PULMONARY BIOGENIC AMINE-CONTAINING CELLS

by

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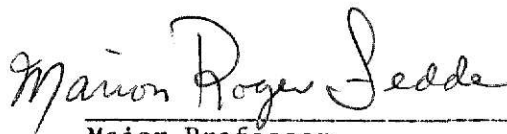
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ABSTRACT

Specialized, non-ciliated, columnar epithelial cells were identified as evaginations into the lumen of a rat respiratory bronchiole. Staining by silver impregnation suggested innervation of a group of such cells. Formaldehyde-induced fluorescence demonstrated the presence of two types of biogenic amine containing cells in the mouse lung. These results were consistent with those of others and verify the validity of the techniques used here.

INTRODUCTION

Increased concern with the effects of environmental pollutants and a rising incidence of diseases of the respiratory system have stimulated a closer examination of the functional anatomy of the lung. Lauweryns et al. ('72) have described distinct groups of innervated, biogenic amine-containing epithelial cells in the lungs of man and various other mammals. They used the formaldehyde condensation technique of Falck and Owman ('65) to produce fluorescence of biogenic amines. Ultrastructurally they were able to identify neurons associated with the basalar aspect of such cells and to describe dense cored vesicles contained within those cells. Lauweryns and Cokelaere ('73) proposed that these specialized groups of epithelial cells were hypoxia-sensitive chemoreceptors.

This laboratory has been interested in peripheral control of breathing in birds. It has been shown that the primary control of eupnea in the chicken is not the inflation-deflation or Hering-Breuer reflexes as is the case in mammals (Eaton et al. '71), but rather peripheral control of

breathing is a function of CO_2 levels in the lung (Peterson and Fedde, '68; Fedde, '70; Fedde and Peterson, '70). Walsh and McLelland ('74) have demonstrated the presence of biogenic amine-containing cells in the extrapulmonary respiratory tract of the embryo and chick of the domestic fowl. We postulated that there may be a relationship between the CO_2 receptors, which have only been identified functionally, and the biogenic amine-containing cells of the chicken lung. The first step toward locating these cells in the intrapulmonary region of the chicken respiratory system was to reproduce some of the light and fluorescent microscopic techniques used to identify them in mammals.

METHODS AND MATERIALS

Light Microscopic Techniques

An eight-week old rat was anesthetized with pentobarbital sodium (26.4 mg/Kg B.W.). A mid-ventral incision was made from the manubrium of the sternum to the tip of the xiphoid process. The heart was located and a 25 gauge needle was inserted into the right ventricle using a micromanipulator. A 3-way stopcock was attached to the needle joining it to two infusion sets. Each infusion set was connected to a bottle of perfusate. The bottles were hung with a constant head pressure of 115 mm Hg (Fig. 1). Two perfusates were used: (A) a heparinized Tyrode's solution which was maintained at 37-39°C by a water bath; and (B) Bouin's fixative (Humason, '62) maintained at 0-2°C by an ice bath.

Once the needle was in the right ventricle, infusion of the Tyrode's solution was begun. A small incision was made in the left atrium through which the fluids from the circulatory system escaped; the fluid was then aspirated from the thoracic cavity. Perfusion with Tyrode's solution

continued until the fluid leaving the left atrium was colorless. The valve on the 3-way stopcock was then turned to permit the fixative to enter the circulatory system. The fixative was perfused for thirty minutes.

A tracheotomy was performed and a tracheal cannula was inserted while the Tyrode's solution was being infused into the circulatory system. When the perfusate was changed from the Tyrode's solution to the fixative, a syringe was attached to the tracheal cannula. This syringe was used to inject fixative into the lungs for rapid fixation of the epithelial surfaces and also to fix the air passages in an open state. The lungs were removed in toto, placed in fresh Bouin's fixative and stored at 6°C for 24 hours. The lungs were then cut into blocks (approximately 8 mm^3) and washed in 50% ethanol to remove all yellow coloration. The small blocks of lung were then dehydrated, infiltrated with paraffin under vacuum and blocked. The tissue blocks were cut in to 5-7 μm thick sections which were mounted on microscope slides precoated with a 2% solution of egg albumin. The sections from one lobe were stained with Mayer's Hematoxylin and Eosin Y (H&E). Another lobe was sectioned and stained with van Campenhout's modification of Bodian's silver stain (van Campenhout, '51) and a Lissamine Fast Red counter stain (Luna, '68).

Fluorescent Microscopic Technique

A nine-week old mouse was surgically prepared in the same manner as the rat. The circulatory system of the mouse was perfused in the same manner as the rat. Instead of Bouin's fixative, however, a phosphate buffered paraformaldehyde fixative, pH 7.2, (Laties et al., '67), was used for perfusion in the mouse and a 2:1 solution of buffered paraformaldehyde and O.C.T. embedding medium (Lab-Tek Products) was injected into the respiratory system. The paraformaldehyde - O.C.T. solution accomplished three functions:

(1) it aided in fixation of respiratory tissue; (2) it provided a good freezing matrix which held the air-ways in an open state; and (3) it provided paraformaldehyde for amine condensation. This technique was a modification of that used by Prince and Porter ('75).

Ten minutes after the air-passeges had been infused via the trachea, the lungs were removed in toto. Sample blocks were cut and immediately quenched in 2-methyl butane cooled to -160°C with liquid nitrogen. The frozen blocks were sealed in small plastic bags and stored at -85°C until they were sectioned. Sections, 10 μm thick, were cut at -25 to -28°C on a cryostat (Ames Lab-Tek) and mounted on coverslips. The tissues were allowed to air dry for 15 minutes. They were then placed in a Coplin jar containing 1 gm of paraformaldehyde, the Coplin jar was capped and heated at 65°C for 3 hours. The paraformaldehyde, prior to use, was equilibrated with water vapor in an atmosphere with a relative humidity of 50%. Equilibration was accomplished by placing the paraformaldehyde in a closed vessel containing sulfuric acid with a density of 1.34 for a minimum of ten days. This level of water vapor-saturated paraformaldehyde was reported by Falck and Owman ('65) to produce an optimal balance between the strength of the fluorescence and the extracellular diffusion of fluorogenic amines from the cells. After the tissues cooled, the coverslips on which they were mounted were attached to microscope slides using Entellan (E. Merck, Darmstadt). The sections were then examined for fluorescence using a Lietz-Ortholux fluorescent microscope with a 200 watt lamp, a BG 38 activating filter, a KP 490 dichromic mirror and a K 510 barrier filter. Fluorescing areas were photographed. Five mice were required to develop this technique.

RESULTS

Light Microscopy

A very distinct group of parallel, non-ciliated, columnar epithelial cells were seen in a rat respiratory bronchiole (Fig. 2A). These cells evaginated into the lumen of the respiratory bronchiole near the origin of an alveolar duct. The nucleus of each cell was oval and vesicular (Figs. 2B and 2C). The cells in this group were readily distinguished from the normal squamous epithelium of the respiratory bronchiole and alveolar duct. Comparison of the non-ciliated columnar cells (Fig. 2C) with the epithelial cells of a terminal bronchiole (Fig. 3) dispells the possibility that the group of columnar cells was simply a terminal bronchiolar-respiratory bronchiolar junction. This body of columnar cells is distinctly different morphologically from the ciliated cuboidal cells and Clara cells of the terminal bronchiole.

Another group of parallel, columnar epithelial cells was seen at the origin of a terminal bronchiole (Fig. 4A). This group of cells evaginated slightly into the bronchiolar lumen. The cells in this group were non-ciliated and slightly taller than surrounding epithelial cells. There was a unique "capping" cell resting on the lumenal surface of this group (Fig. 4B). In an adjacent section, a nerve was seen underlying this group of cells (Fig. 4C).

Fluorescent Microscopy

A yellow-green fluorescence was seen in the epithelium of a mouse bronchiolar lumen (Fig. 5). The shape of the fluorescent region closely resembled the morphology of the body of epithelial cells shown in Fig. 2. In another section, two fluorescent areas were seen in the bronchiolar epithelium: a yellow-green and a bright yellow (Fig. 6).

DISCUSSION

The groups of non-ciliated columnar cells seen in the rat lung

(Figs. 3 and 4) are very similar morphologically to those described by

Hung and Loosli ('74); they consider these cells to be neuroepithelial

bodies, a term used by Lauweryns et al. ('72) to describe biogenic

amine-containing cells which were innervated. The "capping" cell and

the nerve seen at the base of these cells (Fig. 4B) lends further

evidence that the unique groups of cells I identified are indeed

identical to the neuroepithelial bodies described by (Hung and Loosli

('74).

The fluorescence (Figs. 5 and 6) appeared to be consistent with the

expected emission from formaldehyde condensation of biogenic amines

(Falck and Owman, '65). The yellow-green fluorescence may have been

produced by a catacholamine (Jonsson, '67) and the yellow fluorescence

by serotonin (Lauweryns et al., '73). My results, therefore, indicate

that at least two types of biogenic-amine containing cells are present

in the bronchiolar epithelium of the mouse lung.

The results I obtained from the light microscopy of the rat lung

and the fluorescent microscopy of the mouse lung are consistent with

those obtained by other investigators: (a) unique, non-ciliated columnar

epithelial cells in the bronchiolar epithelium; and (b) formaldehyde-

induced fluorescence, indicating the presence of fluorogenic amines, in

the bronchiolar mucosa. I believe that the techniques presented in this

paper are adequate to use in a search for biogenic amine-containing cells

in birds.

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PART II: BIOGENIC AMINE-CONTAINING CELLS IN THE CHICKEN LUNG

ABSTRACT

Formaldehyde induced fluorescence was used to identify biogenic amine-containing cells in the adult chicken and baby chick lung. Such cells were found in the parabronchial region of the adult and the intrapulmonary primary bronchus and parabronchial regions of the chick. This type of cell appears to be very sparsely distributed in both the adult and chick lung. Visual emission spectra from the fluorescing cells indicates the presence of at least two different types of biogenic amine-containing cells in both the adult and young chicken lung: one type of cells probably contains serotonin; the other appears to contain a catecholamine. The location and content of the fluorescing cells provides evidence to suggest either a humoral or hormonal regulation of pulmonary ventilation/perfusion or a receptor involved in peripheral control of breathing.

INTRODUCTION

The lung can no longer be considered a simple organ for gas and heat exchange. Recent histochemical, fluorescent and electron microscopic studies indicate several diversified functions. One rapidly emerging area of study has focused on the content and possible function of specialized epithelial cells in the lung. These cells have been described in man as argyrophilic and argentaffin (Hage, '71, '72a,b) and have been induced to fluoresce (Lauweryns and Peuskens, '69, '72; Lauweryns et al., '70; Hage, '73a). Ultrastructurally, these cells demonstrate certain basic characteristics: a) they are non-ciliated; b) they contain vesicular

nuclei each usually containing a nucleolus and regions of heterochromatin; c) they contain biogenic amines which can be induced to fluoresce (fluorogenic); and d) they contain dense cored vesicles similar to those found in biogenic amine-containing endocrine cells, in presynaptic elements of synapses, and in glomus cells of the aortic body (Lauweryns et al., '70; Hage, '73b).

Less evidence exists for the presence of such cells in the lungs of birds. Individual, non-ciliated columnar cells, which possibly contain biogenic amines, have been found in the intrapulmonary primary bronchus of the chicken using electron microscopy (Cook and King, '69; King et al., '74) and in the extrapulmonary tubular system of chick embryos and newly hatched chicks using formalde-induced fluorescence (Walsh and McLelland, '74). Although only a very few cells were identified, the possible existence of such biogenic amine-containing cells may provide an insight into the peripheral control of respiration and into the regulation of ventilation/ perfusion in birds. This study was initiated in an attempt to provide further evidence for the existence of biogenic-amine containing cells and their location in the chicken lung.

METHODS AND MATERIALS

Fluorescent Techniques

Two formaldehyde-induced fluorescent techniques were used to detect the presence of intracellular, biogenic amines in the lungs of chickens.

1. Formaldehyde-induced fluorescence following lyophilization.

A modification of the formaldehyde-induced fluorescent technique of Falck and Owman ('65) was applied to lung samples from 3 adult, male chickens (White Leghorn-type, Babcock strain). The lungs were removed

while the animals were in a surgical plane of anesthesia (phenobarbital sodium, 0.167 g/kg). The sample blocks of tissue were cut from the parabronchial region adjacent to the mediodorsal secondary bronchi, then immediately quenched in isopentane cooled to -160°C with liquid nitrogen. The frozen specimens were then placed in a lyophilizer (Fig. 2).

The tissues were held at -40°C for four days to produce a slow lyophilization. The temperature was gradually raised to room temperature during the fifth day, then increased to 35°C using a water bath in place of the dry ice-ethanol mixture. The increased temperature retarded water vapor condensation on the dry tissues. The lyophilized tissues were then placed in a 2 liter dessicator with an open Petri dish containing 5 gm of paraformaldehyde. The dessicator was immediately sealed and heated at 65°C for 3 hours. The paraformaldehyde, prior to use, was equilibrated with water vapor in an atmosphere of 50% relative humidity. That was accomplished by placing the paraformaldehyde in a closed vessel containing sulfuric acid with a density of 1.34 for a minimum of ten days. This level of water vapor-saturated paraformaldehyde was reported by Falck and Owman ('65) to produce an optimal balance between the strength of the fluorescence and the extracellular diffusion of fluorogenic amines from the fluorescent structures.

Paraformaldehyde treated specimens were infiltrated with paraffin in vacuo followed by embedding in paraffin blocks. Approximately one thousand 5-10 μm thick sections were cut, mounted on slides and coverslipped using Entellan (E. Merck, Darmstadt, Germany). They were then examined for fluorescence with a Lietz-Ortholux fluorescence microscope using a 200 watt mercury lamp, a KP 490 dichromic mirror, a BG 38 activating filter, and a K 510 barrier filter. Fluorescing areas were photographed. Nine birds were required to develop this technique.

2. Formaldehyde-induced fluorescence following perfusion.

A nine-day old, male chick was surgically anesthetized with
phenobarbital sodium (0.167 g/kg). A mid-ventral incision was made from
a point 1 cm cranial to the furcula to a point 1 cm caudal to the tip of
the sternal carina. This permitted easy accessibility to the heart and
lungs. A 25 gage needle was inserted into the right ventricle using a
micromanipulator. A 3-way stopcock was attached to the needle and joined
to 2 infusion sets. Each infusion set was connected to a bottle of
perfusate. The bottles were hung with a constant head pressure of 115 mm Hg.
Two perfusates were used: (A) a heparinized chicken Ringer's solution
containing the following composition in mM: NaCl, 136.9; KCl, 2.7; $CaCl_2$,
1.8; MgCl, 0.05; NaH_2PO_4, 11.9; $NaHCO_3$, 4.2; Glucose, 5.55 and maintained
at 41-43°C by a water bath; (B) a phosphate buffered paraformaldehyde
fixative, pH 7.2, (Laties et al., '67) maintained at 0-2°C by an ice bath.

Once the needle was introduced into the right ventricle, infusion of
the chicken Ringer's was begun. A small incision was made in the left
atrium through which the fluids from the circulatory system escaped; the
fluid was then aspirated from the thoracic cavity. Perfusion of the
Ringer's continued until the fluid leaving the left atrium cleared. The
valve on the 3-way stopcock was then turned to permit the fixative to
enter the circulatory system.

A tracheotomy was performed and a tracheal cannula was inserted while
the Ringer's was being infused into the vascular system. When the perfusate
was changed from Ringer's to fixative, a syringe was attached to the
tracheal cannula and a 2:1 solution of the phosphate buffered paraformaldehyde
fixative and O.C.T. embedding compound was introduced into the lungs. This
procedure accomplished three functions: a) it provided paraformaldehyde for
amine condensation; b) it aided in the fixation of the respiratory tissue; and

c) it provided a freezing matrix which held the lungs in an open state, a modification of the technique used by Prince and Porter ('75).

After fixation, the lungs were removed in toto and each was bisected into a cranial and caudal block. The blocks were immediately quenched in isopentane cooled to -160°C with liquid nitrogen. The frozen blocks were sealed in small plastic bags and stored at -85°C until they were sectioned. Approximately one hundred fifty 8-10 μm thick sections were cut at -25 to -28°C on a cryostat (Ames Lab-Tek) and mounted on coverslips. The tissues were allowed to air dry for 15 minutes then placed in a Coplin jar containing 1 gm paraformaldehyde. The paraformaldehyde was pretreated identically as in the lyophilization technique. The Coplin jars with the tissues were placed in a 2 liter dessicator; then the dessicator was capped and placed in a 65°C oven for 3 hours. After the tissues cooled, they were attached to slides with Entellan. The sections were then examined for fluorescence using a Lietz-Ortholux fluorescence microscope with 200 watt mercury lamp, a BG 12 activating filter, and a KP 510 barrier filter. Fluorescing structures were then photographed. Five birds were required to develop this technique.

Light Microscopic Techniques

Sections containing fluorescent structures were remounted for staining with Mayer's Hematoxylin and Eosin Y (H&E). This was accomplished by floating the tissue off the slide using a 2% egg albumin solution and gently heating the slide. Once the tissue was floated, the albumin solution was allowed to evaporate leaving the tissue attached to the slide by an albumin adhesive. This adhesive prevented the tissue from separating from the slide during H&E staining.

General orientation of the pulmonary air-ways was obtained from H&E stained sections of both chick and adult lung. A chick lung and an adult lung were perfused as previously described for formaldehyde-induced fluorescence except that in the chick, Bouin's fixative (Humason, '62) was used and in the adult bird, Karnovsky's fixative (Karnovsky, '65) was used. These techniques preserved the air passages of the lung in their natural state.

RESULTS

Intracellular Biogenic Amines in the Parabronchial Region

The general organization of the parabronchial region of the chicken lung is shown in Fig. 8A. The lumen of a parabronchus, atria, infundibula and air and blood capillaries can be seen. Figure 8B shows the epithelial lining of the parabronchus, the underlying smooth muscle, an atrium, an infundibulum and a small area of air and blood capillaries.

Fluorescent microscopy indicated the presence of biogenic amines. The well defined boundaries of fluorescence indicates intracellular biogenic amines.

Thirty six yellow fluorescing regions and eighteen yellow-green fluorescing regions were seen. Seldom were more than one fluorescing region seen on the same section of tissue. On two sections, both a yellow region of fluorescence and a yellow-green region of fluorescence were seen. All the fluorescing regions appeared to be in the parabronchial region and not in the epithelium of the primary or secondary bronchi. However, I was not able to correlate these fluorescing structures with cellular structures after H&E staining.

A yellow-green fluorescence was observed in the parabronchial region of an adult bird (Fig. 9A). This fluorescence was seen in the same region on the two sections adjacent to this one. A red autofluorescence outlined the section of tissue. After H&E staining of the same section, a photograph (Fig. 9B) was taken at the same magnification as Fig. 9A; an arrow indicates the region which corresponds to the area of fluorescence. From this photograph, it is evident that the tissue has separated somewhat. However, it can still be ascertained that the area of fluorescence reaches the atrium and is just under the smooth muscle which surrounds the parabronchus. The fluorescent region of Fig. 9A was photographed at a higher magnification to show three individual fluorescing structures (Fig. 9C). Two of these structures were in sharp focus while the third appeared to be at a slightly different plane, probably due to the thickness (10 μm) of the section. After H&E staining, a photograph of this region, at a magnification equal to that in Fig. 9C, shows several large, vesicular nuclei in the area found to be fluorescing. Only three can be seen in this photograph. However, several others were seen by changing the focus.

Intracellular Biogenic Amines in the Intrapulmonary Primary Bronchus

A section of chick lung taken from the intrapulmonary primary bronchus was stained with H&E and used for general orientation. Figure 10 illustrates the respiratory epithelium in this region. A bright yellow fluorescing cell was found in a similar region from a section prepared by the perfusion technique (Fig. 11). The fluorescence appears to be concentrated in a single epithelial cell. There is, however, some minor diffusion of fluorogenic amines from the fluorescing cell.

An unique, clear non-ciliated, columnar epithelial cell was observed in a H&E section from the chick intrapulmonary, primary bronchus (Fig. 12A). Figure 12B is a higher magnification of this cell. The morphology of this cell closely resembles those identified as amine-containing (King et al., '74). It is columnar with a very light staining cytoplasm and a large, vesicular nucleus which contains some heterochromatin.

Non-Epithelial Fluorescing Structures

It should also be noted that on rare occasions, bright green elongated structures (characteristic of adrenergic nerve fibers) were seen. These structures did not appear to be in the epithelium. This is consistent with the findings of Walsh and McLelland ('74).

DISCUSSION

Consideration of the Methodology

The lyophilization technique used for tissue preparation presented two basic problems when used on chicken lung: (a) collapsing and compressing of the air-ways; and (b) possible exocytosing of fluorogenic amines. The first problem is quite evident from microscopic examination. Such compression of the tissue makes orientation very difficult. The second problem may or may not exist, depending upon the sensitivity of these biogenic amine-containing cells to hypoxia. If these cells do secrete biogenic amines in response to a hypoxic state, as suggested by Lauweryns and Cokelaere ('73a,b), the fluorogenic contents may be lost before the cellular structures can be fixed. However, this did not appear to be a problem in mammalian species (Lauweryns et al., '72). The perfusion technique was used with the chick to preserve the lung in its normal state and to provide a more rapid fixing of the tissue in situ. Furthermore, the preparation time

was reduced from the six days required for lyophilization to one day
for the perfusion method.

Content of Fluorescing Cells

I believe that those fluorescing cells which I have seen contain
biogenic amines. My conclusions are based on the vast amount of work
that demonstrates the formaldehyde condensation of certain amines to
produce fluorophores with characteristic activation and emisison spectra
(Corrodi and Hillarp, '64; Falck and Owman, '65; Corrodi and Jonsson, '65,
'67; Jonsson, '67; Axelsson et al., '72; Axelsson et al., '73; Laszlo, '74).
It was not possible to spectrophotometrically measure the emission spectra;
therefore, I am able to report only visual characteristics of the fluorogenic
structures seen. The yellow fluorescence seen tends to indicate the presence
of serotonin (Corrodi and Jonsson, '67; Lauweryns et al., '73). The yellow-
green fluorescence is more characteristic of catecholamines such as
norepinephrine or dopamine (Jonsson, '67; Corrodi and Jonsson, '67) or
5-hydroxytryptophan (Cutz et al., '74).

Significance of Biogenic Amine-Containing Cells in the Lung

1. General effects of biogenic amines.

There are several biogenic amines which can be induced to fluoresce
using hot formaldehyde vapors and which are of physiological interest with
respect to respiration: norepinephrine; dopamine; and serotonin.
Norepinephrine can act as a neurotransmitter; it can stimulate receptors on
smooth muscle; and it can regulate secretion of various glands (Ganong, '73).
Dopamine also can act as a neurotransmitter and regulate secretion of various
glands (Ganong, '73; Owman et al., '73). In addition to being a
neurotransmitter, serotonin can depolarize cells resulting in release of
cellular contents, eg. serotonin can influence the release of histamine from
mast cells (Owman et al., '73). Serotonin can also increase capillary
permiability (Schayer, '70).

2. Presence in various animals.

Biogenic amine-containing cells have been found in the tracheal mucosa
of man, lambs, rabbits and an armadillo (Cutz et al., '75) and in the
bronchi, bronchioles or alveoli of man, rabbit, cat, lion, monkey, rock
badger, pig, hedge-hog, mouse, guinea-pig, rat and sheep (Hebb and Mann, '68;
Lauweryns et al., '72; Hung and Loosli, '74; Cutz et al., '74; Hage, '74).
Such cells have been reported in the chicken (Cook and King, '69; Walsh and
McLelland, '74; King et al., '74) but appear to be much fewer than in mammalian
lung. The cytology and ultrastructure of such cells are very similar among
species. However, the nomenclature used to describe this special type of cell
varies and includes: a) neurosecretory appearing cells (Terzakis et al., 72);
b) endocrine-like cells (Cutz and Conen, '72); c) Kultschitzky (K) cells
(Bensch et al., '65); d) amine precursor uptake and decarboxylating (A.P.U.D)
cells (Hage, '73); and f) neuro-epithelial bodies (NEBs) (Lauweryns and
Cokelaere, '73). The latter term was derived in response to evidence that
groups of these cells were innervated. Lauweryns and Goodneeris ('75) contend
that the innervation and grouping of NEBs delineate them as separate
morphological structures.

3. Function in the normal lung.

The physiological characteristics of biogenic amines allow for
speculation pertaining to the cells which contain them. The ability to be a
neurotransmitter predicts a possible neurite-receptor complex. Lauweryns and
Cokelaere ('73a,b; '74) suggest that the NEBs are chemoreceptors sensitive to
hypoxia. Such neurite-receptor complex has found in the intrapulmonary
primary bronchus of the chicken (Cook and King, '69; Walsh and McLelland, '74;
King et al. '74). These may or may not be chemoreceptors as suggested by
Lauweryns and Cokelaere ('73a,b). Chemoreceptors sensitive to CO_2 have been
identified physiologically, (Fedde, '70; Peterson and Fedde, '71; Fedde et al.,
'74 a.b.) but not morphologically. However, none of the 60 CO_2 receptors

located physiologically by Scheid et al. ('74) were in the primary bronchus. This does not eliminate the possibility that the neurite-receptor complexes described by King et al. ('74) are CO_2 receptors involved in peripheral control of breathing, but it does reduce the probability of such involvement.

A second possible function of these cells might be to act as a humoral regulatory mechanism for the micro-environmental of the lung. This suggests action of the biogenic amines upon either the bronchial smooth muscle or the vascular smooth muscle or both. The effect of such action might be to alter the ventilation/perfusion ratio as specific local conditions may dictate. Serotonin acts to produce pulmonary vasoconstriction and bronchiolar smooth muscle constriction (Page, '68) and, therefore, could produce such ventilation/perfusion changes.

The third possible function of biogenic amine-containing cells is one of regulation of synthesis and turnover of polypeptide hormones or other vasoactive substances such as prostaglandins and histamine. Such action may explain the presence of serotonin, dopamine and norepinephrine in cells also containing polypeptide hormones (Owman et al., '73; Ganong, '73).

In birds, either a humoral or a hormonal mechanism may be of some importance. The bird relies on its respiratory system not only for gas exchange, but, also, for heat exchange. The minute volume of air passing through the respiratory system of a duck can be increased 6-7 fold without producing a PCO_2 deficit in the arterial blood (Bouverot et al., '74). This infers that there is some shunting of the air from the gas exchange surfaces. Such shunting could possibly be produced by a hormonal or a humoral mechanism controlling the diameter of the secondary bronchi at their origin or the size of the ostia of the atria. Such a shunting would cause air to flow through the primary bronchus to air sacs and out again with very little ventilation of the gas exchange regions.

This raises a very important question: are the biogenic amine-containing cells in the chicken lung numerous enough to be effective as a humoral, hormonal or chemoreceptor controlling respiration? This cannot be answered with the data at hand. It is possible that the technique needs further modification for effective detection of fluorogenic amines in avian tissue. More effort must be spent before a conclusion can be definitely drawn.

4. Function in the pathological lung.

Shunting, also, may be caused by the action of these amines in hypoxic or hypercapnic regions of the lung resulting from emphysema or vascular occlusion. They could produce a redistribution of either blood or air to those pulmonary regions which are capable of optimal ventilation/perfusion; that is, the most efficient gas exchange possible in that lung (Lauweryns and Cokelaere, '73).

The cell Bensch et al. ('65a) described as the normal precursor to bronchial carcinoid adenoma appears to be the same biogenic amine-containing cell with which we are dealing. Work describing the ultrastructure of oat-cell carcinoma proved that it was also composed of Kultsckitzky-like cells (Bensch et al., '68) and, associated with this condition are such endocrine disturbances as elevated serum serotonin and/or its metabolites (Bensch et al. '65b), Cushing's Syndrome (Cheng et al., '58) and the carcinoid syndrome (Parish et al., '64). Gmelich et al. ('67) have described the same type of cell in peripheral adenoma of the lung.

Another pathological condition with which this type of cell may be associated is thrombo-embolism. The work of Butler ('71) implies that some of the symptomatology seen resulting from aortic thrombo-emboli is due, not to a simple occlusion of the vessel, but to some action by components released or activated during coagulation. The best candidate for this role seems to be serotonin. The same may be true for pulmonary thrombo-emboli.

The circulatory deficiency and edema seen in such cases may result from action of biogenic amines rather than a mere mechanical occlusion.

It should be evident that the biogenic amine containing cells of the lung surely play a significant role in more than one pathological condition and may have a major role in normal respiration. Both areas need further investigation.

To continue the investigation of biogenic amine-containing cells in the bird, several modifications should be made. The experimental animal should be a bird which is large enough to readily perfuse but has a small lung. This would permit examination of the entire lung in a reasonable period of time. The histochemical and fluorescent histochemical techniques must be selected based upon the facilities and equipment readily available. The perfusion technique described in this paper should be substituted for the lyophilization technique and samples of the pineal body, adrenal medulla or small intestine could be used for positive controls for formaldehyde-induced fluorescence. Several different stains should be used to try to demonstrate argyrophilia. These modifications should allow: (a) the air-ways of the lungs to be preserved in their natural state, (b) feasible examination of the entire lung (c) better correlation between fluorescing regions and cytological structures.

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    Acta anat. $\underline{75}$:263-275.

APPENDIX I

Solutions, Fixative and Stains

A. Solutions

1. Avian Ringer's Solution - R. D. Cook 1971. Ph D Thesis, University

    of Liverpool, England

    To make: 1 Liter

Sodium chloride	8.00 g
Potassium chloride	0.20 g
Calcium chloride	0.20 g
Magnesium chloride	0.10 g
Sodium hydrogen carbonate	1.00 g
Sodium dihydrogen phosphate	0.05 g
Glucose	1.00 g
Q.S. with distilled water	

2. Mammalian Tyrode's - C. Best and N. Taylor Ed. 1955. The physiological
    basis of medical practice, 6th Ed. The Williams
    and Wilkins Co., Baltimore, Md. p. 192.

    To make: 1 Liter

Sodium chloride	9.00 g
Calcium chloride	0.20 g
Potassium chloride	0.20 g
Sodium hydrogen carbonate	1.00 g
Glucose	1.00 g
Sodium dihydrogen phosphate	0.05 g
Q.S. to 1 liter with distilled water	

B. Fixatives

1. Karnovsky's Fixative - J. M. Karnovsky, 1965. A formaldehyde-
    glutaraldehyde fixative of high osmolarity
    for use in electron microscopy. J. Cel.
    Biol. 27:21-40.

    Materials: Paraformaldehyde
                50% Glutaraldehyde
                Sodium cacodylate (M.W. 160)
                Calcium chloride

    To make: 1100 ml

    a. Cacodylic Buffer (0.2 M)

Sodium Cacodylate	25.6 g	
Distilled water	800	ml

b. <u>Stock Fixative</u>

        Paraformaldehyde   8.0 g
        Distilled water   100.0 ml
        Cacodylate buffer   60.0 ml

Heat in a 70° water bath, under a hood. Swirl intermittently until

clear. If clearing does not occur, add several drops of 10 N sodium

hydroxide. Cool to room temperature.

Mix:

        50% Glutaraldehyde   20 ml
        Calcium chloride   0.1 g
        Cacodylate buffer   20 ml

c. <u>Dilute to 1:4.5</u>

        Stock fixative   200 ml
        Distilled water   200 ml
        Cacodylate buffer 700 ml

Adjust pH to 7.2

Store at 4°C

Filter before use

2. Bouin's Fixative - G. L. Humason, ed. 1962. Animal Tissue Techniques.
                       W. H. Freeman and Co., San Francisco, Calif. p. 13.

  a. Picric acid, saturated aqueous   75 ml
  b. Formalin   25 ml
  c. Glacial acetic acid   5 ml

Wash in 50% alcohol; complete clearing using 70% alcohol plus a few
drops of saturated lithium carbonate.

3. Phosphate Buffered Paraformaldehyde - A. M. Laties et al. 1967. A
                       simplified method for the histochemical localization
                       of cardiac catecholamine containing nerve fibers.
                       J. Histochem. Eytochem. 15:534-542.

To make 100 ml:

        83 ml of 2.26% NaH_2PO_4
        17 ml of 2.52% NaOH

Heat to above 60°C (under a hood)

Add 4 gm paraformaldehyde

Continue heating until solution is clear

pH should be approximately 7.2

C. Stains

1. Van Campenhout's Modified Bodian's Silver Stain - E. Van Campenhout
                1951. Une Nouvelle méthod D´imprégnation
                Argentique. Bull. Microsc. Appl. 1:53-59.

    a. Protargol   1%

    b. Reducing Solution

        2% Hydroquinone
        10% Sodium sulfite
        1% Pyrogallol

    c. Silver Nitrate

        5-10% for CNS
        20% for peripheral nervous system

    d. Gold chloride   0.5%

    e. Sodium hyposulfate   5%

2. Lissamine Fast Red - L. G. Luna, ed. 1968. Manual of Histologic
                Staining, Methods of the Armed Froces.
                Institute of Pathology, 3rd ed. McGraw-Hill
                Book Co., New York, N.Y. pp. 195-196.

    a. Lissamine Solution

        Lissamine fast red   1.0 g
        Glacial acetic acid   1.0 ml
        Distilled water   100.0 ml

    b. Tartazine Solution

        Tartazine   1.5 g
        Glacial acetic acid   1.5 ml
        Distilled water   100.0 ml

    c. Phosphomolybdic acid   1%

3. Mayer's hematoxylin – G. L. Humason, ed. 1962. Animal Tissue
                      Techniques. W. H. Freeman and Co., San
                      Francisco, Calif. p. 125.

      Distilled water   1

      Hematoxylin   1 g

Heat gently while adding:

Sodium iodate   2 g

Potassium alum   50 g

$(Al_2 (SO_4)_3 K_2 SO_4 . 24H_2O$

Heat until dissolved then add:

Citric acid   1 gm

chloral hydrate   50 gm

Ripen 6–8 weeks; can be used in 1–2 weeks.

APPENDIX II

PARAFORMALDEHYDE SATURATION

ADJUSTMENT OF RELATIVE HUMIDITY WITH H_2SO_4 - A.G.E. Pearse, 1972.
Histochemistry, 3rd Ed. II: Churchill-Livingstone. London, England. p. 1394.

Relative Humidity %	Density	(H_2SO_4) g/l
10	1.58	1064
20	1.49	887
30	1.44	779
40	1.39	678
50	1.34	586
60	1.29	503
70	1.25	428

APPENDIX III

FORMALDEHYDE-INDUCED FLUORESCENT
CHARACTERISTICS OF BIOGENIC AMINES
A.G.E. Pearse 1972. Histochemistry, 3rd Ed. II:
Churchill - Livingstone, London, England, p. 1106

Amine	Excitation	Emission
Dopamine	410	480
Epinephrine	410	480
Norepinephrine	410	480
Tryptamine	370	490
5 Hydroxytryptamine	385–410	520–540
5-Methoxytryptamine	380	525

Fig. 1. Diagramatic arrangement of perfusion set-up used for intravascular

infusion.

PERFUSATE A   PERFUSATE B

MANOMETER

MICROMANIPULATOR
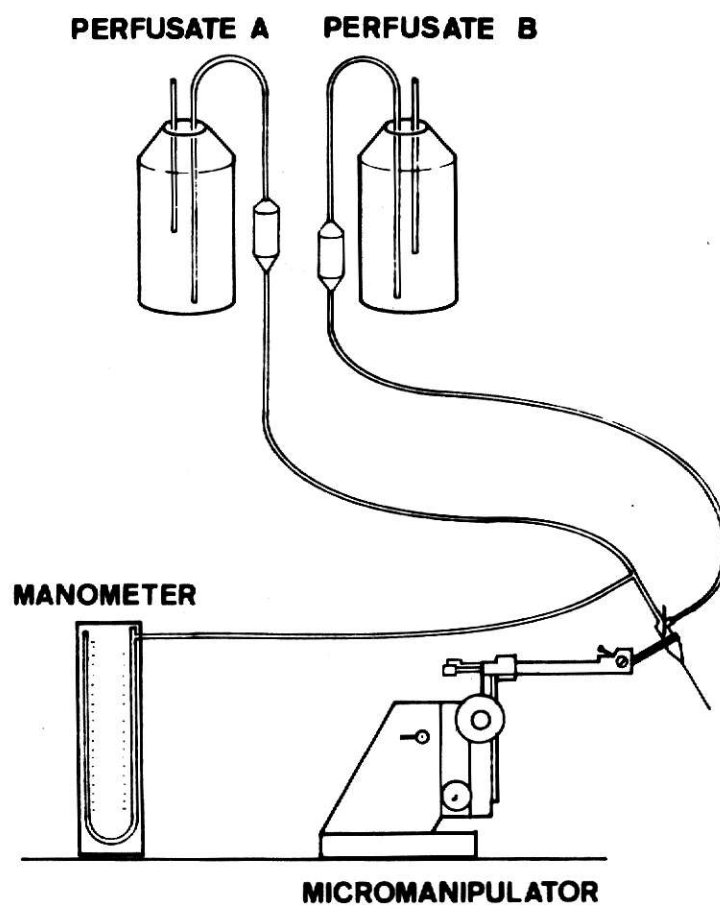
PLATE 1

Explanation of Figures

Fig. 2 . Neuroepithelial body in a rat respiratory bronchiole.

Fig. 2A. Cross section of a rat respiratory bronchiole with a group of
parallel, non-ciliated columnar cells evaginating into the
bronchiolar lumen near the origin of an alveolar duct. BL,
Respiratory bronchiolar lumen (H&E, X160).

Fig. 2B. Higher magnification of the region within the square in Fig. 2A
(H&E, X650).

Fig. 2C. Higher magnification of the columnar cells of Fig. 2A. Note the
large vesicular nucleus of each cell (H&E, X1625).

Fig. 3 . Cross section of a rat terminal bronchiole. Note that none of
these cells correspond to the columnar cells seen in Fig. 2.
C, ciliated cuboidal epithelial cell; NC, non-ciliated Clara
cell (H&E, X2050).

2A

BL

2C

BL

BL

BL

NC →

C →

3
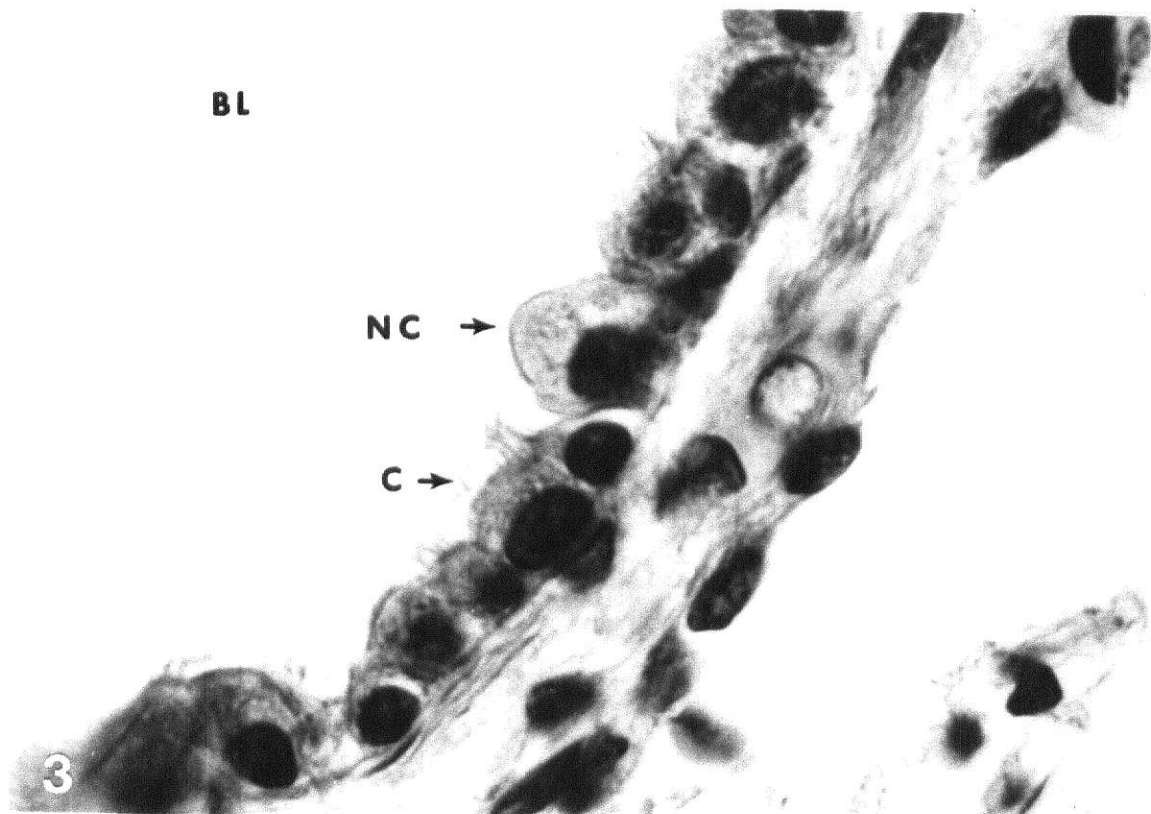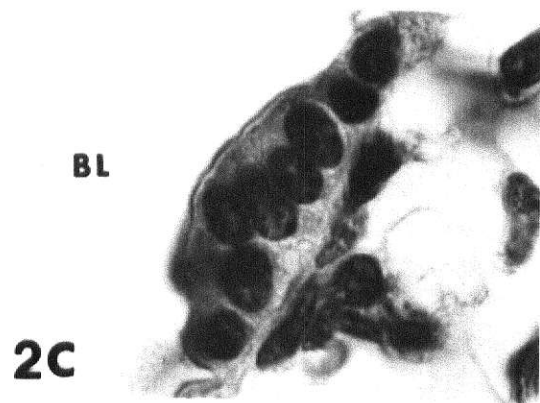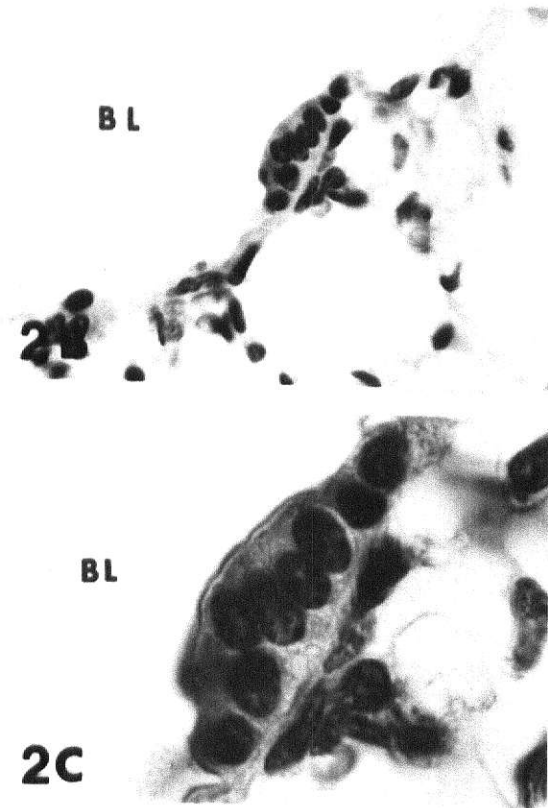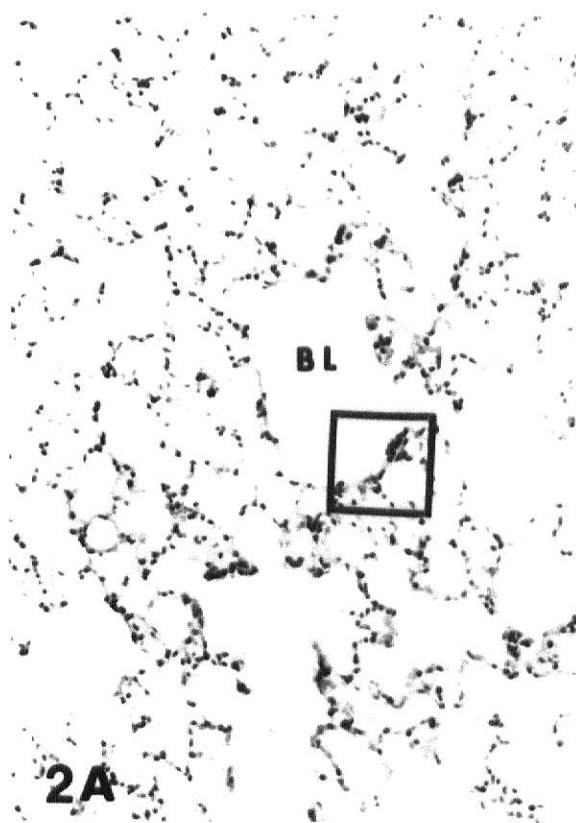
PLATE 2

Explanation of Figures

Fig. 4 . Neuroepithelial body in a rat terminal bronchiole.

Fig. 4A. Longitudinal section of a rat terminal bronchiole (TL) opening

into a respiratory bronchiole. A unique structure is contained

within the squared area (Modified Bodian's Silver Stain, X160).

Fig. 4B. A higher magnification of the area seen within the square in

Fig. 4A. Note the taller, parallel non-ciliated columnar cells

and the "capping" cell (arrow) over them. V, veins. (Modified

Bodian's Silver Stain, X650).

Fig. 4C. An adjacent section to the one seen in Fig. 4B. Note the nerve

(N) beneath the columnar cells (Modified Bodian's Stain, X650).

Fig. 5 . Fluorescent photomicrograph of a mouse bronchiole. The arrow

indicates an area of yellow-green fluorescence which evaginated

into the bronchiolar lumen (BL) (X160).

Fig. 6 . Fluorescent photomicrograph of a mouse bronchiole. Two regions

of fluorescence are indicated by the arrows. The upper region

produced a bright yellow emission while the lower region was

yellow-green (X160).
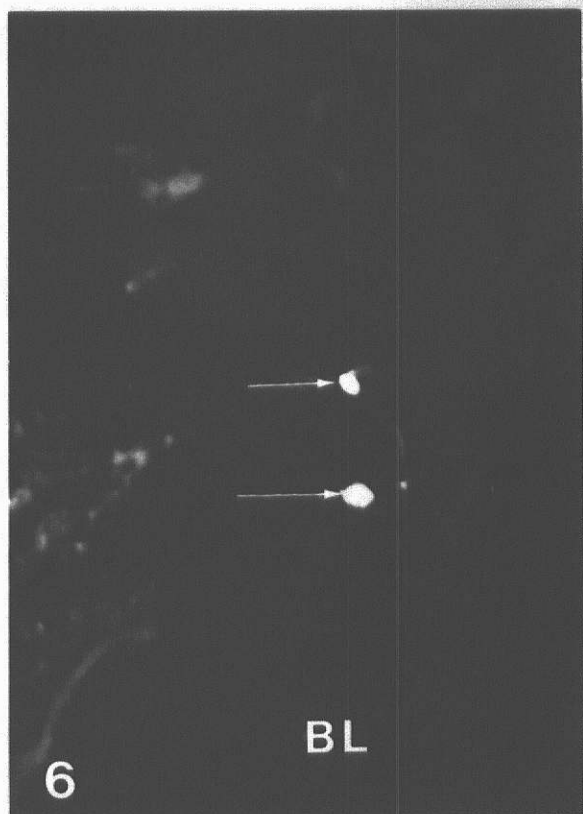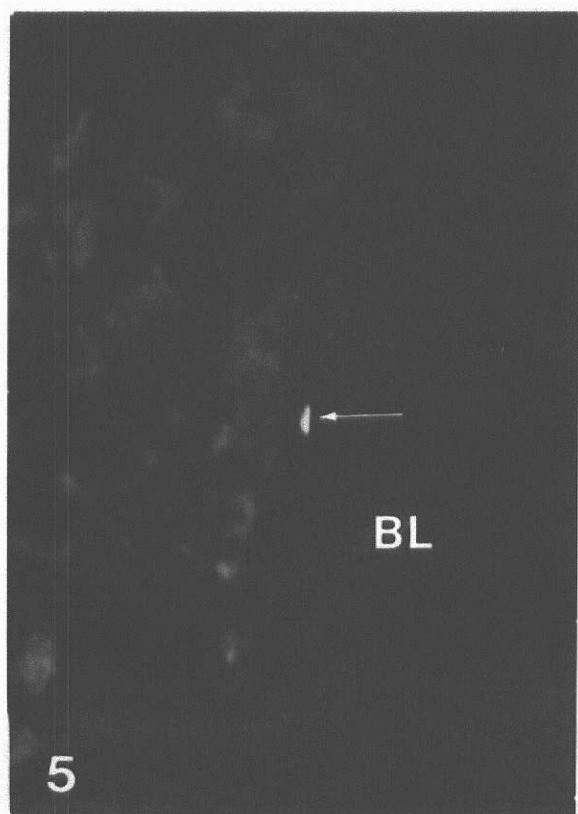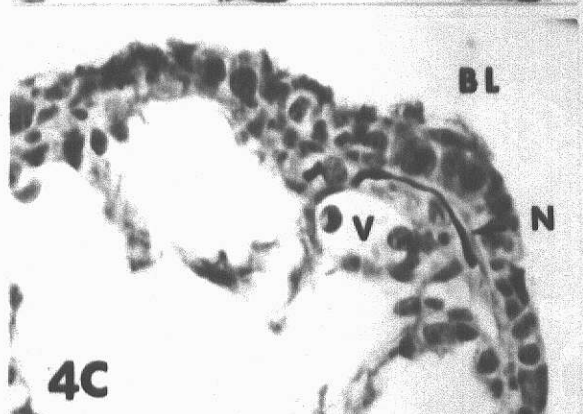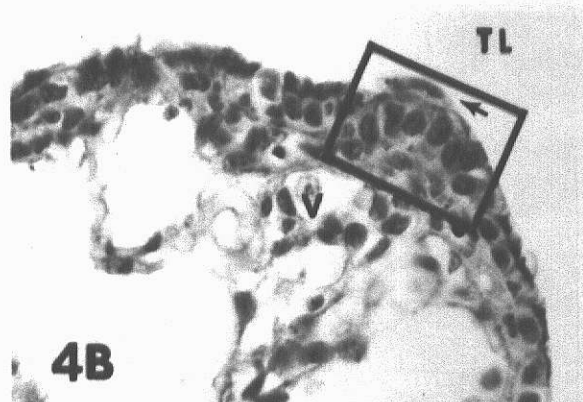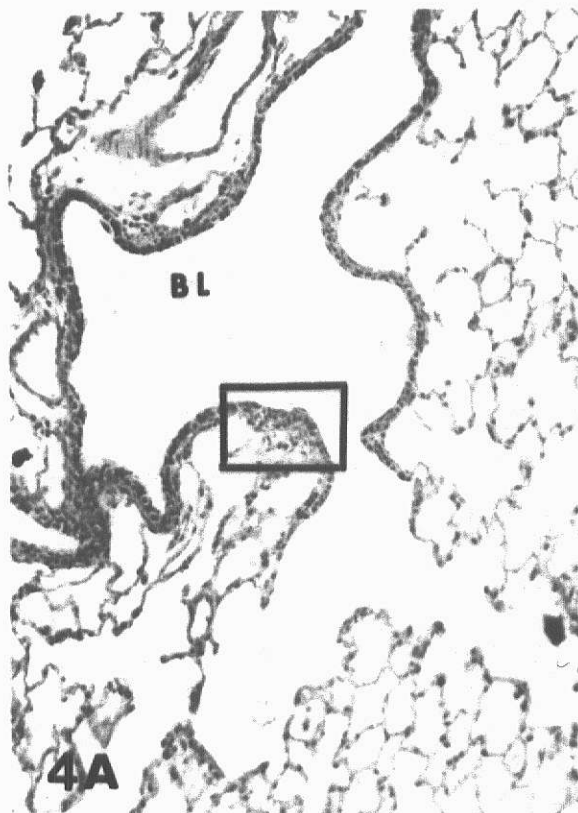
Fig. 7. Schematic diagram of the lyophilizer used to study biogenic
amine-containing cells in the avian lung. The frozen specimens
were placed in a VirTis flask which was then connected to a
manifold leading to the vacuum pump. Air evacuated from the
flask passed through cold trap no. 1 to remove the moisture then
out the pump. When the tissues were dry, the pressure in the
flask was equilibrated to atmospheric pressure by slowly opening
stopcock no. 1, permitting air to enter through port no. 2,
through cold trap no. 2, then into the manifold. The air then
passed through cold trap no. 1. This prevented rehydration of
the lyophilized tissue.

STOP COCKS

P-1

P-2

COLD TRAPS

AIR

ETOH
DRY + ICE

V.F.

TISSUES

ETOH + DRY ICE

V.F. = VIRTIS FLASK
P-1 = PORT-1
P-2 = PORT-2
V.P. = VACUUM PUMP
—— = EVACUATION
----- = EQUILIBRATION

V.P.
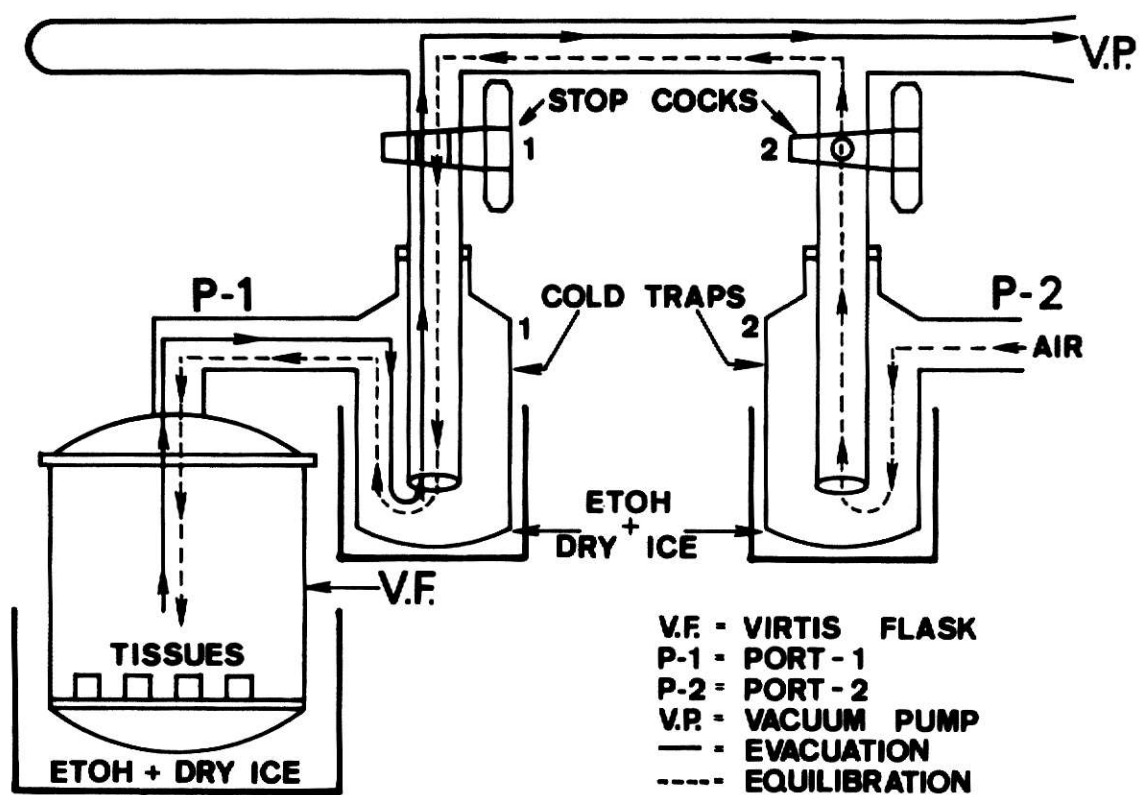
PLATE 3

Explanation of Figures

Fig. 8 . H&E section of a parabronchus.

Fig. 8A. Cross section of a chicken parabronchus. A, Atrium; CT,

Connective tissue; PL, Parabronchial lumen (H&E), X160).

Fig. 8B. Higher magnification of the region contained in the

rectangle in Fig. 8A. A simple squamous epithelium (E)

covers the smooth muscle (SM) which surrounds the atrium (A).

An infundibulum (I) leading from the atrium to the exchange

tissue is also shown. A few red blood cells (RBC) have been

displaced into the parabronchial lumen (H&E, X650).

Fig. 9 . Comparison of fluorescence and H&E staining of an identical

section of chicken lung.

Fig. 9A. Fluorescent photomicrograph showing a region of yellow-green

fluorescence (single arrow) evaginating into the atrium (A).

A red autofluorescence (double arrows) was also seen (X160).

Fig. 9B. Identical section as Fig. 9A, but after staining. The area

corresponding to the region of fluorescence is indicated by

the arrow (H&E, X160).

Fig. 9C. Higher magnification of the fluorescent region of Fig. 9A.

Three distinct regions of fluorescence are indicated by the

three arrows (X650).

Fig. 9D. A higher magnification of Fig. 9B. The epithelium (E) covering

the smooth muscle (SM) can be seen. The three arrows point to

three large vesicular nuclei which evaginate into the atrium (A)

and correspond to the fluorescent regions seen in Fig. 9C (X650).
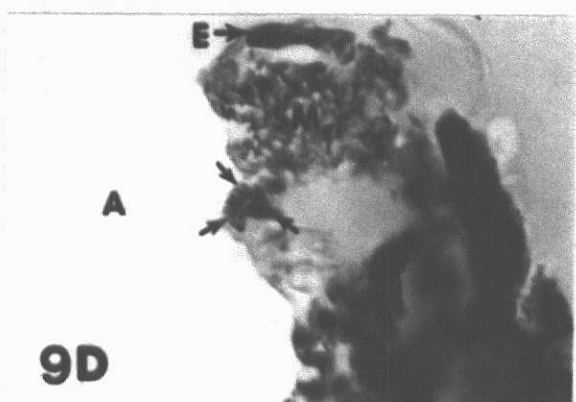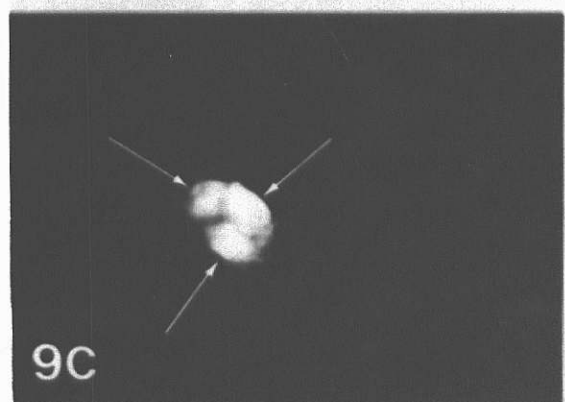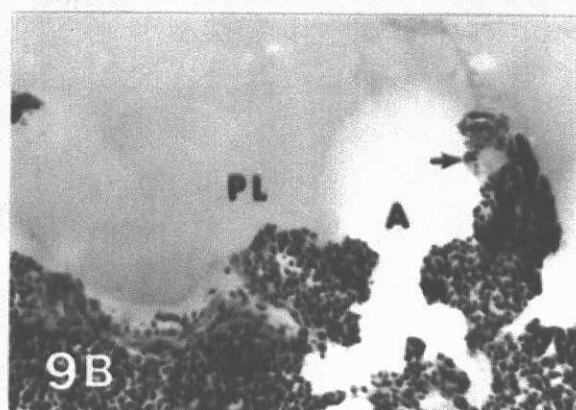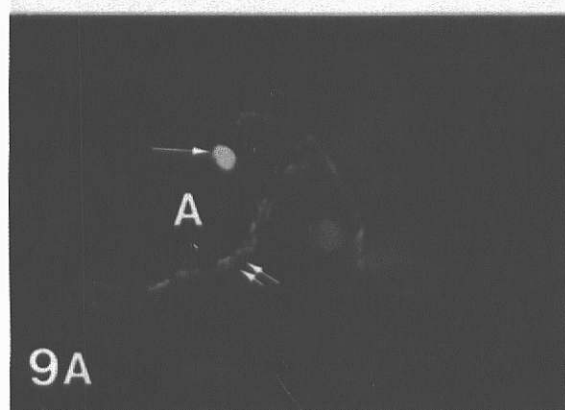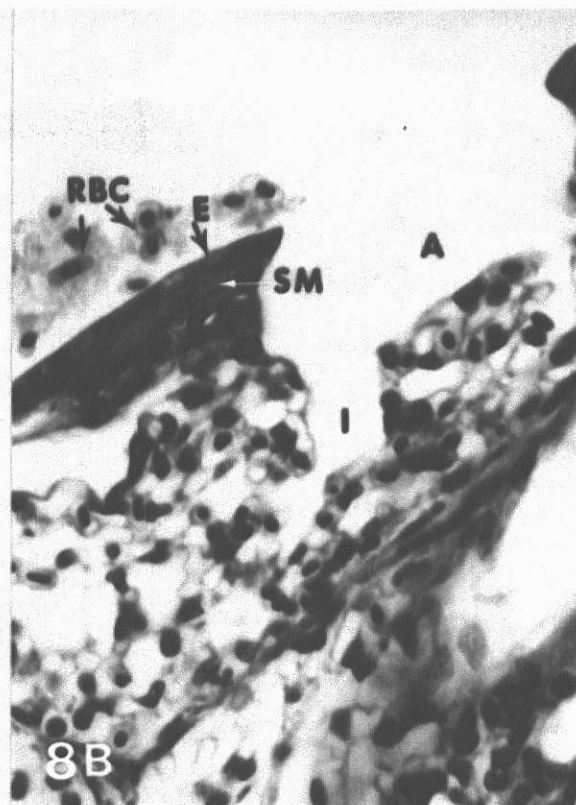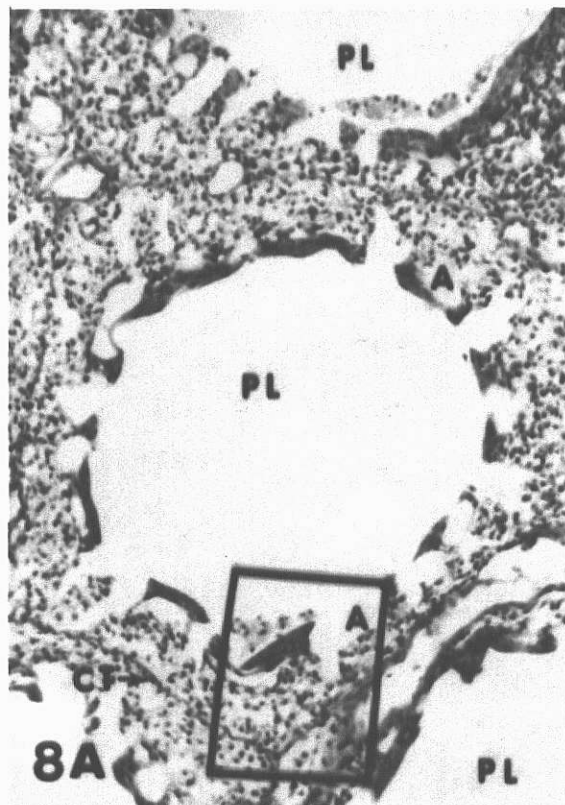
PLATE 4

Explanation of Figures

Fig. 10 . Longitudinal section of an intrapulmonary primary bronchus

from a chick showing the typical respiratory epithelium (E)

with underlying smooth muscle (SM) and veins (V). A few

red blood cells and thrombocytes can be seen in the lumen

of the primary bronchus (IL) (H&E, X650).

Fig. 11 . Fluorescent photomicrograph with an intense region of

fluorescence in the epithelium (E) of the primary bronchus.

IL, Intrapulmonary primary bronchus; SM, Smooth muslce (X650).

Fig. 12 . Unique columnar cell appearing in the chick intrapulmonary

primary bronchus.

Fig. 12A. Cross section of an intrapulmonary primary bronchus taken from

a chick. An unique cell is contained in the square (H&E,

X160).

Fig. 12B. Higher magnification of the region contained within the square

in Fig. 12A. The unique cell is centered and can be compared

with a typical mucous cell (MC) (H&E, X1625).
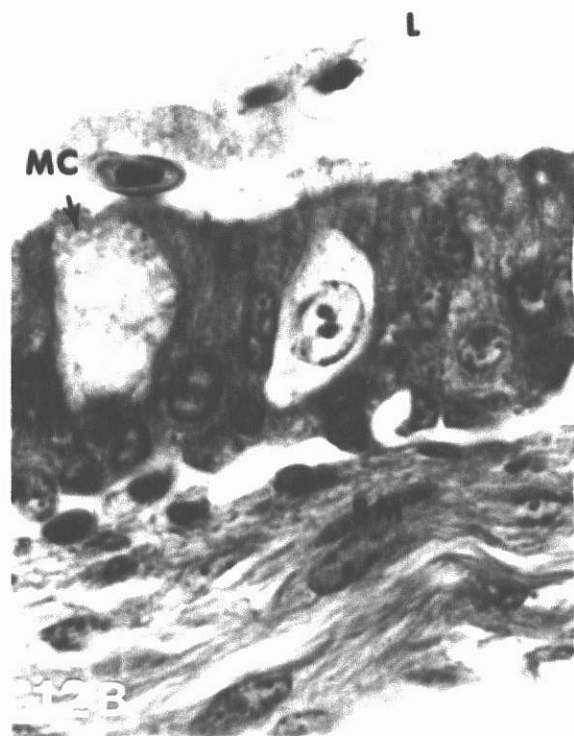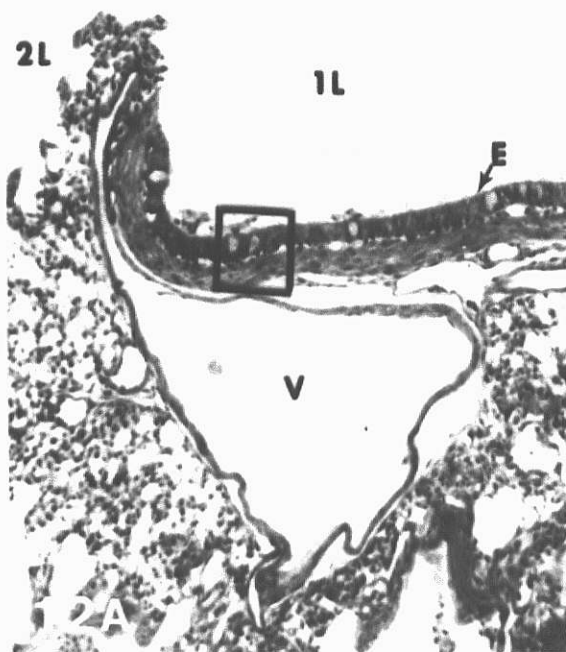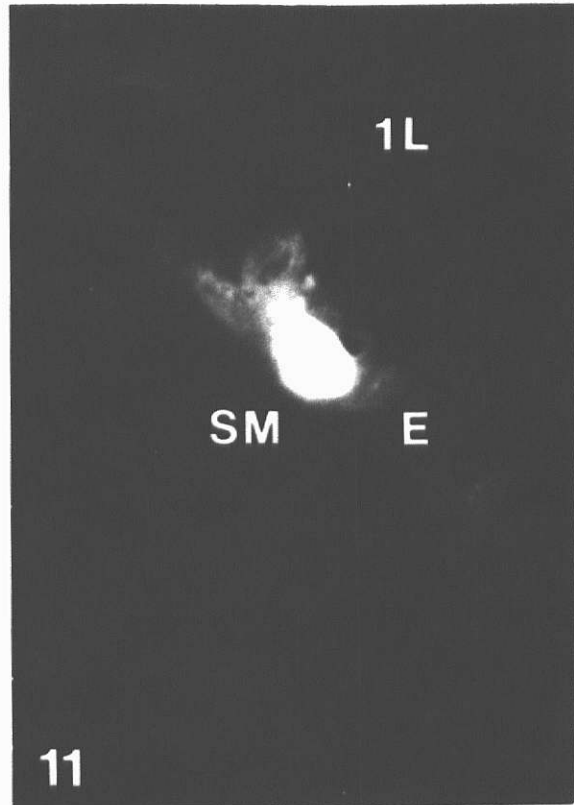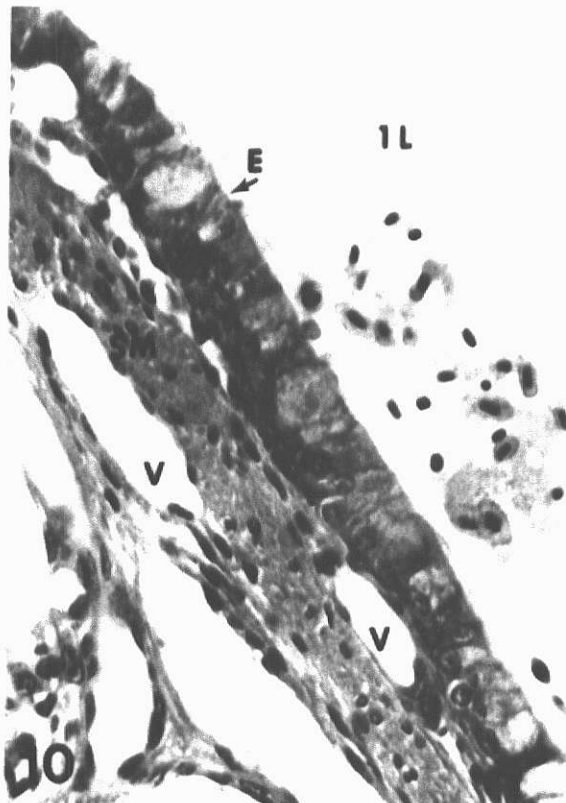
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PULMONARY BIOGENIC AMINE-CONTAINING CELLS

by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Specialized, non-ciliated, columnar epithelial cells were identified as evaginations into the lumen of a rat respiratory bronchiole. Staining by silver impregnation indicated possible innervation of such cells. Formaldehyde-induced fluorescence elicited in the bronchiolar epithelium of the mouse established the presence of intracellular biogenic amines. These results in mammalian lung acted as controls to verify techniques which would be used to identify biogenic amine-containing cells in the avian lung.

Adult chicken and baby chick lungs were examined using formaldehyde-induced fluorescence to identify and locate biogenic amine-containing cells. Such cells were found in the parabronchial region of the adult chicken and baby chick lung and in the epithelium of the intrapulmonary primary bronchus of the chick. This type of cell appears to be more sparsely distributed in both the adult and chick lung than in the mammalian lung. Visual emission spectra suggest that the fluorescing cells seen in the adult chicken and chick lungs probably contained at least two types of amines: a catecholamine; and serotonin. The location and content of these cells suggest a possible humoral or hormonal regulatory mechanism for peripheral regulation of ventilation/perfusion of the lungs or a pulmonary receptor for peripheral control of breathing.