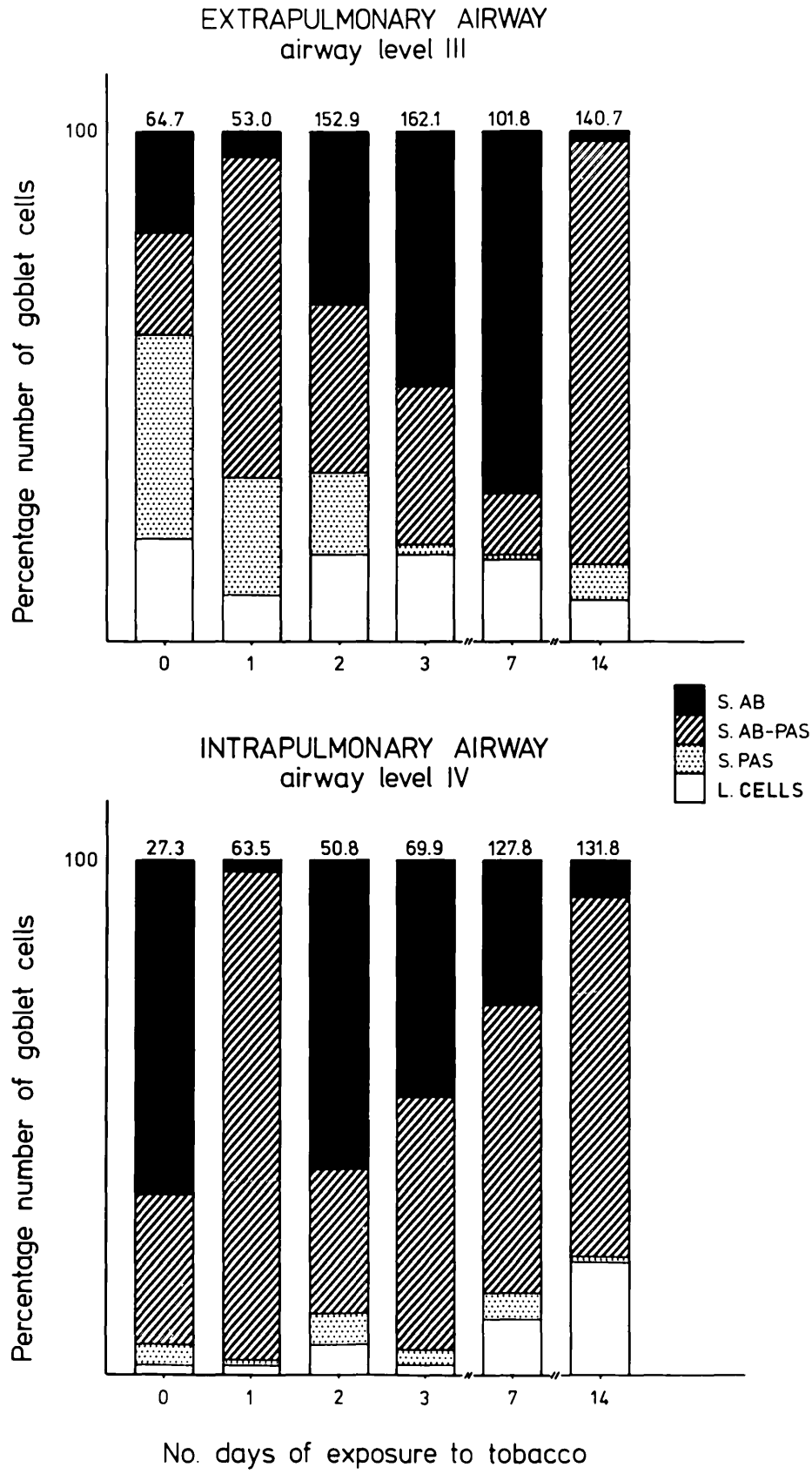
Fig. V.6 Percentage of goblet cells staining with PAS, AB-PAS or with AB at airway levels III and IV in rats exposed to tobacco for periods up to 14 days.

Day 0 = Control value

* Total number of goblet cells.

Cells containing small amounts of glycoprotein (S.AB, S.AB-PAS and S.PAS cells) are illustrated as separate groups: cells containing a large amount of glycoprotein (L.Cells) are grouped together.

Fig. V.6


EXTRAPULMONARY AIRWAY
airway level III
100
64.7
53.0
152.9
162.1
101.8
140.7
Percentage number of goblet cells
0
1
2
3
7
14
S. AB
S. AB-PAS
S. PAS
L. CELLS
INTRAPULMONARY AIRWAY
airway level IV
100
27.3
63.5
50.8
69.9
127.8
131.8
Percentage number of goblet cells
0
1
2
3
7
14
No. days of exposure to tobacco

periods). In addition, the relative number of cells containing either acid and neutral (S.AB-PAS cells) or acid glycoprotein alone (S.AB cells) differed after periods of exposure to tobacco, irrespective of any increase in the proportion of the total goblet cell number represented by the S.AB-PAS+S.AB cell group as a whole (cf. the response after 1 day with that after Day 2 and Day 3).

After 1 day's exposure proportionally more cells contained a small amount of a mixture of acid and neutral than only acid glycoprotein, while after 2 and 3 days they were present in approximately equal numbers. After 7 days of exposure the number containing a small amount of only the acid form was the greater while the reverse wae found after 14 days.

Airway level IV (Fig. V.6 - intrapulmonary)

The response at airway level IV differed from that at Level III in that exposures to tobacco did not produce any increase in the proportion of the total goblet cell number occupied by cells containing small amounts of acid glycoprotein (S.AB-PAS+S.AB cells) - although there was an increase in the total goblet cell number. The response was similar, however, in that there was a change in the relative numbers of cells containing either the mixed or pure form of glycoprotein after different periods of exposure, without any increase in the proportion of the goblet cell number occupied by the acid glycoprotein group as a whole.

After 1 day of exposure the majority of cells contained only a small amount of acid glycoprotein (S.AB), but this increased considerably by Day 2. Subsequently, the proportion of cells containing acid glycoprotein fell progressively on Days 3, 7 and 14 while cells containing both acid and neutral glycoprotein (S.AB-PAS) increased - a reversal of the pattern seen at Level III. At this airway level, as at Level III, the pattern of change from cells containing a mixture to a pure form of glycoprotein was independent of any change in the total goblet cell number, which usually was increased.

The Effect of Tobacco + PMO

Airway level III (Fig. V.7 - extrapulmonary)

The general response of the airway epithelium at Level III was similar after exposure to tobacco + PMO to that seen in the animals exposed to tobacco alone. There was, in addition, an increase in the proportion of the total goblet cell number occupied by cells containing a small amount of acid glycoprotein (S.AB-PAS+S.AB cells) even when the goblet cell number was dramatically reduced (cf. the response after 1 day with that of control animals).

In response to subsequent periods of exposure to tobacco + PMO there was also a fluctuation in the pattern of the predominant cell type that was similar to that seen after exposure to tobacco; with the exception of the response after 7 and 14 when the pattern was reversed, there being more cells containing a mixture of acid

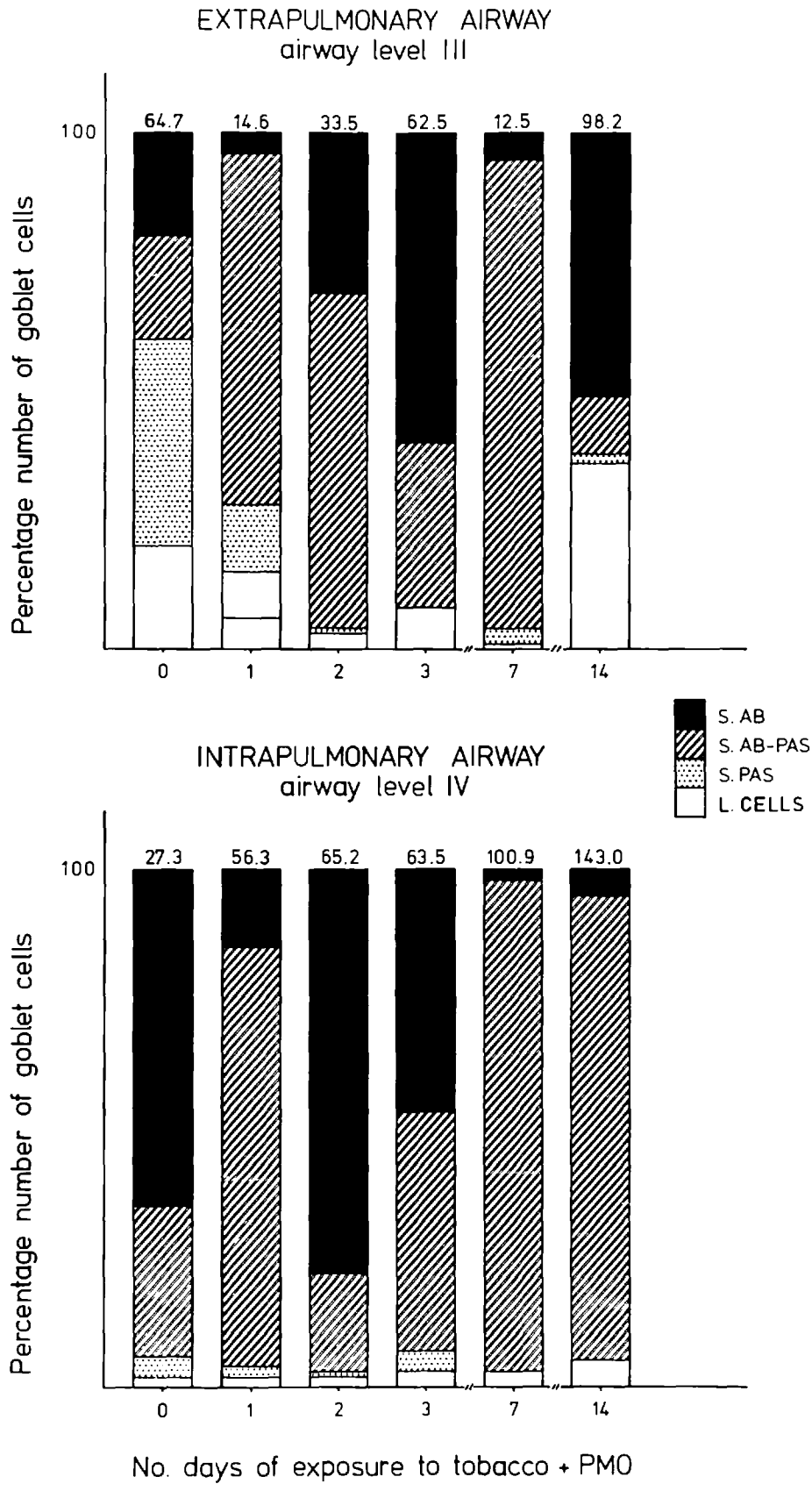
Fig. V.7 Percentage of goblet cells staining with PAS, AB-PAS or with AB at airway levels III and IV in rats exposed to tobacco + PMO for periods up to 14 days.

Day 0 = Control value

* Total number of goblet cells

Cells containing small amounts of glycoprotein (S.AB, S.AB-PAS and S.PAS cells) are illustrated as separate groups: cells containing a large amount of glycoprotein (L.Cells) are grouped together.

Fig. V.7


EXTRAPULMONARY AIRWAY
airway level III
100
64.7
14.6
33.5
62.5
12.5
98.2
Percentage number of goblet cells
0
1
2
3
7
14
S. AB
S. AB-PAS
S. PAS
L. CELLS
INTRAPULMONARY AIRWAY
airway level IV
100
27.3
56.3
65.2
63.5
100.9
143.0
Percentage number of goblet cells
0
1
2
3
7
14
No. days of exposure to tobacco + PMO

and neutral and more cells containing only the acid form on these days respectively, in animals exposed to tobacco + PMO. There was some evidence at this airway level that where the total goblet cell number was reduced the cell containing a mixture of acid and neutral glycoprotein predominated and when it increaeed subsequently there was also an increase in the relative number of cells containing only the acid form (cf. the response after Days 1 and 7 with that after Days 2, 3 and 14).

Although the group of cells containing a small amount of any acid glycoprotein was reduced after Day 14, the increase in 'large' cells seen at this time is due mainly to those containing acid glycoprotein and, in particular, to those containing only the acid form.

Airway level IV (Fig. V.7 - intrapulmonary)

At this airway level also, the general features of the response were similar after exposure to tobacco + PMO to those seen after tobacco.

Again, the fluctuating pattern of change in cells of each type after exposure to tobacco + PMO resembled that seen at this airway level after exposure to tobacco. After Days 1 and 14 the predominant cell contained a mixture of acid and neutral glycoprotein and after Days 2 and 3 it contained only the acid form, but after 7 days more cells now contained the mixed type in response

to tobacco + PMO than in response to tobacco alone. Here also, the change from the goblet cell containing the mixed to the pure form of glycoprotein was independent of any change in the total goblet cell number, which usually was greater the longer the period of exposure.

Change in the Type of Glycoprotein produced by Goblet Cells after Exposure to Tobacco or to Tobacco + PMO including Type of Acid Group

Analysis of the results for the goblet cell number for this experiment (reported in Chapter IV) showed that in the extrapulmonary airways there was a variation in the number of cells depending on the timing of the period of exposures, while in the intrapulmonary airways the number steadily increased. Further analysis was required to determine how the change in goblet cell number and change in type of glycoprotein were related. The distribution of goblet cells containing different types of glycoprotein, including analysis of the acid groups present, was assessed at airway levels III (extrapulmonary) and IV (intrapulmonary) in control rats and in those exposed to tobacco or to tobacco + PMO. The analysis was carried out after only selected periods of the number of exposures given - at Level III after 1, 3, 7 and 14 days, and at Level IV after 1 and 14 days.

For airway level III, Days 1 and 7 were selected because of the fall in goblet cell number at these times (i.e. many cells had discharged their secretory product). This time was chosen so that

the type of glycoprotein within cells which had discharged could be identified as well as the glycoprotein within the existing cell population. The glycoproteins present within goblet cells after 3 days of exposure were analysed since this was the time of greatest increase in goblet cell number between the times of discharge (Days 1 and 7) as well as after 14 days - the longest period of exposure. Since at Level IV there was a steady increase in goblet cell number after subsequent periods of exposure, at this airway level the glycoproteins were analysed after 1 and after 14 days.

The method of analysis of glycoproteins within goblet cell populations was as described previously in Chapter II. The total number of goblet cells of each type after staining with ABpH 2.6-PAS is summarised in Table V.17 (the mean number of each cell type after this staining technique has been given in Tables V.1-6 for each animal group - the total cell numbers being required here for analysis of glycoproteins). The absolute number of goblet cells staining with PAS, AB-PAS or with AB in tissue sections stained with sialidase ABpH 2.6-PAS or with ABpH 1.0-PAS is given in Table V.18 for control animals and in Tables V.19-22 for those exposed.

For these analyses the data was grouped into cell populations containing glycoprotein staining with PAS, AB-PAS or with AB. This was because of the small number of cells in some of the 6 cell groups after certain periods of exposures; small numbers of cells within a group precluded analysis by the chi-squared test.

...../Text continue p. 295

Table V.17 Total absolute number of goblet cells of each type in 3 mm airway epithelium in control rats and in rats exposed to tobacco or to tobacco + PMO.

Animal group	Airway level	No. days' exposure	GOBLET CELLS					
			L.PAS	S.PAS	L.AB-PAS	S.AB-PAS	L.AB	S.AB
Control	III	-	22.02	156.78	42.84	76.56	6.66	78.42
	IV	-	0	6.00	1.14	47.88	1.62	107.22
Tobacco	III	1	2.10	37.09	11.07	100.71	0	8.31
		3	2.68	10.56	79.80	201.96	29.00	325.32
		7	1.41	39.90	37.29	37.59	7.59	217.56
		14	2.58	27.72	33.57	350.16	0	6.96
	IV	1	0	1.65	0	180.84	3.96	4.65
		14	0	3.36	9.87	275.85	75.84	30.54

Tobacco + PMO	III	1	2.43	5.79	1.71	29.88	2.28	1.95
		3	0	0	6.76	78.92	14.84	113.01
		7	0	1.08	0.51	33.99	0	1.89
		14	0	5.01	10.83	31.86	95.31	151.80
	IV	1	0	4.41	3.63	136.38	0	24.60
		14	0	4.86	8.16	384.09	14.04	20.10

Table V.18 Number of goblet cells in 3 mm airway epithelium after staining with (i) sialidase ABpH 2.6-PAS or (ii) ABpH 1.0-PAS - Airway levels III and IV in control rats.

		GOBLET CELLS					
		L.PAS	S.PAS	L.AB-PAS	S.AB-PAS	L.AB	S.AB
(i) Sialidase ABpH 2.6-PAS							
Level III	Total	12.00	198.42	12.96	113.64	0	89.52
	Mean	2.00	31.57	2.16	18.94	0	14.92
Level IV	Total	0	6.01	0	56.46	0	128.16
	Mean	0	1.00	0	9.41	0	21.36
(ii) ABpH 1.0-PAS							
Level III	Total	29.61	111.36	0	64.20	0	14.34
	Mean	4.86	18.56	0	10.70	0	2.39
Level IV	Total	11.64	132.60	0	43.26	0	8.88
	Mean	1.94	22.10	0	7.21	0	1.48

Table V.19 Number of goblet cells in 3 mm airway epithelium after staining with (i) sialidase ABpH 2.6–PAS or (ii) ABpH 1.0–PAS – Airway level III in rats exposed to tobacco.

			GOBLET CELLS					
			L.PAS	S.PAS	L.AB–PAS	S.AB–PAS	L.AB	S.AB
(i)	Day 1	Total	1.20	33.72	4.20	110.58	0	9.33
		Mean	0.40	11.24	1.40	36.86	0	3.11
	Day 2	Total	2.44	103.28	11.48	225.64	5.88	248.40
		Mean	0.61	25.82	2.87	56.41	1.47	62.10
	Day 3	Total	0	15.88	37.04	288.52	13.60	365.60
		Mean	0	3.97	2.10	72.13	3.40	91.40
	Day 7	Total	0	7.80	14.91	58.26	5.82	267.28
		Mean	0	2.60	4.97	19.42	1.94	89.76
	Day 14	Total	6.42	89.58	11.94	387.78	0	3.45
		Mean	2.14	29.86	3.98	129.26	0	1.15

(ii)	Day 1	Total	6.51	76.50	0	25.26	0	3.12
		Mean	2.17	25.50	0	8.42	0	1.04
	Day 2	Total	5.40	255.76	0	24.84	0	16.52
		Mean	1.35	63.94	0	6.21	0	4.13
	Day 3	Total	11.72	379.12	8.68	77.88	0.96	64.40
		Mean	2.93	94.78	2.17	19.47	0.24	16.10
	Day 7	Total	6.21	189.63	0	42.93	0	58.23
		Mean	2.07	63.21	0	14.31	0	19.41
	Day 14	Total	12.45	362.76	1.44	104.37	2.43	3.72
		Mean	4.15	120.92	0.48	34.79	0.81	1.24

Table V.20 Number of goblet cells in 3 mm airway epithelium after staining with (i) sialidase ABpH 2.6-PAS or (ii) ABpH 1.0-PAS - Airway level IV in rats exposed to tobacco.

			GOBLET CELLS					
			L.PAS	S.PAS	L.AB-PAS	S.AB-PAS	L.AB	S.AB
(i)	Day 1	Total	0	20.79	3.00	172.92	0	12.27
		Mean	0	6.93	1.00	57.64	0	4.09
	Day 2	Total	0	16.84	0	73.16	4.96	144.64
		Mean	0	4.21	0	18.29	1.24	36.16
	Day 3	Total	0	12.56	0	157.88	0	144.12
		Mean	0	3.14	0	39.47	0	36.03
	Day 7	Total	0	23.82	8.79	284.70	4.38	66.90
		Mean	0	7.94	2.93	94.90	1.46	22.30
	Day 14	Total	0	17.58	6.42	266.07	15.03	106.96
		Mean	0	5.86	2.14	88.69	5.01	35.62

(ii)	Day 1	Total	0	158.40	3.63	22.02	0	6.27
		Mean	0	52.80	1.21	7.34	0	2.09
	Day 2	Total	8.04	127.60	1.16	45.20	0	24.56
		Mean	2.01	31.90	0.29	11.30	0	6.14
	Day 3	Total	1.84	218.44	0.68	69.68	0	33.88
		Mean	0.46	54.61	0.17	17.42	0	8.47
	Day 7	Total	1.05	283.83	4.83	77.61	0	34.23
		Mean	0.35	94.61	1.61	25.87	0	11.41
	Day 14	Total	12.75	224.64	3.63	91.20	0	30.84
		Mean	4.25	74.88	1.21	30.40	0	10.28

Table V.21 Number of goblet cells in 3 mm airway epithelium after staining with (i) sialidase ABpH 2.6-PAS or (ii) ABpH 1.0-PAS - Airway level III in rats exposed to tobacco + PMO.

			GOBLET CELLS					
			L.PAS	S.PAS	L.AB-PAS	S.AB-PAS	L.AB	S.AB
(i)	Day 1	Total	0.93	2.87	0.63	67.26	0	0
		Mean	0.30	0.96	0.21	22.42	0	0
	Day 2	Total	2.96	25.64	0	119.12	8.01	60.88
		Mean	0.74	6.41	0	29.78	2.00	15.22
	Day 3	Total	0	38.88	0	58.80	0	201.76
		Mean	0	9.72	0	14.70	0	52.69
	Day 7	Total	0	15.48	11.70	27.63	0	20.25
		Mean	0	5.61	3.90	9.21	0	6.75
	Day 14	Total	10.23	40.83	4.29	53.04	31.26	199.14
		Mean	3.41	13.61	1.43	17.68	10.42	66.38

(ii)	Day 1	Total	0	18.57	0	10.23	0	0
		Mean	0	6.19	0	3.41	0	0
	Day 2	Total	0	120.52	0	18.96	0	20.40
		Mean	0	30.13	0	4.74	0	5.10
	Day 3	Total	15.72	241.96	0	29.00	0	12.40
		Mean	2.93	60.49	0	7.25	0	3.10
	Day 7	Total	0	37.23	0	18.63	0	8.85
		Mean	0	12.41	0	6.21	0	2.95
	Day 14	Total	6.30	175.02	7.29	58.11	12.30	37.02
		Mean	2.10	58.34	2.43	19.37	4.10	12.34

Table V.22 Number of goblet cells in 3 mm airway epithelium after staining with (i) sialidase ABpH 2.6–PAS or (ii) ABpH 1.0–PAS – Airway level IV in rats exposed to tobacco + PMO.

			GOBLET CELLS					
			L.PAS	S.PAS	L.AB–PAS	S.AB–PAS	L.AB	S.AB
(i)	Day 1	Total	0.48	11.70	0.69	106.26	0	46.47
		Mean	0.16	3.90	0.23	35.42	0	15.49
	Day 2	Total	0	8.40	0	83.44	4.00	140.08
		Mean	0	2.10	0	20.86	1.00	35.02
	Day 3	Total	5.20	24.04	8.68	140.56	0	83.64
		Mean	1.30	6.01	2.17	35.14	0	20.91
	Day 7	Total	0	16.02	7.68	235.83	0.42	69.93
		Mean	0	5.34	1.60	78.61	0.14	23.21
	Day 14	Total	0	102.51	6.24	336.30	7.47	57.90
		Mean	0	34.17	2.08	112.10	2.49	19.30

(ii)	Day 1	Total	0.34	112.68	0	28.74	0	6.42
		Mean	0.10	37.56	0	9.58	0	2.14
	Day 2	Total	8.56	170.80	0	17.28	0	27.64
		Mean	2.14	42.70	0	4.32	0	6.91
	Day 3	Total	0.84	200.96	0	12.44	0	33.80
		Mean	0.21	50.24	0	3.11	0	8.45
	Day 7	Total	0.92	237.36	0	25.62	0	20.34
		Mean	0.30	79.12	0	8.54	0	6.78
	Day 14	Total	7.23	375.00	0	41.94	0	12.96
		Mean	2.41	125.20	0	13.98	0	4.32

The Effect of Tobacco

(i) Changes in the number of cells containing acid or neutral glycoprotein

Analysis by the chi-squared test showed a significant difference in the distribution of goblet cells containing acid or neutral glycoprotein, alone or in combination, after each period of exposure to tobacco, at both airway levels III and IV (Table V.23). As compared with the control distribution there was a shift to cells producing acid glycoprotein; to some extent at the expense of cells producing neutral glycoprotein alone, but due also to shifts within the cell populations producing either a mixed (AB-PAS) or pure (AB) form of glycoprotein. Only at airway level III after 3 days of exposure to tobacco was there a shift in cells producing either the mixed or pure forms.

In the airway epithelium at Level III, after 1 day's exposure, although the goblet cell number was reduced there was, nevertheless, a shift to cells producing acid together with neutral glycoprotein. Since it is likely that many goblet cells discharged their secretory product in response to the first exposure to tobacco smoke, the findings suggest that in the following 16-hour period, in cells now actively forming secretory product, the synthesis of glycoprotein was already modified. The goblet cell number was again reduced after 7 days but on this occasion the goblet cell population produced mainly the pure form. This suggested that when entering a further period of synthesis after discharge, those cells producing the modified glycoprotein retained their pattern of

Table V.23 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. ABpH 2.6-PAS

TOBACCO/CONTROLS

		Actual value	Expected value	A-E
Airway level III				
	L.PAS+S.PAS	39	74	- 35
Day 1	L.AB-PAS+S.AB-PAS	112	49	+ 63
	L.AB+S.AB	8	35	- 27
	$Px^2 <$			0.001
	L.PAS+S.PAS	13	303	- 290
Day 3	L.AB-PAS+S.AB-PAS	282	202	+ 80
	L.AB+S.AB	354	144	+ 210
	$Px^2 <$			0.001
	L.PAS+S.PAS	41	159	- 118
Day 7	L.AB-PAS+S.AB-PAS	75	106	- 31
	L.AB+S.AB	225	76	+ 149
	$Px^2 <$			0.001
	L.PAS+S.PAS	30	197	- 167
Day 14	L.AB-PAS+S.AB-PAS	384	131	+ 253
	L.AB+S.AB	7	93	- 86
	$Px^2 <$			0.001
Airway level IV				
	L.PAS+S.PAS	2	7	- 5
Day 1	L.AB-PAS+S.AB-PAS	181	57	+ 124
	L.AB+S.AB	9	128	- 119
	$Px^2 <$			0.001
	L.PAS+S.PAS	4	14	- 10
Day 14	L.AB-PAS+S.AB-PAS	286	118	+ 168
	L.AB+S.AB	106	263	- 157
	$Px^2 <$			0.001

production of the pure form. After the 14-day period, however, the increase in goblet cell number was again accompanied by a shift to cells producing the mixed type of glycoprotein, suggesting a difference in the goblet cell population at 14 days as compared with that at 7 days. It is of interest that in animals from the same experiment Jeffery (1973) reported an increase in the number of cells (basal) in mitosis after 1 and after 7 days of exposure, and this finding is discussed, with relation to the changes within the goblet cell populations at these times, in Chapter IV.

At Level IV after 1 and 14 days of exposure the majority of cells were producing a mixture of acid and neutral glycoprotein.

(ii) Changes in the number of cells containing different types of acid glycoprotein

After sialidase ABpH 2.6-PAS staining, at both airway levels III and IV there was a significant difference in the distribution of acid glycoprotein in the goblet cells in the animals exposed to tobacco as compared with the distribution in control animals (Table V.24). At airway level III, after each exposure period there was a shift away from cells containing a glycoprotein staining with PAS, and shifts to or from cells containing mixed or only acid glycoprotein, which corresponded to the direction of the shift in these cells after ABpH 2.6-PAS. Using the method of analysis described in detail in Chapter III, it can be concluded that virtually no cells contained sialomucin sensitive to the enzyme sialidase and that where there was an increase in either the absolute

Table V.24 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. Sialidase ABpH 2.6-PAS

TOBACCO/CONTROLS

		Actual value	Expected value	A-E
Airway level III				
	L.PAS+S.PAS	35	76	- 41
Day 1	L.AB-PAS+S.AB-PAS	115	48	+ 67
	L.AB+S.AB	9	34	- 25
$Px^2 <$				0.001
	L.PAS+S.PAS	16	371	- 355
Day 3	L.AB-PAS+S.AB-PAS	377	235	+ 142
	L.AB+S.AB	379	166	+ 213
$Px^2 <$				0.001
	L.PAS+S.PAS	8	171	- 163
Day 7	L.AB-PAS+S.AB-PAS	73	108	- 35
	L.AB+S.AB	275	77	+ 198
$Px^2 <$				0.001
	L.PAS+S.PAS	96	240	- 144
Day 14	L.AB-PAS+S.AB-PAS	400	152	+ 248
	L.AB+S.AB	4	108	- 104
$Px^2 <$				0.001
Airway level IV				
	L.PAS+S.PAS	21	7	+ 14
Day 1	L.AB-PAS+S.AB-PAS	176	62	+ 114
	L.AB+S.AB	12	141	- 129
$Px^2 <$				0.001
	L.PAS+S.PAS	18	13	+ 5
Day 14	L.AB-PAS+S.AB-PAS	272	121	+ 151
	L.AB+S.AB	122	278	- 156
$Px^2 <$				0.001

or relative number of cells conteining ecid glycoprotein it was in cells conteining a sialomucin resistent to sialidase. The results of anelysis after ABpH 1.0-PAS staining at this eirwey level (see Table V.25), confirm that en increase in cells containing sulphomucin contributed little to the overall increase in the number of cells conteining acid glycoprotein; and that this type of glycoprotein wes present mainly in cells containing only the acid form (AB cells - e.g. efter 3 end 7 days of exposure).

At airway level IV (see Tables V.24 end 25), efter 1 and 14 days of exposure the response wes, in generel, similar to that at Level III; seve that there wes also a shift to cells containing a glycoprotein staining with PAS efter sialidese ABpH 2.6-PAS - thet is, to cells containing neutrel glycoprotein or sialomucin sensitive to sialidase - and the mein contribution from cells containing sulphomucin, was after 14 days of exposure.

...../Text continues p. 301

Table V.25 Goblet cell types in rat airway epithelium. Actual value - TOBACCO - compared with that Expected on the basis of the control distribution. ABpH 1.0-PAS

TOBACCO/CONTROLS

		Actual value	Expected value	A-E
Airway level III				
	L.PAS+S.PAS	83	71	+ 12
Day 1	L.AB-PAS+S.AB-PAS	25	32	- 7
	L.AB+S.AB	3	7	- 4
$Px^2 <$				N.S.
	L.PAS+S.PAS	391	350	+ 41
Day 3	L.AB-PAS+S.AB-PAS	87	159	- 72
	L.AB+S.AB	65	35	+ 30
$Px^2 <$				0.001
	L.PAS+S.PAS	196	191	+ 5
Day 7	L.AB-PAS+S.AB-PAS	43	87	- 44
	L.AB+S.AB	58	19	+ 39
$Px^2 <$				0.001
	L.PAS+S.PAS	375	313	+ 62
Day 14	L.AB-PAS+S.AB-PAS	106	142	- 36
	L.AB+S.AB	6	31	- 25
$Px^2 <$				0.001
Airway level IV				
	L.PAS+S.PAS	158	140	+ 18
Day 1	L.AB-PAS+S.AB-PAS	26	42	- 14
	L.AB+S.AB	6	8	- 2
$Px^2 <$				N.S.
	L.PAS+S.PAS	237	267	- 30
Day 14	L.AB-PAS+S.AB-PAS	95	80	+ 15
	L.AB+S.AB	31	17	+ 14
$Px^2 <$				0.001

The Effect of Tobacco + PMO

(i) Changes in the number of cells containing acid or neutral glycoprotein

After each period of exposure to tobacco + PMO there was a significant difference in the distribution of goblet cells containing neutral, mixed or only acid glycoprotein, at both airway levels III and IV, as compared with the corresponding distribution in control animals (Table V.26): at each airway level there was a shift to cells producing acid glycoprotein which was mainly to cells containing a mixture of acid and neutral types. The exception was after 3 and 14 days of exposure at Level III, where the shift was mainly in cells producing only the acid form, while after 14 days at Level IV, it was equally toward both cell types.

Where there were decreases and increases in the goblet cell number similar changes in the type of glycoprotein were found irrespective of any change in goblet cell number in response to tobacco + PMO to those described after exposure to tobacco alone.

(ii) Changes in the number of cells containing different types of acid glycoprotein

After sialidase ABpH 2.6-PAS staining there was a shift away from cells containing glycoprotein staining with PAS at airway level III and some shift to cells of this type at Level IV, particularly after 14 days of exposure (Table V.27): at each airway

Table V.26 Goblet cell types in rat airway epithelium. Actual value - TOBACCO + PMO - compared with that Expected on the basis of the control distribution. ABpH 2.6-PAS

TOBACCO+PMO/CONTROLS

		Actual value	Expected value	A-E
Airway level III				
	L.PAS+S.PAS	8	21	- 13
Day 1	L.AB-PAS+S.AB-PAS	32	14	+ 18
	L.AB+S.AB	4	10	- 6
	$Px^2 <$			0.001
	L.PAS+S.PAS	0	100	- 100
Day 3	L.AB-PAS+S.AB-PAS	86	66	+ 20
	L.AB+S.AB	128	47	+ 81
	$Px^2 <$			0.001
	L.PAS+S.PAS	1	18	- 17
Day 7	L.AB-PAS+S.AB-PAS	35	12	+ 23
	L.AB+S.AB	2	8	- 6
	$Px^2 <$			0.001
	L.PAS+S.PAS	5	138	- 133
Day 14	L.AB-PAS+S.AB-PAS	43	92	- 49
	L.AB+S.AB	247	65	+ 182
	$Px^2 <$			0.001
Airway level IV				
	L.PAS+S.PAS	4	37	- 33
Day 1	L.AB-PAS+S.AB-PAS	140	50	+ 90
	L.AB+S.AB	25	112	- 87
	$Px^2 <$			0.001
	L.PAS+S.PAS	5	16	- 11
Day 14	L.AB-PAS+S.AB-PAS	392	129	+ 263
	L.AB+S.AB	34	286	+ 252
	$Px^2 <$			0.001

Table V.27 Goblet cell types in rat airway epithelium. Actual value - TOBACCO+PMO - compared with that Expected on the basis of the control distribution. Sialidase ABpH 2.6-PAS

TOBACCO+PMO/CONTROLS

		Actual value	Expected value	A-E
Airway level III				
	L.PAS+S.PAS	4	35	- 31
Day 1	L.AB-PAS+S.AB-PAS	68	22	+ 46
	L.AB+S.AB	0	16	- 16
$Px^2<$				0.001
	L.PAS+S.PAS	39	144	- 105
Day 3	L.AB-PAS+S.AB-PAS	59	91	- 32
	L.AB+S.AB	202	65	+ 137
$Px^2<$				0.001
	L.PAS+S.PAS	15	36	- 21
Day 7	L.AB-PAS+S.AB-PAS	39	22	+ 17
	L.AB+S.AB	20	16	+ 4
$Px^2<$				0.001
	L.PAS+S.PAS	51	163	- 112
Day 14	L.AB-PAS+S.AB-PAS	57	103	- 46
	L.AB+S.AB	230	73	+ 157
$Px^2<$				0.001
Airway level IV				
	L.PAS+S.PAS	12	5	+ 7
Day 1	L.AB-PAS+S.AB-PAS	107	49	+ 58
	L.AB+S.AB	46	111	- 65
$Px^2<$				0.001
	L.PAS+S.PAS	103	16	+ 87
Day 14	L.AB-PAS+S.AB-PAS	343	151	+ 192
	L.AB+S.AB	65	344	- 279
$Px^2<$				0.001

level there was a shift to cells producing acid glycoprotein. Unlike the response to tobacco, however, the shift to cells other than those staining with PAS did not correspond to the same shift after ABpH 2.6-PAS staining alone (cf. the response of cells staining with AB-PAS or AB after 3 days of exposure to tobacco + PMO at airway level III, and after 14 days of exposure at Level IV - Tables V.26 and 27).

The findings suggest that, as in animals exposed to tobacco, most of the acid glycoprotein present within goblet cells was a sialomucin resistant to sialidase with sulphomucin contributing very little (see Table V.28). The main shift to cells producing a sulphomucin was after 14 days of exposure at airway level III.

...../Text continues p. 306

Table V.28 Goblet cell types in rat airway epithelium. Actual value – TOBACCO+PMO – compared with that Expected on the basis of the control distribution. A8pH 1.0–PAS

TOBACCO+PMO/CONTROLS

		Actual value	Expected value	A–E
Airway level III*				
Day 3	L.PAS+S.PAS	258	193	+ 65
	L.A8–PAS+S.AB–PAS	29	87	– 58
	L.AB+S.AB	12	19	– 7
	$Px^2 <$			0.001
Day 14	L.PAS+S.PAS	181	190	– 9
	L.AB–PAS+S.AB–PAS	65	86	– 21
	L.AB+S.AB	49	19	+ 30
	$Px^2 <$			0.001
Airway level IV				
Day 14	L.PAS+S.PAS	382	321	+ 61
	L.AB–PAS+S.AB–PAS	42	96	– 54
	L.AB+S.AB	13	20	– 7
	$Px^2 <$			0.001

* Values given only where the distribution was significantly different from that of control animals.

HYPOTHETICAL FLOW CHART SYSTEM

The results reported here and in Chapter III show patterns of change in both the type and amount of glycoprotein produced by goblet cells of airway epithelium in response to irritation. The main changes are first a reduction in S.PAS cells with an increase in the number of S.AB-PAS and S.AB cells and finally an increase in L.AB-PAS and L.AB cells.

The figures given in Table V.29 illustrate the changes within a population of secreting cells over a period of 6 weeks. (This is concerned with percentage of population and does not take into account an increase in goblet cell number). The figures are based on the observed distribution of each type of goblet cell in the normal airway and the change in response to irritation. Using these values it was possible to suggest the pattern of change from one cell type to another. This is illustrated in Fig. V.8. Of the population of goblet cells moving away from the S.PAS group (-56 cells) only an occasional cell increases its glycoprotein to become a L.PAS cell (+1 cell) the majority of cells would move along the pathway into the cell groups that are AB +ve (55 cells). This ultimately gives rise to an increase in the number of cells in each of these groups, roughly of the order S.AB-PAS (+13 cells), S.AB (+25 cells), L.AB-PAS (+12 cells) and L.AB (+5 cells). The increase in the proportion of goblet cells occupied by each cell type most likely arises from the movement of cells along certain

Fig. V.8 Suggested flow chart system illustrating the most likely pathways along which goblet cells may be converted from one having a particular amount and type of glycoprotein to one of another type: the figures are based on values given in Table V.29 and are described further in the text.

Fig. V.8

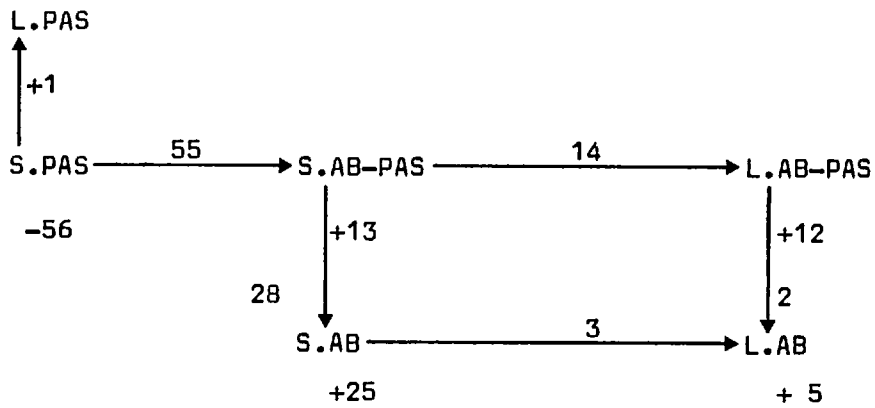


Table V.29 Distribution of cells by type as a % of total goblet cell population after 6 weeks' exposure to an inhaled irritant.

	GOBLET CELL TYPES					
	PAS		AB-PAS		AB	
	Large	Small	Large	Small	Large	Small
Control	2	63	5	15	3	12
Exposed	3	7	17	28	8	37
	+ 1	-56	+12	+13	+ 5	+25

pathways. For example, it is unlikely that a S.PAS cell gives rise directly to a L.AB cell but that it modifies and increases its glycoprotein content to give rise to a L.AB-PAS cell (shown in Fig. V.8 by a movement of $\xrightarrow{14\ \text{cells}}$ in this direction). The glycoprotein within a S.PAS cell may be modified but not increased and thus gives rise to a S.AB cell ($\xrightarrow{28\ \text{cells}}$), and the latter cell may increase its glycoprotein content to give rise to a L.AB cell ($\xrightarrow{3\ \text{cells}}$). It may also be that a L.AB-PAS cell modifies its glycoprotein to give rise to a L.AB cell ($\xrightarrow{2\ \text{cells}}$). The degree of movement of cells along the pathways suggested ultimately increases the proportion occupied by cells of each type in a modified goblet cell population.

From the figures given in Table V.29 the rates of flow per unit interval of time are imputed along the various branches of this model. The rates are given in Fig. V.9 and are based on the proportions of the various cell types in the control population and the rate, at weekly intervals of time, at which each type moves along the various paths in the system.

The results of applying the rates of transfer are shown in Table V.30, and it can be seen that using them the actual values achieved are similar to those expected.

The result of this analysis indicates that the main stream of movement of goblet cells is from S.PAS to S.AB-PAS and to S.AB. The other weekly changes as proportions of the population are much smaller.

Fig. V.9 Probability that a particular goblet cell type could move in the direction shown during one week of exposure to tobacco, i.e. the imputed rate of movement during this time interval.

Fig. V.9

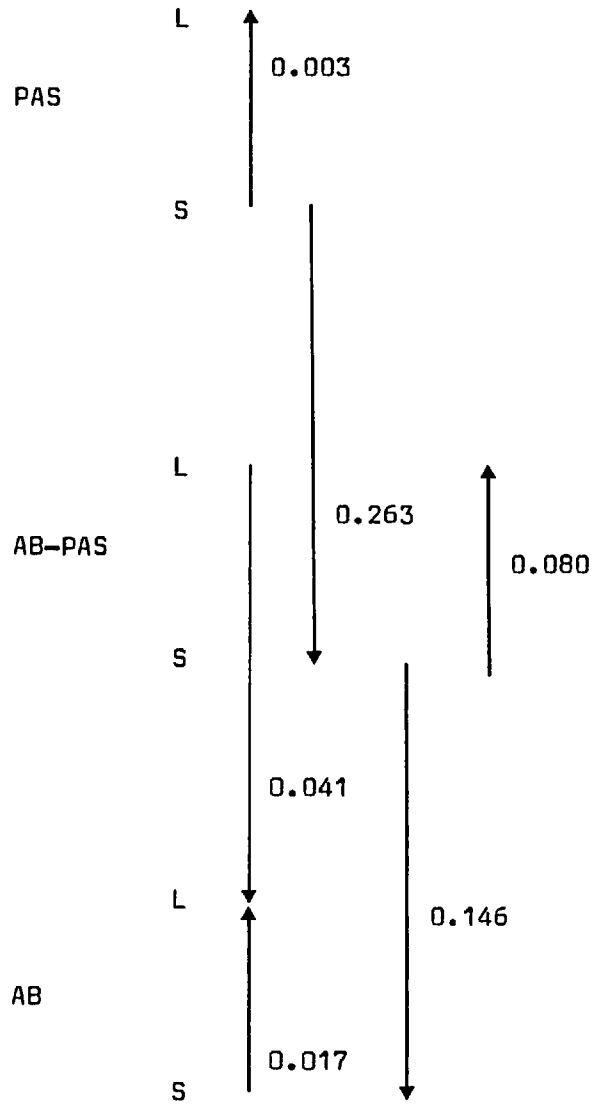


Table V.30 Application of set rates of transfer of cells from one group to another for a population of goblet cells.

		Test run for 6 weeks		
		Start	Finish	
			Actual	Expected
PAS	Large	20*	28	30*
	Small	630	99	70
AB–PAS	Large	50	171	170
	Small	150	288	280
AB	Large	30	74	80
	Small	120	360	370

* For ease, to calculate the set rates of transfer the value for each cell type at the start and at the end of the test run has been multiplied by 10.

Subsequently the results of an experiment in which the exposure time was 2 weeks fitted well with the prediction from the model in that it confirmed that the early and main stream of movement is between 'small' cells even when the total goblet cell number is increased.

...../Text continues p. 315

SUMMARY AND COMMENT

1. In normal rat epithelium there is a variation in the distribution of goblet cells producing neutral or acid glycoprotein, alone or in combination, at different airway levels. In the extrapulmonary airways, cells are mainly those producing neutral glycoprotein alone, or in combination with the acid form - cells producing only the acid type being less frequent. In the intrapulmonary airways cells produce mainly the combined or only the acid type. The distribution along the axial pathway differs in that of cells containing a single type of glycoprotein, those containing the acid form occupy the proximal region of the airway and those containing the neutral form the peripheral. At any airway level most of the goblet cells contain only 'small' amounts of glycoprotein.

2. Exposure to tobacco smoke for periods up to 14 days produces a reduction in the cells containing only neutral glycoprotein and an increase in those containing the acid form, at each airway level. In the epithelium of the extrapulmonary airway this represents a shift within the total goblet cell population, so that an increase in total goblet cell number is accompanied by modification of secretory product to a more acid type, within newly appearing goblet cells which contain only small amounts of acid glycoprotein. In the intrapulmonary airways an increase in goblet cell number is also the result of an

increase in cells containing acid glycoprotein, but here the increase represents not a shift but an increase in the goblet cell type already predominant at this airway level; and there is increase in the number of cells containing a large amount of acid glycoprotein.

In both the extra and intrapulmonary airways the absolute increase in cells containing acid glycoprotein was due mainly to an increase in cells containing a sialomucin resistant to sialidase with only a small increase in those containing sulphomucin.

3. Exposure to tobacco smoke with the addition of PMO produced each of the changes described above that occur in response to to tobacco alone. The addition of PMO did not thus prevent the changes occurring in the nature of the glycoprotein. As after tobacco alone, most cells contain only small amounts of glycoprotein save in the extrapulmonary airway (Level III) where tobacco with PMO ultimately produces a greater increase in goblet cells containing a large amount of acid glycoprotein.

4. From the patterns of change in goblet cell type in response to irritation, it seems that the most sensitive feature is a modification of secretory product whether or not the goblet cell number is increased, unchanged or even reduced. So that the modification will take place within an existing population

of specialised cells and within 'new cells'.

It seems that there is a conversion of goblet cells from from one type to another only along certain pathways or cell lines. Although in response to exposure for periods up to 14 days there is, in general, no increase in the number of cells containing a large amount of secretory product, the response after 6 weeks of exposure (Chapter III) suggests that together with a modification to acid glycoprotein there is ultimately an increase in secretory product within goblet cells of the airway epithelium.

CHAPTER VI

DISCUSSION

This study of rat airway epithelium has shown that increase in goblet cells after irritation occurs at an earlier time than previously reported. Change in the type of intracellular glycoprotein, however, precedes increase in goblet cell number and thus is the earliest and most sensitive index of response to a stimulus by the epithelial secretory cell population. The nature of the change can be predicted. In the early period of response to irritation (up to 2 weeks of exposure), cell populations in both the extrapulmonary and intrapulmonary airway epithelium that normally produce neutral glycoprotein, shift to a pattern of production that includes either a mixture of neutral and acid or acid glycoprotein alone. In the intrapulmonary airway epithelium where the cell populations normally secrete mainly an acid glycoprotein, its synthesis is maintained but many cells additionally synthesise the neutral form. Ultimately, in response to longer periods of irritation (after 6 weeks' exposure) most cells, throughout the airways, produce acid glycoprotein alone. It is the acid glycoprotein sialidase resistant sialomucin that is mainly produced with only some increase in sialidase sensitive sialomucin or sulphomucin. Granules containing various types of glycoprotein are located in particular cell regions.

During the first 2 weeks of exposure to an irritant, the balance between synthesis and discharge is such that the volume of secretory product within the cell is not increased: after 6 weeks of exposure the baseline is modified so that an increase

in the amount of intracellular secretion is apparent in many cells.

The use of an anti-inflammatory agent has shown that each of these intracellular changes in glycoprotein can occur in response to irritation without any increase in goblet cell number - confirming modification within an existing goblet cell population.

A detailed summary of findings has been presented at the end of each chapter (see p. 185 Chapter III, p. 233 Chapter IV and p. 315 Chapter V).

...../Text continues p. 321

MODIFICATION OF EPITHELIAL GLYCOPROTEINS IN RESPONSE TO IRRITATION - AIRWAY

The normal surface epithelium and submucosal gland of human airways shows a variety of types of glycoprotein within the secretory cells (McCarthy and Reid, 1964b; Lamb and Reid, 1969b, 1970) as well as in hypersecretory states such as chronic bronchitis and cystic fibrosis (de Haller and Reid, 1965; Lamb, 1969; Lamb and Reid, 1972a). No abnormal type of glycoprotein has been detected either in disease or in experimental studies, either histochemically or biochemically (Reid, 1970b), but quantitative histochemical methods have revealed a change in the proportion of cells producing the various types of glycoprotein both in the gland (de Haller and Reid, 1965; Lamb, 1968; Jones, Baskerville and Reid, 1975) and in the surface epithelium (Lamb and Reid, 1968; Sturgess and Reid, 1973).

Goblet Cell Glycoproteins of Normal Rat Airway Epithelium

The present study has confirmed that the same range of glycoproteins is seen in the mucus-secreting cells of normal rat airway epithelium as in human airways: including neutral glycoprotein and of the acid varieties - sialomucin either sensitive or resistant to sialidase and sulphomucin.

Within the normal rat airway there is a regional difference in the type of glycoproteins synthesised by goblet cell populations,

but at any airway level a single cell may produce only neutral or acid glycoprotein or a mixture. Each type of glycoprotein, be it neutral or any one of the three acidic types present, is packaged within a separate granule. Thus, within a cell, a single or mixed population of granules may occur.

In the normal extrapulmonary airway epithelium, the goblet cell population preferentially secretes either neutral glycoprotein alone or this in combination with an acid type. In contrast, within the lung, most cells secrete either acid glycoprotein alone or this in combination with the neutral. In one particular region within the lung - the medial wall of the lower axial pathway - mainly neutral glycoprotein is produced by the goblet cell population. These findings confirm and extend in detail the earlier descriptions of the range of glycoproteins within the goblet cells of normal rat airway epithelium (McCarthy and Reid, 1964a; Lamb and Reid, 1968). Only in the present study, however, has a regional difference in the production of neutral and acid glycoprotein been described, as well as the range of glycoproteins within a cell.

McCarthy and Reid (1964a) reported a regional difference in acid radicles throughout the airways, with sulphomucin and sialidase resistant sialomucin in goblet cells of the trachea and main bronchi and sialidase sensitive sialomucin in peripheral airways. These findings were from a batch of Wistar strain rats

(of a wide range in body weight - 55-350 g) and included both male and female animals, whereas the present study reports the findings from a group of Sprague-Dawley (SPF) male animals. Differences in the amount of each type of acid glycoprotein may reflect differences in age, sex and strain of rats studied and, perhaps more importantly, difference between non-SPF and SPF stock. It might be expected that non-SPF stock would have more acid glycoprotein since this is the direction of the change in infection. In addition, the earlier study used uptake of ^{35}S-sodium sulphate to identify sulphomucin while in the present study identification has been by Alcian Blue staining at pH 1.0; and the latter may perhaps be considered the more specific technique for the demonstration of sulphate (vide infra).

The range of acid radicles within goblet cell glycoproteins of rat airways resembles that of airway goblet cell populations of other rodents, e.g. guinea-pig (Jenetuinen and Korhonen, 1969), Syrian hamster and rabbit (Luke and Spicer, 1965) but seems to differ from that of the mouse where only sialidase sensitive sialomucin is synthesised (McCarthy and Reid, 1964a). In certain larger mammals, e.g. cat (Gallagher, Kent, Passatore, Phipps, Richardson and Lamb, 1975) and dog (Spicer, Chakrin, Wardell and Kendrick, 1971) the greater amount of sulphomucin produced more closely resembles the pattern of synthesis seen in goblet cells of human airways (McCarthy and Reid, 1964b; Lamb, 1969; Jones and Reid, 1973a), while in others - e.g. pig - while sialomucins

predominate, sulphomucin also is present in a substantial amount (Jones, Baskerville and Reid, 1975; Baskerville, 1976).

Differences in the type of glycoprotein within individual goblet cells might suggest that these represent phases of a secretory cycle and indeed this has been suggested to be the case in secretory cells of the salivary glands (Ravetto and Bellomi, 1966; Eversole, 1972). In normal rat airway epithelium, however, constant regional differences in the glycoproteins would argue against this. It seems that under normal conditions a goblet cell population secretes a particular range of glycoproteins but that in response to irritation the product may be modified.

Rate of Change in Glycoprotein within a Secretory Cell

The rapid change in the type of glycoprotein implies an even more rapid modification of enzyme syetems within the cell. The present results suggest that by 18 hours (the earliest time studied) there was a shift in glycosyltransferase activity within the goblet cells of the stimulated airway epithelium. This was seen in the extrapulmonary airways as a shift from neutral to acid glycoprotein and, in the intrapulmonary airways, as a shift to include neutral. In the extrapulmonary airways after a single day's exposure to tobacco smoke, and despite a fell in the total number of goblet cells, the relative number of cells synthesising both acid and neutral glycoprotein was increased. Within the lung, where the initial fall in number aseociated with discharge of glycoprotein was

not seen, there was still a relative increase in goblet cells synthesising a mixture of glycoprotein; hence it was the same synthetic enzymes that had been activated. It seems that any secretory cell, no matter its age or position within the airways, responds at this time to stimulation by a similar selection of enzymes. It would thus seem that there is modification of glycoprotein in both new and existing goblet cells.

By 48 hours, since relatively more goblet cells now synthesise only acid glycoprotein, it seems that there has been a further shift. In the extrapulmonary airways, where the total goblet cell number was increased, this shift appeared to take place in newly formed secretory cells whereas in the intrapulmonary airways, where the goblet cell number was not increased, it was within existing cells. The numbers of goblet cells synthesising only acid glycoprotein at this time now re-established the normal proportion of these cells.

In the period up to 14 days of exposure to tobacco, this 'normal' proportion was not maintained. There followed a phase when further increase in goblet cell number was associated with the reappearance of cells synthesising both acid and neutral forms of glycoprotein - particularly in the intrapulmonary airway epithelium. In the first 2 weeks of exposure to an irritant, there is a complex and fluctuating pattern of change in glycoprotein synthesis within the goblet cell populations. This calls for further

analysis of the pattern and timing of the changes during this period, which will be the subject of a future study.

Development of Predominantly Acid Glycoprotein in Secretory Cells of Airway Epithelium

This study has established the range of glycoproteins that may be present within a single goblet cell in rat airway epithelium. In addition to cells synthesising neutral glycoprotein, there are five goblet cell types, as based on their intracellular granules of acid glycoproteins. A cell may synthesise a single type or a mixture of several, in any of the combinations summarised below:

	Goblet Cell Type				
	1	2	3	4	5
Sialidase sensitive sialomucin	+	+			
Sialidase resistant sialomucin		+	+	+	
Sulphomucin				+	+

This resembles the intracellular pattern of acid glycoproteins described by Lamb and Reid, (1969b) within the mucous cells of the

human bronchial gland; but such a range identified within goblet cell populations has not previously been reported.

Ultimately within goblet cell populations in rat airway epithelium there is, in response to irritation, the preferential synthesis of acid glycoprotein. While in some airway regions this represents a shift in enzyme activity, in others it means that the normal pattern is maintained. The former applies mainly to goblet cells of the extrapulmonary airway epithelium and the latter, in general, to those of the intrapulmonary airways. Within the lung, however, in the lower axial pathway in the normal airway where the goblet cell population is synthesising a neutral glycoprotein, there also is a shift.

In the rat tracheal gland also it is acid glycoprotein - at this site the normal secretion - that is produced in hypertrophy. These findings in the present study confirm earlier reports of the synthesis of this type of glycoprotein both in the normal gland and in the gland hypertrophied in response to irritation (McCarthy and Reid, 1964a; Lamb and Reid, 1968).

In the goblet cells of rat airway, synthesis of acid glycoprotein results from activation of sialyltransferases leading to the production of sialomucin resistant to sialidase. Within the goblet cells the production of the predominent type of acid glycoprotein is thus unchanged in response to irritation. In the gland the enzymes responsible for the addition of sulphate are maintained.

In the case of the goblet cells, this finding differs from that of Lamb and Reid (1968). In their study of goblet cell hyperplasia in rat airway epithelium irritated by sulphur dioxide, there was a shift in glycoprotein production - to a sulphomucin. The glycoprotein was demonstrated by uptake of ^{35}S-sodium labelled sulphate rather than by special stains. While it may be that sulphomucin is synthesised in rat airway goblet cells stimulated by this irritant, an alternative explanation could be that uptake of this marker is less specific to identify glycoprotein than has been thought. In the human lacrimal gland, 'type-A' cells which failed to stain for sulphomucin showed positive uptake, evidently due to binding of this label to lysozyme (Allen, Wright and Reid, 1972). Furthermore, the differences between the staining reaction for sulphomucin in serous cells of human bronchial gland, together with high uptake of ^{35}S-sodium sulphate (Lamb and Reid, 1970) by most serous cells, may now also perhaps be explained by the presence of the lysozyme that has recently been reported in these cells (Klockars and Reitamo, 1975; Mason and Taylor, 1975).

In a later experimental study, Lamb and Reid, (1969a) used tobacco smoke as the agent to produce goblet cell hyperplasia but, after a 6-week period of exposure, found no shift to sulphomucin in rats exposed to their highest (20 cigarettes/day) and lowest (5 cigarettes/day) levels of tobacco smoke. These two groups of exposed animals and their control group were each from the same stock, and were of a similar age and weight range to those used in

the present study. In older animals (exposed to 10 cigarettes/day) that were from another batch of animals - obtained from the same supplier but on a separate occasion - evidence of a shift to sulphomucin was found. The relatively high numbers of goblet cells in the control group of this batch of animals would suggest that the difference may have been an age-related effect and the likelihood of infection in older animals. No quantitative results were reported for the goblet cell glycoproteins in this study.

Other studies have similarly reported the synthesis of the normal - and acid - glycoprotein in response to stimulation of airway epithelium where goblet cell hyperplasia was produced. In the dog, when the normal epithelium has been more damaged so that a transitional or even squamous epithelium has developed after exposure to sulphur dioxide, while their content of glycoprotein was reduced the vestigial goblet cells maintained their production of sulphomucin, as was normal (Spicer, Chakrin and Wardell, 1974). In pig bronchial epithelium, where goblet cell hyperplasia has been induced by administration of isoprenaline, Baskerville (1976) also has reported continuation of the normal synthesis of an acid glycoprotein - mainly a sialomucin resistant to sialidase.

Evidence from the present study, combined with that from others, suggests that within airway epithelium any neutral glycoprotein within a goblet cell population will be modified to an acid after irritation and that it is likely that the usual type of acid

glycoprotein will be produced - be it sialomucin or sulphomucin. The contrasting findings of the study by Lamb and Reid (1968) indicate that in response to irritation change in goblet cell glycoprotein in rat airways cannot always be predicted with regard to the type of acid group.

Secretory Cell Glycosyltransferase Activity

It is not known how secretory cells change the nature of the synthesis of their glycoproteins in response to irritation.

The polypeptide chain, first formed on the ribosomes, passes into the cisternae of the rough endoplasmic reticulum and migrates to the Golgi lamellae where the addition of sugars is sequential and is catalysed by the appropriate enzyme (Spiro and Spiro, 1966; Schacter, 1974). The addition of each sugar determines subsequent additions to the side chains. Since the formation of the polypeptide is under genetic control, it is unlikely that it is this part of the molecule that is altered in response to irritation. While the enzyme complement within the cell is also genetically determined, because of the variety of enzymes present and the assembly of the oligosaccharide chins, it seems that a change in glycosyltransferase activity in response to irritation will modify the end-product of the cell.

Since modification of the glycoprotein results from changes in the oligosaccharide chains, a change probably occurs at the Golgi membrane since it is here that most of the sugars are added to the polypeptide chain - galactose (Neutra and Leblond, 1966; Whur, Herscovics and Leblond, 1969), glucosamine (Droz, 1966), fucose (Bennett and Leblond, 1970) and sialic acid (Lawford and Schacter, 1966) - as is sulphate (Lane, Caro, Otero-Vilardebo and Godman, 1964; Berg and Young, 1971). An increase in the rate of synthesis and transport of the polypeptide through the Golgi has been suggested to lead to modification of glycosylation (Spiro, 1966).

An increase in glycoprotein synthesis within secretory cells has been associated with increase in enzyme activity. Baker, Chakrin, Sawyer, Munro and Hillegrass (1973) produced goblet cell hyperplasia and gland hypertrophy in the airways of beagle dogs exposed to sulphur dioxide and reported an increase in the glycosyltransferase activity in microsomal fractions prepared from respiratory tract tissue. The increase was mainly in sialyltransferase and galactosyltransferase activity. Since fucose and sialic acid are both terminal sugars, it may be that there is competition for their attachment to the glycoprotein structure. Increase in sialyltransferase activity would favour a sialomucin at the expense of fucomucin (neutral glycoprotein) and possibly lead to the modification of airway goblet cell glycoproteins seen in the present study in response to irritation.

In the case of the hypertrophied rat tracheal gland, continued sulphomucin production is perhaps secondary to an increase in galactosyltransferase activity. It has been suggested that the addition of more galactose molecules leads to increase in the number of sites available for sulphation (Degand, Roussel, Lamblin and Havez, 1973).

Location of Intracellular Granules

The present study has shown that within a goblet cell in either normal or stimulated airway epithelium, there is a regional difference in the location of granules of different glycoproteins. In cells containing granules of neutral and acid glycoprotein, the acid is found at the apex. In the case of the goblet cell containing a large amount of secretory product, two compartments that are both acid may sometimes be distinguished. In this instance, the resistant form of sialomucin is found near the cell apex, the sensitive near the nucleus. On occasion, the two acid compartments may contain sialidase resistant sialomucin and sulphomucin when it is the sulphomucin that is confined to the cell apex. Here also, the two compartments containing these acid glycoproteins are present in cells of both the normal and stimulated airway. The number of cells with these two compartments of granules, however, is greater in the latter and mostly found after the longest period of exposure to an irritant that has been studied - that of 6 weeks.

From histochemical analysis there is apparently an orderly transition within the cell between populations of granules. This transition of granules is less well defined in secretory cells identified by the electron microscope. Granules of any density that is, either electron-dense or electron-lucent - may be seen at any position within a cell and each type of granule may vary in size.

The basis of the location of these populations is not clear. It may be that different regions of the Golgi complex produce populations of granules containing different glycoproteins; the region nearer to the apex of the cell producing the acid, and that more central the neutral. It is perhaps more likely that enzymes present on the granule membrane continue to modify its secretory product during migration to the cell apex. Since the granule membrane is derived largely from the Golgi lamellae (Jamieson and Palade, 1967, 1971), it could be expected to have a similar complement of enzymes.

It would seem that there is a morphological basis to support the concept that, in response to a stimulus, differences in the granule population are induced within airway secretory cells. Transitional forms of granules - that is, granules with an electron-dense core and electron-lucent halo - have been described in an ultrastructural study of the secretory cells of rat airway epithelium (Jeffery and Reid, 1975). Cells containing these

granules are found at all airway levels. Within a cell, granules of homogenous density - either electron-dense or lucent - are present in addition to those of the transitional type. The number of cells containing transitional granules is increased in stimulated airway epithelium (Jeffery, 1973). Furthermore, there is an increase in the confluence of the electron-lucent granules towards the apex of the cell, suggesting that there is some further modification to their product.

The Effect of Phenylmethyloxadiazole (PMO)

A consistent finding has been that, throughout the airways, the addition of PMO to the tobacco smoke does not prevent the modification to glycoprotein that occurs in the goblet cells. Further discussion of other aspects of the effect of PMO is included later in this Chapter (vide infra).

...../Text continues p. 335

MODIFICATION OF EPITHELIAL GLYCOPROTEINS - OTHER EXAMPLES

In normal and physiological conditions in different organs, a change in intracellular glycoprotein in secretory cells is seen - as in fetal growth, as part of the reproductive cycle, in response to various stimuli and in human disease.

Fetal Epithelia

During embryological and prenatal development and maturation, there is a change in the type of intracellular glycoprotein in human airway epithelium. In the goblet cells of the fetus, both neutral and acid glycoprotein are synthesised while in the adult almost all cells synthesise only the acid form (de Haller, 1969). In the tracheal gland of the fetus, the glycoprotein is sulphated and sialomucin appears only after birth. At first, only sialomucin resistant to sialidase is produced with the sensitive form appearing later during childhood (Lamb and Reid, 1972b). The functional implications are by no means clear. It may be that sulphomucin is less viscid than sialomucin. A sulphomucin without any sialic acid is rarely found in epithelial sites but a recent study of plaice skin mucus which produces sulphated glycoprotein (Fletcher, Jones and Reid, 1976) shows that it has very low viscosity (Lopez-Vidriero, Bhaskar, Jones, Fletcher and Reid, 1976). It may be that the glands in the fetus produce a secretion of low viscosity at a time when the airway is not patent and yet the cells are active (Lamb and Reid, 1972b). The production of only sulphated glycoprotein in the fetal gland

does not reflect a general lack of sialyltransferase activity since other sites are known to produce sialomucins, e.g. submandibular gland (see Lamb and Reid, 1972b). Indeed at another site, human fetal gut, the glycoprotein is mainly sialomucin with only a small amount of sulphomucin present (Lev, 1968) and is the reverse of the pattern of synthesis in goblet cells of the normal adult large intestine (Filipe and Branfoot, 1974).

The Effect of Hormones

Another shift in glycoprotein synthesis that can be considered normal is that produced in certain tissues by hormones. In female mouth-breeding Cichlid fish (Tilapia mossambici), there is both increase in goblet cell number and change in glycoprotein synthesis as part of the normal functional change (Varute and Jirge, 1971). Cyclic increase in goblet cell number within the oral mucosa occurs early in the breeding season and is accompanied by a change from neutral to acid glycoprotein. Both sialomucin and sulphomucin are synthesised and there is an increase in both the size of the goblet cells and their staining intensity. The change to acid glycoprotein and its type is linked closely to the gestation cycle. At the onset of the breeding season, goblet cells synthesising sialomucins increase considerably while sulphomucin first appears in only a few cells. With the laying of eggs and oral gestation, the concentration of cells containing either type of acidic glycoproteins increases. Here, regression in cell number and glycoprotein type is also the pattern of the

change in the post-breeding season.

Hormonal control of glycoprotein synthesis is also represented by sexual dimorphism of the submaxillary gland in the Syrian hamster (Shackleford and Klapper, 1962). Before sexual maturity the sialic acid content of the gland is similar in male and female animals but after six weeks of age this changes. In the now adult male the amount of sialic acid per gm of gland, assessed by the Erhlich reaction, is less than in the female. Furthermore, in ovariectomised females the amount resembles the male value while in oestradial-treated males it is increased. These changes may also be detected histochemically by the intensity of Alcian Blue staining of tissue.

Other changes in acidic glycoproteins induced by sex hormones have been described. In mucus from the cow cervix (Gibbons, 1959) and in mucus taken from rat vagina during pregnancy (Warren and Spicer, 1961) biochemical analysis has shown a change in glycoproteins, with an increase in sialic acid. The latter study also showed that oestradial given to pre-ovulatory mice caused a reduction in sialomucin which could be restored by administration of progesterone - thus reproducing the pattern of the normal cycle.

More recently, Huber and his colleagues have shown that in normal rat tracheal epithelium there are more goblet cells in female than in male animals (Hayashi, Phelps and Huber, 1975a & b; Huber, 1976 - personal communication). The increase in goblet

cell number in the female rat above that of the male was found to be significantly greater in proestrus and oestrus. In dioestrus, while a trend was apparent, the increase was not significant. They further reported that at each stage of oestrus significantly more tracheal goblet cells synthesised neutral glycoprotein than in male animals; in proestrus the number of cells synthesising acid glycoprotein was significantly decreased. These studies suggest that hormonal control of mucous cell number and glycoprotein type , typical of secretory cell populations in the female mammalian reproductive tract (see Brenner and West, 1975) can also influence in a similar manner secretory cell populations of airway epithelium.

The effect of an irritant on the type of glycoprotein produced by tracheal goblet cells has also been studied in rats of different sex (Hayashi, Sornberger, Phelps and Huber, 1976). In both male and female animals exposure to tobacco smoke produced a relative increase in cells synthesising acid glycoprotein although the increase was greater in the females than in the males.

These findings confirm an earlier report of the stimulatory effect of sex hormones on the bronchial tree by El-Heneidy, Helmy and Michael (1966). In their experimental study, 5 mg of ethinyl oestradiol given daily to guinea-pigs produced a substantial increase in goblet cell number in bronchial epithelium. After oestradiol goblet cells were hypertrophied and from their photomicrographs it is apparent that there was an increase in the

intracellular volume of secretory product, but they did not analyse the type of glycoprotein synthesised.

The Effect of Nerve Stimulation and Neuromimetic Drugs

Chemical analysis of secretion from the salivary gland has shown that the secretory product can be influenced by the manner and type of stimulation.

In the cat, stimulation of the cervical sympathetic or parasympathatic nerves (chordalingual) has been reported to produce differences in glycoprotein from the submaxillary gland (Dische, Kahn, Rothschild, Danilchenko, Licking and Wang, 1970). Ratios of neuraminic acid to fucose and of neuraminic acid to hexosamine were greater in saliva produced by sympathetic than by parasympathetic nerve stimulation. The change was not due to differences in the volume of secretion in response to each type of stimulation.

From the same site - in the dog - different dose levels of pilocarpine also produced glycoproteins in which the amount of sugars differed (Dische, Pallavicini, Kawasaki, Smirnow, Cizek and Chien, 1962). Johnson and Winzler (1972) have reported a difference in the composition of glycoproteins from the dog submaxillary gland after electrical stimulation (no further details were given), or to administration of pilocarpine. They identified two different glycoprotein components recovered from

the collected secretion. In each, the amino acid fractions were identical but the oligosaccharides differed. Both included fucose, galactose and N-acetylgalactosamine while, in addition, one included N-acetylneuraminic acid and the other N-acetylglucosamine -4-sulphate.

Since each of these studies was by chemical analysis of the secretory product, the authors were not able to establish the cell of origin. Histochemically, a wide range of glycoproteins has been identified in secretory cells of salivary glands. In dog submaxillary gland, as in the airways, the product of the mucous cells is not uniform. Of the mucous cells most secrete both sialomucin and sulphomucin (75% of the gland volume) and some sialomucin alone (25%), while serous cells secrete only sialomucin (Jones and Reid, 1973a; Jones, 1971). Histochemical analysis of cat submaxillary gland glycoproteins also has shown that different cells of similar populations may produce different secretory products (Harrison, 1974).

Within the salivary gland, the changes in glycoprotein in response to stimuli via nervous pathways or by drug effect may represent a difference in the proportion of a glycoprotein secreted by different cell types, the implication being that a different cell population responds directly to a different neural stimulus. Or, as the result of response to the various stimuli, there may have been modification of the intracellular secretory product of all cells. The early study of Rawlinson (1933) has suggested

the former is the case since the mucous acini were reported to be under parasympathetic and serous acini under sympathetic control: stimulation of the given nerve produced a loss of intracellular content and vacuolisation of the appropriate cells. Histochemical evidence supports the idea that the change in salivary gland produced in response to stimulation via nervous pathways results from release of different glycoproteins from different cells rather than any modification of intracellular secretion.

The influence of neuromimetic drugs on secretory cells of airway epithelium has also been reported and here a different pattern seems to emerge. In rat airway epithelium, stimulation by pilocarpine has been shown, by electron microscopy, to produce exhaustion of the secretory cells while isoprenaline increased their number (Jeffery, 1973). Sturgess and Reid (1973) reported a difference in the type of glycoprotein produced after each of these stimuli. After pilocarpine the number of cells producing neutral or acid glycoprotein was each increased while isoprenaline preferentially increased the number of acid-secreting cells.

The fetal changes doubtless represent a stage in maturation with cells programmed to activate different enzyme systems at various stages in growth. The development of the adult pattern of glycoprotein synthesis takes a matter of months. Lability of the enzyme system is demonstrated by cyclic hormonal control. These changes may be seen over a period of days to weeks and may

represent the induction of new enzyme systems or activation of those present within existing cells. Both the fetal and hormonal changes lead to modification of glycoproteins: this would seem to be adaptive and their change can be predicted. On the other hand, nervous stimulation produces differences in glycoprotein over a short period - minutes to hours - but the change has not been directly assessed as the result of intracellular modification to the glycoprotein. The influence of neuromimetic drugs on airway epithelial cells will be the subject of a future extended study.

Changes in Disease

In disease, intracellular change in goblet cell glycoprotein often occurs and it seems that its direction may sometimes be predicted. For example, in carcinoma of the large intestine, it is sialomucin that increases at the expense of sulphomucin (Filipe and Branfoot, 1974). In the transitional mucosa adjacent to the carcinoma, where crypt size may be double that of the normal, the goblet cells are hypertrophied, distended with secretion, and the regional difference of the normal crypt glycoproteins is reversed. Sialomucin is now synthesised in cells in the lower half of the crypt: there may be either an abrupt or gradual change to sulphomucin towards the top of the villus. Similarly,

in human gall bladder disease such as adenocarcinoma, cholecystitis and in cystic fibrosis, the normal sulphomucin of the goblet cells is replaced by sialomucins and even by neutral glycoprotein (Esterly and Spicer, 1968).

Within human airway epithelium, it is well established that, in chronic bronchitis,hypertrophy of the submucosal gland is accompanied by modification of mucous cell glycoproteins (de Haller and Reid, 1965; Reid, 1967; Lamb, 1968). There is a reduction in the number of mucous cells producing sialidase sensitive sialomucin and an increase in those producing the resistant form. Thus, within the gland where a range of glycoproteins normally is produced, including each acid type and even neutral glycoprotein, there is a pattern of shift that can be predicted. In cystic fibrosis similar changes in glycoprotein are also produced by the submucosal gland where hypertrophy is associated with recurrent periods of infection (Lamb and Reid, 1972a). There have been fewer reports of change in the glycoprotein produced by the goblet cell population within airway epithelium in disease. In the normal human airway, the goblet cells may produce the same range of glycoproteins as mucous cells of the submucosal glands, although many goblet cells produce an acid glycoprotein that is sulphomucin or sialomucin resistant to sialidase. In chronic bronchitis, similar glycoproteins are produced in an increased secretory cell population (Lamb, 1969), although there is a loss of cells containing neutral glycoprotein

and sialidase sensitive sialomucin, with a corresponding increase in the resistant form. More cells show an increase in their intensity of staining with the high iron diamine stain, suggesting increased sulphation.

The timing of the changes in glycoprotein that occur, in disease, in secretory cell populations cannot be assessed. The present studies have aimed to show the pattern of change in airway goblet cells in an experimental situation that - in terms of mucus hypersecretion - is the counterpart of that seen in human chronic bronchitis.

The pattern of change in the animal model may, perhaps, resemble that of the human situation.

...../Text continues p. 345

INCREASE IN MUCUS-SECRETING TISSUE IN RESPONSE TO IRRITATION

The clinical evidence of mucus hypersecretion is sputum production. In hypersecretory states, there is an increase in goblet cell number and gland size - changes that have been produced in the present study.

Change in Epithelial Cell Population - Increase in Secretory Cells

Increase in goblet cell number has been found at an earlier time than previously reported in airway epithelium exposed to tobacco smoke - within 48 hours - in the extrapulmonary airway epithelium. The pattern of increase in goblet cells was found to differ between extrapulmonary and intrapulmonary airway regions. The maximum increase in goblet cell number took longer in the latter but, even here, the increase occurred earlier than previously shown.

A dose-related increase in secretory cell number has previously been described when the daily dose of tobacco smoke administered to rats was different for the three groups of animals, 5, 10 or 20 cigarettes a day being administered over a period of 6 weeks (Lamb and Reid, 1969a). In the present experiment, while the animals had the same daily dose of 25 cigarettes, because of different duration - up to 2 and 6 weeks - the total dose was different. The effect at 2 weeks was the same as at 6 weeks. A cumulative dose effect was seen only during the first 2 weeks.

Regional variation

It is perhaps to be expected that, after exposure to an irritant, increase in goblet cell number is not uniform throughout the airway epithelium. As judged by number, however, there was a striking difference between the increase in goblet cells in different regions of the airways.

Proximal airways After irritation, the greateet numbers of goblet cells were present in the same region as in the normal - that is, in the lower trachea, main bronchi and in the proximal region of the lung. This may simply reflect a higher dose of smoke to the more proximal airways.

It seems that the carinae are particularly susceptible to goblet cell increase. It is considered that it is the particulate matter in tobacco smoke rather than its gas phase that produces the irritative effect on airway epithelium (Falk, Tremer and Kotin, 1959). Deposition of smoke products occurs within airways (Hilding, 1956; Mellors, 1958) but, more particularly, at the carina and bronchial bifurcations where there are regions of constriction (Ermala and Holsti, 1955; Sato, Suzuki and Fukuyama, 1962). At this site smoke may strike the walls of the airways and the increase in turbulence lead to an increase in deposition of the particulate matter. Hilding (1963) also has suggested that it is at the carina that the upward flow of the ciliary stream concentrates particulate matter deposited from tobacco smoke.

In some epithelial regions the increase in goblet cell number exceeded that of other regions that, in the normal airway, contained a similar number of goblet cells. For example, the increase in the epithelium of the third lateral pathway exceeded that in the axial pathway at the same level.

A similar effect has been found in other studies. With increase in the number of goblet cells in airway epithelium after systemic administration of isoprenaline (Sturgess and Reid, 1973), the increase is greatest in the same airway region as the response to tobacco (personal observation - unpublished results). Thus, it would seem unlikely that in these regions the greater increase arises only from the local dose of smoke; more probably it reflects an intrinsic property of the airway epithelium. Despite the stimulus by which the increase in goblet cell number comes about, it would seem that the increase will be greater in particular regions of the airways.

Peripheral airways Both Reid (1960) and Lamb and Reid (1968) have reported an extension of goblet cells into the smallest airways within the lung (i.e. bronchioli < 0.4 mm) after exposure of rats to sulphur dioxide. Such an extension has not been found in the present study, nor was it reported in a later study by Lamb and Reid (1969) where, similarly, tobacco smoke was the irritant used. In the later study they found the number of goblet cells to be increased in the peripheral region of the axial pathway, and a similar response has been found in the present study. The smaller

dose of smoke to the peripheral bronchioli epithelium is probably responsible for absence of goblet cell metaplasia in this region. In rats from the same experiments as those used in the present study, Jeffery (1973) did not find any increase in secretory cells in peripheral bronchioli, identified either from 1 μm toluidine blue stained sections or by electron microscopy. In other animals, he reported that they were present after administration of isoprenaline. From the results of the study of Lamb and Reid (1968) and other studies (Mawdesley-Thomas and Healey, 1969; Chakrin and Saunders, 1974) it would seem that the penetrating and irritant effect of sulphur dioxide in the peripheral airway regions is greater than that of tobacco smoke - at least at the dose levels of tobacco smoke that are used in studies within our Department.

Rate of increase

At each airway level affected, there was a burst of increase in the goblet cell number at some time during the early period of exposure to tobacco smoke. The time of this response was not the same in each airway region. In extrapulmonary airways this early rate of increase in the goblet cell number was greater than at any other airway level. It was most marked in the upper trachea and mid-tracheal region and took place between the first and second days of exposure. In the lower trachea, the rate of increase was less striking, but it was more sustained, in that it was similar between the first and second and the second and third days of exposure.

In the intrapulmonary airwaye the fastest rate was seen earlier, after the firet day, and was greater in the lower than in the upper axial pathways. The delayed response in the trachea, as compared with that in the axial pathway, may reflect the discharge effect on the extrapulmonary epithelium. The elowest response occurred in the lateral pathways and could be the result of a delay in given dose which ie perhaps necessary to achieve the faatest rate. Having achieved this, the increase in goblet cell number might then be at a elower rate until a plateau is achieved at two weeks.

Secretory cell differentiation

Many studies, including the present, have reported increase in goblet cell number within airway epithelium after expoeure to an irritant. The mechanism by which regions of epithelium with relatively few goblet cells develop goblet cell metaplasia in reeponse to irritation is not known. A poseible role for Vitamin A in epithelial cell differentiation and maturation wae firet suggested by the claseic experiments of Fell and Mellanby (1953). They showed that in cultures of chick ectoderm, excess of Vitamin A produced mucous cell metaplasia. After transfer to a normal tissue culture medium, the basal celle of the transformed epithelium then ceased to form new secretory cells and differentiated into a squamoue keratinising epithelium beneath the secretory cell layer, until eventually the secretory and ciliated cells were shed. Other studies have confirmed thie

effect of Vitamin A, in vivo, in hamster cheek pouch (Lawrence and Bern, 1963) and have shown, in rat trachea, that its deficiency gives rise to a squamous rather than mucus-secreting epithelium. This has been shown in studies both in vivo (Marchok, Cone and Nettesheim, 1975) and in vitro (Wong and Buck, 1971). While there was some de-differentiation of superficial cells, as in the studies of Fell and Mellanby (1953), most of the effect was produced by basal cell hyperplasia and sloughing of degenerating superficial cells. While the effect of Vitamin A is thus established for the basal cell population of the extra-pulmonary airways, its effect on cell populations within the lung has not been reported.

De Luca and Wolf (1972), studying the effect of Vitamin A (retinol) on the development of the secretory product of goblet cells in rat small intestine, suggested that it acted as a carrier of monosaccharides during glycoprotein synthesis and thus favoured conditions for glycoprotein production within the cell.

Other factors are known to affect the development of a secretory cell population. The effect of hormones on mucus cell differentiation has already been described. Hilding (1932) has demonstrated that air flow across an epithelial surface may influence the differentiation of its cells. Reduction of air movement across the surface epithelium in rabbit nares on one side, and thus doubling it on the other, produced epithelial changes. While squamous epithelium developed in the open nares

the epithelium of the closed side became ciliated, columnar and contained many goblet cells. In vitro studies also have shown that high levels of carbon dioxide favour differentiation to ciliated mucus-producing cells (Moscona, 1965). The contribution of any of these factors, however, to goblet cell metaplasia produced in response to irritation can only be speculative.

The Effect of Irritation on Epithelial Cell Mitosis and Differentiation

The basal cell has, in general, been considered the source of new goblet cells in normal airway epithelium (Miller, 1947; Blenkinsopp, 1967) although Miller (1947), Rhodin (1966) and Bindreiter, Schuppler and Stockinger (1968) suggested that differentiation into the ciliated or goblet cell occurred via the intermediate cell.

More recently, a study of the frequency of cells in mitosis in normal rat airway has emphasised that the pathway through which a new goblet cell may arise differs in the extrapulmonary and intrapulmonary airways (Bolduc and Reid, 1976a). Because of the frequency of the basal cell in the large airways, these can be considered the stem cell compartment: it is in this cell population that cells in mitosis in the normal airway are found. In the distal airways, where the basal cells comprise only 1% of the total epithelial cell population (Jeffery and Reid, 1975), the epithelial cells form a single layer that has the functions both

of division and differentiation (Bolduc and Reid, 1976a). In the intrapulmonary airways it is not known whether the normal renewal of cells such as the ciliated or secretory types is by direct division or via the intermediate cell.

The study of Jeffery (1973) of epithelial cell types and their frequency, in both the normal and stimulated airway, has shown that, in response to stimulation, new secretory cells may indeed be derived by conversion of an intermediate cell and that within the secretory cell population there is even a pattern of cell conversion.

Interpretation of findings in stimulated airway in the present study

Comment In this section of the Discussion, the term goblet cell refers to the context of the present study, and refers to secretory cells identified by light microscopy. Within this cell population three cell types have been identified ultrastructurally (see p.33): these are termed here the 'serous' cell or 'Clara' cell (those containing electron-dense granules) and the 'goblet' cell (which contains electron-lucent granules), to distinguish specific cell types.

Extrapulmonary airways In the extrapulmonar airways, it would seem that the early increase in goblet cell number (48 - 72 hrs) could arise in three ways - (i) by refilling of the cells that

discharged on the first day after initial exposure to tobacco smoke, (ii) by conversion of cells already present within the superficial layer, or (iii) from daughter cells of the basal layer of the epithelium.

(i) Refilling would take the goblet cell number back to the control value but would not account for the increase above it. Kanda, Mayfield and Ghidoni (1968) have reported the process and time taken to refill secretory cells with granules following their exhaustion from the cell. One hour after administration of isoprenaline to rats, the secretory cells of the submaxillary gland were exhausted of secretion. After four hours, the cytoplasm was packed with rough endoplasmic reticulum and a few secretory granules were found adjacent to the Golgi complex. Granules had accumulated in the cytoplasm by 12 hours, where their number increased between 24 and 36 hours. While no similar detailed study of granule discharge and re-accumulation has been made in secretory cells of airway epithelium, it seems likely that a similar process would occur, and in a similar period of time. That emptying and refilling does occur during the first 36 hours of exposure to tobacco smoke is substantiated by the finding, in animals from the same experiment, of an increase in the relative number of non-secretory cells without secretory granules on the day of discharge (24 hours), with a reduction in this cell type (after 36 hours) on the following day (Jeffery, 1973).

(ii) In the extrapulmonary airway epithelium, there may be conversion of the non-secretory intermediate cell to a secretory cell; or of one secretory cell type - the serous cell - to another - the 'goblet'. Conversion of intermediate cells would certainly contribute to the increase in goblet cell number seen by light microscopy. Increase in the number of secretory granules within serous cells that previously contained but a few, might introduce new secretory cells not previously identified by light microscopy. Only then would the second pathway of conversion contribute to the overall increase in goblet cell number - the transformation of serous to 'goblet', otherwise merely representing a shift within the goblet cell population (from those cells staining with PAS to those staining with AB respectively).

The contribution to increase in goblet cell number through conversion of existing superficial cells is difficult to assess in terms of number, because of an apparent increase made to cells within the superficial layer by daughter basal cells (vide infra). It would seem, however, that the conversion of a goblet cell from a 'labile' precursor cell already present within the superficial cell layer of the airway epithelium, might be faster than from a basal cell.

(iii) By the second day, when the goblet cell number in the extrapulmonary airway epithelium is well above the control value, and the mean cell concentration is known to be increased (Jeffery, 1973), it may be suggested that the wave of basal cells in mitosis

seen on the previous day (Bolduc and Reid, 1976b) has already contributed daughter basal cells to the superficial cell layer. Although refilling and conversion of the existing cells contribute, it seems they do not account for the total increase in goblet cell number.

Although new cells evidently are present within the superficial layer of the airway epithelium as early as 48 hours, the timing or pattern of their differentiation is not known - neither for the normal or stimulated airway epithelium.

The study of Lane and Gordon (1974) suggests that it takes between 3 - 4 days for the differentiation of a goblet cell from a basal cell. They removed cells of the epithelial surface in rat airway by lightly stroking its surface with a probe. Basal cells mostly were left intact, and the pattern of cell replacement within the epithelium was followed by electron micrsocopy. Forty-four hours after injury, some cells were columnar and had a well-developed Golgi complex but they contained only a few or no secretory granules. Granules were first found in any number at 60 hours but only by 90 hours were typical goblet cells seen.

The study of Shorter, Titus and Divertie (1966) was concerned only with migration of labelled cells from the basal to the superficial layer. They reported a time of at least 5 days. Each of these times is faster than those reported in experiments in

which the basal cell layer has been removed and 12 - 14 days are required for goblet cell formation (Wilhelm, 1953; Greenberg and Willms, 1962).

Evidently within the extrapulmonary airwey epithelium there is acceleration of the time of mitosis of basal cells and differentiation of the daughter cells which, with conversion of cells, together leed to the increese in goblet cell number seen in the present study. Nevertheless, it appears that the 'normal' pattern is maintained in that it is through mitosis of basel cells that most new cells within the epithelium arise. Secretory cells already present within the airway epithelium - even a 'goblet' cell - may divide but this pathway is uncommon. Secretory cells in mitosis are rarely seen, even in the stimulated airway epithelium when their number is greater than in the normal (Jeffery, 1973).

Intrapulmonary eirways Within the intrapulmonery airway epithelium, eventual increase in the goblet cell number after irritation arises in e similar manner from (i) cell conversion, or (ii) mitosis. In each case, however, there are differences from the pattern of the extrapulmonary airway epithelium.

(i) In eddition to the pethways of cell conversion found in extrapulmonery eirway epithelium, in the intrapulmonary airways there may be the transformetion of the Clara cell to the serous end, perhaps, eventuelly to the goblet (Jeffery, 1973). In the present

study, such a pattern of change within the goblet cell population of the lower axial pathway would seem most likely.

(ii) A wave of cells in mitosis after 24 hours has also been reported for the intrapulmonary airways, although its extent was less marked (Bolduc and Reid, 1976b). There is thus a possible contribution to goblet cell increase from new cells, but it is not known which cells divide within the intrapulmonary airway epithelium in response to irritation, whether all cell types enter mitosis equally or some preferentially. Again, it would seem probable that the conversion of cell types within the existing cell population would precede any increase in the goblet cell number through mitosis - except perhaps by increase from the mitosis of goblet cells.

While other studies have provided much information of the possible pathways through which goblet cell increase may arise, further information is still needed. A major problem has been the inability to identify cell types within the epithelial surface because of the thickness of the tissue section. Most previous studies have mentioned only the position of dividing cells within the epithelium. The use of autoradiography with labelled

1 um sections is required to identify individual cell types. By such techniques 4 cell types - basal, ciliated, goblet and intermediate - have been identified by incorporation of ^{3}H-5-uridine (into RNA), the remaining cells being grouped together and classed as "unidentified" (Kaufman, Baker, Harris, Smith, Boren, Sporn and Saffiotti, 1972). Even these methods do not identify the 8 epithelial cell types now known to be present within rat airway epithelium but their future use will undoubtedly add further information to the timing and cell pathway pattern of goblet cell formation.

Irritation - its Effect on Synthesis and Discharge from Secretory Cells

The secretory cells of the airway epithelium differ in the type of glycoprotein synthesised but the amount of secretion within the cell at any one time represents a balance between synthesis and discharge. In the normal population the balance is similar, most cells containing a small or moderate amount of secretory product near the apex. Exposure to tobacco smoke, after 6 weeks, causes a striking increase in secretory product within the cell. Such cells contain a large amount of secretory product as compared with the normal. In the early period of exposure to tobacco, however, large cells are not seen. If judged by the amount of retained secretion, the balance between synthesis and discharge appears similar to the normal although it is known that at this time that an increase in secretory cell rate from the cell occurs (Coles and Reid, 1976). The later increase in cells containing

a large amount of secretion suggests either that the balance remains the same but that synthesis and discharge have each increased, or it may be that synthesis now exceeds discharge. Organ culture studies will be needed to establish which of these two processes brings about the new balance in the amount of intracellular secretion within the goblet cells.

In both rat and pig airway epithelium, the rapid increase in goblet cell number, after 6 days' administration of isoprenaline, is accompanied by an increase in amount of intracellular secretion in many cells (Sturgess and Reid, 1973; Baskerville, 1976). Other in vitro studies have shown that isoprenaline does not cause any increase in the secretory rate of mucous cells of the human bronchial gland (Sturgess and Reid, 1972). Evidently the balance of synthesis and discharge is altered within the goblet cells by this β-adrenergic drug, so that either synthesis exceeds discharge or there is retention of secretion within the cells. Here the balance of synthesis and discharge within the goblet cells is quite different from the balance found in response to irritation at a similar time.

Structural studies have shown how secretory cells may discharge their product. Goblet cells show apocrine secretion. In mucous cells of the rat sublingual gland, Kim, Nasjleti and Han (1972) have described the process by which granules fuse and then, perhaps by increase in volume, rupture the cell membrane. By contrast, serous cells discharge their product by merocrine secretion, in which the membrane of individual granules fuses with the cell membrane and an opening forms through which the content of the granule is discharged, as - for example - in pancreas (Ichikawa, 1965) or parotid gland (Amsterdam, Ohad and Schramm, 1969). The mechanisms that control the release of product from secretory cells are not fully understood, although many aspects of the process have been identified (see Douglas, 1968; Rasmussen, 1970; Rubin, 1970). Matthews, Peterson and Williams (1973) have suggested from studies of mouse pancreatic acinar cells that the release of their product involves a series of steps initiated by the action of released acetylcholine acting on specific receptors: there follows an increase in membrane permeability, depolarisation and calcium efflux and the release of secretory product. From studies of rat parotid slices, Schramm and Selinger (1974) also have postulated the control of cell formation of its secretory product and discharge by the action of neurotransmitter substances on cell receptors. Within the airway epithelium it is not known whether a similar process causes release of goblet cell contents. It was suggested in the early study by Florey, Carlton and Wells (1932) that, unlike the submucosal gland, the goblet cells of the airways were not

under direct nervous control and, to date, no studies have suggested otherwise.

In the present study, it was striking that within the first 24 hours tobacco smoke produced overwhelming discharge of goblet cells. Then tolerance seemed to develop to the discharge effect by the second day. One day's rest, however, seemed to abolish the tolerance so that a further overwhelming discharge of cells, within an increased cell population, had occurred.

It seems that in airway epithelium 'tolerance' to a damaging irritant is a well established feature (Fairchild, 1967; Evans, Stephens and Freeman, 1971). For example, at 400 ppm sulphur dioxide produces sufficient damage to epithelial cells to cause shedding after the first period of exposure (Lamb and Reid, 1968). Although the same daily exposures continued, the epithelium then regenerated and differentiated into a 'normal' ciliated columnar mucus-secreting epithelium - with the modification of increase in goblet cell number. The new epithelium is evidently more tolerant of the given dose. An early burst in mitotic activity seen in the first 24 hours of exposure to an irritant is not maintained within the airway epithelium (Kleinerman, 1970; Wells and Lamerton, 1975): despite continuing exposure, the normal level of activity is re-established by about 36 hours. After a day's from tobacco smoke, however, a second wave of mitosis is induced by a single further exposure (Bolduc and Reid, 1976b). In this respect, the pattern of mitosis

and goblet cell discharge are similar. It may be mentioned that only in the extrapulmonary airway epithelium did the discharge effect occur - where the presence of intra-epithelial nerves has been reported (Jeffery and Reid, 1973).

It was also in the regions containing the greatest number of nerves - the upper and mid-trachea - that the greatest discharge effect occurred. The nerves described by Jeffery and Reid (1973) in their study were considered to be both sensory and motor, and were associated not only with basal but also with ciliated and secretory cells.

The Effect of Phenylmethyloxadiazole

In the extrapulmonary airway epithelium, PMO does not protect against the immediate discharge of secretion that occurs after the first exposure to tobacco. Presumably it is through re-filling that by 48 hours the number of goblet cells seen in control animals is virtually regained. In the upper and mid-trachea, however, the number of goblet cells is again reduced 24 hours later when their number now remains below control levels. In the lower trachea the control number is regained, but it would seem that in the extrapulmonary epithelium PMO does prevent the formation of new goblet cells in response to tobacco. The addition of PMO to the tobacco would thus seem to prevent both conversion of the non-secretory population of cells present within the superficial layer to goblet cells, and increase in goblet cell number through

mitosis. Although mitosis is delayed at this time (Bolduc and Reid, 1976b), it is not prevented - indeed, after 48 hours, the number of cells in mitosis exceeds that seen maximally after tobacco alone. Thus, at least at this time, PMO it seems is acting at the level of differentiation or the 'switch-on' of the secretory process within existing and newly formed cells of the extrapulmonary airway epithelium.

Anti-inflammatory agents are known to inhibit glycoprotein synthesis (Musil, Weissova, Adam and Prokopec, 1968; Lukie and Forstner, 1972). It appears that, to some extent, they inhibit polypeptide formation but, more importantly, assembly of the oligosaccharide chains through the incorporation of glucosamine.

A certain number of goblet cells, particularly in the lower trachea, by-pass the suppressing effect on glycoprotein formation and, in these cells, the addition of PMO did not protect against alteration to the oligosaccharide chain. In both the extrapulmonary airways there was mainly increase in sialyltransferase activity leading to the formation of sialidase-resistant sialomucin. A similar glycoprotein was produced within the intrapulmonary airways where the goblet cell number was not suppressed by PMO.

Other studies have shown PMO to have a protective effect on airway epithelium via a systemic route (Dahlgren and Dalhamn, 1972) as well as by inhalation (Dalhamn and Rylander, 1971). In the present study, the greater protective effect of PMO in the proximal

than distal regions of the airways suggests that it may act directly upon the airway epithelium.

In addition to its failure to protect against the discharge produced in response to tobacco after 24 hours, in the lower tracheal epithelium PMO did not protect against the second discharge after 7 days' exposure, where the goblet cell number had regained the control value.

When PMO is added to the tobacco smoke, the amount of retained secretion increases even further. More cells with a large amount of secretion are seen and within a cell the secretory mass tends to be larger. Organ culture studies will be needed to show whether this new balance arises from retention of secretion rather than in even further increase in its synthesis above the level of discharge.

A number of anti-inflammatory agents have been shown to cause an increase in membrane stability. Catenese, Lisciani and

Piccinelli (1969) and Mizushima and Sakai (1969), studying the plasma membrane of erythrocytes, have demonstrated increased resistance to hypotonic haemolysis, and Mizushima, Sakai and Yamaura (1970) have suggested that anti-inflammatory drugs such as indomethacin and phenylbutazone have a stabilising effect on cell membrane proteins. Brown and Tong (1971) have demonstrated that PMO also confers stability on the erythrocyte membrane. In the case of the secretory cell, such an effect either on the cell membrane or that of the secretory granules could be manifest as a slowing in discharge.

The Effect of Irritation on Gland Size and Secretory Product

In addition to increase in goblet cell number in airway epithelium, tobacco smoke has, in these studies, been shown to increase submucosal gland size. In previous experimental studies an increase in gland size has been shown in the rat after exposure to sulphur dioxide (Lamb and Reid, 1968) and in other species after tobacco smoke (Mawdesley-Thomas and Healey, 1973) but this is the first demonstration of gland hypertrophy in the rat after tobacco smoke.

In the hypertrophied rat gland the mucous cells increase both in size and number although no similar change was found for the serous cells. This is similar to the pattern of gland hypertrophy in human chronic bronchitis (de Haller and Reid, 1965). The way in which hypertrophy develops and the mechanisms that

induce and control the degree of change need further study.

Phenylmethyloxadiezole did not protect against the increase in gland size. It may be here also that this enhancement effect of PMO is secondary to its prevention of discharge of secretory product from the cell. Since acinar, lumen and cell size were similar to those found after exposure to tobacco smoke without the addition of PMO, it seems that cell multiplication was greater in the gland after exposure including the anti-inflammatory agent.

The use of animal stock with clean lungs for use in experimental studies cannot be too strongly emphasised. The method of goblet cell counting is tedious but, when goblet cell populations of similar airway levels are compared, quantitative analysis gives precise and significant information. It is hoped that a recently developed and modified technique of image analysis will provide a quicker and equally reliable method for their counting. Image analysis (Optomax - supplied by Micro-Measurements Ltd., Cambridge) techniques, however, will not allow the identification of the different histochemical types of glycoproteins.

The basis of the present study of airway secretory cell response to irritation is an intracellular variety of types of glycoprotein. The same histochemical types can be identified outside the cell, free within the airway lumen (de Haller and Reid, 1965; Reid, 1970b). The application of histochemical staining techniques and quantitative analysis to identify the glycoproteins of airway goblet cells has shown the pattern of change in cell populations and thus the way in which the intracellular secretory product is modified in response to irritation, and given information which could not have been obtained from biochemical analysis of secretion.

The presence of each type of glycoprotein has been identified in sputum; they are not identified singly but as mixtures of the groups in which fucose, sialic acid or sulphate are all present but each in different amounts (Degand, Roussel, Lamblin, Durand and Havez, 1973). Using biochemical techniques, epithelial glycoproteins have been found to have a large molecular weight (Roberts, 1974) and, by density gradient methods, an even large macromolecule has been characterised (Creeth, Bhaskar, Horton, Das, Lopez-Vidriero and Reid, 1976). It is presumably in the airway lumen that this macromolecule is formed, by polymerisation, from the variety of smaller molecules produced by secretory cells.

ACKNOWLEDGEMENTS

I wish to thank Professor Lynne Reid for her guidance, encouragement and help during this study. I am grateful to Dr. Sheila Haworth and to Miss Barbara Meyrick for helpful discussion and to Professor B. Benjamin, formerly Professor of Actuarial Science at the City University, for guidance with the statistical analysis and for the analysis of probability of the hypothetical flow chart system.

I would also like to thank Professor T. Dalhamn of the Karolinska Institute, Stockholm, for providing the cigarettes used in the study, Mr. T. Betts for analysis of the tobacco smoke and Dr. P. Jeffery and Dr. P. Bolduc, colleagues with whom the experiments presented here were shared.

I am grateful to Miss Geneviève Leballeur for the diagrams; to Mrs. Milena Potucek and the staff of the Royal Marsden Hospital Photographic Department for the prints; and to Mr. P. Bishop, Librarian of the Cardiothoracic Institute, and to his staff for helping me to obtain the necessary references.

I particularly wish to thank Mrs. Margaret Mansell for both typing the final copy and photocopying this thesis and for helpful advice during its preparation. In addition, I would like to thank my husband R.A.J. for his help and patience and to extend my personal thanks to Mrs. Margaret Jarman who has spent extra time caring for our active two-year old son.

BIBLIOGRAPHY

The full title and pagination are given for each article.

The full title also is given for each periodical.

ABRAHART, E.N. (1968)
Dyes and their Intermediates. Pergamon Press, London.

ADA, G.L., FRENCH, E.L. & LIND, P.E. (1961)
Purification and properties of neuraminidase from Vibrio cholerae.
Journal of General Microbiology, 24, 409-421.

ALLEN, M.A., WRIGHT, P. & REID, L. (1972)
The human lacrimal gland. A histochemical and organ culture study of the secretory cells.
Archives of Ophthalmology, 88, 493-497.

AMSTERDAM, A., OHAD, I. & SCHRAMM, M. (1969)
Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle.
Journal of Cell Biology, 41, 753-773.

AUERBACH, O., GERE, J.B., FORMAN, J.B., PETRICK, T.G., SMOLIN, H.J., MUESHAM, G.E., KASSOUNY, D.Y. & STOUT, A.P. (1957)
Changes in bronchial epithelium in relation to cigarette smoking and cancer of the lung. A report of progress.
New England Journal of Medicine, 256, 97-104.

AUERBACH, O., HAMMOND, E.C., KIRMAN, D. & GARFINKEL, L. (1970)
Effects of cigarette smoking on dogs. II. Pulmonary neoplasms.
Archives of Environmental Health, 21, 754-768.

AUERBACH, O., HAMMOND, E.C., KIRMAN, D., GARFINKEL, L. & STOUT, A.P. (1967)
Histologic changes in bronchial tubes of cigarette smoking dogs.
Cancer, 20, 2055-2066.

AUERBACH, O., PETRICK, T.G., STOUT, A.P., STATSINGER, A.L., MUESHAM, G.E., FORMAN, J.B. & GERE, J.B. (1956)
The anatomical approach to study of smoking and bronchogenic carcinoma: preliminary report of 41 cases.
Cancer, 9, 76-83.

AUERBACH, O., STOUT, A.P., HAMMOND, E.C. & GARFINKEL, L. (1961)
Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer.
New England Journal of Medicine, 265, 253-267.

AZZOPARDI, A. & THURLBECK, W.M. (1969)
The histochemistry of the nonciliated bronchiolar epithelial cell.
American Review of Respiratory Diseases, 99, 516-525.

BAKER, A.P., CHAKRIN, L.W., SAWYER, J.L., MUNRO, J.R. & HILLGRASS, L.M. (1973)
Levels of glycosyltransferases in canine respiratory tissue in an experimentally induced hypersecretory state.
Federation Proceedings: Federation of American Societies for Experimental Biology, 32, 560 Abstr. 1932.

BASKERVILLE, A. (1976)
The development and persistence of bronchial gland hypertrophy and goblet cell hyperplasia in the pig after injection of isoprenaline.
Journal of Pathology, 119, 35–47.

BENNETT, G. & LEBLOND, C.P. (1970)
Formation of cell coat material for whole surface of columnar cells in the rat small intestine, as visualised by radioautography with L-fucose-^{3}H.
Journal of Cell Biology, 46, 409–416.

BERG, N.B. & YOUNG, R.W. (1971)
Sulphate metabolism in pancreatic acinar cells.
Journal of Cell Biology, 50, 469–483.

BETTS, T. (Personal communication).

BLACKLOCK, J.W. (1961)
An experimental study of the pathological effects of cigarette condensate in lungs with special reference to carcinogenesis.
British Journal of Cancer, 15, 745–762.

BINDREITER, M., SCHUPPLER, J. & STOCKINGER, L. (1968)
Zellproliferation und Differenzierung im Trachealepithel der Ratte.
Experimental Cell Research, 50, 377–382.

BLENKINSOPP, W.K. (1967)
Proliferation of respiratory tract epithelium in the rat.
Experimental Cell Research, 46, 144–154.

BLIX, G. (1957)
Proposed nomenclature in the field of neuraminic and sialic acids.
Nature, 179, 1088.

BLIX, G. & LINDBERG, E. (1960)
The sialic acids of bovine and equine submaxillary mucins.
Acta chemica scandinavica, 14, 1809–1814.

BOLDUC, P. & REID, L. (1976a)
Motitic index of the bronchial and alveolar lining of the normal rat lung.
American Review of Respiratory Diseases (In press).

BOLDUC, P. & REID, L. (1976b)
The effect of tobacco smoke with and without phenylmethyl-oxadiazole (PMO) on mitotic index of rat respiratory epithelium.
(In preparation)

BONOMO, L. & D'ADDABBO, A. (1964)
^{131}I-albumin turnover and loss of protein into the sputum in chronic bronchitis.
Clinica chimica acta, 10, 214-222.

BREEZE, R.G., WHEELDON, E.B. & PIRIE, H.M. (1976)
Cell structure and function in the mammalian lung: the trachea, bronchi and bronchioles.
The Veterinary Bulletin, 46, 319-337.

BRENNER, R.M. & WEST, N.B. (1975)
Hormonal regulation of the reproductive tract in female mammals.
In: Annual Review of Physiology, 37, 273-302. Ed. J.H.Comroe, R.R.Sonnenschein and I.S.Edelman. Annual Reviews Inc., California.

BROWN, H. & TONG, H.S. (1971)
Erythrocyte membrane stabilization by Compound III.
Lorillard Research Center, USA, P.Lorillard Co.

BURNET, F.M., McCREA, J.F. & STONE, J.D. (1946)
Modification of human red cells by virus action.
1. The receptor gradient for virus action in human red cells.
British Journal of Experimental Pathology, 27, 228-236.

CARLTON'S HISTOLOGICAL TECHNIQUE (1973)
Fourth Edn. Revised and rewritten by R.A.B.Drury and E.A.Wallington. Oxford University Press, London.

CATANESE, B., LISCIANI, R. & PICCINELLI, D. (1969)
Erythrocyte membrane stabilisation and protein binding of some anti-inflammatory drugs and of deoxycholic acid.
Biochemical Pharmacology, 18, 1707-1710.

CHAKRIN, L.W. & SAUNDERS, L.Z. (1974)
Experimental chronic bronchitis. Pathology in the dog.
Laboratory Investigation, 30, 145-154.

CHANG, S.C. (1957)
Microscopic properties of whole mounts and sections of human bronchial epithelium of smokers and non-smokers.
Cancer, 10, 1246-1262.

CLOUGH, G., HILL, A. & BLACKMORE, D.K. (1973)
Evaluation of a filter rack for laboratory rodents.
Laboratory Animals, 7, 149-159.

COLES, S.J. & REID, L. (1976)
The effect of drugs on the incorporation of glycoprotein precursors into goblet cells of normal and hypertrophied human airway epithelium.
In: "Lung Cells in Disease". Proceedings of the Brook Lodge Conference, Kalamazoo, Michigan, USA, 21-23 April 1976.
Ed. A.Bouhuys. Elsevier Excerpta Medica, The Netherlands.

CONN, H.J. (1961)
Biological Stains.
7th Edn. The Williams & Wilkins Co., Baltimore, Maryland USA.

CREETH, J.M., BHASKAR, K.R., HORTON, J., OAS, I., LOPEZ-VIDRIERO, M.T. & REID, L. (1976)
The separation and characterisation of bronchial glycoproteins by density-gradient methods.
(In preparation).

CURRAN, R.C. & KENNEDY, J.S. (1955)
The distribution of sulphated mucopolysaccharides in the mouse.
Journal of Pathology and Bacteriology, 70, 449-457.

CUTZ, E. & CONEN, P.E. (1971)
Ultrastructure and cytochemistry of Clara cells.
American Journal of Pathology, 62, 127-142.

DAHLGREN, S.E. & DALHAMN, T. (1967)
The effect of oxalamine citrate on experimentally produced inflammation and cough.
Excerpta Medica International Congress Series, No.163.
Inflammation. Aetiopathogenetic, Clinical and Therapeutic Problems. Proceedings of an International Symposium, Bologna, November 7-8, 1967.

DAHLGREN, S.E. & DALHAMN, T. (1972)
The anti-inflammatory action of Phenyl-Methyl-Oxadiazole (PMO): an experimental study on the guinea-pig trachea.
Acta pharmacologica et toxicologica, 31, 193-202.

DALHAMN, T. (1956)
Mucous flow and ciliary activity in the trachea of healthy rats and rats exposed to respiratory irritant gases (SO_2, H_3N , HCHO). A functional and morphologic (light microscopic and electron microscopic) study, with special reference to technique.
Acta physiologica scandinavica, 36, Suppl.123, 1-161.

DALHAMN, T. (1964)
Studies on tracheal ciliary activity. Special reference to the effect of cigarette smoke on living animals.
American Review of Respiratory Diseases, 89, 870-877.

DALHAMN, T. (1966)
Inhibition of ciliostatic effect of cigarette smoke by oxalamine citrate (3-phenyl 5β-diethylaminoethyl-1, 2, 4-oxadiazole).
American Review of Respiratory Diseases, 94, 799-800.

DALHAMN, T. (1969)
The anticiliostatic effect of cigarettes treated with oxalamine citrate.
American Review of Respiratory Diseases, 99, 447-448.

DALHAMN, T. & RAUD, A. (1968)
The antitussive effect of oxalamine citrate and codeine phosphate in guinea-pigs.
Scandinavian Journal of Respiratory Diseases, 49, 23-25.

DALHAMN, T. & RYLANDER, R. (1971)
Reduction of cigarette smoke ciliotoxicity by certain tobacco additives.
American Review of Respiratory Diseases, 103, 855-857.

DEGAND, P., ROUSSEL, P., LAMBLIN, G., DURAND, G. & HAVEZ, R. (1973)
Donnes biochimiques et rhéologiques dans l'expectoration.
1. Definition biochimique des mucines dans l'expectoration.
Bulletin de Physio-pathologie Respiratoire, 9, 199-216.

DEGAND, P., ROUSSEL, P., LAMBLIN, G. & HAVEZ, R. (1973)
Purification et études des mucines de kystes bronchogeniquee.
Biochimica et biophysica acta, 320, 318-330.

DISCHE, Z., KAHN, N., ROTHSCHILD, C., DANILCHENKO, A., LICKING, J. & WANG, S.C. (1970)
Glycoproteins of submaxillary saliva of the cat: differences in composition produced by sympathetic and parasympathetic nerve stimulation.
Journal of Neurochemistry, 17, 649-658.

DISCHE, Z., PALLAVICINI, C., KAWASAKI, H., SMIRNOW, N., CIZEK, L.J. & CHIEN, S. (1962)
Influence of the nature of the secretory stimulus on the composition of the carbohydrate moiety of glycoproteins of the submaxillary saliva.
Archives of Biochemistry, 97, 459-469.

DOLL, R. & HILL, A.B. (1956)
Lung cancer and other causes of death in relation to smoking. A second report on the mortality of British doctors.
British Medical Journal, 2, 1071-1081.

DORLING, J. (1969)
"Critical electrolyte concentration" method in histochemistry.
Journal of Medical Laboratory Technology, 26, 124-130.

DOUGLAS, W.W. (1968)
Stimulus-secretion coupling: the concept and clues from chromaffin and other cells.
British Journal of Pharmacology, 34, 451-474.

DROZ, B. (1966)
Elaboration de glycoproteines dans l'appareil de Golgi des cellules hépatiques chez le rat; étude radioautographique en microscopie électronique après injection de galactose-^{3}H.
Compte rendu de l'Academie des sciences, Paris, 262, 1766-1768.

DRZENIEK, R. (1973)
Substrate specificity of neuraminidases.
Histochemical Journal, 5, 271-290.

EL-HENEIDY, A.R., HELMY, I.D. & MICHAEL, M.A. (1966)
Experimental diffuse interstitial fibrosis of the lungs and capillary trapping.
The Alexandria Medical Journal, 12, 275-307.

ELMES, P.C. & BELL, D. (1963)
The effects of chlorine gas on the lungs of rats with spontaneous pulmonary disease.
Journal of Pathology and Bacteriology, 86, 317-327.

ELSON, L.A., BETTS, T.E. & PASSEY, R.D. (1972)
The sugar content and the pH of the smoke of cigarette, cigar and pipe tobaccos in relation to lung cancer.
International Journal of Cancer, 9, 666-675.

ELSON, L.A. & PASSEY, R.D. (1963)
Biochemical effects of tobacco smoke and nicotine inhalation.
Unio Internationalis contra Cancrum Acta, 19, 715-717.

ESTERLY, J.R. & SPICER, S.S. (1968)
Mucin histochemistry of human gall bladder: changes in adenocarcinoma, cystic fibrosis and cholecystitis.
Journal of the National Cancer Institute, 40, 1-10.

ETHERTON, J.E. & CONNING, D.M. (1971)
Early incorporation of labelled palmitate into mouse lung.
Experientia, 27, 554-555.

EVANS, M.J., STEPHENS, R.J. & FREEMAN, G. (1971)
Effects of NO_2 on cell renewal in rat lung.
Archives of Internal Medicine, 128, 57-60.

EVERSOLE, L.R. (1972)
The histochemistry of mucosubstances in human minor salivary glands.
Archives of Oral Biology, 17, 1225-1239.

FAIRCHILD, E.J. (1967)
Tolerance mechanisms: determinants of lung to injurious agents.
Environmental Health, 14, 111-126.

FALK, H.L., TREMER, H.M. & KOTIN, P. (1959)
Effect of cigarette smoke and its constituents on ciliated mucus-secreting epithelium.
Journal of the National Cancer Institute, 23, 999-1012.

FAILLARD, H. (1959)
Die Wirkung der Neuraminidase auf verschiedene Formen des Rinder-Submaxillarismucins.
Hoppe-Seyler's Zeitschrift für physiologische Chemie, 317, 257-268.

FELL, H.B. & MELLANBY, E. (1953)
Metaplasia produced in cultures of chick ectoderm by high Vitamin A.
Journal of Physiology, 119, 470-488.

FIELD, W.E.H., DAVEY, E.N., REID, L. & ROE, F. (1966)
Bronchial mucus gland hypertrophy: its relation to symptoms and environment.
British Journal of Diseases of the Chest, 60, 66-80.

FILIPE, M.I. & BRANFOOT, A.C. (1974)
Abnormal patterns of mucus secretion in apparently normal mucosa of large intestine with carcinoma.
Cancer, 34, 282–290.

FISHER, R.A. & YATES, F. (1963)
Statistical tables for biological, agricultural and medical research.
Oliver and Boyd, Edinburgh & London.

FLETCHER, T., JONES, R. & REID, L. (1976)
Identification of glycoproteins in goblet cells of epidermis and gill of plaice (Pleuronectes platessa L.), flounder (Platichthye flesus (L.) and rainbow trout (Salmo gairdneri Richardson).
Histochemical Journal (In press).

FLOREY, H., CARLTON, H.M. & WELLS, A.Q. (1932)
Mucus secretion in the trachea.
British Journal of Experimental Pathology, 13, 269–284.

FRANKENHAEUSER, C. (1879)
Untersuchungen über den Bau der Tracheobronchialschleimhaut.
Thesis, St.Petersburg (Buchdruckerei der Kaiserlichen Akademie der Wisseenschaften).

FREEMAN, G., CRANE, S.C., FURIOSI, N.H., STEPHENS, R.J., EVANS, M.J. & MOORE, W.D. (1972)
Covert reduction in ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide.
American Review of Respiratory Diseases, 106, 563–579.

FREEMAN, A. & HAYDON, G.B. (1964)
Emphysema after low level exposure to NO_2.
Archives of Environmental Health, 8, 125–128.

FREY, H. (1874)
The Histology and Histochemistry of Man.
A treatise on the elements of composition and structure of the human body, p.449. Translated from the 4th German Edn. by A.E.J.Barker. J. & A. Churchill, London.

GALLAGHER, J.T., KENT, P.W., PASSATORE, M., PHIPPS, R.J., RICHARDSON, P.S. & LAMB, D. (1975)
The composition of tracheal mucus and the nervous control of its secretion in the cat.
Proceedings of the Royal Society (London) (Series B), 192, 49–76.

GIBBONS, R.A. (1959)
Chemical properties of two mucoids from bovine cervical mucin.
Biochemical Journal, 73, 209-217.

GIBBONS, R.A. (1963)
The sensitivity of the neuraminosidic linkage in linkage in mucosubstances towards acid and towards neuraminidase.
Biochemical Journal, 89, 380-391.

GIDDENS, W.E. & WHITEHAIR, C.K. (1969)
The peribronchial lymphocytic tissue in germfree, defined-flora, conventional and chronic murine pneumonia-affected rats.
In: Advances in Experimental Medicine, 3, 75-84. Germ-Free Biology. Ed. Mirand and Bock. Plenum Press, New York.

GIDDENS, W.E., WHITEHAIR, C.K. & CARTER, G.R. (1971)
Morphologie and microbiologic features of trachea and lungs in germfree, defined-flora, conventional and chronic respiratory disease-affected rats.
American Journal of Veterinary Research, 32, 115-129.

GOLDRING, I.P., COOPER, P., RATNER, I.M. & GREENBURG, L. (1967)
Pulmonary effects of sulphur dioxide exposure in the Syrian hamster. I. Combined with viral respiratory disease.
Archives of Environmental Health, 15, 167-176.

GOLDSTEIN, D.J. (1961)
Mechanism of differential staining of nucleic acids.
Nature (London), 191, 407-408.

GOLDSTEIN, D.J. (1962)
Correlation of size of dye particle and density of substrate, with special reference to mucin staining.
Stain Technology, 37, 79-93.

GOTTSCHALK, A.E. & LIND, P.E. (1949)
Product of interaction between influenza virus enzyme and ovomucin.
Nature (London), 164, 232-233.

GREENBERG, S.D. & WILLMS, R.K. (1962)
Regeneration of respiratory epithelium. An experimental study in dogs.
Archives of Pathology, 73, 65-70.

HAAG, H.B., LARSON, P.S. & WEATHERBY, J.H. (1960)
The effect on rats of chronic exposure to cigarette smoke.
Annals of the New York Academy of Sciences, 90, 227-238.

HADDOCK, N.H. (1948)
Alcian Blue, a new phthalocyanine dyestuff.
Research, 1, 685-689.

de Haller, R. (1969)
Development of mucus-secreting elements.
In: The Anatomy of the Developing Lung, 94-115.
Ed. J.Emery. Heinemann Medical Books, London.

de HALLER, R. & REID, L. (1965)
Adult chronic bronchitis, morphology, histochemistry and vascularisation of the bronchial mucous glands.
Medicina thoracalis, 22, 549-567.

HALLY, A.D. (1958)
The fine structure of the Paneth cell.
Journal of Anatomy, 92, 268-277.

HARRISON, J.D. (1974)
Salivary glands of the cat: a histochemical study.
Histochemical Journal, 6, 649-664.

HAYASHI, M., PHELPS, P. & HUBER, G. (1975a)
Quantitative differences in airway morphology secondary to sexual function.
Chest, 68, 414.

HAYASHI, M., PHELPS, P. & HUBER, G. (1975b)
Quantitative differences in airway morphology of male and female rats.
Physiologist, 18, 241

HAYASHI, M., SORNBERGER, C., PHELPS, P. & HUBER, G. (1976)
Differential response of male (♂) and female (♀) airway epithelium to chronic tobacco smoke inhalation.
Clinical Research, 23, 386A.

HEATH, I.D. (1962)
Observations on a highly specific method for the histochemicel detection of sulpheted mucopolysecchericles end its possible mechenisms.
Querterly Journal of Microscopicel Science, 103, 457-475.

HENLE, J. (1837)
Symbolae ad enatomiam villorum intestinalium imprimis eorum epithelii et vasorum lecteorum.
Commentatio ecademice. A.Hirschwald, Berolini.

HILDING, A.C. (1932)
Experimentel surgery of the nose end sinuses: chenges in the morphology of the epithelium following veriations in ventiletion.
Archives of Otoleryngology, 16, 9-18.

HILDING, A.C. (1956)
On cigerette smoking, bronchiel cercinome end ciliery ection. III. Accumulation of cigarette tar upon artificielly produced deciliated islends in the respiratory epithelium.
Annels of Otology, Rhinology end Leryngology, 65, 116-130.

HILDING, A.C. (1961)
Cigarette smoke end physiologic dreinege of the bronchial tree.
Diseeses of the Chest, 39, 357-362.

HILDING, A.C. (1963)
Phegocytosis, mucus flow, end ciliery ection.
Archives of Environmentel Heelth, 6, 61-73.

HOLLAND, R., KOZLOWSKI, E.J. & BOOKER, L. (1963)
The effect of cigarette smoke on the respiretory system of the rebbit. A finel report.
Cancer, 16, 612-615.

HOTCHKISS, R.D. (1948)
A microchemicel reection resulting in the staining of polysecchericle structures in fixed tissue preperetions.
Archives of Biochemistry, 16, 131-141.

HUBER, G. (1976) - Personal communicetion .

HUYEN, V.N. (1973)
Étude histochimique chez l'animel de l'ectivité de la S-cerboxy-méthyl-cysteine.
Bulletin de Physio-pathologie Respiretoire, 9, 463-464.

ICHIKAWA, A. (1965)
Fine structural changes in response to hormonal stimulation of the perfused canine pancreas.
Journal of Cell Biology, 24, 369-385.

IDE, G., SUNTZEFF, V. & COWDREY, E.V. (1959)
Comparison of histopathology of tracheal and bronchial epithelium of smokers and non-smokers.
Cancer (Philadelphia), 12, 473-484.

IRAVANI, J. & van AS, A. (1972)
Mucus transport in the tracheobronchial tree of normal and bronchitic rats.
Journal of Pathology, 106, 81-93.

JAMIESON, J.D. & PALADE, G.E. (1967)
Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules.
Journal of Cell Biology, 34, 597-615.

JAMIESON, J.D. & PALADE, G.E. (1971)
Synthesis, intracellular transport and discharge of secretory proteins in stimulated pancreatic exocrine cells.
Journal of Cell Biology, 50, 135-158.

JANATUINEN, M. & KORHONEN, L.K. (1969)
The effect of a substituted benzylamine (Bisolvon) on mucosubstance production.
Sonderdruck aus "Naunym-Schmiedebergs Archiv für Pharmakologie", 265, 112-117.

JEFFERY, P.K. (1973)
Goblet Cell Increase in Rat Bronchial Epithelium arising from Irritation or Drug Administration - an Experimental and Electron Microscopic Study.
Ph.D. Thesis, University of London.

JEFFERY, P. & REID, L. (1973)
Intra-epithelial nerves in normal rat airways: a quantitative electron microscopic study.
Journal of Anatomy, 114, 35-45.

JEFFERY, P.K. & REID, L. (1975)
New observations of rat airway epithelium: a quantitative and electron microscopic study.
Journal of Anatomy, 120, 295-320.

JEFFERY, P.K. & REID, L. (1976)
Ultrastructure of airway epithelium and submucosal gland during development.
Chapter 3 in "Development of the Lung". Monograph 1 of the Series Lung Biology in Health & Disease. Ed. W.A.Hodson. Marcel Dekker, New York.

JOHNSON, E.A. & WINZLER, R.J. (1972)
Two types of acid oligosaccharide in canine submaxillary mucin.
Federation Proceedings. Federation of American Societies for Experimental Biology, 31, A.870. 3714.

JONES, R.C. (1971)
The Effect of pH on Alcian Blue Staining of Sialomucin and Sulphomucin at Selected Epithelial Tissue Sites.
Thesis submitted for Fellowship of Institute of Medical Laboratory Sciences.

JONES, R., BAETJER, A.M. & REID, L. (1971)
Effect of extremes of temperature and humidity on the goblet cell count in the rat airway epithelium.
British Journal of Industrial Medicine, 28, 369-373.

JONES, R., BASKERVILLE, A. & REID, L. (1975)
Histochemical identification of glycoproteins in pig bronchial epithelium: (s) normal and (b) hypertrophied from enzootic pneumonia.
Journal of Pathology, 116, 1-11.

JONES, R. & REID, L. (1973a)
The effect of pH on Alcian Blue staining of epithelial acid glycoproteins. I. Sialomucins and sulphomucins (singly or in simple combinations).
Histochemical Journal, 5, 9-18.

JONES, R. & REID, L. (1973b)
The effect of pH on Alcian Blue staining of epithelial acid glycoproteins. II. Human bronchial submucosal gland.
Histochemical Journal, 5, 19-27.

KAMINSKI, E.J., FRANCHER, D.E. & CALANDRA, J.C. (1968)
In vivo studies of the ciliostatic effects of tobacco smoke.
Archives of Environmental Health, 16, 188-193.

KANDA, T., MAYFIELD, E.D. & GHIDONI, J.J. (1968)
Ultrastructural alterations in submaxillary acinar cells following isoproterenol administration: a new form of secretion granule.
Experimental and Molecular Pathology, 9, 189-196.

KAUFMAN, D.G., BAKER, M.S., HARRIS, C.C., SMITH, J.M., BOREN, H., SPORN, M.B. & SAFFIOTTI, U. (1972)
Co-ordinated biochemical and morphologic examination of hamster tracheal epithelium.
Journal of the National Cancer Institute, 49, 783-792.

KEITH, C.H. (1962)
Abstracts of 16th Tobacco Chemists' Research Conference, Richmond, Virginia.

KENNAWAY, E.L. & KENNAWAY, N.M. (1947)
Further study of incidence of cancer of the lung and larynx.
British Journal of Cancer, 1, 260-298.

KIM, S.K., NASJLETI, C.E. & HAN, S.S. (1972)
The secretion processes in mucous and serous secretory cells of the rat sublingual gland.
Journal of Ultrastructure Research, 38, 371-389.

KLEINERMAN, J. (1970)
Effects of NO_2 in hamsters: autoradiographic and electron microscopic aspects.
Inhalation Carcinogenesis Atomic Energy Symposium Series, 18, 271-281.

KLEINERMAN, J., SORENSEN, J. & RYNBRANDT, D. (1976)
Chronic bronchitis in the cat produced by chronic methacholine administration.
American Journal of Pathology, 82 (2). No.48 in Scientific Proceedings.

KLOCKARS, M. & REITAMO, S. (1975)
Tissue distribution of lysozyme in man.
Journal of Histochemistry and Cytochemistry, 23, 932-940.

KNAUFF, -. (1867)
Das Pigment der Respirationsorgane.
Virchow's Archiv für pathologische Anatomie und Physiologie und für klinische Medecin, 39, 442-475.

LABORATORY ANIMALS CENTRE MANUAL (1974)
The accreditation and recognition schemes for suppliers of laboratory animals.
Medical Research Council Laboratory Animals Centre Manual, Series 1, 2nd Edition.

LAMB, D. (1968)
Mucous secretion in hypersecretory states.
Les Bronches, 18, 453-465.

LAMB, D. (1969)
Intracellular Development and Secretion of Mucus in the Normal and Morbid Bronchial Tree.
Ph.D. Thesis, University of London.

LAMB, D. (1975)
Rat lung pathology and quality of laboratory animals: the user's view.
Laboratory Animals, 9, 1-8.

LAMB, R.A. (Personal communication).

LAMB, D. & REID, L. (1968)
Mitotic rates, goblet cell increase and histochemical changes in mucus in rat bronchial epithelium during exposure to sulphur dioxide.
Journal of Pathology and Bacteriology, 96, 97-111.

LAMB, D. & REID, L. (1969a)
Goblet cell increase in rat bronchial epithelium after exposure to cigarette and cigar tobacco smoke.
British Medical Journal, 1, 33-35.

LAMB, D. & REID, L. (1969b)
Histochemical types of acidic glycoprotein produced by mucous cells of the tracheobronchial glands in man.
Journal of Pathology, 98, 213-229.

LAMB, D. & REID, L. (1970)
Histochemical and autoradiographic investigations of the serous cells of the human bronchial glands.
Journal of Pathology, 100, 127-138.

LAMB, D. & REID, L. (1972a)
The tracheobronchial submucosal glands in cystic fibrosis: a qualitative and quantitative histochemical study.
British Journal of Diseases of the Chest, 66, 239-247.

LAMB, D. & REID, L. (1972b)
Acidic glycoproteins produced by the mucous cells of the bronchial submucosal glands in the fetus and child: a histochemical autoradiographic study.
British Journal of Diseases of the Chest, 66, 248-253.

LANE, B.P. & GORDON, R. (1974)
Regeneration of rat tracheal epithelium after mechanical injury. 1. Relationship between mitotic activity and cellular differentiation.
Proceedings of the Society for Experimental Biology and Medicine, 145, 1139-1144.

LANE, N., CARO, L., OTERO-VILARDEBO, L.R. & GODMAN, G.C. (1964)
On the site of sulfation in colonic goblet cells.
Journal of Cell Biology, 21, 339-351.

LAURENTIUS, A. (1602)
Historia anatomica humani corporis etc. Frankfurt. (Cited by Frankenaeuser, 1879).

LAWFORD, G.R. & SCHACTER, H. (1966)
Biosynthesis of glycoprotein by liver. The incorporation in vivo of ^{14}C-glucosamine into protein bound hexosamine and sialic acid of rat liver subcellular fractions.
Journal of Biological Chemistry, 241, 5408-5418.

LAWRENCE, D.J. & BERN, H.A. (1963)
Vitamin A and mucous metaplasia.
Annals of the New York Academy of Sciences, 106, 646-653.

LEBLOND, C.P. (1950)
Distribution of periodic acid-reactive carbohydrates in the adult rat.
Journal of Anatomy, 86, 1-49.

LEUCHTENBERGER, C., LEUCHTENBERGER, R. & DOOLIN, P.F. (1958)
A correlated histological, cytological and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. I. Bronchitis with atypical epithelial changes in mice exposed to cigarette smoke.
Cancer, 11, 490-506.

LEUCHTENBERGER, C., LEUCHTENBERGER, R., RUCH, F., TANAKA, K. & TANAKA, T. (1963)
Cytological and cytochemical alterations in the respiratory tract of mice after exposure to cigarette smoke, influenza virus and both.
Cancer Research, 23, 555-565.

LEUCHTENBERGER, C., LEUCHTENBERGER, R., ZEBRUN, W. & SHAFFER, P. (1960)
A correlated histological, cytological and cytochemical study of the tracheobronchial tree and lungs of mice exposed to cigarette smoke. II. Varying responses of major bronchi to cigarette smoke, absence of bronchogenic carcinoma after prolonged exposure and disappearance of bronchial lesions after cessation of exposure.
Cancer, 13, 721-732.

LEV, R. (1968)
A histochemical study of glycogsn and mucin in developing human foetal epithelia.
Histochemical Journal, 1, 152-165.

LEV, R. & SPICER, S.S. (1964)
Specific staining of sulphate groups with Alcian Blue at low pH.
Journal of Histochemistry and Cytochemistry, 12, 309.

LIGHTOWLER, N.M. & WILLIAMS, J.R.B. (1969)
Tracheal mucus flow rates in experimental bronchitis in rats.
British Journal of Expsrimental Pathology, 50, 139-149.

LISON, L. (1954)
Alcian Blue 8G with chlorantine fast red 5B, a technic for sslective staining of mucopolysaccharides.
Stain Technology,29, 131-138.

LOPEZ-VIDRIERO, M.T., BHASKAR, K.R., JONES, R., FLETCHER, T. & REID, L. (1976)
Biochsmical, rhsological and histochemical analysis of sulphated glycoproteins from plaice (Platessa pleuronectes) cutaneous mucus.
(In preparation).

LOPEZ-VIDRIERO, M.T., DAS. I., PICOT, R., SMITH, A.P. & REID, L. (1976)
Bronchial ssoretion from normal airways after inhalation of $F_2\alpha$, acetylcholine, histamine and citric acid.
(In preparation).

de LUCA, L. & WOLF, G. (1972)
Mechanism of action of Vitamin A in differentiation of mucus-ssoreting epithelia.
Agricultural and Food Chsmistry, 20, 474-476.

LUCAS, A.M. & DOUGLAS, L.C. (1934)
Principles underlying ciliary activity in the respiratory tract. II. Mucous clsarance in man, monkey, and other mammals.
Archives of Otolaryngology, 20, 518-541.

LUKE, J.L. & SPICER, S.S. (1965)
Histochemistry of surface epithelial and pleural mucins in mammalian lung. Ths demonstration of sialomucin in slvsolar cuboidal epithelium.
Laboratory Investigation, 14, 2101-2109.

LUKIE, B.E. & FORSTNER, G.G. (1972)
Synthesis of intestinal glycoproteins. Inhibition of I-^{14}C glycosamine incorporation by sodium salicylate in vitro.
Biochimica et Biophysica Acta, 273, 380-388.

McCARTHY, C. & REID, L. (1964a)
Acid mucopolysaccharide in the bronchial tree in the mouse and rat (sialomucin and sulphate).
Quarterly Journal of Experimental Pathology, 49, 81-84.

McCARTHY, C. & REID, L. (1964b)
Intracellular mucopolysaccharides in the normal human bronchial tree.
Quarterly Journal of Experimental Pathology, 49, 85-94.

McMANUS, J.F.A. (1946)
Histological demonstration of mucin after periodic acid.
Nature (London), 158, 202.

MADOFF, M.A., ANNENBERG, S.M. & WEINSTEIN, L. (1961)
Production of neuraminidase by L forms of Vibrio cholerae.
Proceedings of the Society for Experimental Biology and Medicine, 107, 776-777.

MALPRADE, M.L. (1934)
Étude de l'action des polyalcools sur l'acide periodique et les periodates alcalins.
Bulletin de la Société chimique de France, 1, 833-852.

MARCHOK, A.C., CONE, M.V. & NETTSHEIM, P. (1975)
Induction of squamous metaplasia (Vitamin A deficiency) and hypersecretory activity in tracheal organ culture.
Laboratory Investigation, 33, 451-460.

MASON, D.Y. & TAYLOR, C.R. (1975)
The distribution of muraminidase (lysozyme) in human tissues.
Journal of Clinical Pathology, 28, 124-132.

MATTHEWS, E.K., PETERSEN, O.H. & WILLIAMS, J.A. (1973)
Pancreatic acinar cells: acetycholine-induced membrane depolarisation, calcium afflux and amylase release.
Journal of Physiology, 234, 689-701.

MAWDESLEY-THOMAS, L.E. & HEALEY, P. (1969)
The quantitative evaluation of experimental chronic bronchitis: a preliminary study.
American Review of Respiratory Diseases, 100, 231-233.

MAWDESLEY-THOMAS, L.E. & HEALEY, P. (1973)
Experimental bronchitis in lambs exposed to cigarette smoke.
Archives of Environmental Health, 27, 248-250.

MAWDESLEY-THOMAS, L.E., HEALEY, P. & BARRY, D. (1971)
Experimental bronchitis in animals due to sulphur dioxide and cigarette smoke. An automated quantitative study.
In: Inhaled Particles III, vol. I. Proceedings of an International Symposium - British Occupational Hygiene Society, Cambridge. Ed. W.H.Walton. Unwin, London.

MEDICAL RESEARCH COUNCIL (1965)
Definition and classification of chronic bronchitis for clinical and epidemiological purposes.
Lancet, 1, 775-779.

MELLORS, R.C. (1958)
Microscopic localisation of tobacco smoke products in the respiratory tract of animals exposed to cigarette smoke.
Proceedings of the American Association for Cancer Research, 2, 325.

MEYRICK, B., STURGESS, J.M. & REID, L. (1969)
A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland.
Thorax, 24, 729-736.

MILLER, W.M. (1947)
The Lung. Charles C. Thomas, Springfield, Illinois.

MIZUSHIMA, Y. & SAKAI, S. (1969)
Stabilisation of erythrocyte membrane by non-steroid anti-inflammatory drugs.
Journal of Pharmacy and Pharmacology, 21, 327-328.

MIZUSHIMA, Y., SAKAI, S. & YAMAURA, M. (1970)
Mode of stabilising action of non-steroid anti-inflammatory drugs on erythrocyte membrane.
Biochemical Pharmacology, 19, 227-234.

MOE, H. (1968)
The goblet cells, Paneth cells and baeal granular celle of the epithelium of the intestine.
Internationel Review of Generel and Experimental Pathology, 3, 241-287.

MONTREUIL, J. & BISERTE, G. (1959)
Acide eialique et epecificité de la reaction e l'acide periodique - fucheine de Schiff eppliquée a l'electrophorèse eur papier. Example particulier de l'orosomucoide.
Bulletin de la Societe de Chimie Biologique, 41, 959-973.

MORGAGNI, J.B. (1712)
Nova institutionum medicarum idea.
Patevii. (Cited by Frankenhaeuser, 1879).

MOSCONA, A.A. (1965)
How celle aseociate.
In: The Living Cell, pp. 213-221. Readinge from Scientific America. W.H.Freeman & Co., San Francisco.

MOWRY, R.W. (1956)
Alcian Blue techniquee for hietochemical etudy of acidic carbohydratee.
Journal of Histochemistry and Cytochemistry, 4, 407.

MOWRY, R.W. (1960)
Revised method of producing improved colouration of acidic polyeaccharidee with Alcien Blue 89X supplied currently.
Journal of Hietochemistry end Cytochemistry, 8, 323-324.

MOWRY, R.W. & MORARD, J.C. (1957)
Dietribution of acid mucopolyaaccharidea in normal kidneye as ehown by Alcian Blue - Feulgan and Alcian Blue-periodic acid Schiff etaine.
Americen Journel of Pathology, 33, 620.

MOWRY, R.W. & WINKLER, C.H. (1956)
The colouration of acidic carbohydratee of bacteria and fungi in tiseue sectione with epecial reference to cepsulee of Cryptococcus neoformans, Pneumococcus and Stephylococcus.
American Journal of Pethology, 32, 628-629.

MUSIL, J., WEISSOVA, J., ADAM, M. & PROKOPEC, J. (1968)
The influence of anti-inflammatory drugs on the glycoprotein bioeyntheeis in vitro.
Phermacology, 1, 295-302.

NEGUS, V.E. (1963)
The function of mucus.
Acta oto-laryngologica, 56, 204-214.

NEUBERGERGER, A. & MARSHALL, R.D. (1966)
Structural analysis of the carbohydrate group of glycoproteins.
In: Glycoproteins, their Composition, Structure and Function.
Ed. A.E.Gottschalk. Elsevier Publishing Co., Amsterdam.

NEUTRA, M. & LEBLOND, C.P. (1966)
Radioautographic comparison of the uptake of galactose-^{3}H and glucose-^{3}H in the Golgi region of various cells secreting glycoproteins or mucopolysaccharides.
Journal of Cell Biology, 30, 137-150.

NIDEN, A.H. (1967)
Bronchiolar and large alveolar cell in pulmonary phospholipid metabolism.
Science, 158, 1323-1324.

NIDEN, A.H. & YAMADA, E. (1966)
Some observations on the fine structure and function of the nonciliated bronchiolar cells.
In: VIth International Congress for Electron Microscopy, Kyoto, p. 599. Maruzen, Tokyo.

PARADINE, C.G. & RIVETT, B.H.P. (1953)
Statistics for Technologists. English Universities Press, Ltd., London.

PEARSE, A.G.E. (1960)
The Feulgen reaction.
In: Histochemistry Theoretical and Applied, Appendix 8, p.822.
2nd Edition. J. & A. Churchill Ltd., London.

PETRIK, P. & COLLET, A.J. (1974)
Quantitative electron microscopic autoradiography of in vivo incorporation of ^{3}H-choline, ^{3}H-leucine, ^{3}H-acetate and ^{3}H-galactose in non-ciliated bronchiolar (Clara) cells of mice.
American Journal of Anatomy, 139, 519-534.

QUEVAUVILLER, A., HUYEN, V.N. & GARCET, S. (1970)
Methodes d'études expérimentales des modifications des sécrétions bronchiques.
Le Poumon et le Coeur, 26, 71-80.

QUINTARELLI, G., SCOTT, J.E. & DELLOVO, M.C. (1964a)
The chemical and histochemical properties of Alcian Blue.
II. Dye binding of tissue polyanions.
Histochemie, 4, 86-98.

QUINTARELLI, G., SCOTT, J.E. & DELLOVO, M.C. (1964b)
The chemical and histochemical properties of Alcian Blue.
III. Chemical blocking and unblocking.
Histochemie, 4, 99-112.

QUINTARELLI, G., TSUIKI, S., HASHIMOTO, Y. & PIGMAN, W. (1961)
Studies of sialic acid containing mucus in bovine submaxillary and rat sublingual glands.
Journal of Histochemistry and Cytochemistry, 9, 176-183.

RASMUSSEN, H. (1970)
Cell communication, calcium ion and cyclic adenosine monophosphate.
Science, 170, 404-412.

RAVETTO, C. (1968)
Histochemical identification of N-acetyl-O-diacetylneuraminic acid resistant to neuraminidase.
Journal of Histochemistry and Cytochemistry, 16, 663.

RAVETTO, C. & BELLOMI, C. (1966)
Histochemical identification of acid mucopolysaccharides in cat lingual glands.
Folia Histochemica et Cytochemica, 4, 267-272.

RAWLINSON, H.E. (1933)
Cytological changes after autonomic and adrenaline stimulation of the cat's submaxillary gland.
Anatomical Record, 57, 289-301.

REID, L.McA. (1954)
Pathology of chronic bronchitis.
Lancet, 1, 275-279.

REID, L. (1960)
Measurement of the bronchial mucous gland layer: a diagnostic yardstick in chronic bronchitis.
Thorax, 15, 132-141.

REID, L. (1963)
An experimental study of hypersecretion of mucus in the bronchial
British Journal of Experimental Pathology, 44, 437-445. tree.

REID, L. (1967)
Bronchial mucus production in health and disease.
In: The Lung. Chapter 8, pp. 87–108. International Academy of Pathology Monograph No.8. The Williams & Wilkins Co., Baltimore.

REID, L. (1970a)
Evaluation of model systems for study of airway epithelium, cilia, and mucus.
Archives of Internal Medicine, 126, 428–434.

REID, L. (1970b)
Chronic bronchitis – A report on mucus research.
Proceedings of the Royal Institution of Great Britain, 43, 438–462.

RHODIN, J.A.G. (1966)
The ciliated cell. Ultrastructure and function of the human tracheal mucosa.
American Review of Respiratory Diseases, 93 Suppl., 1–15.

RHODIN, J. & DALHAMN, T. (1956)
Electron microscopy of the tracheal ciliated mucosa in rat.
Zeitschrift für Zellforschung und mikroscopische Anatomie, 44, 345–412.

RITTER, H.B. & OLESON, J.J. (1950)
Combined histochemical staining of acid polysaccharides and 1, 2 glycol groupings in paraffin sections of rat tissues.
American Journal of Pathology, 26, 639–645.

ROBERTS, G.P. (1974)
Isolation and characterisation of glycoproteins from sputum.
European Journal of Biochemistry, 50, 265–280.

ROCKEY, E.E., SPEER, F.D., AHN, K.J., THOMPSON, S.A. & HIROSE, T. (1962)
The effect of cigarette smoke condensate on the bronchial mucosa of dogs.
Cancer, 15, 1100–1116.

ROYAL COLLEGE OF PHYSICIANS (1972)
Smoking and Health now. A new report and summary on smoking and its effects on health from the Royal College of Physicians, London. Pitman Medical and Scientific Publishing Co.Ltd., (London).

RUBIN, R.P. (1970)
The role of calcium in the release of neurotransmitter substances and hormones.
Pharmacological Reviews, 22, 389–428.

SADÉ, J., ELIEZER, N., SILBERBERG, A. & NEVO, A.C. (1970)
The role of mucus in transport by cilia.
American Review of Respiratory Diseases, 102, 48-52.

SADOWSKY, D.A., GILLIAM, A.G. & CORNFIELD, J. (1953)
Statistical association between smoking and cancer of the lung.
Journal of the National Cancer Institute, 13, 1237-1258.

SATO, T., SUZUKI, T. & FUKUYAMA, T. (1962)
Cigarette smoke: mode of adhesion and haemolyzing and SH-inhibiting factors.
British Journal of Cancer, 16, 7-15.

SCARPELLI, E.M. (1968)
The Surfactant System of the Lung.
Lea & Febiger, Philadelphia.

SCHACHTER, H. (1974)
Glycosylation of glycoproteins during intracellular transport of secretory products.
Advances in Cytopharmacology Vol.2, 207-218.
In: Cytopharmacology of Secretion. Ed. B.Ceccarelli, J.Meldolesi and F.Clementi. Proceedings of the NATO Advanced Study Institute, Venice & Milan, June 16-23, 1973. Raven Press, New York.

SCHRAMM, M. & SELINGER, Z. (1974)
The function of α and β-adrenergic receptors and a cholinergic receptor in the secretory cell of rat parotid gland.
Advances in Cytopharmacology Vol.2, 29-32.
In: Cytopharmacology of Secretion. Ed. B.Ceccarelli, J.Meldolesi and F.Clementi. Proceedings of the NATO Advanced Study Institute, Venice & Milan, June 16-23, 1973. Raven Press, New York.

SCHULZE, F.E. (1871)
Die Lungen.
In: Handbuch Lehre von Geweben des Menschen und der Thiere, pp. 464-485. Ed. S.Stricker. W.Englemann, Leipzig.

SCHUR, M.O. & RICKARDS, J.C. (1957)
Design and operation of a multiple cigarette smoking machine.
Tobacco Science, 1, 13-20.

SCOTT, J.E. & DORLING, J. (1965)
Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian Blue in salt solutions.
Histochemie, 5, 221-233.

SCOTT, J.E., QUINTARELLI, G. & DELLOVO, M.C. (1964)
The chemical and histochemical properties of Alcian Blue.
I. The mechanism of Alcian Blue staining.
Histochemie, 4, 73-85.

SHACKLEFORD, J.M. & KLAPPER, C.E. (1962)
A sexual dimorphism of hamster submaxillary mucin.
Anatomical Record, 142, 495-503.

SHORTER, R.G., TITUS, J.L. & DIVERTIE, M.B. (1966)
Cytodynamics in the respiratory tract of the rat.
Thorax, 21, 32-37.

SLEIGH, M.A. (1974)
Metachronism of cilia of metazoa.
In: Cilia and Flagella. Pergamon Press, Oxford.

SPAIN, D.M., BRADESS, V.A. & MATERO, A. (1970)
Metaplasia of bronchial epithelium. Effect of age, sex and smoking.
Journal of the American Medical Association, 211, 1331-1334.

SPICER, S.S. (1960)
A correlative study of the histochemical properties of rodent acid mucopolysaccharides.
Journal of Histochemistry and Cytochemistry, 8, 18-35.

SPICER, S.S. (1965)
Diamine methods for differentiating mucopolysaccharides histochemically.
Journal of Histochemistry and Cytochemistry, 13, 211-234.

SPICER, S.S., CHAKRIN, L.W. & WARDELL, J.R. (1974)
Effect of chronic sulfur dioxide inhalation on the carbohydrate histochemistry and histology of the canine respiratory tract.
American Review of Respiratory Diseases, 110, 13-24.

SPICER, S.S., CHAKRIN, L.W., WARDELL, J.R. & KENDRICK, W. (1971)
Histochemistry of mucosubstances in the canine ahd human respiratory tract.
Laboratory Investigation, 25, 483-490.

SPICER, S.S. & HENSON, J.G. (1967)
Methods for localizing mucosubstances in epithelial and connective tissues.
In: Methods and Achievements in Experimental Pathology, Vol. 2, pp. 78-112. Ed. E.Baynsz & G.Jasmin. Karger, New York.

SPICER, S.S., LEPPI. T.J. & STOWARD, P.J. (1965)
Suggestions for a histochemical terminology of carbohydrate-rich tissue components.
Journal of Histochemistry and Cytochemistry, 13, 599-603.

SPICER, S.S. & MEYER, D.B. (1960)
Histochemical differentiation of acid mucopolysaccharides by means of combined aldehyde fuchsin-alcian blue staining.
American Journal of Clinical Pathology, 33, 453-460.

SPICER, S.S. & WARREN, L. (1960)
The histochemistry of sialic acid containing mucoproteins.
Journal of Histochemistry and Cytochemistry, 8, 135-137.

SPIRO, R.G. (1969)
Glycoproteins: their biochemistry, biology and role in human disease.
New England Journal of Medicine, 281, 991-1001.

SPIRO, R.G. & SPIRO, M.J. (1966)
Glycoprotein biosynthesis: studies on thyroglobulin. Characterisation of a particulate precursor and radioisotope by thyroid slices and particle systems.
Journal of Biological Chemistry, 241, 1271-1282.

STALEY, M.W. & TRIER, J.S. (1965)
Morphologic heterogeneity of mouse Paneth cell granules before and after secretory stimulation.
American Journal of Anatomy, 117, 365-384.

STANTON, M.F., MILLER, E., WRENCH, C. & BLACKWELL, R. (1972)
Experimental induction of epidermoid carcinoma in the lungs of rats by cigarette smoke condensate.
Journal of the National Cancer Institute, 49, 867-873.

STEEDMAN, H.F. (1950)
Alcian blue 8GS; a new stain for mucin.
Quarterly Journal of Microscopical Science, 91, 477-479.

STOWARD, P.J. (1963)
Studies in fluorescent microscopy.
M.S.D.Phil. Thesis, University of Oxford.

STURGESS, J.M. (1970)
The Control of the Bronchial Glands and their Secretion.
Ph.D. Thesis, University of London.

STURGESS, J. & REID, L. (1972)
An organ culture study of the effect of drugs on the secretory activity of the human bronchial submucosal gland.
Clinical Science, 43, 533-543.

STURGESS, J. & REID, L. (1973)
The effect of isoprenaline and pilocarpine on (a) bronchial mucus-secreting tissue and (b) pancreas, salivary glands, heart, thymus, liver and spleen.
British Journal of Experimental Pathology, 54, 388-403.

SVENNERHOLM, L. (1956)
On the isolation and characterisation of N-acetylsialic acid.
Acta Societatis medicorum upsaliensis, 61, 75.

THURLBECK, W.M., ANGUS, G.E. & PARÉ, J.A.P. (1963)
Mucus gland hypertrophy in chronic bronchitis and its occurrence in smokers.
British Journal of Diseases of the Chest, 57, 73-78.

de TOMASI, J.A. (1936)
Improving the technic of the Feulgen stain.
Stain Technology, 11, 137.

VARUTE, A.T. & JIRGE, S.K. (1971)
Histochemical analysis of mucosubstances in oral mucosa of mouthbreathing. Cichlid fish and seasonal variations in them.
Histochemie, 25, 91-102.

WALLER, C. & BJÖRKMAN, G. (1882)
Studien über den Bau der Tracheal Schleimhaut mit besonderer Berucksichtigung des Epithels.
Biologische Untersuchungen, 2, 71-96.

WARREN, L. & SPICER, S.S. (1961)
Biochemical and histochemical identification of sialic acid containing mucins of rodent vagina and salivary glands.
Journal of Histochemistry and Cytochemistry, 9, 400-408.

WELLS, A.B. & LAMERTON, L.F. (1975)
Regenerative response of the rat tracheal epithelium after acute exposure to tobacco smoke: a quantitative study.
Journal of the National Cancer Institute, 55, 887-891.

WHUR, P., HERSCOVICS, A. & LEBLOND, C.P. (1969)
Radioautographic visualisation of the incorporation of galactose-^{3}H and mannose-^{3}H by rat thyroid in vitro relation to the stages of thyroglobulin synthesis.
Journal of Cell Biology, 43, 289-311.

WILHELM, D.L. (1953)
Regeneration of tracheal epithelium.
Journal of Pathology and Bacteriology, 65, 543–550.

WONG, Y.C. & BUCK, R.C. (1971)
An electron microscopic study of metaplasia of the rat tracheal epithelium in Vitamin A deficiency.
Laboratory Investigation, 24, 55–66.

WRIGHT, B.M. (1972)
A cigarette smoking machine for animal experiments.
Laboratory Practice, 21, 881 and 884.

YAMADA, K. (1964)
Effects of oxidation upon Alcian Blue staining of acid mucopolysaccharides.
Nagoya Journal of Medical Science, 26, 217–220.