CELL TURNOVER OF THE BRONCHIAL AND ALVEOLAR LINING IN THE RAT LUNG IN VARIOUS TYPES OF HYPERTROPHY

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ABSTRACT

The turnover rate of the epithelial lining of the airways and alveoli of the normal rat lung has been studied using both colchicine, a drug that arrests mitosis in metaphase and tritiated thymidine that is incorporated into DNA during synthesis.

Three age groups of rat were studied, each at five airway levels from trachea to lateral bronchi. The turnover time was faster in central than peripheral airways and in young than in old animals. Only in the young animals, a difference between male and female, was detected, turnover time in the male being faster. No difference was seen between the colchicine and the thymidine.

Isoprenaline and pilocarpine each produced an increase in mitotic activity and an increase in goblet cell number.

Finally, the effect of smoke from tobacco with and without an anti-inflammatory agent (PMO) was determined. Tobacco alone increased mitotic activity, goblet cell number, and epithelium thickness. After tobacco + PMO, some protection was offered against the increase in both mitotic number and epithelium thickness.

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SECTION A: LUNG STRUCTURE

The dynamics of growth and regeneration of lung can only be fully understood after study of its constituent tissues.

During the past two centuries, anatomists have given progressively more detailed descriptions of the lung structures but it is only during the last two decades that its cell turnover has been investigated more deeply.

I - The earliest studies on the structure of the lung

For over two hundred years, the structure of the respiratory region of the lung has been the subject of vehement discussion. In fact, it is only 15 years ago that the exact composition of the lining epithelium of the airways and of the alveolar wall became finally established by electron microscopic investigation.

It was in the middle of the sixteenth century that the physiological role of the lung was first appreciated. In 1533 Miguel Servetus declared in his Christianismi Restitutio: "The vital spirit has its origin in the left ventricle of the heart, and the lungs assist greatly in its generation. It is a rarefied spirit, elaborated by the force of heat...".

The first detailed investigations into the structure of the lung were those by Marcello Malpighi in 1686. He described the capillary network around the vesicles of the frog lung and also announced the discovery that the trachea terminated in dilated vesicles. While

some descriptions of the bronchial tree were given by other anatomists, it was only a century and a half later that more attention was paid to the gross pattern of the bronchial tree.

II - Structure of the normal airway

In the late 19th century Schulze (1871) and Verson (1872) gave one of the first histological descriptions of the tracheal mucosa. They found that the inner surface of the trachea was lined by a mucous membrane, having a thickness of 0.13 to 0.15 mm and was characterized by a plexus of longitudinally arranged elastic fibres. The mucosa was covered by a ciliated columnar epithelium resting upon a basement membrane. Schulze also gave a description of the cells constituting the tracheal mucosa. He described and measured the height of the ciliated cell and gave one of the first descriptions of the goblet cell that he called a "cup, goblet, or chalice cell". He also noticed that in small bronchi, near the point of transition of the bronchi into the alveolar passage, the cells were flatter, free of cilia and that the "cup cells" were no longer present. Early in the 20th century the histological structure of the tracheal epithelium was well-established. Two authors, Maximow and Bloom, in the first edition of their textbook of Histology in 1930, summarized the histological structure of the human tracheal epithelium:

> "It is ciliated pseudo-stratified columnar in type, rests on a distinct basement membrane and is present in most of the respiratory tract. Numerous goblet cells are

scattered through it. The lamina propria contains many elastic fibers and numerous small glands, like those of the larynx. These glands, most of which are external to the elastic fibers, open by a short duct onto the free surface of the epithelium. In the bronchial tubes the epithelium consists of columnar ciliated cells with scattered goblet cells. In the more distal airways, the goblet cells are no longer present and the ciliated columnar epithelium gives way to a non-ciliated cuboidal or flattened epithelium. "

In 1937 Miller gave a complete description of the macroscopic and microscopic anatomy of the lung. He noticed little difference between the lining epithelium of the trachea and that of the bronchi, both containing several rows of cells in which he recognized four types:

1) basal cells
2) intermediate cells
3) ciliated cells
4) goblet cells

Later, in 1956, Rhodin and Dalham, examining the upper respiratory tract of the rat under the electron microscope, noticed that the intermediate or supporting cells, as previously described by Miller and Kolliker in human trachea and bronchi, were not found in the material they were studying. They proposed therefore, that

the tracheal epithelium of the rat should be described as a simple ciliated columnar epithelium. They mentioned that they had not seen the development of basal cells via supporting cells or intermediate cells to goblet or ciliated cells, and even concluded that the cells considered with the light microscope, to be supporting or intermediate cells, were brush or goblet cells containing a varying number of mucous granules. Thus Rhodin and Dalham denied the presence of the intermediate or supporting cell in the rat, but in a later publication, Rhodin (1966), described this cell in the human tracheal mucosa. Moreover these authors declared that the only cell type that did not reach the surface of the epithelium was the basal cell. The basal cells which were relatively few in the animal material examined, were considered to represent - or at least some of them - white blood cells or lymphocytes on their way through the epithelium from the blood vessels to the tracheal surface.

Rhodin and Dalham also described in normal tracheal epithelium of rat, a brush cell that might represent a young ciliated cell. However, they found no intermediate stages between the brush and ciliated cell and therefore they considered this brush cell to be a cell type per se.

A few years later, Nagaishi studying mice and rat lungs with the electron microscope, did not find brush cells.

Besides describing these brush cells, Rhodin and Dalham

noticed the presence of a great number of non-ciliated cells that represented for them goblet cells in different stages of intracellular synthesis of mucous. In their studies they found no evidence of transformation of one type of cell into another.

So by the beginning of the last decade, the histological structure of the tracheo-bronchial epithelium of the rat was known to be made of:

1) A basement membrane principally consisting of elastin, collagen and reticulin.
2) Basal cells near the basement membrane, some of which might be white blood cells or lymphocytes.
3) Goblet cells containing varying numbers of mucous granules and therefore often mistaken for the intermediate or supporting cells (as in the human bronchial epithelium).
4) Ciliated cells, the most numerous - having a high columnar appearance in the upper trachea, a more flattened and cuboidal look in the lower bronchial tree.
5) A controversial brush cell described by Rhodin and Dalham as being a cell type per se and not an intermediate from between the basal cells and the fully developed adult ciliated cell.

In 1955 Karrer gave a complete description of the electron microscopic aspect of the bronchiolar epithelium of the normal mouse lung, as did Nagaishi and Okada in 1960. Numerous cilia

and microvilli on the surface of the ciliated cells were measured and fully described, together with the nature of the vacuole-like structures of the goblet cells (Fig I-1). In 1958, Reid gave more details about goblet cells of the human tracheal epithelium, and mentioned that these cells could be in a resting phase showing only mucinogen granules at their base, or be distended with mucous.

In 1966, Rhodin mentioned that in regeneration of the human tracheal epithelium it appeared obvious that new cells arose by division of basal cells. More recently, variations in the epithelium structure at each level of the upper respiratory tract have been described in detail. In the 1968 Emphysema Conference in Aspen (USA), Sorokin mentioned that in larger bronchi, the epithelium is composed mainly of ciliated cells, followed in number by goblet cells, immature non-ciliated cells and an occasional argentaffin cell. He also noticed that the goblet cells and other elements extend down the airway as far as the terminal bronchiolus. At the level of the terminal airway he mentioned that the columnar epithelium was composed of two types of cells:

1) Ciliated cells in greatest number
2) A non-ciliated cell that showed many ultra-structural similarities to those of the so-called CLARA cell.

This non-ciliated, secreting cell described earlier by Kolliker in 1881, Clara in 1937, and more recently by Policard and others, was, and is still, a subject of controversy in the medical literature.

Figure I-1: Diagramatic representation of the ultrastructure of the tracheobronchial epithelium of the rat.

C = Cilia

MV = Microvilli

B = Basal bodies

M = Mitochondria

N = Nucleus

ER = Endoplasmic reticulum

G = Golgi Complex

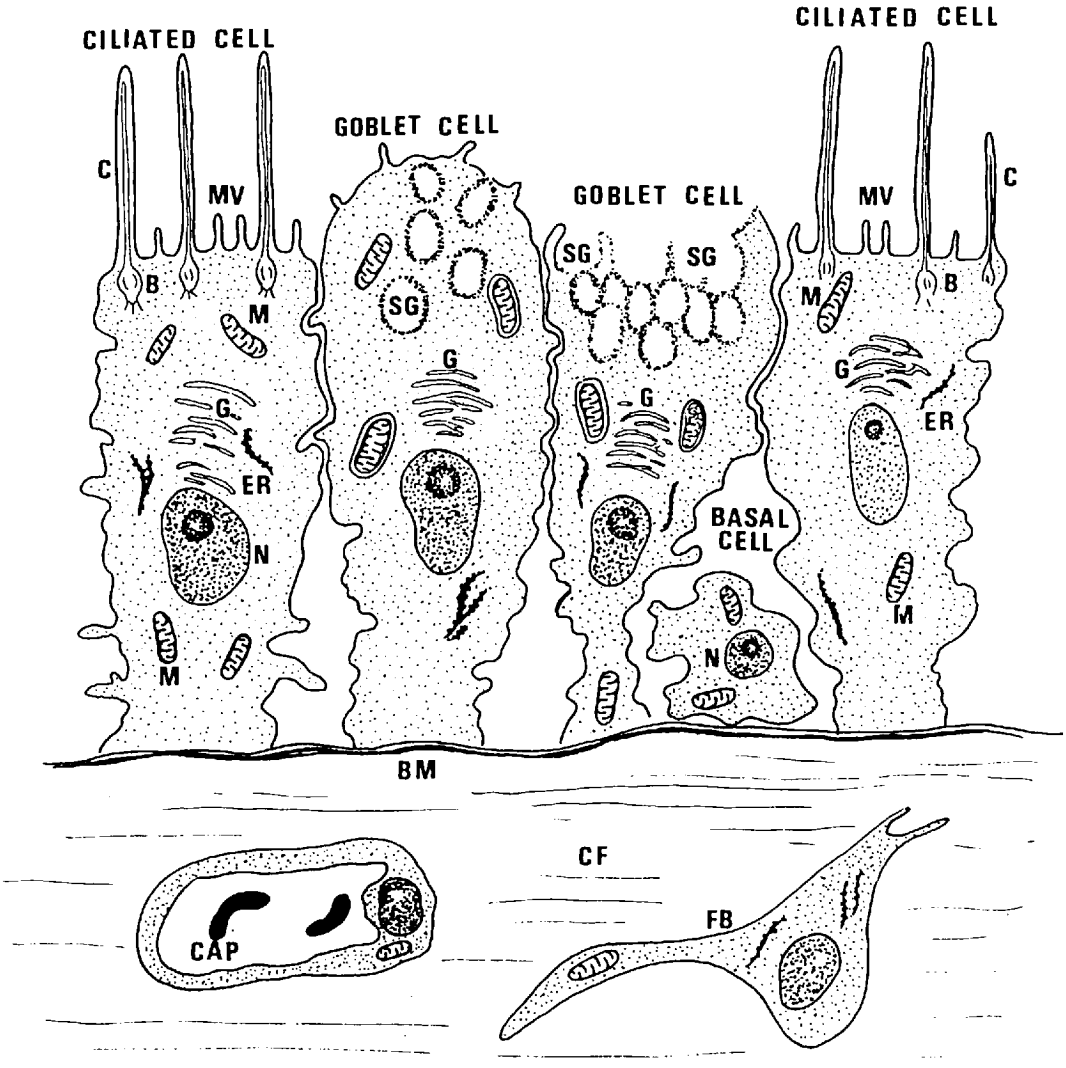
SG = Secretory granules

BM = Basement membranes

CF = Collagen fibres

CAP = Capillary

FB = Fibroblast


CILIATED CELL
GOBLET CELL
GOBLET CELL
CILIATED CELL
C
MV
B
M
G
ER
N
SG
BASAL CELL
BM
CF
CAP
FB

III - Structure of the alveolar wall and its constituents

If a review of the literature reveals few complete histological descriptions of the extra and intrapulmonary airways, it is not so for the structure of the alveolar or respiratory tissue of the lung. After the first description by Malpighi in 1686 of the so-called "air vesicles", many anatomists, Rossignol, Kolliker and Schulze, among them, described this "structure intime" of the lung. After mercury or silver nitrate injection into the lung via the trachea, they described these air vesicles as being dilated and blind air sacs amidst a network of blood capillaries.

Following these descriptions, many discussions followed about the possible intercommunication between these vesicles. Rossignol, in his thesis on "Recherches sur la structure intime du poumon" in 1846, gave a description of the pulmonary lobules after injecting the pulmonary artery of a cat's lung. He was one of the first anatomists to introduce the notion of airway branching by dichotomy or trichotomy He also distinguished parietal from terminal alveoli.

Then in the late 19th century, Schulze (1871) and Kolliker (1881), described more thoroughly the structure of the alveolar wall. To them, the fundamental layer of the alveolar wall was composed of a transparent structureless membrane that only in the thicker parts, exhibits a distinctly fibrous character. This membranous wall, which was entirely devoid of muscular tissue was lined by continuous epithelium, consisting of small nucleated cells and large non-nucleated

plates. For these anatomists, this alveolar structure was the respiratory epithelium. The respiratory capillaries, whose importance is so great for the performance of the lungs, are partly connected to the alveolar wall.

For many years after, the existence and nature of the respiratory epithelium was much debated. Whereas, some authors (Kolliker and Colberg), claimed it to be a continuous pavement or squamous epithelium, formed by one type of cell, others declared the epithelium to be partly interrupted or even to consist of isolated cells.

At the beginning of the 20th century, controversy concerned itself with the nature of the epithelial cells. In 1935 Josselyn, using the silver nitrate impregnation method declared that:

1) The occasional nuclei seen in alveolar walls in addition to those of the capillary endothelium, belong to histiocytes and possibly other connective tissue cells.
2) The phagocytic cells which occur in such profusion in the alveoli in case of need, are histiocytes from extravenous origin.
3) The alveolar wall, in addition to the capillary vessels and cells, consists of a membrane composed of reticular and elastic fibres, and a homogenous transparent ground substance.

In 1939, Ross in an attempt to find the origin and the nature of the alveolar epithelium cell, stimulated their production by injecting a bacterial suspension into the rabbit trachea. This is the first time in this review of the literature that an experimental method is used as is the case in the present study.

The first reaction observed by Ross after the injection of a bacterial suspension is a moderate exudation of poly-morphonuclear cells. This was rapidly followed by an outpouring of a large number of macrophages, which ingested the poly-morphonuclear cells. Then within the first 32 hours, degenerative changes became evident and mild epithelial desquamation was manifest. During the second day, the epithelial damage became more evident and proliferation of macrophages increased. By the third day the reaction began to resolve followed by a maximal alveolar epithelial proliferation at day four. After eight days, the only evidence of the damage sustained by the tissue was a moderate thickening of alveolar walls because of proliferation of fibroblasts.

From this cytodynamic study of alveolar epithelial cells, Ross concluded that the epithelial response to a stimulus like the one described, went through a cycle lasting about eight days, with a peak of epithelial proliferation at four days.

By 1940 therefore, the general consensus of opinion was that the alveolus was a polyhedron containing a network of capillary blood vessels, and a ground substance containing elastin and reticulin fibres.

Two types of cell contribute to the lining of the alveolar wall:

1) A large, exceedingly thin, flat plate devoid of a nucleus.

2) A small, nucleated cell, located in the mesh of the capillaries and considered to be either epithelial in origin or mesenchymal.

In 1937 Miller, in his book, entitled "The Lung", described a technique in which, by pushing the epithelium from the alveolar wall by the accumulation of an exudate behind it (as in a case of pneumonia), he noticed that in the normal lung the epithelium lining the alveolar wall was continuous and made up of thin, flattened, nucleated squames, which were closely applied to the alveolar wall.

Recently, however, modern histochemical methods and electron microscopy have supplied precise evidence in favour of the existence of a simple squamous epithelium lining the surface of the alveolar wall.

In 1952, Low published electron photomicrographs of alveolar tissue which indicated that the alveolar walls were covered by a continuous squamous epithelium composed of long flat cells with exceedingly attenuated cytoplasm. Bertalanffy and Leblond in 1955 concluded that the alveolar walls were lined by two juxtaposed structures - a squamous epithelium and its basement membrane. They also mentioned that the cell population of the alveolar wall consists of:

1) A simple squamous epithelium supported by a basement

membrane and covering the capillaries. This squamous epithelium was responsible for 56% of the total cell population of the alveolar wall.

2) Leucocytes, accounting for 13% of the alveolar wall cell population.

3) Alveolar cells ("epicytes", "alveolar eipthelial cells", "septal cells", "alveolar phagocytes", "macrophages", "dust cells"). These alveolar cells were of two types:

    A) Vacuolated alveolar cells characterized by a variable number of vacuoles filled with lipoid material.

    B) Non-vacuolated.

These two types of cells had in common two important properties:

1) The ability to ingest foreign particles which explains the frequent presence of particulate matter in their cytoplasm.

2) The ability to progress by amoeboid movement which explains the desquamation of these cells into the lumen of the alveoli.

Concerning the origin of these cells, Bertalanffy and Leblond believed that they originated from the reticulo-endothelial system because of the ability of these cells to phagocytose and to show amoeboid movement. Later, in an attempt to clarify a complex misunderstanding about the nomenclature of the alveolar wall cells, Bertalanffy mentioned again the presence of an "alveolar cell" capable of amoeboid motion and phagocytic activity, and co-existing with the squamous epithelial

cells previously described by Low.

In 1960, Policard compared the cellular components of the bronchiolar and alveolar regions and mentioned that there was a zone of transition between the two areas. He also described the alveolar cells.

Following the same idea, Brooks in 1966 also gave a list and concluded:

"It is possible to list the following cell types encountered in normal pulmonary alveolar tissues:

1) Type A alveolar epithelial cell corresponding to the pulmonary epithelium mentioned by Bertalanffy and Low.
2) Type B alveolar epithelial cell which, contrary to the description of Bertalanffy is not a phagocytic pleomorphic type of cell but a cell having a definite and relatively invariant morphology.
3) Alveolar macrophages.
4) Capillary endothelial cell and blood elements within the capillary.
5) Connective tissue cell (fibroblast).
6) Septal macrophage or histiocyte (rare in normal lung).
7) Leucocyte in connective tissue space (rare in normal lung)."

This "mise au point" summarized the work of previous authors - Low in 1953, Swigart in 1954, Driessens in 1959, Nagaishi in 1960. Moreover in 1970,Meyrick and Reid, gave a complete historical

and anatomical description of the alveolar wall constituents and mentioned the various terms applied to the epithelial alveolar lining cells. In Table I-1 is summarized their findings. These two authors also described the ultrastructure of these cells and calculated that according to nuclear counts, the type I pneumocyte and type II pneumocyte were present in a proportion of 40 to 60. Furthermore, in a previous communication, Meyrick and Reid (1968), described, in the rat, the presence of an alveolar brush cell which they called a "third pneumocyte". This brush cell differed remarkably from the other alveolar cells in its ultrastructure. Moreover, according to Meyrick and Reid, this cell type was definitely less frequent; only 13 cells out of a total population of 135 alveolar cells were considered to be brush cells.

In 1970 O'Hare, after giving a complete historical review, concluded that the endoderm is the source of the type I and type II pulmonary cells, and mesoderm, the origin of the interstitial pulmonary cells of the rat lung.

Table I-1

Terms Applied to the Epithelial Alveolar Lining Cells*

Low & Daniels	(1952)	Pulmonary epithelium	
Macklin	(1953)	Pneumonocytes	
Clemens	(1954)	Alveolar epithelial cells	

Policard was probably the first to recognize two types of cells.

Policard et al.	(1954)	Septal	Alveolar
Yasudo	(1958)	Type A	Type B
Policard et al.	(1959)	Small alveolar	Large alveolar
Campiche	(1960)	Type I	Type II
Bertalanffy	(1965)	Pulmonary surface	Alveolar
Meyrick & Reid	(1968)	Type I pneumocyte	Type II pneumocyte

*Meyrick, B., Reid, L. Brit. J. Chest (1970), 64:122.

SECTION B: THE PROCESS OF CELL RENEWAL

I - General considerations

Cell division is a major function of the living organism from the time of fertilization of the ovum. Growth, differentiation, replacement, and repair all depend upon it.

Both plant and animal cells are renewed in much the same fashion: by mitosis or karyokinesis. Rusconi (1826) was probably the first to describe segmentation of the ova of frogs and salamanders, thus laying the foundation for the study of cell proliferation. Shortly afterwards, in 1844, Kolliker from his investigations into the development of the cephalopods, concluded that even in the embryo, all cells are derived by repeated division from the segmentation spheres. In 1845, he assigned to the cell such functions as growth, sensibility, contractility, ingestion, and digestion with excretion and metabolism, an impressively comprehensive statement of cell individuality. Henle in 1841 claimed that cells could multiply in three ways:

1) By budding, as in certain lower plants.
2) By endogenous development, the cell contents of the mother cell becoming the cytoblastema of the daughter cells.
3) By division or segmentation, which is not found in animals except in yolk cells.

In 1857, Rudolf Virchow maintained that following what might be called "formative irritation" there is enlargement of the nucleolus which elongates to a rod and becomes indented in its middle portion

so as to take on a disc shape. Later the indentation increases and the nucleolus divides into two. Soon afterwards, the nucleus elongates, is constricted like a figure of eight into two parts while, in its turn, the protoplasmic body constricts and divides into two equal parts, each containing a nucleus.

So by the 1850's the "humoral theory" long defended by Schleiden and Schwann, in which living things had a free, humoral formation, was finally refuted. For many years and many decades thereafter, studies flourished giving, in the medical literature, greater precision to our knowledge of the mitotic cycle. However, as it is not the aim of this study to comment on the details of these interesting discussions, a brief discription of the mitotic cycle follows.

A - Description and explanation of each phase of cellular mitosis

Today, the fundamentals of cell division are well-documented in many Histology and Pathology textbooks (Cameron, 1952; Ham and Leeson, 1956), and also in more recent publications (Mazia, 1961; Becker, 1969). Various stages are well-recognized in the complicated mechanism of mitosis. Basically, the reproductive cycle of the cell consists of the doubling of all components of the cell, followed by a division that distributes the components to the daughter cells. During prophase, which initiates the process, the nuclear network or reticulum which is made up chiefly of thread-like chromosomal elements, becomes thicker. Then these chromosomes appear shorter and split in their longitudinal axis to give two parallel and identical structures.

The replication of these chromosomal elements, which carry the genetic code is the fundamental part of the process because it is responsible for the conservation of the character and potentialities of each cell. At the end of the prophase, the nucleolus is no longer visible and the nuclear membrane usually vanishes. The centrosome, if not already double, divides and the daughter centrosomes move apart to opposite sides of the nuclear region. Each of the centrosomes becomes surrounded by astral rays and is connected with the other by a central spindle with fine fibrils lying parallel to the longitudinal axis of the developing mitotic figure (Fig I-2A).

In metaphase, chromosomes become grouped around the equatorial plane of the spindle to which they are attached by a specially differentiated and constantly located attachment region (Fig I-2B).

In anaphase the two halves of each chromosome move apart, passing by the summit of the spindle and moving towards the poles (Fig I-2C).

Finally in telophase the chromosomes uncoil and the nuclear membranes and nucleoli of the daughter cells are formed; a new centriole is also produced (Fig I-2D). The completion of the division is accomplished by a cleavage furrow which grows inwards to form the cell periphery. Newsome in 1966 mentioned that the prophase lasted between 10 to 15 minutes, the metaphase 20 to 30 minutes and the anaphase-telophase 3 to 5 minutes. Therefore, the total mitotic

Figure 1-2: Phases of Normal Mitosis

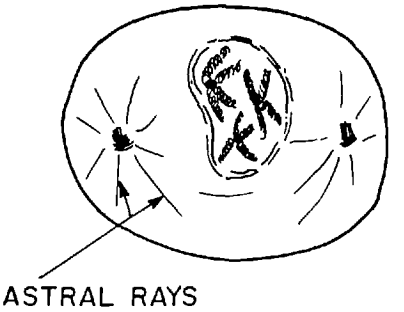
A - In prophase, the chromosomes coil, becoming highly condensed, the nuclear membrane and nucleolus break down and the centrosomes move apart to establish the poles toward which the chromosomes will move.

B - In metaphase, the chromosomes move to the cell equator.

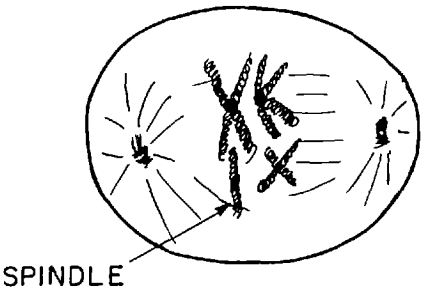
C - In anaphase, sister chromosomes move toward poles.

D - In telophase, the chromosomes uncoil and the nuclear membranes and nucleoli of the daughter cell are formed. Each centrosome has produced a new centrosome.

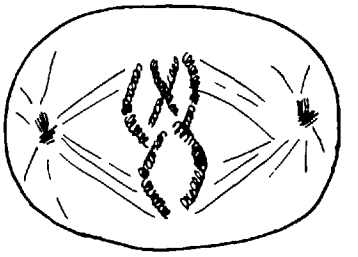
E - In interphase, the period between divisions, the chromosomes are thin, extended threads. If mitosis takes place, the chromosomes will duplicate, the centrosomes separate and the spindle form.


ASTRAL RAYS

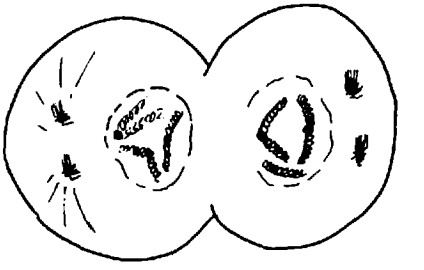
A


SPINDLE

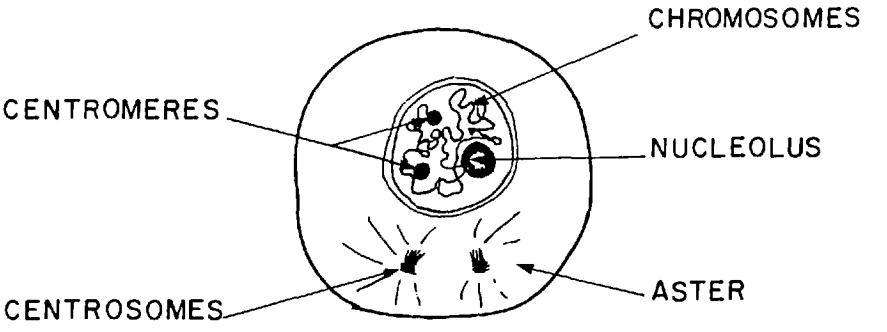
B



C



D


CHROMOSOMES
CENTROMERES
NUCLEOLUS
CENTROSOMES
ASTER

E

process according to Newsome and Mazia takes approximately one hour - with wide variations. After this mitotic process, the cells go into a period of rest called the interphase or the inter-mitotic phase (Fig 1-2E). This phase, according to Leblond and Walker (1956), may last as long as the life of the organism, or, sooner or later, it may be terminated by the onset of another mitosis.

It is important at this stage of our study, to discuss briefly the relevant literature concerning the various phases in the life cycle of cells and the renewal of cell populations, as the understanding of these basic subjects is prerequisite for our experimental studies.

B - Phases of the cell life cycle

Since the early works of Henle in 1866, many biologists attempted to elaborate precise methods for determining the various phases of the life cycle of the cells, but it was only in 1947, and more effectively in 1959, that Pelc and Doniach introduced autoradiography as a biological tool for these investigations.

By labelling with radioactive phosphorus, the precursors of DNA, they investigated the kinetics of cell division of the Vicia Faba bean. They divided the generation cycle of the cell into the following metabolic periods:

1) Postmitotic interphase or G1 phase

2) DNA synthesis or S phase

3) Premitotic interphase or G2 phase

4) Mitosis or M phase

This division of the life cycle of mammalian cells was accepted for many years, and in 1963 Patt and Quastler, studying the radiation effects on cell renewal, claimed that in typical mammalian cell systems, the S phase lasted from 4 to 10 hours depending on species, G2 phase, 1 to 2 hours and the M phase, about an hour. They also postulated that the duration of G1 varied widely, that, being responsible for the very large differences in the cell cycle times among various tissues.

More than ten years after his original communication on the metabolic phases of the life cycle, Pelc (1963), studying the problem of renewal of differentiated cells in tissues and organs of fully grown organisms, proposed a modified concept of his original hypothesis. Mitosis being very rare in muscle, brain, and in some columnar epithelium, it has to be assumed that cells can survive and remain functional for years and even decades without the advantages of cell division. Therefore, Pelc proposed that cells can be in four distinct metabolic stages:

1) Not incorporating precursors into DNA.

2) Incorporating at the full rate equivalent to doubling of the DNA content, with subsequent cell division.

3) Incorporating at the full rate, without cell division.

4) Incorporating at the low rate of approximately 2% of that

of the full rate, and not leading to mitosis.

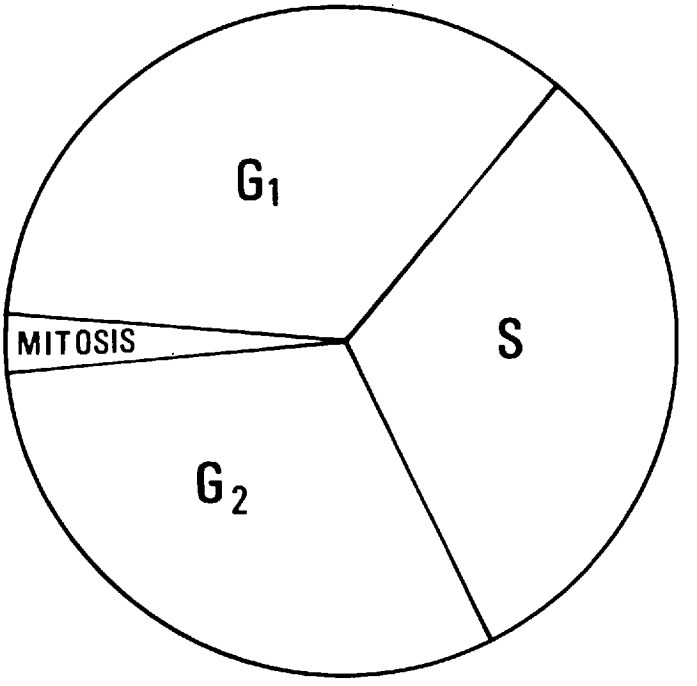
Pelc suggested that in some differentiated tissues, parts of the total amount of the DNA of a cell can be renewed without subsequent cell division.

Two years later, in 1965, Cleaver, using tritiated thymidine as a marker, and calculating the relationship between the duration of the S phase and the fraction of cells which incorporate ^{3}H-T during exponential growth, proposed a schematic representation of the various phases of the cellular cycle (Fig I-3). The results of those investigators are well-accepted by most workers (Mazia; Evensen; Leblond and Walker) and a review of the relevant literature reveals that many other research workers attempted, either to establish a mathematical model for cell division (Koch and Schaechter; Smith and Dendy) or to investigate the mechanisms involved in the initiation of cell division.

In summarizing these, Mazia in 1961 concluded as follows:

> "It is now well-known that in plant and animal cells, the actual replication of the genetic material - the doubling of DNA - takes place only between divisions. This can best be shown by experiments in which a population of cells is fed for a brief interval with some radioactive substance (thymidine) that is built into the newly formed DNA."

Fig I-3: The Phases of the Cell Cycle


G1
S
MITOSIS
G2

C - Dynamics of cell renewal

It is now necessary to consider the dynamics of cellular renewal. In the adult mammal, a large number of cells is produced and lost every day. In many tissues, birth, maturation, and death of cells are delicately balanced so as to maintain a steady state. The cell production is balanced by a cell loss and in such an instance the cells of the tissue are said to undergo "renewal". However, in other tissue, the periodic outburst of mitosis, occurring for instance in ovarian follicles, is a witness of their periodic development or "growth" (Leblond and Walker, 1956 and Patt and Quastler, 1963).

Since the early work of Henle in 1866, who mentioned the presence of mitotic figures in mammalian tissues, considerable progress has been made particularly in recent years, in our understanding of normal cell population kinetics.

Three authors (Leblond and Walker, 1956 and Bertalanffy, 1962, 1967), in studies on renewal of cell populations gave the basic definitions commonly used for the measurement of this renewal.

$$\text{I - Mitotic index of a cell population} = \frac{\text{Number of cells in mitosis}}{\text{Total number of cells present}}$$

Knowing the mitotic index, the renewal of a cellular population is calculated:

$$\text{II - Turnover time or renewal time of a cell population} = \text{Time taken for the replacement of a number of cells equal to that in the whole population}$$

The simplest approach to measuring the mitotic index is to estimate, on histological sections, the number of nuclei in dividing and in non-dividing cells. However, in tissues with a low mitotic activity, this method requires considerable work to assemble enough counts to be statistically significant. Another major disadvantage of this method, is that no measurement of time is involved, and therefore the rate at which the cells of a tissue are replaced cannot be calculated. With the discovery (Lits and Dustin, 1934) of the remarkable property of colchicine to block cell division in metaphase and with introduction in 1947 by Pelc of radioactive DNA precursor labelling agents, it became possible to estimate more precisely the turnover time of cell populations.

The detailed study on the uses, advantages and disadvantages of these two techniques is discussed in the next section (see section II-B-5). It is important to mention at this stage, other workers, who suggested different concepts and methods of evaluating the cellular renewal and growth.

While mathematical models were proposed to evaluate growth rates (Quastler,and Sherman, 1959; Riddle, 1971), some workers used spectrometry to determine the volume distribution spectrum of cells in culture (Anderson and Petersen, 1967).

Using radioactive material Watts in 1964, studied the turnover of RNA in a multiplying animal cell and Fliedner in 1968, studied the

complete labelling of all cell nuclei in newborn rats.

Another aspect of cell renewal, the transit time, was studied in human epidermis by Epstein in 1965. Using an elaborate mathematical formula for the determination of renewal time, he calculated the transit time, that is the time required for a basal cell to reach the prickly and the granular layer of the epidermis.

In 1959, Leblond, Messier and Kopriwa, and in 1960, Messier and Leblond, and later in 1968, Post and Hoffman, studied different cell renewal patterns and claimed that the adult mammalian cell populations may be divided into three groups according to their renewal pattern.

1) Continuous replicators: the cells of these tissues divide throughout the life of the host (e. g. the gastrointestinal tract, the hemato-poietic system).
2) Discontinuous replicators: these are the cell populations that are active from birth until adult life (e. g. the skeletal and renal system).
3) Non-replicators: replication activity will decrease shortly after birth and then stop (e. g. the nervous system).

Another application of cellular renewal is to determine the growth of organs and tissues. In 1962, Enesco and Leblond investigated the growth of young male rats by measuring the total weight and the DNA content of the cells of a series of tissues and organs.

They concluded that:

1) Until about 17 days of age growth of organs and tissues is due to rapid cell proliferation, with little or no change in cell size.
2) Between about 17 and 34-48 days of age cell proliferation continues in all locations but at a slower rate than in the early period. Meanwhile cell size increases in most organs, and even more so in certain tissues.
3) After 34-48 days of age, cell proliferation slows down or even stops.

In the same year, Evensen, made the assumption that the application of carcinogenic substances on the surface of the skin of hairless mice could affect the mitotic duration and the DNA synthesizing time. She, therefore, concluded that the estimation of cell renewal by the technique of evaluating the mitotic index could lead to erroneous results.

More recently, in 1969, Johnson devised a mathematical formula to determine the theoretical value of the mitotic index at any time after partial hepatectomy, based on only three assumptions:

1) That the cell division is triggered when a cell reaches a certain critical mass or size.
2) That the rate of regenerative growth is proportional to the organ deficit.
3) That the liver cells of the intact organ have a normal size or mass distribution.

Complex as the formula is, it has the outstanding advantage of measuring many variables: cell growth, relative cell mass and cell volume.

Finally, in 1967, Bertalanffy claimed that the determination of cell renewal time could be used as a tool to diagnose maligancy.

D - Influences on cell turnover

1) Diurnal Variations

The intensity of proliferation of the total cell population undergoes regular variation during the 24 hours of the day. Many authors (Leblond and Walker, 1953; Leblond, Vulpe and Bertalanffy, 1955; Bertalanffy, 1962; Echave, Llanos and Piezzi, 1963; and Alov, 1963), studied the 24 hour rhythm in the mitotic activity of normal diurnal animals and claimed that maximal mitotic activity was observed at night and minimal activity in the morning.

To study these variations, animals were studied both under normal conditions and in an artificially induced daylight inversion cycle (Alov and Echave Llanos, 1963). By some of these authors, the diurnal variations in mitotic activity were related to the fact that during the resting period when the physical activity is reduced, the cell proliferation increases, and vice-versa (Bertalanffy, 1962 and Alov, 1963).

2) Hormonal Influence

According to Leblond, and Walker, Alov, Epifanova, endocrinolo-

gical influences are numerous. The administration of oestrogen to spayed mice induced an increase in mitotic activity (Leblond and Walker). However, according to Epifanova, the injection of oestrogen to mice can influence cell division in different ways in various tissues that are specific or non-specific to it. Moreover, the adrenal cortex may participate in the regulation of the mitotic cycle of the uterine epithelium of the mice.

3) Temperature and seasonal variation

In 1950, the effect of temperature on mitosis was well studied by Storey and Leblond, and more recently in 1959 by Evans and Savage, who mentioned that an increase in temperature induced an increase in the duration of mitosis and of interphase. Studying the renewal of the root Meristem cells of Vicia Faba, by arresting mitosis in metaphase with colchicine, they claimed that an increase in temperature produced a reduction in the lag time, that is the time required before colchicine effectively blocked all cells in metaphase.

In 1956, Leblond and Walker showed that when rats were kept with their feet on a heated surface, the mitotic activity of the foot-pad epidermis was increased. They also mentioned that mitotic activity was higher in summer than in winter.

4) Nutritional influences-the role of Vitamin A

In 1945, Friedenwald, Buschke and Morris, studying the mitotic activity in the corneal epithelium of Vitamin A-deficient rats, found a reduction of about 30% in the overall mitotic rate. The speed of the

mitotic cycle was also found to be reduced. These findings were corroborated by Lowe, Morton and Harrison in 1953 and by Sherman in 1961, who concluded that: "Vitamin A is a factor in the regulation of the mitotic activity of epithelial cells".

II - Methods of calculation of cell turnover

A - The use of colchicine

The cytotoxic effect of colchicine was mentioned for the first time in the literature by Dixon and Malden in 1908. They injected a rabbit with a dose of colchicine and noticed a leucopenia, followed shortly afterwards by an intensive polynucleosis.

Twenty-five years later, two workers, Dustin and Lits (1934), injected the drug to mice and concluded that the drug:

1) paralyzed some nervous fibres and intoxicated the central nervous system,
2) had an anti-inflammatory effect,
3) produced shortly after injection into a living animal: "Une véritable explosion de mitoses, dès la métaphase",
4) arrested the maximum number of mitosis at about nine hours after injection and a progressive return to normal number after 48.

They also noticed that after this artificial arrest in metaphase, the cells either became pycnotic and died, or carried on their normal mitosis.

Then followed the works of Brues in 1936, Sentein in 1943 and more specifically those of Ludford in 1936 and 1945, about the effect of colchicine upon the caryokinesis.

1) Chemical structure of colchicine

In his original paper in 1936, Ludford mentioned that colchicine was an alkaloid present in the corm, seeds and flowers of a crocus plant found in Autumn and called "Colchicium Autumnale" and that the extract of this plant was the most potent of mitotic poisons, being active in vitro in a diluted dose of 1 part in 100, 000, 000 (see detailed chemical formula, in chapter II, section C).

2) Pharmacological action of colchicine

Many workers claimed that colchicine acted chiefly by interfering with the spindle formation at the stage of metaphase in dividing cells (Ludford, Taylor, and Chakraborty). Ludford, noted in 1945 that although the chromosomes were formed, split and ready to separate, the spindle which is required to separate them into two groups could not be seen, neither in living cultures nor after various fixing and staining methods had been employed. He then stated that this condition was attributed to a lowering of cytoplasmic viscosity preventing the coagulation of the spindle substance. He finally described the characteristic appearance of the so-called "colchicine mitosis" previously mentioned by Dustin. Typically, the chromosomes of an arrested mitosis are clustered together in the centre and the cytoplasm and the cell exhibit: "Une turgescence et un aspect clair",

(Dustin). "In the colchicine mitosis, the cytoplasm is in a condition of prolonged and thereby exaggerated anaphase although the chromosomes are still in metaphase".

It was therefore well-accepted that colchicine could be used to estimate the turnover rate of a given cell population by determining the number of cells in that population that enters the process of mitosis over a period of time allowed for the experiments, (Ham, Leblond, Walker, Bertalanffy, Storey and Leblond, and Blenkinsopp).

Besides this effect on cell mitosis, colchicine has also some pharmacological action described in 1908 by Dixon and Malden and more recently by Ludford. Briefly, there are two types of physiological and pharmacological action.

1) An immediate effect which is exerted on plain muscle and resembles the action of pilocarpine.
2) A remote effect gradually inducing paralysis of the central nervous system.

More recently, Chakraborty and Biswas (1965), in a study on the effect of colchicine on nucleic acid and cellular protein synthesis, showed that colchicine had an effect on adenylate-Kinase, RNA polymerase and produced a 2-3 fold increase in the synthesis of RNA, DNA and protein. Inducing tumoral formation on the "Albium" root tip, they concluded that colchicine was most probably interfering with the secondary or tertiary structure of the Spindle Protein and more precisely with the S-S and S-H formation. In the same year (1965), Taylor

described the effect of colchicine on cellular metabolism, on the kinetics of inhibition of mitosis, and on the uptake and binding of radioactive colchicine. His study revealed that the action of colchicine is on a special nuclear target found only when the cells are preparing for division. "The cell contains a set of sites capable of binding colchicine and a normal mitotic spindle cannot be formed if a critical fraction of these sites is occupied by colchicine."

B - The use of tritiated thymidine

1 - DNA precursors

In 1951, two Swedish workers, Reichard and Estborn, studying the utilization of Desoxyribosides in the synthesis of polynucleotides, (by injecting into a rat ^{15}N-deoxyribosides), concluded that desoxycystidine was utilized for the synthesis of thymidine and cytosine in DNA and that thymidine was utilized for the synthesis of thymidine in DNA. Freidkin, Tilson and Roberts (1956), in their experiments on chicken embryos, showed that thymidine ^{14}C was the precursor of the thymidine of DNA with negligible diversion of the radioactivity towards other components of DNA or RNA. They also claimed that the uptake of thymidine ^{14}C into DNA could be used as a measure of the instantaneous rate of DNA synthesis in chicken embryos. Moreover in 1956, Taylor, Woods and Hughes, utilizing a different radioactive marker, showed that ^{14}C labelled thymidine could be used to study DNA synthesis. They also demonstrated, in another experiment

with tritium labelled thymidine, that both daughter chromosomes resulting from duplication in the presence of the radioactive marker appeared equally and uniformly labelled. Finally in 1958, Hughes and Bond, suggested that thymidine entered the cells via phosphorylation to thymidylic acid and that the labelling of the nuclei with tritiated thymidine occurred within the first hour after intraperitoneal injection. By 1960, it was therefore well-accepted by biologists that thymidine was a precursor in the synthesis of nuclear DNA, and that tritium labelled thymidine could be used for studying the process of DNA synthesis and cellular renewal.

2 - Nuclear aspects of autoradiography

It was in 1957 that Pelc gave the first description of the autoradiographic technique using the thyroid gland of a rat that he had previously injected with radioactive iodine (^{131}I). He brought histological sections of the gland into contact with a photographic film and concluded that the silver grains of these autoradiographs represented iodine in the thyroid follicles. He added a few notes about the stripping film technique, one that is still in use today. It is important at this stage to describe in more detail, the basic nuclear aspects of autoradiography.

In 1968, Van Der Borght suggested that the physical principle behind autoradiography depended on the formation of photographic impressions on the sensitive emulsion overlying a radioactive histological section. To obtain these photographic impressions, radioactive

disintegration from natural or artificially produced unstable isotopes is used. One of the most common of those isotopes utilized in autoradiography is the unstable isotope of hydrogen known as tritium and conventionally represented as in Figure I-4.

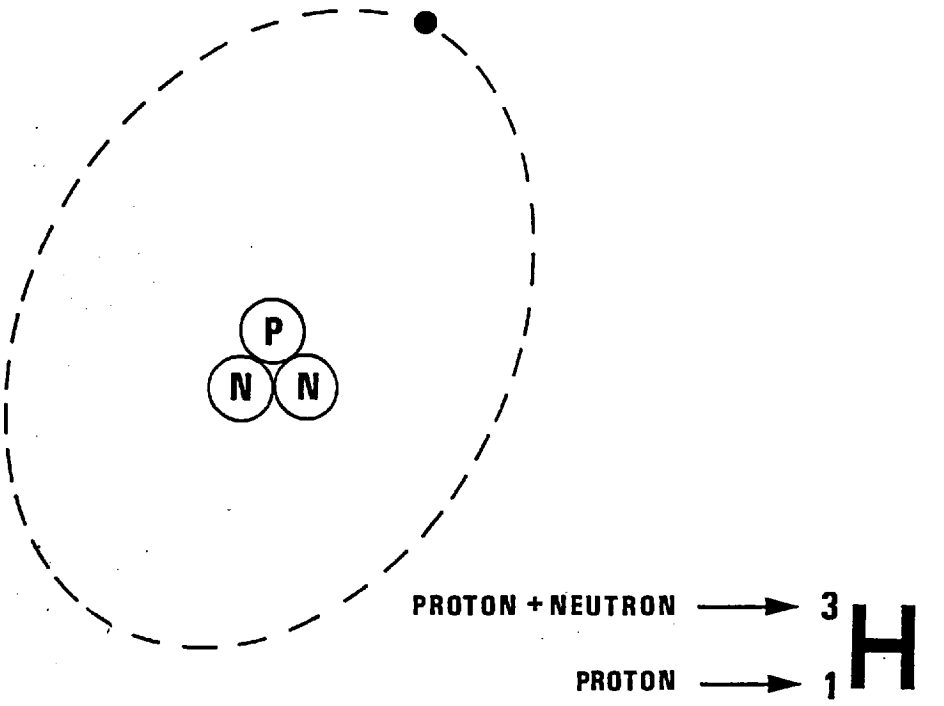
The ratio of protons to neutrons being unstable, the nucleus will undergo alterations to achieve a more stable form. As a result of the conversion of a neutron to a proton, energy will be produced and a β-ray will be emitted (Fig I-5). The energy of this β-emission is 18-KeV (Samuels and Kisieleski), and its range in a tissue will be only 6 μ, and half of the β-rays will travel less than 1 μ. It is suggested by Hughes and Bond (1958), that the overall distance travelled by the β-emission is less than 1 μ because of the constant changes in directions of the particle. It is, therefore, concluded that for autoradiography, a cell labelled with ^{3}H could be localized as laying within about 1 μ of the silver grain it has produced in the emulsion.

3 - Toxicity of tritiated thymidine

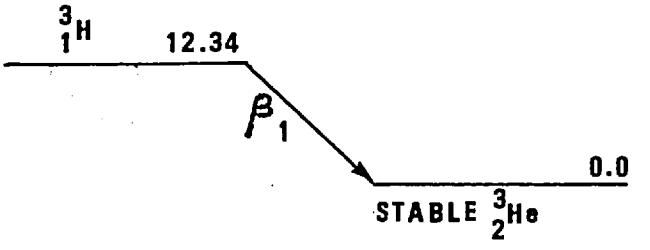
In 1960, Kraus and Plaut, studying the effect of tritiated thymidine on the incorporation of thymidine into chromosomal DNA of Vicia Faba root, showed that tritiated thymidine enhanced the incorporation of thymidine labelled with ^{14}C. They concluded that the incorporation of tritiated thymidine into DNA cannot be assumed to measure normal amounts of synthesis of the acid. Later in 1962,

FIG 1-4: THE ATOMIC PRESENTATION OF TRITIUM

FIG 1-5: H_3 NUCLEAR DECAY SCHEME



HYDROGEN - 3 BETA-MINUS DECAY



RADIATION	% DISINTEGRATION	TRANSITION ENERGY (MeV)	OTHER NUCLEAR PARAMETRES
BETA-1	100	0.0186*	ALLOWED

*ENDPOINT ENERGY (MeV)

Ref: Journal of Nuclear Medicine, 10, supp. 2, p. 15, 1969.

Bateman and Chandley, claimed that tritiated thymidine was mutagenic for the nuclei of male mice sperm cells. In 1963, Samuels and Kisielski, mentioned that the toxicity of ^{3}H-T was more related to the uptake and retention than to the dose administered and that other factors influencing uptake and retention played an important role in evaluating toxicity.

4 - Estimation of cell renewal time and DNA synthesis time

Basically, the simplest and most commonly used method for calculation of the renewal time of a cell population is the estimation of the mitotic index, that is the proportion of radioactive labelled nuclei in a given population, (Messier and Leblond, 1960; Susi, 1968; Fabrikant, 1969).

$$\text{Mitotic index} = \frac{\text{Total number of labelled nuclei}}{\text{Total number of nuclei present}}$$

However, as mentioned by Pelc (1959), although DNA is metabolically stable "the results of study on cell division in different organs of mammals and plants showed a bewildering diversity in the behaviour of DNA, independent of cell division". Later in 1960, Messier and Leblond, added that the radioactive index (% labelled cells) is only a "rough index of the rate of cell division, since it is also influenced by the rate of incorporation of the label into the intermediate substances leading to DNA synthesis and by

the duration of the period of DNA synthesis."

The use of radioactive DNA precursors is not only to study cellular kinetics but also to evaluate more precisely the duration of the different phases of the cell cycle. In 1960, Schultze and Oehlert, studying the incorporation of ^{3}H-Thymidine into cells of the rat and mouse, claimed that there was a "dose relationship between the time interval during which deoxyribonucleic acid (DNA) is synthesized, the duration of the microscopically detectable stages of the metaphase and the anaphase of the mitosis, and with the life span of the cell." They proposed that the index of tritium labelling and the mitotic index could be expressed as:

$$^{3}H \text{ index} = \frac{\text{Duration of DNA synthesis}}{\text{Life span of cell}}$$

$$\text{Mitotic index} = \frac{\text{Duration of mitosis}}{\text{Life span of cell}}$$

Assuming that cell formation in a tissue is due to mitosis only, the final equation could be:

$$\frac{^{3}H \text{ index}}{\text{Mitotic index}} = \frac{\text{Duration of DNA synthesis}}{\text{Duration of mitosis}}$$

By calculating the ^{3}H index and the mitotic index they concluded that the DNA synthesis time was approximately ten times longer

than the duration of mitosis. Following this work, many statistical analysis and mathematical models were proposed to determine the numerous interactions involved in cellular renewal, e.g. Kastenbaum, (1965) and Bronk,(1969).

It was in 1968, and in 1969 that Blenkinsopp, studied the duration of availability of tritiated thymidine after intraperitoneal injection and also compared multiple injections with continuous infusion of the isotope. He confirmed what was previously an assumption that tritiated thymidine does not label cells which enter the desoxyribonucleic acid synthesis phase (S) more than one hour after injection and that multiple injections of the radioactive element could cause a sufficient stress to reduce cell proliferation.

Finally, it can be mentioned that the utilization of tritiated thymidine can serve to study the kinetics of cellular proliferation in normal and malignant tissues (Fabrikent, 1969), the topographical variations in cellular renewal (Christophers, 1969), and to assess the progression of labelled nuclei in a desquamative type of tissue (Susi, 1968).

5 - Tritiated thymidine versus colchicine techniques

In 1964, Bertalanffy in a comparative study on the uses of ^{3}H-T and colchicine gave a complete series of figures in which discrepancy in the various turnover times were found. As mentioned by the author himself, it is difficult to draw any conclusions on these differences as the type of animal, the time of experiment, and the

can vary from one author to the other. In conclusion, he described advantages and disadvantages in both techniques.

Finally in 1965, Vant'hof, compared the results obtained by the use of tritiated thymidine and colchicine on the duration of the mitotic cycle in root meristem cells of Pisum sativum. He found that the elongated cycle as measured with ^{3}H-T was probably the result of chronic β -irradiation from the tritium incorporated to DNA.

III - Pulmonary cell renewal

A - Early studies

Henle in 1866 was one of the first anatomists to mention the presence of mitotic figures in the tracheo-bronchial epithelium. In his textbook of anatomy, he claimed that these mitoses were scattered through the tracheal epithelium and that the renewal of this epithelium, under normal conditions was slow. Twenty years later, in 1885, Bockendahl also wrote a paper about the renewal of the tracheal epithelium but it is only since the fifties that more detailed investigations have been reported in the literature.

B - Present knowledge

In 1951, Macklin, injecting 0.1 mg of colchicine to mice found mitotic figures in the alveolar space, but it was only in 1953 that Bertalanffy, and Leblond , and later in 1964 and 1967, Bertalanffy, using the technique of mitotic counting after colchicine arrest, des-

cribed a continuous renewal of the two types of alveolar cell in the lung of the albino rat. They suggest, after "somewhat speculative calculations", that the turnover time of the vacuolated alveolar cells of the lung was approximately 29.4 days and that of the non-vacuolated cells, 8.1 days.

Earlier in 1951, (Bertalanffy, Thesis), estimated the turnover time of the tracheal epithelium of the rat at 47.6 days. However, there was some disagreement upon the nature and the origin of the alveolar cell types described by Bertalanffy and Leblond (1953).

Using a totally different approach, Greenberg and Willms (1962), observed the extent and degree of regeneration of the respiratory epithelium and its structures on a Mersilene window graft performed on a dog's trachea. Five days after the operation, they described the presence of fibrin and tissue debris partly covered by a squamous-like epithelium one to three cell layer thick. It was only after 10 to 14 days that they observed the cells of the squamous-like epithelium to become similar to mature stratified squamous epithelium.

In 1962, Spencer and Shorter, using the method of labelling with tritiated thymidine, studied the cell turnover in pulmonary tissues in albino mice. They claimed that the turnover time of epithelial cells in the small bronchi and respiratory bronchioli was about seven to ten days, and that contrary to the conclusions of Bertalanffy and Leblond (1953), the scattered, labelled cells in the walls of pulmonary alveoli were macrophages and that it was impossible to differentiate histologically between the vacuolated and non-vacuolated cells.

Also in 1962, two important publications, one from Koburg, and the second from Meyer, Gottesberge, gave more data concerning the rate of cellular regeneration in the mice respiratory epithelium. For these two authors, the basal generative layer had a generation time of 19.5 days, the large bronchi of 18.1 days, and the small bronchi 58.9 days. These figures were obtained by counting 1,000 cells or more in the respiratory tract of albino mice previously injected with tritiated thymidine. In 1964 and two years later in 1966, Shorter, Titus, and Divertie, using tritiated thymidine on mice and on rats found that:

1) In adult Sprague-Dawley rats weighing between 150 to 160 gm, no differences were observed between animal of either sex.
2) Despite the low incidence of labelling of cells in the epithelia of the trachea (migration time of labelled cells six to seven days), and main bronchi (migration time seven to eight days), the occurrence of a small but definite wave of labelled mitotic figures, the apparent migration of labelled cells from basal to superficial position in the epithelia of the trachea and large bronchi followed by their disappearance indicated that these cell populations may be included in the general classification of renewing cell population (Leblond and Walker, 1956).
3) Nuclear labelling in the epithelial cells of the small bronchi persisted for five to six days.
4) Two populations of alveolar macrophage exist, one with a life span of seven days, the other with one up to two to three weeks.

In 1967, Simnet and Heppleston, studied the factors controlling organ growth and mitotic activity in newborn and adult mouse lung in organ culture. Using the colchicine method on lung explants, they concluded that an inherent mitotic inhibitor persists in culture. Pursuing their study, Simnet, Fisher, and Heppleston claimed in 1969 that in the alveolar tissue, this inhibitory agent was a stress hormone-chalone complex. They found also that a high concentration of adrenaline in the culture medium caused a large depression in mitosis. Finally, they claimed that in organ culture technique, sex and age differences were very much reduced.

Using a different approach, that is a continuous infusion of tritiated thymidine, Blenkinsopp, in 1967, found the same turnover time for tracheal and main bronchial epithelium as previous workers using single injections. He suggested that the turnover time of the basal cells of the tracheal epithelium of 330 gm. Black-hooded rats was 82 days and that of the superficial cells of the same epithelium 131 days. He added finally: "In younger rats with a higher rate of growth, the turnover is faster."

Another group of workers (Kury, Craig, and Carter, 1967), and later (Kury, Rev-Kury, and Carter, 1969), studied lung development of rat, before and after birth and also human adult and foetal pulmonary tissues. These workers employed the "in vitro" technique and used tritiated thymidine as a labelling agent. They found that the proliferative rate of alveolar lining cells of the rat was about twice the rate

of adult animals in the neonatal period and three times that in the late foetal. They claimed also that the uptake in the human alveolar wall cells was from 0.05% to 0.65% and that most of these cells were macrophages. In the bronchioli the labelling of epithelial cells varied from 0.20% to 0.70%.

Finally, Wells in 1970, studied the kinetics of cell proliferation in the tracheobronchial epithelium of rats with and without chronic respiratory diseases. Studying upper and lower tracheal levels in five-week old animals (their sex is not stated), he found that the turnover time is slower in those with less disease. He reported finding cells in mitosis in both the basal and superficial layer.

Electron microscopic studies were performed by Bindreiter, Schuppler, and Stockinger in 1968. They injected 25 rats with tritiated thymidine and calculated the turnover time of the different types of cell of the trachea. They concluded that only the basal cells and the so-called intermediate cells were identified and that ciliary cells developed from intermediate or supporting cells.

Electron microscopic studies were also performed by Evans and Bils, first in 1968 and later in 1969. They studied the renewal of the pulmonary alveolar wall cells in mice previously injected with tritiated thymidine. Leucocytes in the capillaries were the cells most often labelled. Endothelial cells were the next largest group (18.2%), and the type II alveolar cells (6%). Finally, the alveolar macrophage represented (5.2%) and the type I ?neumocyte (2.0%). The residual

group was mainly composed of interstitial cells. The turnover time of the alveolar wall cells was 20 to 25 days, the endothelial cells, 10 days, the free alveolar phagocytes, 5 to 10 days, and the type I cells, 30 days. After oxygen poisoning, they noticed a decrease in the rate of cell division in the alveolar wall, and the most affected group of cells was the endothelial type of cells.

IV - Oesophageal cell renewal

As the mitotic rate of the oseophageal epithelium is mentioned in some of our experimental studies, it is relevant to describe briefly the histology of the oesophagus and to report the results of some workers who have studied the cellular renewal of this stucture.

In 1965, Marques-Pereira,described the oesophageal epithelium of the rat and mentioned that it was composed of:

1) A malpighian region including a basement membrane on top of which is found a single layer of cells or Stratum Basale. Many mitoses are frequently seen in this region.
2) The Stratum Spinosum composed of typical spinous cells which are intermediate between the spinous cells and the basal cells and called "transitional cells."
3) The Stratum Granulosum.
4) The Keratinized Region or Stratum Corneum.

He also mentioned that up to one hour after tritiated thymidine administration, numerous labelled nuclei were seen in the basal layer, and

that the cells progressed from the basal layer to the surface in less than 48 hours.

In 1969, Blenkinsopp, studying cell proliferation in the epithelium of the oesophagus of mice, published data that will be discussed later in the discussion chapter.

SECTION C: EFFECT OF PHYSICAL AND CHEMICAL AGENTS ON MITOSIS - TYPES OF HYPERTROPHY

Many physical (endotracheal curettage, irritative inhalants) and chemical (isoprenaline, pilocarpine) agents are known to interfere with normal mitotic activity and to induce cellular and glandular hypertrophy in the respiratory tract.

I - Physical agents

A - Endotracheal curettage

In 1953, and later in 1954, Wilhelm reported his experiments on regeneration of the tracheal epithelium after curettage. He described that the regeneration of the trachea proceeds in four stages:

1) A stage of thrombosis and acute inflammatory changes at 24 and 36 hours after curettage.
2) A stage of regeneration of epithelium over the curetted surface lasting between two to eight hours with an acute mitotic wave in the surrounding epithelium at 24 hours.
3) A stage of simple stratification of the new epithelium after 72 hours. This cellular proliferation was increased and

and sustained when inflammatory changes were prominent.

4) A stage of differentiation of the newly formed epithelium. Presence of goblet cells and ciliated cells at nine to ten days.

Later in 1965, Hilding studied the regeneration of the respiratory epithelium of calves following minimal surface trauma. He found that within an hour all of the cells, except those in the basal layer exfoliated and that regeneration from this layer began after 12 to 24 hours, but was not complete until at least five days.

B - Exposure to sulphur dioxide

Using a different type of irritant, Lamb and Reid, in 1968, studied the effect of sulphur dioxide exposure on mitotic rates, goblet cell increase and histochemical changes in intracellular mucus in rat bronchial epithelium. They found an increase in the mitotic count, particularly in the animals exposed only for a short period of time (four days) and mostly in the central airways rather than in the distal branches. They also noticed an increase in goblet cell number, both at the periphery and centrally.

II - Chemical agents - isoprenaline, pilocarpine

A - Isoprenaline

It has previously been shown (Sturgess and Reid, 1972), that isoprenaline - a substitute amine for the biogenic cathecholamines, adrenaline and nor-adrenaline - increased the number of goblet cells at all levels of the tracheobronchial tree.

In 1964, Evensen claimed that high doses of adrenaline immediately increased the mitotic rate and decreased the mitotic duration in the epithelium of hairless mice in vivo. These results are in accordance with the study of Bicker, in 1969, who showed that a single dose of isoproterenol was sufficient to stimulate a large number of parotid acinar cells to commence cell division. More recently, Malamud and Malt, (1971), mentioned that isoproterenol stimulated cell proliferation in mouse kidney and induced a maximal six fold increase in DNA synthesis. This proliferation occurred 34 hours after a single injection of isoproterenol and they suggested that cyclic 3', 5'-adenosine monophosphate was involved in the initiation of cell proliferation.

However, in 1966, Chiakulas, Scheving,and Winston, studying the effect of exogenous epinephrine and environmental stress stimuli on the mitotic rates of Larval Urodele tissues, declared that catecholamine inhibited mitosis and depressed the overall mitotic rates. They also concluded that the characteristic diurnal variation in mitotic activity was suppressed after injection of the drug. Finally, they claimed that environmental stress stimuli increased production of endogenous epinephrine and therefore, decreased the mitotic rates in the tissues examined.

B - Pilocarpine

The role of a parasympathicomimetic drug (pilocarpine) in the control of bronchial gland was previously investigated by Sturgess and

Reid (1972), who showed that after six and twelve injections of the drug; there was an increase in number of all types of goblet cells. (Detailed chemical formulas of these drugs are given in chapter II, section C.)

III - Cigarette smoke

The increase in the number of mucus secreting cells after cigarette smoke in rats was first mentioned by Mellors in 1958. The first quantitative study was published by Lamb and Reid in 1969. They found that cigar and cigarette smoke produced an increase in the number of goblet cells in the rat trachea and intrapulmonary airways. This increased was proportional to the number of cigarettes smoked and was greatest in the proximal intrapulmonary airways.

IV - Phenyl-Methyl-Osadiazole

The first clinical trial of this antitussive drug, which has also been used for the treatment of tracheobronchial inflammation was first published by DeGregorio, (1960, 1962). More intensive investigations of its pharmacological action, toxicity, and metabolic routes were described by Palazzo and Corsi (1962), Silvestrini, Bignami, Carau, and Pozzatti (1963), Barron (1963), Silvestrini, Catanese, Corsi, and Ridolfi (1963), and Catanese, Palazzo, Pozzatti, and Silvestrini (1963).

Although a minimum percentage of the drug is found in the urine after administration to rats and human beings, some of its metabolites are known to induce urinary bladder irritation that is reversible

(Silvestrini, Bignami, Carau, and Pozzatti, 1963), or to have a carcinogenic effect on the transitional epithelium (Barron, 1963).

More recently, Dalhamn (1960-1969), Dalhamn and Rylander, studying the effect of tobacco smoke on the tracheal ciliary action of cats found that the ciliostatic effect of the tobacco smoke was inhibited by the addition of oxolamine citrate. (A detalied chemical formula of that drug is given in chapter II, section C.)

CHAPTER II

MATERIALS AND METHODS

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SECTION A: ANIMAL STUDIED

I - Type of animal

Albino Sprague-Dawley Pathogen-Free rats were used throughout this study. They were supplied by Carworth-Europe save for the PMO Experiment, when they were supplied by Anticimex, Sweden. The animals were shipped to us by van or by air freight in cages covered by filtre wool.

II - General care

Animals were kept in a well-ventilated and heat controlled environment. The bedding, of sawdust and wood chippings, was changed twice or three times weekly. The animals were weighed daily, fed with the standard pasteurized breeding diet (Oxoid) and given ordinary tap water.

III - Preparation of the animals for experimental studies

A prerequisite for studying experimental variation in cellular turnover in the respiratory epithelium is to study this turnover time under controlled conditions. Since environmental difference has a large effect on cell turnover, in all studies, conditions were standardized as follows. The day prior to the injection with colchicine or tritiated thymidine, half of the animals in each group were carried in their own cages from the animal house to the main laboratory: they were then allowed to rest for at least 24 hours before the injection. In this way, we minimized the effect of an abrupt change in routine,

which could have influenced the rate of cellular turnover. On the next morning, all our injection schedules of colchicine or tritiated thymidine were started at ten o'clock and the animals were injected every half hour. Special attention was paid to alternate at every injection, either the type of drug or the sex of the animal. After being injected, the animals were placed in different and separate cages according to their sex and to the drug injected; they had no more food or water after injection. Colchicine and tritiated thymidine injections were each given by the intraperitoneal route using disposable syringes and 23-gauge disposable needles. Four hours after injection, the animals were killed by intraperitoneal injection of 1.0 to 1.5 ml of veterinary Nembutal (Abbott, 60 mg/ml). The next day the remaining animals were injected and killed in a similar fashion.

SECTION B: EXPERIMENTAL STUDIES

I - Cell turnover in normal animals - Variation with age and sex

For this experiment, two groups of young animals and one of middle and one of old age were studied. For each of those groups, save for the middle aged animal group, two animals, one male and one female were sacrificed 24 to 48 hours after arrival to check on the state of the lungs (section F-1). The mean age, weights, sex distribution, and number of animals used are shown in Table II-1. All these animals were kept in the animal room of the department.

Table II-1

Details of Animals Studied

Group	Age (Days)	Weight (gm)		NUMBER OF ANIMALS											
				Sex		Clean Control*		Colch		^{3}H-T		Control**		Total	
		M	F	M	F	M	F	M	F	M	F	M	F	M	F
Young I	33	133.5	114.5	12	12	2	2	4	4	4	4	2	2	12	12
Middle II	47	241	199	5	5	-	-	2	2	2	2	1	1	5	5
Old III	105	555	334	6	6	1	1	2	2	2	2	1	1	6	6
Total				23	23	3	3	8	8	8	8	4	4	46	

* Clean Control: These animals were killed between 24 and 48 hours after their arrival to establish that the stock was clean.

** Control: These animals were killed after an intraperitoneal saline injection at the same time as the colchicine and the thymidine-treated animal.

Only animals of the same sex were kept in a cage with no more than six animals in a cage.

In each experiment the following routine was followed:

1st day	1 male & 1 female injected with colchicine
	1 male & 1 female injected with ^{3}H-T
	1 male injected with saline (control animal)
2nd day	1 male & 1 female injected with colchicine
	1 male & 1 female injected with ^{3}H-T
	1 female injected with saline (control animal)

II - Effect of isoprenaline and pilocarpine

Twenty-four animals were included in this experiment and grouped as follows:

10 animals (5 males & 5 females) - Isoprenaline injection

10 animals (5 males & 5 females) - Pilocarpine injection

2 animals (1 male & 1 female) - Checked for "cleanliness"

At the beginning of the experiment, the animals were 20 days old. The experiment lasted for 19 days and the weights of all groups of animals are shown in Table II-2. Injections were made daily on consecutive days, up to six for the Isoprenaline Group and up to 12 for the Pilocarpine and Saline Groups. On the seventh day of the experiment, one of the male rats in the PCP group died from a drug overdosage. The two animals for cleanliness control were killed

Table II-2

Weights (in gms) of the Animals at the Start and at the End of the IPN and PCP Experiment

	IPN		PCP		CONTROLS	
	Start	End	Start	End	Start	End
Males	45-60 (m = 52)	130-185 (m = 157)	50-65 (m = 53)	125-185 (m = 145)	45	175
Females	50-55 (m = 52)	135-145 (m = 139)	45-60 (m = 53)	110-150 (m = 134)	55	175

five days prior to the end of the experiment.

The next part of this experiment, that is the injection of colchicine and tritiated thymidine, was performed over two consecutive days. This part of the experiment was carried out as previously described in experiment I, page 64 . In the IPN group, six animals received colchicine and four received ^{3}H-T, and in the PCP group, five animals received colchicine and four received ^{3}H-T. The two controls received colchicine.

III - PMO Experiment I: Long term exposure

Forty-four male rats from Carworth-Europe were used for this experiment. Most animals were completely free from lymphocytic cuffing. Each rat weighed approximately 150 gm. These animals were kept in a room on their own before the experiment started. The rats were divided into four groups.

Group I: Fifteen animals exposed to cigarettes without any additive "tobacco".

Group II: Fifteen animals exposed to cigarettes to which the anti-inflammatory agent (PMO) had been added, "tobacco + PMO".

Group III: Seven animals given a "sham" exposure in that they spent the same time in an exposure chamber as Groups I and II, but were subject only to air flow and not to added tobacco smoke.

Group IV: Seven animals kept in cages.

For the first two days Groups I and II were exposed to 15 cigarettes a day and later to 25 cigarettes. For exposure to tobacco smoke, each group of animals was placed in an aluminum chamber in which the air flow was between 10 and 11 litres per minute. The cigarettes were burnt in a Wright auto-smoker at four puffs a minute (each puff consisted of 30 cc of air drawn through the cigarette), each cigarette taking five to six minutes to burn. A new cigarette was started each ten minutes. Two cages were used for the cigarette combustion, the groups being changed each day, so that on alternate days a chamber burnt the same type of cigarette. The animals were exposed for four days a week for six weeks (Fig II-1).

The temperature was measured in the laboratory and, in each chamber, at the beginning and end of each exposure, the humidity at the end. A rise in humidity and temperature occurred in each chamber, less in the "sham" exposure than in the other two cages. The mean value for each chamber used for tobacco exposure were similar and also the mean value to which each tobacco group had been exposed. The difference between the cages used for tobacco and "sham" exposure was greater with respect to the humidity, than temperature.

The control animals were moved from the room during the

Figure II-1: Wright Auto-smoker

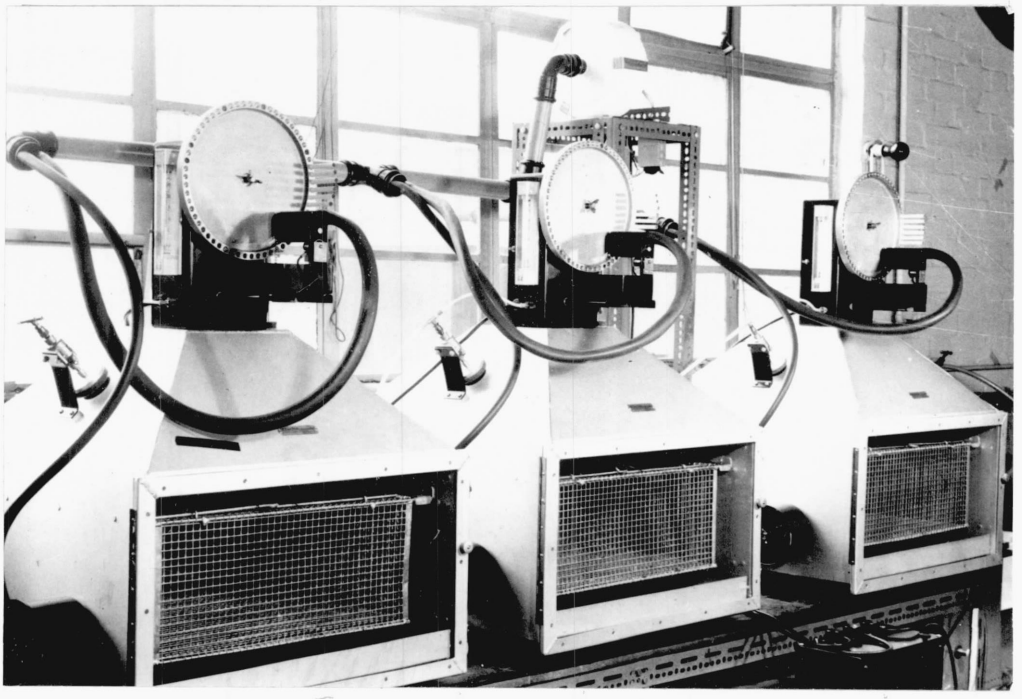
Picture (A): Three aluminum cabinets with the automatic smoking machine on top. Each chamber has a clear perpex front door for easy loading and cleaning of the metal cage inside the cabinet.

Picture (B): Control box with electric clock controlling the puff duration and the time interval between each puff.

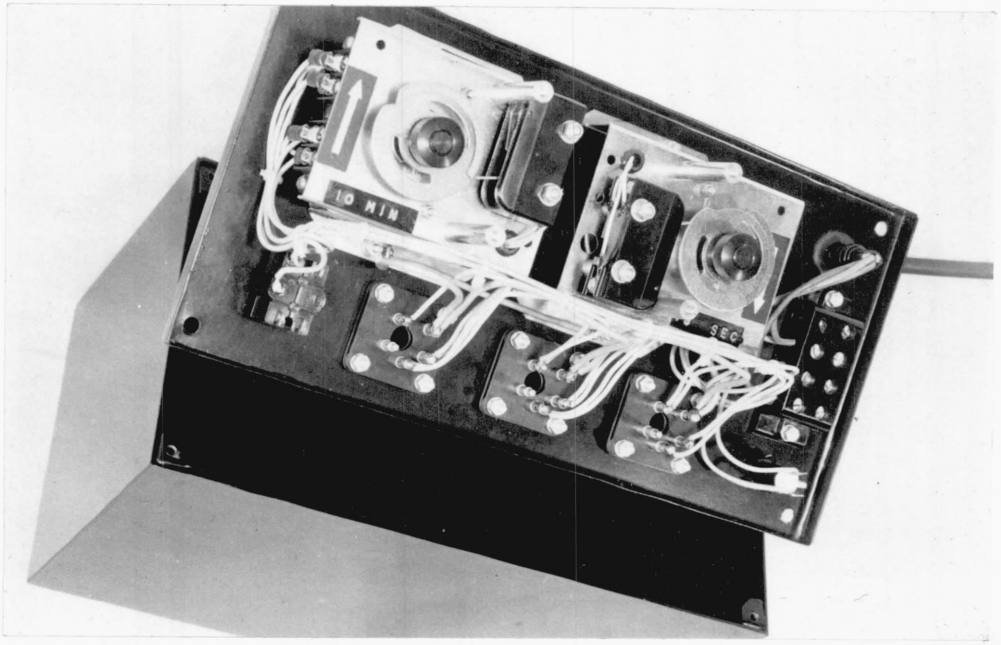
Picture (C): Sixty cigarettes are distributed in sockets around the rim of a wheel which is rotated intermittently by an electric clock motor. The machine is operated pneumatically and the cycle of operation controlled by time switches and electromagnetic valves.

Compressed air is blown intermittently through a jet situated at the end of a cross passage leading to the cigarette socket. Air is thus drawn through the cigarette, mixed with the air from the jet and blown into the chamber.

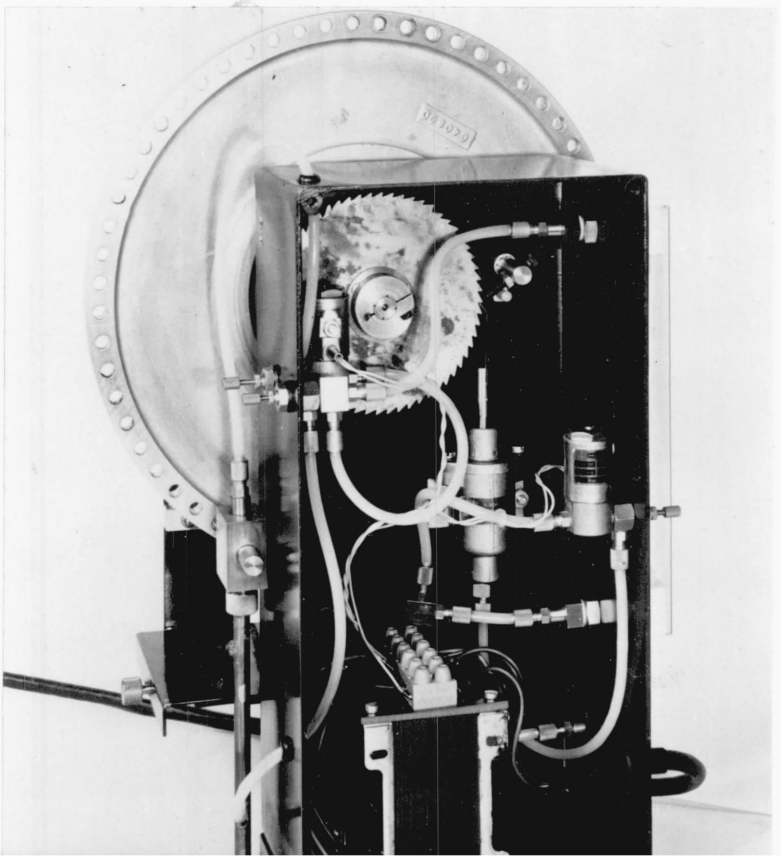
A



B



C



four and one half hours or so of exposure. The animals, including the controls, received no food or water during the time of exposure. Apart from this time, all animals were in the same type of cage, in the same room, with food and drink ad libitum. Each day, the chambers and auto-smoker were cleaned.

The two types of cigarettes, as supplied to us were identified by a code number whose key was not known to any of us; it was not broken until it had been decided which cigarette had the most clinical effect on the animals. The experiment was so arranged that, in the last weeks exposure to cigarette smoke, took place only on Monday and Tuesday; half of the animals in each of the four groups was killed on Wednesday, the other half on Friday. Any difference between the animals killed 24 and 72 hours after exposure was looked for in each of the features studied, but no significant difference was found. All animals were killed by the intraperitoneal injection of 1 to 1.5 cc of pentobarbitone sodium.

III - PMO Experiment II: Short term exposure

Forty-four male rats from Anticimex were used for this experiment. There were 20 animals in each of the smoking groups and 4 animals in the control group. Because no difference in the different features studied in the previous experiment between the "sham" and the control group was apparent, it was therefore decided not to include a "sham" group.

At the beginning of the exposure the animals weighed between 110 - 135 gm (mean = 123.6 gm). This experiment lasted for 16 days: during the first two weeks the animals were exposed for six consecutive days. Monday to Saturday inclusive and in the third week they were exposed only for two days. Each day of exposure, the animals smoked 25 cigarettes, except for the control animals which were transferred from the "smoking room" into a separate adjoining room during the four to five hours or so of exposure. The same routine concerning the care of the animals and the smoking procedures was observed as previously described.

As the purpose of this second smoking experiment was to quantify respiratory epithelial damage and repair in relation to time of exposure, the animals were killed after different periods of exposure.

After one day of exposure, four animals from each tobacco group and two animals from the control group were killed. Among these animals, half received colchicine and half tritiated thymidine. on the second, third, and eighth day of exposure, four animals from each tobacco group were killed and again half of these animals received colchicine and half received thymidine. After 15 days of exposure the remaining animals were killed, that is four animals in each of the smoking groups and two controls.

SECTION C: DRUGS

I - Colchicine

Colchicine ($C_{22}H_{25}NO_6$, Molecular Weight 399.43, British Drug House) was delivered in a small aluminum container in the form of a yellowish powder. Ampoules containing 0.1 mg or 0.2 mg/cc saline solution were made up at the Brompton Hospital Pharmacy, kept in a cold room at 4°C and used within a month. The dose for intraperitoneal injection was 0.1 mg/100 gms of body weight.

II - Tritiated Thymidine

Tritiated Thymidine (Thymidine - $^6H_3$, Code No: TRA61, Specific Activity 5.0 curries/MM0L) from Radiochemical Centre in Amersham was used.

The radioactive concentration was of 1.0 mci/ml and the solution was contained in a sterile flask protected by a special delivery box. The thymidine ($C_{10}H_{14}N_2O_5$; Molecular Weight: 242.23) had the unstable β emitting hydrogen atom on position 6. The dose for intraperitoneal injection was 1 μc per gram of body weight.

III - Pilocarpine

Pilocarpine Nitrate ($C_{11}H_{16}N_2O_2 \cdot HNO_3$; Molecular Weight:

208.25, The British Drug House) was prepared in ampoules containing 40 mg in a 2 ml sterile water solution by the Brompton Hospital Pharmacy. The injections were made subcutaneously and the dosage was 4 mg/100 mg of body weight. The animals in this group received 4 mg for the first eight injections and then 6 mg for the last four injections.

IV - Isoprenaline Sulphate

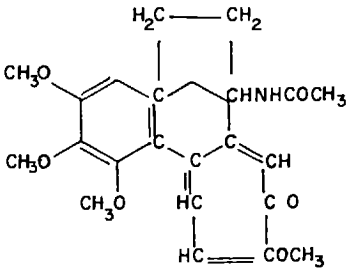
Isoprenaline Sulphate ($C_{11} H_{17} NO_3$; Molecular Weight: 211.24) was supplied by MacCarthy's and ampoules containing 100 mg in 2 ml sterile water solution were made by the Brompton Hospital Pharmacy. Injection was made subcutaneously and the dosage was 10 mg for the first two injections, the animals being under 150 gm of body weight, and 15 mg thereafter.

V - PMO

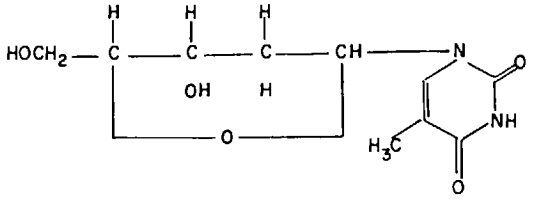
Phenyl-methyl oxadiazole is a derivative from phenyl-vinyl oxadiazole (PVO), a substance that proved to be unstable with a strong tendency to polymerize. This substance (PVO), is one of the three subgroups contained in the molecule of the oxalamine citrate. The amount of PMO contained in each cigarette was 2.4 mg/cigarette or 2% of the total weight of each cigarette. (Detailed formulae of substances referred to in this section are shown in Fig II-2).

Figure II-2: Molecular Structure of the Drugs Used in This Study

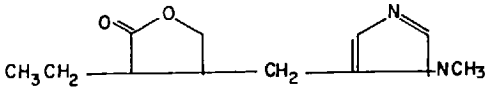
COLCHICINE: $C_{22}H_{25}NO_6$



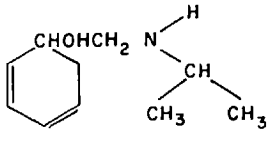
THYMIDINE: $C_{10}H_{14}N_2O_5$



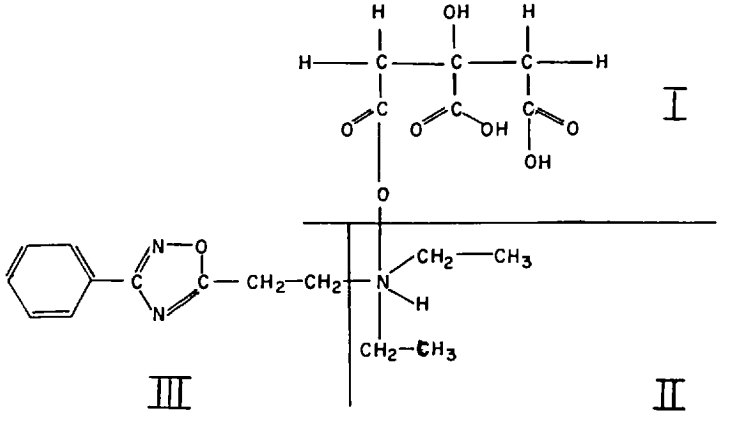
PILOCARPINE NITRATE: $C_{11}H_{16}N_2O_2HNO_3$



ISOPRENALINE SULPHATE: $C_{11}H_{17}NO_3$



P.M.O



I CITRIC ACID
II DIETHYLAMINE
III PHENYLVINYL-OXADIAZOL

SECTION D: PREPARATION OF TISSUES FOR MICROSCOPY

I - Anesthetic procedures

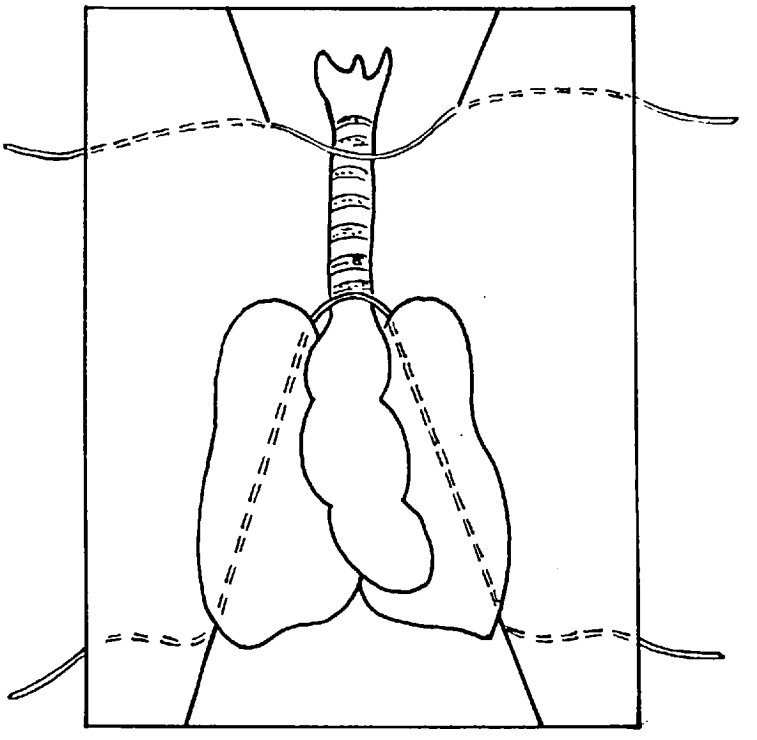
All animals were anesthetized by an intraperitoneal injection of 1 - 1.5 cc of nembutal (Pentobarbitone Sodium, Abbot veterinary preparation 60 mg/cc). When the superficial reflexes were no longer present, thoracotomy was performed. This was usually three to five minutes after the injection.

II - Dissection procedures

A midline ventral incision was used and the thoracic cage opened by dividing the anterior arch of the ribs, taking special care to avoid any damage to the underlying respiratory tissue. A silk suture was then passed underneath the oesophagus and trachea and a knot tied at the level of the cricoid cartilage. While the trachea, main bronchi, and lungs were still in the chest cavity, an intra-tracheal injection was performed with a 25-gauge needle, of 10% formol saline fixative for tritiated thymidine-treated animals, or of Heidenhein-Susa fixative for colchicine-treated animals. Both lungs were inflated until the pleura was tense and within a few minutes, all the intra-thoracic organs were removed en bloc.

The upper trachea and the two lungs were then attached to a piece of cardboard to prevent the trachea from curling and to maintain its length,(Fig II-3). The tissues were fixed overnight and the

Figure II-3: The Trachea and Both Lungs Fixed to a Card with the Heart and Thymus Still Attached.



next morning, after removal of the heart, great vessels, and thymus, blocks were taken.

III - Histological techniques

Five blocks were taken from the lungs of each animal.

A) Upper trachea

One centimeter of trachea immediately below the cricoid cartilage and oesophagus was removed. This was embedded sideways so that a section would include both its cartilaginous and membranous parts.

B) Mid trachea

Three mm long with the oesophagus attached. Embedded with a cut edge down to give a horizontal section of both the trachea and the oesophagus.

C) Lower trachea

Half to one cm long, depending on the overall length of the trachea. No oesophagus attached. Embedded as the upper trachea.

D) Left lung

The left lung was embedded as a whole and in such a way that a section included the main axial intrapulmonary bronchus and some of its lateral airways, (Fig II-4).

E) Lower lobe of the right lung

The right lung was embedded like the left but kept in reserve. All tissues were processed using a Histokinette Automatic Tissue Processing Machine. The program for all specimens is given in

Figure II-4: Ideal Section of the Left Lung

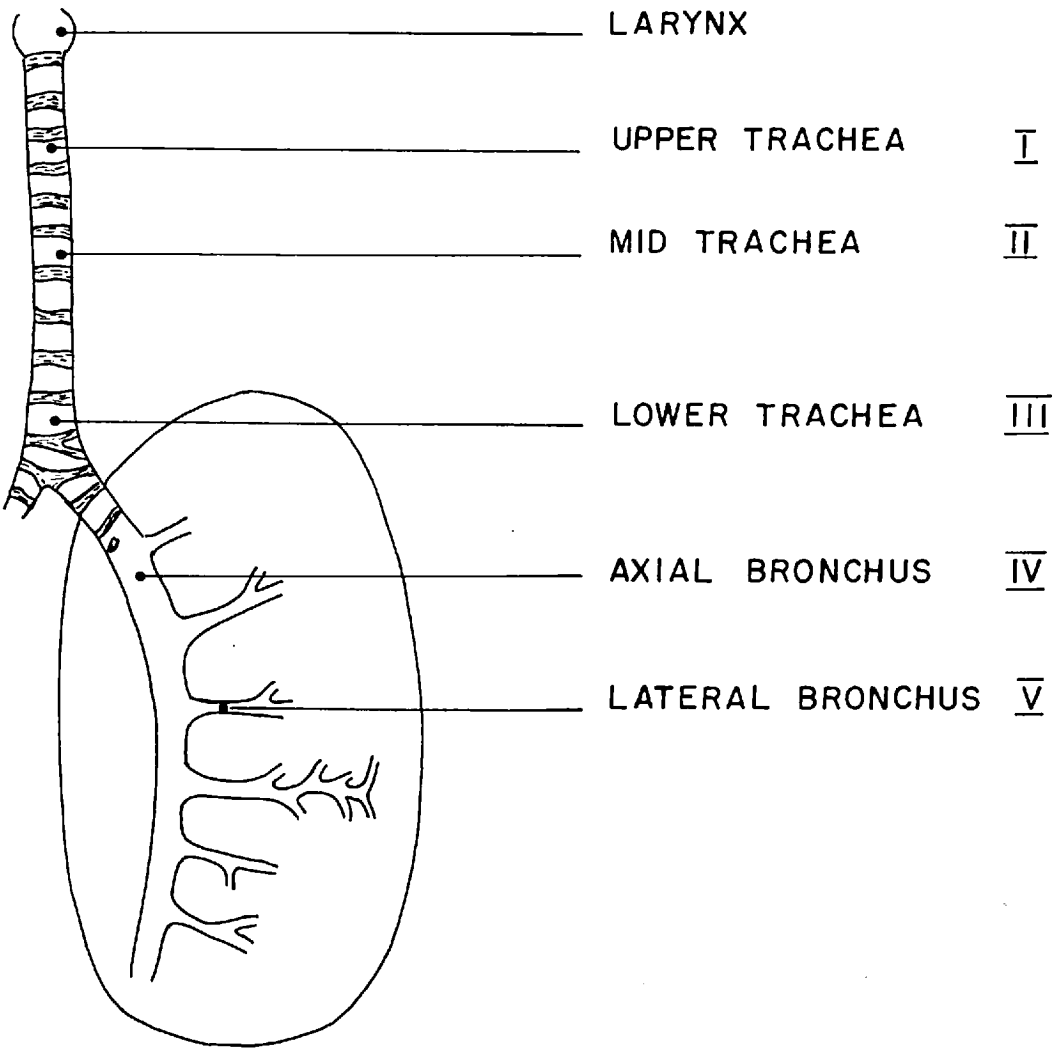

LARYNX
UPPER TRACHEA I
MID TRACHEA II
LOWER TRACHEA III
AXIAL BRONCHUS IV
LATERAL BRONCHUS V

Table II-3. Tissues were given a final change in wax under vacuum and embedded in fresh wax (Fibrowax, melting point: 57° - 58° C).

IV - Cutting

Sections were cut with a Leitz microtome using a base sledge knife. Routinely, 4 μ sections were cut and were mounted on albuminized glass slides. Before staining, the sections were dewaxed in Xylol, dehydrated in graded alcohols and washed in water.

V - Staining

The following staining techniques were applied to the tissues examined microscopically. These are all standard techniques and references are given for each staining method.

A) Haematoxylin and Eosin (H/E)

Acidophilic substances stained red with eosin, basophylic substances, e.g., proteins and nucleic acids, stained blue with haematoxylin (Culling).

B) Alcian Blue/Periodic Acid Schiff (AB/PAS)

Acid glycoprotein, with either carboxylic acid or sulphate groups, reacted with alcian blue to form a blue compound: in conjunction with PAS a spectrum of colours from blue, blue-red, red-blue to red were produced (Spicer and Warren, 1960).

Table II-3: Time Schedule for Processing of Tissues

Treatment	Time in Hours
I - Fixation	
Formol Saline or Heidenhein Susa	24
II - Dehydration	
Formol S. fixed material / H. Susa fixed material	
Alcohol 50%	2
Alcohol 70%	2
Alcohol 90%	2
Alcohol 100% I	2
Alcohol 100% II	3
Alcohol 100% III	1 1/2
III - Clearing	
Chloroform I	2 1/2
Chloroform II	3
IV - Embedding	
Paraffin Wax I (60°C)	2
Paraffin Wax II (60°C)	2

C) Harris's Haematoxylin for Autoradiography

The slides for autoradiography were rinsed in distilled water, and stained with Harris's Haematoxylin for ten minutes. They were washed in running tap water for ten minutes, differentiated in 0.25 HCl in distilled water, washed again in running tap water for thirty minutes, and finally stained in 0.2% Eosin (aqueous solution) for eight minutes (Culling).

After being washed in tap water for six minutes, they were dried in the incubator overnight. The next day, the slides were rinsed in absolute Ethyl Alcohol and Xylene and mounted in neutral Canada Balsam in Xylene.

SECTION E: AUTORADIOGRAPHY

I - Description of the technique employed

Sections were mounted and dewaxed as described above. In a dark room, with a Kodak Wratten No 1 red filtre, the autoradiographs were prepared by the stripping film technique (Pelc, 1947). A fine grain stripping film, AR 10 Kodak Ltd. was used. The film was cut into strips approximately 20 - 30 mm long, and these were floated onto distilled water. The distilled water had previously been boiled and cooled to 20°C. Each strip was floated face down on the surface of the water and allowed to expand for at least three minutes. A strip was then picked up on to the mounted section so that the emulsion was in direct contact with the tissue. The films were allowed to

dry on the sections, the slides being placed in a wooden rack and in a stream of cool air for 20 to 30 minutes. The slides were then transferred to light-tight boxes, containing silica gel as a dessicant. The boxes were wrapped in a black plastic bag and stored at 4°C, and the AR film exposed for a period varying between 20 to 24 days. At the end of the exposure period, the slides were transferred into glass staining racks and developed using a high contrast developer, D-19 Kodak Ltd. at 20°C for six minutes: the developer was freshly prepared and filtred before use. The slides were rinsed in distilled water (two changes) and fixed in Johnsons Fix-Sol, diluted 1:10 for 14 minutes. After fixation the slides were washed in running tap water for 30 minutes, and then dried overnight in an incubator at 37°C.

The above technique originates from the early work of Pelc in 1947, although, during the last two decades, many modifications have contributed to improve the original work. The sections were then stained with Harris's haematoxylin (see page 84).

SECTION F: QUANTIFICATION OF RESULTS

I - Assessment of the respiratory tract cleanliness

The lungs of all animals used in these experiments could be expected to be sterile on culture. We have found a histological grading based on cell infiltration a more sensitive index of clean lung. A scale based on lymphocytic infiltration, grade I to IV,

has been used (Fig 11-5). A slight lymphocytic infiltration along the main bronchus or at a carina of the axial pathway was considered grade I, while a more extensive lymphocytic infiltrate in five or more foci was considered graded III-IV. Any lymphocytic infiltration was usually located in the sub-epithelial and submucosal sites along the main bronchial pathways. This type of cleanliness assessment was found to be reliable, simple, and accurate and studies reported later (p.161) will demonstrate that goblet cell counts correlated with the degree of cleanliness.

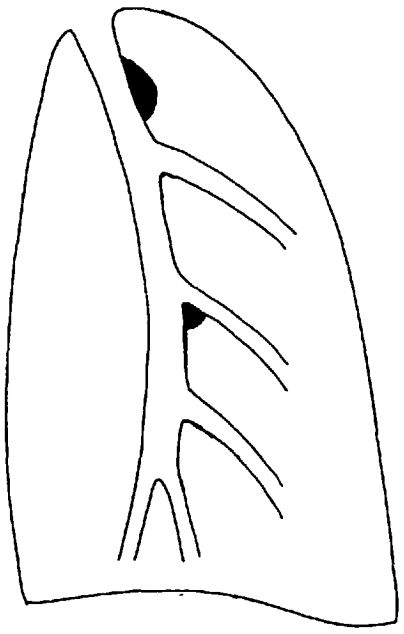
II - Counting technique

All tissue sections were examined microscopically with a Zeiss binocular microscope. All specimens were examined under oil immersion (100 X) using two wide-angle eye-pieces (12.5 X). An ordinary laboratory counter (Clay-Adams), was used in counting.

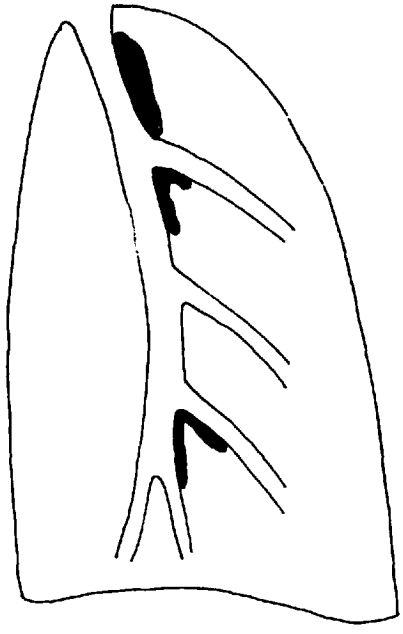
III - Number of cells counted - "Positive" cells in the colchicine and tritiated thymidine techniques - Location of mitoses within the epithelium

To calculate the mitotic index, that is the number of cells in mitosis/the total number of cells present, a total of 1,000 cells were counted for each calculation. Depending on the level, this index was estimated two or four times as shown in Table II-4. Altogether, for each animal, a total of 18,000 (18 x 1,000) cells were

Figure II-5: Grading of Lung Cuffing



GRADE I



GRADE II

GRADE III

GRADE IV

Table II-4

Total Number of Cells Counted in Each Animal

Level	Plane of Sections	Number of Cells Counted	Total
Trachea Upper 1/3	Frontal	4 x 1000	4000
Trachea Mid 1/3	Horizontal	2 x 1000	2000
Trachea Lower 1/3	Frontal	2 x 1000	2000
Axial Br.	Frontal	2 x 1000	2000
Lateral Br.	Frontal	2 x 1000	2000
Alveoli	Frontal	4 x 1000	4000
Oesophagus	Horizontal	2 x 1000	2000
Total		18 x 1000	18000

counted. Results, however, are given as the number of cells in mitosis per 1,000 cells present. For the colchicine-treated animals, a cell was considered to be "positive" when mitosis was arrested in metaphase. These cells were generally more voluminous and the chromosomes, surrounded by a clear halo, were thicker, and grouped together in the centre of the dividing cell (Fig II-6).

In the tritiated thymidine animals, a cell was considered to be "positive" when the number of silver grains within the cell was eight or more.

Positive cells were located in the following way within the epithelium:

a) A positive cell in contact with the basement membrane was said to be "basal".

b) A positive cell not in contact with either the basement membrane or the surface of the epithelium was said to be in a "mid" position.

c) A positive cell, in contact with the epithelial surface was said to be "superficial", (Fig II-7).

IV - The airway levels - oesophagus and alveoli

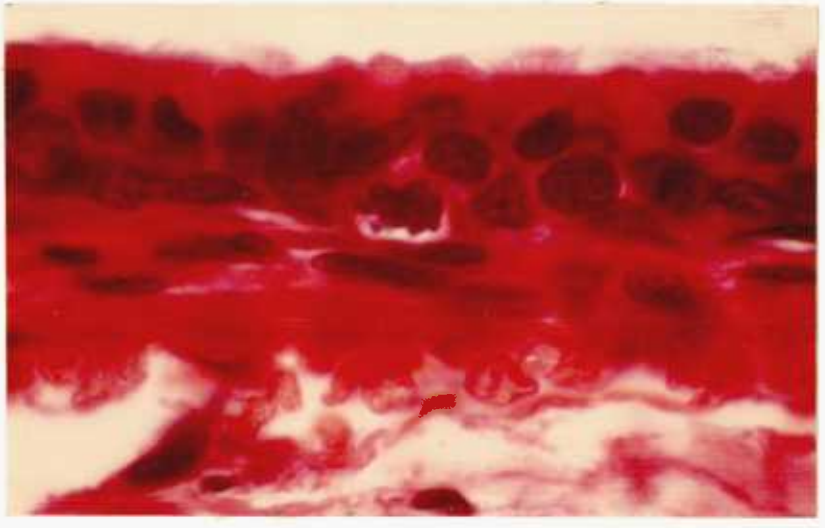
At each airway level, the cells were counted on two non-consecutive sections from the same block, usually the second and the sixth sections.

Figure II-6: Colchicine Arrested Metaphase

1) In a "basal" position in the trachea

2) In the alveolar wall

1



2

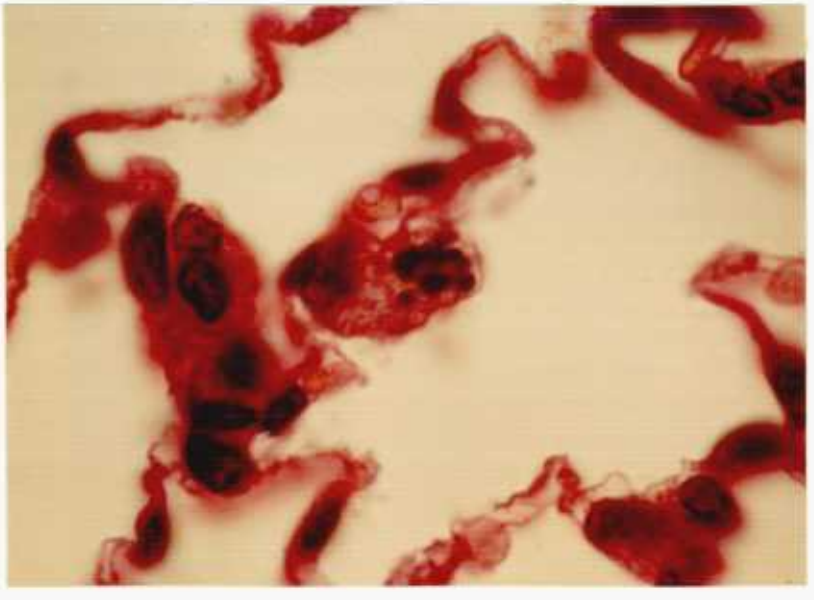
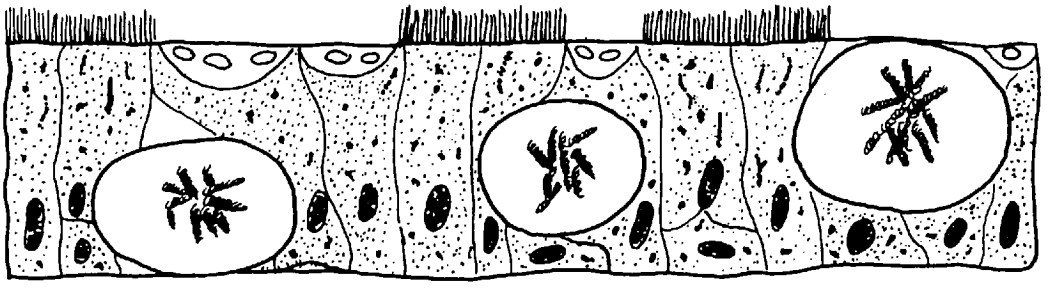


Figure II-7: Position of Mitoses within the Epithelium

1) Basal

2) Mid

3) Superficial



1 2 3

1) Trachea Upper Third - Level I: The epithelium was thick and along a perpendicular to the basement membrane, two, three, and often four nuclei could be seen. A length of 6 mm contained about 1, 000 cells.

2) Trachea Mid Third - Level II: In this only horizontal section, the tracheal epithelium had usually the same appearance as the epithelium of the upper level. Unfortunately, technical difficulties in manipulating this section created artefacts. The oesophagus which was attached behind the trachea and which is known to have a higher mitotic index than the respiratory epithelium was used as a control for both the colchicine and tritiated thymidine techniques.

3) Trachea Lower Third - Level III: Generally, the section included the carina. There, the epithelium was rather thinner than in the upper trachea. It was here that lymphocytic infiltration of the epithelium was more likely to create difficulty of identification of cells.

4) Axial and Lateral Bronchi - Level IV-V Respectively: These intrapulmonary airways were included with the lung. The cells were counted in the medial part of the axial brocus to avoid any cuffing at the carinae of each lateral bronchus. In the lateral airways, the epithlium was thinner and contained only one row of cuboidal cells resting on a rather

irregular basement membrane.

N. B. Subsequently, these levels are referred to by their Roman Numeral.

5) Alveoli: To count the cells in the alveolar epithelium, the section was scanned from top to bottom and from left to right. Those cells that could be identified as macrophages, polymorphs, and endothelial cells were not counted.

V - The observer error

The number of cells in mitosis in the middle aged group was assessed on three different occasions, two and three weeks after the first count. Statistical analysis showed that the difference between the counts was insignificant (Table II-5).

VI - Calculation of the turnover time

The mitotic index was derived by dividing the number of cells in mitosis present during the period between the time of injection and the time of killing the animals, i. e., four hours, by the total number of cells present, i. e., 1, 000. From this mitotic index, the turnover time expressed in days of the total population of cells can be easily calculated.

$$\text{Turnover time in days} = \frac{4 \times 1,000}{24 \times n}$$

n = number of cells in mitosis

Table II-5

Observer Error

The figures represent the mean mitotic index of four animals. Three separate counts were done at two to three week intervals and at three levels in the trachea. Figures in brackets represent the standard error of the mean.

Site	$\bar{X}_1$	$\bar{X}_2$	$\bar{X}_3$
Trachea Upper 1/3	4.56(0.50)	4.06(0.74)	4.50(0.76)
Trachea Mid 1/3	4.00(0.22)	2.50(0.38)	3.38(0.46)
Trachea Lower 1/3	4.25(0.58)	3.00(0.50)	4.13(0.58)

SECTION G: STATISTICAL ANALYSIS

The following statistical methods have been applied to the results in these studies:

- Mean, variance, standard error of the mean, standard deviation, the "t" distribution test of Student, the correlation coefficient.

The significance of the results were determined using statistical tables (Fisher and Yates, 1963).

CHAPTER III

CELL TURNOVER IN NORMAL ANIMALS

THE INFLUENCE OF AGE AND SEX

CONTENTS

NOTE: THE USE OF COLCHICINE AND TRITIATED THYMIDINE

In each experiment and for each group of animals, the colchicine and tritiated thymidine techniques were both used. However, we found that the colchicine technique was easier to employ and the results more readily available.

At the beginning of this study, we compared colchicine and tritiated thymidine injected in young and middle age animals and we found similar results (see p. 103-104, 109, 111, 118).

It was then decided to use only the colchicine technique for further experiments.

In this experiment we studied young, middle age, and old animals. The distribution of these animals in the three experiments is shown in Table III-1. The weights of those animals are shown in page 63 . The mitotic index was studied at five levels of the respiratory tract and also in the alveoli and in one level of the oesophagus. The mitotic index was calculated using both the colchicine and tritiated thymidine and the turnover time was compared in both males and females.

Control Animals

These animals received no drug. In lung sections, no mitotic activity was found, while in the oesophagus cells in mitosis and pycnotic nuclei were both seen. No further mention will be made of these controls.

Oesophagus

One level only of the oesophagus (middle third) was studied in each animal. Because of the high mitotic activity, this tissue was used as an evidence that the technique was satisfactory. The oesophagus showed disparate results between colchicine and tritium, probably because of the small sample on which mitotic activity was assessed.

Alveoli

The mitotic activity was very high at the alveolus level due probably to the fact that cell identification was difficult in the light

Table III-1

Details of Animals Studied

Group	Age (Days)	Weight (g)		TOTAL NUMBER OF ANIMALS									
				Sex		Clean*		Colch		^{3}H-T		Control**	
		M	F	M	F	M	F	M	F	M	F	M	F
Young A	33	134	123	6	6	1	1	2	2	2	2	1	1
Young B	33	133	106	6	6	1	1	2	2	2	2	1	1
Middle	47	241	199	5	5	-	-	2	2	2	2	1	1
Old	105	555	334	6	6	1	1	2	2	2	2	1	1
TOTAL				23	23	3	3	8	8	8	8	4	4

*Clean: These animals were killed between 24 to 48 hours after their arrival to establish that the lungs of the stock were clean.

**These animals were killed after an intraperitoneal injection of saline at the same time as the experimental animals.

microscope and because cells other than epithelial ones were included in the count. Counts were similar with both the colchicine and the tritium techniques.

SECTION A: YOUNG ANIMALS

I - Mitotic index in the colchicine-treated group of animals at different airway levels

In both the male and female groups, the total number of mitoses decreased progressively from the upper trachea to the axial bronchus. The number of mitoses was increased in the male as compared with the female, except in the axial bronchus and oesophagus; but nowhere did this difference reach significance (Fig III-1).

II - Variation in position of the mitoses within the epithelium

In the three tracheal levels of both male and female animals, most of the mitoses were located at the base of the epithelium, while in the axial and lateral bronchi, they were more superficial (Table III-2).

III - Total number of mitotic figures at different airway levels, in both the colchicine and the tritiated thymidine-treated animals

For this experiment, in the colchicine group, four animals (two males and two females) were used. In the tritiated thymidine group, only two animals (one male and one female) were found to

Figure III-1: Young animals - histograms showing the mitotic index (number of mitoses/1, 000 cells) in the male and female colchicine-treated animals at different airway levels.

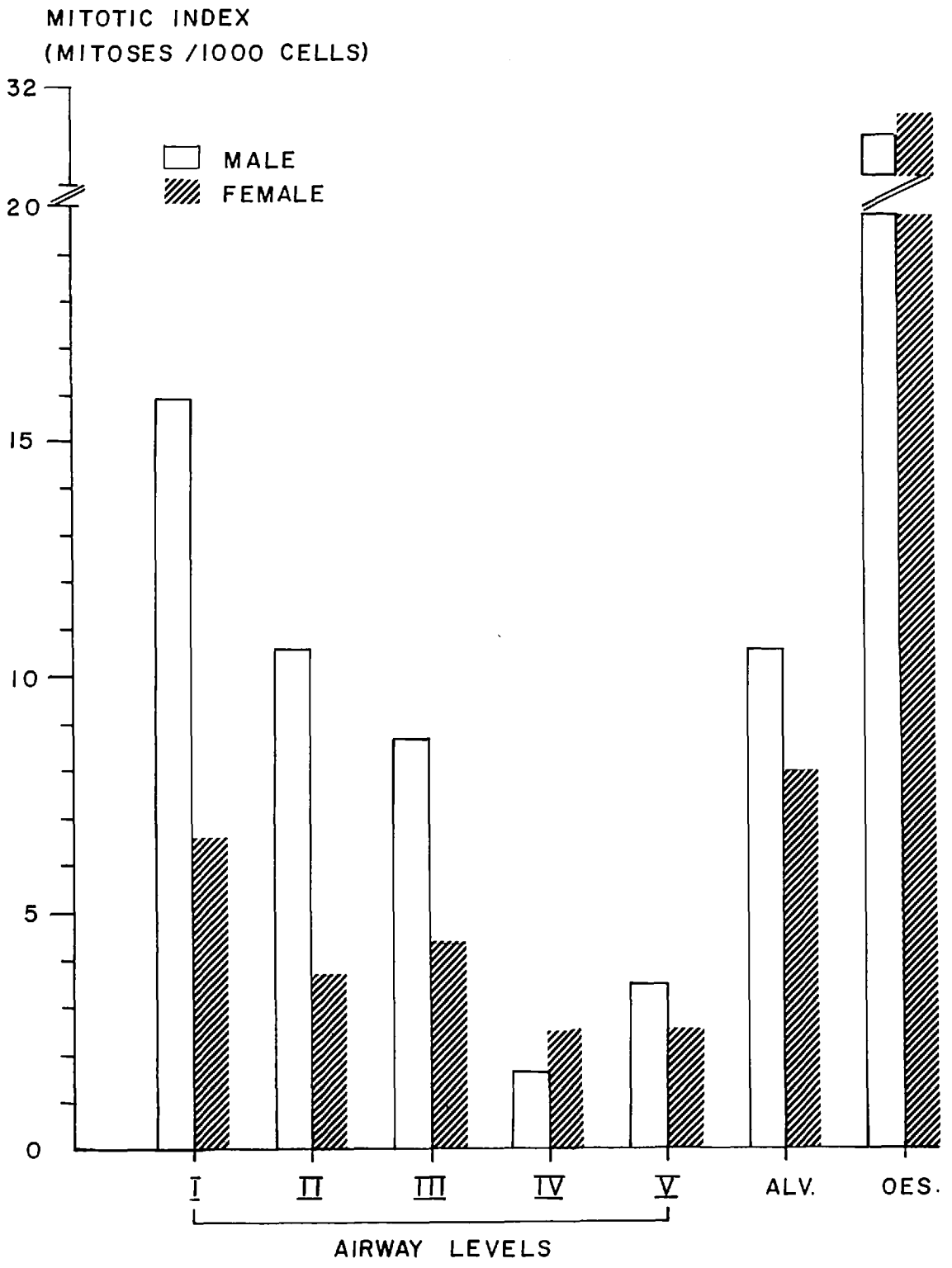

MITOTIC INDEX
(MITOSES /1000 CELLS)
32
20
15
10
5
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Table III-2

Position of mitotic figures in relation to basement membrane of respiratory epithelium of young animals treated with colchicine and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	M	F	M	F	M	F
Trachea						
Upper 1/3	11.4(71)*	4.2(64)	3.4(21)	2.1(31)	1.2(7.5)	0.3(4.6)
Mid 1/3	7.3(69)	3.1(82)	2.4(23)	0.4(11)	0.8(7.5)	0.2(6.6)
Lower 1/3	4.8(61)	3.3(78)	2.3(29)	0.8(18)	0.8(10)	0.1(3)
Axial Bronchus	0.6(41)	1.1(47)	0.5(33)	0.6(26)	0.4(25)	0.6(26)
Lateral Bronchus	0.6(18)	1.5(52)	0.9(26)	0.4(13)	1.9(55)	1.0(35)

( )* Expressed as a percentage of cells at each intra-epithelial site.

be suitable for analysis. Table III-3 shows the number of mitotic figures in both the colchicine and tritiated thymidine-treated animals at all levels of the respiratory tract.

At all airway levels, there was no significant difference between the two groups. In the level of the alveoli and oesophagus, the differences were significant. In Figure III-2, the progressive decrease in mitotic figures in both the colchicine and tritiated thymidine groups, from the upper part of the trachea to the level of the axial bronchus, is shown.

IV - Turnover time in days at different airway levels in both males and females, and in both the colchicine and tritiated thymidine groups

The turnover time in days in both the male and female, and in the colchicine and tritiated thymidine groups of animals is shown in Figure III-3.

SECTION B: MIDDLE AGED ANIMALS

I - Mitotic index in the colchicine-treated animals and at different airway levels

In both the male and the female group, the total number of mitotic figures fell progressively from the upper tracheal level to the lateral bronchus, although the difference in the tracheal levels is not as striking as in the young animal group. A difference between the male and female groups is no longer apparent

Table III-3

Number of mitoses(/1, 000 cells) in the colchicine and tritiated thymidine groups at all airway levels.

Airway Level	TOTAL		P
	Colchicine	^{3}H-T	
Trachea			
Upper 1/3	14.2	16.5	0.9
Mid 1/3	8.9	20.2	0.2
Lower 1/3	7.0	-	-
Axial Bronchus	2.2	4.7	0.1
Lateral Bronchus	2.6	7.5	0.02

Figure III-2: Number of mitoses at different airway levels in the young animal group. Comparison of the results using colchicine and tritiated thymidine.

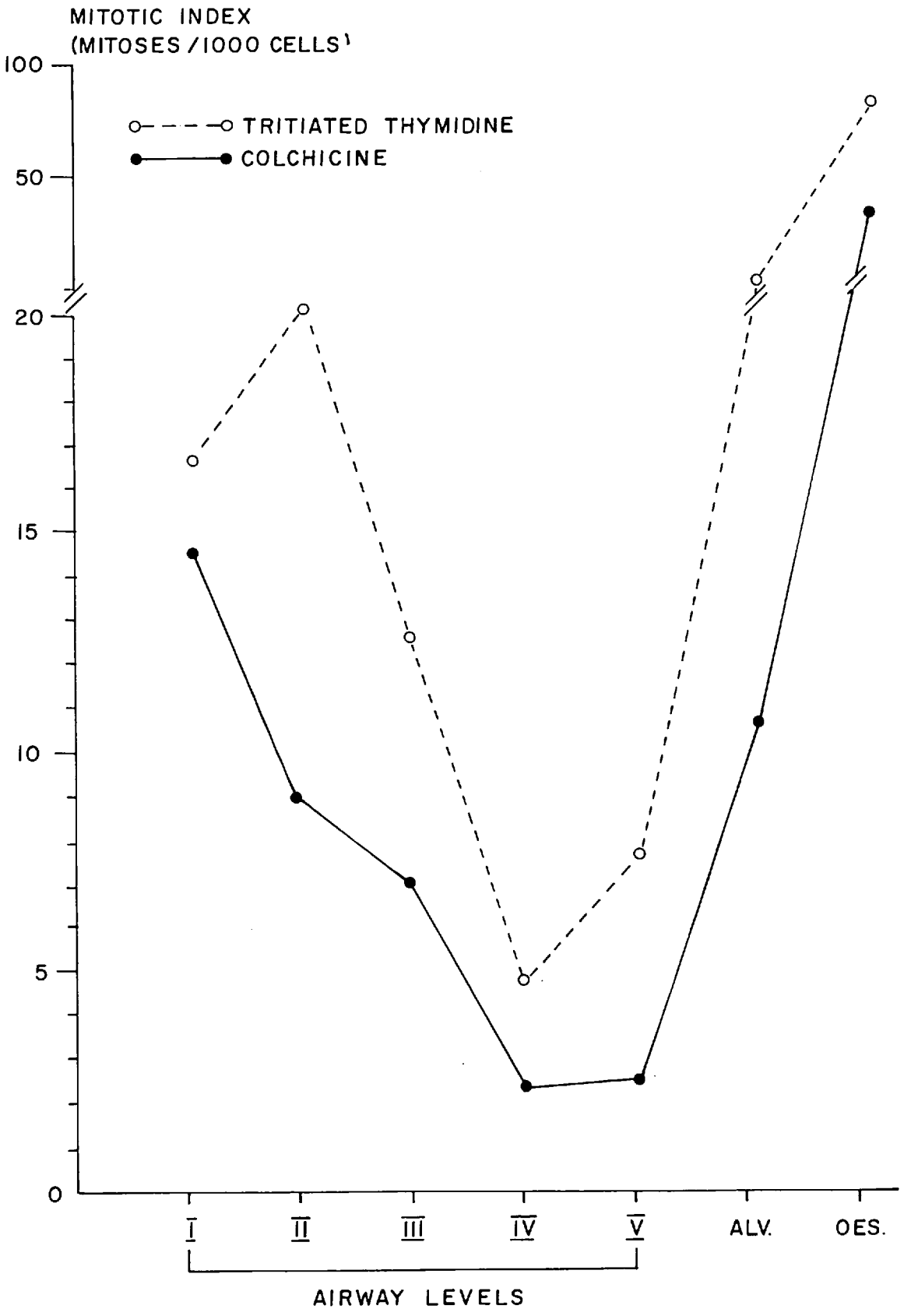
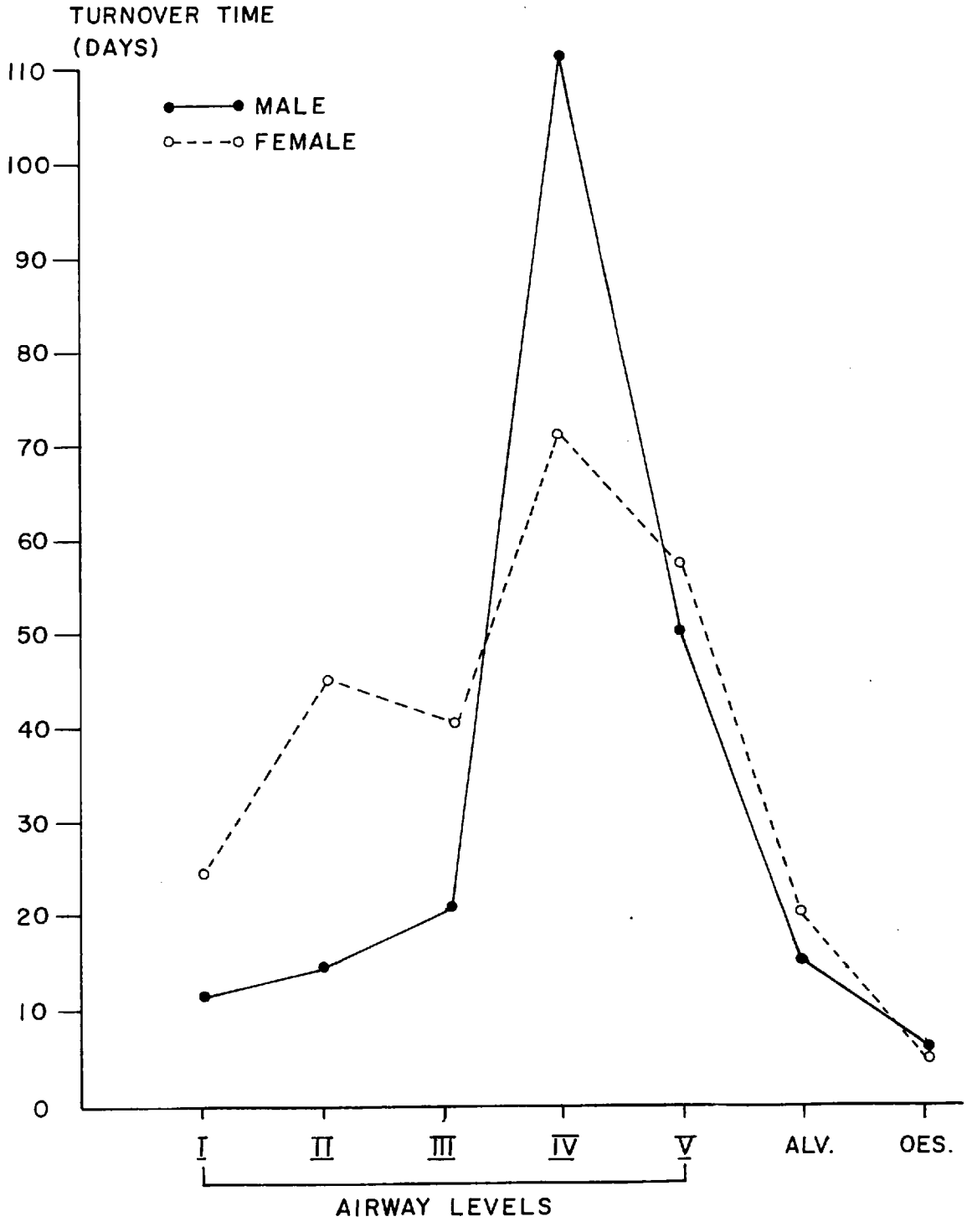

MITOTIC INDEX
(MITOSES / 1000 CELLS)
100
50
20
15
10
5
0
TRITIATED THYMIDINE
COLCHICINE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Figure III-3: Turnover time in days in young animals. The turnover time of the male and the female groups is presented.


TURNOVER TIME
(DAYS)
110
100
90
80
70
60
50
40
30
20
10
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

(Fig III-4).

II - Variation in position of the mitoses within the epithelium

As in the young animals, at the tracheal levels of both male and female animals, most of the mitoses were at the base of the epithelium, while in the axial and lateral bronchi, they were more in the superficial layers (Table III-4).

III - Total number of mitotic figures at different airway levels, in both the colchicine and the tritiated thymidine-treated animals

For this experiment, the four colchicine-treated animals were compared to two tritiated thymidine animals. Figure III-5 shows the mitotic index at all airway levels in both the colchicine and the tritiated thymidine-treated animals. There were no significant differences between the number of mitotic figures in the colchicine animals compared with the tritiated thymidine group except at the level of the oesophagus where "P" was less than 0.05. In this experiment, the progressive decrease in mitotic number from the upper level of the trachea to the peripheral airways is also shown.

IV - Turnover time in days, at different airway levels in both males and females and in both the colchicine and tritiated thymidine groups

The turnover time expressed in days in both the male and female and in the colchicine and tritiated thymidine groups of animals is shown in Figure III-6.

Figure III-4: Middle aged animals - histograms showing the mitotic index (number of mitoses/1, 000 cells) in the male and female colchicine-treated animals at different airway levels.

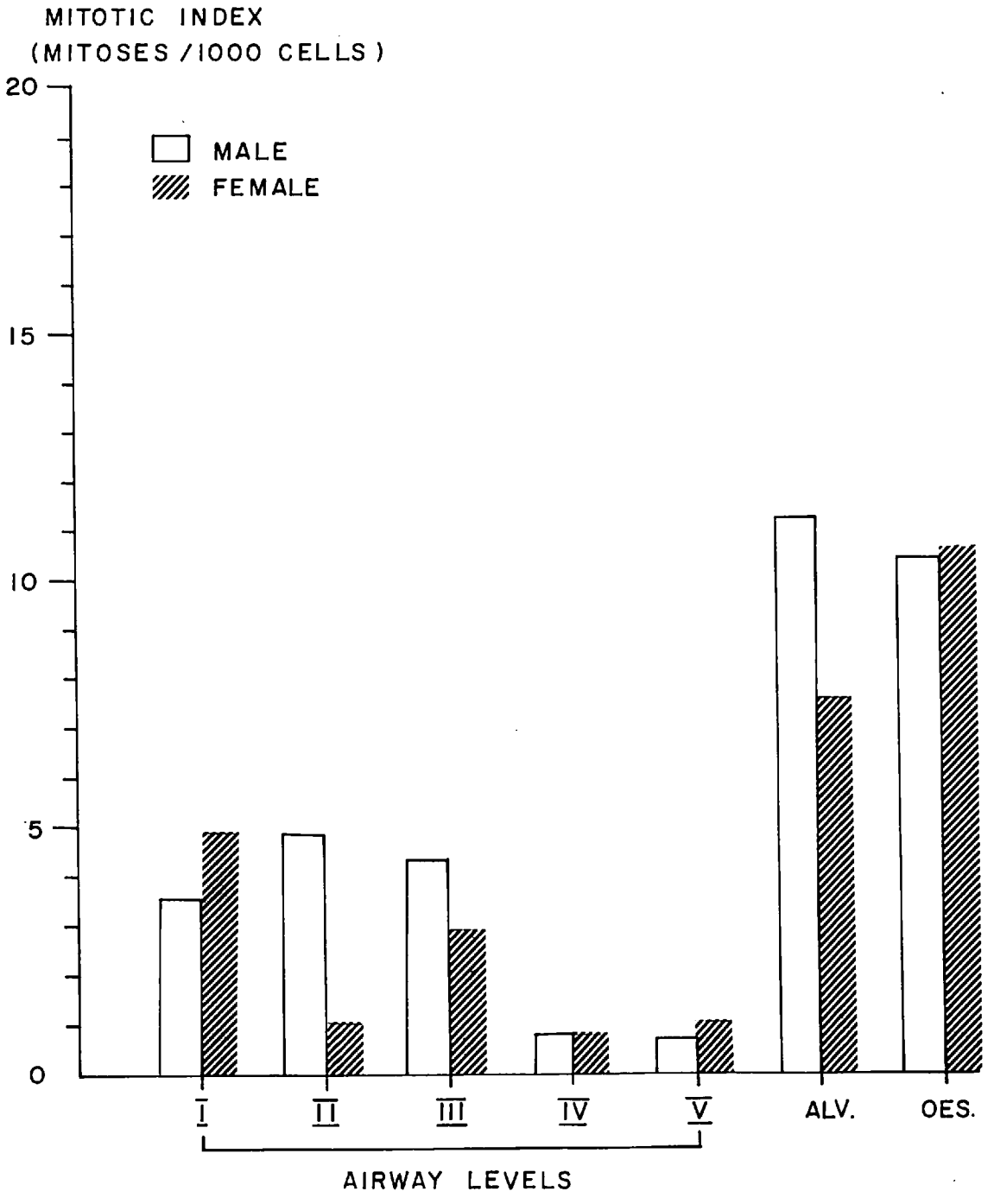

MITOTIC INDEX
(MITOSES / 1000 CELLS)
20
15
10
5
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Table III-4

Position of mitotic figures in relation to basement membrane of respiratory epithelium of middle aged rats treated with colchicine and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	M	F	M	F	M	F
Trachea						
Upper 1/3	2.6(71)*	2.5(52)	0.9(24)	1.9(40)	0.2(5)	0.4(8)
Mid 1/3	4.0(82)	0.9(87)	0.7(15)	0.1(12)	0.1(3)	0.0(0)
Lower 1/3	2.7(65)	1.7(61)	1.2(29)	0.6(22)	0.2(6)	0.5(17)
Axial Bronchus	0.2(33)	0.2(33)	0.2(33)	0.2(33)	0.2(33)	0.2(33)
Lateral Bronchus	0.2(33)	0.5(50)	0.0(0)	0.2(25)	0.5(66)	0.2(25)

( )* Expressed as a percentage of cells at each intra-epithelial site.

Figure III-5: Number of mitoses in the middle aged animal group. Comparison of the results obtained using both the colchicine and the tritiated thymidine methods at different airway levels.

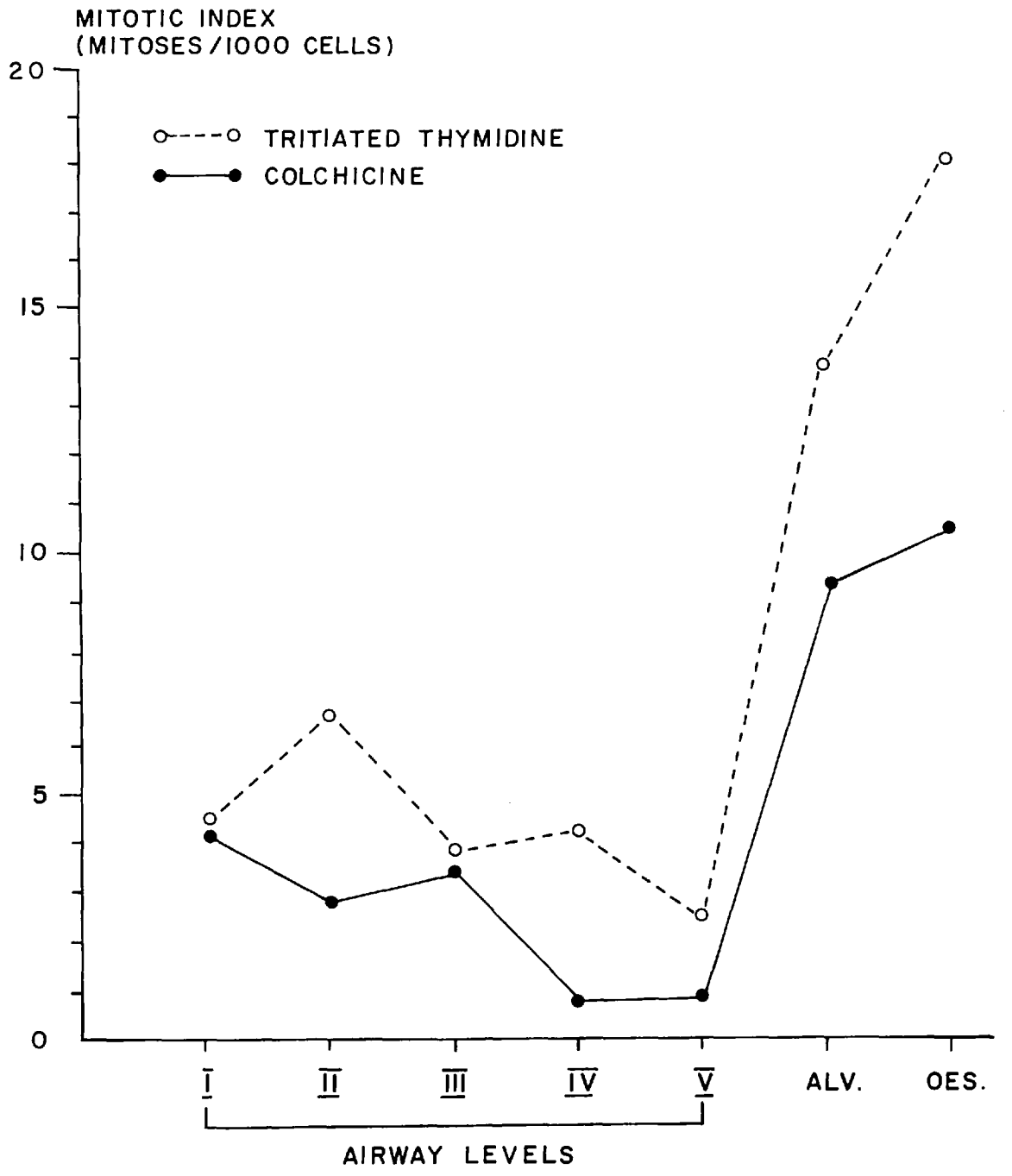
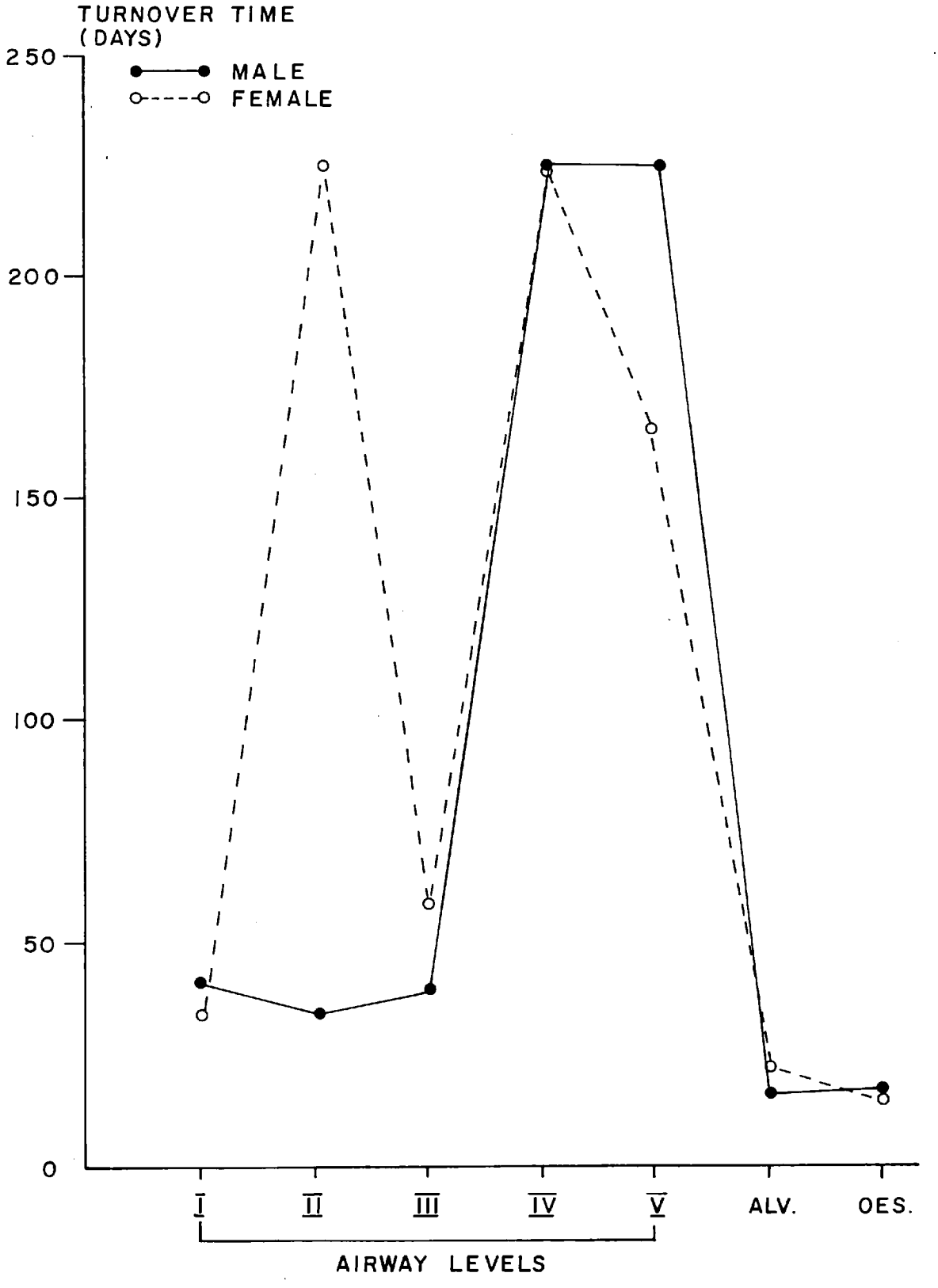

MITOTIC INDEX
(MITOSES/1000 CELLS)
20
15
10
5
0
TRITIATED THYMIDINE
COLCHICINE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Figure III-6: Middle aged animals - turnover time in days. The comparison in turnover time between the male and the female groups.


TURNOVER TIME
(DAYS)
250
200
150
100
50
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

SECTION C: OLD AGED ANIMALS

NOTE: Tritiated thymidine results were not available in this experiment because of a faulty technique.

I - Mitotic index in the colchicine-treated animals, at different airway levels

In comparison with the preceding two groups, there was no progressive decrease in the total number of mitotic figures from the upper level of the trachea to the axial bronchus and in both the male and the female groups.

In these animals, no difference was seen between male and female in the various airway levels, save in the lateral airways. Here, more mitoses were found in male animals and the differences was significant ($p < 0.05$) (Fig III-7).

II - Variation in position of the mitoses within the epithelium

In contrast to the other two age groups, cells in mitosis were mainly located at the base of the epithelium in both the central and the peripheral airways.

III - Turnover time in days, at different airway levels in both the male and female animals

Figure III-8 shows the turnover time in days in both the male and female animals. The wide fluctuation in the number of mitotic figures is due to the fact that very few mitoses were present. It

Figure III-7: Old aged animals - histograms showing the mitotic index (number of mitoses/1, 000 cells) in the male and female colchicine-treated animals and at different airway levels.

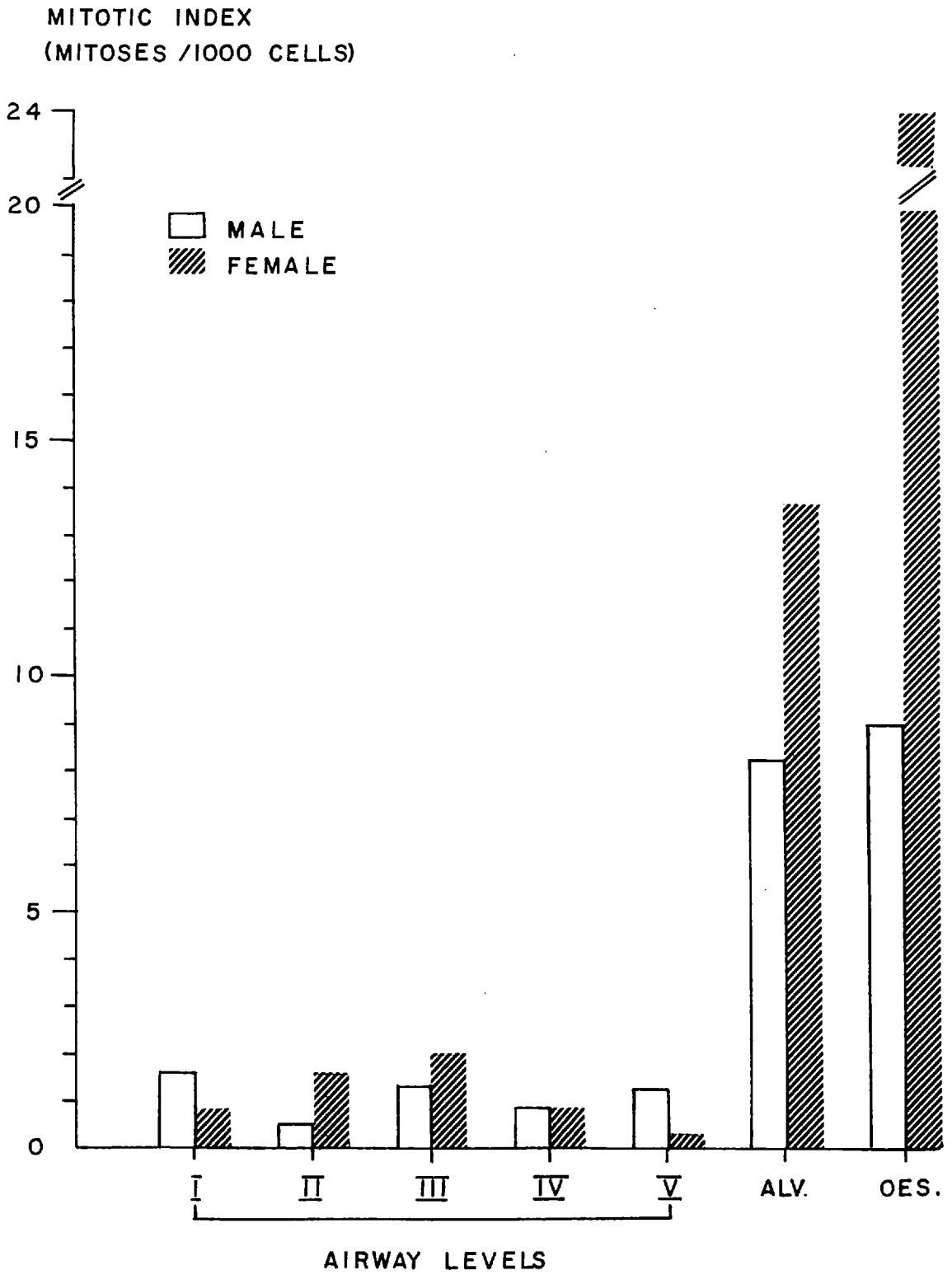

MITOTIC INDEX
(MITOSES /1000 CELLS)
24
20
15
10
5
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

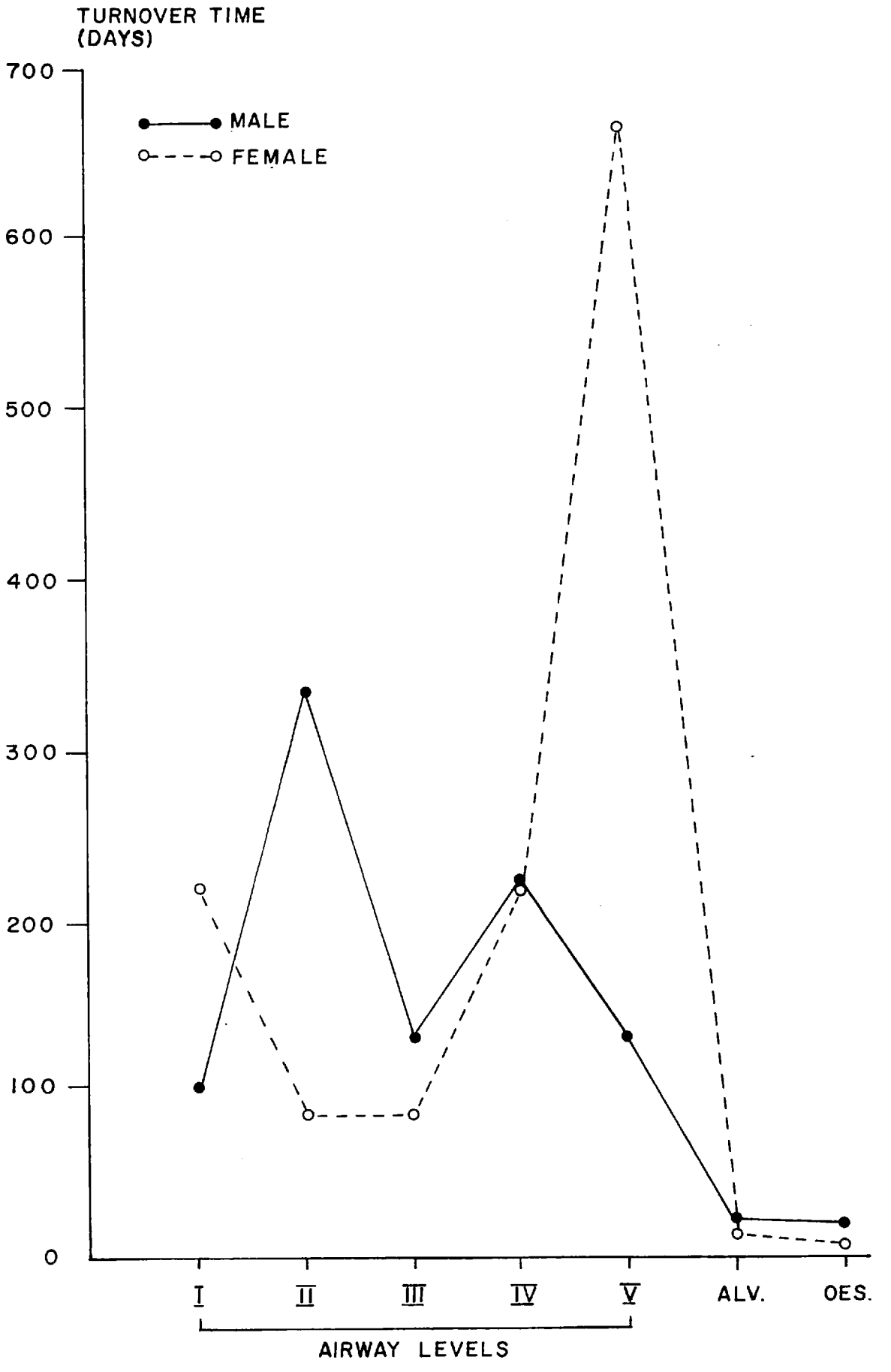
Table III-5

Position of mitotic figures in relation to basement membrane of respiratory epithelium of old aged animals treated with colchicine and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	M	F	M	F	M	F
Trachea						
Upper 1/3	1.2(77)*	0.6(83)	0.4(23)	0.0(0)	0.0(0)	0.1(16)
Mid 1/3	0.2(50)	1.5(75)	0.2(50)	0.2(12)	0.0(0)	0.2(12)
Lower 1/3	0.7(60)	1.5(75)	0.2(20)	0.0(0)	0.2(20)	0.5(25)
Axial Bronchus	0.5(66)	0.5(66)	0.0(0)	0.0(0)	0.2(33)	0.2(33)
Lateral Bronchus	1.0(80)	0.0(0)	0.0(0)	0.2(100)	0.2(20)	0.0(0)

( )* Expressed as a percentage of cells at each intra-epithelial level.

Figure III-8: Turnover time expressed in days in the old aged group of animals. The comparison in turnover time between the male and the female groups is illustrated.


TURNOVER TIME
(DAYS)
700
600
500
400
300
200
100
0
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

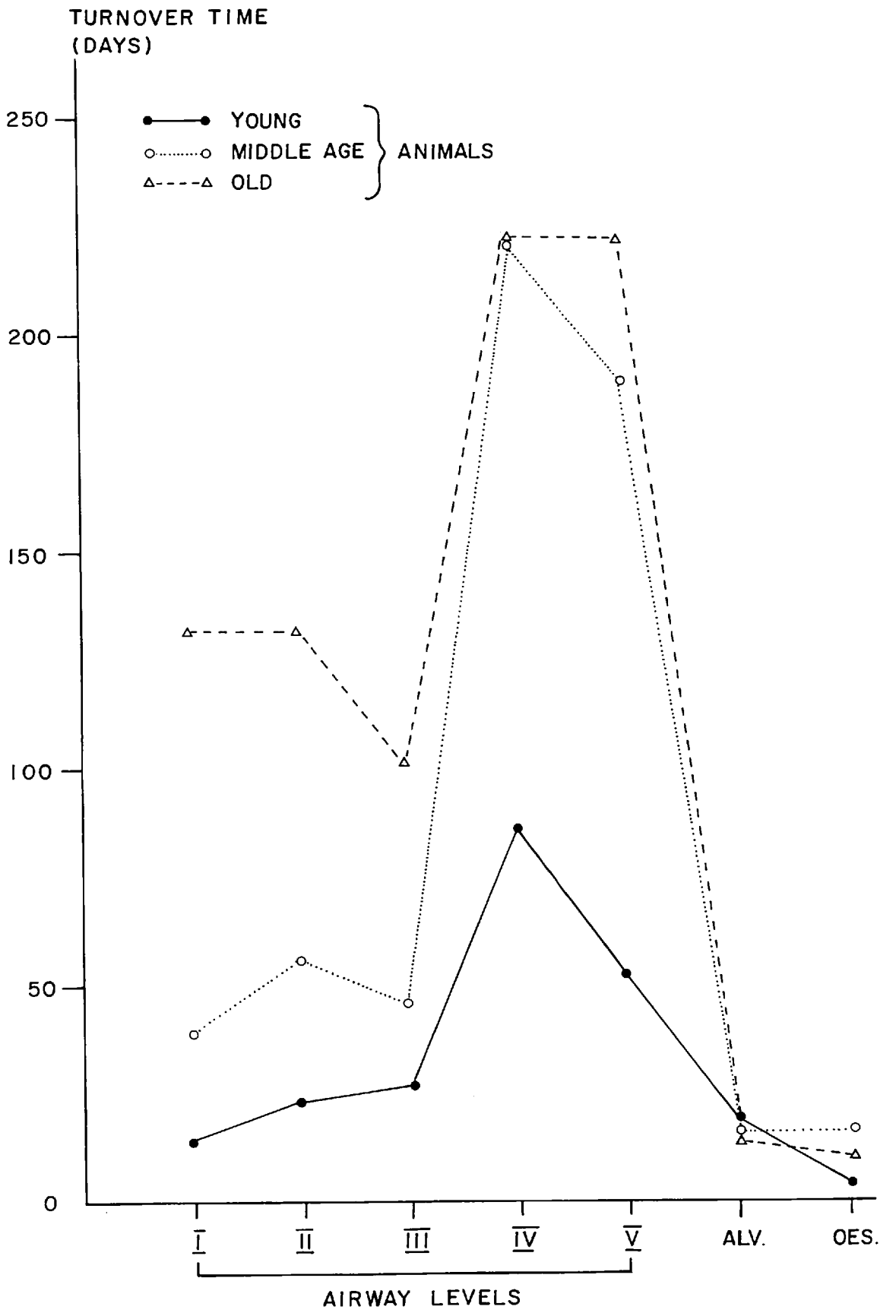
has to be noticed that in the female group of animals, the turnover time of the lateral bronchus epithelium was more than 600 days.

SUMMARY

Comparing the three age groups, the following emerged:

1) There was a higher mitotic activity in the epithelium of the upper levels of the respiratory tract compared with that of the peripheral branches, in the young and middle aged groups of animals, and in both the male and the female animals.
2) There was an increase in mitotic activity in the young males compared with the young females in three out of five airway levels.
3) In the young and middle aged group of animals, 61% to 87% of the cells in mitosis at the level of the upper part of the respiratory tract are located at the base of the epithelium, while in the peripheral airways, only 18% to 52% of the mitoses occupied this site.
4) In all experiments, except in the old aged animal group, there was no significant difference between the results obtained with either the colchicine or the tritiated thymidine techniques, except for the oesophagus.
5) As shown in Figure III-9, there was a significant difference between the turnover time in the young animals compared

Figure III-9: Turnover time in days at all airway levels and in the three groups of animals.


TURNOVER TIME
(DAYS)
250
200
150
100
50
0
YOUNG
MIDDLE AGE
OLD
ANIMALS
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

with the old animals at all airway levels. This was not seen in the alveoli. There was a significant difference between the first three levels of the trachea and the oesophagus in the young animals compared with the turnover time at the same level in the middle aged group animals. There was a significant difference between the middle aged and the old aged animals in the first and third tracheal levels.

CHAPTER IV

THE EFFECT OF ISOPRENALINE AND PILOCARPINE ON WEIGHT GAIN, MITOTIC INDEX AND TURNOVER TIME, AND THE GOBLET CELL INCREASES IN THE RESPIRATORY EPITHELIUM

CONTENTS

Introductory Note

To follow the effect of IPN and PCP, 24 albino rats from Carworth-Europe were used, grouped as in Table IV-1. Details of the experimental program are given in Table IV-2.

Table IV-1

Total number of animals used in the IPN and PCP group, the controls, and in the cleanliness group.

		Sex M	Sex F	Total
IPN	Colchicine	3	3	6
	Tritium	2	2	4
PCP	Colchicine	2*	3	5
	Tritium	2	2	4
Controls	Colchicine	1	1	2
	Tritium	0	0	0
Animals for cleanliness control		1	1	2
Total		11	12	23

*One animal died from a drug overdose after the fourth injection of PCP.

Table IV -2

IPN and PCP: Experimental program

Drug	Sex	Animal NB	DAYS OF INJECTION 3	4	5	6	7	8	9	10	11	12	13	14	15	16	Mitotic Count Agent
IPN	M	1															Colch
		2															Colch
		3															^{3}H-T
		4															Colch
		5															^{3}H-T
	F	1															Colch
		2															^{3}H-T
		3															Colch
		4															^{3}H-T
		5															Colch
PCP	M	1															Colch
		2															^{3}H-T
		3						-	-	-	-	-	-	-	-	-	-
		4															Colch
		5															^{3}H-T
	F	1															^{3}H-T
		2															Colch
		3															Colch
		4															^{3}H-T
		5															Colch
Control	M	1															Colch
	F	2															Colch
Cleanliness Control	M	1										+	-	-	-	-	-
	F	2										+	-	-	-	-	-
Doses in mg	IPN										10	10	15	15	15	15	
	PCP		6	8	10	10	4	4	4	4	4	6	6	6	6	6	

SECTION A: WEIGHT GAIN

The animals were weighed daily during the experiment and the weight of those in each of the groups (isoprenaline, pilocarpine, and control) is given in Figure IV-1. Statistical analysis showed that:

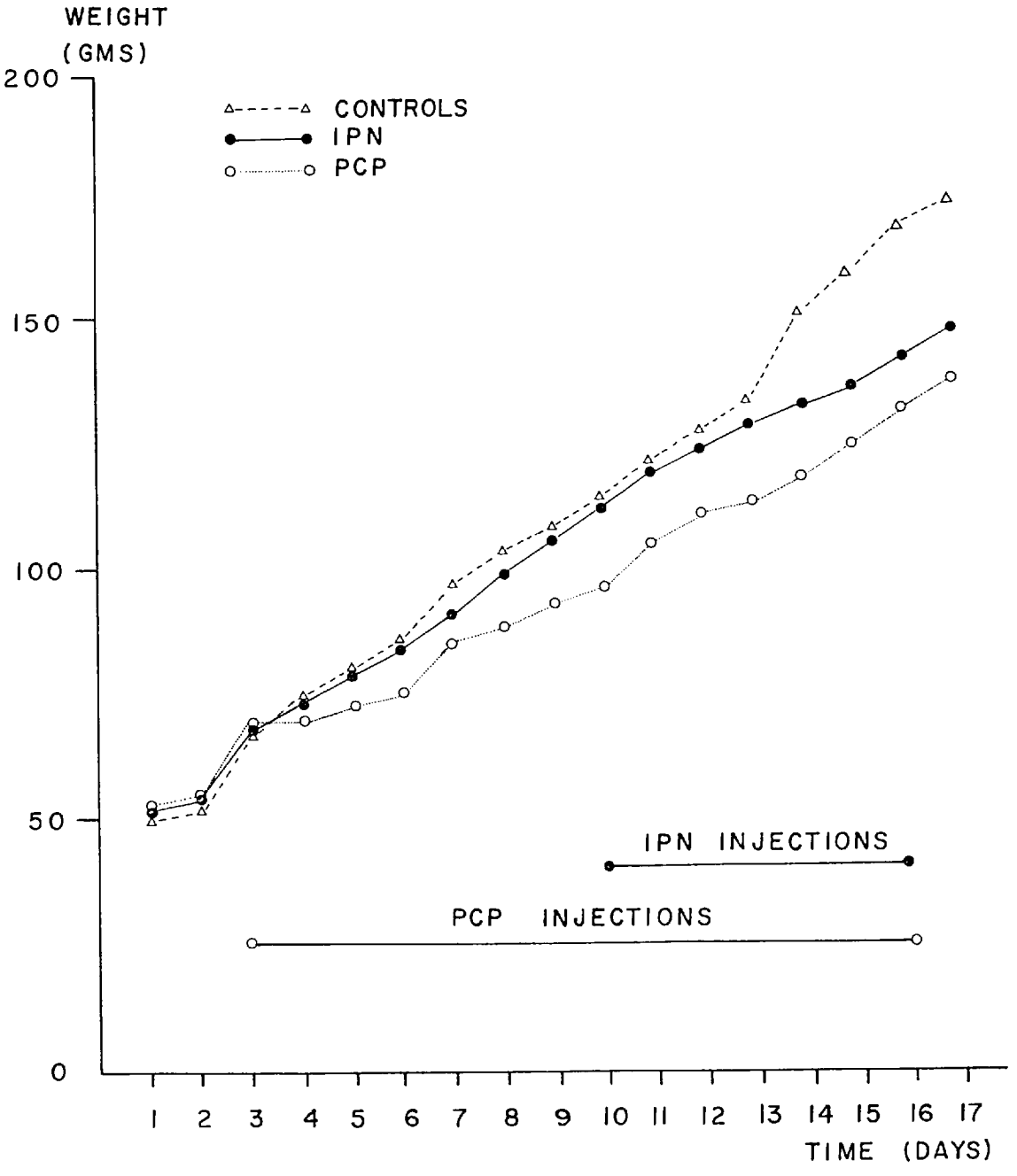
1) In the IPN group, the animals were significantly lighter than those in the control group after four injections ($p < 0.01$).
2) In the PCP group, the animals were lighter than in the control group, but this reached significance only after seven injections ($p < 0.05$). The difference in weights between these two groups increased with the number of injections, and by the middle of the second week of injections, this difference reached a highly significant level ($p < 0.001$).
3) There was no significant difference in weight between the IPN and the PCP groups of animals.
4) There was no significant difference in weight between the males and the females in the three groups of animals.

SECTION B: MITOTIC INDEX

I - The effect of isoprenaline

The total number of mitotic figures in the colchicine-treated

Figure IV-1: Weight gain in each of the three groups of animals


WEIGHT
(GMS)
200
150
100
50
0
CONTROLS
IPN
PCP
IPN INJECTIONS
PCP INJECTIONS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TIME (DAYS)

animals, in both males and females and at all the levels of the respiratory tract and oesophagus is shown in Table IV-3. These results are also presented as histograms in Figure IV-2.

From these figures, it is seen that:

1) Isoprenaline produced an increase in mitotic activity in the first four levels of the respiratory tract and in the oesophagus, and in both the male and the female group.
2) As in the normal animals, there was a progressive decrease in the number of mitoses from the first level in the trachea to the distal airways and in both male and female animals.
3) Although there was a greater number of mitotic figures in the male group compared with the female animals, except at the level of the alveoli, statistical analysis showed no significant difference.
4) The mitotic activity was more evident in the superficial layers of the respiratory epithelium and this at all levels, and in both male and female animals.

II - The effect of pilocarpine

The total number of mitotic figures, in the colchicine-treated animals, in both males and females and at all levels of the respiratory tract and oesophagus, is shown in Table IV-4. These results are also

Table IV-3

Position of mitotic figures in relation to basement membrane of respiratory epithelium of the IPN-colchicine-treated animals and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	M	F	M	F	M	F
Trachea						
Upper 1/3	6.3(52)*	3.7(58)	2.0(16)	1.8(30)	3.9(32)	0.7(12)
Mid 1/3	4.8(55)	2.3(88)	2.3(26)	0.2(6)	1.7(19)	0.2(6)
Lower 1/3	6.8(58)	1.5(47)	2.0(17)	0.7(21)	3.0(25)	1.0(32)
Axial Bronchus	2.0(40)	1.0(60)	0.7(15)	0.7(40)	2.2(45)	0.0(0)
Lateral Bronchus	1.0(24)	0.7(79)	0.5(11)	0.2(21)	2.8(65)	0.0(0)

( )* Expressed as a percentage of cells at each intra-epithelial site.

Figure IV-2: Mitotic index in the IPN-COLCHICINE-treated animals, at all levels and in both the male and the female groups of animals.

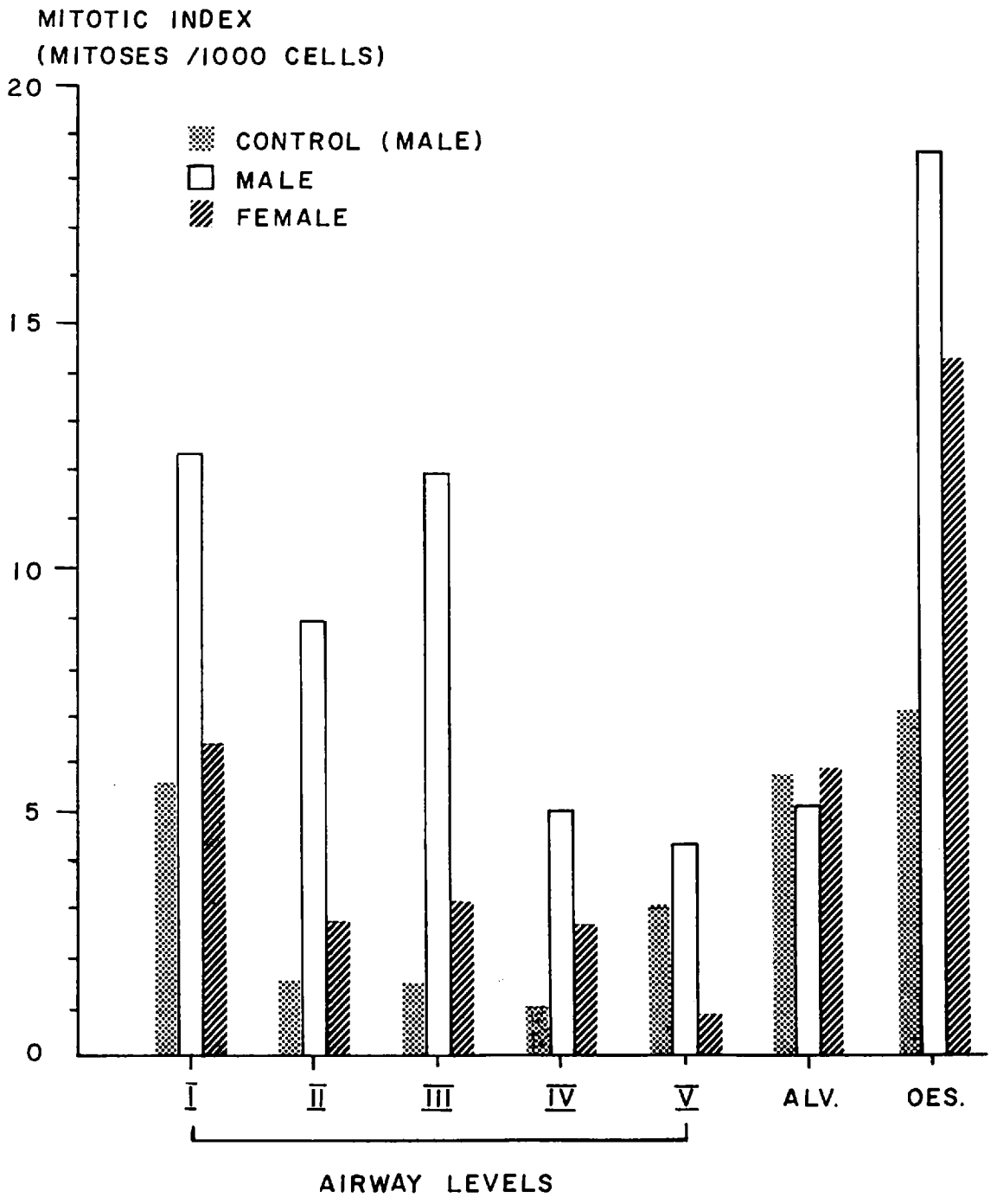

MITOTIC INDEX
(MITOSES /1000 CELLS)
20
15
10
5
0
CONTROL (MALE)
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Table IV-4

Position of mitotic figures in relation to basement membrane of respiratory epithelium of the PCP-colchicine-treated animals and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	M	F	M	F	M	F
Trachea						
Upper 1/3	4.9(60)*	3.7(49)	0.9(11)	3.2(42)	2.4(29)	0.7(9)
Mid 1/3	3.5(67)	2.7(32)	0.0(0)	4.7(56)	1.7(33)	1.0(12)
Lower 1/3	2.2(28)	3.7(35)	1.5(19)	3.7(36)	4.2(53)	3.0(29)
Axial Bronchus	1.2(28)	0.8(19)	0.2(5)	2.2(50)	3.0(67)	1.3(31)
Lateral Bronchus	0.5(22)	1.1(54)	0.2(11)	0.3(15)	1.5(67)	0.7(31)

( )*Expressed as a percentage of cells at each intra-epithelial site.

presented as histograms in Figure IV-3.

These figures showed that:

1) Pilocarpine produced an increase in mitotic activity in the first four levels of the respiratory tract and in the oesophagus, and in both the male and female groups.
2) There was a smaller number of mitoses in the distal airways compared to the first three levels of the respiratory tract.
3) There was no difference between the male group of animals and the female.
4) The mitotic activity was more evident in the superficial layers of the respiratory epithelium and this at all levels, and in both male and female animals.

III - Comparison between the effect of isoprenaline and pilocarpine on the mitotic activity of the respiratory epithelium

The mean number of mitoses in the three groups of animals is shown in Table IV-5. The corresponding turnover time in days is also presented in the table and in Figure IV-4. Although there was an increased number of mitoses in both the IPN and PCP groups, compared to the control group, statistical analysis showed a significant difference in the second, third and fourth levels of the airway and in the oesophagus. The control group showed an increase in

Figure IV-3: Mitotic index in the PCP-COLCHICINE-treated animals, at all levels and in both the male and the female groups of animals.

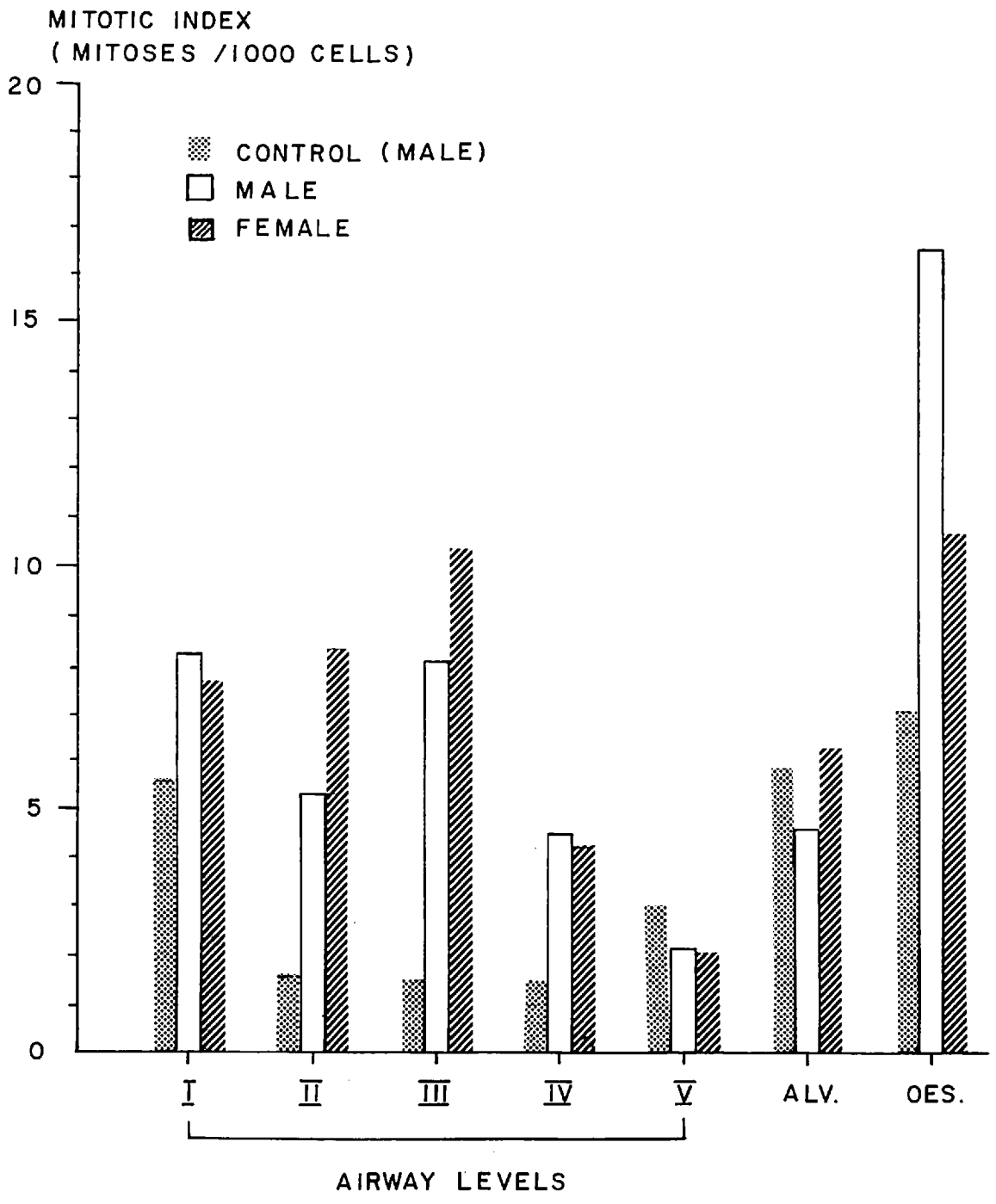
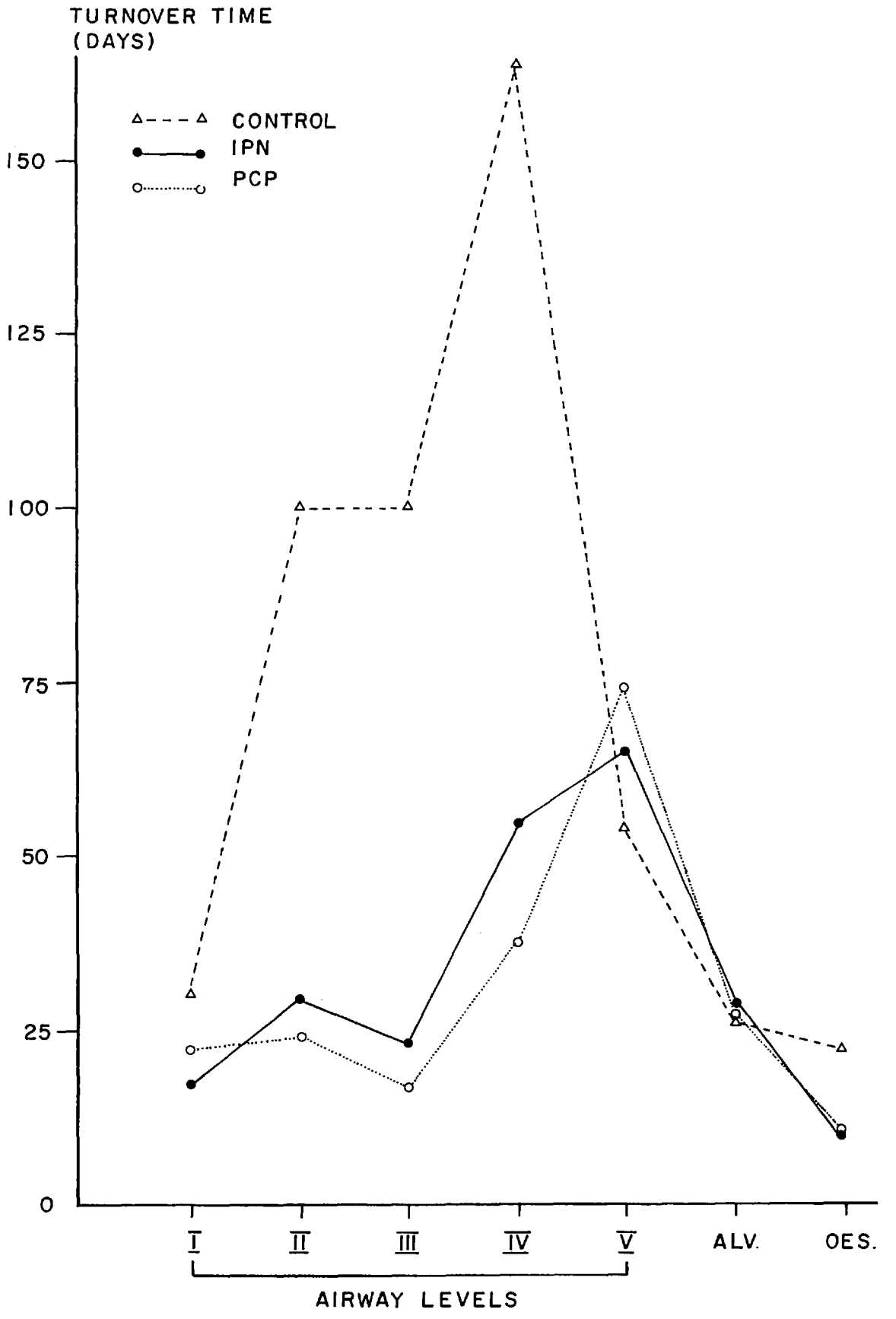

MITOTIC INDEX
(MITOSES /1000 CELLS)
20
15
10
5
0
CONTROL (MALE)
MALE
FEMALE
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

Table IV-5

Mean number of mitoses in the three groups of animals and the corresponding turnover time in days.

	MITOTIC COUNTS			TURNOVER TIME		
	IPN	PCP	Control	IPN	PCP	Control
Trachea Upper 1/3	9.2	7.8	5.5	18.1	21.3	30.2
Trachea Mid 1/3	5.7	7.1	1.5	29.2	23.4	111.0
Trachea Lower 1/3	7.5	9.4	1.5	22.2	17.7	111.0
Ax. Bronch.	3.0	4.4	1.0	55.5	37.8	166.6
Lat. Bronch.	2.6	2.2	3.0	66.6	75.7	55.5
Alveoli	5.5	5.6	5.7	30.2	29.7	29.2
Oesophagus	16.4	15.0	7.0	10.2	11.1	23.8

Figure IV-4: Turnover time in days in the three groups of animals.


TURNOVER TIME
(DAYS)
CONTROL
IPN
PCP
150
125
100
75
50
25
0
I
II
III
IV
V
ALV.
OES.
AIRWAY LEVELS

mitotic activity at the level of the lateral airways compared to the IPN and PCP groups. There was no significant difference in the turnover time at all levels either in the pilocarpine, or the isoprenaline animals, and no difference either at the alveoli level in the three groups of animals.

SECTION C: GOBLET CELLS

The number, size, and staining characteristics of the goblet cells were quantified in three regions of the bronchial tree: the trachea (upper third), the axial and lateral airways of the lung. As previously described by Sturgess and Reid, four types of goblet cells are described.

1) Large blue: Secretory granules occupying a large area in the goblet cell and staining blue with AB.
2) Small blue: Secretory granules occupying only the apex of the goblet cell and staining blue with AB.
3) Large red: Secretory granules occupying a large area in the goblet cell and staining red with PAS.
4) Small red: Secretory granules occupying only the apex of the goblet cell and staining red with PAS.

I - Goblet cell increase with isoprenaline

After the six injections of isoprenaline, there was an increase in goblet cell numbers at all levels in the bronchial tree (Table IV-6).

Table IV-6

Total number of goblet cells in both the IPN and the control groups and at the three levels of the airway.

Group	Trachea Up 1/3	Axial Bron.	Lateral Bron.
IPN [redacted]	70.16	84.20	51.66
Control	14.00	38.00	36.00
p	$p < 0.001$	$p < 0.02$	$p < 0.3$

In the trachea, the increase in small and large blue goblet cells was highly significant. In the axial bronchus, the significant increase in goblet cell numbers was mainly due to the increase in the small blue and small red goblets. In the lateral bronchus, the overall increase in goblet cell numbers did not reach a significant level. However, the small blue goblet cells were predominant (Fig IV-5).

II - Goblet cells increase with pilocarpine

After the twelve injections of pilocarpine, there was an increase in goblet cell number in the upper trachea and in the axial bronchus. However, this increase was significant only at the level of the trachea as shown in Table IV-7. At this level, the increase in the number of small and large blue goblet cells was significant. In the axial bronchus, although the overall increase in goblet cells did not reach significant level, mainly small and large blue goblet cells were increased. In the lateral bronchus, there was no significant increase in the total number of goblet cells (Fig IV-6).

SECTION D: GOBLET CELLS AND MITOSES

I - Relation between the goblet cell increase and the number of mitoses in the IPN group

As shown in Figure IV-7, there was a good correlation between

Figure IV-5: Goblet cells increase (four types) in the IPN group of animals and at the three levels of the respiratory tract.

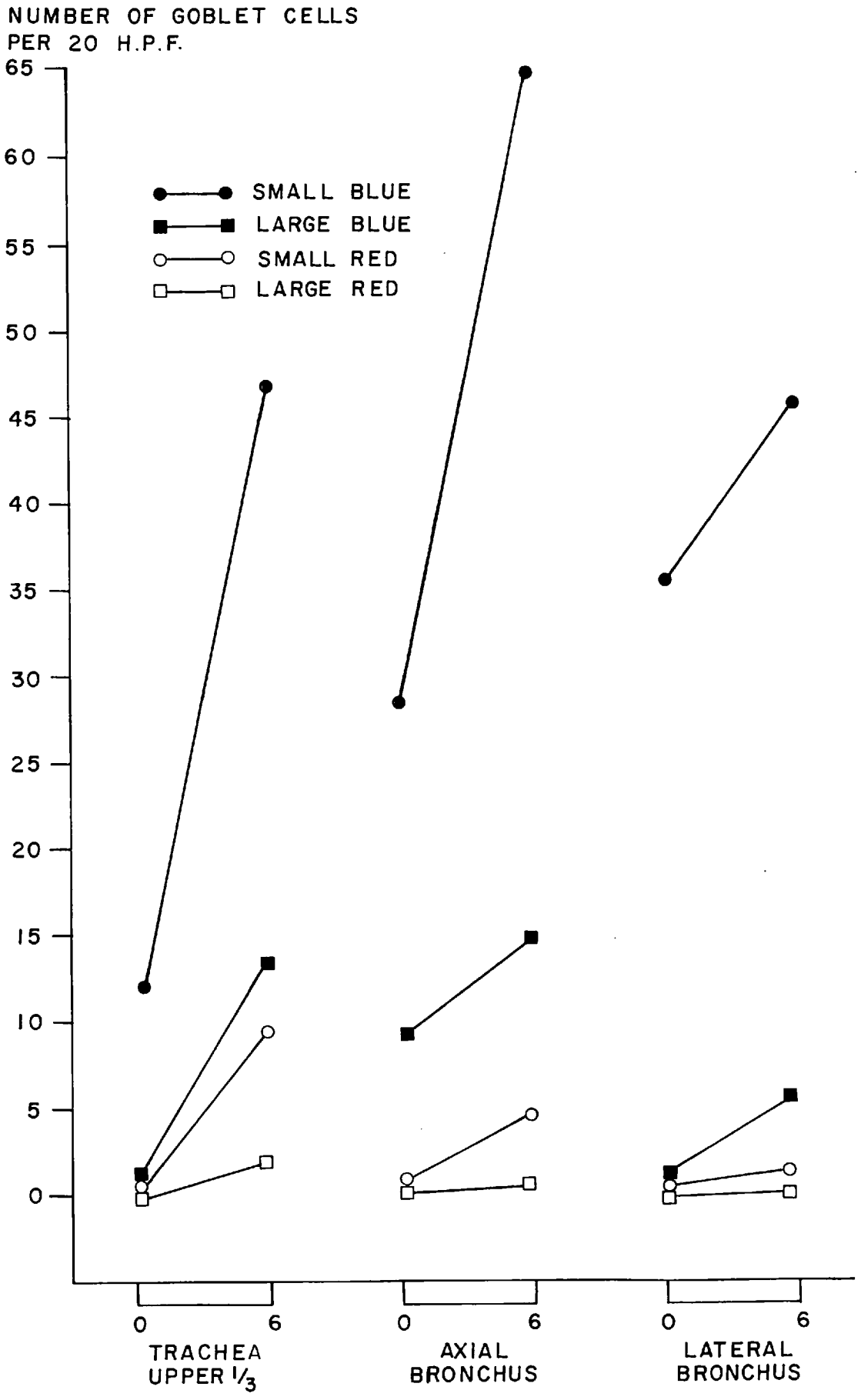

NUMBER OF GOBLET CELLS
PER 20 H.P.F.
65
60
55
50
45
40
35
30
25
20
15
10
5
0
SMALL BLUE
LARGE BLUE
SMALL RED
LARGE RED
0
6
TRACHEA
UPPER 1/3
0
6
AXIAL
BRONCHUS
0
6
LATERAL
BRONCHUS

Table IV-7

Total number of goblet cells in both the PCP and the control groups and at all three levels of the airway.

Group	Trachea Up 1/3	Axial Bron.	Lateral Bron.
PCP	77.40	63.40	30.80
Control	14.00	38.00	36.00
p	$p < 0.01$	$p < 0.4$	-

Figure IV-6: Goblet cells increase (four types) in the PCP group of animals and at the three levels of the respiratory tract.

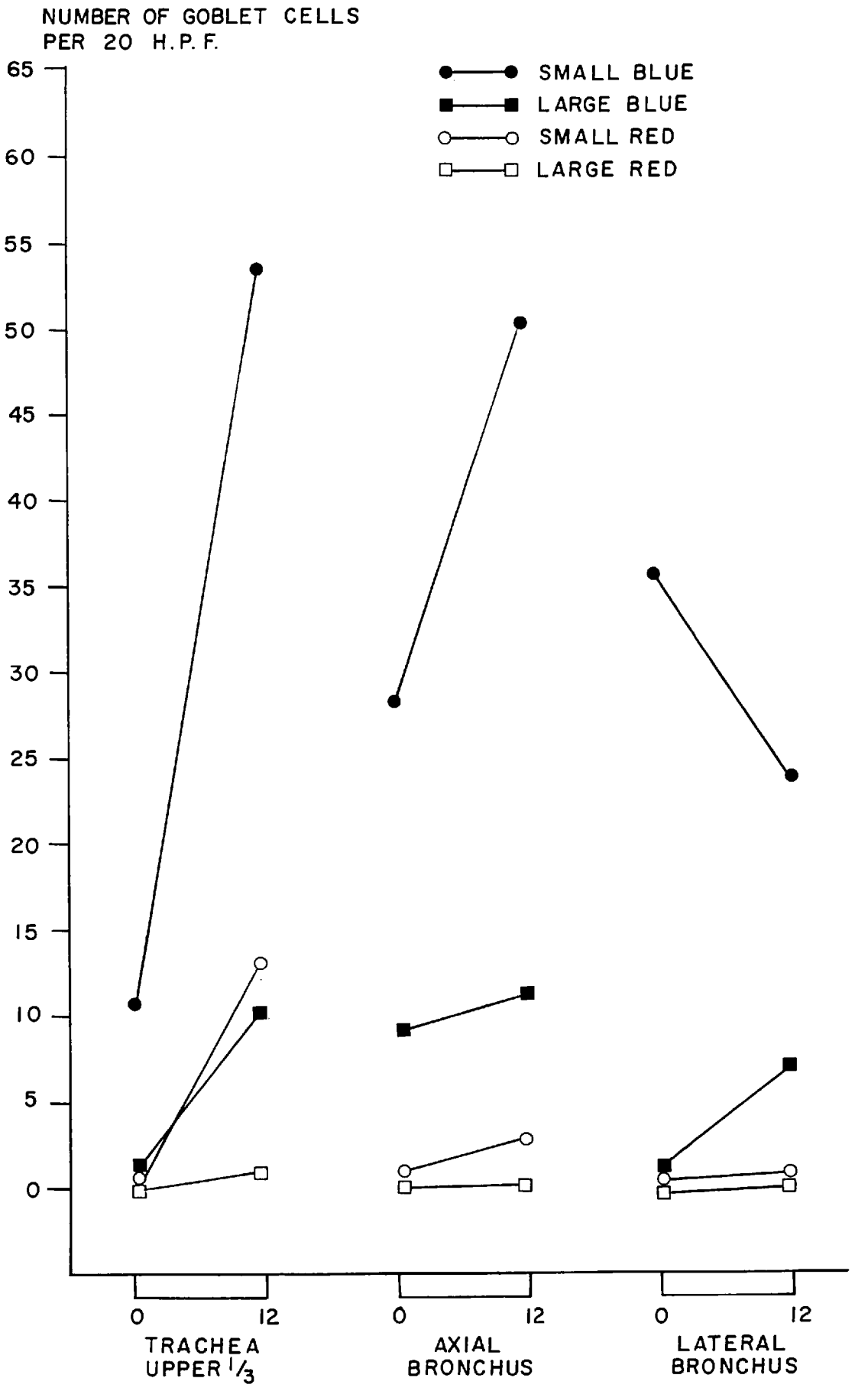
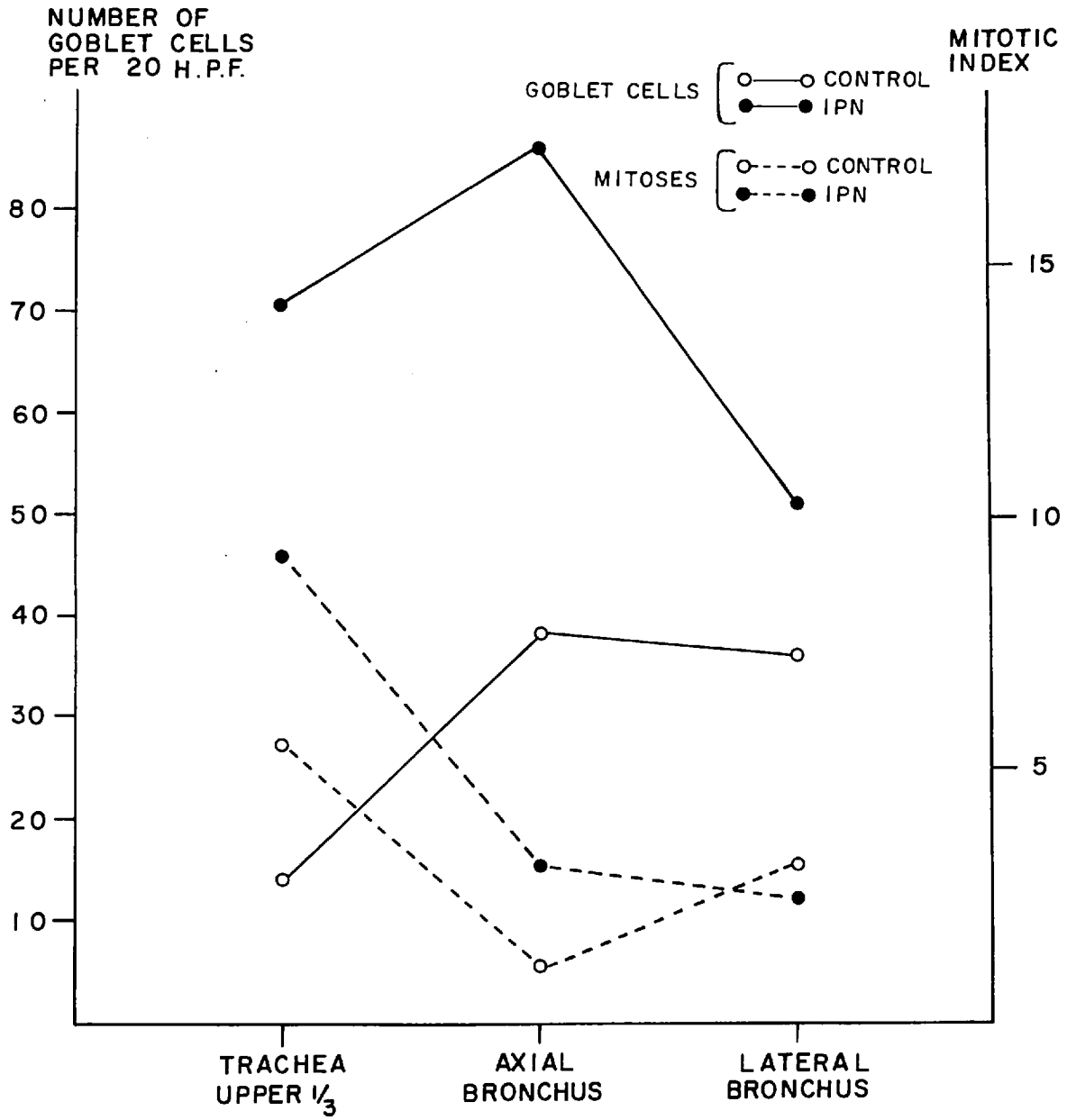

NUMBER OF GOBLET CELLS
PER 20 H.P.F.
SMALL BLUE
LARGE BLUE
SMALL RED
LARGE RED
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
12
TRACHEA
UPPER 1/3
0
12
AXIAL
BRONCHUS
0
12
LATERAL
BRONCHUS

Figure IV-7: Total number of goblet cells and mitotic figures in the IPN-treated animals and at three levels in the airway.


NUMBER OF
GOBLET CELLS
PER 20 H.P.F.
MITOTIC
INDEX
GOBLET CELLS
CONTROL
IPN
MITOSES
CONTROL
IPN
80
70
60
50
40
30
20
10
15
10
5
TRACHEA
UPPER 1/3
AXIAL
BRONCHUS
LATERAL
BRONCHUS

the number of mitoses in the upper trachea and lateral bronchus levels. From the upper part of the airway to the distal lateral airway, except at the level of the axial bronchus, where an increase in goblet cells was found, there was a fall in goblet cell numbers and a similar fall in the mitotic activity.

II - Relation between the goblet cell increase and the number of mitoses in the PCP group

As shown in Figure IV-8, there was a good correlation between the number of goblet cells and the number of mitoses in all the three levels.

As for the IPN group, there was a progressive fall in both the goblet cells and the mitotic activity from the upper trachea to the lateral bronchus.

SECTION E: AN INFECTED ANIMAL

One of the control animals was found to be infected and on histological examination, the left upper lobe presented an area of consolidation with polynuclear cell infiltration. The bronchial tree showed cuffing with lymphocytes, and purulent secretions were seen in the airway. We studied the mitotic activity of this animal's bronchial tree and also the goblet cells.

There was an increase in both the number of goblet cells and the number of mitoses in the three airway levels, and mainly in the axial bronchus near the origin of the left upper lobe lateral bronchus.

Figure IV-8: Total number of goblet cells and mitotic figures in the PCP-treated animals and at three levels in the airway.

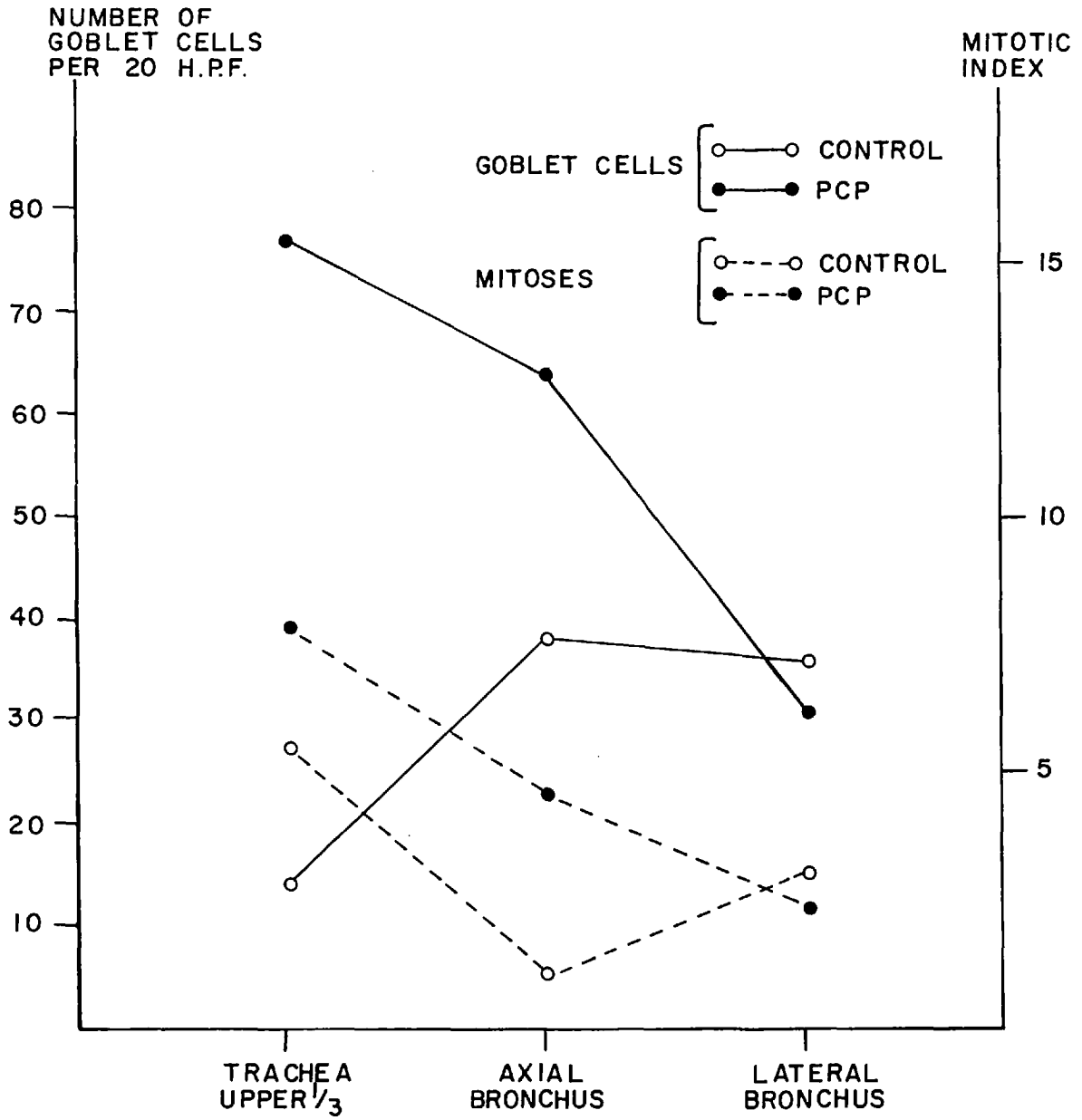

NUMBER OF
GOBLET CELLS
PER 20 H.P.F.
MITOTIC
INDEX
GOBLET CELLS
CONTROL
PCP
MITOSES
CONTROL
PCP
80
70
60
50
40
30
20
10
15
10
5
TRACHEA
UPPER 1/3
AXIAL
BRONCHUS
LATERAL
BRONCHUS

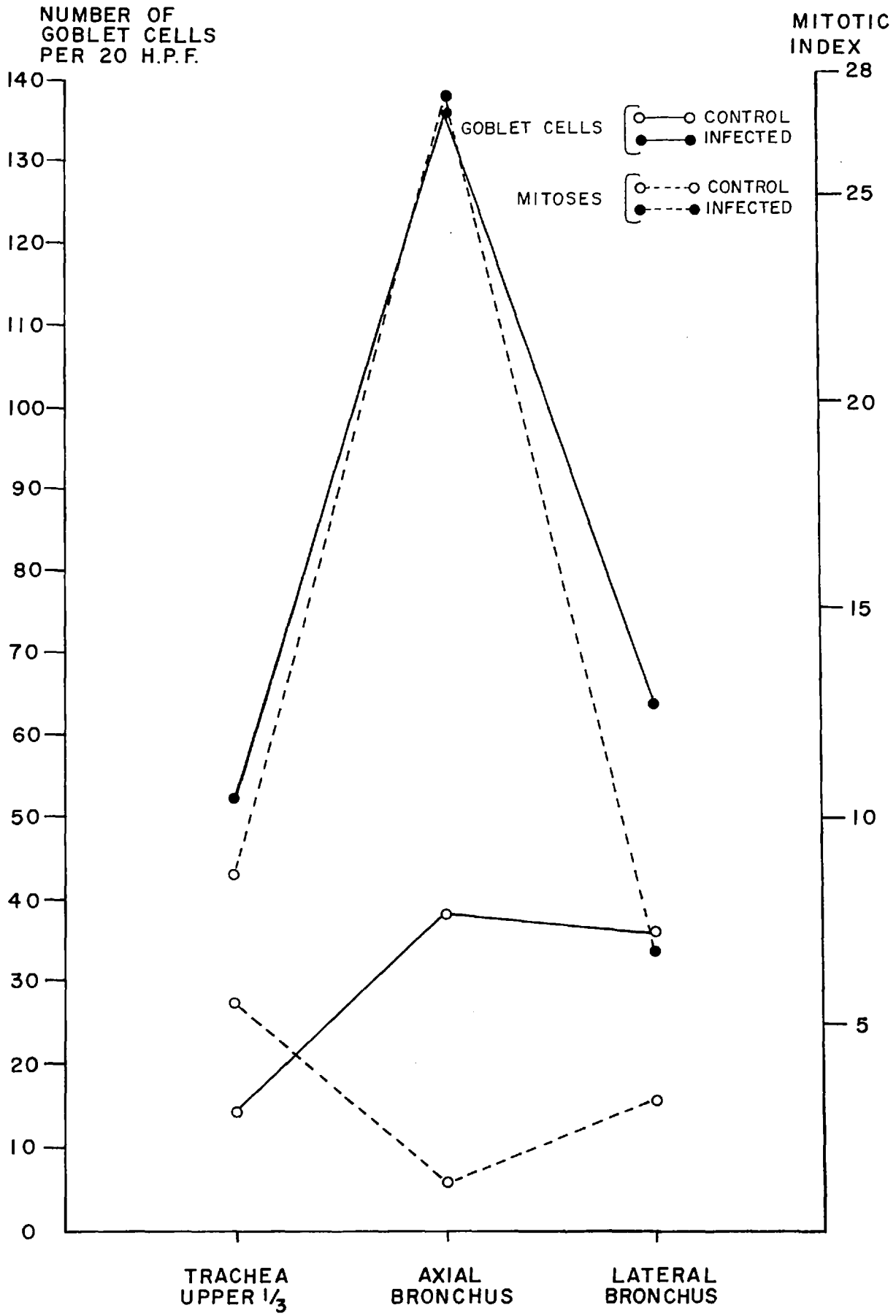
Figure IV-9 shows the similarity between the mitotic index and the number of goblet cells present at the three airway levels.

SUMMARY

After six injections of isoprenaline and twelve injections of pilocarpine, the following changes were observed:

1) Both the IPN and PCP animals were significantly ligher in weight than the controls; the isoprenaline one, after two injections of the drug and the pilocarpine ones, after seven injections.
2) There was no significant difference in the weights between the IPN and PCP groups of animals.
3) In both the isoprenaline and pilocarpine-treated animals, there was:
    a) an increase in mitotic activity in the first four levels of the respiratory tract and in the oesophagus, and in both the male and female groups
    b) As in the normal animals, there was a progressive decrease in mitotic activity from the first levels in the trachea to the distal airways in both the males and the females
    c) As for the middle aged animals in the first experiment, there was no significant difference in the mitotic activity between the males and the

Figure IV-9: Comparison between the mitotic index and the goblet cells count in an infected control animal.


NUMBER OF
GOBLET CELLS
PER 20 H.P.F.
MITOTIC
INDEX
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
28
25
20
15
10
5
GOBLET CELLS
CONTROL
INFECTED
MITOSES
CONTROL
INFECTED
TRACHEA
UPPER 1/3
AXIAL
BRONCHUS
LATERAL
BRONCHUS

females.

d) Mitotic figures were located more frequently in the superficial layer within the epithelium at all airway levels, and in both males and females.

4) There was a significant increase in the mitotic activity of both the IPN and PCP groups, when each was compared with the control animals, at the second, third and fourth airway levels and in the oesophagus.

5) There was no significant difference in the mitotic activity of both the pilocarpine and isoprenaline-treated animals.

6) In the isoprenaline group:

There was a significant increase in goblet cell number at all levels in the bronchial tree. In the trachea, this increase was due to the increase in the small and large blue goblet cells and in the axial bronchus to the increase of the small blue and small red goblet cells.

7) In the pilocarpine group:

There was a significant increase in goblet cell number in the upper third of the trachea and this increase was due to the increased number of small and large blue goblet cells.

8) In the isoprenaline, pilocarpine and the infected control animal, there was a similarity between the goblet cell number and the mitotic activity.

CHAPTER V

THE EFFECT OF TOBACCO SMOKE ON THE RAT BRONCHIAL EPITEHLIUM AND THE PROTECTION GIVEN BY PMO

SHORT TERM EXPERIMENT

CONTENTS

INTRODUCTION

The aim of this experiment is to study the short term effect of tobacco and tobacco + PMO on the mitotic activity of the rat respiratory epithelium. In Chapter VI, the results of a long term exposure are presented. For this present study, 22 male albino rats from Anticimex, Sweden, were used.

The distribution of these animals in groups and the duration of exposure are summarized in Table V-1. All animals were male and the colchicine method was used to study the mitotic activity. Details of the smoking cabinets, humidity and temperature readings are to be found in Chapter II (p. 66-69).

Table V-1

Number of animals in the tobacco, tobacco + PMO and control groups, in the various periods of exposure.

Duration of Exposure (Days)	Tobacco	Tobacco + PMO	Controls	Totals
1	2	2	1	5
2	2	2	0	4
3	2	2	0	4
7	2	2	0	4
16	2	2	1	5
TOTAL	10	10	2	22

SECTION A: THE EFFECT OF TOBACCO AND TOBACCO + PMO ON BODY WEIGHT

As shown in Figure V-1, after three days of exposure, the control animals were significantly heavier than either the tobacco group ($p < 0.02$), or the tobacco + PMO group ($p < 0.01$). After eight days of exposure, the control animals were still heavier than the animals of the two smoking groups, but the difference while significant between the control and the tobacco + PMO group ($p < 0.001$), was not significant between the control and tobacco alone group ($p < 0.3$).

On the last day of exposure to cigarette smoke, the controls were heavier than either the tobacco alone group ($p < 0.05$), or the tobacco and PMO group ($p < 0.001$), and a significant difference was seen between the two smoking groups ($p < 0.001$).

SECTION B: THE EFFECT OF TOBACCO AND TOBACCO + PMO ON THE MITOTIC ACTIVITY

The mitotic index was estimated in each animal for each of the five airway levels. The results shown in this section represent the mean of the five days of exposure.

I - Effect of tobacco

The mitotic index and the localization of the mitoses within the respiratory epithelium at all airway levels are presented in

Figure V-1: Weight gain in the three groups of animals

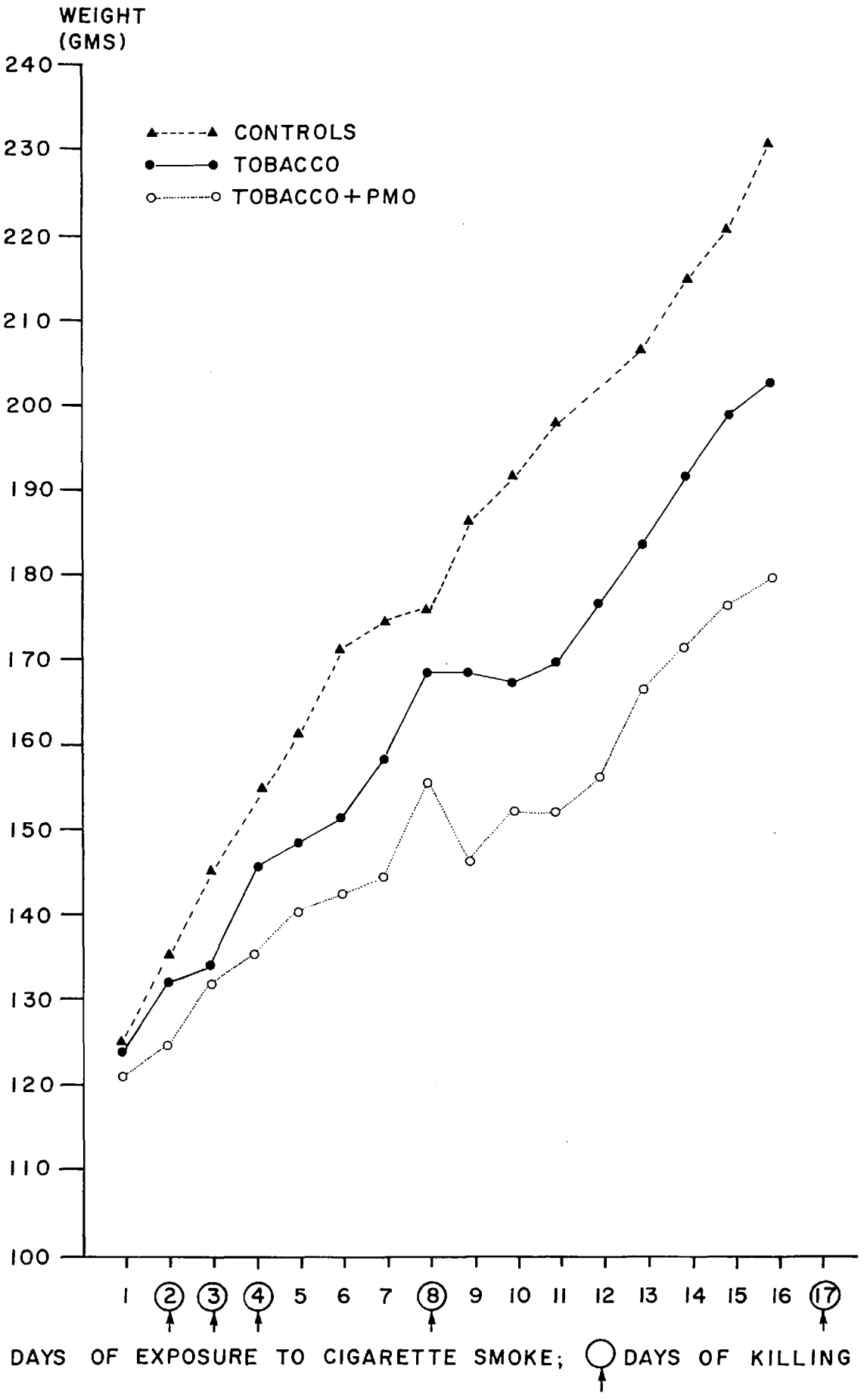

WEIGHT
(GMS)
240
230
220
210
200
190
180
170
160
150
140
130
120
110
100
CONTROLS
TOBACCO
TOBACCO + PMO
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
DAYS OF EXPOSURE TO CIGARETTE SMOKE; DAYS OF KILLING

Table V-2. There was an increase in mitotic activity at all the airway levels in the tobacco group compared to the controls.

In the first three levels of the airway, in both the tobacco and control groups, 56% to 89% of the mitoses were located at the base of the epithelium, and in axial and lateral bronchi, only 1% to 30% of the cells occupied this site.

II - Effect of tobacco + PMO

At all the airway levels, there were more mitoses in the tobacco + PMO group than in the control group, save for the lateral bronchus (Table V-3). At all levels, there were fewer mitoses in the tobacco + PMO group than in the tobacco alone group.

In the first three levels of the airway, in both the tobacco and control groups, 52% to 89% of the mitoses were situated at the base of the epithelium, while in the axial and lateral bronchi, only 1% to 23% occupied this site (Table V-3).

III - Comparison between the effect of tobacco and tobacco + PMO

The mitotic index in both the extra and intrapulmonary airways, at various times of exposure and in the three groups of animals is presented in Figure V-2 (A to G).

After one day of exposure, the animals in the tobacco alone group, showed a marked increase in mitotic counts compared to the tobacco and PMO group and to the control group. This increase was found in all airway levels, except at the alveolar level, and reaches

Table V-2

Position of mitotic figures in relation to basement membrane of respiratory epithelium of the tobacco group, treated with colchicine and at selected airway levels.

Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	Tobacco	Control	Tobacco	Control	Tobacco	Control
Trachea						
Upper 1/3	12.5(56)*	4.2(68)	9.0(41)	1.2(20)	0.7(3)	0.7(12)
Mid 1/3	17.5(77)	4.0(89)	4.4(20)	0.5(11)	0.7(3)	0.0(0)
Lower 1/3	19.0(73)	4.5(75)	5.3(21)	1.0(17)	1.5(6)	0.5(8)
Axial Bronchus	1.7(30)	0.0(0)	0.8(14)	0.2(14)	3.2(56)	1.5(86)
Lateral Bronchus	0.2(5)	0.0(0)	0.4(8)	1.2(21)	4.6(87)	4.7(79)

( )* Expressed as a percentage of cells at each intra-epithelial site.

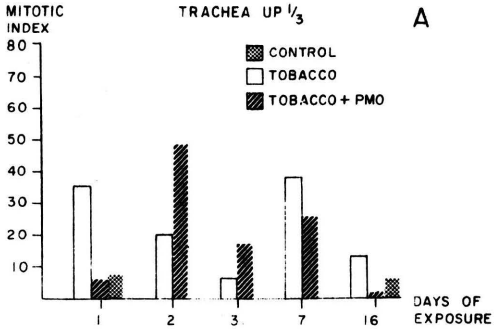
Table V-3

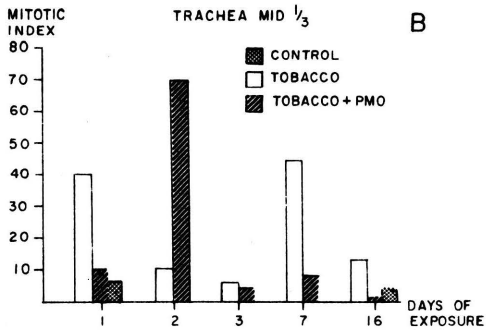
Position of mitotic figures in relation to basement membrane of respiratory epithelium of the tobacco + PMO group, treated with colchicine and at selected airway levels.

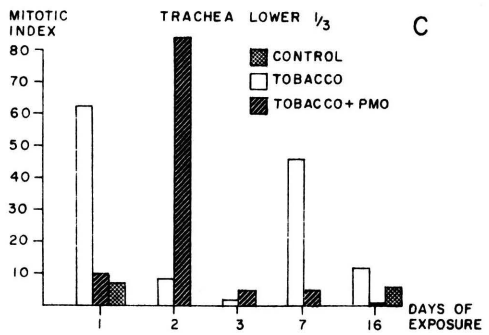
Airway Level	INTRA-EPITHELIAL SITES					
	Base		Mid		Superficial	
	T + PMO	Control	T + PMO	Control	T + PMO	Control
Trachea						
Upper 1/3	12.9(66)*	4.2(68)	5.1(26)	1.2(20)	1.5(8)	0.7(12)
Mid 1/3	10.5(57)	4,0(89)	5.9(32)	0.5(11)	2.0(11)	0.0(0)
Lower 1/3	10.7(52)	4.5(75)	6.8(33)	1.0(17)	2.9(15)	0.5(8)
Axial Bronchus	0.6(23)	0.0(0)	0.5(20)	0.2(14)	1.6(57)	1.5(86)
Lateral Bronchus	0.3(8)	0.0(0)	0.3(9)	1.2(21)	3.2(83)	4.7(79)

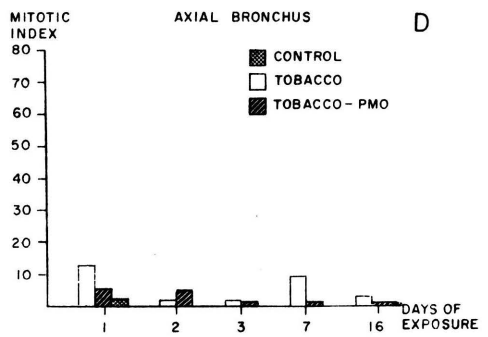
( )* Expressed as a percentage of cells at each intra-epithelial site.

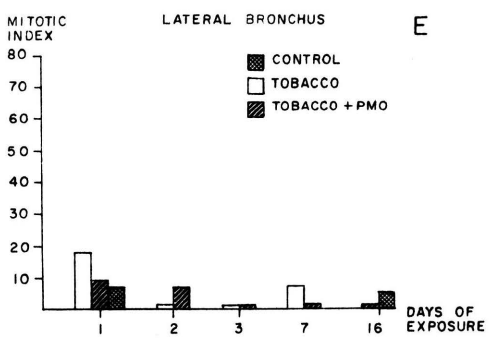
Figure V-2 (A to G): Mitotic index in the three groups of animals (tobacco - tobacco + PMO and controls), and at different airway levels and at various times of exposure.

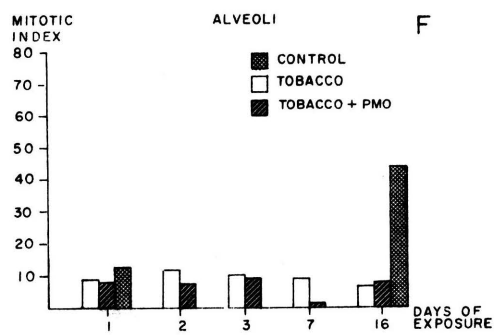

MITOTIC INDEX
TRACHEA UP 1/3
A
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE

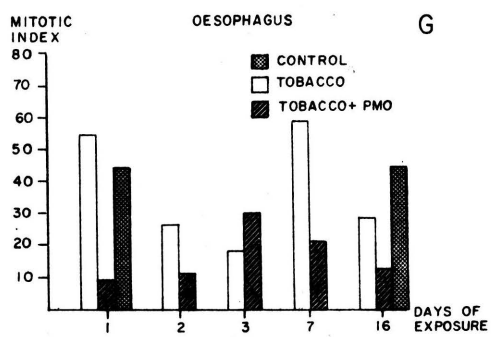

MITOTIC INDEX
TRACHEA MID 1/3
B
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE


MITOTIC INDEX
TRACHEA LOWER 1/3
C
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE


MITOTIC INDEX
AXIAL BRONCHUS
D
CONTROL
TOBACCO
TOBACCO - PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE


MITOTIC INDEX
LATERAL BRONCHUS
E
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE


MITOTIC INDEX
ALVEOLI
F
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE


MITOTIC INDEX
OESOPHAGUS
G
CONTROL
TOBACCO
TOBACCO + PMO
80
70
60
50
40
30
20
10
1
2
3
7
16
DAYS OF EXPOSURE

significant levels in the upper trachea ($p < 0.05$), and in the oesophagus ($p < 0.001$).

After two days of exposure, the animals in the tobacco and PMO group showed, at all airway levels, except in the alveoli, a marked increase in mitotic counts, compared to the animals in the tobacco alone group.

On the third day, the animals in both smoking groups presented a decrease in mitotic activity, although at the oesophageal level, there was still a slight increase.

After a week of exposure, a second wave of increased mitotic activity was found in both the smoking groups, but greater in the tobacco alone group.

Finally after two weeks, the mitotic index was again reduced in both the smoking groups, more in the tobacco + PMO group than in the tobacco alone group, and in all the extra and intrapulmonary airways.

There was a decrease in the mitotic activity in the tobacco + PMO group compared to the control group at all the levels (except in the axial bronchus), and the difference was highly significant ($p < 0.001$), in the inferior part of the trachea, the alveoli and oesophagus.

SUMMARY

A short term exposure to cigarette smoke from either a plain tobacco cigarette or a cigarette containing tobacco with PMO added,

revealed the following changes:

A) Weight changes

1) After three days of exposure to cigarette smoke, there was a significant decrease in weight in the tobacco and the tobacco + PMO groups compared to the controls. This difference persisted throughout the whole experiment, but after eight days only the tobacco + PMO group was statisfically different from the control animals. At the end of the exposure to cigarette smoke, both groups were statistically different from the controls.

B) Effect on mitotic activity

1) At all tracheal levels, more mitoses were located in the basal layer of the epithelium than in the superficial while in the distal airways, more mitoses were found in the superficial layers than in the basal layer. This was found in the tobacco, tobacco + PMO and in the control groups.

2) Two waves of high mitotic activity were identified in animals exposed to tobacco alone, one after one day of exposure to cigarette smoke, the other after a week. The tobacco and PMO group presented only one peak of high mitotic activity, after two days of exposure.

3) After either one week or two weeks of exposure, the animals in the tobacco alone group had a higher mitotic

rate than the animals in the tobacco + PMO group, and that at all levels in the airways.

4) In the two groups, as in the controls, the mitotic index was smaller in the peripheral airways than in the trachea.

CHAPTER VI

PMO LONG TERM

PMO LONG TERM

A long term experiment with either tobacco or tobacco and PMO was carried out over a period of 24 days, and two groups of 15 rats were exposed to 25 cigarettes a day for 24 days. This experiment has already been reported in the British Medical Journal, April 15, 1972.

The group whose cigarettes included PMO showed less immediated distress after exposure than the group exposed to tobacco alone.

The animals in the two tobacco groups failed to gain weight on the days of exposure. There was a significant difference between the weights of those animals and the controls ($p < 0.001$).

In the trachea, the goblet cell count in the group of animals exposed to tobacco alone was significantly above that found either in the group exposed to tobacco and PMO ($p < 0.001$), or in the control animals ($p < 0.01$); there was not significant difference between the control animals and those exposed to tobacco and PMO.

At each of the three tracheal levels in the two tobacco groups, the number of cells in mitosis was found to be increased when compared with that of the controls, but the increase in the tobacco alone group was higher than that of the tobacco + PMO group. When all values were taken in combaination, PMO had caused a significant reduction in cell division.

It seems that tobacco alone led to failure to gain weight and to an increase in goblet cells, and cells in mitosis. PMO gave complete protection against the increase in goblet cells, partial protection against the increase in the number of cells in mitosis, and had no effect on the slower weight gain.

CHAPTER VII

DISCUSSION

CONTENTS

SECTION D: THE EFFECT OF TOBACCO AND TOBACCO + PMO

I - The effect on weight

II - The effect on mitotic activity

A - Short term exposure

SECTION A: TECHNIQUE AND METHODS

I - The use of rats

The decision to use the albino rat as the experimental animal for the study of pulmonary epithelial cell renewal was made in consideration of several factors.

1) Although previous workers had studied pulmonary cell renewal in rats and in mice, no complete study, including pulmonary cellular turnover in different age groups and in both male and female rats, has yet been available. Moreover, although pulmonary cell renewal was determined at more than one level of the respiratory tract, by Bertalanffy in 1960 (trachea and bronchus), and by Shorter, Titus and Divertie in 1966 (trachea, large bronchus and small bronchus), these studies were never carried out in more than one age group of animals.

Finally among the above-mentioned workers, no studies compared the results from colchicine with those from tritiated thymidine for cellular renewal determinations.

2) Previous work in the Department on the effect of irradiation on rat submucosal gland hypertrophy (Jones, Bolduc, et al, 1973), and goblet cell increase in the rat bronchial epithelium (Lamb and Reid, 1968, 1969), provided a good experimental model for the study of mitotic activity in both

the normal animal and after experimentally induced hypertrophy.

3) For pulmonary studies, it is of prime importance that only animals with clean lungs be used, although it is difficult to assess the lung "cleanliness" of a given strain of animal (Lane-Petter, 1970). In the Department, lungs are only regarded as satisfactory if lymphocytic infiltration, in the airway submucosa is virtually absent, since it has been found a more demanding criterion than bacteriological cultures. A good correlation between degree of infection, submucosal lymphocytic cuffing, goblet cells and mitoses number increase, was shown in the one case of an infected animal, in Chapter IV. By availing ourselves of this reliable and simple histological test, the albino rat was found to be a suitable experimental animal.

All animals used in this study were specific pathogen free animals, and they were kept in a separate animal room, where the routine care of bedding, cages, walls and floors was maintained.

For these reasons, at the beginning of each experiment, lungs from a few animals from the stock supplied were examined histologically and only those with minimal

degree of submucosal lymphocytic infiltration were used.

II - The airway levels

Difference in acid glycoprotein content at various levels of the rat respiratory tract has been shown previously (McCarthy and Reid, 1964). More recently, a study of the incidence of various histological types of lung cancer within airway levels showed that epidermoid and oat cell carcinoma were more frequently seen in the large bronchi, while bronchiolar or broncho alveolar carcinoma were more common in distal airways (Melamed, 1968). It is, therefore, of interest to relate the epithelial mitotic activity to various airway levels.

Mitotic activity was also assessed in the oesophagus. Up to one hour after ^{3}H-Thymidine administration, labelled nuclei are numerous in the basal layer of the oesophageal epitehlium (Marques-Periva, 1965). Because of this rapid incorporation of thymidine into the DNA synthesizing basal cells, the oesophagus was useful as evidence of the satisfactory working of colchicine or uptake of tritium for our autoradiographic studies. Also, since the turnover time of the oesophageal epithelium has been established in normal animals, it offered a basis for comparison of our results with those of other workers.

III - The use of colchicine and tritiated thymidine

A - Comparison of the two techniques

For the experiments reported in this study, both colchicine and tritiated thymidine were used to determine cellular renewal. Since drugs can influence the nuclear metabolism of a dividing cell, each drug has its advantages as well as disadvantages.

1) Tritiated thymidine: Greulich, Cameron and Thrasher (1961), observed a significant increase of the mitotic activity of duodenal epithelium following thymidine administration, irrespective of whether or not the thymidine was tritiated. The possibility of re-utilization by cells of ^{3}H-T labelled DNA, derived from cell fragmentation was discussed by Robinson and Brecher (1963).

In the gastrointestinal tract, Baserga and Kineleski (1962), suggested the possibility of an absorption of ^{3}H-T (arising from catabolized DNA of labelled exfoliated cells) and its subsequent re-utilization by cells. In this present study, some technical difficulties were encountered when using autoradiography technique, although proper doses of the tracer, time between injection and sacrifice, and processing of the autoradiographs were done according to the generally accepted method.

Four sections prepared for autoradiography turned out to be completely blank and no silver grains were seen under the microscope. On some other occasions, the background

count of silver grains was so high that it was impossible to distinguish between positive cells and artefacts.

On the other hand, tritiated thymidine technique requires a smaller number of animals than the colchicine technique. Autoradiographs resulting from the use of this technique are evaluated more speedily than those after colchicine treatment.

2) Colchicine: Regarding the colchicine technique, some early workers assumed from the large number of mitoses they observed after colchicine administration, that the drug would stimulate cells to enter mitoses. Also, the incidence of colchicine-arrested metaphase in tissue can decrease with time, if a long interval is allowed between colchicine administration and sacrifice.

On the other hand, the colchicine technique has the advantage that it is easier to use, in that the tissue is more easily prepared, and it does not include the risk of irradiation. Although the mitotic counts are more laborious to make, the colchicine metaphase with its dark clumped chromatin, surrounded by a halo of light cytoplasm, is readily recognized.

It has also been reported (Bertalanffy, 1964), that there may be discrepancies in results obtained for the colchicine and tritiated thymidine.

These differences were found in studies where different species of animals were compared, and where the sex of these animals was not taken into consideration. In the present study, the sex, age group and the species were all carefully checked and assessed beforehand. A good correlation between the results obtained with colchicine and those obtained with tritiated thymidine was reached.

B - The calculation of the turnover time

For the tritiated thymidine technique, the time between injection of the animals and the killing can vary from 5 minutes to 12 days, depending upon the design of the experiment.

The turnover time or generation time of cell populations can be determined by two different methods:

1) Estimation of the transit time: It is the time between administration of ^{3}H-T and the disappearance of all labelled cells from a tissue, for instance by exfoliation. The transit time corresponds to the renewal or turnover time of the tissue, and can be ascertained by sacrificing groups of animals at different intervals, after ^{3}H-T administration. Transit times have been estimated in cases of certain epithelia, such as the tongue (Walker, 1960), the vagina (Peckham, 1962), the gastro-intestinal tract (Creamer, 1961, Fry, 1962), and cornea (Hanna, 1960). The transit time can also be used in cases where the

tissue samples are limited, such as in human biopsy material.

2) Another approach for estimating generation times is the enumeration of ^{3}H-T labelled cells. This procedure supplies information on the duration of the phases of the cell cycle, and quantitative data on the magnitude of cell proliferation (Koburg, 1962).

By using the latter method of calculation and by sacrificing animals four hours after the injection, an approximate value of the turnover time was obtained, but no corrections for diurnal variations were made. However, the object of this study being the measurement of the effect of irritation on mitotic activity and technical basal conditions being observed in each experiment, this estimation of cell renewal was quite satisfactory. For the colchicine technique, the turnover time of a cell population is based upon the time required for division of 100% of cells within the population. Here again, no corrections were made for diurnal variation, which in the case of the mouse epidermis can fluctuate from 1.6 mitoses at 20.00 to 9.0 at 08.00.

3) Other techniques - Vinblastine: Vinblastine resembles colchicine in that it stops cell mitosis in metaphase, and has been used by some workers to study cellular replace-

ment (Williams, 1967; Chernozemski, 1968).

According to Chernozemski, striking stability of the prophase counts characterized the results obtained from all tissues examined in the hamster. Williams, however, using various compounds such as colchicine and Demecolcin, which is a cytotoxic drug, similar in structure to colchicine, studied mitotic activity in the embryonic rat in utero. He found great differences in the mitotic index obtained by these various methods.

SECTION B: THE MITOTIC COUNTS IN THE NORMAL ANIMAL

As shown in Chapter III, pulmonary cell renewal is subject to variations depending on the age and sex of the animals and also to variations in the level of the airway and to the level within the bronchial epithelium.

I - Factors influencing mitotic activity

A - Growth

Most organs and tissues of the male rat grow in proportion to the body weight and the number of cells increases in a parallel manner. Bertalanffy, in 1951, mentioned that in a 200 gm rat, a mitotic activity of between 0.8 to 1.5% could be considered to serve for growth, and a mitotic activity above 1.5% for renewal. However, in this present study, these figures of assessing growth and renewal

do not seem applicable. In the young group of animals, mitotic activity was above 1.5% only in the upper trachea level of the male group, despite a weight gain of nearly 5 gm a day.

B - Sex and age

It is well shown in this study that sex and age influenced the rate of cell renewal in the epithelial lining of the respiratory tract.

In 1967, Simnet and Heppleston, using an in vivo method, concluded that strain, age and sex played an important role in the mitotic incidence of the mouse alveolar wall cells. This influence of age and strain was also shown in a study on mice prostatic epithelium (Simnett and Morley, 1967). They found that the mitotic incidence in the prostate was high in young mice (three weeks old), and low in the older animals (six weeks old). However, Shorter, Titus and Divertie in 1966, using albino rats weighing between 150 and 160 gm, did not observe any differences between the animals of either sex, but did find a slight difference in the migration time between the cells in the trachea, large bronchi and small bronchi. The migration time of the tracheal epithelial cells was six to seven days, and in the large bronchi from seven to eight days. In the small bronchi, this migration time was lower, from five to six days. Although Schultz and Oehlert (1960), used animals of both sexes in their study, figures of cellular renewal for each sex are

not given separately. In conclusion, therefore, as mentioned by Leblond and Stevens (1948), cellular replacement is a constant physiological phenomenon, which occurs continuously and which is not only a response to damage of the epithelium.

II - Variation in mitotic activity

1) Mitotic activity varies in the respiratory tract according to the level and the site within the epithelium. Many studies on the skin show that mitotic activity varies according to site.

The only work available for the lung is that of Shorter, Titus and Divertie (1964). They showed little difference in the turnover time between the cells in the trachea, large and bronchi. There is no work in this respect on the gut at present, available in the literature.

2) Mitotic activity varies also, according to the site within the epithelium. As early as 1882, zones of mitotic activity in the gastric mucosa were described by Patzelt and in 1953, Stevens and Leblond, using the colchicine technique, mentioned that the mitotic activity in the gastric mucosa of the rat was higher in the surface epithelial cells than in the mucous neck cells of this mucosa.

3) Finally, it is also well known that mitotic activity can be localized in centres, in foci. This was not apparent in our study because the mitotic counts were performed on many

samples of tissue. This phenomenon cannot be the cause of variation.

III - Mechanisms regulating mitotic activity

The possible mechanisms of the control of mitotic activity in various organs of different species of animal have been studied in the past.

In 1959, a study by Bullough and Laurence suggested that in the epidermis of the adult mouse, specific tissue-inhibitors controlled the growth of these tissues. Later in 1961, and 1964, they called these chalone-adrenaline complexes, mitotic inhibitors. During physical activity, stress or starvation, these complexes are released and inhibit the mitotic activity. In contrast, during rest periods, the level of this circulating substance is lower and the mitotic activity increased.

More recently, Jeffery and Reid (1972), studying the distribution of intra-epithelial nerves in the rat airways, under the electron microscope, showed several interesting features:

1) Nerves are present in all extrapulmonary airways, but not in the intrapulmonary bronchi and bronchioli.

2) There are more nerves in the upper trachea than in the lower segments.

3) They found no sex difference in nineteen day old animals (45 to 50 gm).

4) 87% of the nerves within the epithelium were located

close to the basement membrane and 13% were located away from the basement membrane and above it.

5) The nerves were closely associated with basal cells.

These findings correlated with the mitotic activity of the respiratory epithelium of the normal rat.

In the young and middle aged group of animals, 61% to 87% of the cells in mitosis, at the level of the upper respiratory tract, were located at the base of the epithelium. Because of the fact that the animals used in the present study were older (33 and 47 days or 106 to 241 gm), a sex difference in mitotic activity was found.

In conclusion, therefore, to explain the difference between young males and young females, it seems likely that the mitotic activity in the respiratory epithelium of the rat is influenced by hormones, and is also, under the control of chalone-adrenergic substances that may be closely related to the presence of nerve fibres within the epithelium. It has been shown, for the rat thymocytes, that cyclic AMP may be involved in the control of cell proliferation (Whitfield, 1970).

SECTION C: THE EFFECT OF IPN AND PCP

The effect of IPN and of PCP on the mitotic activity of the tracheobronchial epithelium are reported here for the first time.

I - The effect of isoprenaline

A - The effect on mitotic activity

Isoprenaline produced an increase in the number of cells in mitosis in the tracheobronchial epithelium of the rat, and in both males and females. Contrary to what was found in the normal animals, the mitotic activity was higher in the superficial layers of the respiratory epithelium than in the basal layers. It may be that because of an increase in the rate of cellular renewal, more mitotic figures were seen in the superficial layers of the epithelium than in the basal. It will be of interest, to study the rate of cellular migration in a subsequent experiment, to explain the increase in mitotic figures in superficial layers of an epithelium. The effect of catecholamines on cell proliferation has been studied in the epidermis (Evensen, 1964), and in the kidney (Malamud, 1971). According to Malamud, a six-fold increase in DNA synthesis within the mouse kidney occurred 34 hours after a single injection of 9 mg of isoproterenol. In the salivary gland also, Barka (1965 and 1970), studied the stimulation of DNA synthesis by isoproterenol, and found that the incorporation of tritiated thymidine was greatly stimulated after a single dose of isoproterenol. The peak of this stimulation occurred 24 hours after the administration of the drug. In a subsequent study, he suggested that a prolonged treatment with isoproterenol, which led to an enlargement of the submandibular gland, decreased the sensitivity of the gland to the stimulatory effect of a single injection of the drug. As our study included a series of isoprenaline injections, it would be interesting to assess the effect of a single dose on the

mitotic activity of the respiratory epithelium of the rat. Our findings are at variance with studies of Chiarulas (1966), in the corneal epithelium and epidermis of urodele larvae. He found that exogenous epinephrine acts as a mitotic inhibitor and depresses the overall mitotic rates.

B - The effect on goblet cells

The effect of isoprenaline on the submucosal tracheal glands and on the respiratory epithelium goblet cells was first described by Sturgess in 1970. She found an overall submucosal tracheal gland hypertrophy with both serous and mucous cell hypertrophy, an increase in goblet cell number at all levels in the tracheobronchial tree and a change in the histochemical properties of goblet cells. As previously mentioned by McCarthy and Reid in 1964, the goblet cells staining blue with AB contain an acid type of glycoprotein, and those staining red with PAS contain a neutral type of glycoprotein.

In the present study, there was an increase in goblet cell number at all intra and extrapulmonary levels of the airway. This increase was mainly due to an increase in small and large blue goblet cells. These results are similar to those of Sturgess and Reid (1973). In the axial airway, however, an increase in both small blue and small red cells was responsible for the overall increase in the total goblet cell number.

As in the Sturgess study (Thesis), the demonstration of increase

in the goblet cells by isoprenaline was performed under germ-free conditons which justifies the assumption that both the increase in mitotic index and goblet cell number represents a direct effect of the drug and not any secondary effect from dormant infection.

C - Possible mechanisms of action

Although this unusual drug action of IPN has been studied in past years, in some detail (Barka, 1965, 1968, 1970); Malamud and Malt, 1971; Sturgess and Reid, 1973, and others), its mechanism remains unknown. It was suggested by Sturgess that isoprenaline had a specific effect on β adrenergic receptors in the bronchial gland, but more recently, a different mechanism was suggested (Barka, 1970). Pyrogallol is a known inhibitor of catechol-o-methyl transferase (COMT) which is involved in the catabolism of isoprenaline. When given both before and after the administration of isoprenaline, pyrogallol not only inhibits the stimulatory effect of isoprenaline, but also inhibits the incorporation of tritiated thymidine into the submandibular glands of the rat. However, this mechanism of action was denied by Baserga, who claimed that pyrogallol enhanced rather than inhibited the stimulation of DNA synthesis by isoproterenol in the salivary glands of mice.

It seems, therefore, that the exact role of catecholamine derivatives on DNA synthesis remains unclear and the subject of discussion. Further electron microscopic studies, on nerve distribution within tissues and further biochemical and pharmacological investigation

will probably bring a clearer answer.

II - The effect of pilocarpine

A - The effect on mitotic activity

The effect of pilocarpine on cells in mitosis was similar to that of isoprenaline; there was an increase in the number of mitoses at all levels of the airway, except in the lateral bronchus. The mitoses were found more in the superficial layers of the epithelium than in the basal layers. However, contrary to the findings in the isoprenaline-treated animals, there was no progressive decrease in mitotic activity from the upper trachea to the axial bronchus. On the contrary, there was a slight increase in the number of mitoses in the third level of the trachea compared to the first level.

B - The effect on goblet cells

It was previously shown by Sturgess and Reid (1973), that PCP produced an increase in goblet cell size and number. In this study, there was an increase in goblet cell number in the trachea and axial bronchus after twelve PCP injections. At these levels, there was an increase in small blue AB positive and small red PAS positive goblet cells, but the percentage increase in small red PAS positive goblet cells was greater than for the blue. These results correspond to the findings of Sturgess and Reid (1973).

C - Mechanisms of action

As in the isoprenaline group of animals, no epithelial ulceration was seen in the airways after treatment with PCP. The presence

of an increased number of goblet cells and mitotic figures does not reflect, therefore, a repair mechanism for a damaged epithelium, but a pharmacological response of the organism to stimulation. It is not known yet by which mechanism pilocarpine produced such a response. As suggested by Sturgess and Reid (1973), an alternation in the cell metabolism could provide an answer.

As mentioned by Jeffery and Reid (1972), it is possible that the difference in the number of nerve fibres at different levels in the tracheal epithelium could explain variation in mitotic activity at the tracheal level and that PCP is acting by stimulating goblet cells at heavily innervated sites only.

More recently, however, Tiber (1970), suggested that pilocarpine could have an effect on protein synthesis in the submandibular gland of the hamster. It could then be postulated that administration of this drug to the animal produced overactivity of the secretory function of the surface epithelium with increased in mitotic index as a secondary effect to this.

SECTION D: THE EFFECT OF TOBACCO AND TOBACCO + PMO

I - The effect on weight

Previous studies (Elson and Passey, 1963), showed that loss of weight or failure to gain weight is usual in experimental animals exposed to tobacco smoke. They found that the nicotine present in

sustained irritation. In the tobacco alone group, the mitotic activity was reduced three days after the beginning of the exposure. That period of reduced activity could correspond to a state of cellular exhaustion after a period of high mitotic activity. After a week of exposure in the PMO group, there was a new peak in the mitotic rate, that, as in the tobacco alone group, could represent a period of healing and slow replacement of damaged epithelium. This period is followed by a new episode of reduced mitotic activity in which, the mitotic rate is still higher in the tobacco alone group, than in the tobacco + PMO group.

3) The oesophagus:

At the level of the oesophagus, the same pattern is present in the tobacco alone group, as in the tobacco + PMO, but in the PMO group, the peak of the mitotic rate is at a later time. The rapid response to irritation did not appear on the second day of exposure as in all the tracheal levels, but on the third day.

It seems, therefore, that in the oesophagus, PMO counteracted the effect of tobacco smoke for a longer period.

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GOBLET CELL GLYCOPROTEIN AND TRACHEAL GLAND HYPERTROPHY IN RAT AIRWAYS: THE EFFECT OF TOBACCO SMOKE WITH OR WITHOUT THE ANTI-INFLAMMATORY AGENT PHENYLMETHYLOXADIAZOLE

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Summary.—A quantitative analysis has been made of tracheal gland size and of histochemical changes occurring in goblet cells of the respiratory epithelium, in rats exposed either to tobacco smoke alone or to tobacco smoke with phenylmethyloxadiazole (PMO).

Exposure to tobacco smoke alone causes an increase in goblet cell number with a shift from the production of neutral to acid glycoprotein, mainly sialidase resistant sialomucin but some sialidase sensitive sialomucin and sulphomucin. Acid glycoprotein, and each of its types, appears first at the cell apex. The addition of PMO to the tobacco protects against the increase in goblet cell number but gives no protection against the shift from neutral to acid glycoprotein and causes a larger secretory mass within the goblet cell.

In the tracheal gland exposure to smoke from either tobacco alone or tobacco with PMO causes a significant increase in cell size and acinar diameter and a lesser increase in lumen diameter. There is also an increase in the thickness of the gland and its depth. Each of these gland changes is more pronounced in those animals receiving PMO with the tobacco.

THE administration of phenylmethyloxadiazole (PMO) has recently been shown to protect against the ciliostatic effect of tobacco smoke (Dalhamn and Rylander, 1971) and also against other lung damage caused to the rat by tobacco smoke, although the degree of protection afforded against each of the changes that occurred was not the same (Jones, Bolduc and Reid, 1972). The addition of PMO to the tobacco prevents any increase in goblet cell number and partly prevents increase in epithelial thickness and cell number and the number of cells in mitosis, but does not affect the decrease in the animal's weight gain, a decrease produced at least in part by nicotine (Elson and Passey, 1963).

In the normal rat, goblet cells are more numerous in the large airways and sparse in peripheral airways (McCarthy and Reid, 1964). Glycoproteins within the goblet cells may be neutral or acidic, including in the rat as in man either sialic acid (sensitive or resistant to the enzyme sialidase) or sulphate. Sialidase resistant sialomucin and sulphomucin are found more commonly in central airways and sialidase sensitive sialomucin toward the periphery (McCarthy and Reid, 1964).

The present studies are concerned with the effect on the rat airways of exposure to smoke of either tobacco alone or of tobacco with PMO, particularly with the

size of the secretory mass in the goblet cells and the type of glycoprotein present, and with the hypertrophy of the tracheal gland.

MATERIAL AND METHODS

Rats were exposed to smoke from one of 2 types of cigarette, each made from the same tobacco (a mixture of flue cured and naturally cured leaf) but to the tobacco of one type 2% by weight of PMO was added. Analysis showed little difference between those cigarettes containing tobacco alone or tobacco + PMO. For example, for cigarettes smoked to a butt length of 10 mm, the measurements for tobacco and tobacco + PMO respectively were pH 4·85 and 4·83, 6% and 5·9% sugar content, 0·77 and 0·82 buffering capacity, 13·70 and 16·85 mg/cigarette tar content and 1·98 and 1·94 mg/cigarette nicotine content.

The animals were from specific pathogen-free stock (Carworth-Europe, CFY strain) and weighed approximately 150 g at the start of the experiment. They were divided into groups —tobacco alone (9 rats exposed to tobacco without any additive), tobacco + PMO (9 rats exposed to tobacco to which PMO had been added), controls (6 rats). The animals were amongst those for which the total goblet cell number has previously been reported (Jones *et al.*, 1972). In that paper, details of the exposure and tissue preparation are also given; briefly, the animals were exposed to 25 cigarettes a day, 4 days a week for 6 weeks, in aluminium cabinets with a Wright Autosmoker (Wright, 1972).

Goblet cells

Sections of the left lung—in the rat a single lobe—were cut to include the full axial pathway and several of the lateral pathways (Lamb and Reid, 1968): in all sections used for quantification the second and third branches were included. Goblet cells were examined under oil immersion along the full length of the trachea, save for the upper 3 mm which was used for gland measurement (between 70 and 100 HPF), the medial wall of the axial pathway (between 60 and 90 HPF) and 20 HPF in each of the second and third lateral airways, the mean of these two counts being taken as the " lateral " pathway value.

Histochemical analysis.—Within the goblet cells the presence of granules of neutral or acid glycoprotein was identified by the Alcian blue–periodic acid Schiff (AB/PAS) technique: granules of acid glycoprotein are AB positive and those of neutral glycoprotein PAS positive. The AB/PAS technique thus identifies the region containing secretory granules but not the cell edge. In this study a "small " cell is one containing granules only at its apex and a " large " cell one filled with granules above the level of the nucleus, both types being identifiable with the electron microscope (Jeffrey, personal communication). Four types of goblet cell were recognized here—a large and small PAS positive cell and a large and small AB positive cell. The PAS positive cell contained only one type of granule, but whereas the AB positive cell may have contained only AB positive granules, a few PAS positive ones were often present toward the cell base.

All animals in this study were examined to identify the number of goblet cells with either neutral or acid glycoprotein; detailed histochemical analysis was carried out on 6 animals from each group.

The various acid radicles were identified by comparing a section stained with AB/PAS at pH 2·6 with adjacent sections treated with sialidase (receptor destroying enzyme, RDE) and stained with AB/PAS at pH 2·6 or stained with AB/PAS at pH 1·0. The presence of a sialomucin was identified by its failure to stain either with AB after dialidase digestion (sialidase sensitive sialomucin) or with AB at pH 1·0 (sialidase sensitive and sialidase resistant sialomucin); by either of these techniques sulphomucin was AB positive. The details of the staining techniques and their application for quantitative studies have been reported elsewhere (Jones and Reid, 1973*a*, *b*).

Tracheal gland

In the rat trachea most of the gland is present immediately below the larynx and hence the upper 3 mm was used to study hypertrophy of the tracheal gland.

Measurement of tracheal gland.—Two animals were chosen at random from each group for tracheal gland measurement; serial transverse sections of 4 μm thick were prepared and from one animal were stained by haematoxylin and eosin and from the other by AB/PAS at pH 2·6.

Gland measurements were made on sections 160 μm apart. Using an eyepiece graticule, in each section examined 3 measurements were recorded for each tubule—along the maximum diameter, perpedicular to the lumen of the trachea, the lumen diameter and the cell height of each wall (× 2). From these the acinar diameter, from basement membrane to basement membrane, was calculated.

RESULTS

Goblet cells

Cell number.—Exposure to tobacco smoke causes a significant increase in the total number of goblet cells in rat tracheal epithelium (tobacco/controls $P < 0{\cdot}01$), while the addition of PMO prevents such increase (tobacco + PMO/controls P, N.S; tobacco + PMO/tobacco $P < 0{\cdot}001$). In the axial and lateral pathways neither smoke from tobacco alone nor from tobacco + PMO causes a significant increase in the number of goblet cells (Jones *et al.*, 1972).

Histochemical analysis.—Table I shows the number of each of the 4 types of goblet cell, in sections stained by AB/PAS at pH 2·6, RDE AB/PAS at pH 2·6 and AB/PAS at pH 1·0, at each airway level, for each of the 3 groups of animals.

TABLE I.—*Goblet Cells/6 mm Airway after Selective Staining: Control (C), Tobacco Alone (T) and Tobacco + PMO (T + PMO): Airway Levels*—1 (*Trachea*), 2 (*Axial*) *and* 3 (*Lateral*)

Staining technique	Animal group	Airway level	Goblet cells: PAS positive Large	PAS positive Small	AB positive Large	AB positive Small
	C	1	1·87	30·96	2·06	22·87
		2	7·81	51·47	11·01	34·42
		3	7·88	52·03	19·55	39·84
AB/PAS	T	1	2·82	16·68	41·64	95·53
pH 2·6		2	7·54	54·98	16·36	49·12
		3	5·98	53·20	19·14	53·67
	T+PMO	1	2·76	19·57	8·95	21·53
		2	66·36	48·28	20·36	72·16
		3	3·85	34·98	20·15	57·48
	C	1	1·79	23·67	1·57	25·65
		2	1·43	31·45	0·51	28·94
		3	0·93	43·66	1·23	29·64
RDE	T	1	0·19	9·87	17·29	132·88
AB/PAS		2	4·90	63·55	4·00	85·19
pH 2·6		3	8·85	81·68	4·68	54·06
	T+PMO	1	0·00	9·83	1·33	23·30
		2	3·37	51·35	2·91	72·53
		3	11·08	61·08	7·48	59·83
	C	1	3·00	29·75	0·00	1·24
		2	9·93	61·65	0·00	2·25
		3	5·46	68·40	0·13	1·64
AB/PAS	T	1	18·29	90·57	1·58	10·54
pH 1·0		2	13·00	111·72	0·00	2·04
		3	19·64	133·04	1·66	1·75
	T+PMO	1	4·43	21·05	4·44	16·74
		2	16·40	109·32	0·63	9·68
		3	17·70	122·46	0·00	2·08

From these results the following conclusions may be drawn: At each airway level, in both the tobacco group alone and the tobacco + PMO group a highly

significant shift occurs to cells secreting acid glycoprotein ($P_{\chi^2} < 0{\cdot}001$). This shift occurs whether or not there is an increase in the absolute number of goblet cells. It is particularly the small PAS positive cells that change to small AB positive but both types of AB positive cell are increased and both types of PAS positive cell decreased (Table II).

TABLE II.—*Types of Goblet Cell at Three Airway Levels. Actual Values—(i) Tobacco Alone and (ii) Tobacco + PMO. Expected values—Controls*

		Trachea			Axial pathway			Lateral pathway		
		Actual	Expected	Diff.	Actual	Expected	Diff.	Actual	Expected	Diff.
(i) *Tobacco/Controls*										
PAS positive	Large	6	11	−5	37	47	−10	33	47	−14
	Small	37	186	−149	270	309	−39	288	312	−24
AB positive	Large	92	12	+80	80	66	+14	104	117	−13
	Small	211	137	+74	241	207	+34	291	239	+52
	P_{χ^2} <			0·001			0·01			0·001
(ii) *Tobacco + PMO/Controls*										
PAS positive	Large	18	11	+7	31	47	−16	24	47	−23
	Small	128	186	−58	205	309	−104	215	312	−97
AB positive	Large	59	12	+47	86	66	+20	124	117	+7
	Small	141	137	+4	307	207	+100	353	239	+114
	P_{χ^2} <			0·001			0·001			0·001

In the tobacco alone group, the absolute number of both types of AB positive cell in both the trachea and axial pathway is increased, but in the lateral pathway only the small AB positive cell shows an increase. In the trachea, the absolute number of small PAS positive cells is reduced; at the axial and lateral levels both large and small PAS positive cells remain the same (Table I). In the tobacco + PMO group the absolute number of large AB positive cells in the trachea is increased, that of small PAS positive cells being reduced. In the axial pathway, the absolute number of both small AB and large PAS positive cells is greatly increased, while in the lateral pathway the number of small AB positive cells is increased and small PAS positive cells reduced (Table I). At each level of the airway, there are variations in response between animals within the group. Each animal shows a striking increase in the proportion of AB positive cells at some level of the airway

Types of acid glycoprotein granules within a single cell.—From histochemical analysis of the intracellular types of acid glycoprotein the following types of goblet cell can be identified: those containing (i) sialidase sensitive sialomucin only or (ii) sialidase resistant only, (iii) those containing both sialidase sensitive and resistant sialomucin, and (iv) those containing sialidase resistant sialomucin and sulphomucin. Sulphomucin, and sometimes sialidase resistant sialomucin, are found only at the apex of the cell, but they may come to fill the whole cell. This

suggests that as a cell begins to secrete a new type of acid glycoprotein, it appears first at the cell apex.

Types of goblet cells containing acid glycoprotein (AB positive).—In the trachea of the control animals the few large AB positive cells are sialidase sensitive and resistant sialomucin; in the axial and lateral pathways they contain both the sensitive and the resistant forms, demonstrated by a significant shift from large to small AB positive cells after the action of sialidase ($P_{\chi^2} < 0{\cdot}001$, Table I). The small AB positive cells are usually sialidase resistant: this group includes the sulphomucin-containing cell that is occasionally seen.

In the animals exposed either to smoke from tobacco or from tobacco + PMO most large AB positive cells contain both types of sialomucin ($P_{\chi^2} < 0{\cdot}001$, Table I) but some, particularly in the trachea of those exposed to tobacco alone, may contain only sialidase resistant sialomucin. The small AB positive cell (as in the controls) contains sialidase resistant sialomucin and includes the group of cells containing sulphomucin, the frequency of the latter being increased over the control value only in the trachea of the tobacco alone group, and in the trachea and axial pathway of the tobacco + PMO animals.

Area of secretory mass within AB positive cells.—In all animals exposed to tobacco + PMO the large AB staining goblet cells appeared more distended because of an increase in the area of granules, due to an increase in their granule number rather than in size. This was quantified by measuring the height and width of the secretory mass of acid glycoprotein within a cell (judged as the area of granules staining with AB). At each level of the airway, measurements were made when either the height or width of the secretory mass was 5 units or more of the eyepiece graticule (5 units = 6·5 μm). The product of height and width was used as an " index " of the area of the secretory mass. One animal was chosen at random from each of the tobacco alone and control groups, and 2 animals from the tobacco + PMO group. In animals exposed to tobacco alone or to tobacco + PMO usually about 15 cells containing a large secretory mass were present within the length of airway measured but in the control animal only about 7 cells. Of the 2 animals exposed to tobacco + PMO, one had shown a marked and one a lesser shift to cells secreting acid glycoprotein but both gave a high area index with little difference between their area size.

The result of measurements for one animal from each group is given in Table III. At each level of the airway the area of the AB positive cells is substantially

TABLE III.—*Width and Height (μm) of Secretory Mass Staining with AB, and their Product as an Index of Area (μm_2); Three Airway Levels, in Control, Tobacco Alone and Tobacco + PMO Animals*

Animal group	Trachea			Axial pathway			Lateral pathway		
	Width	Height	Area	Width	Height	Area	Width	Height	Area
Control	7·51 (0·80)	8·23 (0·75)	56·44 (3·98)	6·31 (0·53)	5·75 (0·48)	36·21 (4·27)	5·59 (0·93)	4·47 (0·44)	28·16 (4·87)
Tobacco	5·20 (0·69)	10·53 (1·43)	47·99 (3·92)	6·31 (0·38)	6·50 (0·40)	49·92 (3·53)	6·12 (0·25)	5·01 (0·50)	30·66 (3·22)
Tobacco +PMO	8·71 (0·47)	7·15 (0·39)	61·35 (3·26)	8·07 (0·43)	7·80 (0·23)	63·01 (3·92)	7·61 (0·28)	6·96 (0·35)	53·91 (3·81)

( ) s.e. of the mean

larger in the tobacco + PMO animal than in either the tobacco alone or the control, between which there is no significant difference. This increase in area in the tobacco + PMO animal above both other animals is highly significant in both axial and lateral pathways (tobacco + PMO/tobacco, tobacco + PMO/control $P < 0{\cdot}001$) but does not achieve significance in the trachea. Evidently the addition of PMO to the tobacco causes an increase in the size of the secretory mass of acid glycoprotein within the cell.

Tracheal gland

The result of tracheal gland measurements of animals from each of the 3 groups is given in Table IV. Compared with the control animals, there is a

TABLE IV.—*Tracheal Gland Measurements in Control, Tobacco Alone and Tobacco + PMO Animals*

	Mean acinar diameter (μm)	Mean lumen diameter (μm)	Mean cell size (μm)
Control	36·55 (2·32)	24·09 (2·54)	6·26 (0·19)
Tobacco	42·79 (1·83)	27·92 (1·73)	7·50 (0·18)
Tobacco +PMO	43·66 (1·67)	29·16 (1·67)	7·23 (0·13)

( ) s.e. of the mean

similar increase in mean acinar diameter in animals exposed either to smoke from tobacco alone or from tobacco + PMO (tobacco/controls $P < 0{\cdot}05$; tobacco + PMO/controls $P < 0{\cdot}02$) and a similar and highly significant increase in cell size (for both tobacco/controls and tobacco + PMO/controls, $P < 0{\cdot}001$). While there is also an increase in mean lumen diameter in both groups of animals exposed to tobacco smoke, this increase is not significant when compared with the control group. Evidence of increase in cell width was sought by comparing the number of nuclei included per unit length of tubule, but there was no statistical difference between the values for each animal group (mean number of nuclei per unit length of gland: control 1·66; tobacco alone 1·48; tobacco + PMO 1·56).

In the exposed animals, the overall increase in mean acinar diameter is due to an increase in the range of the size of the acini, that is, acini greater than 70 μm diameter appear. In the animals exposed to tobacco alone acini up to 120 μm diameter appear and in those exposed to tobacco + PMO, up to 130 μm (Fig. 1)

Hypertrophy of the gland was also assessed by comparing the mean number of tubules in a tracheal section and the proportion of sections that included gland. Many more tubules are found in the animals exposed to tobacco + PMO than in either the animals exposed to tobacco alone or the control animals (tobacco + PMO/tobacco alone $P < 0{\cdot}05$; tobacco + PMO/controls $P < 0{\cdot}01$). While there are more tubules in the tobacco alone group than in the control animals, the increase does not achieve significance (Fig. 2). Similarly, it was in the animals exposed to tobacco + PMO that most sections included gland, although in the animals exposed to tobacco alone more sections included gland than in the controls (units of tracheal length containing gland: controls, 57·57; tobacco alone 69·04; tobacco + PMO 75·86).

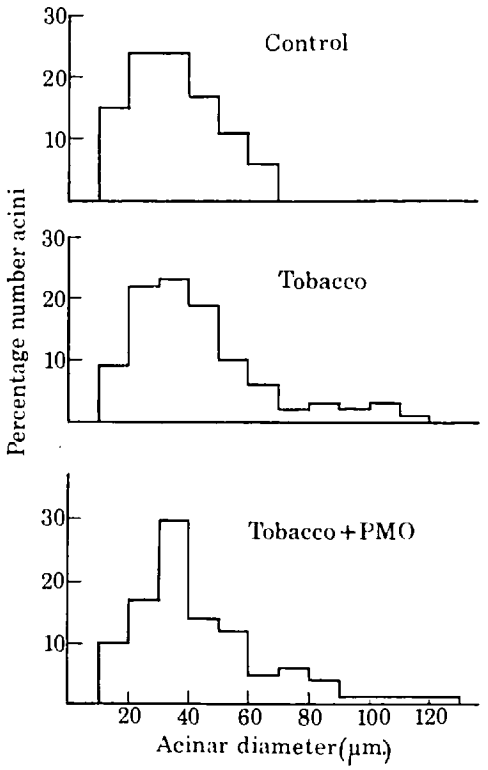

FIG. 1.—Tracheal gland size distribution of acini (as % of total).

DISCUSSION

Goblet cells

The present study has shown that following exposure to tobacco smoke to the airway epithelium histochemical changes in glycoprotein within bronchial goblet cells can occur without any increase in goblet cell number; previously such changes have been reported only with an increase in goblet cell number (Lamb and Reid, 1968, 1969). The administration of either isoprenaline or pilocarpine to rats has been found to increase goblet cell number but only after isoprenaline is there a histochemical shift (Sturgess, 1970; Sturgess and Reid, 1973). Thus a stimulus to airway epithelium may result either in a change in the nature of glycoprotein within pre-existing goblet cells or in an increase in goblet cell number, both associated or unassociated with a shift in the goblet cell type of glycoprotein. This suggests that change within a goblet cell is mediated differently from the development of new cells. Since a histochemical shift to the production of acid glycoprotein has been found in regions of the bronchial tree where goblet cell number is not increased, change within a cell that is already secreting could be the most sensitive evidence of damage to epithelium.

At all levels of the bronchial tree in the control animals used in this study, more than half the goblet cells contained only neutral glycoprotein. Of those

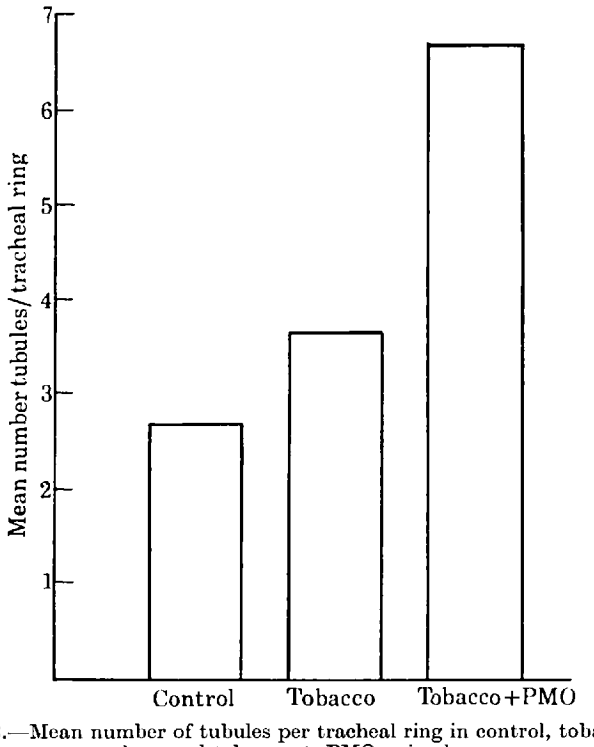

FIG. 2.—Mean number of tubules per tracheal ring in control, tobacco alone and tobacco + PMO animals.

cells containing acid glycoprotein, sialidase resistant sialomucin and sulphomucin were present in the trachea, and both as well as sialidase sensitive sialomucin in the axial and lateral pathways, most sialidase sensitive sialomucin being present in the lateral airways. Only an occasional cell contained sulphomucin at any level of the bronchial tree. A similar pattern of distribution of acid glycoproteins has been described by McCarthy and Reid (1964) but the preponderance of cells containing neutral glucoprotein has not been reported previously.

After exposure to tobacco smoke, either with or without the addition of PMO to the tobacco, there was a shift to cells producing acid glycoprotein; the distribution through the bronchial tree of types of acid glycoprotein remained the same as that found in control animals, although there was an increase in cells secreting sulphomucin, particularly in the trachea, and few cells contained sialidase sensitive sialomucin alone but this was now usually in combination with the resistant form. This combination of sialomucins within a cell resulted in a greater number of " large " cells containing acid glycoprotein in the exposed than in the control animals.

Previously, histochemically assessed shift in acid glycoprotein has been reported only for the cell population; here it has been possible to analyse the change within a single cell. Only certain combinations of glycoprotein are found. When neutral glycoprotein is found with acid glycoprotein, the acid glycoprotein is at the cell apex; when sialidase sensitive and sialidase resistant forms are together,

the resistant form is at the cell apex and when sialidase resistant sialomucin and sulphomucin are together, the sulphomucin is at the cell apex. In respiratory goblet cells the new type of acid glycoprotein appears first at the cell apex, then the granules increase in number so that ultimately the cell changes in type.

The present studies thus indicate that the addition of acid radicles to the glycoprotein takes place in the apical region. However, the use of autoradiography and electron microscopy suggests that for the goblet cells it is in the Golgi apparatus that terminal sugars (Neutra and Leblond, 1966; Whur, Herscovics and Leblond, 1969) and sulphate (Lane *et al.*, 1964; Neutra and Leblond, 1966) are added. In the airway goblet cell it is in the apical region that the granules become confluent (Jeffrey, personal communication) and it may be that changes before discharge are responsible for converting sialic acid to the enzyme resistant form and perhaps for modifying the sulphate, so that it is only at the apex that these two become susceptible to staining by the histochemical techniques used here.

In the animals exposed to tobacco + PMO, some goblet cells included more granules of acid glycoprotein than in either the control animals or the animals exposed to tobacco alone. It seems that PMO does not prevent any change in the nature of the glycoprotein synthesized by the goblet cells but prevents, or partially suppresses, the discharge of the secretory granules. This suggests that change in the type of glycoprotein is not the result of increased secretion from the cell. The reason for this PMO effect is not clear.

A number of anti-inflammatory agents have been shown to increase membrane stability. Catenese, Lisciani and Piccinelli (1969) and Mizushima and Sakai (1969), studying the plasma membrane of erythrocytes, have demonstrated increased resistance to hypotonic haemolysis, and Mizushima, Sakai and Yamaura (1970) have suggested that acidic anti-inflammatory drugs such as indomethacin and phenylbutazone have a stabilizing effect on cell membrane proteins. Brown and Tong (1971) have demonstrated that PMO, a basic compound, also confers stability on the erythrocyte membrane.

It may be supposed that irritation causes mucus discharge from the cell and, with increased stimulus, increases synthesis and discharge so that cell turnover is raised. If an agent were to protect against discharge by stabilizing the membrane of either the cell or its granules, a cell might maintain synthesis, or even increase it, a larger amount of secretion being retained within the cell, which would explain the increase in the secretory product found within the cells of the animals receiving PMO. It would seem that it is only in the AB positive cells that this occurs, that is, in those cells synthesizing acid glycoprotein. The addition of PMO would seem both to prevent a non-secretory cell from developing into a secretory cell and discourage discharge from those cells producing only acid glycoprotein, leading to retention of their secretion.

Tracheal submucosal glands

It may not be surprising that exposure to tobacco smoke produces hypertrophy of the submucosal glands, the gland volume being increased as well as the average diameter of acinus and lumen and of the individual cell. What is surprising is that the addition of PMO to the tobacco led to greater increase in gland size although acinus, lumen and cell diameters were similar to those found after exposure to tobacco smoke alone, suggesting that cell multiplication was greater

in the gland after exposure to PMO. Thus it would seem that in the trachea the stimulus to gland activity from the surface was greater in the PMO " protected " animals, which seems paradoxical since the effect on surface epithelium of the PMO was to prevent any increase in goblet cell number, although in certain types of goblet cell the volume of intracellular secretion was increased. A simple explanation might be that distended cells are themselves a source of continuing stimulation. The recent demonstration of intra-epithelial nerve axons in the rat extrapulmonary airways, both sensory and motor, make this suggestion more reasonable (Jeffrey and Reid, 1972).

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Protection of Rat Bronchial Epithelium against Tobacco Smoke

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British Medical Journal, 1972, 2, 142–144

Summary

Addition to tobacco of phenylmethyloxadiazole (PMO) protects rats against some of the adverse effects of exposure to cigarette smoke. Two groups of 15 rats were exposed to 25 cigarettes a day for 24 days; the group whose cigarette included PMO showed less immediate distress after exposure, a smaller tracheal goblet cell count, less thickening of the tracheal epithelium, and less cells in mitosis than those exposed to ordinary tobacco.

Introduction

Human epidemiological studies have shown that tobacco smoking causes hypersecretion of mucus which is typical of chronic bronchitis and that it induces bronchial cancer. The fundamental structural changes seem to be hypertrophy of the bronchial submucosal mucous glands and an increase in the number of goblet cells and their extension into peripheral small airways that are normally free of them. Such changes can be produced experimentally in animals by tobacco smoke and other irritants such as sulphur dioxide (Lamb and Reid, 1968, 1969; Mawdesley-Thomas, Healey, and Barry, 1971) and nitrous oxide (Freeman and Haydon, 1964). The similarity to the morphological changes in man provides a satisfactory animal model for the study of chronic bronchitis (Reid, 1970).

In animals the mitotic rate also is increased during exposure to an irritant. This increase may, in part, reflect the repair of ulcerative damage seen in the first weeks after exposure to sulphur dioxide (Lamb and Reid, 1968), but cell division does not always seem proportional to such damage. For example, in one series of experiments, though two types of tobacco produced a similar increase in goblet cells flue-cured tobacco produced many more mitoses than air-cured tobacco (Lamb and Reid, 1969). In rats these changes have not been seen to develop into bronchial cancer, but a high mitotic count offers evidence of disturbance of cell division and presents features of bronchial damage additional to the increase in goblet cells. Detailed mitotic counts after exposure to tobacco smoke (Lamb and Reid, 1969) were not given in the previous report and so are reported here for the first time. Furthermore the higher mitotic count after exposure to flue-cured or barn-cured tobacco rather than to air-cured or Burley tobacco accords with the higher prevalence of human bronchial cancer after exposure to the former type of tobacco (Passey, Blackmore, Warbrick-Smith, and Jones, 1971).

Because of the relevance to human disease of the changes produced experimentally by tobacco smoke—increase in goblet cells and cells in mitosis—each count offers a suitable test for screening tobacco substitutes or additives.

Dalhamn (1966) and Dalhamn and Rylander (1971) have shown that phenylmethyloxadiazole (PMO), a substance first used as an antitussive agent, offers protection against the ciliostatic effect of tobacco smoke. At their suggestion we undertook the experiments reported here to test the effect of this additive on the increase in goblet cells and on the nature of the distribution of acid glycoprotein they contain, on the cells in mitosis, and on the epithelial thickness. The histochemical study of the intracellular mucus is reported separately (Jones, Bolduc, and Reid, 1972).

Material and Methods

The effect of two types of cigarette was investigated. Each was made with the same tobacco, but to the tobacco for one type 2% by weight of PMO was added.

In the experiments described here 41 Sprague-Dawley rats (Carworth Europe, CFY strain) from specific pathogen-free stock were used. The animals weighed about 150 g at the start of the experiment. Lungs from additional animals of the same batch were examined microscopically to ensure that lymphocytic infiltration was minimal (Reid, 1970).

The rats were divided into three groups—(1) tobacco, 15 animals exposed to cigarette without any additive; (2) tobacco and PMO, 15 animals exposed to cigarettes to which the anti-inflammatory agent PMO had been added; and (3) control, (a) 6 rats given a "sham" exposure in that they spent time in an exposure chamber as groups 1 and 2 but were subjected only to airflow and not to added tobacco smoke, and (b) 5 animals kept in cages. Since statistical analysis showed that groups 3a and 3b were indistinguishable for any of the features studied here, in this paper the results are presented with groups 3*a* and 3*b* added as the control group.

Groups 1 and 2 were exposed to 25 cigarettes a day. The animals were placed in an aluminium chamber and the cigarettes were burnt in a Wright Autosmoker (Wright, 1972). The details of exposure are similar to those reported by Lamb and Reid (1969). The animals were exposed four days a week for six weeks. The mean values of humidity and temperature for each chamber used for tobacco exposure were similar. The difference between the cages used for tobacco and sham exposures was greatest with respect to humidity, doubtless due in part to increased water loss from the animals induced either by the greater rise in temperature or by a nicotine effect, and perhaps because of the water vapour in the tobacco smoke. It has been shown that neither extremes of temperature nor humidity influence the number of goblet cells (Jones, Baetjer, and Reid, 1971).

During the four and a half hours or so of exposure no animal received food or water and the control animals were removed from the room. Apart from this period all animals spent their time in the same type of cage, in the same room, and with as much food and drink as required. Each day the chambers and the Autosmoker were cleaned.

The two types of cigarette supplied to us were identified by a code number whose key was not known to any one of us; it was not broken until it had been decided which cigarette had most effect on the animals.

The animals on whom the mitotic studies were to be made were left quietly in the same room for 18 hours before they were

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killed. Injection of colchicine (0·1 mg/100 g body weight), always at the same time of day, was given four hours before the animal was killed.

All animals were killed by intraperitoneal injection of 1-1·5 ml of pentobarbitone sodium. Six of the animals were used for electron microscopical studies, two from each of groups 1 and 2 and one from each of groups 3*a* and 3*b*. The results of the studies are reported elsewhere. The trachea, main bronchi, and lungs from all the animals were removed en bloc after being inflated with fixative through a tracheal injection until the pleura was tense. The lungs from the animals used for goblet cell counts (nine animals from each exposure group and six controls) were inflated with formol saline, and those from the animals injected with colchicine (four animals from each exposure group and three controls) for mitotic counts were inflated with Heidenhain Susa's solution. Lung sections were cut to include the full length of the main intrapulmonary bronchus and several of its side branches.

For goblet cell counts sections were stained with alcian blue at pH 2·6 and periodic-acid Schiff. Goblet cells were counted under oil immersion (field size 0·18 mm) at three airway levels (*a*) along the full length of the trachea, (*b*) along the medial wall of the axial pathway, and (*c*) in 20 fields each of the second and third lateral airways. By the alcian blue and periodic-acid Schiff technique goblet cells may stain blue or red or as a mixture of these two colours.

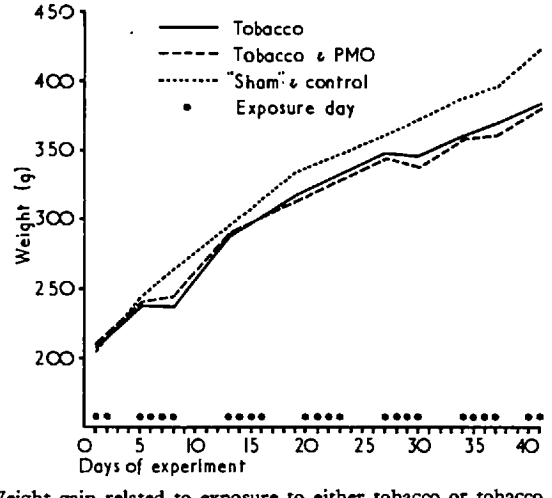
Sections for mitotic counts were stained with haematoxylin and eosin. Cells in mitosis were counted along the same levels of the airway as for goblet cell studies, save that the trachea was considered at upper, middle and lower levels. A section of oesophagus at the mid-tracheal level was included.

Results

During the first eight exposures it was clear that as judged by their general condition of exhaustion one group of animals was affected more than the other. Within a few minutes of removal from the cabinet the animals that we subsequently designated group 2 recovered quickly and started cleaning themselves and moving about; the group 1 animals took about a quarter of an hour before starting this activity. This assessment was made in ignorance of the type of tobacco used, and it emerged only later that the less affected animals were receiving PMO. After further exposures the difference was no longer apparent. The mild conjunctivitis seen during the first days of exposure to tobacco smoke was similar in both groups.

WEIGHT GAIN

The pattern of weight gain for each animal group is illustrated in the Chart. There was no significant difference in this respect between the two tobacco groups but a significant difference was seen between these and the control animals ($P < 0·001$). The animals exposed to tobacco smoke failed to gain weight on the days of exposure; in the days between they gained weight faster and, at least in the first couple of weeks, their weight virtually regained the normal value.



Weight gain related to exposure to either tobacco or tobacco and PMO.

GOBLET CELL COUNT

In the trachea the goblet cell count in the group of animals exposed to tobacco alone was significantly above that found either in the group exposed to tobacco and PMO ($P < 0·001$) or in the control animals ($P < 0·01$) (Table I); there was no significant difference between the control animals and those exposed to tobacco and PMO. In the axial and lateral pathways in the animals exposed to tobacco goblet cells were increased, the increase being greater centrally than distally, although there was no significant difference between any of the groups.

TABLE I—*Mean Goblet Cell Count (S.E. of Mean) per 6 mm of Airway Epithelium in Animals Exposed to Tobacco Alone (Group 1) or Tobacco and PMO (Group 2)*

	Control Group	Group 1	Group 2
Trachea	59·45 (14·48)	156·70 (24·18)	52·92 (8·60)
Axial pathway	104·84 (25·51)	128·11 (17·04)	148·33 (24·91)
Lateral pathways 2nd ..	87·50 (15·80)	92·77 (16·94)	88·50 (22·74)
Lateral pathways 3rd ..	57·00 (11·45)	66·55 (14·07)	60·22 (8·66)

EPITHELIAL THICKNESS AND CELL NUMBER

In the upper trachea the epithelial thickness was measured with an eyepiece graticule as the vertical distance between the superficial surface of the basement membrane and the epithelial surface. Ten measurements were made over the plates of cartilage and 10 between, and the mean for each site was analyzed both for each animal and for each group. These were assessed separately because of an apparent increase between the plates, but no difference in any group was found between the values for the two sites (Table II).

TABLE II—*Epithelial Thickness (μm) and Cell Number in the Upper Trachea (S.E. of Mean given in Parentheses)*

	Epithelial Thickness		Cell Number	
	Over	Between	Over	Between
Control group	12·18 (0·66)	14·35 (1·64)	2·30 (0)	2·70 (0·17)
Group 1	21·37 (0·67)*	21·17 (0·57)†	3·47 (0·07)*	3·40 (0·10)§
Group 2	15·68 (0·90)‡‖	18·41 (0·79)§¶	2·9 (0·19)‡¶	3·16 (0·18)§••

Over = Measurements made over cartilage plates. Between = Measurements made between cartilage plates.
* $P < 0·001$ against controls.
† $P < 0·01$ against controls.
‡ $P < 0·02$ against controls.
§ N.S. against controls.
‖ $P < 0·01$ against group 1.
¶ $P < 0·05$ against group 1.
•• N.S. against group 1.

Heidenhain-Susa's solution being a better cytological fixative than formalin, the measurements of epithelial thickness and cell number were made on material fixed with this solution. Evidence of an increase in cell number contributing to the increase in thickness of the surface epithelium was sought by counting the number of nuclei transected by the vertical line along which the thickness measurement was made. In each tobacco group there was a highly significant increase in thickness compared with the controls, and this was greatest in group 1. The difference between group 1 and group 2 was also highly significant. The results from the animals whose lungs had been fixed in formalin were always similar, although the degree of significance was not as high. The number of cells was also increased in groups 1 and 2 compared with the controls, although only the counts obtained over the cartilage plates achieved significance.

CELLS IN MITOSIS

The number of cells in mitosis for the three groups of animals is given in Table III. At each of the three tracheal levels in groups 1 and 2 the number of cells in mitosis was found to be increased when compared with that of the controls, but the increase in group 1 was higher than that in group 2. At each level the mitotic count was lower in group 2 than in group 1, but at no single level did this achieve significance. When all values were taken in combination PMO had caused a significant reduction in cell division.

TABLE III—*Mitotic Count per 1,000 Cells for the Three Animal Groups*

	Control Group	Group 1	Group 2
Total trachea ..	0·996	6·650 ($P < 0·02$)*	2·943 ($P < 0·05$)
Upper third ..	0·830	7·310 ($P < 0·05$)	3·500 ($P < 0·02$)
Middle third ..	0·660	6·250 ($P < 0·02$)	2·830
Inferior third ..	1·500	6·120 ($P < 0·05$)	2·500
Axial bronchial pathway	1·166	0·875	1·167
Lateral bronchial pathway	1·500	1·625	1·166
Alveoli	7·916	11·000	7·583
Oesophagus	12·330	32·325	14·166

* Value of P given only where count in exposed animals was significant when compared with control animals.

In the alveolar region group 2 was not different from the controls but the mitotic number in group 1 was higher, although not significantly so. The oesophageal count showed a progressive increase from the controls to group 2 to group 1, but these differences were also not significant.

It seems that tobacco alone led to failure to gain weight and to an increase in goblet cells, cells in mitosis, and epithelial thickness. PMO gave complete protection against the increase in goblet cells, partial protection against the increase in both number of cells in mitosis and epithelial thickness, and had no effect on the slower weight gain.

Discussion

For the first time an effective means of protection has been found against some of the changes produced in bronchial epithelium by tobacco smoke. In this, the first detailed quantification of mitotic numbers in rat lung after the application of tobacco smoke, the increase of cells in mitosis was found in large airways—the trachea and main bronchi—and not in the intrapulmonary airways. In the alveolar region there was no significant difference between any of the groups investigated, although the animals exposed to tobacco alone showed some increase in cells in mitosis. With light microscopy it is possible to identify dividing epithelial cells in the airways; electron microscopy is necessary to identify cells in the alveolar wall.

In the airways after exposure to sulphur dioxide it has been shown (Lamb and Reid, 1968) that in the first two days epithelial ulceration was much more severe than later and that the associated healing led to a great increase in cells in division. Preliminary analysis of the number of cells in mitosis after short-term exposure to tobacco smoke has shown a similar effect (P. Bolduc, unpublished observations). Since healing is usually complete within 14 days (Wilhelm, 1953) and the experiment described here was of six weeks' duration the early acute damage is unlikely to have been the cause of the increase. In the larger airways during later weeks of exposure to sulphur dioxide the mitotic count was somewhat raised, suggesting some continuing damage. It may be that such a situation occurs also after exposure to tobacco smoke, but it is likely that there is, in addition, some effect on mitosis peculiar to tobacco smoke. This is suggested by the striking difference in the number of cells in mitosis arising from exposure to different types of tobacco although goblet cell increase was similar (Lamb and Reid, 1968).

A variety of irritants have been shown to produce an increase in goblet cells. This increase is seen in epithelium where the mitotic count is not necessarily raised. It is also induced by the parenteral administration of isoprenaline and pilocarpine (Sturgess, 1970; Sturgess and Reid, 1972)—that is, experimentally induced by irritation or as a result of a pharmacological effect. Histochemical analysis (Jones *et al.*, 1972) has shown that in addition to the increase in goblet cells tobacco produces a change in the distribution of acid glycoprotein throughout the goblet cell population.

The failure of animals exposed to tobacco smoke to gain weight was investigated by Elson and Passey (1963). They found a similar effect from nicotine even without tobacco smoke.

Not all these changes resulting from the effect of tobacco smoke were modified by PMO to the same extent, confirming that various mechanisms are concerned in their production, either singly or in combination. While PMO does not prevent the metabolic-mediated or nicotine-mediated effect it completely prevented an increase in goblet cells. If there is a single cause of mitotic increase it may be modified by PMO; if, as seems likely, at least two factors are operating, one peculiar to tobacco smoke, the other non-specific, then it may be that only one is susceptible. In view of the suppression of goblet cell increase it may be that it is the non-specific effect which is susceptible. But until the effect of PMO on the epithelial response to other irritants has been followed this is not certain.

The preliminary results of the histochemical studies indicate that the effect of PMO does not prevent all effects of tobacco smoke on goblet cells. For example, the intracellular shift in type of acid glycoprotein occurs although goblet cell increase is suppressed. This is being analysed further, as is the effect of PMO on the changes produced by tobacco smoke in the early stage of exposure.

In the airways the influence of PMO on the ciliostatic effect of tobacco smoke has been investigated by administering PMO mixed with the tobacco in the cigarette (Dalhamn and Rylander, 1971) and also by oral administration of oxolamine citrate, the parent compound of PMO (Dalhamn, 1966). By both routes a protective effect was shown, suggesting that these compounds modify the tissue not the tobacco smoke.

This substance justifies further study as a possible additive to tobacco, but whether or not it ultimately proves of value for this purpose it does permit a more effective analysis of the damage done to airway epithelium by tobacco smoke.

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