THE EFFECT OF TOBACCO SMOKE AND NICOTINE ON RAT AIRWAY EPITHELIUM AND THE RESPONSE OF TOBACCO SMOKE-INDUCED SECRETORY CELL HYPERPLASIA TO ANTI-INFLAMMATORY DRUGS

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by

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ABSTRACT

An experimental model of human bronchitis is used to examine the effect of six anti-inflammatory and one mucolytic drug on epithelial secretory cell hyperplasia induced by inhalation of cigarette tobacco smoke (TS). The time taken for secretory cell hyperplasia to return to normal after cessation of tobacco smoke exposure is established and the effect of two anti-inflammatory drugs on the speed of recovery is examined.

Exposure of rats to the smoke from 25 cigarettes daily for 14 days increases the number of secretory cells in the trachea and throughout the intrapulmonary airways: the degree of hyperplasia is proportional to the amount of smoke delivered. Two non-steroidal anti-inflammatory drugs, indomethacin and flurbiprofen, three steroidal drugs, dexamethasone, prednisolone, hydrocortisone and the mucolytic agent, N-acetylcysteine (NAC) inhibit TS-induced secretory cell hyperplasia. The inhibitory effect of indomethacin is proportional to the degree of hyperplasia induced. When given by intraperitoneal (ip) injection, inhibition is greatest with indomethacin, flurbiprofen and dexamethasone and least following prednisolone and hydrocortisone. When given by gavage, flurbiprofen is partially effective, indomethacin is not effective and aspirin exacerbates the effect of TS. NAC is effective when given in the drinking water.

Following cessation of TS exposure, it takes a minimum of 21 days for the number of secretory cells to return to normal. When indomethacin or flurbiprofen are given by ip injection during the recovery period, the time taken to recover is significantly reduced: the effect is restricted to the intrapulmonary airways.

Nicotine, given daily by aerosol has no effect on secretory cell number. However, when given by injections there is a 'bi-phasic' effect depending on dose. At a dose which gives a mean plasma concentration of 650 ng per ml there is a reduction in secretory cell number whilst at a dose giving a mean plasma concentration of over 2000 ng per ml there is an increase.

It is concluded that a number of anti-inflammatory drugs and a mucolytic agent inhibit experimental bronchitis induced by tobacco smoke and reduce the time taken to recover after cessation of exposure. Some of the drugs merit clinical investigation. It would appear that nicotine alone is not responsible for TS-induced secretory cell hyperplasia.

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CHAPTER 1

INTRODUCTION

It is now proved beyond doubt that smoking
is one of the leading causes of statistics

FLETCHER KNEBEL
Readers Digest, December, 1961

Increased numbers of mucus-secreting cells (i.e. secretory cell hyperplasia) are found in the airways of patients with bronchial diseases associated with mucus-hypersecretion (see Anderson, 1976; Crofton and Douglas, 1981; Dunnill, 1982). Chronic bronchitis, in particular, is a prime example of a condition characterised by excessive mucus-secretion. Mucus mixes with other substances which together are removed from the airways by coughing and expectorated as sputum (Mitchell-Heggs, 1982). Cystic fibrosis is a clinical condition which also has hypersecretion as one component. The lungs of the cystic patient become infected early in the course of the disease leading to the production of a purulent and viscid secretion. Asthma is defined as a recurrent, reversible narrowing of the airways in response to stimuli of an intensity not inducing such narrowing in most individuals (Dunnill, 1960; Dunnill et al., 1969). A thick, viscid, eosinophilic sputum is often associated with asthmatic attacks. After many years of asthmatic attacks the patient may also become 'bronchitic' (Burr et al., 1975) and be categorised as a 'wet' asthmatic. At autopsy the airways of patients with chronic bronchitis, cystic fibrosis or asthma (status asthmaticus) are found to show secretory cell hyperplasia both in the surface epithelium and in the sub-mucosal glands which are consequently increased in size (i.e. hypertrophy) (Reid, 1954; Reid and de Haller,

1964; Dunnill et al., 1969; Crofton and Douglas, 1981; Lopez-Vidriero and Reid, 1983). Emphysema, bronchorrhoea and bronchiectasis are bronchial diseases in man which may also be associated with mucus-hypersecretion (see Whitwell, 1952; Anderson, 1976; Crofton and Douglas, 1981; Dunnill, 1982). However, secretory cell hyperplasia and the production of an excessive amount of mucus are secondary to the main characteristics of these diseases; i.e. permanent enlargement of the respiratory airways distal to the terminal bronchiole in emphysema, excessive secretion of a watery fluid (which may be transudate) in bronchorrhoea, and localised chronic dilatation and infection of the bronchi in bronchiectasis.

Thus, a number of bronchial diseases in man have mucus-hypersecretion as a component: secretory cell hyperplasia is a characteristic of a few of them. The experimental studies described herein are concerned primarily with an animal model of chronic bronchitis, a condition which has mucus-hypersecretion and secretory cell hyperplasia as two well-defined characteristics.

A. CHRONIC BRONCHITIS

Chronic bronchitis is defined as a clinical entity characterised by cough with sputum production (in the absence of cardiac or other pulmonary disease) on most days for three months of the year during at least two consecutive years (Oswald et al., 1953; Oswald, 1954; Glynn and Michaels, 1960; Reid, 1960, 1961;

Medical Research Council, 1965). Many patients who are recurrent sputum producers fall outside such a strict definition and may be thought of as having 'acute bronchitis' or 'catarrhal bronchitis' (Anderson, 1976). Accumulation of excessive mucus tends to encourage colonisation of retained secretions by bacteria, especially Haemophilus influenzae and Streptococcus pneumoniae, which are of secondary importance in the prime causation of the disease (see Jeffery, 1983 a). However, secondary infections during acute exacerbation cause a change in the type of sputum produced from mucoid to mucopurulent or purulent and may also accompany mucosal inflammation, ulceration, scarring and destruction of bronchiolar walls with narrowing of airways, multiple stenoses and irreversible airway obstruction (i.e. small airways disease) (ibid). The volume of sputum produced correlates strongly with smoking, frequency of chest episodes and FEV_1, but not with the rate of decline of FEV_1 with age indicating that air-flow obstruction and mucus hypersecretion are largely independent disease processes (Fletcher et al., 1976; Fletcher and Peto, 1977; Peto et al., 1983).

I. Aetiology

Chronic bronchitis results from chronic irritation of the bronchial epithelium (Anderson, 1976). Epidemiological studies have implicated exposure to irritants associated with atmospheric pollution in the aetiology of the disease (Oswald et al., 1953; Reid et al., 1964, Holland et al., 1965; Gregg, 1974; Marini, 1980). Dampness,

fog and photochemical smog (containing sulphur dioxide) may aggravate the disease. Personal pollution by inhaling tobacco smoke, particularly from cigarettes, has been shown by many statistically-based studies to be also a major cause of chronic bronchitis (Oswald and Medvei, 1955; Royal College of Physicians, 1962, 1971, 1977; Hammond, 1962; U.S. Surgeon General, 1964; Fletcher and Horn, 1971; U.S. Department of Health, Education and Welfare, 1968 and 1972). Consequently, chronic bronchitis is exceptionally common in heavy smokers and in people from industrialised areas. It is especially prone to occur in middle-aged men and is a major cause of absenteeism from work and so is of great economic and social importance (Anderson, 1976). Chronic bronchitis is rare in life-long non-smokers in an unpolluted atmosphere (Huhti, 1982).

II. Histological features

The most obvious histological correlate of the clinical hallmarks of chronic bronchitis, starkly seen by light microscopy is the increase in both the number (i.e. hyperplasia) and size (i.e. hypertrophy) of mucus-secreting cells throughout the airways, both in the surface epithelium and in the sub-mucosal glands (Reid, 1954, 1958, 1967; Thurlbeck et al., 1975). Bronchiolitis, smooth muscle hypertrophy and a loss of airway patency may also be associated with the disease (Hogg et al., 1968; Karpic et al., 1970; Matsuba and Thurlbeck, 1973). Possibly the most important pathological change is seen in the 'small' airways (i.e. bronchioli of less than 2mm in diameter) which are normally only sparsely populated with epithelial mucus-secreting cells (Miller, 1932; Reid, 1954). In disease these airways show mucous cell metaplasia (i.e. a change in the function of epithelial cells, in this

case to one of mucus-production) followed by mucous cell hyperplasia. If mucous cell hyperplasia correlates with increased mucus production in the small, distal airways, then the defect may have a greater functional implication in terms of airflow limitation than the increase in amount of mucus-secreting tissue seen in the larger, more proximal airways. In the large airways, cough and expectoration will dislodge and remove excess mucus and aid muco-ciliary clearance (Hogg et al., 1968; Thurlbeck, 1973; Mossberg et al., 1978; Macklem, 1979; Bateman et al., 1981; Camner, 1981). In the smaller airways, coughing does not remove mucus. In fact the small airways rely on an intact muco-ciliary apparatus for the efficient clearance of particles. However, it has been found in general that there are fewer cilia in the more distal airways and that they are shorter and beat at a slower rate than those in the upper airways of both Man and experimental animals (Rhodin, 1966; Greenwood and Holland, 1972; Jeffery and Reid, 1975; Serafini et al., 1976; Serafini and Michaelson, 1977; Rutland et al., 1982). Muco-ciliary clearance has also been found to be slower in the peripheral airways than in the more proximal airways (Asmundsson and Kilburn, 1970; Iravani and Van As, 1972; Yeates et al., 1973, 1975; Serafini et al., 1976). Consequently, excess mucus-production in the distal airways may interfere with a muco-ciliary clearance mechanism which is normally less efficient than that in the upper airways. In addition, other changes occur in the small airways of smokers and bronchitics which may further exacerbate the problem caused purely by mucous cell hyperplasia. For example, there may be a reduction in the amount of small molecular weight anti-protease which may allow for destruction of the airways by protease enzymes (Eriksson, 1978; Carp et al., 1982; Mooren et

al., 1983). Oedema and epithelial thickening are also seen (Niewoehner et al., 1974) which may further decrease airway calibre.

In human smokers and patients with chronic bronchitis a histological change is seen in the type of intracellular glycoprotein of the mucus-secreting cells (McCarthy and Reid, 1964; de Haller and Reid, 1965; Lev and Spicer, 1965; Lamb, 1969; Lamb and Reid, 1969 a; Kollerstrom et al.,1977). The sparse mucus-secreting cell population in both the surface epithelium and sub-mucosal glands comprises a mixture of types containing either neutral or acidic-staining glycoprotein. The latter may be sialomucin, sulphomucin or a mixture of the two. In disease there is a shift in the population with secretory cell hyperplasia in the epithelium and glands due mainly to an increase in the number of those cells containing acidic glycoprotein whilst the number of cells containing neutral glycoprotein may remain virtually unchanged or even be reduced. The clinical implications of the shift to a predominance of acidic mucus-secreting cells is unclear, suffice to say that acidic mucus (containing neuraminic acid) may be more viscid and less 'pourable' than neutral mucus (Odin, 1958; Gibbons, 1959; Gibbons and Glover, 1959; Munies et al., 1968; Keal, 1971; Lopez-Vidriero et al., 1973).

Thus it may be seen that the histological features of chronic bronchitis in Man are complex and the importance of any one feature in the pathogenesis and progress of the disease must be considered in the light of the whole.

B. EXPERIMENTAL BRONCHITIS

Inhalation of various irritants will produce many of the histological characteristics of lung disease in experimental animals. For example, gases such as sulphur dioxide (Reid, 1963; Lamb and Reid, 1968; Chakrin and Saunders, 1974; Spicer et al., 1974; Morgenroth, 1980), nitrogen dioxide (Freeman and Haydon,1964), chlorine (Elmes and Bell, 1963) and tobacco smoke (see below) produce histologically 'bronchitic' changes in experimental animals including epithelial secretory cell hyperplasia, increased epithelial thickness and sub-mucosal gland hypertrophy. Other agents such as ozone (Boatman and Frank, 1974; Stevens et al., 1974, a and b; Shami et al., 1982) and chemical aerosols such as sulphuric acid (Alarie et al., 1975; Brownstein, 1980) damage the lungs of experimental animals extensively, causing epithelial desquamation and alveolar haemorrhage and inflammation. Consequently some irritants are unsuitable for use in experimental animal models of chronic bronchitis, whereas others are suitable because they cause 'bronchitic' changes without widespread damage.

I. Tobacco Smoke

The airways of animals exposed for relatively short periods to comparatively 'low' concentrations of whole cigar tobacco, cigarette tobacco, or even marijuana smoke exhibit many of the histological characteristics of human chronic bronchitis including epithelial thickening and secretory cell hyperplasia, increases in the volume and density of intracellular mucin and a change in the epithelial secretory cell population to a predominance of mucous cells (i.e. cells containing acid-staining mucin) (Lamb, 1967; Lamb and Reid, 1969 a; Walker, 1977; Hayashi et al., 1978; Huber et al., 1981). More

severe regimes of cigarette tobacco smoke exposure, for example over a longer period or with more cigarettes, produce changes beyond the histologically 'bronchitic' stage such as pulmonary fibrosis and emphysema, epithelial desquamation, squamous cell metaplasia, possible production of tumours and may even lead to death of the animal (Auerbach et al., 1967, 1970, a and b; Passey and Blackmore, 1967; Hammond et al., 1970; Kendrick et al.,1976; Bernfield et al., 1979; Le Bouffant et al., 1980; Dalbey et al., 1980; Betts et al., 1981).

Tobacco smoke is therefore a very good irritant for use in experimental lung pathology because of the current interest in its harmful effects, its relative ease of administration and because it will cause different airway changes when given at different 'concentrations'. In particular, a short exposure of experimental animals to relatively lower concentrations of smoke produces histologically 'bronchitic' changes and avoids the severely detrimental effects on the health of the animals seen with higher concentrations ofsmoke and with many other inhaled irritants. However, the use of tobacco smoke as a bronchial irritant does have a number of disadvantages. Automatic cigarette smoking machines are specialist equipment and so can be expensive. Similarly, cigarettes are expensive and organising relief of tobacco-duty to reduce costs is difficult and time-consuming. Quantifying the amount of smoke being delivered is also more difficult than quantifying the concentration of other 'simpler' irritant gases such as sulphur dioxide.

Many experimental studies do not quantify the amount of smoke

used. It would be desirable to know the amount of smoke to which experimental animals have been exposed as this would allow comparison of the changes seen in animals between different studies and possibly allow for more reliable extrapolation to Man. A desire for standardisation of smoke-exposure dosage has already been expressed (Dalhamn; 1966) and various standardising criteria have been suggested (Tobacco Manufacturers Standing Committee, 1961; Tobacco Research Council, 1972). However, many of these criteria apply to the smoke delivered by smoking machines in terms of puff frequency or leaving a standard length cigarette butt. Such standardisation does not give a measure of the amount of smoke in the immediate vicinity of the animals, nor the amount of smoke actually inhaled by an animal. Consequently, some of the studies reported herein are concerned with determining the concentration of tobacco smoke which produces airways change.

Tobacco smoke is a complex mixture of more than 1500 chemical constituents (Stedman, 1968) and may be thought of as being an aerosol comprised of two parts, namely a continuous 'gaseous phase' and a discontinuous 'particulate phase' (Stills and Stedman, 1956; Roy, 1975). Major toxic agents in the gas phase of cigarette smoke include ammonia, acetaldehyde, crotonaldehyde, acrolein, dimethylnitrosamine, nitrosopyrrolidine and oxides of nitrogen (Wynder and Hoffman, 1979). The particulate or 'tar' phase of tobacco smoke contains numerous toxic agents including phenol, arsenic, naphthalenes, polynuclear aromatic hydrocarbons such as benzo(a)pyrene, and nicotine (ibid.). Carbon dioxide (CO_2) and carbon monoxide (CO) are

two gases which are produced in large quantities by burning tobacco (Keith and Tesh, 1965; George and Keith, 1967; Wynder and Hoffman, 1967), and may be suitable measures of the amount of smoke in the exposure cabinets. However, a pilot study, using a mass spectrophotometer, to determine the concentration of CO_2 in cabinets of rats during an experimental smoke-exposure study could not demonstrate a significant difference in CO_2 concentration between smoke-filled cabinet air and room air (authors unpublished observations). Consequently, only CO concentrations were measured in subsequent experiments. Direct determination of the density of particles of smoke in experimental exposure cabinets is another measure of smoke concentration. Combining measurements of CO concentration and particle density should allow the concentration of smoke in the vicinity of the rats to be quantified for both its gaseous and particulate phases.

CO is avidly incorporated into the haemoglobin molecule and forms carboxyhaemoglobin (COHb) (Ayers and Granelli Jr., 1960; Turino, 1981). The amount of CO produced by many brands of cigarette is known (Russell et al., 1975 a; Wald et al., 1981) as are the concentrations of COHb in the plasma of human smokers (Russell et al., 1973; Wald et al., 1977). Similarly, nicotine is a major constituent of both tobacco (Wynder and Hoffman, 1967; Akehurst, 1968) and tobacco smoke (Harlan and Moseley, 1955). The nicotine content of many brands of cigarette is known (Russell et al., 1975 b; Laboratory of the Government Chemist, 1973 to 1982) as are the concentrations of nicotine in the plasma of human smokers (Ashton et al., 1970; Russell et al., 1975 b and 1980). The concentrations of COHb and

nicotine in human smokers vary with the cigarette and rise as successive cigarettes are smoked (Russell et al., 1973; Wald et al., 1977) and so produce a range of plasma concentrations of between 1 and 13% COHb and 9 and 46 ng/ml nicotine (Russell et al., 1973, 1975 b, 1980; Gilman et al., 1980). Thus, measuring the concentration of COHb and nicotine in the plasma of experimental animals exposed to tobacco smoke should allow a degree of comparison with the 'smoke intake' of human smokers.

II. <u>Drugs and chemical agents</u>

A number of drugs and other chemical agents have been found to increase the amount of mucus-secreting tissue in the airways of experimental animals when observed by light and electron microscopy. The drugs have been given by a number of different routes of administration.

Isoprenaline (synonyms isoproterenol and isopropylnorepinephrine) is one of the most active sympathomimetic catecholamines which acts almost exclusively on β receptors (Gilman et al., 1980; British National Formulary, 1983). When given in 'high' doses of 40 or 100 mg/Kg daily for 6 or 12 days, subcutaneously to rats (Sturgess and Reid, 1972 and 1973; Jeffery, 1973; Jones and Reid, 1979) or at a lower dose of 4 mg/Kg for 6 days to pigs, intramuscularly (Baskerville, 1976) isoprenaline causes both epithelial secretory cell hyperplasia and hypertrophy and sub-mucosal gland hypertrophy. In the rat, the effect is dose related.

Pilocarpine is a naturally occurring alkaloid and cholinomimetic

which causes secretion of mucus from sub-mucosal glands (Florey et al., 1932). When given at a daily dose of 4 mg/Kg, subcutaneously to rats for 6 or 12 days, pilocarpine nitrate causes epithelial secretory cell hyperplasia and hypertrophy, and sub-mucosal gland hypertrophy (Sturgess and Reid, 1973). Electron microscopy of randomly-selected animals from the same study showed an increase in number of cells without secretory granules, many of which gave the impression of recent discharge of secretion (Jeffery, 1973).

Salbutamol (syn. albuterol) is a sympathomimetic drug which is used in aerosol form for asthma attacks of moderate severity (Gilman et al., 1980). When given subcutaneously at a dose of 100 mg/Kg to rats it caused a small and limited secretory cell hyperplasia (Jones and Reid, 1979; Reid and Jones, 1980). This result could not be confirmed by electron microscopy (Dr. P.K. Jeffery, unpublished observations).

Methacholine (acetyl-B-methylcholine) is a synthetic choline derivative with greater selectivity and duration of action than acetylcholine (Gilman et al., 1980). When given daily for 90 days to cats, intramuscularly at a dose of 3 mg/Kg, methacholine causes secretory cell hypertrophy and a hyperplasia which extends into the distal bronchioli (Kleinerman et al., 1976).

Chronic oral or intramuscular administration of the 'female hormone oestrogen (as ethanyloestradiol, oestradiol dipropionate, ethinyl ostradiol or stilboestrol) at daily doses of between 10 μg and 5 mg to guinea pigs initially produced histologically

'bronchitic' changes which were subsequently replaced by a stratified squamous epithelium (El-Heneidy et al., 1966; El-Ghazzawi et al., 1979).

Intratracheal instillation of the proteolytic enzymes papain and elastase induces 'emphysematous' changes in the lungs of experimental animals (Gross et al., 1964; Goldring et al., 1968; Pushpakom et al., 1970; Johanson et al., 1971, 1973; Kaplan et al., 1973; Sherter et al., 1974; Hayes et al., 1975). However, the studies did not produce the 'bronchitic' component of emphysema, an important feature of the disease in Man. Modification of the model has consistently produced a mucosal lesion in the airways of the hamster which resembles mucous cell metaplasia in Man (Christensen et al., 1977; Hayes and Christensen,1978). Only a single intratracheal installation of 2 mg/Kg (activity of 26.8 units/mg) of elastase was required to produce 'bronchitic' and 'emphysematous' changes.

Thus, a variety of drugs and chemical agents, many of which are not normally associated with the pathogenesis of bronchitis in Man, will produce many of the histological features of 'bronchitis' in experimental animals.

III. Animal Models

The gross pulmonary anatomy of many animal species has been described and light microscopic, and transmission and scanning electron microscopic studies of the airways have enabled identification of those species which might be suitable as animal models of bronchial disease in Man (reviewed by Jeffery, 1983 b). Anatomically, the horse has the most similar lung to Man (McLaughlin et al., 1961, a and b) and in fact the

donkey has been used as a model for muco-ciliary clearance in Man (Albert et al., 1969, 1971, 1974; Berger et al., 1978; Schlesinger, 1978, 1979). However, any large animal model of lung disease has both practical and economical limitations.

Fortunately, a variety of smaller animals are available. Many have been described in terms of their airway structure and ultrastructure including the rat (Reid, 1963, 1970; Kuhn and Finke, 1972; Jeffery and Reid, 1977), dog (Groniowski et al., 1972; Spicer, 1974; Drazen et al., 1982), pig (Baskerville, 1976), cat (de Haller, 1969; Jeffery, 1978), hamster (Nowell and Tyler, 1971; Homburger et al., 1978), and mouse (Leuchtenberger et al., 1960; Greenwood and Holland, 1972). However, the airway ultrastructure of some animals is very different to Man, making them suitable as models only for some aspects of lung research. For example, the goose does not have any submucosal glands in the trachea and has instead abundant mucous cells, many of which form shallow depressions or 'intraepithelial glands' (Jeffery, 1978). The goose would be useful in the study of a purely epithelial secretory cell population. The airways of cat, ferret and oppossum have sub-mucosal glands which extend into the bronchioli (Sorokin, 1965; Jeffery, 1978; Hyde et al., 1979; Jacob and Poddar, 1981; Tom-Moy et al., 1982). In fact the cat may be thought of as being naturally 'bronchitic' by human standards because it has numerous epithelial secretory cells in all extra- and intrapulmonary airways, and abundant submucosal glands which extend into the bronchioli (Jeffery, 1978). The vast mucous cell population of the cat is therefore particularly useful

in research involving mucus-secretion (reviewed by Richardson and Phipps, 1978). However, quantification of increases in secretory cell number after exposure of cats to irritants would be difficult in an epithelium already 'normally' highly populated with secretory cells. Conversely, the rat, mouse, hamster and rabbit have few epithelial secretory cells. In particular, the specific pathogen-free (SPF) rat has few airway epithelial secretory cells and has been recommended as being a suitable model of bronchial hyper-secretory disease in Man (Reid, 1963, 1970).

A small study of 10 patients dying of heart disease has failed to show sex differences in tracheal epithelial mucous cell number (Ellefsen and Tos, 1970). However, counts of tracheo-bronchial cytological preparations in women have shown that the numbers of mucous cells recoverable by 'brushing' exhibit cyclical changes during the menstrual cycle (Chalon et al., 1971). Animal studies support this finding and also suggest that there are sex differences in the tracheal epithelial secretory cell population in rats (Hayashi and Huber, 1977): the tracheal epithelium is thicker in male than in female rats. Female rats in dioestrus had a slightly higher mean number of epithelial secretory cells containing neutral glycoprotein (i.e. serous cells) than male rats. However, oestrus and proestrus females had a significantly greater number of serous cells than either males or dioestrus females. In addition, the number of secretory cells containing acidic glycoprotein (i.e. mucous cells) was consistently lower in female than in male rats. The differences were accentuated after exposure for 30 days to cigarette smoke (Hayashi et al.,

1978) when the smoke-induced increase in epithelial secretory cell number was greater in female than in male rats. However, in the females the increase was due to an increase in the number of serous cells whereas in males it was due to an increase in the number of mucous cells. The authors suggest that the higher proportion of mucous cells and thicker epithelium found in male rats might, if also true in Man, be important factors in the pathogenesis of chronic bronchitis. The female rat would not be the best animal to use in smoke inhalation studies because of the variation in secretory cell number during the menstrual cycle. Consequently, the male SPF rat was the experimental animal of choice for the studies to be presented herein.

In the rat, sub-mucosal glands are sparse and generally confined to the upper third of the trachea (Lamb and Reid, 1968). In fact sub-mucosal glands may be even less abundant in this region in some commercially available strains of rats than in other strains (author's unpublished observations). Therefore, it is the secretory cell population in the surface epithelium which almost completely contributes to the production of mucus for muco-ciliary transport in the rat. Consequently, the major response by the airways of the SPF rat to irritants such as SO_2 or whole cigarette tobacco smoke is a hyperplasia of surface epithelial mucus-secreting cells which can be quantitated by counting these cells along a given length of airway both before and after exposure to smoke (Reid, 1963; Lamb and Reid, 1968 and 1969 b).

Many of the gases, for example SO_2, which produce airway epi-

thelial secretory cell hyperplasia are unfortunately often both difficult and hazardous for the experimenter to use. Cigarette tobacco smoke is not easy to use but is less hazardous to the experimenter. Consequently many systems have been designed to deliver tobacco smoke to experimental animals (for example see Proetz, 1939; Dalhamn, 1963; Bretthauer et al., 1972; Wright, 1972; Betts and Betts, 1979). In addition, it has been shown that daily exposure of rats for as little as two to six weeks to cigarette tobacco smoke produces airway epithelial secretory cell hyperplasia in both extra- and intra-pulmonary airways (Lamb and Reid, 1969 b; Jeffery et al., 1982). Tobacco smoke also causes the appearance and subsequent increase in number of epithelial secretory cells in the distal bronchioli of experimental animals such as the rat. Consequently, counting mucus-secreting cells in the surface epithelium of the airways of SPF rats which have been exposed to whole cigarette tobacco smoke is a reproducible and quantitative technique against which the effect of other experimental procedures can be compared.

C. NOMENCLATURE AND STAINING OF AIRWAY SECRETORY CELLS

The secretory cells containing acidic mucin are thought to be equivalent to those cells described by early light microscopists as 'goblet' cells due to distension into a chalice-like shape by their secretory content (Schaffer, 1927). By electron microscopy, these cells have been shown to contain electron-lucent secretory granules (Rhodin, 1966). However, 'goblet' cells are not always distended by their secretory content and the term 'mucous' cells

is preferred (McCarthy and Reid, 1964; Jeffery and Reid, 1975; Jones and Reid, 1978; Jeffery et al., 1982). The secretory cells containing neutral mucin seen by light microscopy are thought to be equivalent to those cells seen by electron microscopy which contain electron-dense secretory granules. The latter cells have been termed 'serous' cells due to their resemblance, by electron microscopy, to the serous cells of the sub-mucosal glands (Jeffery and Reid, 1975). The two groups of secretory cells (mucous and serous) can in fact each be further sub-divided into different types on the basis of the complex-carbohydrate content of their secretory granules (Spicer et al., 1980). In general, the secretory granules of the sub-groups of mucous cells all contain a form of intracellular glycoprotein, whereas not all of the secretory granules of the serous cell sub-groups contain glycoprotein. The present thesis is concerned primarily with airway mucus-secreting cells which can be detected by mucus-specific stains and these sub-groups will not be discussed further.

I. Alcian Blue

The phthalocyanine dye Alcian Blue (AB) was described as early as 1948 by Haddock and was first used as a stain for acidic glycoproteins by Steedman in 1950. Steedman's technique was later improved (Lison, 1954; Mowry, 1956). The copper core of the dye provides the blue colouration, but copper phthalocyanine itself is insoluble and is made soluble by the addition of isothiourium groups which produce up to four positively charged groups on each dye molecule and allows polyanions to be stained in tissue sections (Abrahart, 1968). The variant of Alcian Blue currently commercially

available is AB 8GX (Scott et al., 1964; Quintarelli et al., 1964 a and b).

The mechanism or mechanisms suggested for the staining of glycoproteins by Alcian Blue are many and various (Spicer, 1960; Goldstein, 1961 and 1962; Stoward, 1963; Yamada, 1964; Dorling, 1969). However, electrostatic attraction is thought to be the most likely explanation (Scott et al., 1964). For example, attraction between polyanions, such as acidic glycoproteins, and positively charged groups on the dye molecule ('Coulombic' attraction) would lead to the formation of 'salt-links' between the two. However, 'weak' bonds (such as London-van der Waals forces), hydrogen and 'hydrophobic' bonds may also contribute to the binding process.

The staining properties of acidic glycoproteins by Alcian Blue can be modified by alterations in the acid pH of the dye in the range 0.5 to 2.6 (Jones and Reid, 1973 I and II). The effect is linked to the ionisation or dissociation of the acid groups in glycoproteins, a measure of which is the dissociation constant (or pK_a value). The pK_a value for the carboxyl radicle of sialic acid is 2.6 (Svennerholm, 1956), whereas the pK_a value of the sulphate groups of sulphomucins may vary because sulphuric acid has two pK_a values (one dissociation being almost immediate at an extremely acid pH, the other at a value of 1.9). Thus maximum staining of each acidic group takes place at distinct pH values where the different acidic radicles dissociate and bind to the dye.

II. Periodic Acid-Schiff

The periodic acid-Schiff (PAS) technique for staining muco-

polysaccharides including mucin was developed in the 1940's (McManus, 1946; Hotchkiss, 1948). Subsequent developments of the technique have allowed demonstration of glycoproteins (see Pearse, 1972).

Periodic acid is an oxidant and will break the carbon-carbon bonds of polysaccharides converting them to high molecular weight polyaldehydes without hydrolysing nucleic acids. The polyaldehydes may then be stained with Schiff's reagent, a fuchsin-sulphurous acid, to form coloured compounds in the tissue (Hotchkiss, 1948). Of importance to the studies reported in the present thesis is the finding that the sialic acid moiety of acidic glycoproteins will also stain with PAS. However, AB-positive acidic glycoproteins which are also PAS positive will not react with PAS when stained first with AB.

There are several variations in the method of preparation of Schiff's reagent (Pearse, 1960). The most common of these is that of de Tomasi (1963) which is the method used by the suppliers, R.A. Lamb, of the reagent used in the studies presented herein.

III. Alcian Blue/Periodic Acid-Schiff

Modifications of Steedman's (1950) method of Alcian Blue staining by Lison (1954) and Mowry (1956, 1963) lowered the pH of the staining solution to between 2.4 and 2.6 to impose selectivity for staining acidic glycoproteins and allowed neutral carbohydrate components to be sequentially stained with PAS. Sequential staining by Alcian Blue and periodic acid-Schiff (AB/PAS) was indicated by the work of Ritter and Oleson (1950), but was first used for the demonstration of

both the neutral and acid components of glycoproteins in the 1950's (Mowry and Winkler, 1956; Mowry and Morard, 1957). The epithelial glycoproteins (Fig. 1) of interest in these studies are those which are 'neutral' (i.e. containing sugars without acidic radicles) and those which are acidic (i.e. containing sugars with acidic radicles). There are two main types of glycoprotein containing acid radicles: those containing sialic acid (N-acetylneuraminic acid) are termed 'sialomucins', and those containing sulphate esters are termed 'sulphomucins' (Spicer, 1965). The sialomucins may be sub-divided into those which are either sensitive or resistant to removal by sialidase (i.e. neuraminidase) (Warren and Spicer, 1961). Consequently, four main types of epithelial glycoprotein may be demonstrated histochemically;

1. Neutral
2. Sialomucin sensitive to sialidase
3. Sialomucin resistant to sialidase
4. Sulphomucin

Staining with Alcian Blue (AB) at different pH values and periodic acid-Schiff (PAS) can be used to demonstrate neutral mucin, sialomucin and sulphomucin:

Mucin	AB (pH 1.0)	AB (pH 2.6)	PAS
Neutral	-	-	+
Sialo-	-	+	+
Sulpho-	+	+	+

PAS will stain all three types of mucin, but prior staining with Alcian Blue will make most of the sialo- and sulpho-mucins unavailable for staining with PAS. In these studies,

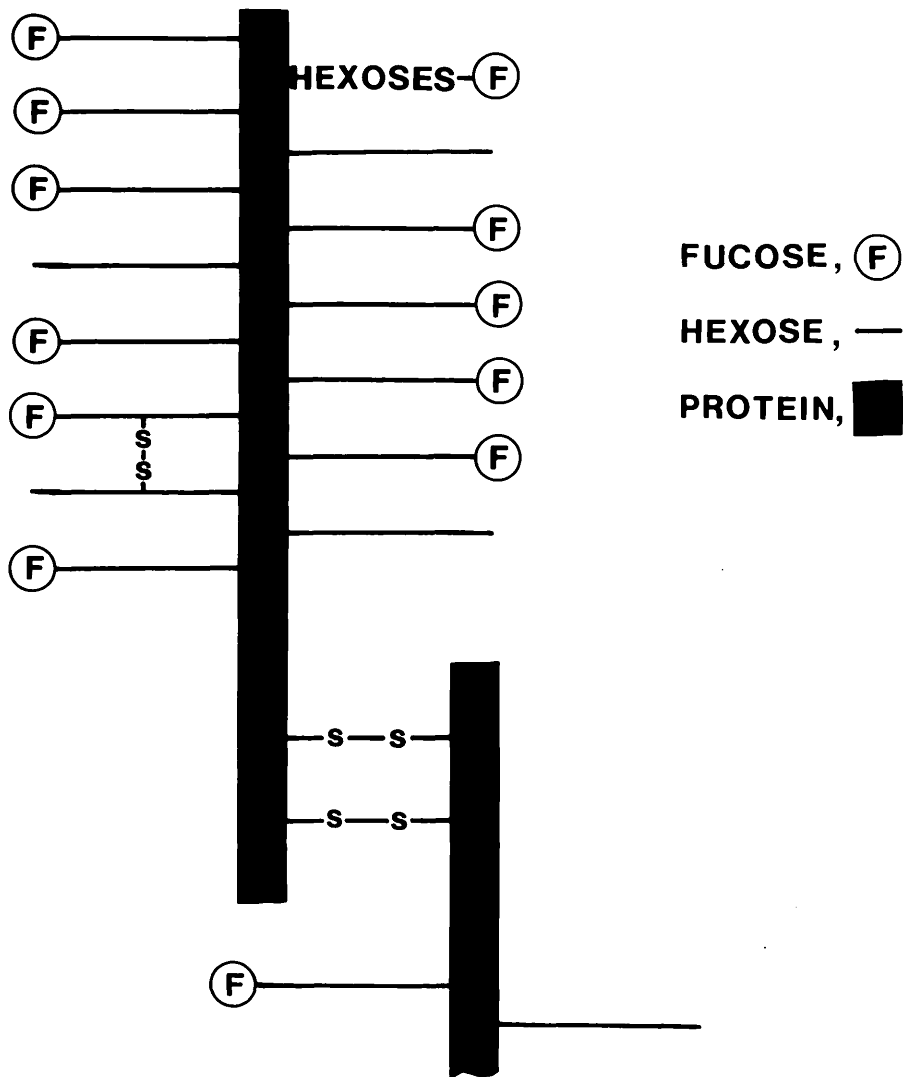


Fig. 1. Mucus-glycoprotein molecule.

Mucus glycoproteins are long, high molecular-weight molecules comprised of many sub-units, each sub-unit joined by di-sulphide bridges (-S-S-). The protein core has sugar side-chains and other groups attached at one end. Hexose sugars comprise the greater proportion of the side chains with fucose the predominant terminal sugar (Meyer et al., 1977; Kent, 1978; Meyer and Silberg, 1978; Schrager and Cumming, 1978).

the shift in the epithelial secretory cell population from a mixture of types containing either neutral or acidic mucin to a predominance of those cells containing acidic mucin after tobacco smoke exposure is of interest as changes in the chemistry of mucus may affect its viscoelastic properties. AB/PAS has already been shown to stain distinct histochemical types of airway secretory cells in the rat (LeBlond, 1950; McCarthy and Reid, 1964 a; Jones and Reid, 1978) the mouse (McCarthy and Reid, 1964 a) and Man (McCarthy and Reid, 1964 b; de Haller, 1969). Staining with Alcian Blue at pH 2.6 was chosen for the studies presented here because it would stain the majority of the acidic glycoprotein in the secretory cells. In combination with PAS the following types of glycoprotein in the secretory granules of the cells would be demonstrated.

Glycoprotein	Predominant Colour
Neutral (AB-ve, PAS + ve)	Red
Acidic (AB + ve, PAS-ve)	Blue
Mixtures of neutral and acidic (AB + ve, PAS + ve)	Purple

D. NICOTINE

Inhalation of tobacco smoke has been implicated in the aetiology of bronchitis in Man and has been shown consistently to produce 'bronchitic' changes in the airways of experimental animals (see above). However, few of the individual components of tobacco smoke have been investigated in terms of their effects on the mucus-secreting tissue of the airways.

Nicotine, for example, is a naturally occurring alkaloid which was first isolated from the leaves of the tobacco plant Nicotiana

tobacum and is a major component of both tobacco (Wynder and Hoffman, 1967; Akehurst, 1968) and inhaled tobacco smoke (Harlan and Mosely, 1955). Nicotine may in fact be one of the most important factors in the physiological dependance of the smoker to his/her smoking habit (Jarvik et al., 1970; Jarvik 1977; Stepney, 1978). Specifically, individual smokers have been found to maintain a 'personal' plasma nicotine concentration despite being given cigarettes of differing nicotine yields (Ashton and Watson, 1970; Russell et al., 1973, 1980; Schmidt, 1976). Individual smokers appear to be able to maintain a 'desired' nicotine level by adjusting their intake of nicotine by varying the frequency of 'puffing' a cigarette (or cigar), the 'draw strength' of each 'puff' and the number of cigarettes (or cigars) smoked; non-inhaling cigar smokers absorb nicotine through the oral mucosa (Armitage and Turner, 1970). It has therefore been suggested that cigarettes with a 'high tar' content, and thus a high nicotine content (Bentley, 1975), may be 'less harmful' than 'low tar' cigarettes (especially those with 'ventilated' filters) for continual smokers because fewer cigarettes have to be smoked to reach a 'desired' nicotine level and they also have a lower yield of the poisonous gas carbon monoxide (Russell et al., 1973, 1975 a and b; Wald et al., 1977, 1981; Wynder and Hoffman, 1979). Artificially raising the nicotine content of cigarettes by the further addition of nicotine has consequently been discussed as a way of producing a 'safer cigarette'. In addition, chewing gum containing nicotine and snuff have both been advocated as possible aids to smokers wishing to break the smoking habit (Brantmark et al., 1973; Ferno et al., 1973; Russell et al., 1976, 1977; Kozlowski et

al., 1975, 1982; Fee and Stewart 1982; McNabb et al., 1982). The 'average' plasma nicotine concentration in subjects who have chewed experimental nicotine containing gum is 11 ng/ml for a 2 mg gum and 23 ng/ml for a 4 mg gum (Russell et al., 1976; McNabb et al., 1982). These concentrations are lower than the 'average' plasma nicotine concentration of 30 ng/ml found in human cigarette smokers (Russell et al., 1975 b, 1980). However, in one subject who had chewed a 4 mg nicotine gum ad libitum, plasma nicotine concentrations were as high as 81 ng/ml; especially interesting because Man is thought to be particularly intolerant of nicotine: the lethal dose of nicotine in Man has been calculated as being about 60 mg (Taylor, 1980) (i.e. about 1 mg/Kg). However, despite the current interest in nicotine as a tobacco additive or as a gum substitute for cigarette smoking, the effect of nicotine alone on the lung is not known. It was therefore the purpose of part of the work presented herein to determine experimentally the effect of nicotine on the number of airway epithelial secretory cells.

E. INHIBITION OF TOBACCO SMOKE-INDUCED AIRWAY CHANGES

Preceding sections of the introduction have shown that irritants such as tobacco smoke, gases (e.g. SO_2), and chemical aerosols (e.g. H_2SO_4) as well as drugs(e.g. isoprenaline) and chemical agents (e.g. elastase) produce 'bronchitic' changes in experimental animals, and some in Man also. Much research is also aimed at investigating possible cures for lung diseases such as chronic bronchitis. For example, three drugs have been investigated experimentally in regard to their potentially 'beneficial' effects on tobacco smoke-induced secretory cell hyperplasia; vinblastine, phenylmethyloxadiazole and indomethacin.

I. Vinblastine

Vinblastine (vincale coblastine) is one of the naturally occurring 'vinca alkaloids' derived from the periwinkle plant (Vinca rosea) which is used clinically in the treatment of a number of human malignant diseases including certain cancers and Hodgkin's disease (British National Formulary, 1983). The vinca alkaloids are 'anti-mitotic' drugs in that they are cell-cycle-specific agents which block mitosis at metaphase, probably by inhibiting the re-polymerisation of microtubules which are necessary for continuation into telophase (Gilman et al., 1980; Rollason et al., 1983). Experimentally, vinblastine sulphate (given by intraperitoneal injection at a dose of 0.05 mg/Kg, daily for 21 days to rats) completely inhibited tobacco smoke-induced secretory cell hyperplasia in the airways of the lung and reduced the number of secretory cells to below control values in the trachea (Jeffery et al., 1984 a). The results are preliminary and need verification. Because there was no accumulation of metaphase-arrests with time, vinblastine (two experiments) was thought to cause inhibition of cell hyperplasia by a mechanism other than the inhibition of mitosis (P.M. Evans, unpublished observations).

II. Phenylmethyloxadiazole (PMO)

PMO is structurally related to its parent compound oxolamine citrate (3-phenyl-5-diethyl-aminoethyl- 1, 2, 4, oxadiazole), an anti-inflammatory and anti-tussive agent which is a component of some cough mixtures commercially available in Italy (Corbella, 1960; Deidda, 1960; Silvestrini and Pozzatti, 1960; Tropia, 1961; Dahlgren and Dalhamn, 1966;

Dalhamn and Raud, 1968).

a. Anti-ciliostatic effect

Experimentally, tobacco smoke has been found to reduce the rate of muco-ciliary transport in a variety of preparations (reviewed by Wanner, 1977). Specifically, tobacco smoke has been shown to be ciliostatic (Dalhamn, 1959, 1964; Dalhamn and Rylander, 1965). When cigarette smoke was passed over the exposed tracheal surface of rats *in situ*, through the trachea of rabbits and cats *in situ*, or over extirpated human tissue *in vitro*, the cilia stopped beating in the majority of preparations. However, when cats were given oxolamine citrate perorally the smoke from more cigarettes than would normally produce ciliostasis had to be administered before their tracheal cilia would stop beating, and the time taken for the recovery of ciliary activity was also reduced (Dalhamn, 1966). Interestingly, when oxolamine citrate was incorporated into cigarette tobacco (as 5% by weight), more smoke had to be administered from the cigarettes before tracheal ciliostasis was achieved than from cigarettes without incorporated drug (Dalhamn, 1969). However, in both studies it was unclear whether the anti-ciliostatic action of oxolamine citrate was due solely to a direct effect on cilia or whether there was an additional effect on mucus secretion.

When each of the three components of the oxolamine citrate molecule, namely citric acid, diethylamine citrate and phenylvinyloxadiazole (PVO) were incorporated into cigarette tobacco smoke and their effects on tracheal

ciliostasis assessed, only PVO was anti-ciliostatic (Dalhamn and Rylander, 1971). However, when PVO was found to be chemically unstable with a tendency to polymerise, a stable derivative, PMO, was examined and found to be equally anti-ciliostatic, especially at a concentration of 2% by weight of tobacco (ibid).

b. Inhibition of secretory cell hyperplasia

The effect of PMO on experimentally induced 'bronchitic' change has been studied (Jones et al., 1972, 1973; Jones and Reid, 1978; Jeffery and Reid, 1981). PMO was incorporated as 2% by weight into cigarette tobacco, the smoke delivered to rats, and its effect on airway changes compared to those found in rats which had been exposed to tobacco smoke without incorporated PMO. Rats which had been given smoke with added PMO showed less immediate distress after exposure, fewer cells in mitosis, an increase in the number of ciliated cells, less thickening of the tracheal epithelium and no increase in secretory cell number in the trachea. However, PMO did not inhibit secretory cell hyperplasia in the airways of the lung and did not prevent the shift in the secretory cell population to a predominance of cells containing acidic glycoprotein.

Although the effect of PMO was limited mainly to the extra-pulmonary airways, the results of the studies showed that a drug could be used experimentally to inhibit tobacco smoke-induced airway changes. It might be suggested that the effect of PMO was limited to the trachea by its inhaled route of administration because PMO is thought to be bound to the particulate phase of tobacco smoke (Dalhamn and Rylander,

1971) and thus would be deposited mainly in the trachea.

c. Mechanism of action

PMO has been found to have anti-inflammatory properties in the airways of guinea pigs (inflamed with acrolein aerosol) when given either orally or by intraperitoneal injection (Dahlgren and Dalhamn, 1972). PMO, when given in vivo to rats, has been shown to block the increased rate of glycoprotein secretion as a response to tobacco smoke exposure, whilst in vitro it has been found to reduce the rates of both glycoprotein discharge and precursor incorporation into the intracellular glycoproteins of the sub-mucosal glands of unexposed rats (Coles et al., 1979). In fact, it has been suggested that the anti-ciliostatic effect of PMO may be due to the secretion of a less viscid mucous which may allow the cilia to beat more freely (Dahlgren and Dalhamn, 1972). However, although PMO has been shown to affect mucus synthesis and secretion and has also been found to 'stabilize' red blood-cell membranes (Brown and Tong, 1971), the exact pharmacological or biochemical mechanisms of action of PMO are not understood. PMO appears to act directly on bronchial tissue as opposed to altering the characteristics of inhaled smoke because its anti-ciliostatic and anti-inflammatory effects have been shown to be intact when given orally or by intraperitoneal injection (see above).

III. Indomethacin

Indomethacin was first synthesized by Shen and his co-workers in 1963 and is a non-steroidal drug (as is PMO) which has anti-inflammatory, antipyretic and mild analgesic properties when tested in classical experimental systems such as the rat paw-oedema and rectal temperature assays (Winter

et al., 1963). In each assay indomethacin was found to be many times more potent than phenylbutazone, aminopyrene, aspirin or hydrocortisone. In addition, when indomethacin was used in conjunction with the steroidal anti-inflammatory drug dexamethasone, the action of the steroid was enhanced (ibid).

Preliminary trials in the 1960's indicated that indomethacin could be used clinically in the treatment of many classically inflammatory diseases such as rheumatoid arthritis, osteo-arthritis and ankylosing spondylitis as well as in cases of fever such as glandular fever and Reiter's disease (Hart and Boardman, 1963; Smyth, 1965; Thompson and Percy, 1966; Pitkeathly et al., 1966; Pinals and Frank, 1967). Today, indomethacin has become a well known drug which may be used clinically in preference to other, more toxic drugs in the treatment of inflammatory diseases (Dickson and English, 1982).

a. Inhibition of secretory cell hyperplasia

Indomethacin has been investigated with respect to its inhibitory effects on tobacco smoke-induced airway epithelial secretory cell hyperplasia (Greig et al., 1980). Rats were exposed to cigarette tobacco smoke and given concurrent intraperitoneal injections of indomethacin (2 and 4 mg/Kg). The number of secretory cells was assessed in the trachea and at a number of intrapulmonary airway levels. In a dose-related way indomethacin inhibited tobacco smoke-induced secretory cell hyperplasia in the trachea and at four airway levels in the lung: the inhibitory effect was greatest in the most distal, intra-pulmonary airways.

b. Mechanism of action

Anti-inflammatory drugs, including indomethacin, have been found to inhibit the biosynthesis of prostaglandins; their 'classical' mechanism of action (Vane, 1971; Ferreira et al., 1971; Flower et al., 1972; Davis and Horton, 1972; Bhattercherjee and Eakins, 1972; Uotilla et al.,1981). In addition, indomethacin and a number of other anti-inflammatory drugs have been found to affect mucin synthesis and secretory processes as well as affecting cell proliferation and maturation (Lukie and Forstner, 1972; Main and Whittle, 1975; Bustos et al., 1975; Sluszkiewicz and Pawlikowski, 1980).

IV. Drugs used in the present study

In order to expand the potential of the inhibitory effects of vinblastine, PMO and indomethacin a number of drugs are assessed in regard to their ability to inhibit tobacco smoke-induced secretory cell hyperplasia.

a. Non-steroidal anti-inflammatory drugs

Indomethacin, the 'reference standard', and two further drugs are examined which have differing anti-inflammatory potencies. Aspirin (acetylsalicylic acid) and flurbiprofen are two drugs with anti-inflammatory, antipyretic and analgesic properties (British National Formulary, 1983). Aspirin was chosen because it is an 'established' and widely used drug which was first introduced into medicine in 1899 by Dreser: in fact, the antipyretic properties of willow bark (*Salix alba*) were known to the ancients. In contrast, flurbiprofen (a derivative of phenylalkanoic acid) is relatively a newly introduced non-steroidal, antirheumatic and anti-inflammatory drug (Chalmers et al., 1972, 1973). Both aspirin and flurbi-

profen inhibit experimentally-induced inflammatory reactions such as the rat paw oedema assay (Ishii et al., 1975) as well as inhibiting prostaglandin biosynthesis (Collins et al., 1975). The inhibition afforded by flurbiprofen in both assays was over 14 times greater than indomethacin which in turn was over 18 times greater than aspirin. Thus, the three non-steroidal anti-inflammatory drugs flurbiprofen, indomethacin and aspirin were used in the studies herein to determine whether they might exhibit a similar range of potencies in inhibiting tobacco smoke-induced secretory cell hyperplasia.

b. Steroidal anti-inflammatory drugs

Dexamethasone, prednisolone and hydrocortisone are three 'corticosteroid' drugs which are extensively used in hormone replacement therapy, in the treatment of inflammatory and allergic disorders and are also given to patients in 'shock' (Gilman et al., 1980; British National Formulary, 1983). Hydrocortisone (synonym, cortisol) is a naturally occurring corticosteroid in that it is the main glucocorticoid hormone secreted by the adrenal cortex (Guyton, 1977). Dexamethasone and prednisolone are two synthetic corticosteroid drugs which are both more potent than hydrocortisone in terms of anti-inflammatory properties and anti-prostaglandin synthesis activity (Nijkamp et al., 1976; Flower, 1980). Dexamethasone is over 5 times more potent than prednisolone, which in turn is over 4 times more potent than hydrocortisone. Thus, using these three steroidal anti-inflammatory drugs might again produce a range of inhibition of secretory cell hyperplasia which may be associated with their relative anti-inflammatory potencies.

c. Mucolytic agents

'Mucolytic' agents are so-called because they are claimed to reduce the viscosity of mucus making it easier for patients to dislodge and remove it by cough (Lieberman, 1970). However, of the many substances which have been reported to be mucolytic only a very few are useful and in many, their effectiveness is limited or held in doubt (Gilman et al., 1980; British National Formulary, 1983).

N-acetylcysteine (NAC), S-carboxymethylcysteine (SCMC), bromhexine and pancreatic dornase are four mucolytic agents which are currently used clinically (British National Formulary, 1983).

N-acetylcysteine is a naturally occurring substance which is used clinically in two diverse ways: as an oral and parenteral mucolytic drug (Gilman et al., 1980; British National Formulary, 1983) and as the treatment of choice to counter paracetamol poisoning (Prescott et al., 1979; Ho and Beilin, 1983). NAC has been found to decrease the viscosity of mucus both in vitro (Sheffner, 1963; Sheffner et al., 1964; Hurst et al., 1967; Lieberman, 1968; Hirsch et al., 1969; Lightowler and Lightowler, 1971; Marriott and Richards, 1974; Martin et al., 1980) and in patients (Paez et al., 1966; Hirsch and Kory, 1967; Hurst et al., 1967; Kory et al., 1968; Hirsch et al., 1970; Pulle et al., 1970). Compounds such as NAC which contain R-S-S-R or R-SH configurations are thought to 'thin' mucus by breaking the disulphide bonds which contribute to the tertiary structure of glycoproteins (Fig. 1) and then attach them-

selves to the displaced glycoprotein chains (Havez et al., 1970). However, whether or not NAC treatment is of benefit to patients and whether or not it reduces the volume of sputum produced is controversial because few prospective studies have included proper controls or assessed in follow-up. Of the more closely controlled studies, as many suggest that NAC has very little effect (e.g. Kory et al., 1968; Hirsch et al., 1970; Pulle et al., 1970) as indicate clinical improvement (Aylward et al., 1980; Chodosh, 1980; Multicenter Study Group, 1980). However, in most studies there is improved self-evaluated patient 'well-being' with few serious side-effects reported.

S-carboxymethylcysteine is, like NAC, a cysteine derivative. However, unlike NAC, it does not have a terminal SH group or a disulphide linkage. Experimentally, SCMC does not reduce the viscosity of mucus or increase the rate of muco-ciliary transport and in this respect is similar in its lack of action to other 'blocked' sulphydryl compounds (Martin et al., 1980). Clinical trials with SCMC have failed to show whether or not SCMC is of benefit to patients with hypersecretory airways disease (see Richardson and Phipps, 1978). Bromhexine is a naturally occurring alkaloid extracted from Adhatoda vasica leaves which has been shown to reduce the viscosity of mucus (Burgi, 1965). However, the clinical use of bromhexine is controversial: at best, some patients may benefit from treatment (see Richardson and Phipps, 1978). Proteolytic enzymes will digest mucus and deoxyribonucleases such as bovine pancreatic dornase (BPD) will make purulent sputum less viscid by breaking

down nucleic acid (Salomon et al., 1954; Charman and Reid, 1972; Dulfano et al., 1971). Again, the evidence that enzyme treatment improves patients is controversial (see Richardson and Phipps, 1978). Of the four 'mucolytic' agents described above, NAC would appear to be truly mucolytic, may benefit patients and has low toxicity. NAC was therefore chosen for study because it is another class of drug which might inhibit TS-induced secretory cell hyperplasia by a mechanism different to that of the anti-inflammatory drugs.

F. REVERSIBILITY OF BRONCHITIS AND SECRETORY CELL HYPERPLASIA

I. Man

In Man there is evidence of recovery from cigarette smoking-related lung disease if the smoking habit is broken before irreversible changes occur. Retrospective studies have shown that the life expectancy of ex-smokers increases with the number of years of abstinence (Hammond, 1965). One of the first detailed studies showed that in a group of age-matched male doctors which included non-smokers, ex-smokers and smokers there was 20 per cent less mortality in those who had stopped compared with those who had continued to smoke (Doll and Hill, 1964; and see Royal College of Physicians, 1971).

Lung function is generally impaired in chronic cigarette smokers (U.S. Surgeon General's Advisory Committee on Smoking and Health, 1964; U.S. Department of Health, Education and Welfare, 1968) with reduced gas transfer and consequent hypoxaemia (Streider and Kazemi, 1967; Streider et al., 1968). However, when most young smokers stop smoking their lung function has been found

to return to normal (Krumholz et al., 1964, 1965; Hammond, 1965; Wilhelmsen, 1967; Peterson et al., 1968; Streider and Kazemi, 1967; Streider et al., 1968; Fletcher and Horn, 1971). The symptoms of cough and expectoration usually abate rapidly in those who stop smoking (Hammond, 1965; Wilhelmsen, 1967; Peterson et al., 1968). In fact, when patients with moderately severe airways obstruction stop smoking there may be a dramatic reduction in breathlessness and cough, despite years of smoking (Fletcher and Horn, 1971). However, when patients with advanced bronchitis and emphysema stop smoking the damage to their lungs may be irreversible and so there is only a slight improvement in breathlessness, although the distressing cough may be relieved (Burrows et al., 1969).

Pulmonary epithelial permeability has been found to be increased in otherwise healthy smokers. There is a significant improvement in this defect in as little as 24 hours after cessation of smoking with a maximum improvement at seven days after cessation which is maintained for at least a further 14 days (Minty et al., 1981). However, the maximum value for recovery of permeability is still about 40 per cent less than non-smokers.

The suggestion that muco-ciliary clearance is reduced in human smokers and bronchitics is controversial. Studies of tracheobronchial clearance are based upon direct or indirect observation of the movement of particles placed on the tracheal mucosa (for example see Santa Cruz et al., 1974) or on the deposition pattern and clearance rates of inhaled radioactive aerosols (for example see Camner et al., 1973). Controversy surrounds the latter type of study, in particular, because the clearance

of inhaled particles from the lungs is affected by the initial deposition pattern, which in turn is dependent upon particle size and the flow characteristics of the airways (Muir, 1972; Agnew et al., 1981). Consequently, mucociliary clearance in patients with chronic bronchitis has been variously reported as being increased (Thomson and Pavia, 1974), unaltered (Luchsinger et al., 1968; Pavia and Thomson, 1970; Pavia et al., 1970) or reduced (Toigo et al., 1963) when compared to 'normal' controls. However, one of the better controlled studies (Lourenço et al., 1971) showed that healthy, non-smokers had the fastest clearance rates, followed by smokers with 'mild' bronchitis. The slowest rates were found in patients with chronic bronchitis and bronchiectasis. However, even here the pattern was not clear cut. The clearance rate of ex-smokers improves following cessation of smoking for about three months (Camner et al., 1973). A small human study has shown that the airway epithelial mucous cell hyperplasia seen by histology in smokers is reversed to the normal after about two years of abstinence from smoking, providing that severe airway changes have not occurred (Bertram and Rogers, 1981).

II. Experimental animals

A few studies have shown that recovery of airways mucus-secreting tissue takes place in the lungs of experimental animals. In an early study on the recovery of various tobacco smoke-induced changes in mice after long-term exposure, Leuchtenberger and co-workers (1960) found that there was a reversal of severe histologically 'bronchitic' changes to

normal in many animals a few months after cessation of exposure. Baskerville (1976), studying the effect of isoprenaline on the main bronchi of pigs killed at intervals up to three months after the last injection found that submucosal gland hypertrophy was returning to normal after four weeks and mucous cell hyperplasia recovered after two months. In the rat, isoprenaline-induced secretory cell hyperplasia is reduced to 22% of its original value 12 weeks after the last injection (Jones and Reid, 1982). Lamb and Reid (1968), studying the airways of rats which had been exposed to 400 ppm sulphur dioxide for three weeks and then killed up to 35 days after cessation of exposure, found that goblet cell numbers were still increased, and the change in type of intracellular mucus persisted, for at least 5 weeks after exposure. Jones (1978) found changes towards normal in the proportions of secretory products in the epithelial secretory cells in the trachea and extra- and intra-pulmonary bronchi of rats nine days after cessation of exposure to cigarette smoke.

III. Accelerated recovery after drug treatment?

In Man, no study has been undertaken to determine whether drug treatment will speed the reversal of airway changes after the smoking habit is broken. Similarly, in experimental animals, "reversal of the bronchitic response by administration of anti-inflammatory agents (has) not yet been explored" (quoted from Reid and Jones, 1983, p. 376).

G. PURPOSE OF STUDIES

The main purpose of the experimental studies described herein may be divided into three main sections. In each study, the numbers

of secretory cells (staining for neutral and acidic mucin) will be assessed in the trachea and at a number of intrapulmonary airway levels.

1. Phenylmethyloxadiazole (PMO) and indomethacin are two non-steroidal anti-inflammatory drugs which have been found to inhibit cigarette tobacco smoke-induced secretory cell hyperplasia when given concurrently with the smoke. In order to confirm and extend the scope of these studies, the effect on tobacco smoke-induced secretory cell hyperplasia of a number of steroidal (dexamethasone, prednisolone and hydrocortisone) and non-steroidal (indomethacin, flurbiprofen and aspirin) anti-inflammatory drugs and a mucolytic agent (N-acetylcysteine) are assessed after concurrent smoke and drug administration.
2. The reversal of 'bronchitic' changes after cessation of irritation is of interest academically, clinically and also to smokers who wish to break the smoking habit. Consequently, the time taken for the number of secretory cells in tobacco smoke-induced secretory cell hyperplasia to return to control values after cessation of smoke exposure is determined. The effect of the most potent drugs from the 'concurrent' smoke and drug studies on the 'normal' recovery time is then assessed.
3. Nicotine is of interest in that it is a major constituent of tobacco smoke as well as being currently considered as both an important factor in the production of a 'safer' cigarette (which may have a 'high' nicotine content) and, when incorporated into chewing gum, as an aid to breaking the smoking habit. However, the effect of nicotine alone

on bronchial tissue is unknown. The effect of exogenously administered nicotine on the airway secretory cell population is therefore examined.

In addition to the main studies outlined above, measurements are made of the concentrations of carbon monoxide and smoke particles in the exposure cabinets and of the plasma concentrations of nicotine (nicotine and smoke-exposure studies) and carboxyhaemoglobin (smoke-exposure studies) in the rats. The values obtained should allow for both comparison of effects with other studies and possible extrapolation to Man.

H. SUMMARY

1. Many of the histological hallmarks of chronic bronchitis in Man can be reproduced in experimental animals. These features include an increase in both the number (i.e. hyperplasia) and the size (i.e. hypertrophy) of mucus-secreting cells throughout the extra- and intra-pulmonary airways both in the surface epithelium and in the sub-mucosal glands, resulting in gland hypertrophy. Possibly the two most important changes are the appearance and subsequent hyperplasia of secretory cells in the distal intrapulmonary airways and the shift in the secretory cell population to a predominance of those cells containing acidic glycoprotein often at the 'expense' of those cells containing neutral glycoprotein.
2. A variety of drugs, (e.g. isoprenaline), gases (e.g. sulphur dioxide) and chemicaal aerosols (e.g. sulphuric acid) can be used to produce histologically 'bronchitic' features in experimental animals. In particular, whole cigarette tobacco smoke has been widely used to consistently produce 'bronchitic'

changes in a variety of animal species and many automatic, experimental smoke exposure systems are available for use. However, the 'dose' of smoke given to animals is not generally measured or standardised and so prevents both comparison between experimental studies and the possible extrapolation of effects to Man.

3. A variety of animal species are available for use in the experimental study of bronchitis with the airways of many being characterised anatomically and morphologically. However, certain species are not ideal for the experimental induction of 'bronchitic' airway changes due to their size (e.g. the donkey) or because the morphology of their airways is suited to more specialised studies (e.g. the normally histologically 'bronchitic' cat is well suited to studies of mucus secretion). Of the remaining small animal species, the specific pathogen free (SPF) rat has a well documented airway morphology which can be made histologically 'bronchitic' by the administration of many agents including cigarette tobacco smoke. The rat has sparse sub-mucosal gland and so the major response by the airways to irritants or other agents **is** epithelial secretory cell hyperplasia which can be quantified by counting the number of these cells before and after treatment.

4. The mucus-secreting cells of the airways can be demonstrated histologically in light microscopic sections by specific staining of their intracellular mucin. Differential staining with the combined Alcian blue (at pH 2.6) and periodic acid-Schiff technique (AB/PAS) allows the secretory cells to be divided into at least three groups on the basis of their intracellular mucin. Neutral mucin stains red (i.e. AB-ve, PAS + ve), acidic mucin stains blue (i.e. AB + ve, PAS-ve), and

mixtures of neutral and acidic mucin within a single cell stain purple (i.e. AB + ve, PAS + ve).

5. Nicotine is a major constituent of tobacco and tobacco smoke and is an important factor in the smoking habit. Consequently, the possibility that it may be given to habitual smokers in nicotine-containing chewing gum or snuff as an aid to breaking the smoking habit or to reduce cigarette consumption by raising its concentration in the cigarettes of continual smokers is receiving considerable attention. However, although nicotine is a potent pharmacological agent, its effect on bronchial tissue, particularly on the number of secretory cells **is unknown.**

6. Experimentally, tobacco smoke is ciliostatic. This effect can be delayed by the non-steroidal anti-inflammatory drug, phenylmethyloxadiazole (PMO), both when the animal is pre-treated perorally with PMO and when PMO is incorporated into cigarette tobacco and the smoke containing PMO is administered to the experimental preparation. Subsequent studies in which cigarette tobacco smoke containing PMO was delivered to rats in vivo have shown PMO to inhibit tobacco smoke induced tracheal epithelial secretory cell hyperplasia. However, although PMO is known to affect mucus synthesis and secretion and 'stabilise' the cell membranes of erythrocytes, its precise mechanisms of action are not known. Indomethacin is another non-steroidal anti-inflammatory drug which exhibits the classical inhibition of prostaglandin biosynthesis of other anti-inflammatory drugs. Indomethacin when given concurrently by intraperitoneal injections to rats exposed to tobacco smoke also inhibits epithelial secretory cell hyperplasia in the trachea and throughout the intra-pulmonary airways. The 'anti-mitotic' drug

vinblastine has also been found to inhibit tobacco smoke-induced secretory cell hyperplasia although by a mechanism other than the inhibition of mitosis.

7. Prospective studies in Man have shown that many of the pathological changes associated with smoking-related lung diseases such as reduced lung function, cough and expectoration, increased pulmonary epithelial permeability and possibly reduced mucociliary clearance may be reversed if the smoking habit in particular is broken before severe damage has occurred. There is also evidence that recovery takes place in the airways of experimental animals: there is a reversal of epithelial secretory cell hyperplasia after cessation of drug or TS administration.

8. The main purpose of the studies described herein are to: (i) determine the effect of nicotine alone on epithelial secretory cell number, (ii) confirm and further determine whether the anti-inflammatory drugs indomethacin, flurbiprofen, aspirin, dexamethasone, prednisolone and hydrocortisone and the mucolytic agent, N-acetylcysteine inhibit tobacco smoke-induced secretory cell hyperplasia, and (iii) determine whether anti-inflammatory drugs will reduce the time taken for recovery after tobacco-smoke induced secretory cell hyperplasia.

CHAPTER 2

MATERIALS AND METHODS

Sweet is the love which Nature brings;
Our meddling intellect
Mis-shapes the beauteous forms of things:-
We murder to dissect.

WILLIAM WORDSWORTH, 1798

This chapter describes the materials and methods used: (i) the animals used and how they were cared for, (ii) the drugs used and how they were administered, (iii) the way in which the animals were exposed to cigarette tobacco smoke (TS), (iv) the methods used to determine blood concentrations of nicotine and carboxyhaemoglobin, (v) the preparation of tissue, followed by sections on (vi) qualtification and (vii) statistical analyses.

Details of individual experiments and the measurements made will be found in the chapter of Results.

The addresses of the companies which supplied the various materials used are given in Appendix 1.

A. ANIMALS

Male, outbred rats (Rattus norvegicus) were used and were obtained either from Olac or Charles River U.K. Ltd. The rats were albino and of the Sprague Dawley strain.

I. Lung cleanliness

All rats were Grade 3 or 4 specific pathogen-free (SPF) (Medical Research Council, 1974) which means they were purchased on the understanding that they were free of important respiratory pathogens such as those of the Mycoplasma and Reckettsia species and Pasturella pneumotropica (Giddens et al., 1971; Hill, 1972; Cassell et al., 1979). Infection by M. pulmonis, in particular, can lead to the infiltration of bronchial associated lymphoid tissue (BALT) into the lungs (Lindsey et al., 1971, 1978; Cassell et al., 1979). Such infiltrations have been found to make the lungs of infected rats unsuitable for

determination of airway epithelial secretory cell number (Reid, 1970). M. pulmonis is thought to be the causative agent in chronic respiratory disease of rodents in which histologically 'bronchitic' changes are seen (Kohn and Kirk, 1969; Lindsey et al., 1971; Wittlestone et al., 1972; Jersey et al., 1973). In addition, experimental infection of piglets with another mycoplasmal species, M. hyorhinis produces 'bronchitic' changes including mucous cell hyperplasia and submucosal gland hypertrophy (Jones et al., 1975).

a. Determination of Mycoplasma infection

Mycoplasma infection was determined directly in fresh homogenates of the right lung from selected animals in each study (Appendix 2). In brief, the right lung from each rat was removed, placed in a plastic 'universal' and kept on ice (for a maximum of an hour). The lungs were homogenised and a sample of each homogenate spread onto selective agar where growth was compared to that of standard mycoplasmal stains.

b. Assessment of BALT

Determination of the amount of BALT was made at a magnification of X100 on sections stained with haemaloxylin and eosin. Confirmation that the areas of BALT contained lymphocytes was made at a magnification of X400. BALT may be found along the entire length of the bronchial mucosa, especially around biurfications (Bienenstock et al., 1973). In the present studies the greatest concentrations of BALT were found at the biurfications between the intrapulmonary lateral and axial bronchioli (Fig. 2). BALT was very occasionally seen extending for a short way along the mucosa beyond the biurfications. Areas of BALT obscure the epithelium in tissue sections

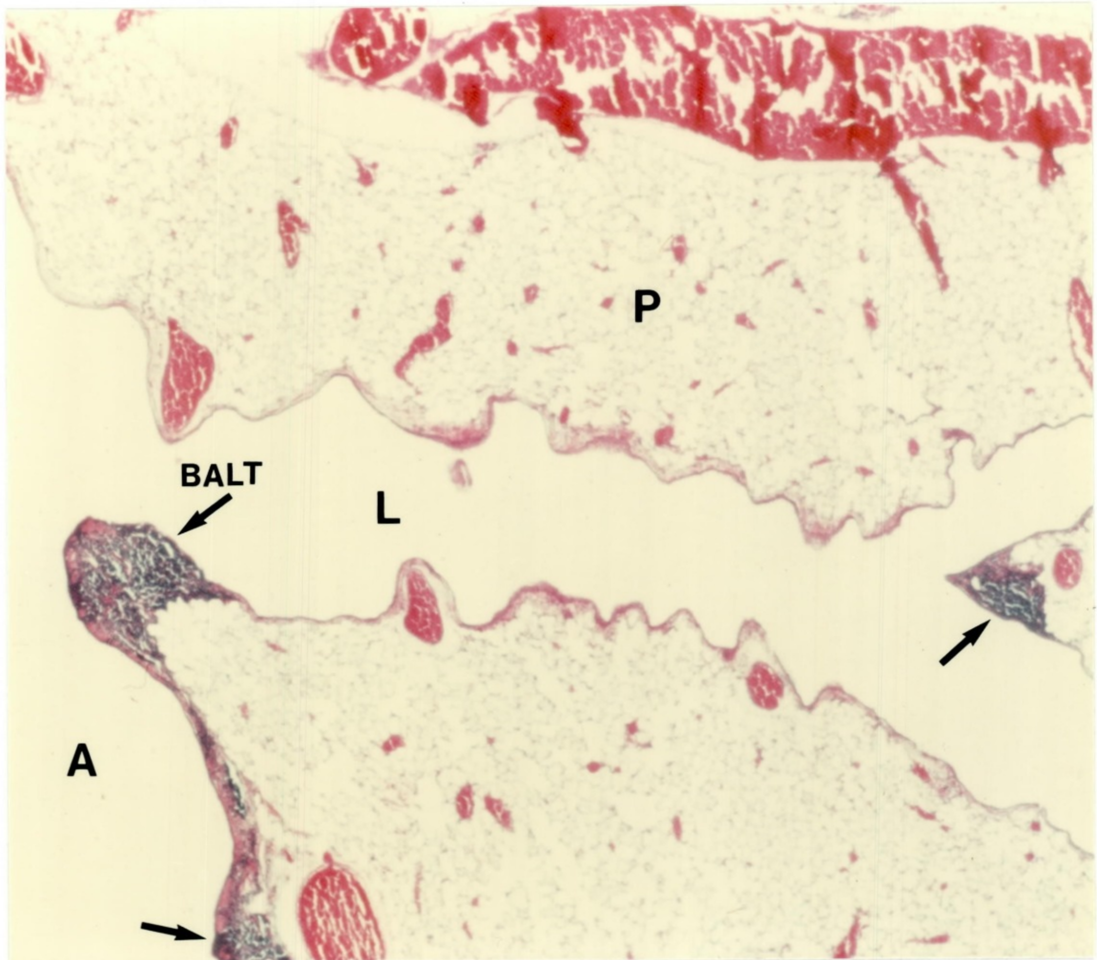
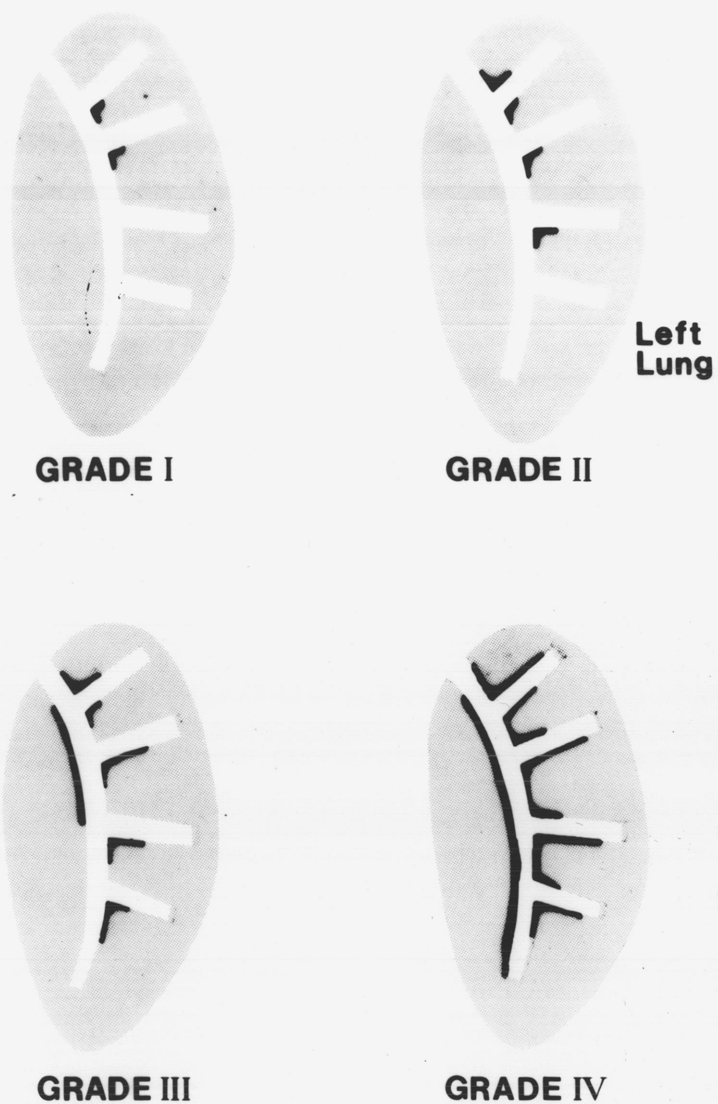


Fig.2. Bronchial associated lymphoid tissue (BALT) infiltrating rat intrapulmonary airways. Haematoxylin and eosin, mag. x 400. A = axial pathway, L = lateral pathway, P = lung parenchyma.

observed by light microscopy and reliable cell counts cannot be made in these areas. In addition, large areas of BALT indicate that the animal may have had a respiratory infection which confounds interpretation of results in regard to the effect of TS or drugs. The degree of infiltration by BALT has been classified (Reid, 1970) (Fig. 3). Grade I infiltration is acceptable and Grade II is the limit of acceptability. Tissue sections with higher grades of infiltration, particularly with extensions of BALT along the airways, are unacceptable and were rejected from the studies reported herein. The majority of the sections in the present studies showed Grade I infiltration; a few were Grade II, but none were in Grades III or IV. The trachea, medial wall of the intrapulmonary axial pathway and the upper walls of the intrapulmonary lateral pathways were completely free of BALT.

II. Care of the animals

After rats were delivered they were allowed to become used to their new surroundings for a few days before starting a study. Animals were kept in cages, not more than five to a cage, with water and a dry pellet food (Heygate and Sons Ltd., or Dixon and Son Ltd.) freely available. Sterilised sawdust bedding was used to absorb urine in the cages, and was changed frequently to prevent the concentration of ammonia in the cages encouraging mycoplasmal infection (Broderson et al., 1976; Lindsey and Conner, 1978). The animals were housed in a specially designed 'clean' room which was maintained at a constant temperature of 22°C. The animals were kept in unidirectional, laminar flow cabinets (Forth-Tech Services Ltd.) (Fig. 4) which filtered the room air before it was passed to the animals. The room was maintained at a positive



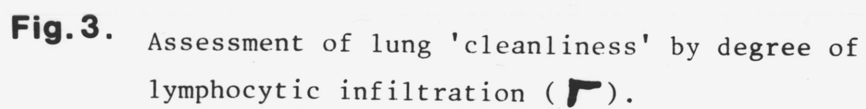


Fig. 3. Assessment of lung 'cleanliness' by degree of lymphocytic infiltration ( ).

GRADES I and II : a few small foci (acceptable).
GRADES III and IV: progressively heavier infiltration (unacceptable).

Fig. 4. Laminar Flow Cabinet. Filtered room air (A) passes over animal cages (C).

air pressure to prevent contaminated air from entering. To further prevent possible infection, only people directly involved in the experiments were allowed into the room. The room was cleaned regularly and, before each experiment, was fumigated with a one per cent aqueous aerosol of Hycolin (Microsol Fogging Machine) (Machinery and Chemicals Ltd.).

a. General condition

In general, the animals remained in good condition during the studies with sleek fur and with skin which retained its elasticity. The fur of rats exposed to tobacco smoke became stained yellow with smoke products. Rats exposed to smoke were often irritable on removal from the exposure cabinets but recovered quickly. Post-mortem examinations were carried out on the small number of animals which died during the studies (see Results).

b. Body weight

The animals were weighed regularly on an electronic balance (Mettler PK480 Delta Range) (Gallenkamp and Co. Ltd.) throughout each study, or daily if a particular treatment appeared to be detrimental to their health. Rats were weighed in the morning, before any treatment. In general, rats gained weight during the period of the studies. The experimental treatment of rats which failed to gain weight normally or which lost weight was checked (see Results). In experiments where treated rats were deprived of food and water (i.e. during exposure to tobacco smoke or nicotine aerosol), control rats were also deprived over the same period.

c. Organ weight

Organs were removed and fixed for a few days in neutral-buffered formal saline (see Drury and Wallington, 1980, p.49). The organs were then 'blotted' dry and weighed on a spring balance (Gallenkamp and Co. Ltd.).

B. ADMINISTRATION OF DRUGS

Drugs were given orally, by injection or in an aerosol.

I. Nicotine

L-nicotine hydrogen (+) tartrate (BDH Chemicals Ltd.) (henceforth referred to as nicotine) was chosen because it is the compound which most closely approximates to the compound of nicotine found in nature which is commercially available, and which is commonly used in nicotine research (for example see Jarvik et al., 1970; Littleton and Umney, 1977; Naquira et al., 1978).

Nicotine was given in two ways: by injection at so-called 'low' and 'high' doses, and by aerosol.

a. Injection : 'low' dose

A suspension of nicotine was made up in arachis (peanut) oil (BDH Chemicals Ltd.) at a concentration of 10g/l which allowed a small and easily calculated volume to be administered. Arachis oil was used because it is the vehicle commonly used when the slow release of a drug is required (The Pharmaceutical Society of Great Britain, 1979). A fresh suspension of nicotine was prepared every few days to avoid degradation leading to reduced potency. The nicotine suspension was given by subcutaneous injection. The site of injection was varied on a rota system to avoid associated local inflammation interfering

with absorption.

b. <u>Injection : 'high' dose</u>

In an attempt to maintain plasma levels of nicotine, a series of injections were given. Suspending nicotine in arachis oil (see above) necessitated the use of a wide-bore needle for injection because a small-bore needle is blocked by the suspension. However, it was decided that rats would not be able to tolerate many subcutaneous injections using a wide-bore needle. Consequently, nicotine was dissolved in sterile (by autoclaving), physiological saline which allowed a small bore needle to be used without blocking. Giving a solution of nicotine in saline would not allow for slow release of the drug, so it was given as repeated intraperitoneal (ip) injections over a period of eight hours (up to a maximum of eight, one hourly injections). Three rats were used to determine the optimum concentration of nicotine for each injection. A dose of 10mg/Kg was considered too high because the rats showed severe behavioural and physiological responses, including loss of balance, tremors, lachrymation and increased breathing rate. Doses of 5 and 7.5 mg/Kg produced none of these effects, and the dose of 7.5 mg/Kg was subsequently used in the study.

A fresh nicotine solution (7.5 g/l) was prepared every few days to avoid reduction in potency of the drug and possible infection of the rats.

c. <u>Aerosol</u>

A fresh solution of nicotine was prepared daily in sterile, distilled water at a concentration of 0.5 g/l. Distilled

water was used to avoid 'salting up' the piping in the exposure chamber, and was sterilised by autoclaving to avoid possible contamination of the rats. Rats were exposed to an aerosol of either sterile, distilled water ('vehicle' controls) or nicotine in water (nicotine-treated rats) in a 150 litre capacity environmental chamber (Tri-R Airborne Infection Apparatus) (Camlab Ltd.) which was adapted for the purpose (Littleton and Umney, 1977) (Fig. 5). Because only one apparatus was available for use, 'vehicle' control rats were exposed first, early in the morning. Treated rats were exposed to nicotine aerosol later in the morning to avoid any possible contamination of the controls with nicotine. At the end of each day's nicotine exposure the chamber and nebulizer were cleaned. The nebulizer was then filled with sterile distilled water and the piping and chamber flushed out in preparation for the following day's exposure.

Each of the rats from the appropriate group were randomly placed in separate sections of the exposure cage. The rats were separated to prevent them huddling together. The nebulizer was filled with a 4 ml aliquot of either water or nicotine solution (containing 2 mg nicotine) and the apparatus switched on. Each aliquot was completely aerosolised in 20 minutes and was immediately replaced by another 4 ml sample. Four of these aliquots (i.e. a total of 8 mg nicotine) were aerosolised during an 80 minute period. During this period the chamber was being cleared by the exhaust at a slower rate than the aerosol was being delivered. Consequently, the concentration of aerosol droplets containing nicotine in the chamber gradually rose to a maximum by the end of the 80 minute period. After the last

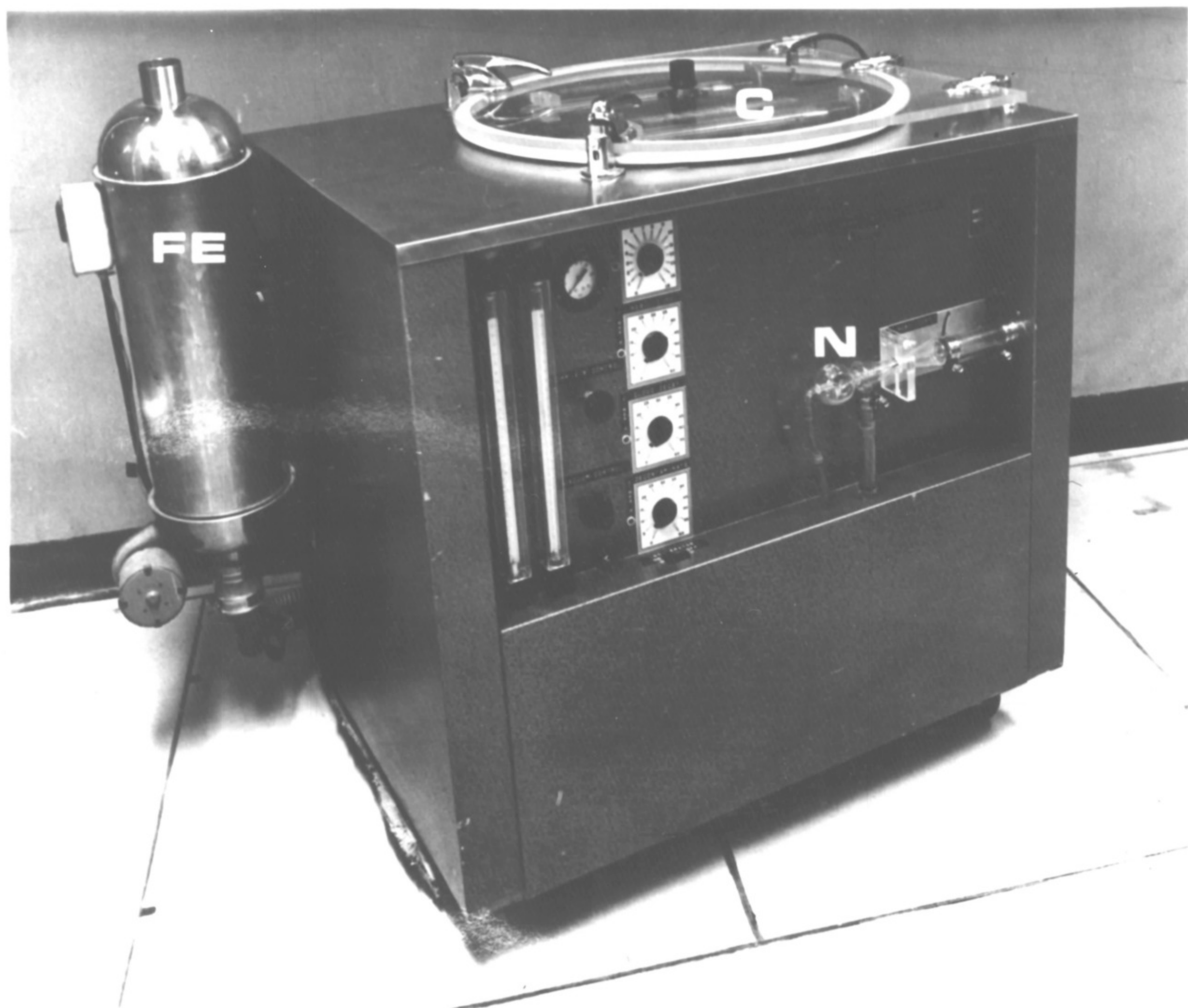


Fig. 5. Aerosol Chamber. Nebulizer (N) generates aerosol which is delivered into chamber (C) containing animals. FE = filtering and exhaust system.

aliquot had been aerosolised the exhaust was allowed to clear the chamber for a further hour during which time the concentration of aerosol droplets declined. No data was available concerning the size of the aerosol droplets delivered by the apparatus, nor could it be measured in our laboratory. After exposure, the rats were 'blotted' dry with tissues to avoid an uncontrolled intake of nicotine during communal fur-licking.

II. Anti-inflammatory and mucolytic drugs

Six anti-inflammatory drugs and one mucolytic agent (Table 1) were given to the rats either orally (by gavage or in drinking water) or by ip injection.

a. Oral administration

Drugs given orally were dissolved in tap water and the pH of the final solution adjusted to 6.5. Tap water was used to avoid gastric irritation additional to that caused by anti-inflammatory drugs (Gilman et al., 1980; British National Formulary, 1983). The effect of N-acetylcysteine in drinking water was to be determined. A fresh solution of each drug was made up every two or three days to ensure maintained activity and to avoid infecting the rats.

For gavage, indomethacin and flurbiprofen were prepared in solution to a concentration of 4 g/l and aspirin was prepared to a concentration of 20 g/l. These concentrations allowed a small and easily calculated volume to be administered (i.e. 0.1 ml per 100 g body weight). Each drug solution was drawn into a 1 ml syringe and delivered into the rats' stomach's through a specially designed gavage needle. The needle had a large bore (1 mm), was chromium-plated to prevent rusting,

Table 1. Drugs used in the studies

Drug	Supplier
Aspirin (NSAID)	Beecham Research Laboratories
Flurbiprofen (NSAID)	The Boots Co. PLC.
Indomethacin (NSAID)	Merck, Sharp and Dohme Ltd.
Dexamethasone (SAID)	Merck, Sharp and Dohme Ltd.
Hydrocortisone (SAID)	Organon Laboratories Ltd.
Prednisolone (SAID)	Merck, Sharp and Dohme Ltd.
N-acetylcysteine (mucolytic)	Zambon S.p.A. (Milan)

NSAID = Non-steroidal anti-inflammatory

SAID = Steroidal anti-inflammatory

curved for ease of insertion into the oesophagus, and had a blunted and smoothed tip to avoid internal damage to the rats.

N-acetylcysteine was administered in the drinking water made up as a 1% (i.e. 1g in 100ml) solution and given to rats in calibrated water bottles.

b. Injection

Indomethacin, flurbiprofen, dexamethasone, hydrocortisone and prednisolone were given by ip injection. Each drug was dissolved in a one per cent (weight to volume) aqueous solution of sodium hydrogen carbonate ($NaHCO_3$) (BDH Chemicals Ltd.). A stock solution was prepared and divided into aliquots which were sterilised by autoclaving. Each aliquot was used for only two consecutive days to avoid infecting the rats. The drugs were readily soluble in this solution. $NaHCO_3$ was recommended (Dr. C. Franklin, personal communication) as being a suitable vehicle because it is commonly used for experimental drug administration and is pharmacologically inactive in 1% aqueous solution. Solutions of the drugs were prepared in concentrations which would allow the same small and easily calculated volume to be injected. For injection of drug at a dose of 0.4 mg/Kg, a concentration of 0.4 g/l was prepared. For injection at a dose of 4 mg/Kg, a concentration of 4 g/l was prepared. Consequently, 0.1 ml of the drug solution was administered per 100g body weight of rat.

C. TOBACCO SMOKE EXPOSURE

Due to demand for space, animals were exposed to smoke in the same room where they were kept. To avoid contamination of control animals by smoke they were housed in laminar flow cabinets (see

above). The smoking machines were designed to exhaust all mainstream and stray smoke to the outside (Air Control Installations).

I. Exposure system

In 1972, Wright published his design for a system which would automatically expose many small animals simultaneously to the smoke from a series of cigarettes. The system has been used to produce histologically 'bronchitic' changes in the airways of rats (for example see Lamb, 1967; Lamb and Reid, 1969 b). A variation of Wright's system has been described (Betts and Betts, 1979). Comparison of the degree of tobacco smoke-induced secretory cell hyperplasia and epithelial thickening in the airways of rats exposed in the two systems did not demonstrate a difference between them (authors unpublished observations). A slightly modified 'Wright' design was chosen for use in the present studies (see below). The system consisted of a series of metal cabinets, each with an automatic smoking machine mounted above (Fig. 6). (Engineering Department, Chelsea College, Manresa Road, London SW3).

a. Smoking machines

Twenty-five cigarettes were held in holes around the periphery of a disc mounted on the front of each smoking machine (Fig. 6). The smoking machines were operated pneumatically, using pumped air (Charles Austen Pumps Ltd.) to drive the machines and generate the supply of air and smoke to the cabinets containing the animals. A constant stream of room air was passed through each cabinet at a rate of 5 l/min. The smoking regime was controlled automatically by a manually pre-set timing box such that the disc moved one hole every ten minutes, when a new cigarette was lit.

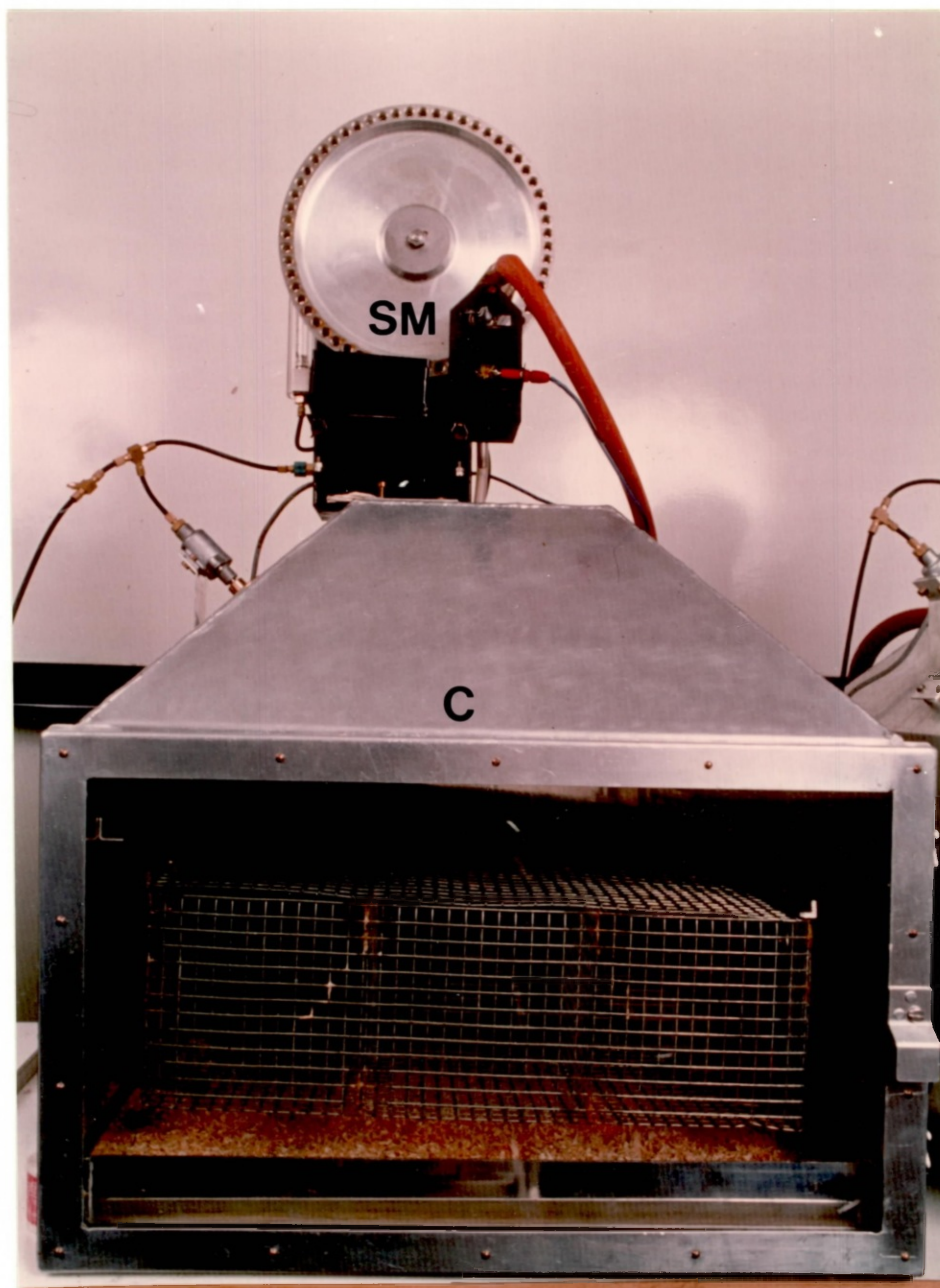


Fig. 6. Cigarette Smoke Exposure System. Automatic smoking machine (SM) mounted above exposure cabinet (C).

Thirty ml 'puffs' of smoke were generated by the action of a 20 l/min jet of air passing down a small bore tube placed at right angles to the cigarette butt (i.e. a 'venturi' effect). In this way, a mixture of air and smoke was blown into the cabinet for 2 seconds, 4 times per minute. Each cigarette burned completely over a 6 minute interval allowing four minutes of air to be passed into and clear the cabinet of smoke before beginning the next.

b. Exposure cabinets and caging

Each side of the exposure cabinets measured about 60 cm^2 giving an internal volume of 165 l. Smoke was exhausted from both sides of each cabinet below the level of the animals. Exhaust rate was monitored by flow meters (Flow Bits Ltd.) and could be adjusted to give the desired 'level' of smoke exposure. Altering the exhaust rate from the cabinets allowed different degrees of secretory cell hyperplasia to be induced in the airways of the rats (authors unpublished observations). An exhaust rate from each side of the cabinets of 1 l/min (henceforth referred to as 2 x 1 l/min) produced a significantly greater increase in secretory cell number ($p < 0.05$) than exhaust rates of 2 x 2 l/min or 2 x 0.2 l/min (which should have respectively lowered and raised the 'levels' of smoke in the cabinets) (see Fig. 7 and Discussion). Exhaust rates of 2 x 1 or 2 x 2 l/min were used in the present studies (see Results).

Animals were kept singly, during exposure, in cages divided into sections. Each animal was placed in a different compartment from day to day to avoid differences in exposure due to unequal distribution of smoke within the cabinets. A droppings tray lined with absorbent disposable material was placed beneath

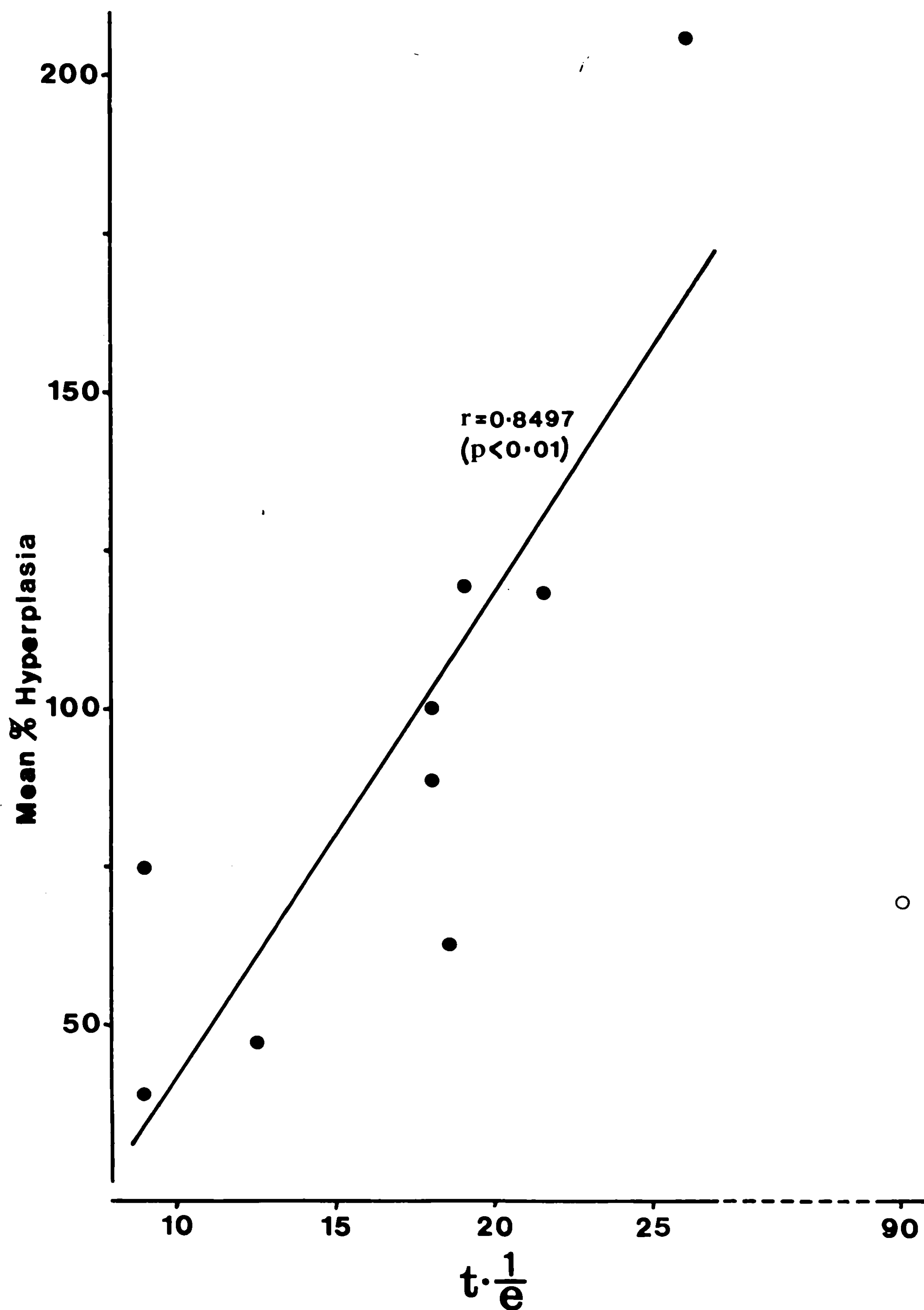


Fig. 7. Relationship between secretory cell hyperplasia and dose of tobacco smoke exposure : tar content of cigarette, mg x reciprocal cabinet exhaust rate, l/min = t· 1/e.
o = not included in regression line.

Table for Fig. 7. Tar and nicotine content (mg) of cigarettes and mean % secretory cell hyperplasia * due to tobacco smoke

Experiment	Cigarette	Tar (t)	Nicotine	Cabinet Exhaust Rate.l/min. (e)	t.[1]/e	Mean % hyper-plasia
1	JPNC	18	1.4	2	9	30.9
2	JPNC	18	1.4	2	9	74.9
3	JPNC	18	1.4	1	18	88.9
4	JPNC	18	1.4	1	18	100.2
5	JPNC	18	1.4	0.2	90	69.4
6	JPNC	18.5	1.4	1	18.5	63.1
7	JPNC	19	1.4	1	19	119.9
8	JPNC	21.5	1.7	1	21.5	118.7
9	JPNC	25	1.9	2	12.5	47.6
10	CFS	26	2.7	1	26	206.7

* calculated as the mean of the % secretory cell hyperplasia at each airway level studied.

Key

Experiment 1, 3, 5 = The effect of different cabinet exhaust rates on TS-induced secretory cell hyperplasia (Materials and Methods, C.I.b.).

2 = Indomethacin and flurbiprofen: 0.4 and 4 mg/Kg (Results, Section B, experiment 1).

4 = Recovery: experiment 2 (Results, Section D).

6 = Comparison of the amount of TS-induced secretory cell hyperplasia in two exposure systems (Materials and Methods, C.I.).

7 = Indomethacin and three steroidal anti-inflammatory drugs (Results, Section B, experiment 3).

8 = Recovery: experiment 1 (Results, Section D).

9 = Oral non-steroidal anti-inflammatory drugs (Results, Section B, experiment 2).

10 = N-acetylcysteine (Results, Section C).

JPNC = John Player Navy Cut unfiltered cigarettes.

CFS = Capstan Full Strength unfiltered cigarettes.

each cage and changed regularly to avoid contamination of cabinet air by ammonia from urine.

c. Temperature and humidity in the exposure cabinets

Temperature and humidity were monitored in the cabinets during exposure periods to determine whether these parameters were **within** an 'acceptable' range. Temperature was measured with thermometers and relative humidity (hereafter referred to as humidity) was measured with hair hygrometers (Gallenkamp and Co. Ltd.), suspended in the middle of the cabinets, just above the rat cage. Half hourly measurements were made during a day's exposure in one cabinet containing rats but with air passing through ('sham' smoking) and in three cabinets containing rats exposed to smoke (Table 2, Figs. 8 and 9). The temperature and humidity after a half hour's equilibration period was different in each cabinet (range: 22.0 to 23.5^{o}C and 65 to 71% respectively). During the exposure period the temperature in all cabinets rose similarly by about 1°C per hour to between 26 and 27°C (Fig. 8). The pattern of change in humidity was different in the cabinet used for 'sham' smoking to the cabinets used for smoke exposure (Fig. 9). In the 'sham' smoking cabinet, the humidity fell from 66 to 52% during the first hour of exposure and then levelled at around 51%. In the 'smoking' cabinets, the humidity rose initially to between 73 and 85% and then fell gradually, although never to the values for the 'sham' smoking cabinet. The humidity in one cabinet (cabinet 4) during smoke exposure was consistently higher than in any of the other cabinets. Thus, cabinets were changed between 'sham' smoking and smoking use during an experiment, and rats were kept in different cabinets on each day to compensate for differences in humidity between cabinets. In

Table 2. Day's change in cabinet temperature (^{o}C) and relative humidity (%)

Cabinet No. & atmosphere		hours								
		0	½	1	1½	2	2½	3	3½	4
1. Air	^{o}C	23.5	23.5	24.5	24.5	25.0	25.5	25.5	26.0	26.0
('sham' smoke)	%	66	57	52	51	52	52	50	50	49
2. Smoke	^{o}C	22.5	23.0	24.0	24.5	25.0	25.0	25.0	25.5	26.0
	%	71	76	69	68	67	64	62	62	61
3. Smoke	^{o}C	22.0	23.0	24.0	24.5	24.5	25.5	25.5	26.0	26.0
	%	70	73	67	65	64	63	60	59	60
4. Smoke	^{o}C	23.0	24.5	25.5	26.0	26.5	26.5	27.0	27.0	27.0
	%	65	85	79	77	77	77	76	75	75

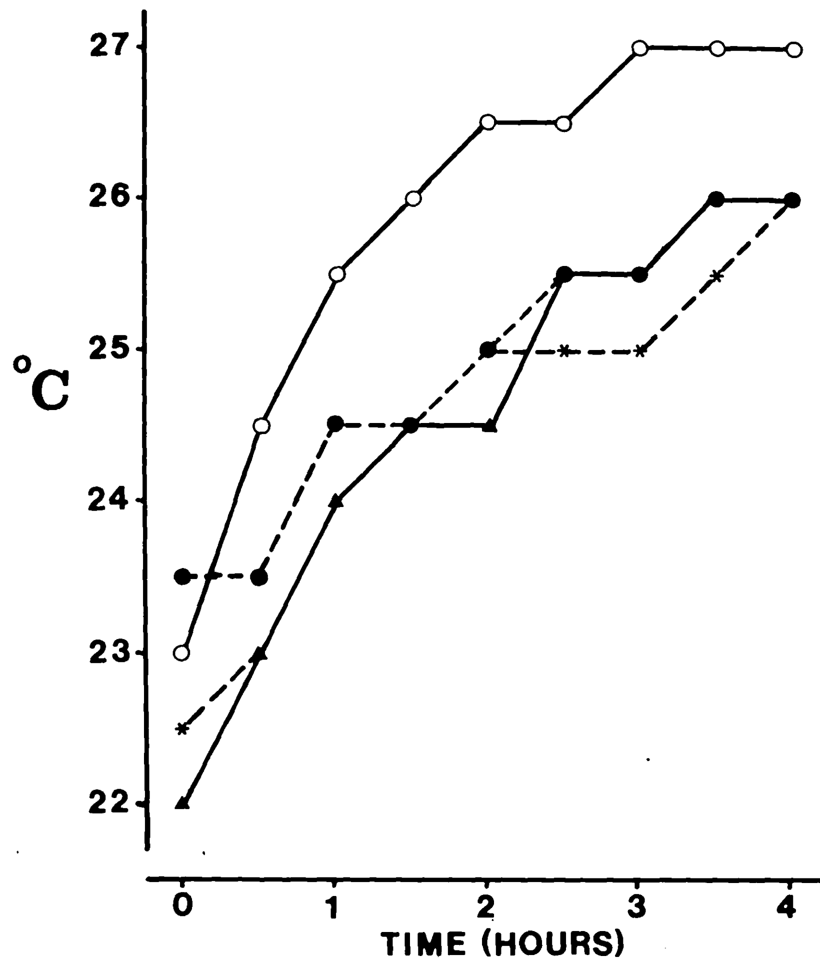


Fig. 8. Change in cabinet temperature (°C) with time of tobacco smoke (TS) or air delivery.

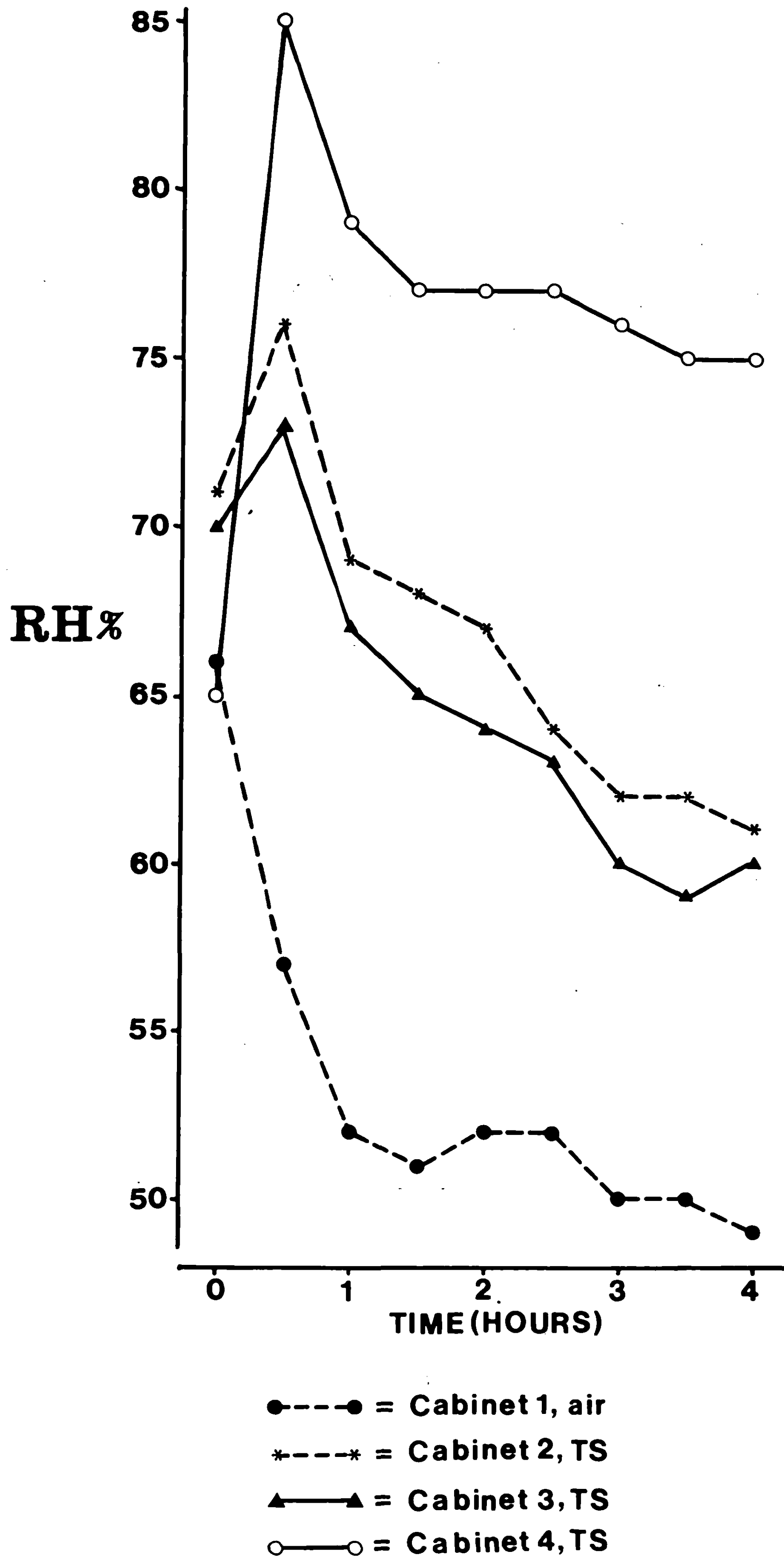
●---● = Cabinet 1, air

--- = Cabinet 2, TS

▲——▲ = Cabinet 3, TS

○——○ = Cabinet 4, TS

Fig. 9. Change in cabinet relative humidity (RH%) with time of tobacco smoke (TS) or air delivery.



subsequent experiments, measurements of temperature and humidity were made at the end of a day's exposure period. Results for four cabinets used at random for 'sham' smoke and smoke exposure during a typical study are given in Table 3. Again, the mean temperature in each cabinet was similar (mean range: 24.9 ± 0.5 to $26.0 \pm 0.5^{\circ}C$) whilst the mean humidity was more variable (mean range: 56 ± 0.0 to $65 \pm 2.2\%$).

d. Cigarettes

Two commercially available brands of cigarette were used in the studies. In all but one study, John Player Navy Cut, Plain (i.e. unfiltered) were used and were rated as 'middle tar' during the period of the studies (August, 1978 to December, 1980): tar content 18-25 mg/cigarette, nicotine content 1.4-1.9 mg/cigarette and carbon monoxide content 12.5 mg/cigarette (3.5 volume % per cigarette) (Laboratory of the Government Chemist, 1978 to 1981). Capstan Full Strength, Plain cigarettes were used in one study (January, 1982) at which time they were rated 'middle to high tar': (content per cigarette) tar 26 mg, nicotine 2.7 mg and carbon monoxide 16.7 mg (4.0%) (Laboratory of the Government Chemist, 1982). The change to the latter cigarettes was made because the tar and nicotine content of the former cigarettes has recently declined (Fig. 10). The degree of secretory cell hyperplasia had similarly declined (see Discussion), and altering the exhaust rate from the cabinets was by itself insufficient to increase the degree of secretory cell hyperplasia (see Fig. 7 and Discussion).

D. MEASUREMENT OF CABINET CARBON MONOXIDE AND SMOKE PARTICLE CONCENTRATION

Carbon monoxide (CO) and smoke particle concentrations in the cabinets were measured to determine whether CO concentrations reflected

Table 3. Daily cabinet temperature (°C) and relative humidity (%)
S = Smoke, A = Air (i.e. 'sham' smoke)

Cabinet No.		Day of exposure 5	6	7	8	9	12	13	Mean (± SEM)
1	S or A	S	S	S	A	S	S	S	
	°C	-	-	23.5	-	-	26.5	-	26.0 (0.5)
	%	-	-	56	-	-	56	-	56 (0)
2	S or A	S	A	S	S	S	A	S	
	°C	-	24.0	-	-	26.0	-	26.0	25.3 (0.7)
	%	53	66	-	69	60	-	-	62 (3.5)
3	S or A	S	S	A	S	S	S	A	
	°C	22.5	-	26.0	26.0	25.5	-	26.0	25.2 (0.7)
	%	-	61	-	-	-	-	58	60 (1.5)
4	S or A	A	S	S	S	A	S	S	
	°C	25.0	23.5	-	25.0	-	26.0	-	24.9 (0.5)
	%	66	-	66	73	66	58	60	65 (2.2)

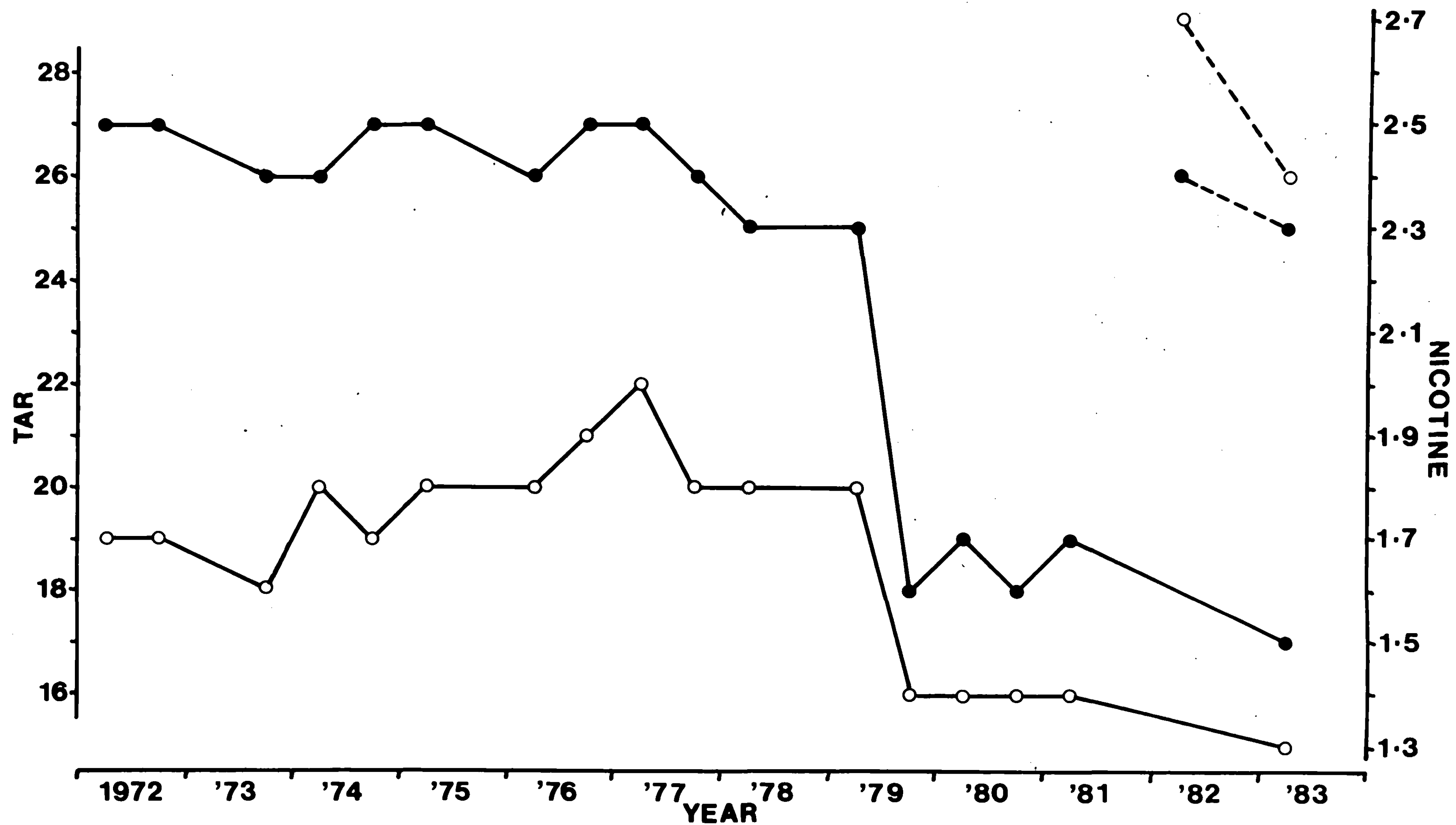


Fig. 10. Bi-annual tar (●) and nicotine (o) yields of cigarettes (mg/cigarette). ——— = John Player Navy Cut, unfiltered; – – – = Capstan Full Strength, unfiltered. (After: Laboratory of the Government Chemist Reports, 1972 to 1983).

particle density, and hence smoke concentration.

Fifteen rats were put into each of three cabinets for a half hour equilibration period, and then exposed to the smoke of 25 cigarettes over a four hour period. CO concentration and particle density in the cabinets were recorded at the end of the equilibration period and whilst the last few cigarettes were being smoked. Measurements of CO concentrations and particle density were made in the room.

I. Carbon monoxide

CO concentrations were measured using a portable analyser (Ecolyser 4000 series) (Energetics Science, Inc.) which had a minimum detectable sensitivity of 1%, and a high degree of specificity for CO (e.g. 10,000 ppm CO_2 is not registered). Samples were continuously pumped through the instrument directly from the cabinets through a gas line whose opening was positioned just above the rats. The instrument was initially calibrated using a standard gas mixture containing 50 ppm CO (Eco-Span) (Energetics Science, Inc.).

II. Particles

Particle density was measured concurrently, in one cabinet, with CO concentrations at the end of the equilibration period and between the sixteenth and nineteenth cigarette. Measurements were made with a unit which continuously monitored and recorded particle density; the Reading Dust Monitor (Fig. 11) (Holt and Young, 1964). The machine uses a collimated beam of light which is scattered by sample particles and the degree of scatter, and hence particle concentration, determined by photomultiplier tubes. The output current of the tubes is

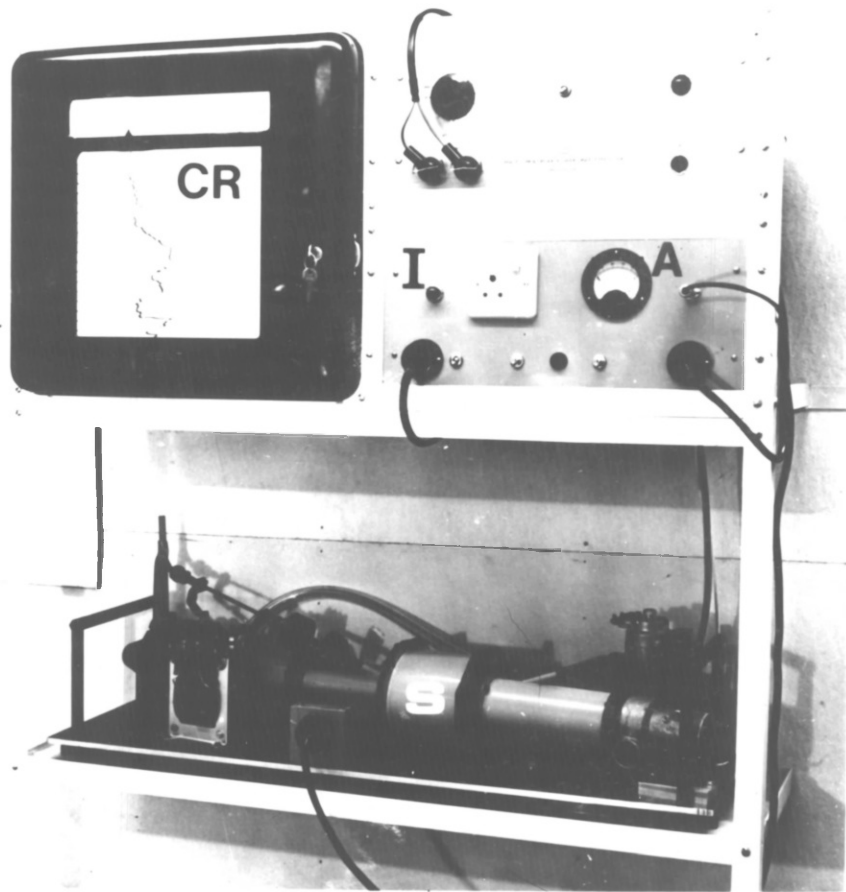


Fig. 11. 'Reading Dust Monitor'. Sampling unit (S) relays particle density to integrating system (I) which displays values on ammeter (A) or chart recorder (CR).

displayed by an ammeter from which readings were noted and expressed in 'arbitrary units'.

The concentration of CO in the room and in the cabinets after half an hours equilibration was 7 ppm (* See footnote)(Table 4). Particles were not detected either in the room or in the cabinets (Table 4).

In general, CO levels rose as successive cigarettes were smoked, from 7 ppm to a maximum of 145 ppm, although during a single cigarette the levels fluctuated and were sometimes lower at the 'end' of a cigarette than before the cigarette was lit.

Particle density also rose as successive cigarettes were smoked and correlated well with CO concentrations ($r = 0.9264$; $p < 0.001$) (Fig. 12). Thus, measuring CO concentrations in the cabinets would appear to be a suitable way of quantifying the amount of whole smoke available to the rats.

E. DETERMINATION OF PLASMA CARBOXYHAEMOGLOBIN AND NICOTINE

Plasma concentrations of carboxyhaemoglobin (COHb) and nicotine were measured to determine the amount of smoke inhaled by the rats. Plasma concentrations of nicotine were measured also in rats given nicotine hydrogen tartrate (i.e. 'nicotine').

---

* Footnote

Conversion factor for CO (National Primary and Secondary Air Quality Standards, 1971):

$$1 \text{ ppm} = 0.0001\ \% = 1.15 \text{ mg m}^{-3}$$

Table 4. Carbon monoxide (CO) concentrations (ppm) and smoke particle density (arbitrary units)

Cabinet 1			Cabinet 2		Cabinet 3	
Cigarette	CO	particles	Cigarette	CO	Cigarette	CO
0	7	0	0	7	0	7
15, lit	70		20, lit	100	24 lit	112
1/4	75		1/4	70	1/4	105
end	60		1/2	80	1/2	102
			end	120	3/4	102
16, lit	60				end	110
1/4	60	5	21, lit	120		
1/2	70	6	1/4	130	25, 1/4	125
end	71	7.5	1/2	115	1/2	120
17, 1/4	80	10	22, lit	110		
	79		1/4	108		
			1/3	105		
18, lit	75	9.5	1/2	110		
1/4	73		3/4	115		
1/2	70	9.5	end	130		
3/4	105	11				
end	110	12	23, 1/4	145		
			1/2	135		
19, 1/4	132	14				
1/2	129					
3/4	120	14				
end	119	13				
20	117	12				

Fractions = amount of cigarette 'smoked'

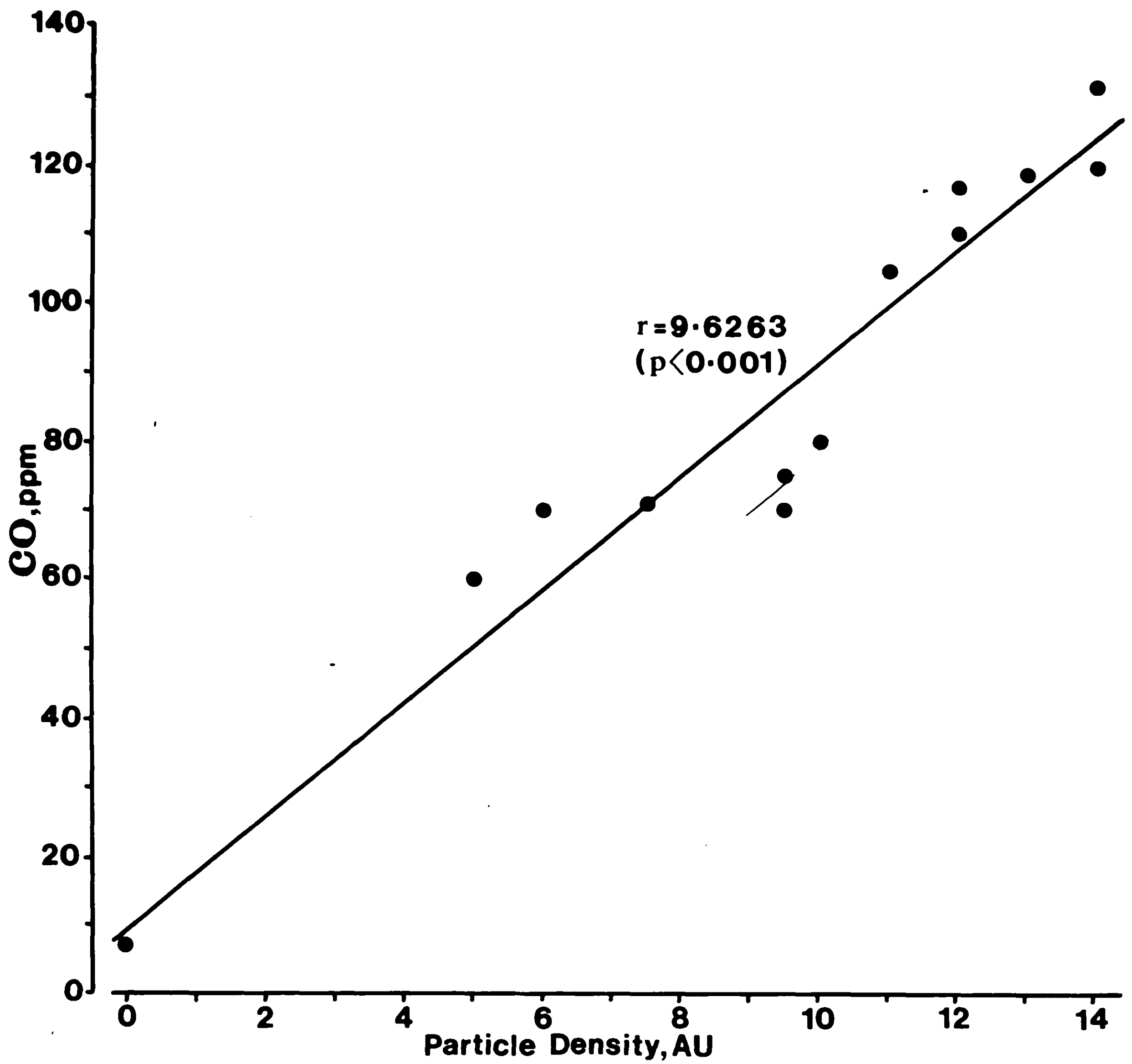


Fig. 12. Relationship between cabinet carbon monoxide (CO) concentration and smoke particle density. Arbitrary Units, AU = ammeter readings from 'Dust Monitor'.

I. Carboxyhaemoglobin

Six rats (mean body weight of 249 ± 9g) were selected at random during the last day of exposure to smoke (i.e. day 14) from a group of rats which had already been exposed to smoke for 13 days. Three were killed after one hour's exposure to 6 cigarettes and three were killed after the last cigarette had been extinguished (i.e. after exposure to the smoke of 25 cigarettes). Rats were anaesthetised with sodium pentobarbitone (60-80 mg/Kg) and approximately 5 ml of blood removed directly from the heart with a needle and syringe after making a small incision in the side of the rib cage. Blood was transferred to ice-cooled, heparinised tubes and centrifuged (1,200g for 5 minutes). The plasma was removed, transferred to fresh tubes and stored frozen at -20°C. Plasma per cent COHb measurements were made on each sample a few days later using an IL 182 Co-Oximeter (Instrumentation Laboratory) (Russell et al., 1973; Wald et al., 1977; Dennis and Valeri, 1980). Variability between samples in a group was assessed by the coefficient of variation (V): V-values above 10% indicate unacceptable variation between samples.

The mean plasma % COHb level was calculated for each group of rats and the difference between them assessed for significance by one-way analysis of variance. The mean plasma concentration of COHb in the rats which had been exposed to the smoke from 6 cigarettes was 11.7% (Table 5). However, variability between these samples was high (V = 9.6%). The mean plasma concentration of COHb in the rats which had been exposed to smoke from 25 cigarettes was 16.4%, with a low variability between samples

(V = 4.0%). The mean values were significantly different ($p < 0.05$), indicating that plasma COHb concentrations in rats exposed to cigarette smoke rise with the number of cigarettes 'smoked'.

Table 5. Mean plasma % COHb concentration (% coefficient of variation)

Time of exposure, hr.	No. cigarettes	COHb
1	6	11.7 (9.6)
4	25	16.4 (4.0)

II. Nicotine

Twenty-nine rats (mean body weight 220 ± 4g) were divided into 5 treatment groups (Table 6). Untreated rats were killed for blood letting before treated rats to avoid contamination with nicotine. Rats exposed to an aerosol of nicotine (8 mg nicotine in 80 min.) (see above) were 'blotted' dry to remove surplus nicotine which may have contaminated the blood samples, and killed at the end of the 80 minute exposure period. Rats given a single subcutaneous injection of 10 mg/Kg nicotine in arachis oil ('low' dose) were killed immediately after the injection and then after 30, 60 and 340 minutes to determine the effectiveness of arachis oil as a 'slow-release' vehicle. Plasma samples from rats given the 'high' dose of nicotine (eight 7.5 mg/Kg intraperitoneal injections of nicotine in saline) (see above) were each divided into three aliquots to determine the reproducibility of the method. The final group of rats were exposed to cigarette smoke for four days. On the fifth day, rats were killed after exposure to the smoke of at least 12 cigarettes.

Table 6. Mean plasma nicotine concentration (± SEM), ng/ml.

Treatment	Time before blood let (min)	No. rats	Nicotine
None	-	6	0.3 (0.2)
Aerosol	5	5	10 (2)
Injection			
'low' dose	5	4	651 (100)
	30	2	860 (223)
	60	2	573 (33)
	345	2	42 (7)
'high' dose	5	1*	3168 (120)
	10	1*	1869 (18)
	15	1*	2030 (36)
Tobacco smoke			
12 cigarettes	5	2	83 (21)
13 "	5	1	70
14 "	5	1	64
15 "	5	1	213

* = sample divided into 3 aliquots

Rats were anaesthetised with ether which does not interfere with analysis of nicotine (Mr. Colin Feyerabend, personal communication). Blood (about 5 ml) was withdrawn, following minimal dissection, by cardiac puncture and transferred immediately to tubes containing EDTA which were centrifuged (1,200 g for 5 minutes) to leave a supernatant of plasma (about 2 ml) containing nicotine. Plasma samples were transferred to new tubes and stored frozen at -24°C. Plasma samples were extracted for nicotine, reduced to a small final volume of butyl acetate (containing nicotine) and injected onto a gas chronatograph which was sensitive enough to detect nicotine concentrations of 0.1 ng/ml. (for details see Feyerabend and Russell, 1979). Mean plasma nicotine concentrations are given in Table 6. Standard errors of the means for the three aliquots taken for each plasma sample from rats given the injected 'high' dose of nicotine were between 1 and 4% of the mean values: the method is acceptably reproducible at high nicotine concentrations. The plasma of untreated rats contained 0.3 ng/ml nicotine. The plasma of rats exposed to the aerosol of nicotine contained 10 ng/ml nicotine, significantly lower than the mean values obtained for any of the nicotine-treated rats ($p < 0.01$). Nicotine in the plasma of rats given the single 'low' dose of injected nicotine was maintained at a mean concentration of 684 ng/ml for at least an hour after injection, there being no significant difference between the mean values 5, 30 and 60 minutes after injection. However, the mean concentration of plasma nicotine had fallen significantly to 42 ng/ml, 5¾ hours after the injection ($p < 0.05$). Rats given the 'high' dose of

injected nicotine showed varying mean plasma nicotine values. However, the values were not significantly different and give an overall mean value of 2356 ± 208 ng/ml. The plasma nicotine concentration in rats exposed to the smoke of 12 to 15 cigarettes also varied. The overall mean value is 106 ± 29 ng/ml.

F. TISSUE PREPARATION

As in previous studies (for example see Lamb and Reid, 1969b; Jones and Reid, 1978) rats were killed on the day following the last day of exposure to TS. Rats were weighed and killed by intraperitoneal injection of sodium pentobarbitone ('Sagatal', 120 mg/Kg) (May and Baker Ltd.). Each rat was quickly dissected to expose the trachea, main extrapulmonary bronchi, lungs and associated cardio-vasculature. A needle was inserted into the ventral surface of the trachea, just below the larynx, and a syringe, containing fixative, attached. The lungs were inflated *in situ* with about 10 ml of neutral buffered formal saline (see Drury and Wallington, 1980, p. 49) by very slowly emptying the syringe until the pleural margins were sharp. After inflation, the needle was gently withdrawn and the trachea ligatured just below the needle hole to prevent fixative leaking out. After the trachea, main bronchi and lungs had fixed *in situ* for a few minutes they were removed *en bloc*, stripped of the heart, associated vasculature, oesophagus and connective tissue, strung onto labelled cards to preserve their shape and relative positions, and immersed in a bath of neutral buffered formal saline.

After a few day's fixation, the trachea and left lung were separated from the left main bronchus and processed. The bronchi were

not used in the light microscopic studies described herein because they were too short for satisfactory determination of secretory cell number. The right lung was not used because it is sub-divided into at least four smaller lobes. The left lung is not so divided and has a main axial pathway which runs for nearly the whole length of the lung from its junction with the left extrapulmonary bronchus at the lung carina to the tip of the lung extremity (Fig. 15). Lateral airway branches extend at right angles from the axial pathway into the periphery of the lung.

I. Processing, section cutting and staining

The tracheas and left lungs were processed conventionally for light microscopy on a 'histokinette' (Reichert-Jung U.K. Ltd.) (see Drury and Wallington, 1980, pp. 64-67). In brief, the tissue was automatically and sequentially washed free of fixative, gradually dehydrated by passage through a range of alcohol solutions, cleared of alcohol and impregnated with liquid paraffin wax.

The larynx was cut away from the trachea and a couple of cartilage rings at the laryngeal (i.e. proximal) end of the trachea separated. Both portions of trachea were embedded together in a block of paraffin wax with the proximal portion laid at right angles to the cranial end of the trachea. The left lungs were embedded in blocks of paraffin wax, ventral surface uppermost to minimise subsequent orientation during section cutting. All tissue was vacuum embedded to remove air bubbles which might affect the quality of the sections.

Sections of each block of tissue were cut at a thickness of

between 4 and 6 μm. Tracheal sections were cut longitudinally (coronally) down the mid-line of the trachea so that 'ideal' sections included each 'wall' with the epithelium not cut obliquely, cartilage plates and a transverse section of the proximal portion of the trachea. The left lungs were cut coronally, and orientated so that 'ideal' sections included the whole length of the intrapulmonary axial pathway and at least four of its lateral branches. Sections were flattened in 5% aqueous alcohol, floated in a bath of distilled water at 50°C, collected and dried onto 1 by 3 inch glass slides, one section per slide. Sections were freed of wax with xylol, re-hydrated with graded alcohol/water solutions and stained. Alternate sections were stained for intracellular mucin with the combined Alcian Blue (pH 2.6) and periodic acid-Schiff technique (AB/PAS) (see Introduction). The remaining sections were stained with Ehrlich's haemotoxylin and eosin (1% aqueous solution) (Pearse, 1972) for estimation of lymphocyte infiltration (BALT). Thus, a pair of adjacent sections, representing a similar portion of airway, could be assessed for BALT and secretory cell number. Stains were purchased from Raymond A. Lamb. Stained sections were mounted under coverslips.

G. DETERMINATION OF SECRETORY CELL NUMBER

The number of surface epithelial mucus-containing secretory cells (henceforth referred to as secretory cells) in the trachea and intrapulmonary airways was determined by light microscopic observation of sections stained specifically for mucin with AB/PAS. AB/PAS does not stain cell membranes or nuclei and so it was the

number of coloured 'spots' of stained intracellular mucin which represented the 'number' of secretory cells (Fig. 13). Tissue sections were observed using an 'Ultraphot' Photomicroscope Mark II (Carl Zeiss Jena Ltd.) equipped with a 'Planapo' 40/0.95 objective lens which is manufactured for good colour discrimination in stained sections. Secretory cell number was determined at a magnification of X 400 (field diameter 0.35 mm).

I. Neutral and acidic secretory cells

The mucins of interest in the present studies are those containing neutral or acidic radicles. Alcian blue stains acidic mucins blue, and subsequent staining with periodic acid-Schiff stains neutral mucin red. By electron microscopy (EM), granules containing acidic mucus (i.e. in mucous cells) are electron-lucent whilst granules containing neutral mucus (i.e. in serous cells) are electron-dense (Jeffery and Reid, 1977). However, it is rare to find a mucous cell by EM which contains exclusively electron-lucent granules (author's observations of Dr. P.K. Jeffery's electron micrographs). Rather, there is a range of granules within a single mucous cell which show varying degrees of electron-lucency. Serous cells consistently contain a predominant proportion of completely electron-dense granules. Consequently, staining mucins in the intracellular granules of mucous cells with AB/PAS will, when observed by light microscopy, produce a variety of coloured 'spots' of mucus ranging from pure blue (i.e. a mucous cell containing only acidic mucus-granules) to various shades of purple (i.e. a mucous cell containing different proportions of acidic and neutral mucus-granules) (Fig. 13). In the present light microscopic studies, very

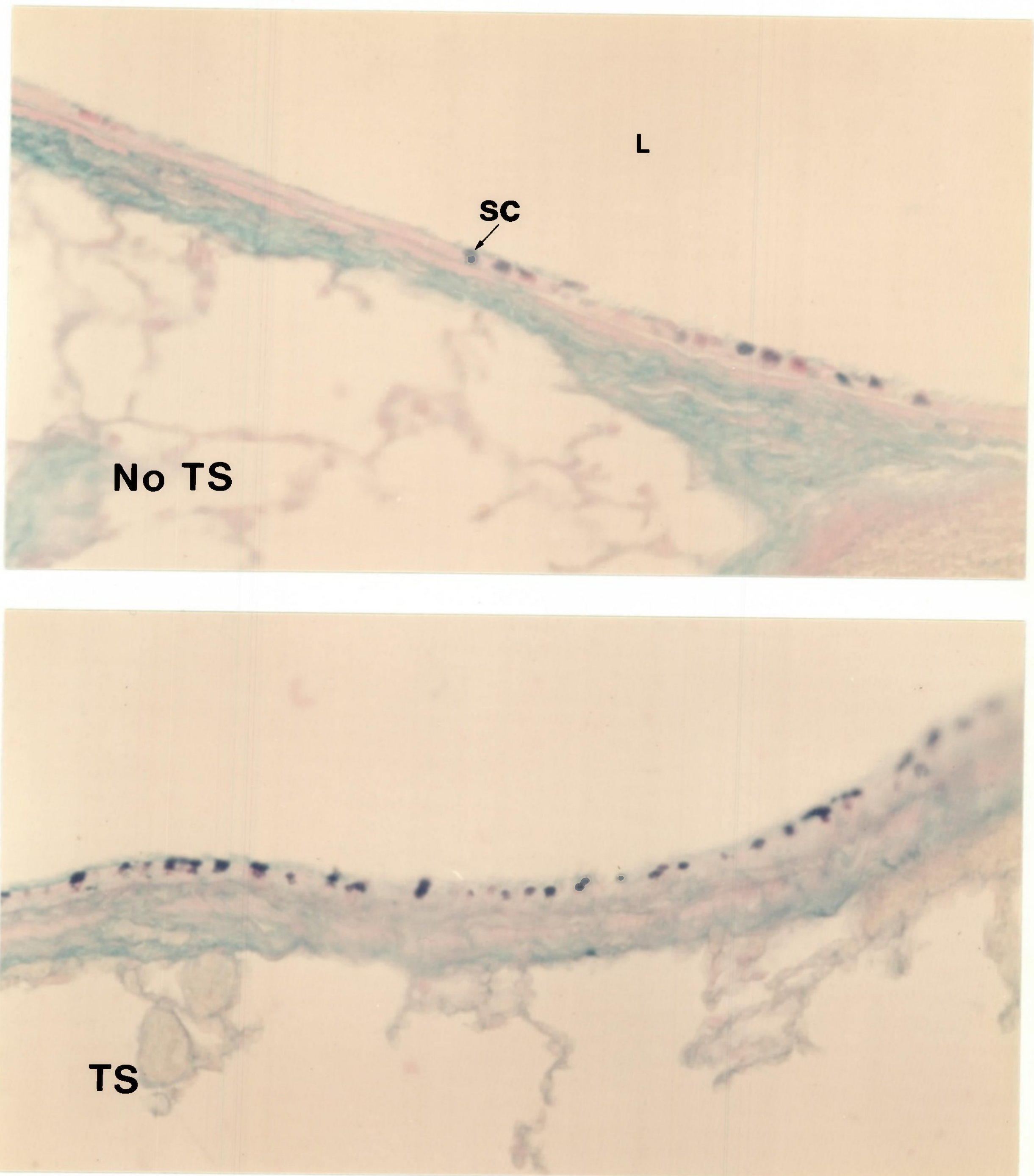
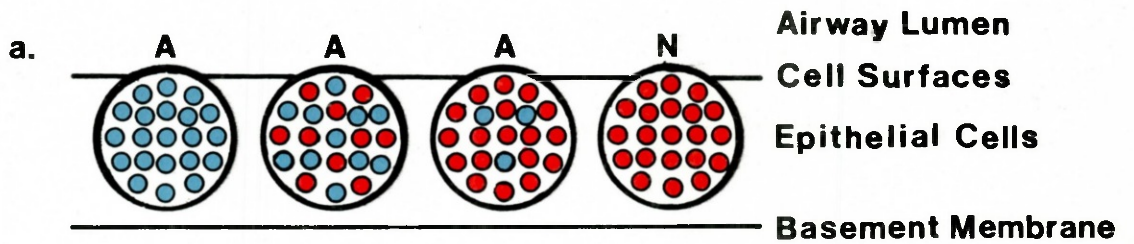


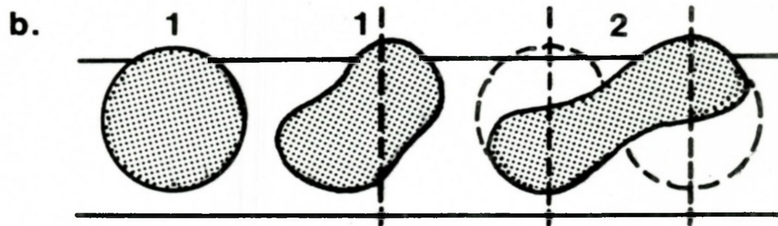
Fig.13. Mucus-containing secretory cells (SC) in rat intrapulmonary airway epithelium. Alcian blue (pH 2.6) and periodic acid-Schiff, mag. x 400. No TS = not exposed to tobacco smoke; TS = exposed to tobacco smoke, showing an increase in number of secretory cells. L = airway lumen.

few pure blue 'cells' were found, confirming the EM studies. Consequently a coloured 'spot' of stained mucus which was either blue or purple (even if the spot was predominantly red) was considered to be an acidic mucus-containing secretory cell (i.e. a mucous cell by EM) and was henceforth termed an 'acidic secretory cell' (Fig. 14a). A coloured 'spot' of stained mucus which was completely red (i.e. without a blue component) was considered to be a neutral mucin-containing secretory cell (i.e. probably a serous cell by EM) and was henceforth termed a 'neutral secretory cell' (Fig. 14a).

By light microscopy of semi-thin sections (1 μm thickness), the epithelium of rat airways is normally pseudo-stratified: although all epithelial cells touch the basement membrane, individual cells may extend at angles to the basement membrane and overlap (Jeffery and Reid, 1977). Consequently, when observed by light microscopy of thicker paraffin sections (4-6 μm in the present studies) the 'spots' of stained mucus assume a variety of shapes and sizes (Fig. 14b). In addition, there are some mucous and serous cells seen by EM which contain only a few mucus-granules, congregated at the apex of the cell (authors observations of Dr. P.K. Jeffery's electron micrographs). By light microscopy the mucin of these secretory cells would appear as a coloured 'cap' (Fig. 14c). Jones (1976) puts such 'caps' into a separate secretory cell category from the larger coloured 'spots'. However, a 'cap' represents a complete secretory cell (perhaps having recently discharged its secretory content) and so was counted in the present studies as a secretory cell. In summary, surface epithelial mucus-containing secretory cells were identified by light

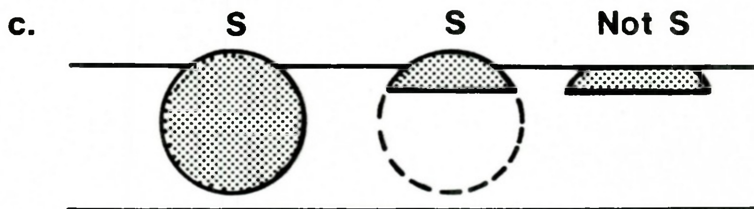


A = Acidic secretory cells (blue or purple).
N = Neutral secretory cell (red).



1 = One secretory cell : apex within base.

2 = Two secretory cells : apex falls outside base.



S = Counted as secretory cells : apex protruding.
Not S = Not counted as a secretory cell.

Fig. 14. Criteria for light-microscopic counting of 'secretory cells' stained with alcian blue (pH 2.6) and periodic acid Schiff.

microscopy of paraffin sections stained with AB/PAS according to the following criteria (Fig. 14):

i. A blue or purple 'spot' of stained mucus was termed an 'acidic secretory cell'.

ii. A red 'spot' of stained mucus was termed a 'neutral secretory cell'.

iii. A secretory cell lying at an angle to the basement membrane was counted as a single cell unless the base of the cell fell outside a perpendicular to the apex of the cell when it was counted as two cells.

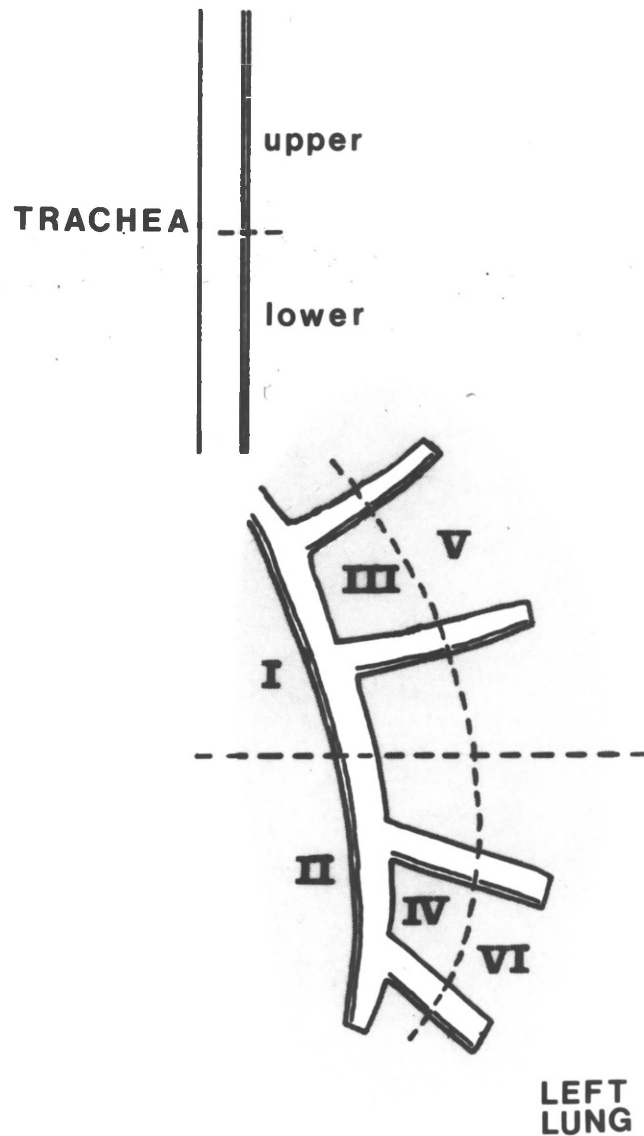
iv. Small stained 'caps' protruding above the cell surface layer were counted as secretory cells; thin, densely staining 'lines' which were not raised above the cell surface layer were not counted.

v. Coloured spots were occasionally seen which fell outside the criteria above but were not included in the counts.

II. Airway 'levels'

Neutral and acidic secretory cells were counted along consecutive lengths of epithelium, orientated across the diameters (0.35mm) of a series of consecutive high power fields (HPF) (i.e. x 400 magnification). Secretory cells were counted in the trachea and at a number of intrapulmonary airway levels (Fig. 15). Portions of the airways where the epithelium was broken, folded or otherwise disrupted were not included in the counts: particularly in the trachea where the epithelium lying between the cartilage plates was frequently folded. In the airways of the lung, the epithelium was infrequently disrupted. Approximately 20 HPF were counted in each half

Fig.15. Airway Levels



I = upper axial
II = lower axial
III = proximal upper laterals
IV = proximal lower laterals
V = distal upper laterals
VI = distal lower laterals

of the trachea and approximately 16 HPF were counted in each sub-division of the intrapulmonary airways. On average, 16,000 secretory cells were counted in each experiment.

III. Presentation of results

a. Secretory cell number

Except for a few instances, secretory cells were counted in at least 16 HPF. Secretory cell counts at each airway level were therefore expressed as the number per 10 HPF (representing a 3.5 mm length of epithelium). A small number of counts made in fewer than 10 HPF were extrapolated to this quantity. The mean number of secretory cells per 10 HPF at each airway level, plus or minus the standard error of the mean (± SEM), was calculated for each animal in a group. SEM was calculated as: $\frac{\text{Std. Dev.}}{\sqrt{n-1}}$.

b. % change in secretory cell number

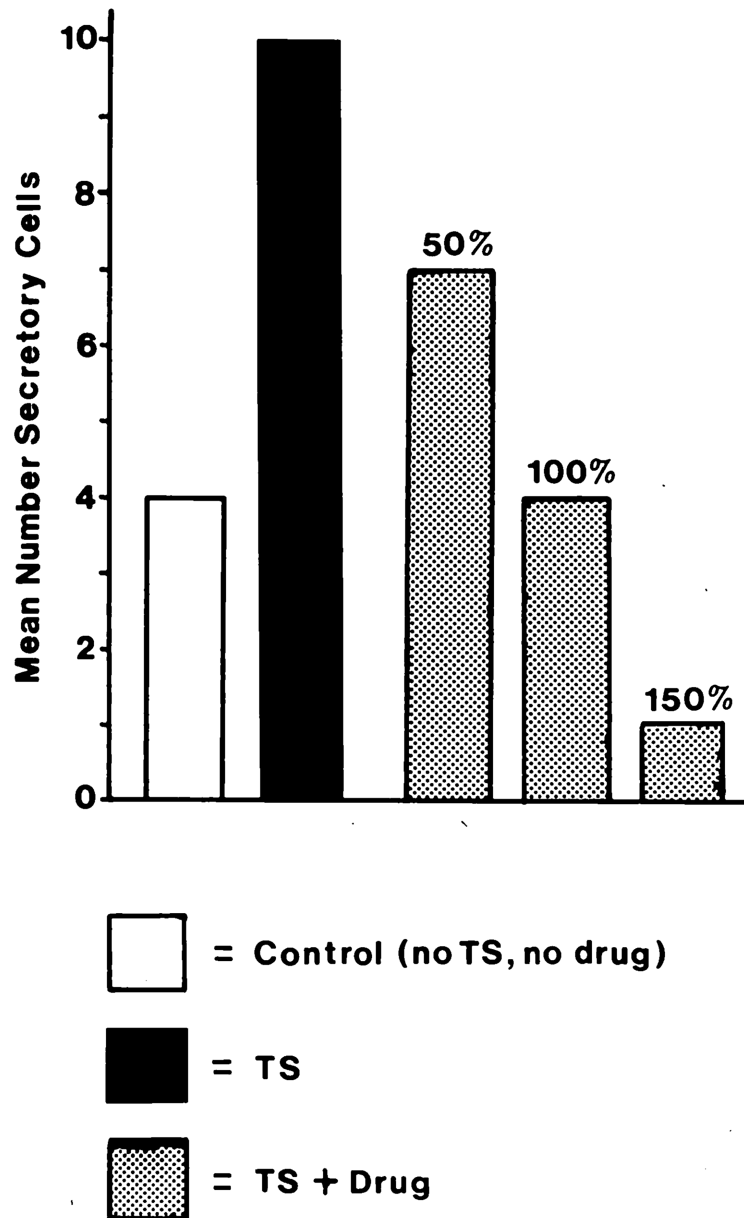
Percentage change (i.e. % increase or decrease) in secretory cell number at each airway level in treated animals (i.e. given nicotine or tobacco smoke) compared to controls was calculated as below:

$$\frac{\text{mean number (treated)} - \text{mean number (controls)}}{\text{mean number (controls)}} \times 100\%$$

c. % inhibition due to drug treatment

Inhibition, due to drug treatment, of tobacco smoke-induced changes in secretory cell number (i.e. increase or decrease) may be represented graphically (Fig. 16). However, graphical representation of inhibition in up to 8 airway levels (i.e. involving up to 24 histogram

Fig.16. % inhibition of tobacco smoke (TS)-induced secretory cell hyperplasia by drug treatment.



bars for one drug alone) is difficult to interpret. Consequently, % inhibition was calculated as below for each airway level:

$$\frac{\text{difference between mean number with TS and mean number with TS+drug}}{\text{difference between mean number with TS and mean number in controls}} \times 100\%$$

From the formula, the greater the % value calculated, the greater the inhibition afforded by the drug.

H. OTHER MEASUREMENTS

I. Body weight

Change in body weight of a rat from the morning on the first day of treatment (i.e. 'first' day) to the morning of the day on which the rat was killed (i.e. 'last' day) was calculated as below:

$$\frac{\text{body weight on 'last' day - body weight on 'first' day}}{\text{mean body weight on 'first' day}} \times 100\%$$

The mean % change, during the period of an experiment, in body weight for each group of rats was then calculated.

II. Organ weight

Organ weight is expressed in g. However, to compensate for differences in body weight between rats, organ weight was calculated as a percentage of body weight for statistical analysis (see below).

I. STATISTICAL ANALYSES

Statistical analyses were performed on three machines :

i. Apple Europlus II 'microcomputer' (Lavenread Computers Ltd.) using a programme written by Bruce Land (Interstat., 1979) (Serendipity Systems, Inc.).

The programme was checked for statistical accuracy by Miss M. Rehahn (Group Statistician, Brompton Hospital) and found to be acceptable. Mann-Witney U-test, Student's t-test.

ii. Hewlett-Packard 9810A Programmable Calculator using programmes written by Miss M. Rehahn. Analysis of co-variance, Student's t-test for difference between slopes of regression lines.

iii. Prime 750 ('Main Frame') Computer using programmes written by Miss M. Rehahn. Frequency distributions, one-way Kruskal-Wallis analysis of variance by ranks.

I. Distribution of data

Appropriate statistical tests, to determine whether there were significant differences between groups, were chosen after inspection of frequency distributions constructed from 'raw' data.

a. Secretory cell number

The frequency distribution for number of neutral, acidic and total (i.e. combined neutral and acidic) secretory cells was 'Normally' (i.e. Gaussian) distributed, but consistently positively skewed (i.e. with the 'tail' extending to the right) so that the mean value did not approximate to the median value. Non-parametric statistical analyses were therefore employed. One-way Kruskal-Wallis analysis of variance by ranks was used to initially assess the variation in the data. The number of animals in a group was too small to allow for a strict median test between groups and subsequent analysis was performed using the Mann-Witney U-test (two tailed) which is a 'flexible' test not invalidated by small

sample numbers. Mean values and standard errors of the means (± SEM) are presented in the Results' tables.

b. Body weight

Change in body weight of rats was calculated from the body weight at the beginning and end of the experiment, the values of which were 'Normally' distributed but varied independently. Differences between the mean % change in body weight were therefore compared by analysis of co-variance: the regression line of starting weight on final weight for each pair of values for rats in a group was constructed, giving a common combined slope. The significance of differences between slopes for each group was determined by Student's t-test.

c. Organ weight

The frequency distribution of organ weight as a percentage of body weight (dependent variables) was 'Normally' distributed. The significance of differences between means was therefore determined by Student's t-test (two-tailed).

II. Levels of significance

The statistical probability value (p-value) at which the Null Hypothesis (i.e. no difference between groups) was either accepted or rejected was 0.05 (i.e. 5%) in the studies presented here. P-values above 0.05 were therefore taken as indicating no significant difference between groups. P-values less than 0.05 were taken as indicating a significant differences between groups. Three levels of significance were chosen: p less than 0.05, 0.01 and 0.001.

III. Control animals

Experiments associated with the studies presented herein (see above) have demonstrated no significant difference in secretory cell number ('total', neutral and acidic) between rats not exposed to TS (i.e.'cage controls') and rats exposed to air to the same regimen as described above for TS (i.e. 'sham' TS). Due to demand for space limiting the number of exposure cabinets, cage control rats were used as the 'baseline' for comparison in the studies presented herein (see Results).

IV. Selection of airway walls

Comparison of the number of secretory cells were made between airway walls at each airway level.

a. Trachea

Coronal sections of the trachea appear as two separate walls, one wall being on the left side of the animal and the other wall the right. Secretory cells were counted per high power field (HPF) in both walls of three, randomly selected tracheas and the mean number per 10 HPF (± SEM) calculated:

Secretory cell number

Neutral		Acidic		Total	
Wall 1	Wall 2	Wall 1	Wall 2	Wall 1	Wall 2
1 5 (5)	1 0 (4)	8 5 (1 4)	8 3 (1 4)	10 0 (1 5)	9 3 (1 5)
7 (2)	6 (3)	8 3 (1 4)	6 5 (1 4)	9 0 (1 3)	7 1 (1 2)
4 6 (1)	5 1 (6)	13 4 (1 2)	10 4 (1 5)	17 9 (1 7)	14 9 (1 5)

There was no significant difference in the mean numbers of each secretory cell category between the walls. Consequently, the least fragmented

wall was chosen for secretory cell counts in the trachea.

b. Intrapulmonary axial pathway

The 'medial' wall was chosen for counts because, unlike the lateral wall, it was generally completely free of BALT and 'unbroken' by lateral branches.

c. Intrapulmonary lateral pathways

Secretory cell counts can be made along the upper or lower 'walls' of the lateral branches of the axial pathway.

Secretory cells were counted along the upper and lower 'walls' of the uppermost and lowermost lateral pathways (see Fig. 15) for two groups of 8 animals. The mean number of each secretory cell category was calculated for the upper and lower 'wall' of each 'lateral' in each group (Table 7).

Table 7. Mean secretory cell number (± SEM) per 10 HPF's in the upper (U) and lower (L) walls of the lateral pathways

Group	Lateral	Neutral		Acid		Total	
1	Upper	3.0	2.8	40.3	48.0	43.3	50.3
		(1.7)	(1.1)	(20.8)	(26.7)	(21.2)	(26.1)
	Lower	3.8	2.8	11.3	12.3	15.0	15.0
		(1.8)	(1.0)	(5.2)	(6.6)	(6.9)	(7.5)
2	Upper	1.3	2.8	65.8	57.8	67.0	60.5
		(1.3)	(1.1)	(34.9)	(30.4)	(34.2)	(3.14)
	Lower	5.0	3.3	21.8	17.0	26.8	20.3
		(2.6)	(2.0)	(1.7)	(4.7)	(4.7)	(3.8)

There was no significant difference between the means

for each 'wall'. Secretory cells were therefore counted along the lower 'walls' of the lateral pathways.

V. Observer errors

a. Error of repeat measurement

Variation between counts of secretory cells by the author was determined by the 'error of repeat measurement' (Table 8). The number of secretory cells in three sections from each of 10 rats (with a range of secretory cell 'counts') was determined in the trachea and in the intrapulmonary airways: the number of secretory cells were combined to give an overall 'airway' value. Each section was 'counted' on 3 consecutive days. The mean 'airway' secretory cell number and standard error was calculated for each set of three counts. The standard error was expressed as a percentage of the mean to determine the error of repeat measurement (Table 8). In general, errors were greatest in counts of neutral cells, particularly in sections with few secretory cells (animals 5, 6 and 7), and least in counts of acidic secretory cells. The latter contributed to lower the error in the total count of secretory cells. The 'total mean % error' for each secretory cell category was; neutral secretory cells 25.2 $\pm$ 5.0, acidic secretory cells 6.5 $\pm$ 1.1 total 7.0 $\pm$ 1.1. However, these are maximum values because the error in repeat counts which were carried out after the author had gained 'experience' in counting secretory cells (animals 8, 9 and 10) was generally less than had been found previously (see below).

Table 8. Standard error as % of mean *

Rat	Section	Neutral	Acidic	Total
1	1	17.2	4.8	5.0
	2	2.5	5.6	5.1
	3	3.2	6.3	6.1
2	1	4.0	2.3	7.7
	2	0.5	14.2	9.3
	3	8.1	3.2	2.1
3	1	37.9	3.8	5.1
	2	13.1	3.0	3.1
	3	18.8	3.5	4.2
4	1	32.0	15.3	17.1
	2	31.6	11.1	8.3
	3	19.3	7.0	6.8
5	1	34.6	1.2	6.5
	2	100.0	16.5	15.3
	3	50.4	8.3	16.6
6	1	34.8	2.7	2.0
	2	17.0	19.2	19.8
	3	69.7	22.2	20.6
7	1	25.6	1.9	3.6
	2	62.0	14.2	12.8
	3	100.0	1.5	3.9
8	1	11.5	1.6	4.1
	2	4.7	1.4	2.2
	3	4.5	7.0	4.6
9	1	11.2	0.8	1.7
	2	16.9	1.8	1.7
	3	1.9	2.9	2.6
10	1	11.3	1.0	3.2
	2	6.0	9.0	7.5
	3	4.5	2.6	1.1

* mean secretory cell number in each section counted on 3 separate days.

b. Variation between observers

On three occasions during the studies, variation in counts between the author and two other observers was investigated. On each occasion, secretory cells were counted along the complete length of a trachea (Table 9) The same counting criteria were agreed upon by all observers (see above, Fig. 14).

Table 9 Secretory cell number per 10 HPF's

Year	Observer	Neutral	Acidic	Total
1978	1	26.7	193.6	220.3
	2	13.0	188.9	201.9
	*3	3.5	151.0	154.5
1980	*1	7.4	79.7	87.1
	2	0	53.9	53.9
	3	3.9	41.9	45.8
1983	*1	50.6	104.4	155.0
	2	6.4	65.0	71.4
	3	10.0	83.3	93.3

* observer = author

In the table, observers 1 to 3 are in order of 'experience' in counting secretory cells with observer 1 being the most experienced. There is a difference between counts made by different observers, and in general, the more 'experienced' the observer, the higher the count of each cell type. Again, the greatest differences are found in the counts of neutral secretory cells.

VI. Intra-animal variation

Variation in distribution of secretory cells within an animal may be divided into two parts: a. the variation in number of secretory cells between each high power field (HPF), and b. the variation between sections.

a. Variation between fields

Secretory cells were counted along the complete length of six tracheal 'walls' and the number of each secretory cell type in each field of view determined (average 39 field/wall). The mean, standard deviation (SD), standard error (SEM) and 'percentage error' (standard error as a percentage of the mean) were calculated for each cell type in each trachea. The overall mean SD and % error (± SEM) was calculated for each cell type:

	Neutral	Acidic	Total
Mean SD	1.9 (0.4)	5.0 (0.3)	5.4 (0.3)
Mean % error	30.9 (5.5)	15.4 (1.4)	13.7 (1.2)

The high mean % error values obtained reflected the subjective observation that the secretory cells were not distributed evenly along a length of epithelium. Some portions of epithelium contain clusters of secretory cells whilst others have no secretory cells. Examination of the 'raw' data revealed that the distribution of counts for each category of secretory cell may be Normal, but more often was either positively skewed Normal or Poisson: i.e. there are many more low than high counts.

In general, the smallest number of fields counted at any airway level in the studies presented herein was 16. To determine the confidence limit for counts in 16 fields, the 'sampling error' (SE) of the means for a set of data with a Poisson distribution ($\bar{X}$ = 2.5) and a set of data with a Normal distribution ($\bar{X}$ = 14.1) were first compared using the Central Limit Theorem:

Poisson (mean approximates to variance) :

$$95\% \text{ confidence limits} = \bar{X} \pm 2 (\sqrt{\bar{X}})$$

Normal (mean does not approximate to variance) :

$$95\% \text{ confidence limits} = \bar{X} \pm 2 (\text{variance})$$

Thus, for 16 fields, the following 'sampling errors' (SE) are found:

Distribution of data	$\bar{X}$	SE
Poisson	2.5	3.2
Normal	14.1	116.5

Normally distributed data, therefore, has greater sampling errors than non-Normally distributed data. To determine the confidence limits of Normally distributed data the following formula may be used (Aherne and Dunnill, 1982):

$$z_{\frac{a}{2}} \left[\frac{s}{\sqrt{n}} \right] < e$$

where n is the number of observations (i.e. HPF)

s is the standard deviation of the sample (7.6 in this example)

z is the Z-variate with the subscript $^{a}/2$ indicating that the 5% significance 'territory' is

shared between the upper and lower tails of the probability distribution(s): 1.96 for 95% confidence.

e is an independent quantity which equals:

[probability level, p; e.g. 0.05] $\cdot \bar{X}$

$$\therefore \text{ when } n = 16; \quad e = 1.96 \left[\frac{7.6}{\sqrt{16}} \right] = \underline{3.7}$$

Solving for the probability level (p), where $p = \dfrac{e}{\bar{X}}$

$$p = \frac{3.7}{14.1} = \underline{0.26}$$

Thus, in <u>Normally</u> distributed data with p = 0.26, it is only 74% certain that the mean (i.e. 14.1) is within ± e (3.7) of the true (population) mean. The degree of confidence in the present studies is probably better than this because i. more fields were usually counted (i.e. up to 20 or more), and ii. the distribution of the data in the study is <u>not</u> Normal. However, the variability of the data ultimately decides the degree of 'confidence' and, in the case of secretory cell number per field, variability is large. Thus, the complete length of epithelium at each airway level was 'counted' in the studies presented herein (see below).

b. <u>Variation between sections</u>

The mean and standard error for the first counts for each section from each of the 10 animals described previously (Table 8) was calculated. Each standard error was expressed as a percentage of its mean. The 'total mean % error' (± SEM) for each cell type is as follows:

Neutral: 13.9 ± 0.9, Acidic: 6.8 ± 3.5, Total: 8.4 ± 2.7

The second and third counts for each section were treated similarly and the values were found to be comparable to those above. There was no significant difference in the counts of each cell type between sections for each animal, except for three instances where there was a difference in number of neutral secretory cells ($p < 0.05$), two instances of a difference in number of acidic secretory cells ($p < 0.05$). and one instance of a difference in total number of secretory cells ($p < 0.05$). Two sections per animal were therefore 'counted' in the studies presented herein.

VII. Variation between animals

The variation in secretory cell number between animals was determined by calculating the mean and standard error of the first count of the first section for a group of 8 animals. The standard error was expressed as a percentage of the mean and the 'total mean percentage error' (± SEM) calculated for each cell type:

Neutral: 30.5 ± 1.3, Acidic: 28.2 ± 2.1, Total: 25.1 ± 2.5

When the total percentage error was calculated for two sub-groups (within the group of 8 above) comprising 3 and 5 animals each, the error became reduced with the greater the number of animals in the sub-group:

No. animals	Mean % error		
	Neutral	Acidic	Total
3	42.1	31.7	28.9
5	31.9	30.3	27.5
8	30.5	28.2	25.1

In addition, when all the counts (i.e. of repeat measurement, intra and inter-animal variation) for the group of 8 animals were combined (i.e. n = 72 'counts' for each cell type) the total mean % errors are as follows:

Neutral: 9.6, Acidic: 8.1, Total 6.6.

In summary, the two main sources of variation when counting the number of epithelial secretory cells are i. the patchy distribution of both neutral and acidic secretory cells along the epithelium, and ii. the variation between animals. Both the observer error and the variation between sections of one animal are small by comparison. 'Counting' the complete length of epithelium at each airway level will reduce the variation in secretory cell number between fields of view. However, although it would be possible to reduce the variation between animals by repeated counting of many sections from a few animals it is doubtful whether this would be a statistically valid quantification technique. It was therefore decided to have comparatively large animal numbers in a group and to only count two sections on one occassion for each animal.

Counts of neutral secretory cells consistently showed the greatest percentage errors and variation which was reflected in the difference in counts between observers. Observers agreed that a greater amount of 'decision-making' was required when counting neutral than when counting acidic secretory cells: many more neutral secretory cells are counted by 'experienced' observers. There are two main consequences of this finding. Firstly, the numbers of cells counted and the composition of the secretory cell

population will change with time over a series of experiments conducted by the same worker. Secondly, strict comparison with counts made by other workers must be made with caution. In addition, when acidic secretory cells are numerous, for example after exposure to smoke, many neutral secretory cells may be 'missed' and an erroneously low number recorded.

CHAPTER 3

RESULTS

There is no more common error than to assume that, because prolonged and accurate mathematical calculations have been made, the application of the result to some fact of Nature is absolutely certain.

A.N. WHITEHEAD

SECTION A

The effect of nicotine on secretory cell number

The effect of nicotine on the number of secretory cells in bronchial epithelium was determined in two experiments: the first to determine the effect of nicotine when given in an aerosol and by injection at a 'low' dose; the second to determine the effect of a 'high' dose of nicotine given by injection.

Forty five rats with a mean body weight of 205g (± 2g) on the first day of treatment were divided into seven different treatment groups (Table 10). The mean starting weight of the rats in the first experiment of 199g (± 2g) was significantly less than that of 221g (± 4g) of the rats in the second experiment ($p < 0.001$).

The mean concentrations of nicotine in the plasma of the rats with each treatment were determined (see Materials and Methods for details) and are summarised below:

Treatment	Nicotine, ng/ml
Controls (untreated and 'vehicle'-treated)	0.3
Nicotine aerosol	10.0
Nicotine injection: 'low' dose	695.0
'high' dose	2356.0

Table 10. Plan of experiment

Treatment (no. days)			Number of animals
None (Pure controls)			6
Sterile, distilled water:	Aerosol	(21)	5
Arachis oil:	2 sc. injections	(21)	5
Physiological saline:	8 ip. injections	(14)	5
Nicotine in sterile, distilled water:	8 mg. for 80 minutes by aerosol	(21)	8
Nicotine in arachis oil:	2 x 10 mg/Kg sc. injections ('low' dose)	(21)	8
Nicotine in physiological saline:	8 x 7.5 mg/Kg ip injections ('high' dose)	(14)	8

sc. = subcutaneous ip. = intraperitoneal

In the first experiment, plasma levels of injected nicotine declined towards control values before the second injection was given (see Materials and Methods for details). Consequently, in the second experiment, a series of injections were given in an attempt to maintain constant plasma nicotine levels.

In the first experiment, rats were treated for 22 consecutive days. In the second experiment, rats were treated for 14 consecutive days to allow comparison of results with rats exposed to tobacco smoke in subsequent studies. The body weight of the rats and the weight of adrenal glands, heart, kidneys, spleen, testes and thymus gland were measured.

I. Animals: General condition

The only animals to show any abnormal behavioural effects with

treatment were those injected with the 'high' dose of nicotine. After the first couple of injections on each day, the rats became irritable but this symptom was reduced with subsequent injections and disappeared before further injections were given. When the rats were dissected at the end of the experiments no abnormal features were seen on the surface of any internal organ.

II. Body weight

All rats gained weight during the two experiments (Table 11).

Table 11. Mean % increase in body weight, g (± SEM)

Treatment	Duration of study (days)			
	15		23	
Pure controls	46.5	(2.4)	65.7	(2.8)
Water aerosol	42.7	(2.5)	71.4	(4.0)
Arachis oil (sc)	33.2**	(2.1)	47.9**	(3.4)
Saline (ip)	38.7	(2.6)	-	
Nicotine aerosol	51.1	(2.9)	71.2	(4.3)
Nicotine in arachis oil, 'low' dose (sc)	40.2	(3.6)	61.0	(4.3)
Nicotine in saline, 'high' dose (ip)	15.3***	(1.2)	-	

sc = sub-cutaneous injection

ip = intra-peritoneal injection

$n \geq 5$

Significant differences compared to pure control values: ** = $p < 0.01$

*** = $p < 0.001$

The only treatments to have a significant effect on body weight were arachis oil and the 'high' dose of nicotine: rats gained less weight than the pure controls (arachis oil, $p < 0.01$; 'high' dose nicotine, $p < 0.001$).

III. Organ weight

The mean organ weights are given in Table 12. When expressed as a percentage of the body weight, the only significant finding was that the thymus glands in rats given the 'high' dose of nicotine (in saline) were lighter than those in control rats given saline ($p < 0.05$).

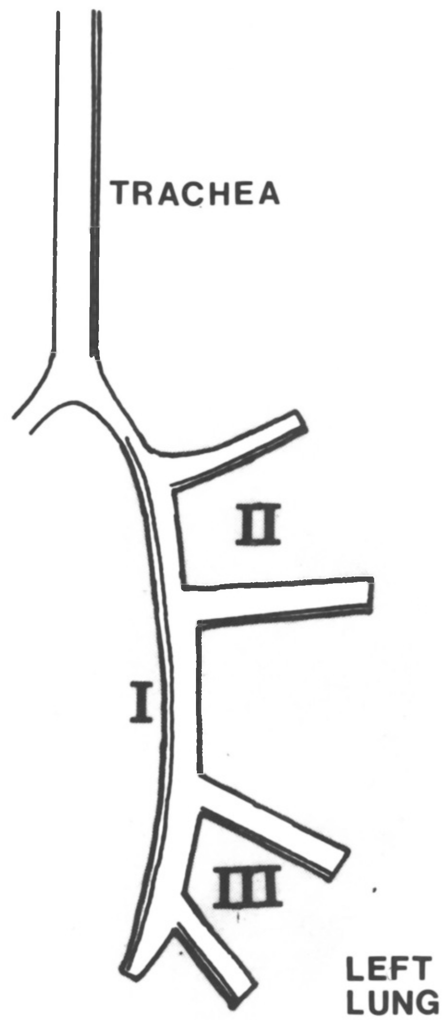
IV. Secretory cell number

Secretory cells were counted at four airway levels: the trachea, and the intrapulmonary axial and upper and lower lateral pathways (Fig. 17). Mean secretory cell numbers ($\pm$ SEM) for experiments one and two are given in Tables 13 and 14 respectively.

In neither experiment were there any significant differences in secretory cell number between control groups. Consequently, in the first experiment the significance of differences in number of secretory cells in animals treated with nicotine was assessed against pure control values. In the second experiment, the significance of differences in secretory cell number with 'high' dose nicotine-treatment was assessed against saline-treated control values.

The effect of nicotine-treatment on the percentage change in secretory cell number varied (Table 15). When given by aerosol, nicotine had no consistent or significant effect. The 'low' dose of injected nicotine in general decreased the number

Fig.17. AIRWAY LEVELS:NICOTINE



I = axial

II = combined upper two laterals

III = combined lower two laterals

Table 12. Mean organ weight, g (± SEM)

Treatment	Adrenal glands	Heart	Kidneys	Spleen	Testes	Thymus glands
Pure controls (untreated)	0.03 (0.00)	1.02 (0.07)	1.24 (0.04)	0.72 (0.12)	1.68 (0.02)	0.50 (0.05)
Water aerosol	0.03 (0.00)	0.90 (0.04)	1.23 (0.04)	0.60 (0.03)	1.47 (0.02)	0.53 (0.05)
Arachis oil (sc)	0.03 (0.00)	0.92 (0.04)	1.20 (0.05)	0.52 (0.04)	1.56 (0.04)	0.49 (0.04)
Nicotine aerosol	0.03 (0.00)	0.84 (0.04)	1.07 (0.05)	0.54 (0.05)	1.54 (0.03)	0.48 (0.04)
Nicotine in arachis oil: 'low' dose(sc)	0.03 (0.03)	0.92 (0.02)	1.16 (0.04)	0.53 (0.04)	1.49 (0.04)	0.40 (0.04)
Saline (ip)	0.03 (0.00)	0.96 (0.04)	1.07 (0.05)	0.50 (0.04)	1.23 (0.08)	0.63 (0.07)
Nicotine in saline: 'high' dose (ip)	0.02 (0.00)	0.96 (0.04)	1.14 (0.04)	0.65 (0.09)	1.33 (0.04)	0.24 (0.04)

sc = sub-cutaneous injection, ip = intra-peritoneal injection, $n \geq 5$

Table 13. Mean secretory cell number per 10 high power fields (± SEM) (first experiment).

Airway level	Secretory cells	Pure controls		Water aerosol		Arachis oil (sc.)		Nicotine aerosol		Nicotine 'low' dose (sc.)	
Trachea	Total	35.6	(4.6)	44.2	(5.5)	35.3	(4.5)	47.1	(7.4)	34.3	(3.5)
	Neutral	10.2	(2.0)	11.9	(1.7)	13.0	(1.9)	12.7	(1.8)	8.0	(1.0)
	Acidic	25.4	(3.2)	32.3	(4.9)	22.3	(2.7)	34.4	(6.0)	26.3	(3.3)
Axial	Total	41.8	(7.0)	61.9	(12.0)	43.7	(5.2)	43.1	(3.1)	32.0	(4.2)
	Neutral	2.8	(0.5)	2.6	(0.7)	3.9	(0.7)	2.5	(0.4)	3.9	(0.6)
	Acidic	38.9	(7.0)	59.3	(11.6)	39.8	(4.8)	40.6	(2.9)	28.1	(3.9)
Laterals: upper	Total	47.6	(11.0)	58.4	(19.5)	41.2	(2.2)	42.8	(9.8)	15.3	(3.6)
	Neutral	2.8	(1.1)	2.1	(0.9)	3.8	(1.8)	3.4	(2.1)	3.0	(0.6)
	Acidic	44.9	(10.4)	56.3	(19.4)	37.4	(8.1)	39.4	(8.9)	12.9	(3.2)
lower	Total	15.0	(7.5)	24.0	(7.2)	40.8	(25.0)	19.9	(10.4)	20.8	(12.2)
	Neutral	2.8	(1.0)	3.5	(2.1)	5.3	(3.6)	0.9	(0.7)	2.8	(1.4)
	Acidic	12.3	(6.6)	17.0	(4.7)	35.8	(21.4)	18.4	(10.5)	18.0	(11.1)

sc = sub-cutaneous injection; n ≥ 10

Table 14. Mean secretory cell number per 10 high power fields (± SEM) second experiment.

Airway level	Secretory cells	Saline (ip.)		Nicotine 'high' dose (ip.)	
Trachea	Total	97.3	(7.1)	111.5	(6.2)
	Neutral	0.6	(0.1)	0.7	(0.2)
	Acidic	96.7	(7.1)	110.8	(6.2)
Axial	Total	304.3	(16.4)	425.3	(20.0)
	Neutral	10.6	(10.0)	6.5	(1.3)
	Acidic	293.7	(13.0)	418.8	(20.0)
Laterals:upper	Total	184.6	(21.7)	287.5	(30.6)
	Neutral	8.7	(3.8)	4.3	(1.9)
	Acidic	175.9	(21.4)	283.2	(30.5)
lower	Total	190.7	(19.6)	188.6	(18.0)
	Neutral	6.9	(3.9)	1.9	(0.6)
	Acidic	183.8	(19.2)	186.7	(18.0)

ip. = intra-peritoneal injection

n $\geqq$ 10

Table 15. % increase (↑) or decrease (↓) in secretory cell number (compared to controls) after nicotine treatment

Airway level	Secretory cells	Aerosol	'Low' dose	'High' dose
Trachea	Total	↑ 32.3	↓ 3.7	↑ 14.6
	Neutral	↑ 24.5	↓ 21.6	↑ 16.7
	Acidic	↑ 35.4	↓ 3.5	↑ 14.6
Axial	Total	↑ 6.2	↓ 23.4	↑ 39.8 *
	Neutral	↑ 47.1	↑ 39.3	↓ 38.7
	Acidic	↑ 4.4	↓ 27.8 *	↑ 42.6 **
Laterals: upper	Total	↓ 6.1	↓ 67.9 *	↑ 55.7 **
	Neutral	↑ 21.4	↑ 7.1	↓ 50.6
	Acidic	↓ 7.9	↓ 71.3 *	↑ 61.0 **
lower	Total	↑ 32.6	↑ 38.7	↓ 1.1
	Neutral	↓ 0.7	0	↓ 72.5
	Acidic	↑ 49.6	↑ 46.3	↑ 1.6

$n \geqq 10$

Significant differences compared to controls: * = $p < 0.05$, ** = $p < 0.01$

of secretory cells, significantly so in the upper lateral pathways of the lung. The reduction was due to a decrease in number of acidic secretory cells which was significant in the axial and upper lateral pathways ($p < 0.05$). No significant change was seen in the number of neutral secretory cells. Conversely, the 'high' dose of injected nicotine in general increased the number of secretory cells, significantly so in the axial and upper lateral pathways ($p < 0.05$ and < 0.01 respectively). The increase was due to an increase in number of acidic cells which was significant in the axial and upper lateral pathways ($p < 0.01$). No significant change was seen in the number of neutral secretory cells.

V. Summary

1. Rats were treated with nicotine given in an aerosol (mean plasma nicotine concentration 10 ng/ml) or by injection at a 'low' dose (mean plasma concentration 695 ng/ml) or 'high' dose (mean plasma concentration 2356 ng/ml) and the number of secretory cells assessed in the trachea and at three intrapulmonary airway levels.
2. Rats given the 'high' dose of nicotine gained less weight and their thymus glands were lighter than controls.
3. Nicotine aerosol had no consistent or significant effect on secretory cell number.
4. When given by injection, nicotine had opposing effects; the 'low' dose significantly reduced, whilst the 'high' dose significantly increased, the number of secretory cells in the proximal intrapulmonary airways ($p < 0.05$).
5. The change in number of secretory cells was due primarily

to significant changes in the number of acidic secretory cells. There was no significant change in the number of neutral secretory cells.

SECTION B

The effect of anti-inflammatory drugs on tobacco smoke-induced secretory cell hyperplasia

Experiment 1.

Parenteral administration of indomethacin and flurbiprofen

To determine,i. whether TS-induced secretory cell hyperplasia could be inhibited by intraperitoneal injections of the non-steroidal anti-inflammatory drugs indomethacin and flurbiprofen given daily with the smoke and, ii. whether any inhibitory effect was dose related.

Sixty eight rats with a mean body weight of 215g (± 2g) on the first day of treatment were divided into 6 different treatment groups (Table 16). A daily dose of 4 mg/Kg indomethacin was chosen because it has been shown previously to be the most potent dose in inhibiting TS-induced secretory cell hyperplasia (Greig et al., 1980): a dose of 2 mg/Kg was less effective. A dose of 0.4 mg/Kg was also given. Flurbiprofen is a more potent inhibiter of certain inflammatory reactions than is indomethacin (for example see Ishii et al., 1975). Consequently, the same daily doses of flurbiprofen (i.e. 0.4 and 4.0 mg/Kg) were chosen to allow direct comparison, on an 'equidosage' basis, of the degree of inhibition by each drug in the system. Each drug was given as a twice daily dose of either 0.2 or 2 mg/Kg in an attempt to maintain plasma levels in the rats. The first injection was given about an hour before the beginning of TS exposure, the second immediately after the exposure period.

Rats were exposed daily to the smoke from 25 John Player Navy Cut, unfiltered cigarettes (see Materials and Methods for details). The exhaust rate from the exposure cabinets was 2 x 2 l/min (see Materials and Methods for details). The animals were treated for 14 consecutive days during which time they were weighed regularly.

Table 16. Plan of experiment

Treatment	Daily drug dosage, mg/Kg	Number of rats
No TS, $NaHCO_3$	-	8
No TS, flurbiprofen in $NaHCO_3$	2 x 0.2	5
	2 x 2.0	5
No TS, indomethacin in $NaHCO_3$	2 x 0.2	5
	2 x 2.0	5
TS + $NaHCO_3$	-	8
TS + flurbiprofen in$NaHCO_3$	2 x 0.2	8
	2 x 2.0	8
TS + indomethacin in $NaHCO_3$	2 x 0.2	8
	2 x 2.0	8

TS = cigarette tobacco smoke

$NaHCO_3$ = sodium bicarbonate

I. Animals : General condition

One animal which had been exposed to TS and treated daily with a 4 mg/Kg indomethacin for 6 days had to be killed before the end of the study. After 6 days of treatment the rat had lost weight and had become thin and 'hunched'. A post-mortem on the rat revealed the liver to be pale in colour. No other gross abnormalities were found.

II. Body weight

Apart from the rat which had to be killed, all rats gained weight during the course of the experiment (Table 17). Control rats which had not been exposed to TS but had been given the two drugs gained more weight than the control rats given sodium bicarbonate, but only significantly so in the case of rats given 0.4 mg/Kg indomethacin ($p < 0.01$). Rats exposed to TS all gained less weight than their respective control groups not exposed to TS. Rats exposed to TS and given 4mg/Kg of either drug gained significantly less weight than control rats given sodium bicarbonate ($p < 0.05$).

III. Secretory cell number

Secretory cells were counted at seven airway levels: the whole length of the trachea and at intrapulmonary airway levels I to VI (see Fig. 15). Mean numbers of secretory cells are given in Tables 18, 19 and 20. In the group of rats given 0.4 mg/Kg flurbiprofen, airway level VI was absent from the plane of the section in all but one animal. The 'mean' value obtained was therefore excluded from subsequent analysis.

a. Control animals

Treatment of animals with the drugs alone had little significant effect on secretory cell number when compared to

Table 17. Mean % increase in body weight, g (± SEM)

TS = Cigarette tobacco smoke

$NaHCO_3$ = Sodium bicarbonate

Administration	Dose, mg/Kg	No TS	TS
$NaHCO_3$	-	26.7 (2.0)	23.9 (0.8)
Flurbiprofen in $NaHCO_3$	0.4	33.4 (3.2)	22.3 (1.1)
	4.0	30.2 (1.7)	*18.3 (2.0)
Indomethacin in $NaHCO_3$	0.4	**37.8 (2.3)	27.5 (2.8)
	4.0	30.4 (1.7)	*16.7 (3.1)

$n \geq 5$

Significant differences compared to No TS, $NaHCO_3$ * = $p < 0.05$

** = $p < 0.01$

Table 18. Mean number secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, F = flurbiprofen, I = indomethacin, 0.4 and 4.0 = dose in mg/Kg

Airway level	No TS $NaHCO_3$	No TS F, 0,4	No TS F, 4.0	No TS I, 0.4	No TS I, 4.0	TS alone	TS + F, 0.4	TS + F, 4.0	TS + I, 0.4	TS + I, 4.0
Trachea	90.7 (12.8)	77.8 (5.1)	66.0 (11.2)	63.4 (14.2)	71.0 (15.0)	90.4 (9.7)	70.4 (10.6)	54.0 (4.0)	63.9 (7.5)	61.8 (6.3)
Lung: I	44.1 (4.3)	37.1 (4.9)	59.9 (9.2)	68.4 (8.4)	59.6 (1.7)	91.6 (12.5)	66.6 (6.6)	60.2 (6.6)	73.3 (10.3)	73.4 (7.2)
II	40.2 (6.0)	23.1 (7.0)	38.2 (10.0)	51.4 (12.6)	47.4 (8.1)	72.3 (11.7)	49.5 (10.4)	74.9 (9.2)	70.0 (11.7)	57.3 (10.8)
III	50.4 (7.7)	50.8 (11.0)	49.8 (6.1)	70.1 (9.0)	68.5 (5.1)	76.9 (9.4)	91.3 (16.8)	82.8 (10.0)	99.9 (11.5)	81.3 (11.6)
IV	23.1 (4.9)	20.0 (7.3)	31.7 (6.1)	39.7 (11.0)	26.9 (9.9)	46.6 (5.0)	58.9 (12.1)	65.7 (11.3)	55.5 (9.6)	36.5 (8.0)
V	14.3 (4.9)	22.6 (2.5)	5.6 (1.8)	29.4 (12.5)	29.5 (8.7)	30.1 (10.6)	41.8 (17.4)	30.5 (6.6)	26.8 (6.8)	17.2 (9.0)
VI	12.7 (2.3)	[5.7]	23.3 (14.0)	10.2 (1.9)	19.8 (7.6)	22.6 (3.8)	31.1 (18.3)	32.2 (8.3)	25.4 (7.9)	7.5 (3.5)

n ≧ 10, except [ ] where n = 2

Table 19. Mean number neutral secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, F = flurbiprofen, I = indomethacin, 0.4 and 4.0 = dose in mg/Kg

rway level	No TS $NaHCO_3$	No TS F, 0.4	No TS F, 4.0	No TS I, 0.4	No TS F, 4.0	TS alone	TS + F, 0.4	TS + F, 4.0	TS + I, 0.4	TS + I, 4.0
rachea	21.5 (4.8)	13.2 (3.9)	12.1 (1.6)	5.5 (1.2)	14.0 (5.6)	16.4 (3.2)	10.0 (1.0)	10.0 (2.1)	12.2 (2.4)	10.1 (2.6)
ung: I	1.6 (0.7)	3.5 (0.9)	7.7 (3.7)	2.4 (0.7)	2.2 (0.5)	2.2 (1.5)	2.7 (0.8)	5.0 (2.0)	3.9 (1.8)	2.8 (0.9)
II	2.1 (1.0)	1.4 (0.3)	1.7 (1.1)	2.5 (0.9)	1.9 (0.5)	1.8 (0.8)	1.8 (0.5)	2.9 (1.2)	4.6 (1.7)	2.6 (0.6)
III	4.0 (0.8)	4.5 (1.9)	2.6 (0.7)	1.0 (0.6)	2.7 (1.4)	4.5 (3.0)	4.7 (2.0)	3.9 (1.6)	5.4 (1.6)	1.8 (0.6)
IV	3.0 (1.3)	0.0 (0.0)	0.6 (0.6)	2.1 (0.8)	0.8 (0.4)	2.6 (1.3)	3.3 (0.9)	3.9 (1.7)	0.7 (0.6)	1.4 (0.5)
V	0.8 (0.5)	4.2 (0.9)	0.3 (0.3)	1.8 (0.6)	2.8 (1.8)	0.6 (0.4)	1.7 (0.7)	2.3 (1.2)	1.8 (0.8)	0.2 (0.2)
VI	0.7 (0.5)	[0.0]	4.3 (2.3)	1.6 (0.8)	1.9 (1.2)	1.8 (0.8)	3.1 (1.9)	2.2 (0.7)	2.0 (0.7)	0.0 (0.0)

n ≧ 10, except [ ] where n = 2

Table 20. Mean number acidic cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, F = flurbiprofen, I = indomethacin, 0.4 and 4.0 = dose in mg/Kg

Airway level	No TS $NaHCO_3$	No TS F, 0.4	No TS F, 4.0	No TS I, 0.4	No TS I, 4.0	TS alone	TS + F, 0.4	TS + F, 4.0	TS + I, 0.4	TS + I, 4.0
Trachea	69.3 (9.2)	64.6 (1.3)	53.9 (11.2)	52.6 (11.1)	57.0 (9.7)	74.0 (7.0)	60.5 (10.7)	44.0 (4.0)	51.7 (6.4)	51.8 (5.3)
Lung: I	42.4 (4.0)	33.5 (4.4)	52.2 (8.5)	66.0 (8.5)	57.4 (1.6)	96.9 (7.3)	64.0 (6.5)	55.2 (7.3)	69.4 (9.9)	70.6 (3.4)
II	38.1 (5.8)	21.8 (7.2)	36.5 (8.6)	48.9 (11.9)	45.6 (7.8)	70.5 (11.9)	47.8 (10.5)	72.0 (9.9)	65.3 (10.2)	54.7 (10.6)
III	46.0 (7.3)	46.3 (10.7)	47.3 (5.9)	69.0 (8.9)	65.8 (5.4)	72.3 (7.7)	87.2 (16.2)	78.6 (10.3)	94.6 (10.2)	79.4 (11.8)
IV	20.0 (3.9)	20.2 (7.3)	31.3 (5.7)	37.6 (10.5)	26.1 (9.8)	44.0 (4.5)	55.5 (11.9)	61.8 (10.7)	54.8 (10.6)	34.9 (10.1)
V	13.4 (4.8)	18.4 (3.4)	5.3 (1.5)	27.6 (12.1)	26.6 (7.5)	29.7 (10.6)	40.0 (17.2)	28.3 (6.5)	24.9 (6.2)	16.5 (8.7)
VI	11.0 (2.1)	[5.7]	19.0 (13.0)	8.6 (2.1)	17.9 (6.5)	20.8 (3.7)	28.0 (16.9)	30.0 (7.7)	23.4 (7.8)	7.3 (3.4)

n ≥ 10 except [ ] where n = 2

those given sodium bicarbonate. The only significant differences were,i. there were fewer neutral secretory cells in the trachea and airway level III in rats given 0.4 mg/Kg indomethain ($p < 0.05$), and ii. there were significantly more total and acidic secretory cells at level I in rats given either dose of indomethacin ($p < 0.05$).

b. Effect of tobacco smoke

Secretory cell numbers were compared between rats given sodium bicarbonate and exposed to TS. TS increased the number of secretory cells at all intrapulmonary airway levels studied, significantly so at levels I, II, III and IV (Table 21). The increase was due primarily to a significant increase in number of acidic secretory cells (at levels I, III, IV and VI (Table 22) because TS had no consistent or significant effect on the number of neutral secretory cells (Table 19).

c. Inhibitory effect of the drugs

The inhibitory effect of the drugs on TS-induced changes in secretory cell number was assessed by comparing rats exposed to TS alone and rats given TS and drug. Complete inhibition was achieved when there was no significant difference between secretory cell number in rats given TS and drug and rats given sodium bicarbonate alone.

Neither drug had an extensive inhibitory effect on tobacco smoke-induced secretory cell hyperplasia (Table 21). Indomethacin was inhibitory at both doses in 4 out of 6 intrapulmonary airway levels, but the effect was not significant (i.e. levels I, II, V and VI). Both doses

Table 21. % inhibition of tobacco smoke-induced secretory cell hyperplasia

Airway level		% increase due to TS	% inhibition			
			flurbiprofen		indomethacin	
			0.4 mg/Kg	4.0 mg/Kg	0.4 mg/Kg	4.0 mg/Kg
Trachea		0	-	-	-	-
Lung:	I	107.7 *	52.6 *	66.1 ** c	38.5	38.3
	II	79.9 *	71.0	0	7.2	46.7
	III	52.6 *	0	0	0	0
	IV	101.7 **	0	0	0	0
	V	110.5	0	0	20.9	81.7
	VI	78.0	0	0	0	152.5

TS = cigarette tobacco smoke

Significant increase due to TS or significant difference to rats exposed to TS (i.e. inhibition):

* = $p < 0.05$

** = $p < 0.01$

c = complete inhibition

Table 22. % inhibition of tobacco smoke-induced increase in number of acidic secretory cells.

Airway level	% increase due to TS	% inhibition flurbiprofen 0.4 mg/Kg	flurbiprofen 4.0 mg/Kg	indomethacin 0.4 mg/Kg	indomethacin 4.0 mg/Kg
Trachea	6.8	348.2	638.3**	474.5	472.3*
Lung: I	128.5**	60.4**	76 5** c	50.5*	48.3*
II	85.0	70.1	0	16.1	48.8
III	57.2*	0	0	0	0
IV	120.0**	0	0	0	37.9
V	121.6	0	8.6	29.5	81.0
VI	89.1 *	0	0	C	137.8

TS = cigarette tobacco smoke

Significant increase due to TS or significant difference to rats exposed to TS (i.e. inhibition):

* = $p < 0.05$

** = $p < 0.01$

c = complete inhibition

of flurbiprofen were significantly inhibitory at airway level I (i.e. 1 out of 6 intrapulmonary airway levels) but had no inhibitory effect in the lateral pathways (i.e. levels III to VI). Neither drug had a consistent or significant effect on the number of neutral secretory cells (Table 19). However, both drugs 'dose-relatedly' inhibited the tobacco smoke-induced increase in acidic secretory cells, significantly so in the more proximal airways (i.e. trachea and airway level I) (Table 22). Inhibition was complete at airway level I with the dose of 4mg/Kg flurbiprofen.

IV. <u>Summary</u>

1. Rats were exposed to cigarette tobacco smoke (TS) (John Player Navy Cut, unfiltered; cabinet exhaust rate of 2 x 2 l/min.) and given concurrent intraperitoneal injections of either indomethacin or flurbiprofen at daily doses of 0.4 and 4.0 mg/Kg. The number of secretory cells was assessed in the trachea and at 6 intrapulmonary airway levels.
2. One rat treated with TS and 4 mg/Kg indomethacin was killed during the period of study because of a loss in body weight and poor general condition.
3. TS did not produce the extensive and significant increases in secretory cell number which had been expected: there was no secretory cell hyperplasia in the trachea, and in only 4 out of 6 intrapulmonary airways was there a significant increase in total and acidic secretory cells ($p < 0.05$). TS had no consistent or significant effect on the number of

neutral secretory cells.

4. Flurbiprofen 'dose-relatedly' and significantly inhibited tobacco smoke-induced secretory cell hyperplasia in the upper axial pathway (i.e. 1 airway level): inhibition was complete at the higher dose. Indomethacin was inhibitory at 4 intrapulmonary airway levels, although not significantly so.
5. Both drugs 'dose-relatedly' inhibited the increase in number of acidic secretory cells in up to 6 airway levels, although only significantly so in 2 of them.
6. Overall, neither drug could be said to have had a dramatic inhibitory effect on TS-induced secretory cell hyperplasia.

Experiment 2.

Oral administration of indomethacin, flurbiprofen and aspirin

To determine whether TS-induced secretory cell hyperplasia could be inhibited by oral administration (i.e. by gavage) of indomethacin, flurbiprofen or aspirin.

Fifty five rats with a mean body weight of 188g (± 2g) on the first day of treatment were divided into eight different treatment groups (Table 23). A total daily dose of 4 mg/Kg for both indomethacin and flurbiprofen was chosen because two studies (Greig et al., 1980; Experiment 1 of this section) have shown this dose to have an inhibitory effect on tobacco smoke-induced secretory cell hyperplasia. Aspirin has been shown to be a less potent inhibitor of certain inflammatory reactions than indomethacin or flurbiprofen (for example see Ishii et al., 1975). It was considered that a daily dose of 4 mg/Kg aspirin would not inhibit 'inflammation' and so a recommended daily dose of 20 mg/Kg was used (Dr. C. Franklin, personal communication). Each drug was given as a split daily dose: half the daily dose being given just before exposure to TS and half immediately after the end of exposure.

Rats were exposed to the smoke from 25 John Player Navy Cut, unfiltered cigarettes (see Materials and Methods for details). The exhaust rate from the exposure cabinets was 2 x 2 l/min (see Materials and Methods for details). The rats were treated for 14 consecutive days and weighed regularly.

I. Animals : General condition

Two animals which had not been exposed to TS died during the period of treatment. One had been given indomethacin for three

Table 23. Plan of Experiment

Treatment	Daily drug dosage, mg/Kg	Number of Rats
None ('Pure' controls)	-	8
No TS, aspirin	2 x 10	5
No TS, flurbiprofen	2 x 2	5
No TS, indomethacin	2 x 2	5
TS alone	-	8
TS + aspirin	2 x 10	8
TS + flurbiprofen	2 x 2	8
TS + indomethacin	2 x 2	8

TS = cigarette tobacco smoke

All drugs dissolved in tap water

Table 24. Mean % increase in body weight, g (± SEM)

Treatment	No TS	TS
None ('Pure' controls)	60.7 (3.8)	***32.9 (3.5)
Aspirin	52.3 (4.6)	***28.0 (5.4)
Flurbiprofen	51.2 (7.2)	* 45.0 (5.1)
Indomethacin	49.6 (6.0)	** 42.4 (2.6)

$n \geqq 4$

Significant differences compared to 'pure' controls:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

TS = cigarette tobacco smoke

All drugs dissolved in tap water

days, the other, flurbiprofen for seven days. Post mortems on the rats revealed no gross abnormalities on the surface of any organ. However, both had haemorrhaged in the throat and thoracic cavity indicating that the gavage tube had entered the trachea. Death was probably due more to haemorrhagic shock or drowning than to any detrimental effects of the drugs.

II. Body weight

All animals gained weight during the experiment (Table 24): even the two rats which died had been gaining weight until the night of their death. There was no significant difference in weight gain between groups of rats which were not exposed to TS. Rats treated with TS and drugs gained significantly less weight than the pure controls ($p < 0.05$ to < 0.001). Rats treated with TS and aspirin gained significantly less weight than rats treated with TS and flurbiprofen ($p < 0.05$).

III. Secretory cell number

Secretory cells were counted at seven airway levels: the whole length of the trachea and at intrapulmonary airway levels I to VI (see Fig. 15). Mean numbers of secretory cells in each treatment group are given in Tables 25, 26 and 27. However, in some treatment groups, airway levels IV and VI were absent from the plane of the sections in some animals (see Tables 25, 26 and 27 for details). Where the 'mean' values so obtained were for less than 3 animals, they were excluded from subsequent analysis.

a. Control animals

Treatment of rats with drugs alone affected secretory cell number in some airways when compared to pure controls.

Table 25 Mean secretory cell number per 10 high power fields (± SEM)

TS = cigarette tobacco smoke

Airway level	'Pure' controls	No TS aspirin	No TS flurbiprofen	No TS indomethacin	TS alone	TS + aspirin	TS + flurbiprofen	TS + indomethacin
Trachea	22.3 (2.4)	92.8 (27.1)	81.7 (36.4)	57.9 (20.4)	55.5 (5.8)	161.0 (29.6)	79.9 (14.5)	66.9 (11.1)
Lung: I	95.0 (7.3)	105.3 (11.7)	77.0 (14.7)	78.3 (15.8)	123.3 (11.2)	168.3 (29.5)	114.5 (16.6)	126.2 (30.6
II	75.9 (8.4)	102.7 (16.3)	86.7 (14.1)	73.2 (18.2)	113.8 (8.1)	111.8 (31.1)	99.3 (12.6)	129.2 (37.8)
III	76.1 (5.7)	139.0 (19.9)	48.1 (10.8)	97.1 (15.2)	125.1 (13.7)	138.2 (20.2)	104.0 (8.6)	141.1 (6.0)
IV	51.9 (18.5)	20.4 † (12.1)	35.0 † (2.5)	37.8 (17.5)	58.7 (14.6)	163.3 (24.0)	40.9 (14.6)	85.6 (20.3)
V	49.2 (10.4)	80.6 (27.4)	49.6 (26.5)	41.7 (11.2)	62.5 (13.5)	89.1 (23.4)	35.2 (7.3)	65.3 (15.2)
VI	13.2 (3.4)	17.4 † (0.1)	[20.0]	-	11.4 (3.2)	110.4 † (62.9)	16.3 † (13.8)	31.1 † (21.4)

n ≥ 8 except † , n = 4; [ ], n = 2, - = no airway present in section

Table 26. Mean number neutral secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke

Airway level	'Pure' controls	No TS aspirin	No TS flurbiprofen	No TS indomethacin	TS alone	TS + aspirin	TS + flurbiprofen	TS + indomethacin
Trachea	4.4 (0.8)	14.5 (5.2)	19.8 (9.7)	11.5 (5.7)	2.4 (0.5)	7.2 (1.9)	4.3 (0.9)	5.4 (1.3)
Lung: I	6.0 (2.8)	5.0 (2.5)	6.3 (1.5)	7.2 (3.3)	2.2 (0.8)	4.1 (1.0)	5.3 (1.0)	5.2 (2.2)
II	5.4 (1.1)	5.7 (0.7)	8.5 (2.8)	7.4 (2.9)	3.5 (0.8)	2.9 (0.6)	5.3 (2.0)	4.6 (1.8)
III	4.6 (1.2)	5.2 (1.7)	5.3 (2.1)	10.9 (2.6)	4.4 (1.2)	4.5 (1.2)	5.5 (1.5)	6.7 (2.1)
IV	4.6 (2.7)	0 † (0)	3.8 † (1.3)	5.6 (2.9)	2.1 (0.8)	5.6 (1.6)	2.8 (1.0)	9.2 (3.6)
V	7.5 (1.8)	9.4 (2.2)	4.7 (4.0)	3.6 (1.4)	7.0 (2.2)	4.4 (1.6)	3.9 (0.9)	3.1 (1.0)
VI	5.2 (0.6)	0 † (0)	[5.0]	-	0.4 (0.4)	8.8 † (1.3)	0 † (0)	4.0 † (1.0)

n ≥ 8 except † , n = 4; [ ], n = 2; - = no airway present in sections

Table 27. Mean number acidic secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke

Airway level	'Pure' controls	No TS aspirin	No TS flurbiprofen	No TS indomethacin	TS alone	TS + aspirin	TS + flurbiprofen	TS + indomethacin
Trachea	17.9	78.3	61.8	46.4	53.2	153.8	75.6	61.5
	(1.7)	(24.6)	(27.1)	(15.1)	(5.9)	(29.0)	(14.5)	(10.6)
Lung: I	87.9	100.0	70.8	71.1	121.5	164.1	108.7	120.6
	(5.5)	(12.8)	(13.9)	(14.0)	(11.6)	(29.2)	(17.1)	(31.6)
II	70.6	97.0	78.2	65.7	109.8	108.7	92.8	124.4
	(7.9)	(15.8)	(11.4)	(16.0)	(7.8)	(32.0)	(13.8)	(39.2)
III	71.5	133.8	42.8	87.4	120.7	133.7	98.5	135.4
	(5.6)	(20.6)	(9.7)	(14.5)	(14.5)	(20.4)	(8.3)	(6.8)
IV	47.3	20.4 †	31.3 †	32.2	56.6	157.8	38.2	75.6
	(16.5)	(12.1)	(1.3)	(14.7)	(14.5)	(23.2)	(14.5)	(22.8)
V	41.7	71.3	44.9	38.1	55.6	84.7	31.3	62.2
	(10.0)	(27.9)	(22.7)	(10.1)	(12.7)	(22.8)	(6.7)	(15.2)
VI	8.0	17.4 †	[15.0]	-	11.0	101.7 †	16.3 †	27.1 †
	(3.0)	(0.1)			(2.8)	(61.7)	(13.8)	(20.4)

n ≧ 8 except † , n = 4, [ ], n = 2; - = no airway present in sections

Rats given aspirin and flurbiprofen had significantly more secretory cells in the trachea ($p < 0.05$), and had more (with aspirin) and fewer (with flurbiprofen) secretory cells at airway level III ($p < 0.05$). Both drugs had a similar effect on the number of acidic secretory cells ($p < 0.05$). The only treatment to significantly affect the number of neutral secretory cells was indomethacin: the number was increased in airway level III ($p < 0.05$). However, although these were the only significant effects of the drugs given alone when compared to the pure controls, aspirin increased the number of total and acidic secretory cells by between 11 and 338% in all airways except level IV. Neither indomethacin nor flurbiprofen had such a consistent effect.

b. Effect of tobacco smoke

TS increased the number of secretory cells at all airway levels, except level VI, when compared to the 'pure' controls, significantly so in 4 out of 7 of them (i.e. trachea and levels I, II and III) ($p < 0.05$ to < 0.01) (Table 28). The increase was due primarily to an increase in the number of acidic secretory cells, (the same levels of significance, in the same airways as the 'total' number of secretory cells). The number of neutral secretory cells was not significantly affected by TS.

c. Effect of drugs

None of the drugs significantly inhibited TS-induced secretory cell hyperplasia (Table 28). In the trachea, drug treatment increased the number of secretory cells above TS values, significantly so in rats given aspirin

Table 28. % change in secretory cell number

Airway level	% increase due to TS	aspirin + TS	flurbiprofen + TS	indomethacin + TS
Trachea	148.9**	↑ 190.1**	↑ 44.0	↑ 20.5
Lung: I	30.0*	↑ 36.3 Δ	↓ 31.6 Δ	↑ 2.2 Δ
II	49.9**	↓ 5.3 Δ	↓ 38.3 Δ	↑ 13.5 Δ
III	64.4**	↑ 10.5	↓ 43.1 Δ	↑ 12.8
IV	13.1	↑ 178.2*	↓ 261.8 Δ	↑ 45.8 Δ
V	27.0	↑ 42.6 Δ	↓ 135.1 Δ	↑ 4.5 Δ
VI	0	-	-	-

TS = cigarette tobacco smoke

↓ = inhibition of TS-induced hyperplasia

↑ = increased above TS-exposed values

\- = no airway present in sections

Significant increase due to TS or significant differences to TS values: * = $p < 0.05$

** = $p < 0.01$

Δ = not significantly different to 'pure' control values

($p < 0.01$). Flurbiprofen was the only drug to inhibit secretory cell hyperplasia in the intrapulmonary airways, although not significantly so. However, flurbiprofen reduced the number of secretory cells at all intrapulmonary airway levels (where there was an increase in number due to TS) to values which were not significantly different to control values. Aspirin and flurbiprofen increased the mean number of secretory cells at all intrapulmonary levels but one, to above the mean values in rats exposed to TS, significantly so at level IV in rats treated with aspirin ($p < 0.05$). However, there was no significant difference between the mean number of secretory cells at a number of airway levels in rats treated with TS and aspirin or indomethacin, and control rats. The effect of the drugs on the number of secretory cells was due primarily to significant effects on the number of acidic secretory cells. The number of neutral secretory cells was not significantly altered by drug treatment.

IV. <u>Summary</u>

1. Rats were exposed to cigarette tobacco smoke (TS) (John Player Navy Cut, unfiltered; cabinet exhaust rate of 2 x 2 l/min) and given concurrent oral administration of aspirin (daily dose 20 mg/Kg), indomethacin (4 mg/Kg) or flurbiprofen (4 mg/Kg) by gavage. The number of secretory cells was assessed in the trachea and at 6 intrapulmonary airway levels.
2. Two rats died during the period of the study due more to the gavage technique than any adverse effects of the drugs.

3. TS did not produce the extensive and significant increases in secretory cell number which had been expected: the number of total and acidic secretory cells was significantly increased in the trachea and in 3 intrapulmonary airway levels ($p < 0.05$). TS had no significant effect on the number of neutral secretory cells.
4. None of the drugs significantly inhibited TS-induced secretory cell hyperplasia:
   a. Flurbiprofen reduced the number of secretory cells to within control values at all intrapulmonary airway levels where there was an increase due to TS.
   b. Indomethacin increased the number of secretory cells above TS values at all airway levels, although not significantly so.
   c. Aspirin increased the number of secretory cells to above TS values in all but one airway level, significantly so in two of them ($p < 0.05$).
5. Overall, administration of flurbiprofen, indomethacin, and aspirin by gavage could not be said to inhibit TS-induced secretory cell hyperplasia.

Experiment 3.

Parenteral administration of indomethacin, dexamethasone, prednisolone and hydrocortisone

To determine i. whether three steroidal anti-inflammatory drugs (dexamethasone, prednisolone and hydrocortisone) would inhibit TS-induced secretory cell hyperplasia when given by intraperitoneal injection, and ii. compare inhibition to that of indomethacin.

Sixty four rats with a mean body weight of 160g (± 2g) on the first day of treatment were divided into 10 different treatment groups (Table 29). Three additional animals were killed on the day of delivery from the breeders (Olac) to examine lung 'cleanliness'. Indomethacin was included in the study as a 'reference standard' which has been shown to inhibit TS-induced secretory cell hyperplasia when given intraperitoneally, twice daily at a total daily dose of 4 mg/Kg (Greig et al., 1980; experiment 1 of this section). Each steroidal anti-inflammatory drug was given in the prescribed form (usually given by injection): 'Decadron' (Merck, Sharp and Dohme), formulated as dexamethasone sodium phosphate; 'Codelsol' (M S & D), as prednisolone sodium phosphate; hydrocortisone as hydrocortisone sodium succinate (Organon Ltd). The same daily dose of 4 mg/Kg of each steroidal anti-inflammatory drug was given for direct comparison of inhibitory effect with indomethacin at this dose. However, (due to its toxicity) the daily dose of dexamethasone had to be reduced to an average of 2 mg/Kg during the period of the study (see I. Animals : General condition). Each drug was given twice daily in an attempt to maintain constant plasma levels: the first daily administration of 2 mg/Kg was given about an hour before the beginning of TS exposure, the second (2 mg/Kg) was given immediately after TS exposure.

Table 29. Plan of experiment

Treatment	Number of Rats
No TS, SW	6
No TS, indomethacin in 1% $NaHCO_3$ (in SW)	5
No TS, dexamethasone in SW	5
No TS, prednisolone in SW	5
No TS, hydrocortisone in SW	5
TS + SW	8
TS + indomethacin in 1% $NaHCO_3$ (in SW)	6
TS + dexamethasone in SW	8
TS + prednisolone in SW	8
TS + hydrocortisone in SW	8

TS = cigarette tobacco smoke

SW = sterile water

$NaHCO_3$ = sodium bicarbonate

The cigarettes used in the studies were John Player Navy Cut, unfiltered (see Materials and Methods for details). The exhaust rate from the cabinets was 2 x 1 l/min (see Materials and Methods for details).

I. Animals : General condition

One rat treated with indomethacin alone began to lose weight after three days of treatment. The rat also became 'hunched' and its fur lost its sleekeness. Reducing the daily dose of indomethacin by half to 2 mg/Kg prevented a further loss in body weight but the rat died on day 13. The condition of the other rats treated with indomethacin, with or without exposure to TS, was good.

The condition of rats given prednisolone and hydrocortisone alone was good, but when also exposed to TS their body weight fluctuated and their general condition deteriorated (i.e. the animals became 'hunched', thin, and their fur lost its sleekness). One rat given TS and prednisolone died on day 13.

Dexamethasone-treatment had a marked detrimental effect on the general condition of the rats, with or without exposure to TS. After six days of treatment the daily dose for all dexamethasone-treated rats had to be reduced by half to 2 mg/Kg. However, the reduction in dose did not prevent three rats dying over the following four days. The dose was therefore reduced by half again to 1 mg/Kg which prevented any further deaths before the end of the experiment.

Post mortems on all animals which died revealed the thymus glands to be dramatically reduced in size. The abdomen was filled with a watery fluid and the gut contents were more liquid than

'normal'. Very little fat was found and there was some evidence of muscle wastage. Death was probably due to a combination of reduced immunocompetency and gastro-intestinal complications.

II. Body weight

The weight of many individual rats given the drugs fluctuated throughout the study. However, apart from the groups of rats given dexamethasone, each group of rats as a whole gained weight over the fourteen day treatment period (Table 30). All rats given dexamethasone lost weight during the period of the study: many of these rats had failed to gain weight normally by the third day of treatment. The order of weight gain with different treatments may be summarised as below:

SW indo.	>	hydro.	>	pred.	>	TS + SW TS + hydro.	>	TS + indo. TS + pred.	>	dex. TS + dex.
	***		**		***		**		***	

**, *** = $p < 0.01$ and $p < 0.001$ respectively

TS = cigarette tobacco smoke

SW = sterile water

indo. = indomethacin in 1% aqueous sodium bicarbonate

hydro. = hydrocortisone in SW

pred. = prednisolone in SW

dex. = dexamethasone in SW

III. Secretory cell number

Secretory cells were counted at eight airway levels: in the upper and lower halves of the trachea, and at intrapulmonary airway levels I to VI (see Fig. 15). Mean numbers of secretory

Table 30. Mean % change in body weight, g (± SEM)

+ = increase in weight, - = decrease in weight

TS = tobacco smoke

SW = sterile water

$NaHCO_3$ = sodium bicarbonate

Administration	No TS	TS
Sterile water (SW)	+ 77.1 (3.8)	+ 36.1 *** (1.7)
Indomethacin in 1% $NaHCO_3$ (in SW)	+ 80.1 (5.0)	+ 29.2 *** (3.3)
dexamethasone in SW	- 22.0 (3.5)	- 27.2 (2.2)
prednisolone in SW	+ 52.8 (3.0)	+ 26.5 *** (3.8)
hydrocortisone in SW	+ 62.1 (1.6)	+ 34.9 *** (4.2)

$n \geq 4$

*** = Significant differences compared to corresponding No TS value, $p < 0.001$

cells are given in Tables 31, 32, 33 and 34.

a. Newly-delivered animals

The mean numbers of 'total', neutral and acidic secretory cells in the airway of newly delivered rats are given in Table 31. There was no significant difference in the mean number of any of these secretory cell categories between newly-delivered, and control animals given sterile water alone (Tables 32, 33 and 34). The secretory cell counts for the newly-delivered animals were not included in the main experiment as these animals were not given the appropriate control treatment.

b. Control animals

There were few significant differences in the mean number of secretory cells between control rats given sterile water and control rats given the drugs (Table 32). None of the drugs had any significant effect on the numbers of neutral secretory cells (Table 33). Significant effects on the number of secretory cells were therefore reflected by similar changes in the number of acidic secretory cells (Table 34). In the upper half of the trachea there were significantly more 'total' and acidic secretory cells in rats given indomethacin ($p < 0.05$) and hydrocortisone ($p < 0.01$) than in rats given sterile water. In airway levels II, III and IV there were fewer 'total' and acidic secretory cells in rats given dexamethasone than in rats given hydrocortisone ($p < 0.05$).

c. Effect of tobacco smoke

Secretory cell number was compared between rats given sterile water but not exposed to TS, and rats given

Table 31. Mean number secretory cells per 10 high power fields in newly delivered rats (± SEM)

Airway level	Secretory cell		
	Total	Neutral	Acidic
Trachea: upper	35.4 (5.2)	2.2 (0.6)	33.2 (4.7)
lower	43.1 (10.7)	5.9 (2.4)	37.2 (8.4)
Lung: I	52.3 (12.2)	4.5 (2.9)	47.8 (9.4)
II	47.4 (12.3)	3.6 (1.3)	43.8 (11.4)
III	61.6 (9.0)	1.7 (0.7)	59.9 (8.3)
IV	54.5 (7.1)	0.7 (0.3)	53.9 (6.9)
V	23.5 (5.4)	0.7 (0.3)	22.8 (5.3)
VI	12.7 (6.9)	0.5 (0.3)	12.3 (6.8)

$n \geqq 6$

Table 32. Mean secretory cell number per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, indom. = indomethacin, dex. = dexamethasone, pred. = prednisolone, hydro. = hydrocortisone.

Airway level	No TS	No TS + indom.	No TS + dex.	No TS + pred.	No TS + hydro.	TS	TS + indom.	TS + dex.	TS + pred.	TS + hydro.
Trachea: upper	39.6 (4.1)	62.1 (5.1)	49.0 (16.2)	56.3 (8.8)	67.0 (6.5)	108.8 (17.3)	35.8 (9.0)	96.2 (8.0)	78.7 (11.4)	77.9 (10.2)
lower	52.2 (6.6)	59.6 (3.1)	57.4 (9.8)	59.8 (7.3)	63.0 (7.6)	108.4 (11.7)	43.8 (9.5)	120.2 (8.2)	80.1 (9.3)	140.1 (15.6)
Lung: I	76.6 (7.0)	70.0 (1.7)	53.0 (6.4)	63.8 (8.2)	55.3 (14.9)	154.0 (8.6)	99.0 (5.9)	93.0 (10.4)	95.3 (8.9)	80.8 (8.8)
II	55.2 (8.1)	62.3 (18.2)	34.8 (4.7)	49.8 (9.2)	60.0 (5.2)	139.0 (11.3)	58.3 (8.4)	79.8 (13.8)	70.8 (8.9)	79.3 (9.1)
III	65.7 (6.7)	60.0 (5.5)	52.0 (5.4)	60.2 (4.6)	70.9 (3.5)	123.4 (8.9)	70.3 (7.0)	78.0 (8.2)	91.9 (12.0)	89.4 (6.8)
IV	49.7 (5.8)	47.1 (8.5)	25.4 (6.4)	46.2 (7.9)	55.9 (9.5)	98.1 (10.6)	49.0 (7.6)	52.7 (10.0)	72.9 (10.0)	64.7 (7.3)
V	24.0 (6.8)	30.3 (9.0)	27.8 (8.1)	30.8 (7.7)	37.5 (8.4)	31.5 (4.9)	25.6 (8.5)	15.3 (3.8)	27.6 (5.6)	44.8 (12.1)
VI	10.4 (3.8)	6.8 (1.7)	7.0 (2.6)	12.5 (3.3)	16.8 (4.3)	32.0 (5.6)	31.1 (10.3)	10.7 (5.1)	24.8 (10.7)	41.4 (10.1)

$n \geq 8$

Table 33. Mean number neutral secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, indom. = indomethacin, dex. = dexamethasone, pred. = prednisolone, hydro. = hydrocortisone

Airway level	No TS	No TS + indom.	No TS + dex.	No TS + pred.	No TS + hydro.	TS	TS + indom.	TS + dex.	TS + pred.	TS + hydro.
Trachea: upper	2.2	3.3	0.3	6.3	1.8	1.4	1.8	0.4	1.7	1.6
	(0.8)	(1.6)	(0.3)	(2.2)	(0.9)	(0.5)	(1.0)	(0.2)	(1.1)	(1.4)
lower	4.2	8.8	0.4	5.0	3.0	1.3	1.4	0.6	1.4	3.8
	(1.2)	(4.2)	(0.3)	(1.3)	(0.7)	(0.6)	(0.8)	(0.3)	(0.6)	(3.3)
Lung: I	4.2	3.7	1.8	3.5	3.3	1.3	0.8	2.2	1.8	2.8
	(1.4)	(1.9)	(1.0)	(1.0)	(0.9)	(0.5)	(0.5)	(0.7)	(1.2)	(1.2)
II	5.0	2.3	1.4	3.2	4.2	1.5	0.8	1.8	0.4	1.3
	(1.9)	(1.5)	(0.8)	(0.7)	(2.0)	(0.7)	(0.5)	(0.8)	(0.2)	(0.7)
III	0.6	1.1	0.3	0.9	0.6	0.3	0.0	0.3	0.3	0.5
	(0.5)	(0.5)	(0.3)	(0.4)	(0.3)	(0.2)	(0.0)	(0.2)	(0.3)	(0.2)
IV	0.6	0.4	0.0	0.3	0.4	0.5	0.1	0.2	0.0	0.5
	(0.4)	(0.4)	(0.0)	(0.3)	(0.3)	(0.3)	(0.1)	(0.2)	(0.0)	(0.3)
V	0.2	0.0	0.2	0.3	1.3	0.0	0.3	0.3	0.0	0.0
	(0.2)	(0.0)	(0.2)	(0.3)	(0.4)	(0.0)	(0.2)	(0.3)	(0.0)	(0.0)
VI	0.0	0.8	0.0	0.0	0.2	0.6	0.0	0.0	0.5	0.0
	(0.0)	(0.6)	(0.0)	(0.0)	(0.2)	(0.5)	(0.0)	(0.0)	(0.5)	(0.0)

$n \geq 8$

Table 34. Mean number acidic secretory cells per 10 high power fields (± SEM)

TS = cigarette tobacco smoke, indom. = indomethacin, dex. = dexamethasone, pred.= prednisolone, hydro. = hydrocortisone

Airway level	No TS	No TS + indom.	No TS + dex.	No TS + pred.	No TS + hydro.	TS	TS + indom.	TS + dex.	TS + pred.	TS + hydro.
Trachea: upper	37.4	58.8	48.8	50.0	65.2	107.4	34.0	95.8	77.1	76.3
	(3.3)	(3.8)	(16.1)	(7.8)	(6.5)	(17.4)	(9.5)	(7.9)	(11.6)	(10.6)
lower	48.0	50.8	57.0	54.8	60.0	107.1	42.4	119.6	778.7	136.5
	(5.4)	(4.0)	(9.8)	(7.4)	(7.2)	(11.5)	(9.6)	(8.2)	(9.5)	(16.6)
Lung: I	72.4	66.3	51.2	60.3	52.0	152.7	98.3	90.8	93.5	78.0
	(5.8)	(3.4)	(7.0)	(7.2)	(14.8)	(8.6)	(5.7)	(10.3)	(8.0)	(8.8)
II	50.2	60.0	33.4	46.6	55.8	137.5	57.5	78.0	70.4	78.1
	(7.4)	(17.0)	(4.5)	(9.2)	(4.5)	(11.8)	(8.6)	(13.9)	(8.8)	(9.6)
III	65.1	58.9	51.7	59.3	70.3	123.1	70.3	77.7	91.6	88.9
	(6.9)	(5.1)	(5.4)	(1.4)	(3.5)	(8.8)	(7.0)	(8.2)	(12.0)	(6.8)
IV	49.1	46.8	25.4	45.9	55.5	97.7	48.9	52.4	72.9	64.2
	(5.9)	(8.6)	(6.4)	(8.0)	(9.5)	(10.7)	(7.7)	(10.0)	(10.0)	(7.2)
V	23.8	30.3	27.6	30.5	36.2	31.5	25.3	15.0	27.6	44.8
	(6.9)	(9.0)	(8.1)	(7.7)	(8.4)	(4.9)	(8.3)	(4.0)	(5.6)	(12.1)
VI	10.4	6.0	7.0	12.5	16.6	31.4	31.1	10.7	24.3	41.4
	(3.8)	(1.8)	(2.6)	(3.3)	(5.5)	(5.3)	(10.3)	(5.1)	(10.6)	(10.1)

n ≥ 8

sterile water and exposed to TS for 14 days. TS reduced the number of neutral secretory cells at all airway levels but one, although not significantly so. Conversely, TS increased the number of 'total' and acidic secretory cells in all airways studied, significantly so in all but one ($p < 0.05$ to < 0.01) (Tables 35 and 36 respectively). The increase for 'total' and acidic secretory cells was between 31 and 202%.

d. <u>Inhibitory effect of drugs</u>

The inhibitory effect of the drugs on TS-induced changes in secretory cell number was assessed by comparison with rats exposed to TS alone. Complete inhibition was achieved when there was no significant difference in secretory cell number between rats given TS and drug and untreated control rats.

All drugs inhibited TS-induced secretory cell hyperplasia to varying degrees (Table 35). The inhibitory effect was due entirely to inhibition of TS-induced acidic secretory cell hyperplasia (Table 36) because none of the drugs significantly changed the number of neutral secretory cells.

Indomethacin was the most effective drug, giving significant and complete inhibition in six proximal airway levels (i.e. trachea to airway level IV) ($p < 0.01$). Dexamethasone gave no significant inhibition in the trachea, but gave significant and complete inhibition in all the airways of the lung where there was a significant increase in secretory cell number after TS alone ($p < 0.01$). Prednisolone and hydrocortisone were much less effective 'inhibitors' than either indomethacin or dexamethasone. Prednisolone gave some inhibition in all airways studied, but only

Table 35. % inhibition of tobacco smoke (TS)-induced secretory cell hyperplasia

Airway level		% increase due to TS	% inhibition			
			indomethacin	dexamethasone	prednisolone	hydrocortisone
Trachea:	upper	174.8 **	105.5 **c	18.2	43.5	30.9
	lower	107.7 **	115.0 **c	0	28.3	0
Lung:	I	101.0 **	71.1 **c	78.8 **c	75.8 **c	94.6 **c
	II	151.8 **	96.3 **c	70.6 **c	81.4 **c	71.2 **c
	III	87.8 **	92.0 **c	78.7 **c	54.6	58.9 *
	IV	97.4 **	101.5 **c	93.8 **c	52.1	69.0 *c
	V	31.3	78.7	216.0	52.0	0
	VI	207.7 **	4.2	98.1 * c	33.3	0

Significant increase due to TS or significant differences to TS values (i.e. inhibition)

* = $p < 0.05$

** = $p < 0.01$

c = complete inhibition

Table 36. % inhibition of tobacco smoke (TS)-induced increase in number of acidic secretory cells

Airway level	% increase due to TS	% inhibition			
		indomethacin	dexamethasone	prednisolone	hydrocortisone
Trachea: upper	187.2 **	104.9 **c	16.6	43.3	44.4
lower	123.1 **	109.5 **c	0	48.1	0
Lung: I	110.9 **	67.8 **c	77.1 **c	73.7 **c	93.0 **c
II	173.9 **	91.6 **c	68.2 **c	76.9 **c	68.0 **c
III	89.1 **	91.0 **c	78.3 **c	54.3	59.0 *
IV	99.0 **	100.4 **c	93.2 **c	51.0	68.9 *c
V	32.4	80.5	214.3	50.7	0
VI	201.9 **	1.4	98.6 *c	33.8	0

Significant increase due to TS or significant differences to TS values: * = $p < 0.05$; ** = $p < 0.01$

c = complete inhibition

significantly so and complete in the intrapulmonary axial pathway ($p < 0.01$). However, although inhibition was not significant in the majority of airways, prednisolone did reduce the numbers of secretory cells to values which were not significantly different to control values ($p > 0.05$). Hydrocortisone gave significant inhibition at airway levels I to IV. However, hydrocortisone gave no inhibition in the lower trachea or at airway levels V and VI. In addition, hydrocortisone, unlike prednisolone, did not reduce the number of secretory cells to within control values. In fact, the inhibition afforded by prednisolone was significantly greater than that of hydrocortisone in the lower trachea ($p < 0.01$). There was no significant difference between the inhibition given by each of the latter two drugs in the other airways.

IV. Summary

1. Rats were exposed to cigarette tobacco smoke (TS) (John Player Navy Cut, unfiltered; cabinet exhaust rate of 2 x 1 l/min) and given concurrent, intraperitoneal injections of either the non-sterioidal anti-inflammatory drug indomethacin (daily dose 4 mg/Kg), or steroidal anti-inflammatory drugs: dexamethasone (average daily dose 2 mg/Kg), prednisolone or hydrocortisone (both at daily doses of 4 mg/Kg). The number of secretory cells was assessed at eight airway levels.
2. Dexamethasone, at doses of 2 and 4 mg/Kg, had severely detrimental effects on the general condition of rats and caused a loss in body weight over the period of study.

Prednisolone and hydrocortisone were also detrimental to the general condition of, and weight gain by, rats. Indomethacin was the least detrimental to the 'health' of rats.

3. TS, when exhausted from the exposure cabinets at a reduced rate of 2 x 1 l/min, produced the largest and most extensive secretory cell hyperplasia reported in the studies thus far: significant in 7 out of 8 airway levels ($p < 0.01$). The effect was entirely due to significant increases in number of acidic secretory cells at the same airway levels ($p < 0.01$). TS had no significant effect on the number of neutral secretory cells.

4. All the drugs inhibited TS-induced secretory cell hyperplasia to varying degrees. Indomethacin was the most potent inhibitor (significant and complete inhibition in 6, more proximal airways), followed by dexamethasone (significant and complete inhibition in 5, more distal airways). Prednisolone and hydrocrotisone were less effective.

5. Overall, indomethacin (at a dose of 4 mg/Kg) inhibited TS-induced secretory cell hyperplasia in the more proximal airways without seriously affecting the 'health' of rats, whilst dexamethasone (at an average dose of 2 mg/Kg) was inhibitory in the more distal airways but was seriously detrimental to the 'health' of rats.

SECTION C

The effect of oral N-acetyl cysteine on tobacco smoke-induced secretory cell hyperplasia

To determine whether the mycolytic agent, N-acetylcysteine, given orally in drinking water, would inhibit TS-induced secretory cell hyperplasia.

Forty rats with a mean body weight of 237g (± 2g) on the first day of treatment were divided into four different treatment groups (Table 37). To check lung 'cleanliness', five rats were killed at the beginning of the experiment for comparison of secretory cell number with control five rats, given tap water alone, killed at the end of the experiment.

NAC was given orally as a 1% w/v solution of drinking water (tap water), the dose suggested by the manufacturers (Zambon S.p.A., Milan). NAC has a disagreeable, sulphurous odour and an unpleasant taste (in the author's opinion!). In an attempt to avoid an expected initial reduction in water intake by the rats, administration of NAC began two days before exposure to TS. A fresh solution of NAC was given daily except for the 'weekend' when the solution was changed after two days. Rats were caged in pairs, and each pair drank the NAC solution from a single, calibrated water bottle. The daily volume of water (and hence the concentration of NAC) consumed /dose by each pair of rats was measured and the combined daily body weight for each pair of rats calculated. The 'average' daily dose of NAC in mg/Kg/rat was calculated from the formula derived below :

$$\frac{\text{volume NAC solution consumed (l)} \times \text{concentration of NAC (g/l)} \times 10^{3}}{\text{total body weight for pair of rats (g)} \times 10^{-3}}$$

$$= \frac{\text{volume 1\% NAC solution consumed (ml)}}{\text{total body weight (g)}} \times 10^{4} \text{ mg/Kg/rat}$$

Table 37. Plan of experiment

Substance(s) administered	Number of rats
No TS, tap water	10 *
No TS, NAC in tap water	10
TS + tap water	10
TS + NAC in tap water	10

TS = cigarette tobacco smoke

NAC = N-acetylcysteine

* 5 rats killed on day 1 and 5 on day 15

The mean daily dose of NAC during the period of the experiment (i.e. days 2 to 16) (Table 38) was 923 mg/Kg (± 46 mg/Kg) for rats given NAC alone, and 1018 mg/Kg (± 27 mg/Kg) for rats given NAC and exposed to TS. There was no significant difference between the two values: the overall mean daily dose was 973 mg/Kg (± 27 mg/Kg).

The cigarettes used in the study were Capstan Full Strength, unfiltered (see Materials and Methods for details). The exhaust rate from the exposure cabinets was 2 x 1 l/min (see Materials and Methods for details). The changed regimen of TS exposure produced carbon monoxide concentrations in the exposure cabinets which rose from 7 ppm to 120 ppm over the four hour exposure period and produced mean plasma concentrations of carboxyhaemoglobin in the rats of 11.7% after one hour of exposure and 16.4% after four hours of exposure (see Materials and Methods for details). Rats were exposed to TS for 14 consecutive days.

I. Animals : General condition

Apart from one rat which died during the course of the experiment, the general health condition of all rats was good. The rat had not been given NAC, and died in the exposure cabinet during the first day of exposure to TS. No abnormal post-mortem features were found and the rat had been gaining weight normally.

II. Water consumption

Rats given NAC solution drank significantly less than those given water ($p < 0.01 - < 0.001$):

Table 38. 'Average' daily dose of NAC, mg/Kg/rat

TS = cigarette tobacco smoke

TS, day 3 ↓ (between day 2 and day 5)

	Day										
	1	2	5	6	7	8	9	10	12	13	16
No TS	343	702	1026	1107	913	1045	971	909	-	817	818
TS	456	1017	1074	1044	938	1000	985	1082	1036	854	1150

Mean water consumption, ml per rat (± SEM)

No TS	34.0 (1.7)	$p < 0.001$
No TS, NAC	23.2 (2.0)	
TS	38.6 (2.1)	$p < 0.01$
TS + NAC	25.3 (1.7)	

III. Body weight

All rats gained weight during the course of the experiment (Table 39). Untreated rats gained the most weight, followed by rats given only NAC, rats exposed to smoke, and rats exposed to TS and given NAC. The changed regimen of TS exposure caused rats to initially lose weight but recover within two days, although not to unsmoked values. Reduced weight gain due to NAC was most probably associated with reduced water consumption.

IV. Secretory cell number

Secretory cells were counted at eight airway levels: in the upper and lower halves of the trachea and at intrapulmonary airway levels I to VI (see Fig. 15). Mean numbers of secretory cells are given in Tables 40 to 43.

a. 'Pure' control animals

Mean numbers of 'total', neutral, and acidic secretory cells for five rats killed on the first day of study and five controls killed on the last day of study are given in Table 40. Only one rat from the 'Last' group had enough airway in level II to allow sufficient counts to be made (i.e. more than three high power fields). Consequently, no valid statistical comparison could be made between groups at this airway level. The only significant difference found was at airway level IV: there were fewer neutral secretory cells in the 'Last' than

Table 39. Mean % increase in body weight, g (± SEM)

Administration			Significantly different to No TS
No TS	50.4	(2.6)	} $p < 0.05$
No TS, NAC	39.9	(2.6)	} $p < 0.001$
TS	26.1	(1.6)	} $p < 0.001$
TS + NAC	20.5	(1.7)	

TS = cigarette, tobacco smoke

NAC = N-acetyl cysteine

Table 40. Mean secretory cell number per 10 high power fields (± SEM) in control rats killed on the first day of the experiment ('First') and the last day ('Last')

Airway level		Total		Neutral		Acidic	
		'First'	'Last'	'First'	'Last'	'First'	'Last'
Trachea:	upper	62.3 (7.4)	41.3 (8.5)	14.4 (2.9)	8.8 (2.0)	47.9 (7.1)	32.5 (6.6)
	lower	67.9 (5.3)	56.7 (11.1)	14.9 (1.8)	13.8 (3.9)	53.0 (5.4)	43.0 (8.0)
Lung :	I	81.3 (8.1)	65.8 (4.6)	9.5 (1.2)	5.5 (4.1)	71.8 (9.1)	60.3 (6.5)
	II	54.9 (13.5)	[54.0]	6.3 (1.5)	[1.0]	48.7 (12.8)	[53.0]
	III	115.8 (11.2)	102.2 (11.5)	13.7 (1.8)	13.5 (4.0)	102.2 (11.4)	88.7 (9.6)
	IV	61.6 (13.0)	24.8 (6.8)	9.4 (2.1)	0.0** (0.0)	52.1 (11.7)	24.8 (6.8)
	V	18.3 (2.9)	37.0 (12.9)	7.1 (1.9)	6.7 (3.1)	11.2 (2.6)	30.3 (10.1)
	VI	17.2 (5.7)	29.8 (11.5)	6.3 (1.3)	4.7 (2.3)	6.8 (3.0)	25.0 (9.6)

$n \geq 10$, except:

[ ], no SEM because n = 2

Significant difference between 'First' and 'Last' groups: ** = $p < 0.01$

Table 41. Mean secretory cell number per 10 high power fields (± SEM)

Airway level		No TS	No TS, NAC	TS	TS + NAC
Trachea:	upper	52.9 (5.8)	57.7 (5.4)	85.9 (15.4)	64.4 (7.8)
	lower	62.2 (5.3)	62.5 (6.4)	127.2 (18.3)	86.7 (6.4)
Lung:	I	75.5 (6.4)	59.4 (5.7)	191.9 (15.4)	135.5 (12.4)
	II	55.8 (10.6)	48.7 (6.5)	191.6 (17.6)	113.4 (10.3)
	III	113.3 (8.4)	45.8 (4.1)	229.1 (9.6)	147.8 (8.2)
	IV	47.7 (10.2)	38.7 (6.6)	200.6 (15.1)	109.3 (10.0)
	V	21.3 (4.0)	19.1 (2.9)	111.0 (19.8)	49.3 (8.4)
	VI	26.1 (8.0)	13.6 (2.9)	90.1 (15.3)	14.8 (2.2)

TS = cigarette tobacco smoke
NAC = N-acetylcysteine
$n \geqq 18$

Table 42. Mean number neutral secretory cells per 10 high power fields (± SEM)

Airway level		No TS	No TS NAC	TS	TS + NAC
Trachea:	upper	11.9 (2.0)	14.3 (2.2)	3.8 (1.3)	1.6 (0.8)
	lower	14.3 (2.0)	15.9 (3.0)	2.6 (0.7)	0.9 (0.5)
Lung:	I	8.0 (1.7)	2.5 (1.3)	0.7 (0.4)	1.2 (0.7)
	II	5.4 (1.5)	1.1 (0.3)	0.7 (0.4)	0.5 (0.2)
	III	13.6 (1.7)	5.8 (0.7)	0.9 (0.5)	1.7 (0.4)
	IV	6.5 (1.9)	4.7 (1.0)	0.6 (0.2)	2.5 (0.7)
	V	7.0 (1.6)	4.2 (0.8)	1.0 (0.5)	1.4 (0.4)
	VI	5.6 (1.2)	3.2 (0.4)	0.8 (0.3)	1.3 (0.4)

TS = cigarette tobacco smoke

NAC = N-acetylcysteine

$n \geqq 18$

Table 43. Mean number acidic secretory cells per 10 high power fields (± SEM)

Airway level		No TS	No TS, NAC	TS	TS + NAC
Trachea:	upper	41.0 (5.4)	43.5 (4.9)	82.1 (15.3)	29.2 (7.5)
	lower	48.0 (4.9)	46.6 (5.7)	124.8 (18.3)	53.0 (9.3)
Lung:	I	67.5 (6.2)	57.0 (5.5)	191.2 (15.4)	117.5 (12.7)
	II	49.4 (10.5)	47.6 (6.5)	191.0 (17.6)	120.5 (10.1)
	III	97.7 (8.2)	40.4 (4.0)	224.7 (10.0)	166.4 (8.2)
	IV	43.7 (8.9)	109.6 (12.4)	200.0 (16.0)	144.6 (11.5)
	V	18.0 (4.5)	15.6 (2.6)	110.0 (19.8)	92.1 (19.4)
	VI	15.1 (5.2)	10.4 (2.9)	89.3 (15.3)	31.9 (8.4)

TS = cigarette tobacco smoke

NAC = N-acetylcysteine

$n \geq 18$

in the 'First' group ($p < 0.01$). However, there was a trend from 'First' to 'Last' for the numbers of neutral secretory cells to be reduced at all airway levels (see also Section D, Experiment 2, Part IIIa of this Chapter). Numbers of total and acidic secretory cells also showed a tendency to be reduced in the proximal airways and increased in the distal airways. For each group, the mean counts for the 'pure' controls were combined.

b. <u>NAC control animals</u>

NAC alone had little significant or consistent effect on the number of secretory cells. NAC significantly reduced the numbers of secretory cells in all three categories (i.e. 'total', neutral and acidic) at airway level III ($p < 0.01$) (Tables 41, 42 and 43) and the number of neutral secretory cells at airway levels I and II ($p < 0.05$) (Table 42).

c. <u>Effect of tobacco smoke</u>

For the first time in the studies, TS significantly reduced the number of neutral secretory cells ($p < 0.01$) (Table 44) and increased the number of acidic secretory cells ($p < 0.01$) (Table 45) at all airway levels. The increase in number of acidic secretory cells was consistently so large that it more than offset the reduction in number of neutral cells and led to a significant increase in the 'total' number of secretory cells at all airway levels ($p < 0.01$) except the upper trachea (Table 46).

d. <u>Inhibitory effect of NAC</u>

NAC inhibited TS-induced secretory cell hyperplasia at all airway levels, significantly so in 6 out of 8 of them ($p < 0.05$) (Table 46). Inhibition was complete at airway

Table 44. The effect of tobacco smoke (TS) and TS + N-acetylcysteine (NAC) on the number of neutral secretory cells

Airway level		% reduction due to TS	% inhibition by NAC (of reduction due to TS)
Trachea:	upper	68.1 **	0
	lower	81.8 **	0
Lung:	I	91.3 **	6.8 *
	II	87.0 **	0
	III	93.4 ***	6.3
	IV	90.8 **	32.2
	V	85.7 **	6.7
	VI	85.7 **	10.4

Significant reduction due to TS or inhibition by NAC:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 45. The effect of tobacco smoke (TS) and TS + N-acetylcysteine (NAC) on the number of acidic secretory cells

Airway level		% increase due to TS	% inhibition by NAC (of increase due to TS)
Trachea:	upper	100.2 **	128.7
	lower	160.0 **	93.5 *
Lung:	I	183.3 **	59.6 **
	II	286.6 **	49.8 **
	III	130.0 ***	45.9 **
	IV	357.7 ***	35.4 **
	V	511.1 ***	19.5 **
	VI	491.4 ***	77.4 **

Significant increase due to TS or inhibition by NAC:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 46. The effect of tobacco smoke (TS) and TS + N-acetylcysteine (NAC) on secretory cell number

Airway level		% increase due to TS	% inhibition by NAC (of increase due to TS)
Trachea:	upper	62.4	34.9
	lower	104.5 **	37.7 *
Lung:	I	154.2 **	51.6
	II	243.4 **	57.6 **
	III	102.2 **	70.2 **
	IV	320.6 **	59.7 **
	V	421.1 **	68.8 **
	VI	245.2 **	117.7 ** c

Significant increase due to TS or inhibition by NAC:

* = $p < 0.05$

** = $p < 0.01$

c = complete inhibition

level VI, the most distal airway studied. Inhibition was due primarily to inhibition of acidic secretory cell hyperplasia, significant at all airway levels but the upper trachea ($p < 0.05$) (Table 45). NAC had little consistent effect on the TS-induced reduction in number of neutral secretory cells (Table 44): inhibition was significant in airway level I ($p < 0.05$).

V. Summary

1. Rats were exposed to the smoke of 'middle to high tar' cigarettes (TS) (Capstan Full Strength, unfiltered; cabinet exhaust rate of 2 x 1 l/min) and given N-acetyl-cysteine (NAC) concurrently as 1% of their drinking water (average daily dose of 973 mg/Kg). Numbers of secretory cells were assessed at eight airway levels.
2. Rats given NAC alone gained significantly less weight than rats not treated with NAC, and rats treated with TS + NAC gained significantly less weight than rats exposed to TS ($p < 0.05$). The reduced weight gain by rats given NAC appeared to be due to reduced consumption of NAC solution.
3. The use of a 'middle to high tar' cigarette caused, for the first time in the studies presented herein, a significant reduction in neutral secretory cells ($p < 0.01$) and a significant increase in acidic secretory cells ($p < 0.01$) at all eight airway levels. The increase in number of acidic secretory cells was so great it more than offset the reduction in number of neutral secretory cells and led to a significant increase in the 'total' number of secretory cells at all airway levels ($p < 0.01$) except the upper trachea.

4. NAC inhibited TS-induced secretory cell hyperplasia at all airway levels, significantly so in 6 out of 8 of them ($p < 0.05$). Inhibition was complete in the most distal airway studied.
5. Inhibition was due primarily to inhibition of acidic secretory cell hyperplasia which was significant at all airway levels ($p < 0.05$) but the upper trachea. NAC had little consistent or significant effect on the TS-induced reduction in number of neutral secretory cells.
6. In conclusion, NAC inhibited secretory cell hyperplasia due to TS but did not prevent a histochemical 'shift' in type of intracellular mucin by failing to inhibit the TS-induced reduction in number of neutral secretory cells.

SECTION D

The effect of indomethacin and flurbiprofen on 'recovery' from tobacco smoke-induced secretory cell hyperplasia

To determine whether indomethacin and flurbiprofen would reduce the time taken for secretory cell numbers to return to control values (i.e. 'recovery') in rats, when given by intraperitoneal injection after cessation of TS exposure.

Two experiments were carried out. The first experiment was to determine the time course within which TS-induced secretory cell hyperplasia returned to control values (i.e. 'recovery time'). The days after cessation of TS on which rats were killed during the recovery period were based upon other studies, not all of them in the rat (see Introduction): 9 (Jones, 1978, 1979), 21, 42 (Baskerville, 1976), and 84 (Leuchtenberger et al., 1960; Baskerville, 1976). The duration of the second study (the effect of drugs on recovery time) and the days on which rats were killed was based upon the results of the first experiment. Flurbiprofen and indomethacin were the drugs of choice, based on previous inhibition with few adverse side effects (Greig et al., 1980; Section B, experiments 1 and 3 of this Chapter).

Experiment 1: Time course of recovery

Thirty seven rats with a mean weight of 223g (± 2g) on the first day of the experiment were divided into two groups: one of 11 rats not exposed to TS and left as 'pure' cage controls for 14 days, the other of 26 rats exposed to TS (John Player Navy Cut, unfiltered, cabinet exhaust rate of 2 x 1 l/min: see Materials and Methods for details) for 14 days. After the exposure period, rats were taken at random from each group for killing on five 'recovery' days (Table 47). Secretory cell number was assessed at three airway levels: trachea, intrapulmonary axial pathway

Table 47. Number of rats killed on each day

	days after cessation of TS exposure				
	1	9	21	42	84
No TS	6	0	0	0	5
TS	6	5	5	5	5

TS = cigarette tobacco smoke

and in four intrapulmonary lateral pathways, the counts of which were combined to give a single value for the 'laterals'.

I. Body weight

Rats exposed to TS were significantly lighter than control rats ($p < 0.01$) at the end of the exposure period (i.e. day 1 after cessation of TS) (Table 48). By 9 days after cessation of TS, there was no significant difference in weight gain between the two groups (Table 48).

II. Lung cleanliness

Only in lungs from two control animals killed 84 days after cessation of TS (i.e. over 14 weeks after delivery from the suppliers) was any noteworthy amount of BALT seen (see Materials and Methods for details); Grade II, the limit of acceptability (see Fig. 3). The secretory cell counts for the two rats were included in the study.

III. Secretory cell number

a. Effect of tobacco smoke

At each airway level, there was no significant difference in mean secretory cell number between control rats killed on days 1 and 84: the values for each group were combined:

Airway level	No TS: combined mean number secretory cells per 10 high power fields (± SEM)
Trachea	39.9 (3.8)
Axial	55.5 (3.3)
Laterals	47.9 (4.8)

TS significantly increased the mean number of secretory cells above the combined control values at all three airway

Table 48. Mean % increase in body weight, g (± SEM) from the first day of exposure to tobacco smoke (TS)

	days after cessation of exposure to TS				
	1	9	21	42	84
No TS	30.7 (1.2)	52.0 (2.4)	76.5 (3.6)	96.0 (4.4)	116.0 (3.8)
TS	26.2 (0.7) **	50.8 (1.2)	77.6 (1.7)	96.2 (3.3)	130.1 (7.6)

n = 5 or 6

Significant difference between No TS and TS: ** = $p < 0.01$

levels ($p < 0.05$ - < 0.01) (Table 49): the increase was progressive from proximal to distal airways (52, 79 and 225% for the trachea, axial and laterals respectively).

b. Recovery

Recovery of secretory cell number was judged to have taken place when there was no significant difference between the mean TS value on each day and the mean for the combined control values (Table 50). In the trachea, recovery took between 2 and 9 days, in the axial pathway between 10 and 21 days, and in the lateral pathways between 43 and 84 days (Table 50). In the trachea and axial pathway of the lung, the number of secretory cells in the animals exposed to TS was below control values on days 42 and 84, although not significantly so. The reduction cannot be explained as being due to infection (i.e. secretory cells obscured by BALT: see Materials and Methods for details) because the amount of BALT was minimal (see Part II of this experiment).

IV. Summary

TS significantly increased the number of secretory cells at all airway levels studied ($p < 0.05$ to $p < 0.01$): the increase was greatest in the distal airways. The time taken for recovery of secretory cell number to control values appeared to be directly related to the initial degree of hyperplasia due to TS:

Airway level	% increase due to TS	days for recovery
Trachea	52	2 to 9
Axial	79	10 to 21
Laterals	225	43 to 84

Table 49. Mean number of secretory cells per 10 high power fields (± SEM)

Airway level		Day after cessation of TS 1	9	21	42	84
Trachea	No TS	43.3 (4.5)	-	-	-	35.3 (6.3)
	TS	60.5 (4.2)	48.4 (1.4)	44.6 (8.0)	36.8 (8.2)	29.0 (6.8)
Axial	No TS	58.6 (4.2)	-	-	-	52.3 (5.1)
	TS	99.5 (6.7)	87.1 (5.7)	62.6 (7.6)	49.7 (7.5)	52.0 (7.7)
Laterals	No TS	48.8 (3.0)	-	-	-	46.8 (10.9)
	TS	155.8 (9.5)	124.5 (17.7)	84.7 (5.9)	116.0 (17.0)	101.2 (38.6)

TS = cigarette tobacco smoke

n = 10 or 12

Table 50. % increase or decrease (-) in number of secretory cells

Airway level	Days after cessation of TS exposure 1	9	21	42	84
Trachea	51.6 *	21.3	11.8	- 7.7	- 27.3
Axial	79.3 **	56.9 **	12.8	-10.5	- 6.3
Laterals	225.3 **	159.9 **	76.8 **	142.2 **	111.3

TS = cigarette tobacco smoke

Significant differences to controls at each day:

* = $p < 0.05$

** = $p < 0.01$

Experiment 2 : Effect of drugs on recovery time

Eighty rats with a mean weight of 197g (± 2g) on the first day of the experiment were divided into two groups: one, of 25 rats, was not exposed to TS and left as 'pure' cage controls for 14 days, the other, of 55 rats, was exposed to TS (John Player Navy Cut, unfiltered; cabinet exhaust rate of 2 x 1 l/min) for 14 days. After the exposure period the two groups of rats were randomly divided into six different treatment groups for study during the recovery period (Table 51). Indomethacin and flurbiprofen were given by intraperitoneal injection (ip) to rats in their respective groups at a total daily dose of 4 mg/Kg, as 2 mg/Kg in the morning and 2 mg/Kg in the afternoon in an attempt to maintain plasma levels. The dose of 4 mg/Kg was chosen as being the 'standard' effective dose used throughout the studies presented herein. In order to minimise the possibility of infection and, for handling reasons, keep body weight to a minimum the following days after cessation of TS were chosen for study: 1, 4, 9 and 21.

I. Animals : General condition and body weight

Apart from one animal which died during the course of the recovery period, the general health and condition of the remaining animals was good. The fur of the animals exposed to TS became stained yellow but slowly returned to its normal colour after cessation of TS exposure. The rat which died had been treated with indomethacin for eight days and had been losing weight from day 5 of recovery. Post mortem examination revealed the abdominal cavity to contain a clear liquid and the gut contents to be fluid. A rat given flurbiprofen, killed at the same time for comparison, did not have these

Table 51. Number of rats killed on each day after cessation of tobacco smoke (TS) exposure

Treatment after cessation of TS	day			
	1	4	9	21
(No TS) $NaHCO_3$	8	0	0	5
(No TS) indomethacin in $NaHCO_3$	-	0	1 *	5
(No TS) flurbiprofen in $NaHCO_3$	-	0	1 **	5
(TS) $NaHCO_3$	10	5	5	5
(TS) indomethacin in $NaHCO_3$	-	5	5	5
(TS) flurbiprofen in $NaHCO_3$	-	5	5	5

$NaHCO_3$ = sodium bicarbonate

(No TS) = 'pure' controls for 14 days before drug treatment

(TS) = TS exposure for 14 days before drug treatment

* died during course of experiment

** killed for comparison with *

features. No other gross abnormalities were found in the rat which died and death was probably due to gastrointestinal complications. All the other rats gained weight during the period of the study (Table 52). Rats exposed to TS gained significantly less weight than the 'pure' controls, both over the period of exposure ($p < 0.001$) and after four days of drug treatment after cessation of TS ($p < 0.05 - < 0.01$). By the ninth day of the recovery period, there was no significant difference in body weight between the six groups.

II. Lung cleanliness

The only noteworthy amount of BALT found was in the lungs of 3 animals which had been killed 21 days after the end of TS exposure. The amount was not unacceptable (see Materials and Methods for details; Fig. 27) and the animals were included in the study.

III. Secretory cell number

Secretory cell number was determined at eight airway levels: the upper and lower halves of the trachea and intrapulmonary airway levels I to VI (see Fig. 15). Mean numbers of secretory cells are given in Tables 53 to 56.

a. Controls

In control animals which had not been exposed to TS but given sodium bicarbonate solution during the 'recovery' period, the number of neutral secretory cells became reduced at all airway levels by an average of 78% (± 8%) from day 1 to day 21. (Table 53): reduction was significant in both halves of the trachea and in airway levels I, II and V ($p < 0.01$). Numbers of 'total' and acidic secretory cells were significantly increased in airway level III

Table 52. Mean % increase in body weight g, (± SEM) from the first day of exposure to tobacco smoke (TS)

Treatment after cessation of TS	Days after cessation of TS exposure			
	1	4	9	21
(No TS) $NaHCO_3$	52.3 (5.3)	62.8 (2.9)	67.1 (3.4)	89.5 (3.7)
(No TS) indomethacin in $NaHCO_3$	-	56.5 (2.5)	57.1 (8.1)	85.7 (7.1)
(No TS) flurbiprofen in $NaHCO_3$	-	55.2 (2.4)	63.7 (2.5)	85.3 (3.1)
(TS) $NaHCO_3$	40.5 *** (1.1)	48.8**(1.9)	59.3 (2.7)	89.5 (4.2)
(TS) indomethacin in $NaHCO_3$	-	50.7 *(2.3)	51.1 (5.2)	80.3 (5.9)
(TS) flurbiprofen in $NaHCO_3$	-	48.5**(2.2)	59.7 (2.6)	85.2 (5.0)

n = 5, 6, 8 or 10

$NaHCO_3$ = sodium bicarbonate

(No TS) = 'pure' controls for 14 days before drug treatment

(TS) = TS exposure for 14 days before drug treatment

Significant differences compared to (No TS) $NaHCO_3$ on each day:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 53. Mean number secretory cells per 10 high power fields (± SEM) in control rats (i.e. No TS, $NaHCO_3$) at days 1 and 21 of recovery

Airway level	Neutral		Acidic		Total	
	1	21	1	21	1	21
Trachea: upper	24.4 (4.9)	2.4 ** (1.0)	37.6 (5.2)	43.2 (15.0)	62.0 (8.8)	45.6 (15.4)
lower	20.6 (3.7)	2.8 ** (0.7)	45.3 (6.4)	39.0 (11.1)	65.9 (7.5)	41.8 (11.7)
Lung: I	16.9 (2.1)	1.2 ** (0)	65.2 (8.9)	64.0 (5.5)	82.0 (9.5)	65.2 (5.6)
II	17.5 (1.7)	1.0 ** (0.3)	59.9 (7.1)	63.8 (5.1)	77.4 (8.0)	64.8 (5.2)
III	2.9 (0.8)	0.9 (0.4)	39.7 (4.0)	53.6 *(4.5)	42.5 (3.8)	54.6*(4.6)
IV	3.0 (1.0)	2.1 (1.5)	35.3 (5.6)	45.6 (5.6)	38.3 (5.9)	47.6 (7.3)
V	3.9 (0.9)	0.1 ** (0.1)	20.6 (3.7)	21.1 (2.8)	24.5 (4.1)	21.2 (2.8)
VI	2.4 (0.8)	0.9 (0.5)	14.6 (3.5)	8.6 (3.2)	17.0 (3.7)	9.4 (3.0)

Significant differences in number of each cell category between days 1 and 21 :

* = $p < 0.05$

** = $p < 0.01$

n = 10 or 16

Table 54. Mean number secretory cells per 10 high power fields (± SEM) in control rats given indomethacin alone for 9 and 21 days of recovery

Airway level		Neutral		Acidic		Total	
		9	21	9	21	9	21
Trachea:	upper	[14.0]	3.2 (1.5)	[23.3]	41.4 (6.6)	[37.3]	44.6 (7.3)
	lower	[6.7]	6.2* (1.0)	[48.0]	42.4 (3.7)	[54.7]	48.6 (3.5)
Lung:	I	[2.9]	0.4 (0.4)	[61.8]	64.8 (12.7)	[64.7]	65.2(12.4)
	II	[2.5]	0.8 (0.6)	[33.0]	64.2 (11.2)	[35.5]	65.0(11.0)
	III	[0.0]	1.3 (0.7)	[35.0]	61.6 (5.1)	[35.0]	62.9 (5.4)
	IV	[0.0]	0.5 (0.2)	[13.8]	42.7 (6.8)	[13.8]	43.2 (6.9)
	V	[3.0]	0.2 (0.2)	[15.9]	16.7 (3.9)	[17.5]	17.0 (3.9)
	VI	[1.3]	0.0 (0.0)	[6.8]	4.6 (2.3)	[7.5]	4.6 (2.3)

n = 10, except:

[ ] = number in one animal

Significant difference in number on day 21 between indomethacin and $NaHCO_3$ treatment (see Table 53): * = $p < 0.05$

Table 55. Mean number secretory cells per 10 high power fields (± SEM) in control rats given flurbiprofen alone for 9 and 21 days of recovery

Airway level		Neutral		Acidic		Total	
		9	21	9	21	9	21
Trachea:	upper	[15.0]	6.4 (1.3)	[38.3]	33.4 (5.0)	[53.3]	39.8 (5.0)
	lower	[13.9]	4.6 (0.7)	[20.0]	34.4 (9.1)	[33.9]	39.0 (9.3)
Lung:	I	[9.4]	1.4 (0.6)	[98.2]	60.2 (5.3)	[107.7]	61.6 (5.0)
	II	[12.1]	1.2 (0.5)	[102.6]	63.8 (10.6)	[114.7]	65.0 (10.5)
	III	[6.3]	1.9 (0.8)	[50.7]	54.5 (6.0)	[51.3]	56.4 (6.3)
	IV	[2.4]	3.7* (0.8)	[28.4]	45.0 (6.3)	[30.7]	48.7 (6.5)
	V	[1.3]	1.1 (0.4)	[15.8]	17.4 (3.2)	[18.5]	18.5 (3.4)
	VI	[2.5]	1.8 (0.7)	[4.2]	8.0 (3.3)	[9.8]	9.8 (4.0)

n = 10, except:

[ ] = number in one animal

Significant difference in number on day 21 between flurbiprofen and $NaHCO_3$ treatment (see Table 53): * = $p < 0.05$

Table 56. Mean number secretory cells per 10 high power fields (± SEM) : 'baseline' values

Airway level		Neutral	Acidic	Total
Trachea:	upper	15.3 (4.3)	39.9 (6.6)	55.2 (8.1)
	lower	13.8 (3.3)	42.8 (5.6)	56.6 (7.0)
Lung:	I	10.8 (2.6)	64.7 (5.7)	75.5 (6.5)
	II	11.1 (2.5)	61.4 (4.7)	72.5 (5.5)
	III	2.1 (0.5)	45.0 (3.3)	47.2 (3.1)
	IV	2.6 (0.8)	39.4 (4.5)	42.0 (4.6)
	V	2.6 (0.8)	20.8 (2.6)	23.3 (2.8)
	VI	2.1 (0.7)	13.3 (2.9)	15.3 (3.0)

n = 26

($p < 0.05$) (Table 53). The change in number of secretory cells cannot be explained as being due to infection (i.e. secretory cells obscured by BALT: see Materials and Methods for details). There were only two significant differences in the number of secretory cells on day 21 of recovery between control rats given sodium bicarbonate and those given the drugs: there were more neutral cells in the lower trachea with indomethacin ($p < 0.05$) (Table 54), and in airway level IV with flurbiprofen ($p < 0.05$) (Table 55). The 'baseline' against which the effects of TS alone or TS + drug was compared was taken as the means of values on days 1 and 21 in rats not exposed to TS and given sodium bicarbonate during the recovery period (Table 56).

b. Effect of tobacco smoke

TS exposure for 14 days produced a secretory cell hyperplasia (between 52 and 177% above 'baseline' values) which was significant at all airway levels ($p < 0.05 - < 0.001$) for at least 9 days after exposure (Table 57). Numbers of secretory cells were still significantly higher in airway levels III and IV after 21 days of recover ($p < 0.01$); the airways which showed the greatest hyperplasia on the first day after cessation of TS exposure.

Secretory cell hyperplasia was due primarily to an increase in number of acidic secretory cells above 'baseline' values (Table 58); significant in all airways for at least 4 days, in all but the upper trachea for at least 9 days, and in airways III and IV for at least 21 days ($p < 0.05 - < 0.01$). The latter airways showed the

Table 57. % change in number of secretory cells after tobacco smoke (TS) exposure

All values are % increase above 'baseline' values except: - = % reduction

Airway level		Days after cessation of TS			
		1	4	9	21
Trachea :	upper	64.9 **	108.3 **	85.5 *	-4.9
	lower	72.6 **	86.2 **	100.7 **	18.9
Lung:	I	68.5 **	83.6 **	55.0 *	-28.5
	II	94.6 **	116.3 **	55.0 *	-26.6
	III	177.3 ***	225.6 **	141.3 **	172.9 **
	IV	188.6 ***	171.4 ***	156.2 ***	135.0 ***
	V	52.4 *	168.2 **	164.0 *	32.6
	VI	82.4 *	287.6 **	161.4 *	-16.3

Significant differences compared to 'baseline' values :

* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Table 58. % change in number of acidic secretory cells after tobacco smoke (TS) exposure

All values are % increase above 'baseline' values except : - = reduction

Airway level		Day after cessation of TS			
		1	4	9	21
Trachea:	upper	87.0 **	132.6 **	71.9	17.8
	lower	100.9 **	102.8 **	93.9 **	40.9
Lung :	I	76.5 **	77.0 **	56.1 **	-22.1
	II	108.6 **	108.1 **	62.5 **	-14.7
	III	185.8 ***	233.8 **	147.1 **	185.6 **
	IV	196.2 **	178.2 **	168.0 ***	140.9 ***
	V	61.5 *	187.5 **	194.7 **	43.8
	VI	91.0 *	329.3 **	199.3 **	-9.0

Significant differences compared to 'baseline' values:

* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

greatest increase in number of acidic secretory cells after TS exposure. TS exposure had no consistent or significant effect on the number of neutral secretory cells.

c. <u>Effect of drugs on recovery time</u>

Recovery after TS-induced secretory cell hyperplasia was judged to have taken place when there was no significant difference between the mean TS value on each day and the 'baseline' value. Both indomethacin and flurbiprofen reduced the recovery time in the intrapulmonary airways, but not in the trachea (Tables 59 and 60). Flurbiprofen had inhibited TS-induced secretory cell hyperplasia at all airway levels by day 4, significantly so in the intrapulmonary airways ($p < 0.05$) which led to recovery in 5 of them. The remaining intrapulmonary airway had recovered by day 9. Indomethacin had inhibited TS-induced secretory cell hyperplasia at all but one airway level by day 4: inhibition was significant at 5 intrapulmonary airway levels ($p < 0.05$) which led to recovery in 4 of them. The remaining intrapulmonary airway had recovered by day 9. Inhibition of secretory cell hyperplasia and subsequent recovery with drug-treatment was due primarily to inhibition of TS-induced acidic secretory cell hyperplasia (Tables 61 and 62). TS had no consistent or significant effect on the number of neutral secretory cells upon which the drugs could act.

IV. Summary

1. Two experiments are described in which secretory cell hyperplasia is induced in rats by exposure to cigarette tobacco smoke (John Player Navy Cut, unfiltered; cabinet

Table 59. Effect of indomethacin on recovery time: % inhibition of tobacco smoke (TS)-induced secretory cell hyperplasia on three days after TS exposure

Airway level		Day		
		4	9	21
Trachea:	upper	53.2	64.8	- R
	lower	0.0	59.7	322.4 * R
Lung:	I	68.5	84.8	- R
	II	79.0 ** R	83.7 R	- R
	III	61.1 **	93.4 ** R	83.2** R
	IV	74.2 * R	115.0 ** R	99.1** R
	V	100.8 * R	118.6 ** R	214.5* R
	VI	94.6 * R	107.3 R	-

Significantly different to TS value:

* = $p < 0.05$

** = $p < 0.01$

R = recovery: i.e. not significantly different to 'baseline' value

- = fewer secretory cells after TS than 'baseline' value

Table 60. Effect of flubiprofen on recovery time: % inhibition of tobacco smoke (TS)-induced secretory cell hyperplasia on three days after cessation of TS exposure

Airway level		Day		
		4	9	21
Trachea:	upper	63.6	6.1	- R
	lower	59.8	43.5	323.4 R
Lung:	I	116.3 * R	59.3 R	- R
	II	84.3 * R	53.1 R	- R
	III	85.9 **	78.9 ** R	83.6 ** R
	IV	83.6 * R	73.9 ** R	91.2 * R
	V	96.9 * R	115.5 * R	131.6 R
	VI	95.7 * R	142.9 ** R	- R

Significantly different to TS value:

* = $p < 0.05$
** = $p < 0.01$

R = recovery; i.e. not significantly different to 'baseline' value

\- = fewer secretory cells after TS than 'baseline' value

Table 61. Effect of indomethacin on recovery time: % inhibition of tobacco smoke (TS)-induced acidic secretory cell hyperplasia on three days after cessation of TS exposure

Airway level		Day 4	Day 9	Day 21
Trachea:	upper	61.2	75.3 R	291.6 R
	lower	14.1	61.2	168.6 * R
Lung:	I	68.9	78.0	- R
	II	77.1 * R	74.7 R	- R
	III	60.2 **	91.4 ** R	82.9 ** R
	IV	72.4 * R	111.9 ** R	98.0 ** R
	V	98.0 * R	114.3 ** R	187.9 ** R
	VI	93.6 * R	115.5 R	- R

Significantly different to TS value:

* = $p < 0.05$

** = $p < 0.01$

R = recovery; i.e. not significantly different to 'baseline' value

\- = fewer secretory cells after TS than 'baseline' value

Table 62. Effect of flurbiprofen on recovery time: % inhibition of tobacco smoke (TS)-induced acidic secretory cell hyperplasia on three days after cessation of TS exposure

Airway level		Day	
	4	9	21
Trachea: upper	67.7	14.3 R	342.3 R
lower	63.6	66.2	169.1 R
Lung: I	116.5 * R	67.8 R	- R
II	81.0 * R	50.5 R	- R
III	85.0 ** R	77.3 ** R	85.4 ** R
IV	80.5 * R	72.7 ** R	89.4 * R
V	93.6 * R	106.9 *	115.4 R
VI	92.7 ** R	140.0 ** R	- R

Significantly different to TS value:

* = $p < 0.05$

** = $p < 0.01$

R = recovery; i.e. not significantly different to 'baseline' value

\- = fewer secretory cells after TS than 'baseline' value

exhaust rate of 2 x 1 l/min) (TS) and the number of days for the increased number of secretory cells to return to control values (i.e. 'recovery time') established. The effect of indomethacin and flurbiprofen on recovery time is then determined.

2. In both experiments, TS caused a significant increase in secretory cell number at all airway levels studied: the increase being due to acidic secretory cell hyperplasia.
3. Recovery took at least 21 days at all airway levels; up to 84 days in some intrapulmonary airways.
4. Indomethacin and flurbiprofen (4 mg/Kg, daily after cessation of TS exposure) more than halved recovery time in the airways of the lung but not in the trachea (Table 63).
5. One rat given indomethacin died.
6. Flurbiprofen was overall, marginally the more effective drug.

Table 63. Summary: Number of days for recovery after tobacco smoke-induced secretory cell hyperplasia

TS = recovery after exposure to tobacco smoke

ind. = recovery with indomethacin

flurb. = recovery with flurbiprofen

		Acidic secretory cells			Total secretory cells		
		TS	ind.	flurb.	TS	ind.	flurb.
Trachea:	upper	9	9	9	21	21	21
	lower	21	21	21	21	21	21
Lung:	I	21	21	4	21	21	4
	II	21	9	4	21	4	4
	III	> 21	9	4	> 21	9	9
	IV	> 21	4	4	> 21	4	4
	V	21	4	4	21	4	4
	VI	21	4	4	21	4	4

SECTION E

Summary of Results

1. The results of studies on the effect of sub-chronic administration of nicotine, cigarette tobacco smoke (TS) or TS + a range of anti-inflammatory drugs and a mucolytic agent on the number of epithelial mucus-containing secretory cells (henceforth referred to as secretory cells) in the airways of the rat are presented in this Chapter.

   The effects of the different treatments is determined by counting the number of secretory cells staining for neutral or acidic intracellular mucin in the trachea at a number of airway levels (I to VI) in the left lung.

2. a. Nicotine, when given twice daily by subcutaneous injection, each dose of 10 mg/Kg (producing plasma concentrations of 695 ng/ml; 'low' dose) significantly reduces the number of secretory cells in one intrapulmonary airway.

   b. When given as eight daily doses of 7.5 mg/Kg by intraperitoneal injection (producing plasma concentrations of 2356 ng/ml; 'high' dose), nicotine significantly increases the number of secretory cells in the more proximal airways of the lung.

   c. The change in secretory cell number is due primarily to changes in the number of acidic secretory cells.

   d. When given by aerosol (producing plasma concentrations of 10 ng/ml) nicotine has no significant effect on secretory cell number.

3. a. TS causes secretory cell hyperplasia in both extra-and intrapulmonary airways. However, the degree of hyperplasia and its extent throughout the airways is dependent upon smoke concentration and the type of cigarette used.

b. Increasing the smoke concentration in the exposure cabinets to a level where it produces plasma nicotine concentrations of 106 ng/ml induces significant secretory cell hyperplasia in 7 out of 8 airway levels; more than is produced by lower concentrations of TS. Hyperplasia is due primarily to an increase in number of acidic secretory cells: the number of neutral secretory cells is not altered at this smoke concentration.

c. The time taken for the number of secretory cells to return to control values (i.e. 'recovery time') after cessation of exposure to the concentration of TS defined in b. above takes up to 21 days in the trachea, intrapulmonary axial pathway and most distal bronchioli: recovery takes up to 84 days in more proximal bronchioli. Recovery time is directly related to the initial degree of hyperplasia due to TS.

d. The greatest effect on secretory cell number is found following exposure to the smoke from higher tar cigarettes than used above (plasma carboxyhaemoglobin concentrations of 14%). The number of neutral secretory cells is reduced, but the reduction is more than offset by a large and extensive increase in the number of acidic secretory cells which leads to an overall secretory cell hyperplasia throughout the airways. Thus, TS from 'high tar' cigarettes causes the 'shift' in the secretory cell population to a predominance of acidic secretory cells which is seen in bronchitic patients.

4. When given concurrently (by intraperitoneal injection) with TS exposure, two non-steroidal anti-inflammatory drugs,

indomethacin and flurbiprofen, and three steroidal anti-inflammatory drugs, dexamethasone, prednisolone and hydrocortisone inhibit TS-induced secretory cell hyperplasia. However, the inhibitory effect is directly related to the initial increase in number of secretory cells due to TS.

i. Indomethacin and flurbiprofen are inhibitory at daily doses of 0.4 and 4 mg/Kg: the inhibitory effect is greatest in more proximal airways and with the higher dose. The effect is due primarily to inhibition of acidic secretory cell hyperplasia. Flurbiprofen is marginally the more effective.

ii. Dexamethasone (at an average daily dose of 2 mg/Kg), prednisolone and hydrocortisone (both at daily doses of 4 mg/Kg) are inhibitory. The effect is again due primarily to inhibition of acidic secretory cell hyperplasia. The potency of inhibition is in the aforementioned order. However, dexamethasone is detrimental to the health of the rats. Indomethacin is a more potent inhibitor than dexamethasone in proximal airways: dexamethasone is more potent than indomethacin in distal airways.

5. When given orally by gavage, concurrently with TS exposure, indomethacin (4 mg/Kg), flurbiprofen (4 mg/Kg) and aspirin (20 mg/Kg) do not significantly inhibit secretory cell hyperplasia. Flurbiprofen reduces the mean number of secretory cells to within control values, indomethacin had no inhibitory effect, aspirin exacerbated the effect of TS.

6. When given concurrently with TS exposure, the mucolytic agent N-acetylcysteine (NAC) inhibits secretory cell hyperplasia throughout the airways when given orally in the drinking water at an average dose of 973 mg/Kg. The inhibitory effect is entirely due to inhibition of acidic secretory cell hyperplasia because NAC does not inhibit the reduction in neutral secretory cells seen after exposure to the 'high tar' cigarette.

7. Indomethacin and flurbiprofen reduce 'recovery time'. When given by intraperitoneal injection at a daily dose of 4 mg/Kg after cessation of TS exposure, both drugs reduce recovery time in the intrapulmonary airways from a minimum of 21 days to 9 days or less. Neither drug is effective in the trachea. Flurbiprofen is marginally the more effective drug.

8. Overall, the order of potency of inhibition of TS-induced secretory cell hyperplasia by the drugs is flurbiprofen and NAC followed by indomethacin, dexamethasone, prednisolone, hydrocortisone and aspirin.

CHAPTER 4

DISCUSSION

He who claims a 'first'
Lives to regret;
There is sure to be a 'firster'
You can bet.

SAMUEL VAISRUB, MD, 1978

The studies reported herein have shown that whole cigarette smoke (TS), a number of anti-inflammatory drugs and a mucolytic agent in combination with TS, and nicotine will all affect the number of surface epithelial mucus-secreting (i.e. secretory) cells in the airways of the specific pathogen-free (SPF) rat.

A. TOBACCO SMOKE-INDUCED CHANGES

Tobacco smoke caused a hyperplasia of secretory cells as has been reported in other studies (Lamb and Reid, 1969 b, Reid, 1970, Reid and Jones, 1983). The increase in number of secretory cells may be assumed to be due to TS and not to other environmental factors in the cabinets because it has been shown that neither extremes of temperature (3 to 33°C) nor humidity (5 to 85%) influence the number of secretory cells in the airways of rats (Jones et al., 1971). The temperature and humidity in the exposure cabinets in the present studies were within the range investigated by Jones and her colleagues; temperature 22 to 27°C, humidity 49 to 85%. In the study by Lamb and Reid (1964) a dose relationship was established between secretory cell hyperplasia and the number of cigarettes to which the animals were exposed: the greater the number of cigarettes (5, 10 and 20) the greater the hyperplasia. A similar dose effect was found in the present studies where the amount of secretory cell hyperplasia was directly related, up to a threshold (see below), to a combination of the tar content of the cigarettes (t) and the exhaust rate (e) from the exposure cabinets; the $t.^{1}/e$ value ($r = 0.8497$, $p < 0.01$) (see Fig. 7). A mean secretory cell hyperplasia of 120% above control values was achieved when the mean plasma concentration of nicotine in the rats was 106 ng/ml. However, even at this 'concentration' of TS exposure

there was no histochemical shift in type of intracellular mucin: i.e. TS exposure did not significantly reduce the number of neutral secretory cells although the number of acidic secretory cells was increased. Changing to a 'middle to high tar' cigarette (yield in mg/cigarette: tar 26, nicotine 2.7, carbon monoxide, CO, 16.7) caused a histochemical shift: i.e. neutral secretory cells were reduced whilst acidic secretory cells were increased. The latter cigarettes produced CO concentrations in the exposure cabinets which rose from 7 ppm at the beginning of exposure to TS to a maximum of 145 ppm at the end of exposure. During this time, mean plasma concentrations of carboxyhaemoglobin (COHb) in the rats rose from 12% after 1 hours exposure to 16% at the end of exposure. This is the first occasion that the amount of TS to which experimental animals have been exposed has been quantified in terms of CO exposure, plasma concentrations of COHb and nicotine and related to the degree of secretory cell hyperplasia. In early studies (Lamb and Reid, 1969 b; Jones et al., 1973), where the tar and nicotine content of the cigarettes was similar to that of the currently used 'middle to high tar' cigarette (see Fig. 10), shifts in the type of intracellular mucin were commonplace. Thus, it would appear that although the amount of hyperplasia produced is related to the amount of TS to which the rats are exposed, it is the 'quality' (in terms of high tar and nicotine) of the smoke which causes the histochemical shift. The extensive study of Huber et al. (1981) on the effect of TS on rat lung tissue quantified numerous parameters. However, because the study was conducted on only one batch of rats over a six month period, the relationship between the 'quality' of TS and the resulting pathological changes was not apparent.

The reduced secretory cell hyperplasia produced at a t.$^{1}/e$ value of 90 (see Fig. 7) may be due to stimulated discharge of intracellular mucin by the 'high dose' of TS. Discharge of intracellular mucin has been found to lead to an apparent reduction in secretory cell number after one day of exposure to TS (Jones et al., 1973). The concentration of TS used in light microscopic experimental studies of secretory cell hyperplasia should therefore be chosen with care to avoid an apparent reduction in cell number.

The range of plasma concentrations of COHb and nicotine in human cigarette smokers has been found to be between 1 and 13% and between 9 and 46 ng/ml respectively (mean 30 ng/ml) (Russell et al., 1973, 1975 b; Gilman et al., 1980). However, the CO yields of cigarettes currently on the market may be higher than those of a few years ago (Wald et al., 1981). In addition, smokers of cigarettes with 'ventilated' filters have been found to have higher plasma concentrations of COHb than smokers of unfiltered or 'normally' filtered cigarettes (Wald et al., 1977). Consequently, current concentrations of plasma COHb in some human smokers may be greater than the upper limit of 13% found over eight years ago. Thus, the concentrations of COHb found in the plasma of the rats in the present studies (12% after 1 hours exposure to 16% at the end of exposure) may be close to the current concentrations in the plasma of some human smokers. Conversely, the nicotine yield of cigarettes has been declining over the past few years (see Fig. 10) and the smoking of 'low tar' (i.e. low nicotine) cigarettes is currently advocated (Gori and Lynch, 1978; Royal College of Physicians, 1977; Wynder and Hoffman, 1979; US Surgeon General, 1979; Kunze, 1980;

Laboratory of the Government Chemist, 1978-1983). Many human cigarette smokers may therefore have lower plasma nicotine concentrations than those of a few years ago causing the current mean concentration to be less than 30 ng/ml. The latter value is over $3\frac{1}{2}$ times less than the mean plasma nicotine concentration of 106 ng/ml found in the rats in the present studies. Thus, although the concentration of TS to which the rats were exposed is similar to that of human cigarette smokers in terms of plasma COHb concentrations, it is considerably higher in terms of plasma nicotine concentrations. Extrapolation of effects to Man must therefore be made with caution.

I. Inhibition

Tobacco smoke-induced secretory cell hyperplasia was inhibited by concurrent, intraperitoneal (ip) injection of two non-steroidal anti-inflammatory drugs (flurbiprofen and indomethacin) and three steroidal anti-inflammatory drugs (dexamethasone, prednisolone and hydrocortisone). When given concurrently orally by gavage, flurbiprofen was partially inhibitory, indomethacin had no inhibitory effect whilst aspirin (non-steroidal anti-inflammatory drug) exacerbated the hyperplasia due to TS in some airways. The mucolytic drug, N-acetylcysteine (NAC) was inhibitory when given concurrently orally in drinking water. With indomethacin and flurbiprofen (ip), the inhibitory effect appeared to be dose related. In the case of indomethacin (three experiments), the degree of inhibition and its extent throughout the airways appeared to be related to the amount of secretory cell hyperplasia due to TS : the greater the hyperplasia the greater the inhibition (Fig. 18). Whether the relationship is biologically significant or not can only be answered by further study as the number of

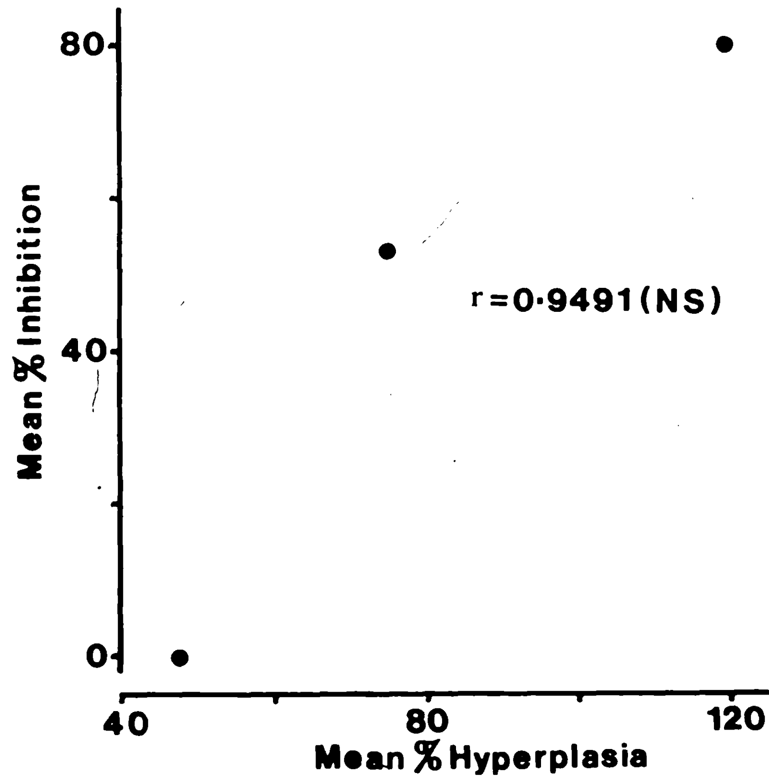


Fig. 18. Relationship between tobacco smoke-induced secretory cell hyperplasia and its inhibition by indomethacin :

Experiment (see Key to Table for Fig. 7)	Mean % hyperplasia	Mean % inhibition
9	47.6	0
2	74.9	53.2
7	119.9	80.9

NS = not significant.

observations is too small to allow for statistical analysis.

The inhibitory effect of the various drugs extends the findings of other workers. The addition of the non-steroidal anti-inflammatory drug phenylmethyloxadiazole (PMO) to tobacco (2% by weight) inhibits both the ciliostatic effect of TS (Dalhamn and Rylander, 1971) and the inflammatory changes produced by inhalation of acrolein aerosol (Dahlgren and Dalhamn, 1972). PMO also inhibits many of the TS-induced changes in the extrapulmonary, but not intrapulmonary airways of rats (Jones et al., 1972, 1973; Jones and Reid, 1978; Jeffery and Reid, 1981). Secretory cell hyperplasia was inhibited completely whilst increases in mitosis and epithelial height were only partially inhibited. Indomethacin has previously been reported to inhibit TS-induced secretory cell hyperplasia in a dose-related manner when given ip (2 and 4 mg/Kg body weight) (Greig et al., 1980). The present studies reported herein support this result and extend the finding that, unlike PMO, indomethacin is inhibitory in both extra- and intrapulmonary airways. It is suggested that the inhibitory effect of PMO is limited to extrapulmonary airways by its inhaled route of administration; PMO absorbed onto smoke particles tending to be deposited proximally (Dalhamn and Rylander, 1971). Systemic administration allows drugs to be carried via the pulmonary arteries to intrapulmonary airways. Inhibition of TS-induced secretory cell hyperplasia by flurbiprofen and the three steroidal anti-inflammatory drugs (when given ip) indicates that the inhibitory effect extends to other anti-inflammatory drugs. In particular, the finding in the present studies that the order of potency of inhibition of TS-induced secretory cell hyperplasia is related to the order of potency of inhibition of other, more 'classical'

inflammatory reactions is of interest. The 50% inhibitory dose of UV-induced erythema and carageenan or yeast-induced paw oedema by oral administration of flurbiprofen and indomethacin has been calculated (Ishii et al., 1975). In each test, flurbiprofen was 14 to 25 times more potent than indomethacin in inhibiting the induced inflammatory reaction. Similarly, dexamethasone is 6 times more potent than is prednisolone which in turn is 4 times more potent than is hydrocortisone (Sayers and Travis, 1970). The same orders of potency of inhibition of TS-induced secretory cell hyperplasia were found in the present studies.

When given orally by gavage, neither indomethacin, flurbiprofen nor aspirin exhibited significant inhibition. The gavage technique appeared to cause irritation of the airways, particularly the trachea, additional to that caused by TS. The number of secretory cells was increased above TS values in gavage and TS-treated animals. The increase was significant in two airways in rats given aspirin but not in flurbiprofen or indomethacin-treated animals. The lack of inhibitory effect of the drugs may be due to the oral route of administration. A higher dose of aspirin, indomethacin or flurbiprofen has to be given orally to achieve the same degree of inhibition of 'classical' inflammatory reactions to that reached when the same drugs are given rectally or by ip injection (Ishii et al., 1975). This finding may explain why indomethacin and flurbiprofen, at the same daily dose of 4 mg/Kg, were inhibitory when given ip but not when given by gavage. Aspirin is up to 31 times less potent than indomethacin and up to 775 times less potent than flurbiprofen in inhibiting 'classical' inflammatory reactions. Thus, although indomethacin and flurbiprofen appeared to partially inhibit 'TS and gavage-

induced' secretory cell hyperplasia, aspirin, at a dose only 5 times greater, would not be expected to be inhibitory.

The inhibitory effect of the mucolytic drug, NAC, extends both the scope of the studies presented here and the findings of other workers. Concurrent administration of another 'mucolytic' drug, S-carboxymethylcysteine (SCMC) to rats exposed to sulphur dioxide (SO_2) (300 ppm for 80-195 hours) has been found to partially prevent airway secretory cell hyperplasia (Quevauviller et al., 1970). SCMC has also been found to prevent the change to increased sialidase-resistant sialylated and sulphated glcoprotein in the secretory cells of rats similarly exposed to SO_2 (Huyen, 1973). The study of NAC presented herein was the only experiment in which TS caused a histochemical shift in type of intracellular mucin by reducing the number of neutral secretory cells whilst increasing the number of acidic secretory cells. Unlike SCMC (ibid), NAC did not prevent the histochemical shift: NAC inhibited the increase in acidic cells but did not prevent the reduction in neutral cells. In this respect NAC was similar in action to PMO which also did not prevent the TS-induced change in type of intracellular mucin: both drugs failed to prevent the decrease in number of neutral cells (Jones et al., 1972, 1973; Jones and Reid, 1978). SCMC would therefore appear to merit further study in an experimental TS-exposure system. NAC has also been found to inhibit the increase in epithelial thickness due to TS (Rogers and Jeffery, 1984) and in this respect is similar in action to PMO (Jones et al., 1972).

II. Recovery and reversibility

The studies presented herein have shown that the increase in number of secretory cells due to TS is reversible and will return

to control values (i.e. recovery) after cessation of TS exposure. In the trachea and at airway levels I, II, V and VI in the lung, the increase in secretory number after TS was similar and was maintained for at least 9 days. Recovery had taken place in these airways by 21 days after cessation of TS. In two intra-pulmonary airways (levels III and VI) where the increase after TS was greatest, the secretory cell increase was maintained for up to 42 days with recovery taking place within 84 days. Recovery time would therefore appear to be related not only to airway level but also to the initial degree of secretory cell hyperplasia. Thus, comparisons with other studies must be made with caution.

Administration (ip, 4 mg/Kg) of indomethacin or flurbiprofen during the recovery period has no inhibitory effect in the trachea but reduces the recovery time to as little as 4 days in the intra-pulmonary airways. Although the recovery of bronchial epithelium after cessation of TS or drug administration has been previously reported, this is the first occasion that anti-inflammatory drugs have been shown to reduce recovery time. In one comparatively small study (Jones, 1978) it has been found that two weeks after inhalation of TS is stopped, there was no recovery of secretory cell number in rat trachea, although there was a shift to the 'normal' (sic.) in type of intracellular mucin. The findings agree partially with the results of the present study where recovery of secretory cell number took up to 21 days in the trachea. However, in the present study, the number of neutral secretory cells in rats which had not been exposed to TS was significantly reduced (in the absence of infection measured by the amount of BALT) 35 days after the start of the experiment when compared to the number 15 days after the start of the experiment

(i.e. the first day of the recovery period). A shift to the 'normal' in type of intracellular mucin (Jones, 1978) would therefore be difficult to detemine as the 'baseline' is not constant. Recovery of TS-induced histological airway changes has been studied in most detail after cessation of administration of isoproterenol in piglet bronchi (Baskerville, 1976) and in rats over a 3-month (i.e. 84-day) period (Jones and Reid, 1979, 1982). In the latter studies in the rat, the pattern of recovery after isoproterenol administration is different to that found in the studies reported herein after TS exposure. After isoproterenol, recovery in the proximal trachea takes place within a week and is maintained: after TS, recovery in this airway region takes at least 9 days. However, the main differences in recovery time between the two administrations is found in the intrapulmonary airways. After cessation of isoproterenol administration there is a rapid fall in secretory cell number followed by a rise in number towards the end of the study period. After TS there is a gradual fall during the period of study without a terminal rise in number. Why the pattern of recovery after the two agents should be different is unclear. In the present study, which was divided into two experiments, the pattern of recovery was slightly different in each. The difference appeared to be related to the degree of initial hyperplasia due to TS. It has been also found that intra-animal variation is greater during recovery after isoproterenol than it is during the increasein secretory cell number in response to the drug (Jones, 1980). Further study on recovery after TS exposure is therefore required. A noteworthy finding is that the increase in hamster epithelial secretory cell number after a single intratracheal insillation of elastase is maintained for at least a year

(Christensen et al., 1977; Hayes and Christensen, 1978). Thus comparison of recovery of bronchial epithelium after administration of different agents is of value and may indicate differences in the pathogenesis of secretory cell hyperplasia.

III. Clinical relevance

The results of the present studies and those of other workers indicate that certain of the anti-inflammatory and mucolytic drugs investigated may have a clinical use in the treatment of patients with hypersecretory diseases. This suggestion is not without support because indomethacin, NAC and methylprednisolone have already been investigated clinically in patients with bronchial hypersecretion. Indomethacin has been administered to five patients with chronic airways obstruction whose symptoms persisted in spite of conventional therapy, including steroid treatment: the patients were considered to be chronic 'asthmatic' (Hume and Eddey, 1977). The results of such a small trial are controversial and proper controls (see NAC below) were omitted. Improvement of symptoms, reduction in the dosage of steroids and improvements in patient 'well being' were found. However, in one report (D'Agostino et al., 1968), the condition of some asthmatic patients was unchanged by indomethacin-treatment.

N-acetylcysteine has been given to patients with hypersecretory pulmonary disease in a number of studies (reviewed by Richardson and Phipps, 1978; Wanner and Rao, 1980). As with the studies using indomethacin, the results of many of the latter studies using NAC are controversial. Control groups given placebo treatments of saline or, better, a low concentration of NAC which still retained its smell were often omitted. Patients were rarely assessed in follow-up. Many of the studies were not

'double blind'. In 1980 a number of new clinical studies on the efficacy of NAC were published (Eur. J. Respir. Dis., 61 suppl. III). Fifteen studies were conducted in adult patients and children with chronic obstructive pulmonary disease (COPD) or chronic asthma, and five further studies reported the use of NAC in cystic fibrosis. Seven studies are left after excluding those studies which are anecdotal, did not include a placebo treatment, were not double-blind, had no follow-up or where the results were not analysed statistically. The parameters measured in these latter seven studies were varied but included sputum volume, viscosity and ease of expectoration, as well as forced expiratory volume in one second (FEV_1), forced vital capacity, peak expiratory flow rate, frequency of cough and subjective estimates of patient well being. Four out of the seven studies concluded that NAC was of benefit, particularly in causing an initially increased expectoration of less viscid sputum which led subsequently to reduced cough, improved lung function, better patient 'well being', and fewer episodes in follow-up (Aylward et al., 1980; Chodosh, 1980; Multicenter Study Group, 1980; Verstraeten, 1980). The remaining three studies did not demonstrate significant differences in the measured parameters between patients treated with NAC and those given placebo; most patients improved with either treatment (Gepts, 1980; Gotz, 1980; Ravez, 1980). In the most recently reported clinical trial (Boman et. al., 1983), oral acetylcysteine was found to reduce significantly exacerbation rate in 203 patients, smokers or ex-smokers, with chronic bronchitis (MRC definition). The trial was multi-center, randomized, double-blind, placebo-controlled (although the nature of the placebo is not stated), and the results were analysed

statistically. The criteria for exacerbation included cough (new or aggravated) and expectoration of mucopurulent or purulent sputum with at least one of the following symptoms present: general malaise, symptoms of common cold, fever ($> 38^{o}c$), breathlessness, increased mucus production, increased sputum-thickness, foul tasting sputum, increased difficulty of expectoration, leucocytosis or pneumonia. Selection of patients (see below) appeared to ensure that there was no significant difference between the placebo and drug-treated groups in regard to the prevalence of adverse side effects.

Methylprednisolone is a steroidal anti-inflammatory drug similar in action to prednisolone (British National Formulary, 1983). When given to 44 patients with chronic bronchitis (sic.) in a double-blind, randomized, placebo-controlled trial (Albert et al., 1980), methylprednisolone improved both pre- and post-bronchodilator FEV_1. However, it is not clear from the report whether or not the patients were chronic bronchitics: sputum production was not included as a criterion in diagnosis! Many of the patients may have been asthmatic. Steroid-treatment, with prednisolone being the current drug of choice, will improve asthmatic symptoms (Crompton, 1983). In addition, some chronic bronchitics will respond to steroid-treatment with increases in FEV_1 which are not related to favourable changes in sputum production (ibid).

In summary, the results of studies on the treatment of bronchitic patients with indomethacin, N-acetylcysteine or methylprednisolone are controversial, either because many of the studies could have been conducted more rigorously or because the results are equivocal. N-acetylcysteine, the drug most studied, is associated with few

detrimental side-effects and would appear to improve patient well-being and reduce exacerbation rate.

The experimental studies presented herein have shown that a number of anti-inflammatory drugs and a mucolytic agent will inhibit TS-induced secretory cell hyperplasia. NAC was inhibitory when given orally. Indomethacin and flurbiprofen reduced the time taken for recovery of secretory tissue after cessation of TS exposure. Bronchitic patients who are prepared to give up the smoking habit might therefore benefit from subsequent treatment with the latter two drugs. The ethics of treating bronchitic patients who would not be prepared to give up smoking with these or any of the other drugs studied experimentally herein would be controversial. The spirometric tests used in the clinical trials on indomethacin, NAC and methylprednisolone summarised above explored only 'large' airway function. However, it would be more reasonable to study changes in the smaller, more peripheral airways with measurements of pulmonary compliance, expiratory flow at 5% of vital capacity (reviewed by Wanner, 1980) or gas mixing (Cumming, 1967, 1980; Cumming and Guyatt, 1981). Few side effects were noted by patients given indomethacin, NAC or methylprednisolone in the clinical studies. However, some patients did complain of side effects including nausea, gastrointestinal bleeding, vomiting and dyspepsia. However, similar symptoms were found in some placebo-treated patients and were not considered to be serious by the clinicians involved in the trials. The lack of serious side effects may be attributed to the low toxicity of the drugs and the selection of patients. In the trial with indomethacin, patients with a history of aspirin sensitivity were excluded. Such patients should be excluded from any study involving the

use of non-steroidal anti-inflammatory drugs (Szczeklik et al., 1977; Mathison and Stevenson, 1979). Asthmatic patients should be excluded from any trial involving NAC because of the possibility of induced broncho-spasm (Waltemath and Bergman, 1973; Ho and Beilin, 1983) albeit that isoprenaline given simultaneously prevents such attacks (Bernstein and Ausdenmoore, 1964; Kory et al., 1968; Pulle et al., 1970). Overdosage or prolonged use of corticosteroid drugs can lead to a number of detrimental side effects including immunosuppression, hypertension, osteoporosis, mental disturbance and peptic ulceration (British National Formulary, 1983). Specifically, prednisolone-treatment has been found to cause a transitory impairment of leucocyte function in patients with ulcerative colitis in complete remission (Clarke et al., 1977). Thus, any clinical trial resulting from the current experimental studies should: i. measure small airways function, and ii. select patients who will not be additionally compromised by the test treatment.

B. <u>THE EFFECTS OF NICOTINE</u>

The effect of nicotine (as a major constituent of tobacco smoke and when included in chewing gum as an aid to breaking the smoking habit) on the bronchial secretory cell population has not been documented. Possibly the most interesting finding of the studies presented herein is that nicotine alone, when inhaled (by aerosol) at a 'dose' which produces mean plasma concentrations of nicotine in the rats of 10 ng/ml, did not change the number of secretory cells. The range of plasma nicotine concentrations in human smokers is between 9 and 46 ng/ml (Russell et al., 1975 b, 1980; Gilman et al., 1980). The mean

plasma concentration of nicotine in subjects who have chewed gum containing 2 and 4 mg nicotine ad libitum are 11 and 23 ng/ml respectively (Russell et al., 1976; McNabb, 1982). Thus, the 'dose' of nicotine administered experimentally to rats is similar to that achieved by some human smokers and gum chewers. Although extrapolation of effects from experimental animals to Man must always be made with caution, it would appear that: 1) the 'bronchitic' changes seen in Man, which are statistically linked to inhalation of cigarette smoke, are due to factors other than the effect of nicotine alone, and 2) chewing nicotine-containing gum would not be expected, of itself, to cause bronchial secretory cell hyperplasia.

The injected 'low' dose of nicotine produced a mean plasma nicotine concentration in the rats of 556 ng/ml for 6 hours after the injection and reduced the number of secretory cells in the airways of the lung. The injected 'high' dose of nicotine produced mean plasma concentrations of 2356 ng/ml and increased the number of secretory cells in the airways of the lung. A 'dose' of TS which has been shown herein to induce secretory cell hyperplasia in the lungs of rats produces a mean plasma nicotine concentration of 106 ng/ml. Thus, although nicotine at plasma concentrations above this latter value exhibits a 'bi-phasic' effect on secretory cell hyperplasia the hyperplasia seen in rats exposed to TS must be due to factors in TS other than nicotine alone.

C. ARE THE CHANGES IN NUMBER OF SECRETORY CELLS 'REAL' OR APPARENT ?

In the studies presented herein, secretory cells were visualised by light microscopy of sections stained specifically for intracellular mucin. Individual cells were not stained. The

identification of a secretory cell was therefore dependent upon its containing stainable intracellular mucin. Observed changes in number of secretory cells may have been due to a 'real' change in cell number or merely due to changes in the presence and amount of intracellular mucin: i.e. an 'apparent' change in number. A real decrease in overall cell number may be due to cell death and loss from the epithelium or a decrease in number of a particular cell type may be due to a change in its morphology to that of another cell type (i.e. differentiation and transformation). A real increase in overall cell number may be due to cell division (proliferation) or an increase in number of a particular cell type may be due to differentiation or transformation from another cell type. Apparent changes in secretory cell number (by light microscopy of paraffin sections) are the result of the balance between mucus-synthesis and secretion (Fig. 19). Increased synthesis with reduced secretion, causing a build-up of retained secretion, would lead to an apparent increase in cell number. Decreased synthesis with increased secretion would lead to an apparent decrease in cell number.

In bronchial epithelium, it is now clear that mitotic activity (leading to cell hyperplasia) is not restricted to the basal cell compartment (long thought to be the stem cell) but is also a feature of secretory cells (Jeffery, 1973; Jeffery and Reid, 1981; McDowell et al., 1978, 1979; Ayers and Jeffery, 1982). Cell differentiation and transformation is also a feature of bronchial epithelial cells. Early studies (Drasch, 1881; Bindretter et al., 1968) suggested that 'daughter' cells of dividing respiratory tract cells differentiate first into mucous cells which in turn become ciliated cells without further division.

G
RER
SC
uptake of
mucus-precursors

a. b. c.

Fig. 19. Synthesis and secretion of mucus (   ) by airway secretory cells (SC). RER = rough endoplasmic reticulum (protein synthesis, initial stages of mucus-synthesis), G = Golgi apparatus (later stages of mucus-synthesis).

a. Mucus-synthesis and secretion balanced.

b. Increased synthesis and reduced secretion ('apparent' increase in secretory cell number).

c. Reduced synthesis and increased secretion ('apparent' reduction in secretory cell number).

More recent studies have shown that a variety of routes of differentiation are available to bronchial progenitor cells, particularly in response to injury (Gordon and Lane, 1977, 1980; McDowell et al., 1978, 1979; Becci et al., 1978; Ayers and Jeffery, 1982; Donnelly et al., 1982). In general, the results of the above referenced studies using semi-thin, electron microscopic and autoradiographic techniques, agree and may be summarised as follows. In 'normal' SPF rat epithelium, cell division and differentiation are very slow with only a small fraction of both the basal and secretory serous cell (containing neutral mucin) population dividing and differentiating. The activity of the mucous cell (containing predominantly acidic mucin) is virtually nil. With irritation there is a change in the dividing cell population with a marked proliferative response now by the mucous cell. In fact the mucous cell divides at a rate which was hitherto unexpected. In addition, there is also a contribution to the increased mucous cell population by an increase in the transformation of serous, through transitional, to mucous cells. In the present light microscopical studies and those of other workers (Lamb and Reid, 1969 b; Jones et al., 1972, 1973; Jones and Reid, 1978), TS-induced secretory cell hyperplasia was due entirely to an increase in the number of acidic secretory cells because the number of neutral cells was either unchanged or reduced. The increase in number of acidic cells was so great that it more than offset any reduction in number of neutral cells. The latter finding indicates that the increase in acidic secretory cell number is due to increased division by the acidic cells with a smaller contribution by the transformation of neutral to acidic secretory cells.

The suggestion that changes in the number of airway secretory cells in response to a stimulus are due to changes in the rates of mucus-

synthesis or discharge has been made previously (McDowell et al., 1983). In the latter study, the effect of intra-tracheal instillation of elastase in saline or saline alone in hamsters was examined at both the semi-thin and ultrastructural levels. The authors concluded that although the number of secretory cells appeared to be increased 24 hours after the instillations, the increase was due to the accumulation of secretory granules in pre-existing but previously unobtrusive secretory cells; i.e. an apparent increase in number. Changes in the number and size of mucin-containing granules within secretory cells during exposure to TS have been reported in studies at the electron microscopic level (Jeffery and Reid, 1981). The initial response to tobacco smoke was a discharge of granules from serous cells followed, on the second and third days of exposure, by a reappearance of granules. The latter granules were divided between electron-dense serous types and electron-lucent mucous types. Subsequent breaks in TS exposure led to discharge followed by the reappearance of granules with an increasing proportion of electron-lucent types. At the light microscopic level, Jones et al., (1973) found a substantial fall in secretory cell number after one day of exposure to TS, followed by a rise on day 3. The pattern was repeated following reintroduction of the irritant after a break in the exposure routine (Jones, 1978). The initial fall in number is thought to parallel the EM studies and be due to a discharge of intracellular mucin which would lead to an apparent decrease in number. The subsequent rise would be due to re-synthesis of mucin leading to an apparent increase in number. In the present studies, two experiments, investigating the reversibility of TS-induced secretory cell hyperplasia, showed that the number of acidic

secretory cells was generally increased on the fourth and ninth days after cessation of TS to values above that found on the first day of cessation of TS. The increased numbers on days 4 and 9 of recovery may have been due to the lack of 'need' or stimulus to secrete but with maintained synthesis (i.e. apparent increase) after removal of the irritant. The increase in secretory cell number on days 4 and 9 was due to an increase in number of acidic secretory cells indicating a synthesis of predominantly mucus-type granules as described above by Jeffery and Reid (1981). Secretory cells were however increased in the present studies on the day after fourteen days of exposure to TS. If discharge of secretory granules follows removal of the irritant, the observation in the present studies that the increase in secretory cell number was directly related to the 'concentration' of TS used (up to a threshold concentration) (Fig. 7) may be due to a balance between discharge and secretory cell proliferation. At 'low' concentrations of TS the increase in number of secretory cells may not be sufficiently large to greatly override the apparent reduction in number due to discharge. At higher concentrations of TS the increase in number may be so great as to mask any apparent reduction. At concentrations of TS above the threshold, the secretory response to TS may be increased to a level which causes an apparent reduction in secretory cell number which overrides the real increase in number. Semi-thin and EM studies may serve to elucidate these questions.

Tobacco smoke has been shown to affect both mucus synthesis and secretion (Richardson et al., 1978; Coles et al., 1979; Jeffery et al., 1984 b). When passed through cat trachea in situ, TS (undiluted and diluted 1:9 with air) increased the output of ^{35}S-

labelled mucin into the airway lumen (Richardson et al., 1978). Coles et al., (1979) studied radioactively labelled glycoprotein precursor incorporation and glycoprotein secretion in an in vitro preparation of laryngeal and tracheal glands from 'normal' and 'bronchitic' rats. Secretion was raised in bronchitic rats whilst the concentration of labelled glycorpotein in mucous cells at unit time after pulse-labelling was similar in control and 'bronchitic' rats. The latter anomaly was explained by the authors: the size of mucous cells was increased in the hypertrophied glands causing the total amount of incorporated precursor per cell to be increased proportionally. Jeffery et al., (1984 b) measured the output of fucose (as a marker for mucus) into in situ tracheal preparations of 'normal' rats and rats made histologically 'bronchitic' by prior exposure to TS: output was significantly greater in bronchitic rats.

However, the latter three studies did not study mucus synthesis and secretion in surface epithelial secretory cells per se. Epithelial secretory cells from denuded explants of canine trachea have been shown, by radiolabelling techniques, to both synthesise and secrete mucin-type macromolecules (Stahl and Ellis, 1973). In cat trachea, in situ and using radioactively-labelled mucin-precursors, ammonia vapour has been shown to stimulate discharge of mucins with low ^{35}S-sulphate and relatively high sialic acid content which are presumed to be of epithelial 'goblet' cell origin (Gallagher et al., 1977). Coles and Reid (1978), using labelled precursors of mucus, investigated synthesis and secretion in epithelial secretory cells in airway explants from 'normal' and clinically bronchitic patients. The results of the study showed that both synthesis and secretion in the epithelial secretory

cells was increased in explants from bronchitics. The authors concluded that in histologically 'bronchitic' tissue (i.e. with secretory cell hyperplasia and gland hypertrophy) from clinically bronchitic patients (i.e. with sputum production), mucus hyper-secretion is associated with increased rates of uptake of glyco-protein precursors, synthesis and discharge of mucus. Long term modification of one or more of the stages of secretion would there-fore appear to lead to an increase in the rates of the other stages. If, in the studies presented herein, mucus synthesis and secretion by secretory cells in rat airway epithelium after exposure to TS were equally balanced, the secretory cells would contain stainable intracellular mucin by the end of the TS exposure. The increase in number of secretory cells with TS would therefore be 'real'. The rates of mucus-synthesis and secretion in histologically 'bronchitic' rats merit further study in order to determine whether or not they are balanced.

Anti-inflammatory drugs have been shown to inhibit cell prolifera-tion in a wide variety of animal species and experimental situations. In particular, anti-inflammatory drugs inhibit the proliferation of cells which have either been acutely stimulated to proliferate (by injury or by the use of chemicals) or are naturally proliferating (foetal or tumour cells). The criteria for inhibition of prolifera-tion used in the majority of the studies includes reduced uptake of ^{3}H - thymidine, reduced DNA synthesis, reduced labelling and mitotic indices, as well as quantitative morphology. Indomethacin, aspirin, prednisolone and hydrocortisone have all been shown to inhibit cell proliferation after mechanical or chemical stimulation in skin (Francis and Marks, 1977; Furstenbergen and Marks, 1978; Fursten-bergen, 1979), liver (MacManus and Braceland, 1976), pituitary gland

(Pawlikowski et al., 1981) and thyroid gland (Sluszkiewicz and Pawlikowski, 1980). Indomethacin was more potent than either aspirin or prednisolone and was the anti-inflammatory drug of choice in the majority of the studies. In rabbit foetal lung, hydrocortisone, prednisolone and dexamethasone all inhibit normal cell proliferation (Carson et al., 1973; Adamson and Bowden, 1975; Kauffman, 1977 a and b, 1980; Lin et al., 1982). Similarly, a variety of non-steroidal anti-inflammatory drugs, including indomethacin and aspirin have been shown to inhibit cell proliferation in tumour cell lines (Powles et al., 1973; Bayer and Beaven, 1979). Again, indomethacin was the most potent inhibitor. However, in one study (Rosner and Cristofalo, 1979) hydrocortisone inhibited cell proliferation in seven vertebrate species (mammals, bird, reptile, amphibian and fish) but stimulated human foetal lung proliferation. Conversely, Heifetz and Snyder (1981) found that hydrocortisone inhibited cell proliferation in human foetal lung. This anomaly could be due to the finding that in Man the number of cellular binding sites for steroidal drugs decreases with age (Rosner and Cristofalo, 1981). The age of the foetal lung cultures (see above) was not stated in the reports. Whether or not different aged foetal lung cultures would react differently to hydrocortisone is not documented. The differing effects of hydrocortisone on lung cell proliferation may also be due to the timing and duration of drug-administration. Differences in the timing and duration of methylprednisolone-treatment of mice with acute lung injury (induced experimentally with butylated hydroxytoluene) produces differing effects on cell proliferation (Smith and Brody, 1981). Treatment with methylprednisolone on days 1 and 2 after hydroxytoluene-induced lung damage either inhibits (after 'mild' damage)

or has no effect on (after 'severe' damage) proliferation of alveolar type II cells. 'Prolonged' methylprednisolone-treatment (days 1 - 5) potentiates proliferation, damage and mortality. 'Late' drug-treatment (days 3 and 4) impairs differentiation of type II into type I cells but speeds the maturation of new type II cells (see below). In addition, the differences in effect with hydrocortisone (see above) could be due to terminology. For example, steroidal anti-inflammatory drugs have been shown to accelerate the <u>maturation</u> of existing lung cells whilst inhibiting <u>proliferation</u> (Liggins 1969; Wang et al., 1971; Mercurio and Rhodin, 1976; Kauffman, 1977 a and b, 1980). The criteria for cell maturation are diverse but generally include ultrastructural analysis of lamellar body volume (by area) and density. Of interest here is the finding that indomethacin will inhibit the maturation of rabbit foetal lung (Bustos et al., 1975) as well as inhibiting proliferation whilst methylprednisolone will impair differentiation but enhance maturation of adult mouse type II alveolar cells (Smith and Brody, 1981). The actions of steroidal and non-steroidal anti-inflammatory drugs on lung cell division, differentiation and maturation would thus appear to be complex and it is beyond the scope of this thesis to consider them further.

Mucolytic drugs in general have not been shown to affect cell proliferation. However, in one study using autoradiographic techniques and semi-thin sections, NAC has been found to have anti-proliferative properties (Jeffery et al., 1984 c). The same regimen of TS exposure and NAC administration, as well as some of the animals used in the latter study were the same as those used in the study presented herein. NAC was found to delay and attenuate the proliferative response of the epithelium, but to extend its

duration. NAC inhibited the proliferation of basal cells but did not effect that of the mucous cells. However, the combined results of the two studies (light microscopic and semi-thin) indicate that although NAC inhibits secretory cell hyperplasia, the extent of the inhibitory effect seen at the light microscopic level must have an additional 'apparent' component (i.e. an effect on mucus-synthesis or discharge).

The discussion above indicates that administration of the anti-inflammatory drugs used herein may cause a 'real' inhibition of secretory cell hyperplasia. However, as the results of the two studies with NAC described above indicate, the inhibition may have an additional 'apparent' component (i.e. be affecting the synthesis or secretion of mucus).

Anti-inflammatory drugs have been shown to affect both mucus synthesis and secretory activity directly. Many anti-inflammatory drugs, including phenylmethyloxadiazole (PMO), hydrocortisone and aspirin, have been shown to inhibit the incorporation of radio-actively labelled precursors of mucin into glycoproteins in a variety of tissues including airway mucosa (Coles, 1977; Coles et al., 1979), human foetal lung (Heifetz and Snyder, 1981), gastrointestinal mucosa (Kent and Allen, 1968; Lukie and Forstner, 1972; Rainsford, 1978), cartilage (Whitehouse, 1965, Smith and Dawkins, 1971) and liver (Musil et al., 1968). Therefore, if mucin synthesis was inhibited by anti-inflammatory drugs, there would be an 'apparent' inhibition of secretory cell hyperplasia. Indomethacin has been found to decrease mucus secretion from gastric mucosa (Nicoloff, 1968) whilst PMO reduces mucus-secretion in rat airways _in vitro_ (Coles et al., 1979). NAC has been shown

to have no effect on secretion of hexose from 'normal' rat trachea in situ, but reduces pilocarpine-stimulated secretion (Turner and Marriott, 1983). However, in the latter study, hexose was measured as a marker for glycoprotein and not as a specific marker for bronchial mucus-glycoprotein (see Jeffery et al., 1984 b). Inhibition of mucin discharge would lead to an apparent increase in secretory cell number. However, the drugs used in the present studies inhibited TS-induced increases in secretory cell number. Therefore, if a drug, which has been shown to inhibit discharge of mucus, is also shown to inhibit secretory cell hyperplasia it must be primarily having an inhibitory effect on cell proliferation (i.e. a real inhibitory effect).

D. FACTORS AFFECTING SECRETORY CELL NUMBER

I. Innervation

The mucus-secreting cells in the bronchial epithelium of the rat are closely associated with nerve fibres, particularly in the central airways (i.e. trachea and main bronchi) (Jeffery and Reid, 1973, 1975). Neurosecretory-like vesicles found in many of the fibres indicate a motor innervation and, if proven, the transmitter has yet to be determined. The control of innervated effectors may be by: (1) direct inhibition, (2) indirect inhibition acting via receptors located on post-ganglionic, parasympathetic, cholinergic neurons, or (3) purinergic or peptidergic nerves (reviewed by Jeffery, 1982). That the release of neutro-transmitters may affect bronchial secretory cell number has been demonstrated by the use of β-adrenergic and cholinergic agonists (Sturgess and Reid, 1973; Kleinerman, 1976; Jones and Reid, 1979).

Isoproterenol, salbutamol and pilocarpine given chronically to rats and methacoline given to cats, cause epithelial secretory cell hyperplasia. The latter drugs alter the histochemical nature of the glycoprotein synthesized by airway secretory cells (depending on the drug used and the airway level studied) as well as affecting synthesis and secretion (Sturgess and Reid, 1973; Kleinerman et al., 1976; Bolduc and Reid, 1978; Jones and Reid, 1979; Jones 1980). Nicotine has long been known to exert actions on autonomic ganglia (both sympathetic and parasympathetic) (Orfila, 1843; Langley and Dickinson, 1889). Subsequent studies have led to the general principle that 'high' doses of nicotine inhibit the activity of structures that are stimulated by 'low' doses of the drug (Comroe, 1960; Nedergaard and Schrold 1977; Taylor, 1980). 'Low' doses of nicotine stimulate the ganglion cells directly and facilitate the transmission of impulses. The excitatory effects on ganglia are rapid in onset, blocked by non-depolarizing ganglionic blocking agents (e.g. hexamethonium) (Barlow and Ing, 1948; Paton and Zaimis, 1949), and mimic the initial excitatory post-synaptic potential. Transmission of nerve impulses is blocked by actions at the primary nicotinic receptor. After an initial stimulation of the ganglia in a similar way to acetylcholine, 'high' doses of nicotine cause a prolonged blockade by persistent depolarization which desensitizes the cholinoceptive site (Volle, 1969; Haefely, 1972). The complex and sometimes unpredictable tissue responses that occur after nicotine treatment are therefore due not only to its effects on a variety of neuroeffector junctions, but also to its stimulatory and inhibitory 'phases' of action. Consequently, the responses of a tissue to nicotine treatment represents the balance between

the different and opposing effects of the drug. For example, in the study presented here a 'low' dose of nicotine reduced the number of bronchial secretory cells whilst a prolonged 'high' dose of the drug increased the number of these cells. The dual effect may be explained by the 'bi-phasic' action of nicotine shown to be effective on exocrine glands (Volle and Koelle, 1975). For example, nicotine causes an initial stimulation of secretion from sweat, salivary and bronchial glands which is followed by inhibition. In a similar way, the 'low' dose of nicotine in the present study might have stimulated discharge of mucin from the secretory cells leading to an 'apparent' reduction in their number (Fig. 20). Conversely, the 'high' dose of nicotine might have blocked secretion, leading to retention of intracellular mucin and an 'apparent' increase in number of mucin-containing cells (Fig. 20).

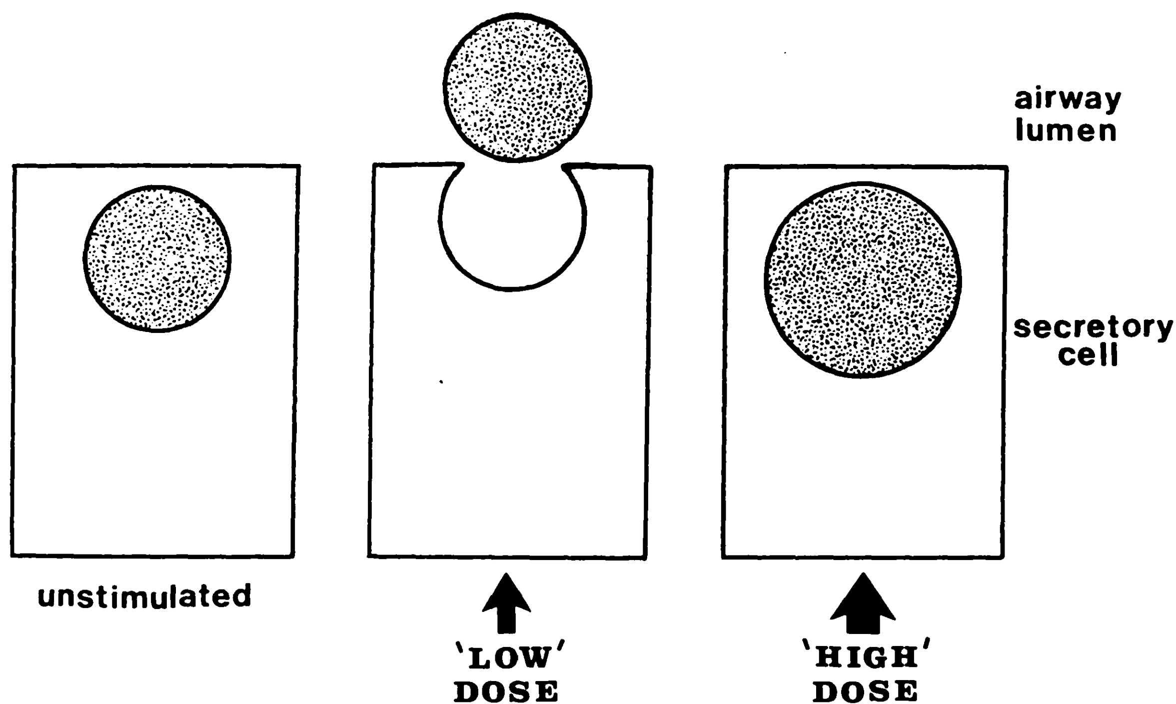
Tobacco smoke has not been shown to affect nervous transmission directly. However, the effects of nicotine on the ganglion described above may contribute to the irritant effect of TS. Irritation by smoke particles impinging on the surface of secretory cells in the airway epithelium may cause discharge of secretion by a local reflex (Richardson and Phipps, 1978; Richardson et al., 1978; Reid et al., 1983). Anti-inflammatory and mucolytic drugs have not been studied in regard to their effects on nervous transmission. Their inhibitory effect on secretory cell proliferation may however be via other mechanisms (see below).

II. Products of arachidonic acid metabolism and cyclic nucleotides

a. Prostaglandins

Arachidonic acid is one member of the family of biologically

Fig.20. Effects of nicotine (↑) on discharge of intracellular mucin (●).



'low' dose nicotine: stimulated discharge.

'high' dose nicotine: discharge inhibited leading to build-up of retained secretion.

active C-20 polyunsaturated fatty acids. From this one compound, by the action of various enzymes, mediators of inflammation such as the prostaglandins (PG's) (Van Dorp et al., 1964; Bergstrom et al., 1964), modulators of platelet aggregation such as thromboxanes (TX's) and prostacyclin (PGI_2) (Hamberg et al., 1975; Moncada et al., 1976; Johnson et al., 1976), 'slow reacting substances of anaphylaxis (SRS-A) such as the leukotrienes (LT's) (Piper and Vane, 1969; Samuelsson et al., 1979), and chemotactic agents such as monohydroxyeicosatetraenoic acid (HETE) (Turner et al., 1975) can be generated (Fig. 21). The lung, in particular, is one of the major tissues which avidly metabolises arachidonic acid and its products: numerous bronchoactive products are synthesised (Bakhle, 1981; Spannhake, 1981), many of which are also inactivated by the lung. For example, in most species including Man, 70-95% of intravenously injected E and F prostaglandins are removed from the circulation during a single passage through the pulmonary circulation (Sors and Even, 1982). Prostaglandins are compounds which are actively metabolised (synthesis and inactivation) by the lung (Bergstrom et al., 1962; Karim et al., 1967; Bakhle, 1981). Prostaglandins are produced in the lungs of numerous animal species, including the rat, under a wide variety of conditions. In particular, stimulating, challenging or injuring the lung causes prostaglandin production. For example, challenging the lung with histamine or other agents which cause anaphylaxis will release prostaglandins (Piper and Vane, 1969; Hitchcock, 1978; Engineer et al., 1978; Kaliner, 1979; Adkinson et al., 1980). Similarly, stimulating the lung with emboli produces prosta-

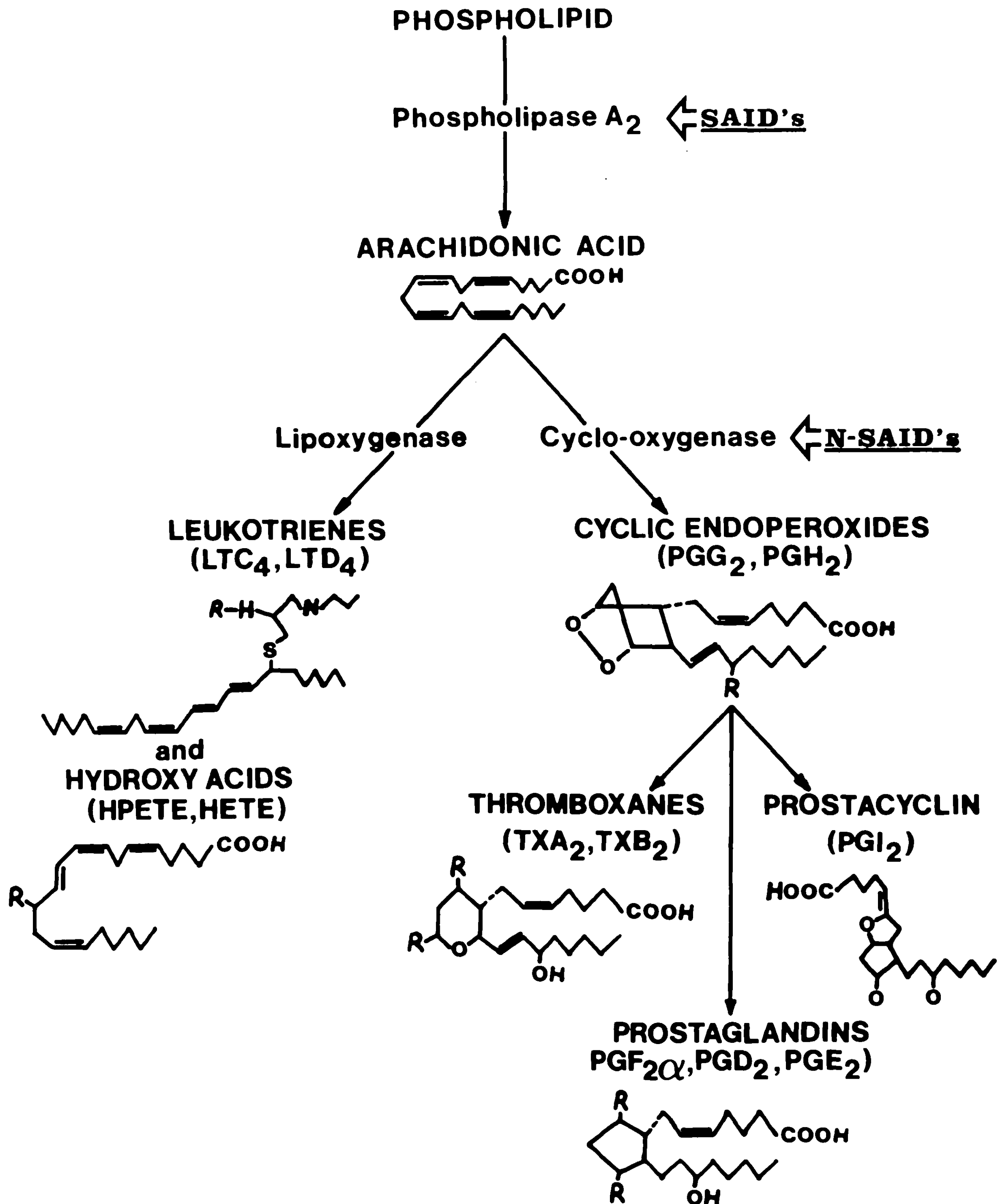


Fig. 21. Pathways of arachidonic acid metabolism

⇐ = Inhibition by steroidal anti-inflammatory drugs (SAID's) or non-steroidal anti-inflammatory drugs (NSAID's).

R = Functional groups.

(Adapted after Eling and Ally, 1981; Moncada and Vane, 1981; Piper et al., 1981).

glandins (Alabaster and Bakhle, 1970; Lindsey and Wyllie, 1970). Prostaglandins are produced not only in the airways, but also in the lung parenchyma (Steel et al., 1979; Taylor, 1979). In the latter studies, prostaglandin production was not by a smooth muscle response.

The first step in the biosynthesis of the biologically active products of arachidonic acid is the hydrolysis of membrane bound phospholipids by phospholipase enzymes (mainly phospholipase A2) which releases free arachidonic acid (Fig. 21). This is a rate-limiting step which is classically inhibited by corticosteroid (steroidal anti-inflammatory) drugs (Fig.21) (Herbaczyñska-Cedro and Staszewska-Barczak, 1974; Gryglewski et al., 1975; Kantrowitz et al., 1975; Tashjian et al., 1975; Flower, 1978; Flower and Blackwell, 1979). Thus, steroidal anti-inflammatory drugs block the formation of free arachidonic acid and all the subsequent biologically active products. Subsequent biosynthesis of the products of arachidonic acid from the free arachidonic acid takes place along two distinct pathways; the 'lipoxygenase pathway' and the 'cyclo-oxygenase pathway'. Enzymes in the lipoxygenase pathway convert free arachidonic acid into the leukotrienes and the monohydroxylated fatty acids (HETE's) (see above). PG endoperoxide synthetase enzymes in the cyclo-oxygenase pathway convert free arachidonic acid into prostaglandins, prostacyclin and thromboxanes (see above) (Fig. 21). Non-steroidal anti-inflammatory drugs classically inhibit the latter pathway (Collier, 1969; Vane, 1971; Ferreira et al., 1971; Flower et al., 1972). The lipoxygenase pathway is not inhibited by non-steroidal anti-inflammatory drugs and so although the production of PG's and thromboxanes

is inhibited, the production of leukotrienes and HETE's is not (Fig. 21). In fact, the lipoxygenase pathway of arachidonic acid metabolism is rather enhanced when the cyclo-oxygenase pathway is inhibited (see Whittle, 1979). For example, the immunological release of SRS-A is potentiated by indomethacin but inhibited by nordihydroguaiaretic acid, eicosatetraenoic acid and phenidone which inhibit both pathways.

Many of the products of arachidonic acid are associated with inflammation, although not specifically with the 'inflammatory' response of bronchial epithelial secretory cell hyperplasia. However, because steroidal and non-steroidal anti-inflammatory drugs classically inhibit the production of these substances and have been shown herein to inhibit secretory cell hyperplasia, the possible involvement of some of these substances in both the genesis and inhibition of secretory cell hyperplasia cannot be ignored. In particular, the prostaglandins, whose production is inhibited by both types of anti-inflammatory drug and which are currently particularly associated with 'inflammatory' reactions, must be considered. The thromboxanes, also inhibited by both types of drug are currently more associated with platelet aggregation and are thus less likely 'candidates' as mediators of secretory cell hyperplasia. The many actions of the products of the lipoxygenase pathway (leukotrienes and HETE's) are only now being documented and their direct effects on secretory cell hyperplasia are uncertain. However, leukotrienes and HETE's have been found to stimulate airways mucus secretion (particularly in inflamed airways) and thereby may contribute to apparent changes in

secretory cell number (e.g. stimulated discharge of stainable intracellular mucin leading to an apparent reduction in cell number) (Marom et al., 1981; Ziporyn, 1981; Peatfield et al., 1982; Shelhamer et al., 1982). In fact, the order of potency of mucin-discharge from human airways in vitro is thought to be leukotrienes followed by HETE's, prostaglandins and histamine (Shelhamer et al., 1982). More work is required on the latter aspect, but the results indicate that leukotrienes and HETE's may assume an important role in the release of mucus.

b. Cyclic nucleotides

Cyclic nucleotides are small intracellular molecules found throughout the animal kingdom, as well as in a number of bacteria. The two most widely studied are cyclic adenosine-3,5'-monophosphate, (i.e. cyclic AMP) and cyclic guanosine-3,5'-monophosphate, (i.e. cyclic GMP). Cyclic AMP appears to be one of the most important regulators of cell, organ and whole body function. In a table drawn up by Robison et al., in 1971, seventy-two enzymes or processes were listed which were associated with a change in the activity of cyclic AMP. The processes listed included activation of enzymes, production and release of hormones, secretion, movement of ions, nervous transmission, protein synthesis, DNA synthesis, and cell growth. In sixty-three of these processes the activity of cyclic AMP was increased, whilst in the remaining nine processes the activity of cyclic AMP was reduced. Cyclic AMP is produced from ATP, catalysed by a hormone-sensitive, membrane-bound enzyme called adenyl cyclase (Rall and Sutherland, 1962). Adenyl cyclase is 'activated' by hormones at the extracellular surface of the cell membrane (Birnbaumer, 1970). The activated enzyme

increases the intracellular rate of production of cyclic AMP from ATP by an order of magnitude or more. The released cyclic AMP is thought to exert its initial physiological effects in the cell via a family of enzymes, the protein kinases (Hittleman and Butcher, 1971). The accumulation of intracellular cyclic AMP is controlled by an enzyme, phosphodiesterase which catalyses the breakdown of cyclic AMP to 5'-AMP (Sutherland and Rall, 1958; Drummond and Perrott-Yee, 1961; Butcher and Sutherland, 1962; Cheung, 1970).

Prostaglandins have been shown to increase intracellular concentrations of cyclic AMP in a number of tissues including lung (Butcher and Baird, 1968; Robison et al., 1971). The increase is thought to be due more to stimulation of adenyl cyclase activity (Zor et al., 1969 a and b; Marsh, 1970; Scott, 1970; Mozkowitz et al., 1971) than suppression of phosphodiesterase activity (Amer and Marquis, 1972; Amer and McKinney, 1973). Thus prostaglandins might stimulate mucus-synthesis and secretion as well as proliferation in airway secretory cells by their actions on cyclic AMP.

Cyclic GMP is another small cyclic nucleolide, similar in structure to cyclic AMP, which is produced from GTP by guanyl cyclase (Hardman and Sutherland, 1969). In the rat, concentrations of guanyl cyclase are particularly high in the lung (White and Aurbach, 1969; Bohme et al., 1969). Cyclic AMP and cyclic GMP have equal and opposite effects in the regulation of cell function: the ratio of the two determining cellular response (Goldberg et al., 1972, 1973). In canine and bovine veins the cyclic GMP/cyclic AMP ratio

increases in response to $PGF_{2\alpha}$ and decreases with PGE_2 (Dunham et al., 1974). Similarly, $PGF_{2\alpha}$ produces a rapid rise in cyclic GMP levels in rat uterus whilst having no effect upon cyclic AMP levels (Goldberg et al., 1973; Kuehl et al., 1973). It has, therefore, been suggested (Kuehl, 1974) that cyclic GMP is linked to the actions of the F prostaglandins whilst cyclic AMP is linked to the E prostaglandins.

The actions of the cyclic nucleotides and the prostaglandins are many and varied and often intimately linked. Of particular importance to the students in this thesis is their involvement in both cell proliferation and secretory activity (see below). The calcium ion (Ca^{2+}) is also important to both these processes (Douglas and Poisner, 1963; Perris et al., 1967, 1971; Selinger and Naim, 1970; Feinman and Detwiler, 1974; Coles et al., 1982), but will not be discussed in detail.

c. <u>Cell Proliferation</u>

DNA synthesis precedes mitosis. Just before DNA synthesis the levels of cyclic AMP and cyclic GMP have been found to change. However, there is controversy about the directions of the change. Some workers have found that the levels of cyclic AMP decrease with a concomitant rise in the levels of cyclic GMP (Otten et al., 1971; Seifert and Rudland, 1974). Other workers have reported the reverse situation (Franks et al., 1971; MacManus and Whitfield, 1971; Boynton et al., 1976). However, the former group is supported by the finding that rapidly dividing cells, such as HeLa cells tend to have lowered levels of cyclic AMP and raised levels of cyclic GMP (Ryan and Heidrick, 1968): the

reverse is true of stationary phase cells. Conversely, PGE given _in vitro_ to rat thymic lymphoblasts causes a 67 to 68-fold increase in the intracellular level of cyclic AMP which is followed by DNA synthesis and cell proliferation. The differences between the two 'camps' may be due to differences between the tissue studied, differences in the amount of experimentally supplied extracellular Ca^{2+}, as well as species differences. However, it is clear that a change in the intracellular concentrations of cyclic AMP and/or cyclic GMP is necessary before DNA synthesis and cell proliferation take place.

d. _Secretory activity_

Synthesis and secretion of numerous secretory products, including mucus, are intimately involved with the cyclic nucleotides and the prostaglandins. However, recent work has indicated that the lipoxygenase products of arachidonic acid (leukotrienes and HETE's) may soon be found to also have an important role in secretory activity (see above) (Marom et al., 1981; Ziporyn, 1981; Peatfield et al., 1982; Shelhamer et al., 1982). Mucus synthesis (i.e., leading to a possible build-up of stainable intracellular mucin) appears to be mediated by cyclic AMP and prostaglandins. Apart from one study, where the concentrations of prostaglandins used were far higher than physiological levels (Malemud and Sokoloff, 1977), addition of cyclic AMP or a wide range of prostaglandins to _in vitro_ cultures of many tissues increases the incorporation of radioactive precursors of mucus into glycoprotein molecules (Goggins et al., 1972; Forstner et al., 1973; Murota et al., 1976, 1977; Kleine and Jungman, 1977).

Cyclic AMP, calcium and prostaglandins are also intimately involved with secretory activity. However, whereas Ca^{2+} is essential for secretory activity (Hokin, 1966; Selinger and Naim, 1970; Case and Clausen, 1973; Coles et al., 1982) and dibutyryl cyclic AMP causes bronchial mucus and gastric acid-secretion (Whimster and Reid, 1973; Main and Whittle, 1975), the prostaglandins generally inhibit secretion (i.e. leading again to a possible build-up of stainable secretory product). Apart from one study where a range of prostaglandins increased the secretion of gastric mucus (Mahoney and Waterbury, 1981) and another study where E prostaglandins had no effect on gastric acid secretion (Benett et al., 1973), many prostaglandins have been shown to inhibit secretion (Robert et al., 1967; Ramwell and Shaw, 1968; Way and Durbin, 1969; Classen et al., 1971; Main and Whittle, 1973). In fact Main and Whittle (1974) concluded that the prostaglandins inhibit secretagogues at a stage prior to stimulation by cyclic AMP. Later, these same authors (Main and Whittle, 1975) postulated a possible negative feedback role for prostaglandins after their study on the opposite effects of cyclic AMP and prostaglandins on secretion from rat gastric mucosa.

In summary, the cyclic nucleotides, cyclic AMP and cyclic GMP, and the prostaglandins are intimately linked in cellular metabolism. In general, altered levels of prstaglandins alter the intracellular concentrations of the cyclic nucleotides in numerous tissues: raised levels of the E prostaglandins increase cyclic AMP concentrations whilst raised levels of the F prostaglandins increase cyclic GMP concentrations. The consequences of these changing concentrations are many

and varied and include either cell proliferation or secretory activity. A change in the intracellular ratio of cyclic AMP to cyclic GMP (most probably mediated by prostaglandins) leads to DNA synthesis and cell proliferation. Conversely, whilst raised levels of cyclic AMP lead to increased synthesis of mucus and secretory activity, raised levels of prostaglandins lead to increased synthesis but will inhibit secretion. The consequence of these effects on the balance of synthesis and discharge will be an overall build-up of stainable intra-cellular mucus and thus more secretory cells counted.

III. The effect of nicotine, tobacco smoke, anti-inflammatory and mucolytic drugs on prostaglandin metabolism

Nicotine has recently been shown to stimulate the production of prostaglandins in vitro in the cardiovascular system (Wennmalm, 1977, 1978 and 1982) and in rat lung (Berry et al., 1979). Consequently, if administration of nicotine in vivo (as in the present studies) also stimulates the production of prostaglandins, then effects on cell proliferation and secretory activity might be expected (see above). For example, nicotine-induced prosta-glandin production may cause an alteration in the intracellular ratio of cyclic AMP and GMP which may lead to cell proliferation (i.e. a 'real' increase in number). Conversely, prostaglandin production may cause a build-up of stainable intracellular mucin by increasing mucus synthesis whilst inhibiting secretion (i.e. an 'apparent' increase in number). These considerations hold true for the 'high' dose of nicotine (which increased secretory cell number), but not for the 'low' dose (which decreased secretory cell number). However, if the effect of nicotine on prostaglandin production is dose dependent, then the reduction in number of

mucus-secreting cells with the 'low' dose may be partially explained. The 'low' dose may not have caused sufficient prostaglandin production to initiate cell proliferation or the retention of mucus. Instead, cyclic AMP may have been able to initiate mucus secretion in the absence of the inhibitory effects of the prostaglandins and so lead to a loss of intracellular mucin. In fact, adrenergic agonists have been shown *in vitro* to be able to increase secretory rate whilst having no effect upon mucus-synthesis (Coles, 1977). Nicotine might therefore have a similar effect and merits further study in regard to mucus-synthesis and discharge. Whole cigarette tobacco smoke has been shown to inhibit the breakdown and inactivation of prostaglandins, particularly PGE_2, in the lung (Bakhle, et al., 1979; Mannisto et al., 1981). Conversely, nicotine will stimulate the production of prostaglandins (see above). Consequently, exposure of the lung to tobacco smoke might be expected to cause a rise in tissue levels of prostaglandins. Thus, the tobacco smoke-induced increase in number of secretory cells could be due to prostaglandin-mediated effects on secretory activity ('apparent' increase) and cell proliferation ('real' increase). Autoradiographic, electron microscopic and semi-thin studies on cell kinetics favour cell proliferation (see above).

Steroidal and non-steroidal anti-inflammatory drugs will inhibit cellular proliferation in many tissues including lung (see above). Whether the effect is direct or mediated by inhibition of substances such as the prostaglandins is equivocal. In theory, the latter appears to be convincing and is supported by one study (Trevsiani et al., 1980). *In vivo* and *in vitro* experiments run in parallel on rapidly proliferating 'Yoshida' hepatoma cells

have shown the cells to have high concentrations of PGE_2. Administration of indomethacin reduces the concentration of PGE_2 in the cells and also reduces the tumour mass, presumably by inhibition of cell division with subsequent cell loss not being replenished. In the present studies, theoretical TS-stimulated increases in prostaglandin concentrations may have been inhibited by the anti-inflammatory drugs used. Subsequent prostaglandin-mediated, TS-stimulation of cell proliferation may therefore have been blocked. Whether anti-inflammatory drugs inhibit mucus synthesis by inhibiting prostaglandin biosynthesis is also equivocal. In one study (Rainsford, 1980) aspirin was found to inhibit mucus synthesis directly.

N-acetylcysteine has not been studied in regard to its effects on prostaglandin biosynthesis. The inhibitory effect of NAC on TS-induced secretory cell hyperplasia may however be due to other actions. For example, it has been suggested that compounds such as NAC which are sulphydryl donors (i.e. contain soluble sulphydryl groups) protect against the damaging effects of oxydising agents in the liver and heart (Mitchell et al., 1973; Olson et al., 1980, 1981). Intracellular glutathione is thought to protect tissues against injury by electrophilic attack (Flohe and Gunzler, 1976). Depletion of glutathione with diethyl maleate (DEM) potentiates acetaminophen-induced necrosis and adriamycin toxicity in the liver and heart (see above): N-acetylcysteine, cysteine and cysteamine reduce the damage. It has been suggested (Dr. V. Ferrari, personal communication) that TS-induced depletion of glutathione in the lung (leading to lung damage by raised levels of free radicals) is prevented by N-acetylcysteine. However, when NAC (same dose as given herein) and DEM (300 mg, twice daily by ip injection) were

given alone and in combination to rats exposed to TS, both drugs inhibited TS-induced airways secretory cell hyperplasia and epithelial thickening (Rogers and Jeffery, in preparation). In some airways, NAC and DEM in combination were more inhibitory than either drug alone. No airway damage (e.g. squamous metaplasia) was seen with DEM although the drug had detrimental effects on the weight gain by, and general condition of, the rats. It was concluded that the inhibition afforded by DEM was likely to be due to factors such as generalised retardation of protein synthesis leading to reduced mucus-synthesis (i.e. 'apparent' inhibition). Whether NAC prevents glutathione depletion (and therefore damage) in the lung requires further study. The inhibitory effect of NAC on TS-induced airways changes may therefore be by other mechanisms. For example, it has been suggested (Sprince et al., 1979) that sulphur-containing compounds, including L-cysteine, protect against the toxic effects of some of the constituents of tobacco smoke in two ways: by direct detoxification of aldehyde toxicants or indirectly by returning toxicant-induced synthesis or release of catecholamines to normal. It is clear therefore, that the mechanism(s) of the inhibitory effect of NAC on TS-induced airways changes requires more research!.

IV. Other factors

The list of other possible factors which might cause changes in secretory cell number is extensive and includes 5-hydroxytryptamine,angiotensin II, bradykinin and other peptides. For example, bombesin and vasoactive intestinal peptide (VIP) have been implicated in secretory activity (Bertaccini et al., 1973; Coles et al., 1981) and Substance-P may be linked with the

effects of cigarette tobacco smoke (Lundberg and Saria, 1983). However, the metabolism and actions of many of these substances are only now being documented and it is beyond the scope of this thesis to consider them at present

E. FUTURE DIRECTIONS

The results and discussion of the studies presented herein indicate a number of directions for future research; either in the form of new work or to clarify existing controversy.

1. Possibly the most controversial aspect of any light microscopic (LM) study of the effect of drugs, tobacco smoke (TS) or other agents on secretory cell number is whether the observed changes in number are 'real' or 'apparent'. Future LM studies on the effect of TS or drugs on secretory cell number should be supported by semi-thin or electron-microscopic (EM) studies. For example, the right main bronchus and lung are available in studies similar to those presented herein for ultrastructural study to distinguish between cell proliferation and effects on mucus-synthesis or secretion.
2. The results of the present study indicate a direct relationship between the degree of TS-induced secretory cell hyperplasia and its inhibition by drugs (particularly indomethacin). Adjusting the exhaust rate from the exposure cabinets and using cigarettes of differing tar ratings may allow for the degree of hyperplasia to be controlled. The relationship between hyperplasia and its inhibition could therefore be studied more accurately.
3. Flurbiprofen and indomethacin merit further study, in regard to inhibition of TS-induced secretory cell hyperplasia, when given orally. Although the gavage technique delivers a

controlled dose of drug, it exacerbates the effect of TS, particularly in the trachea. Administration of the drug in drinking water avoids this problem, albeit that the dose is not controlled. A relatively high dose of drug would have to be given to compensate for reduced potency caused by the oral route of administration.

4. The results of the present studies and those of other workers indicate that a number of drugs (particularly N-acetylcysteine, flurbiprofen and indomethacin) may be of therapeutic value in the treatment of hypersecretory airway disease. In particular, bronchitic patients who were prepared to stop smoking might benefit from drug-treatment: recovery of airway changes may be speeded. A carefully controlled clinical trial would indicate whether drug-treatment of such patients would be beneficial. The trial should be double-blind, randomized, placebo-controlled and the results analysed statistically. Small airways function should be measured. Patients should be carefully selected and those who might be compromised by the drug-treatment should be excluded.

5. The present study and those of other workers have shown that exposure of rats to TS produces many of the histological changes in the airways similar to those found in bronchitic patients. However, it would be of interest to determine whether the histological changes (i.e. secretory cell hyperplasia) are associated with mucus-hypersecretion. Mucus-synthesis and secretion could therefore be measured in 'normal' rats and compared to that in rats made 'bronchitic' by exposure to TS. Mucus-synthesis could be measured by the use of radiolabelled precursors of mucus in <u>in vitro</u>

organ culture or by the administration of precursors _in vivo_ (although the latter method is expensive). Mucus-secretion might be measured _in situ_ using biochemical assays for specific mucus-markers (such as fucose) to determine the amount of mucus produced directly. The effects of drug treatment on mucus-synthesis and secretion could then be studied. The drugs or other agents used might include those used in the present study as well as adrenergic and cholinergic agonists, products of arachidonic acid metabolism (e.g. prosta-glandins and leukotrines) and biologically active peptides (e.g. VIP). The drugs could be given chronically during the TS-exposure period or acutely during the _in vitro_ or _in situ_ portions of the studies. The results of the histological (LM, semi-thin and EM) and physiological studies could then be compared to determine more accurately the contribution of each component to the pathogenesis of 'bronchitis'.

6. The studies outlined in 5. above could be used to study the:
   i. pathogenesis and progression of 'bronchitis' by killing animals, for histological, and using others for physiological study, at various times during TS-exposure.
   ii. reversibility of 'bronchitis' after cessation of TS-exposure.

7. Nerves are associated closely with surface epithelial secretory cells. However, in mammals, these cells appear to be unresponsive to nervous stimulation as regards mucus-secretion. In the small airways, the secretory cells in the epithelium are thought to produce the mucus which is of prime importance in the functional impairment of hypersecretory disease. How,

then, are these cells stimulated to secrete ?. It would therefore be of interest to determine whether innervation is changed in disease: using normal and 'bronchitic' rats. For example, in disease, nerves may have a lowered threshold to stimulation and the number of innervated secretory cells or the numbers of receptors on secretory cells may be changed. Electrical stimulation of nerves or the use of neuromimetic agents might alter nerve or receptor number.

8. Some of the products of arachidonic acid metabolism may be involved in the pathogenesis of 'bronchitis' and its inhibition by anti-inflammatory drugs. Preliminary in vitro studies in 'normal' rat and guinea-pig trachea have indicated that prostaglandins (PGs) may be released in response to acute administration of TS (author's unpublished observations). The tracheal preparation has been adapted by the author and may now be used to study the release of PG's (as well as leukotrienes, HETE's or peptides) from the trachea and lungs of normal and 'bronchitic' animals. The chronic and acute effect of drugs on mediator release could then be examined.

9. Intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) may be involved in the pathogenesis of 'bronchitis' and its inhibition by anti-inflammatory drugs. Preliminary in vitro studies in rat trachea have shown that cAMP concentrations can be measured (author's unpublished observations). Epithelial 'scrapings' from normal and bronchitic bronchial tissue maintained in organ bath may be assayed biochemically for changes in the concentrations of intracellular cyclic nucleotides to elucidate whether they are involved in hypersecretory disease. The effect of drugs, which may increase

or deplete the intracellular concentrations of cyclic nucleotides, could then be investigated. For example, theophylline inhibits phosphodiesterase and so increases the intracellular concentration of cAMP which may increase mucus secretion or synthesis and lead to cell proliferation.

F. GENERAL SUMMARY

1. The specific pathogen-free rat exposed to whole cigarette tobacco smoke (TS) appears to be a good model of histologically 'bronchitic' changes and provides a system for testing the efficacy of drugs: both their anti-bronchitic action and capacity to speed recovery. When the system is used for testing drugs it works best when there is significant TS-induced secretory cell hyperplasia throughout the airways. The use of 'high tar' cigarettes is recommended.

2. Five anti-inflammatory drugs were 'anti-bronchitic' when given parenterally but not when given by gavage. The mucolytic agent, N-acetylcysteine (NAC) was effective when given at a high dose in drinking water.

3. Indomethacin, flurbiprofen and NAC showed marked anti-bronchitic action with few side effects. Dexamethasone was anti-bronchitic but toxic. Prednisolone and hydrocortisone were the least effective but had few side effects.

4. In the intrapulmonary airways, both indomethacin and flurbiprofen more than halved the time taken for recovery after cessation of TS. They were ineffective in the trachea.

5. The experimental results indicate a possible clinical use for some of the drugs in the treatment of hypersecretory disease.

6. Nicotine, when inhaled in an aerosol at a 'dose' which produced plasma nicotine levels similar to human cigarette smokers and subjects who had chewed nicotine-containing gum, did not affect secretory cell number. When given by injection at two much higher,ascending doses, nicotine either reduced

or increased secretory cell number respectively.

7. It is suggested that the changes in secretory cell number with TS alone and in combination with the mucolytic and anti-inflammatory drugs are due more to effects on cell proliferation (i.e. 'real' changes) than to effects on mucus synthesis and secretion (i.e. 'apparent' changes). It is also suggested that the changes in secretory cell number with 'high' doses of nicotine are due to the drug's 'bi-phasic' action on ganglia leading to opposing effects on mucus secretion (i.e. 'apparent' changes).

8. The changes in secretory cell number seen after some of the treatments may be mediated by prostaglandins (or other products of arachidonic acid metabolism) and cyclic nucleotides.

APPENDICES

Appendix 1. Addresses of suppliers (alphabetical listing)

Air Control Installations; supplied by Research Engineers Ltd., Orsman Road, London, N1 5RD.

Analysis Automation Ltd., Southfield House, Eynsham, Oxon., OX8 1JD.

Beecham Research Laboratories, Beecham House, Great West Road, Brentford, Middx. TW8 9BD.

The Boots Co. PLC, 1, Thane Road West, Nottingham, Notts., NG2 3AA.

BDH (British Drug Houses) Chemicals Ltd., Freshwater Road, Dagenham, Essex, RM8 1RZ.

British Oxygen Co. Ltd., Special Gases, 24, Deer Park Road, London, SW19 3UF.

Camlab Ltd., Trinity Hall Farm Industrial Estate, Nuffield Road, Cambs., CB4 1BR.

Carl Zeiss Jena Ltd., P.O. Box 43, 2, Elstree Way, Borehamwood, Herts., WD6 1NH.

Charles Austen Pumps Ltd., 100, Royston Road, Byfleet, Weybridge, Surrey, KT14 7PB.

Charles River U.K. Ltd., Manston Road, Margate, Kent, CT9 4LT.

Dixon and Son (Ware) Ltd., Crane Mead Mills, Ware, Herts., SG12 9PZ.

Energetics Science, Inc.; U.K. distributors - Analysis Automation Ltd.

Flow Bits Ltd., Wella Road, Basingstoke, Hants., RG22 4AQ.

Forth Tech Services Ltd., Mayfield, Dalkeith, Midlothian, Scotland, EH22 4AQ.

Gallenkamp and Co. Ltd., P.O. Box 290, Technico House, Christopher Street, London, EC2 2ER.

Hewlett-Packard, The Quadrangle, 106-118, Station Road, Redhill, Surrey, RH1 1PS.

Heygate and Sons Ltd., Bugbrooke Mills, Northampton, Northants., NN7 3QH.

Instrumentation Laboratory; U.K. distributors - Analysis Automation Ltd.

Lavenread Computers Ltd., 90, Portland Road, London, SE25 4PJ.

Machinery and Chemicals Ltd., 137, Hanworth Road, Hounslow, Middx. TW3 3TP.

May and Baker Ltd., Rainham Road South, Dagenham, Essex, RM10 7XS.

Merck, Sharp and Dohme Ltd., Hertford Road, Hoddesdon, Herts., EN11 9BU.

National Collection of Type Cultures, Colindale Avenue, London, NW9.

Olac, Shaws Farm, Bicester, Oxon., OX6 0TP.

Organon Laboratories Ltd., Cambridge Science Park, Milton Road, Cambs., CB4 4BH.

Raymond A. Lamb, 6, Sunbeam Road, London, NW10 6JL.

Reichert-Jung U.K. Ltd., 820, Yeovil Road, Slough, Berks., SL1 4JB.

Serendipity Systems, Inc.; U.K. distributors - Great Northern Computer Services Ltd., 116, Low Lane, Horsforth, Leeds, Yorks., LS18 5PX.

Zambon S.p.A. (Milan), Corporate Medical Dept., Via L. Del Duca, 10, 20091 Bresso (MI), Italy.

Appendix 2. Determination of Mycoplasma pulmonis

Plates containing 5 ml supplemented mycoplasma agar medium are innoculated with homogenised lung tissue and incubated in an atmosphere of 90% hydrogen and 10% carbon dioxide (BBL Gas Pack System) (British Oxygen Co. Ltd.) in an anaerobic jar at 37°c.

The suitability of the agar for supporting mycoplasma growth was determined by innoculating test plates with either a maintained commensal stock culture or a commercially available strain (National Collection of Type Cultures): the agar was found to support growth of the test cultures.

Plates were examined at x50 magnification, weekly for two weeks, to observe growth.

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