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Fluorescent, Cytochemical, and Ultrastructural Characteristics of Amine Containing Cells in the Laryngeal and Tracheal Epithelium of Adult Albino Rats

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FLUORESCENT, CYTOCHEMICAL, AND ULTRASTRUCTURAL CHARACTERISTICS OF AMINE CONTAINING CELLS IN THE LARYNGEAL AND TRACHEAL EPITHELIUM OF ADULT ALBINO RATS

By

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ABSTRACT

FLUORESCENT, CYTOCHEMICAL, AND ULTRASTRUCTURAL CHARACTERISTICS OF AMINE CONTAINING CELLS IN THE LARYNGEAL AND TRACHEAL EPITHELIUM OF ADULT ALBINO RATS

By

Allan Dorland Pearsall

The extrapulmonary epithelium of adult Sprague Dawley rats contain numerous endogenously fluorescent (weak) cells. These cells are present following the classic histofluorescent method (FIF) of Falck and Hillarp (1962). Cytospectrofluorimetry revealed an emission maximum of 493nm. The excitation maximum could not be calculated but appeared to be below 350nm, which requires a special quartz optical system. In agreement with Hoyt et al. (1979), aqueous formaldehyde fixation and embedding in glycol methacrylate failed to identify these cells even though mast cells (excitation and emission maxima: 401/515nm) were evident. Treatment of the sections with formaldehyde and HCl vapors (LFIF-HCl) (Bjorklund et al., 1968) resulted in the identification of a new population of intensely fluorescent cells (455/537nm) throughout the epithelial lining of the extrapulmonary airways. This spectral shift and increase in fluorescent intensity following acidification is similar to reported values for tryptamine, tryptophan,

and peptides with NH_2-terminal tryptophan (Bjorklund et al., 1968 and Larsson et al., 1975) markedly different from those reported for the phenylethylamines or serotonin (Bjorklund et al., 1968, 70 and 71) duodenal enterochromaffin cells, prepared according to the LFIF-HCl method, displayed the characteristic spectra for the presence of serotonin (425/513nm). The morphology of the fluorescent cells was similar when prepared according to the FIF or LFIF-HCl techniques. Conjunctive light microscopy demonstrated that the fluorescent granules were methylene blue, alcian blue, periodic-acid Schiff and ferric-ferricyanide positive. Fresh cryostat sections an intense monoamine oxidase activity throughout the epithelial lining. These cells were similar to globule leukocytes which previously have been identified in the mucus membranes of the urinary (Cantlin et al., 1972), intestinal (Murrey et al., 1968), and respiratory (Kent, 1966) systems. These results were supported through the conjunctive use of fluorescence and electron microscopy which demonstrated that the fluorescent epithelial cell was indeed a globule leukocyte. Additionally, microspectrofluorimetric recordings and electron microscopic investigation further support Hoyt et al. (1979) findings that aqueous formaldehyde fixed tissues embedded in glycol methacrylate are useful for the histochemical identification of serotonin containing cells.

Furthermore, acid hydrolysis may extend the usefulness of this technique by identifying other closely related β-(3-indolyl) ethylamines.

This work is dedicated to four people who have given me love, support, and encouragement throughout all of my life. To my parents, Ruth and Allan, for providing me with an appreciation for higher education and the means to attain it, and to my sisters, Karen and Sandy, for their invaluable support and understanding, I dedicate this work.

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CHAPTER I

INTRODUCTION

The epithelium lining the lumen of the respiratory tract is composed of many cell types distinguishable by morphologic and cytochemical characteristics (Rhodin, 1956; 1966; Jeffery and Reid, 1975; and Breeze et al., 1977). Three cell types which contain biogenic monoamines have been described in the respiratory epithelium. Histochemical studies by Pearse (1969) demonstrated that many kinds of polypeptide hormone-producing cells were able to take up amine precursors, decarboxylate them and produce biogenic amines. In an attempt to provide a conceptual framework for the diffuse endocrine-like cell system, Pearse (1969) proposed the Amine Precursor Uptake and Decarboxylation concept (APUD). APUD cells share some or all of the following characteristics: (a) amine content or amine precursor uptake and decarboxylation; (b) high levels of α-glycerophosphate dehydrogenase and or non-specific esterases or cholinesterase histochemical activity; (c) masked metachromasia; and (d) endocrine-like granules. In an attempt to extend this conceptual framework, Fujita (1976) proposed the paraneuron concept which was based on morphologic and

physiologic characteristics. Paraneurons are cells which traditionally have not been directly associated with the nervous system but because of their similar structure, metabolism, and function should be regarded as closely related. Paraneurons share to some degree all of the following characteristics: (a) contain neurosecretion-like and/or synaptic vesicle-like granules; (b) contain or produce substances identical with, or related to neurosecretions or neurotransmitters; (c) possess receptosecretory function; and (d) are embryologically derived from neuroectoderm. Fujita and Kobayashi (1979) have subsequently eliminated the neuroectoderm origin characteristic. In the respiratory system, three cell types have been described which fulfill the paraneuron concept.

One of these cell types, the globular leukocyte, is present in the respiratory epithelium of larynx, trachea, and bronchioles (Kent, 1966). This cell is characterized by large electron-dense granules closely encircled by a unit membrane (Kent, 1966; Ewen et al., 1972; and Jeffery and Reid, 1975) and a lack of morphologic attachments to adjacent epithelial cells (Kent, 1966; Cantin et al., 1972). Both endogenous amine content (Murray et al., 1968; Ewen et al., 1972) or induced amine content after administration of an amine precursor (Ewen et al., 1972) have been demonstrated in globular leukocytes. Based on subjective observations of cytofluorescence, the endogenous amine has been

suggested to be dopamine (Murray et al., 1968; Ewen et al., 1972; and Breeze et al., 1977). The exact function of the globular leukocyte in the tracheobronchial epithelium remains unclear. In the gastrointestinal tract, they have been investigated in relation to parasitic infections (Murray et al., 1968; and Miller, 1971). It has been suggested that the biogenic monoamine contained with the globular leukocyte is released to increase the permeability of the gastro-intestinal wall and to allow circulating or locally produced antibodies to pass through the epithelium and gain access to invading antigens (Miller, 1971).

Light and electron microscopy (Kent, 1966 and Jeffery et al., 1975) have demonstrated that the endocrine granules vary in size and shape even within the same cell. Despite this wide range of granule diameter, all of the granules are well above the optical limit of resolution, 2,000 Å (Kent, 1966).

The second type of biogenic amine containing cell occurs singly in the extra and intrapulmonary airways of animals (Ericson, et al., 1972; Cutz et al., 1975; and Dey et al., 1976, 1980) and humans (Cutz et al., 1972 and 1975; and Hage et al., 1977). They are endogenously fluorescent for serotonin (Dey et al., 1976; 1980) and are able to take up amino acid precursors (Hage, 1973a; and Hoyt et al., 1979). The single cells are triangular or flasked shaped (Tateishi

et al., 1973; and Hage et al., 1977), with the base resting along the basement membrane (Cutz et al., 1972, 1974; and Hage et al., 1974). The most characteristic feature of these cells at the ultrastructural level was the presence of small dense-core vesicles (approximately 1,200 Å) surrounded by a clear area and limited by a single unit membrane (Gmelich et al., 1967; Cutz et al., 1972; and Hage 1973b). Recently Echt et al., (1980), reported a significant decrease in the number of detectable tracheal fluorescent cells in neonatal rabbits after exposure to hypoxic hypoxia or carbon monoxide induced hypoxia. The functional role of these cells remains to be determined.

The third cell type containing biogenic amines exists in a closely associated group of similarly fluorescent cells. Because of their unique structural relationships and endocrine-like features, Lauweryns and Peuskens (1972a) called these cell clusters neuroepithelial bodies (NEBs). Endogenous fluorogenic amines have been demonstrated in NEBs of both animals and humans (Hage et al., 1974; and Lauweryns et al., 1972b and 1975). NEBs also have been demonstrated to take up amino acid precursors (Hage 1974; and Cutz et al., 1974). With spectrofluorimetry, the excitation and emission spectra of NEBs were found to be identical with serotonin standards (Lauweryns, et al., 1973a, 1977a). The NEBs appear to be well innervated by both afferent and efferent nerve fibers (Lauweryns et al., 1973b).

However, direct synaptic contacts have not been observed (Lauweryns et al., 1973a). The neuroepithelial bodies are composed of both granulated cells and modified Clara cells (Hung et al., 1979). Lauweryns et al. (1973a; 1973b; 1977d; 1978) have investigated their possible functional roles. In these studies, it was demonstrated that the NEBs responded to reduced alveolar oxygen content by degranulation and therefore were probably intrapulmonary chemoreceptors. Palisano and Kleinerman (1980) have also investigated the response of clusters as well as singly occurring pulmonary endocrine-like cells in rats exposed to NO_2. Using silver staining it was determined that the number of arygyrophilic cells decreased following exposure to NO_2.

Whether singly occurring endocrine-like cells or neuroepithelial bodies respond to the same stimuli remains to be determined. In addition, any relationship with other epithelial cell types such as ciliated or goblet cells has not been investigated. Further investigations will be necessary to confirm and extend the functional roles of these and other intrapulmonary endocrine-like cells.

The classification of endocrine-like cells present many difficulties. These difficulties increase as our knowledge of structure, function and species differences expands. A single staining reaction and/or morphologic description of endocrine-like cells cannot fully characterize the functional significance of these cells. For example, numerous endocrine-like cells in various endocrine glands, and the

gastrointestinal and respiratory system give a positive chromaffin reaction. However, the biochemical content and function of these cells are different. On the basis of morphologic and cytochemical criteria, five uniquely different endocrine-like cells have recently been demonstrated in the intrapulmonary airways of adult hamster (Hoyt, personal communication). In view of these observations, it is imperative that these endocrine-like cells be fully characterized by morphologic and histochemical methods in order to support effective investigations of their functional roles.

Statement of the Problem

The recent discovery of endogenous amine-containing cells in the extrapulmonary respiratory epithelium of adult rats (Ewen et al., 1972), rabbits (Cutz, et al., 1975; Dey et al., 1980), and human fetuses (Cutz et al., 1975), has generated great interest in the morphological and histochemical characterization of pulmonary amine-containing cells.

With the Falck-Hillarp (1962) formaldehyde-induced fluorescent technique, the laryngeal and tracheal respiratory epithelium of albino rats has been found to contain endogenously (weak) fluorescent cells (Ewen, et al., 1972).

These cells also demonstrated the ability to take up amino acid precursors (L-dihydroxyphenylaline and 5-hydroxytryptophan). Amino acid uptake and decarboxylation

is a characteristic feature of cells which belong to the APUD system of endocrine polypeptide cells (Pearse, 1969). Despite the ability of Ewen et al. (1972) to demonstrate the uptake of amino acid precursors, the morphological and cytochemical characteristics of these cells were very different from those of the APUD series (Pearse, 1969), as well as the tracheal amine-containing cells described by previous investigators. Therefore, the purpose of this study will be to investigate the morphologic and histochemical characteristics of amine-containing cells in the laryngeal and tracheal respiratory epithelium of adult rats.

Research Plan and Rationale

The present investigation will further elucidate certain morphologic and cytochemical characteristics of cells in adult rat laryngeal and tracheal respiratory epithelium which are believed to contain an endogenous biogenic monoamine.

The fluorescent histochemical demonstration of endogenous biogenic amines will be accomplished by two methods. Freeze-dried extrapulmonary airways will be treated with formaldehyde vapor and embedded in glycol methacrylate. The second method will involve the fixation of extrapulmonary airways with 6% liquid formaldehyde, embedding in Spurr's resin and subsequently exposing sections to hydrochloric acid vapor. The identity of the HCl-induced fluorophore will be evaluated by microspectrofluorimetric analysis. The

amine handling properties of rat extrapulmonary epithelial cells will be evaluated by administration of the amine precursors L-dihydroxyphenylalanine (L-DOPA) and 5-hydroxy-tryptophan (5-HTP).

The histochemical fluorescent method of Falck and Hillarp (1962) demonstrates the presence of monoamines at the cellular level. In an attempt to correlate directly fluorescence microscopy with subsequent electron microscopic observations of the same cells, tissues will be fixed with liquid formaldehyde, embedded in Spurr's resin and exposed to formaldehyde and HCl-vapors. Ultrathin sections will then be cut for electron microscopy. This will determine if the endogenous biogenic amine containing cell is a small granule cell (less than 2,000 Å in diameter) or a globular leukocyte with large granules (greater than 2,000 Å in diameter).

Cytochemical methods will be used to elicit further information concerning the nature of the fluorescent epithelial cells. The cytochemical procedures will be carried out on freeze-dried, formaldehyde vapor-treated plastic embedded tissue sections so that direct correlation between fluorescent cells and their cytochemical characteristics may be demonstrated. PAS-lead hematoxylin positive cells will demonstrate the presence of endocrine-like cells with small granules. Methylene blue and alcian blue positive cells suggest the presence of acidic proteins and or acid

mucopolysaccharides. Ferric-ferricyanide positive cells will demonstrate the presence of amines.

Acid phosphatase positive cells will demonstrate the presence of hydrolytic enzymes which are frequently associated with lysosomes. Additional cytochemical procedures will also be carried out on cryostat sections of fresh frozen tissues for the demonstration of monoamine oxidase (MAO), and freeze dried cryostat sections for the demonstration of histamine (O-phthalaldehyde method) and tryptamine (ozone method).

Significance

This investigation will further elucidate the current knowledge concerning extrapulmonary endocrine-like cell structure. At present, the potential biological importance of these cells which synthesize and secrete biogenic amines and/or peptide hormones, can only be surmised. The knowledge of how these cells fit into the function of the extrapulmonary airways and the organism as a whole is, as yet, unknown. A morphologic and cytochemical characterization of endocrine-like cells will allow future studies of the function and dysfunction of these cells.

Limitations

1. This study is restricted to the laryngeal and tracheal epithelium of adult albino rats.

2. The fluorescent and light microscopic histochemical characterization of these extrapulmonary epithelial cells is

restricted to freeze-dried and liquid formaldehyde fixed tissues.

3. The cytochemical characterization of the endocrine-like cells in extrapulmonary airways is restricted only to the methods employed in this study. No attempt was made to characterize the full cytochemical potential of these cells.

4. Electron microscopic observation and correlation is restricted to formaldehyde fixed tissue.

5. The microspectrofluorimetric analysis of the fluorophore is restricted to freeze dried or liquid formaldehyde, HCl-induced fluorescence.

6. The microspectrofluorimetric standards used in this study were the fluorescent cells of the duodenum. These cells have been reported to contain serotonin.

CHAPTER II

LITERATURE REVIEW

Feyrter (1938) described a system of epithelial cells in the gastrointestinal tract with characteristic morphologic and histochemical properties. Because of their almost water-clear cytoplasm after hematoxylin and eosin staining, he called them 'Helle-Zellin' cells. He postulated that these clear cells were part of a "diffuse endocrine epithelial organ" and suggested the possibility of finding these cells in the respiratory epithelium. The first description of these clear-cells was reported by Fröhlich (1949) in the bronchial epithelium of man as well as several animal species. Pearse (1966) investigated the cytochemical properties of different endocrine-like cell types in several animals. In an attempt to provide a conceptual framework for the diffuse endocrine-like cell system, Pearse et al. (1968) proposed the APUD (Amine Precursor Uptake and Decarboxylation) concept.

The APUD series of endocrine-like cells described by Pearse and the Helle-Zellin System of endocrine cells described by Feyrter are almost identical. The major difference between these two theories is the embryologic

origin of the endocrine-like cells. Feyrter (1953) suggested that the Helle-Zellin cells arose from epithelial cells. Pearse et al. (1976) demonstrated a neural crest origin of some endocrine-like cells, however, Andrew (1974) presented evidence disproving the neural crest origin of gastrointestinal endocrine cells.

Despite the differences regarding origin, the morphologic, physiologic, and pharmacologic characterization of respiratory endocrine-like cells has continued. The ultrastructural characteristics of a specific type of endocrine-like cell was first described in human bronchial glands by Bensch et al. (1965) and in the bronchial epithelium by Gmelick et al. (1967). Since this time, numerous studies have investigated the histochemical (Hage 1973a; Dey et al., 1980), ultrastructural (Kent et al., 1966; Lauweryns et al., 1972b); and Cutz et al., 1975), and physiologic (Lauweryns et al., 1977b, 1978; Echt et al., 1980) functions of endocrine-like cells in respiratory epithelium.

Trachea and Extrapulmonary Bronchial Respiratory Epithelium of Humans

Fetal and Infant

The presence of endocrine-like cells in the tracheal respiratory epithelium of human fetuses, infants, and children was reported first by Cutz et al. (1975). The histochemical fluorescent method of Falck and Owman (1965)

demonstrated only a few weakly fluorescent yellow-green cells. However, after *in vitro* incubation with L-DOPA, the numbers of fluorescent cells and the intensity of fluorescence increased. Argyrophilic positive cells were scattered throughout the epithelium. The cells were predominantly single, but occasionally groups of two or three cells were observed. Neuroepithelial bodies (Lauweryns *et al.*, 1972b) were never observed in the tracheal epithelium. However, it should be noted that a detailed morphometric study was not performed. The fluorescent and argyrophilic cells were triangularly shaped, with the base resting on the basement membrane and the apex projecting towards the lumen. Argentaffin positive cells were not observed.

Fetal and Infant Ultrastructure

Cutz *et al.* (1975) also investigated the ultrastructural characteristics of endocrine-like tracheal cells in human fetuses, newborn infants, and children. Granulated basal cells were found with an electron-lucent cytoplasm containing numerous dense-core, membrane-bound vesicles of the neurosecretory type. The vesicles had a mean diameter of 1150 Å, while the space surrounding the dense-core was 160-180 Å. Additional cytoplasmic features common to the granulated cells were: small mitochondria, moderate amounts of smooth and rough endoplasmic reticulum, and Golgi complexes.

Adult

The presence of endocrine-like cells in the tracheal respiratory epithelium of human adults has not been reported.

In summary, the tracheal respiratory epithelium of human fetuses, infants, and children contain single occurring fluorescent and argyrophilic positive cells (Cutz et al., 1975). Only a very weak endogenous fluorescence was reported. However, an intense fluorescence was noted after incubation with L-DOPA. Microspectrofluorimetric analysis has not been performed on the extrapulmonary endocrine-like cell of fetal, infant, or adult humans. The most characteristic feature of these cells at the ultrastructural level was the presence of dense-core vesicles (approximately 1150 Å) surrounded by a clear area and limited by a single unit membrane. The cytoplasmic matrix contained small mitochondria, moderate amounts of smooth and rough endoplasmic reticulum and a prominent Golgi complex.

Intrapulmonary Single Endocrine-like Cells and Neuroepithelial Bodies in Humans

Fetal

Endocrine-like cells in the bronchial respiratory epithelium of human fetuses were studied by Hage (1971, 1972). With light microscopy the cells appeared pyramidal or bottle-shaped and were located close to the basement membrane. Formaldehyde induced fluorescence produced a few

Table 1.--Single (†) and groups (***) of endocrine-like cells which have been demonstrated in extrapulmonary airways at various ages in animals and man. Note: (?) indicates that the author did not specify if single or groups of endocrine-like cells were observed.

		EXTRAPULMONARY AIRWAYS		
		Fetus	Neonate	Adult
Mammila	Little brown bat			Sorokin et al. (82)†
	Armadillo		Cutz et al. (75)†	
	Man	Cutz et al. (75)†		Cutz et al. (75)†
	Mouse			Ericson et al. (72)† Sorokin et al. (78)†
	Rat		Hoyt et al. (79)†	Ewen et al. (72)† Jeffery et al. (73,75)† Sorokin et al. (78)† Hoyt et al. (79)†
	Guinea Pig	Sarikas et al. (82)†		Kirkeby et al. (74)†***
	Rabbit	Cutz et al. (75)† Barry et al. (82)†	Cutz et al. (75)† Echt et al. (80,82)† Friedenbach et al. (81)†	Cutz et al. (75)† Dey et al. (79,80,81)† Sonstegard et al. (76)†
	Cat			Hung (75)† Das et al. (78)?
Aves	Chicken	Walsh et al. (74)†	Walsh et al. (74)†	Cook et al. (69)†

weakly fluorescent cells (control), but after incubation with L-DOPA the number of fluorescent cells increased. The number and distribution of control fluorescent cells was similar to the number and distribution of cells that were stained with lead-hematoxylin, HCl-toluidine blue and silver techniques for argyrophilia.

Hage (1973a) investigated the amine handling properties of endocrine-like cells in the bronchial epithelium of human fetuses. Lung segments were incubated at 37°C in Tyrodes solution containing the following agents: pargyline (an irreversible inhibitor of monoamine oxidase); D- or L-3,4-dihydroxyphenylalanine (D- or L-DOPA); D- or L-5-hydroxy-tryptophan (D- or l-5-HTP); Ro4-4602 (an inhibitor of decarboxylating enzyme) followed by L-DOPA or l-5-HTP; dopamine; and 5-hydroxytryptamine (5-HT). Control, formal-dehyde vapor-treated tissues demonstrated a few greenish-yellow fluorescent cells in the bronchial epithelium. The cells were pyramidal or oval shaped and the fluorescence was concentrated in the basal or paranuclear cytoplasm. With the administration of pargyline, the fluorescence intensity increased slightly. After incubation with D-DOPA or 5-HTP, the number of fluorescent cells also increased. Tissues incubated with L-DOPA or 5-HTP resulted in an increased number of green or yellow fluorescent epithelial cells. The L-DOPA incubated specimens emitted the most intense fluorescence and frequently, both nucleus and

cytoplasm appeared to be fluorescent. After L-5-HTP incubation, the fluorescence of most respiratory epithelial cells was restricted to the cytoplasm. Specimens pretreated with Ro4-4602, followed by L-DOPA or 5-HTP produced a weaker fluorescence than those incubated with precursors alone. Incubation with dopamine or 5-HTP produced a weak fluorescence, and the number of cells was smaller than after incubation with precursor amino acid.

Wharton et al. (1978) reported the presence of bobesin-like immunoreactivity in human fetal lung segments. Single cells as well as groups of cells with bombesin-like immunoreactivity were identified in the bronchial and bronchiolar epithelium.

Fetal Ultrastructure

The ultrastructural appearance of granulated cells in the pulmonary epithelium of human fetuses was studied by Cutz et al. (1972). Lung samples were fixed with 5% glutaraldehyde in cacodylate buffer (pH 7.4) for two hours at room temperature and then post-fixed in 1% OsO_4. Endocrine-like cells were found in all lungs and appeared as single cells or in groups. The cells were always located along the basement membrane and frequently formed pseudopod-like processes which extended between adjacent epithelial cells. A consistent feature was the presence of cytoplasmic electron-dense granules (dense-core vesicles) 1000-3000 Å in

diameter, surrounded by a single limiting membrane. The number and size of the granules varied. In some endocrine-like cells, the dense-core vesicles were randomly distributed while in other cells the granules were concentrated at the base close to the basement membrane. Other cytoplasmic organelles included: numerous vesicles of smooth and rough endoplasmic reticulum, many mitochondria, microfibrillar material, and a small Golgi apparatus.

Hage (1973b) also investigated the ultrastructural characteristics of pulmonary endocrine-like cells of human fetuses. All endocrine-like respiratory epithelial cells were adjacent to or resting on the basement membrane with pseudopod-like extensions penetrating deeply into the mesenchymal tissues. The cell shape was variable while the nucleus was round or oval and basally located. Smooth endoplasmic reticulum was present along with some rough endoplasmic reticulum, mitochondria, and a prominent Golgi complex. Contacts between nerve fibers and endocrine-like cells were never observed.

On the basis of the morphology of the secretory granules three types of endocrine-like cells were postulated. Type I cells were extremely variable in shape. The shape ranged from polygonal to narrow columnar or horizontally elongated. The cell seldom reached the luminal surface and the cytoplasm appeared more pale than other endocrine-like cells. Randomly distributed secretory granules were few in number but two

distinct populations of granules could be identified. Some granules were small (approximately 1100 Å) while other granules were considerably larger. The smaller granules contained dense-cores which were separated from their surrounding membranes by narrow, clear spaces (approximately 180 Å). The small granules were also argyrophilic positive. The larger granules did not contain a dense-core and were both argentaffin positive and argyrophilic. This cell type was located individually throughout the bronchial tree or in groups of up to five cells.

Type II cells were pryamidal or polygonal in shape and occasionally reached the luminal surface. Cytoplasmic extensions were also observed. The cytoplasm appeared much darker than other endocrine-like cells due to the presence of thick bundles of microfilaments and a greater number of cytoplasmic granules. The dense-core vesicles were uniformly round with little variability in the electron density of their cores. The granules were confined to the basal part of the cells with a mean diameter of 1400 Å. These granules were argentaffin negative and argyrophilic positive. Type II cells were also located throughout the bronchial tree. In the larger bronchi they were found in groups along with endocrine-like cells of the other types.

Only a few of the Type III cells were observed. This cell type was oval or pyramidal with a high nucleus to cytoplasm ratio and few cytoplasmic organelles. The cell was never observed to make contact with the lumen of the

bronchi. The secretory granules were round, about 1900 Å in diameter, and were nearly always located in the basal part of the cell. The electron dense-core was homogenous and surrounded by a membrane. The argyrophilic reaction was positive while the argentaffin reaction was negative. These cells were only observed in the larger bronchi. A fourth type, Type IV, of endocrine-like cell was also identified in the pulmonary epithelium of fetuses with a crown-rump length between 20 and 54 mm. These cells differed from the Type III cell by having a higher content of cytoplasmic glycogen and by the appearance of the secretory granules. The granules were more variable in both size, shape, and electron density. This cell type was only found in the larger bronchial tubules. No endocrine cells were identified in a fetus with a crown rump length of 20 mm or less.

Infant

Lauweryns et al. (1969 and 1970) reported the presence of endocrine cells, in the bronchial and bronchiolar respiratory epithelium of newborn human infants. The histochemical fluorescent technique according to Falck and Owman (1965) demonstrated the presence of amine containing cells. Argyrophilic staining was also positive. The cells were triangular to pyramidal in shape, with their bases resting on the basement membrane. A predilection in the distribution of the argyrophilic positive cells was not observed. These

investigators were unable to demonstrate argentaffin or chromaffin positive cells.

Many clusters of argyrophilic positive cells in human infant bronchial and bronchiolar epithelium were observed by Lauweryns and Peuskens (1972a). Silver impregnation and Falck's histochemical amine technique were utilized to demonstrate and characterize these groups of cells. Because of the unique structural relationship of these endocrine-like cells, Lauweryns called them neuroepithelial bodies (NEBs).

The spherical to ovoid NEBs were located periodically along the entire respiratory bronchial and bronchiolar tree and usually protruded slightly into the lumen. The height of the NEBs varied from 19 to 74 mu with an average of 35 mu. In hematoxylin-eosin stained sections, the more basal cells had clear cytoplasm and formed intercalated cone-shaped corpuscles. The apical part of the NEB protruded into the lumen above the level of the ciliated epithelial cells and was composed of non-ciliated cuboidal cells. The base of the cone rested on the basement membrane and at times displaced it into the lamina propria. The cells in the peripheral layer were smaller and flatter than the inner cells. The corpuscular cells were both fluorescent and argyrophilic positive but the peripheral cells were not.

Silver impregnation demonstrated numerous nerve fibers ramifying into the NEBs. Fluorescent histochemistry revealed

nerve fibers with a distinct green fluorescence in the immediate vicinity of these corpuscles and thus, suggested adrenergic innervation.

Single cells and groups of cells with bobesin-like immunoreactively have also been observed in the bronchial and bronchiolar epithelium of neonatal human lung (Wharten et al., 1978).

Infant Ultrastructure

The ultrastructural appearance of granulated respiratory epithelial cells in the lungs of human infants has been described by several investigators. A consistent feature was the presence of dense-core secretory granules. Lauweryns et al. (1970) using glutaraldehyde fixed lungs, noted that the endocrine-like cell membrane had typical desmosomal attachments with its neighboring cells. The granulated vesicles ranged from 800 to 1500 Å in diameter. The cytoplasm contained many mitochondria, filaments, free ribosomes, a small Golgi-complex, small amounts of rough and smooth endoplasmic reticulum, and some glycogen. The most striking discovery was the presence of nerve endings in close association with the groups of granulated cells. The intramucosal nerve fibers, which lacked Schwann cell envelopment, were characterized by an electron transparent axoplasm and several beaded enlargements along the course of the axon. The enlargements contained numerous small granular and agranular vesicles. Lauweryns believed that these variosities

were highly suggestive of "direct contacts" between the granulated cells and the nervous system. These nerve fibers were located deep to the basement membrane and in intimate relationship to the granulated cells, being separated by about 200 Å.

Rosan and Lauweryns (1971) noted granulated respiratory epithelial cells in the lungs of prematurely born infants. Lung samples from two infants, 600 and 1,600 g, were fixed in phosphate buffered glutaraldehyde (pH 7.2), post-fixed in osmium and embedded in Epon. The endocrine-like cells were: cuboidal; protruded into the submucosa; closely associated with fenestrated capillaries; not in contact with the lumen; and devoid of long narrow protoplasmic processes between or around cells. The presence of cytoplasmic electron-dense granules (800 to 1000 Å in diameter), surrounded by a clear area and a unit membrane (1100 to 1300 Å in diameter) were the most characteristic features of these endocrine-like cells. The relative frequency of cells was greater in the 600 g infant. In the smaller infant the granules were more numerous, denser, and more uniform in size than in the 1,600 g infant.

Adult

Hage (1973a) investigated the amine-handling properties of adult human main bronchial epithelium. Human bronchial segments were incubated in L-DOPA or dopamine. In both

control and dopamine treated animals, fluorogenic amines were not demonstrated with the formaldehyde-induced fluorescent technique. However, an intense green fluorescence with an exclusively cytoplasmic localization was noted after treatment with the precursor amino acid L-DOPA.

Tateishi et al. (1973) reported that the epithelium of bronchi and bronchioles of forty-five human, Mongolian adults (23 to 71 years of age) contained a number of distinctly argyrophilic cells. The cells occurred singly or in clusters; the cell clusters tended to occur more frequently in the smaller bronchi or in the bronchioles. By light microscopy, triangularly-shaped cells were seen near the basement membrane. Both single and grouped cells had cytoplasmic processes extending along the basement membrane. The number of argyrophilic cells tended to increase as the caliber of bronchi decreased in size. However, the number of cells in the terminal bronchioles was small. The cells frequently occurred in close association with goblet cells. There appeared to be no relationship between age of the subjects and the number of cells present, however, women tended to have more argyrophilic cells, particularly in the larger bronchi.

Hage (1973c) also examined the histological and histochemical features of human surgical lung specimens from six bronchial carcinoid tumor cases. Lung samples were fixed with a variety of fixing agents and subsequently embedded

in paraffin. Sections were stained by lead-hematoxylin, HCl-toluidine blue, Masson-Hamperl's and Grimelius's silver method, zanthydrol, and diazonium reaction to demonstrate endocrine cell granules. Her previous classification (Type I, II, III, and VI) of endocrine cell granules was utilized to characterize the endocrine cells (Hage, 1973b). Cells of the Type II category were unreactive to all staining methods except for the agyrophilic silver method. Type III cells did not conclusively react with any of the stains, however, electron microscopy did reveal their presence. The polymorphic granules, observed only in tumors, were argentaffin, diazonium, argyrophilic, lead-hematoxylin, and HCl-toluidine blue positive.

In 1975, Lauweryns et al. identified NEBs in lung tissue biopsed from children and adults. Lung segments were removed and fixed in Bouin's fluid. Hematoxylin and eosin in conjunction with silver staining revealed clusters of 4 to 10 argyrophilic positive cells in the bronchial, bronchiolar, and alveolar respiratory epithelial lining. The cellular morphology was similar to that previously described by Lauweryns et al. (1972a) for human fetal lung tissue. At the level of the bronchi, the NEBs were 20 to 40 mu wide and 15 mu high; the alveolar NEBs were about the same width (30 to 36 mu) but were shorter (6 mu). The individual cells comprising the bronchiolar NEBs were 4 to 8 mu wide and 15 mu high. The cells of the alveolar NEBs were the same width but only 6 mu high.

Adult human lungs, prepared for routine light microscopy, were studied by Hage et al. (1977) for the presence of endocrine-like respiratory epithelial cells. In the lobar and proximal segmental bronchi, a few singly placed cells were reactive to the argyrophil methods of Grimelius (1968). Argyrophilic cells were rarely present in the bronchiolar epithelium, alveolar lining cells, or in the acini and ducts of the parabronchial glands. The cells were triangular or flask shaped, located at or near the basal lamina, without luminal contact, and only occasionally were observed in groups. No positive reaction was obtained by methods demonstrating biogenic amines (diazonium nor argentaffin stains) or by methods staining endocrine cell granules (lead hematoxylin or HCl-toluidine blue). No endogenous fluorescent cells were demonstrated by the amine formaldehyde-induced fluorescent technique. However, incubation of lung segments with L-DOPA or 5-HTP produced an intense green or weak yellow fluorescence respectively in singly occurring cells. Fluorescent cells in groups or clusters were not observed.

Adult Ultrastructure

Bensch et al. (1964) investigated the ultrastructural characteristics of endocrine-like cells in adult human bronchial glands. Segments of normal human bronchus were

fixed by immersion in glutaraldehyde and subsequently in osmium. A rare cell type was frequently observed at the junction between mucus and serious cells. The characteristic feature of this cell was the presence of electron opaque cytoplasmic granules of the neurosecretory type. The granules were of uniform appearance and size, averaging 1400 Å in diameter. The core was filled with a homogenous electron-opaque substance surrounded by a clear area encircled by a membrane. No pattern of distribution was obvious, but the majority of these granules appeared to be located between the nucleus and the basal surface of the cell. At the base of these cells the cytoplasm frequently formed pseudopod-like processes which extended from the cell body. These cell processes contained granules (1400 Å in diameter) of the neurosecretory type. Similar cell processes were also found in the intercellular space between serous cells. The granules in these cell processes were different from those previously described in that: the neurosecretory granules were smaller (900-1000 Å); and there were large numbers of empty vesicles (500-600 Å diameter) resembling pinocytotic or synaptic vesicles. Synapses of the outer cell membrane were not observed.

The ultrastructural characteristics of small granule endocrine-like cells in adult human bronchiolar surface respiratory epithelium was first reported by Gmelich et al.

(1967). Lung tissue from a 68 year old woman was surgically removed and processed for routine light and electron microscopic observations. Lung segments prepared for electron microscopy were fixed for 24 hours in a 3.5% buffered glutaraldehyde solution. The tissue studied consisted of normal areas and also several tumor nodules. Light microscopy revealed that one nodule appeared to be a mucus-producing adenocarcinoma while the other nodules resembled a peripheral bronchial carcinoma.

Electron microscopy of the normal bronchioles revealed cuboidal ciliated epithelial cells which alternated with cells with relatively short plump pseudopods on a luminal surface. Scattered sparsely throughout the bronchiolar epithelial lining were a number of electron-translucent cells. These cells never reached the luminal surface. The most conspicuous cytologic feature was the presence of great numbers of intracytoplasmic granules, 1200 to 1500 Å in diameter. The cores of these granules were homogeneously electron opaque and surrounded by a zone of electron transparency and encircled by a unit membrane. Single cells, as well as clusters were present above the bronchiolar basement membrane. The carcinoid tumor consisted of all of the normally observed cell types, including the dense-core endocrine-like cell.

Terzakis et al. (1972) investigated the ultrastructural characteristics of endocrine-like epithelial cells in adult

human segmental bronchi. Segments of human bronchi were removed from 14 surgical cases. Four of the cases had no neoplastic disease while ten of the cases had lung carcinoma. The tissues were immersion fixed for at least 24 hours in 0.075M cacodylate-buffered glutaraldehyde followed by 2 hours in veronal acetate-buffered osmium tetroxide, dehydrated in alcohols, and embedded in Epon.

The bronchial respiratory epithelium consisted of ciliated, basal, goblet, and irregularly shaped cells which were more electron translucent than the surrounding cells. These cells of low cytoplasmic density, usually occurred singly but groups of two to twelve were also observed. The cells were located along the basement membrane and had small oval nuclei. The cytoplasm contained numerous electron-dense granules 800 to 1700 Å in diameter. These granules were surrounded by a clear area and a single trilaminar membrane. Other cytoplasmic organelles of these endocrine-like cells included: numerous vesicles of smooth and rough endoplasmic reticulum, mitochondria, Golgi apparatus, free ribosomes, fine filaments, and rare lipid and lipofuchsin granules. No evidence of any synapse was observed in relation to the granule-containing cells. The cell surface interdigitated with neighboring cells but no desmosomes nor terminal bars were noted. However, several goblet cells contained small, dense, membrane-bound granules which appeared identical to those observed in the endocrine-like epithelial cells.

Since carcinoid tumors of the gastrointestinal tract are derived from endocrine cells in the intestinal mucosa, Hage (1973c) investigated the ultrastructural similarities between endocrine-like cells in the pulmonary respiratory epithelium of human fetuses and the cells of bronchial carcinoid tumors. Lung tumors from six adults were fixed in cacodylate buffered glutaraldehyde (3%), post-osmicated (1%), and embedded in Epon. Nearly all of the tumor cells contained secretory granules. The number and appearance of these granules were variable. Three categories of secretory granules were postulated according to her previous classification of endocrine-like cells in the pulmonary epithelium of human fetuses (Hage 1973b). Type I cells were not observed in any tumor material. Cells which contained small, round, membrane-bound granules of uniform shape and size, characteristic of Type II cells, were observed in all carcinoid tumors. The secretory granules were concentrated in the cytoplasmic extensions. In two cases, a few cells contained larger, round granules of uniform size and bound by a discontinous membrane. The granules of these cells (Type III) were evenly distributed in the cell body. A cell type not observed in the bronchial epithelium of human fetuses was observed in three tumor cases. These cells contained polymorphic granules. She concluded that these cells were very similar to enterochromaffin cells of the human gastrointestinal tract.

Electron microscopy examination of normal, adult pulmonary epithelium was also performed by Hage et al. (1977). Four adult lung samples from areas with no obvious macroscopic lesions, were fixed in 3% glutaraldehyde buffered cacodylate (pH 7.4), post-fixed in osmium, and stained with lead-citrate and uranyl-acetate. The characteristic neuroepithelial bodies (Lauweryns et al., 1972a), were only rarely observed. Endocrine-like cells occurred singly in the respiratory epithelium of lobar bronchi, segmental bronchi, acini, and ducts of the parabronchial glands, but more often in clusters of three to six in the epithelium of the lower segmental bronchi and bronchioles. The characteristic feature of the endocrine-like cell was the presence of small round cytoplasmic secretory granules. These granules, 1100-1400 Å in diameter, were enclosed by unit membranes and contained dense-cores surrounded by narrow, clear spaces. The cytoplasm was rich in mitochondria, microfilaments, a prominent Golgi complex as well as smooth and rough endoplasmic reticulum. The lateral cell membranes were straight with a few infoldings into adjacent cells. Several desmosomal structures were noted. The endocrine-like cells observed in the adult lungs were similar to the endocrine-like cells of Type II described previously in the human fetal lung (Hage 1973b).

It has been demonstrated that the intrapulmonary airways of fetal, infant, and adult human lung contain numerous

groups (Hage 1973a; Lauweryns et al., 1972a, 1975) as well as single endocrine-like cells (Cutz et al., 1971; and Tateishi et al., 1973). The single, argyrophilic cells are triangular or flask shaped, located at or near the basal lamina and are without luminal contact (Tateishi et al., 1973; Hage et al., 1977). With the amine tracing fluorescence technique (Falck et al., 1965), no endogenously fluorescent cells have been demonstrated in adult human lung. Incubation of lung segments with L-DOPA or L-HTP, however, has revealed green and yellow fluorescent cells respectively (Hage et al., 1977). In fetal human lung, some singly occurring cells emit an endogenous green fluorescence (Hage, 1973a). An increase in the number of green fluorescent cells has also been demonstrated after incubation with L-DOPA (Hage, 1973a).

Groups of argyrophilic and fluorescent cells have also been identified in infant and adult human lung (Lauweryns et al., 1969, 1970, 1972a, 1975). Because of the unique structural relationship of these endocrine-like cells, Lauweryns et al. (1972a) called them neuroepithelial bodies (NEBs). In infant and adult, the NEBs were: spherical to ovoid, periodically located along the entire respiratory bronchial, bronchiolar and alveolar epithelium, and usually protruded slightly into the lumen (Lauweryns et al., 1972a). Silver impregnation demonstrated numerous nerve fibers ramifying into the NEBs. Fluorescent histochemistry

suggested an adrenergic component in the nerve fibers (Lauweryns et al., 1972a). The presence of single and groups of endocrine-like cells with bombesin-like (polypeptide-like) immunoreactivity has also been demonstrated in the bronchial and bronchiolar epithelium in fetal and neonatal human lung (Wharton et al., 1978). Microspectrofluorimetric analysis of single and groups of fluorescent cells in human intrapulmonary airways has not been reported.

The fine structure of single (Gmelich et al., 1967; Cutz et al., 1971; Hage 1973b) and groups (Cutz et al., 1971; Hage 1973; and Lauweryns et al., 1970) of endocrine-like cells in respiratory epithelium has been described in fetal, infant, and adult lung tissue. Single cells were always located along the basement membrane (Cutz et al., 1972; and Hage 1973b). In addition, pseudopod-like processes were frequently observed to extend between adjacent epithelial cells (Gmelich et al., 1967; Cutz et al., 1971). No relationship between single occurring endocrine-like cells and nerve fibers has been reported. The ultrastructure of groups of endocrine-like cells has been extensively studied by Lauweryns et al. (1970) and Rosan et al. (1971 and 1972). Lauweryns et al. (1970) noted numerous varicose nerve endings in close association with the neuroepithelial bodies, separated by approximately 200 Å. From these observations, it was suggested that "direct

contacts" were present between the granulated cells and the nervous system.

The most characteristic cytoplasmic feature of these endocrine-like cells in respiratory epithelium is the presence of dense-core granules (Cutz et al., 1972; Hage et al., 1973b). Several studies have attempted to classify these granulated cells according to the morphology of their secretory granules (Hage et al., 1973b). However, the size, shape, and distribution of the secretory granules is quite variable. One possible explanation for the variability could be the many different methods of tissue fixation. For example, it has been demonstrated that the distribution pattern and structure of vesicles within the carotid body show specific changes according to the method of tissue preparation for microscopy (Hans, 1977). The diameters of the dense-cored vesicles ranged from 800 Å in the infant (Rosan et al., 1971) to 1900 Å (Hage, 1973b) in the fetus. Additional cytoplasmic organelles included: many small mitochondria, moderate amounts of smooth and rough endoplasmic reticulum, many free ribosomes, filaments, and a small Golgi complex (Lauweryns et al., 1970; Hage et al., 1977).

Laryngeal, Tracheal, and Extrapulmonary Bronchial Respiratory Epithelium of Animals

Fetal

Cutz et al. (1975) investigated the endocrine-like cells of fetal lambs and rabbits as well as infant rabbit and armadillo. In all animals studied, singly occurring argyrophilic cells were observed. The cells were triangular with their bases resting on the basement membrane. Frequently, a thin apical extension was observed to reach the lumen. Argentaffin positive cells were not observed. In control animals, only a few weakly fluorescent cells were demonstrated by the formaldehyde induced-fluorescence technique. However, following injection or in vitro incubation with L-DOPA, a significant number of fluorescent cells were observed in all animals studied.

Fetal Ultrastructure

Cutz et al. (1975) also investigated the ultrastructural characteristics of endocrine-like respiratory epithelial cells in fetal lambs and rabbits, and infant rabbits and armadillo. The general appearance of the cytoplasmic organelles in these cells was similar to that of humans (Cutz et al., 1975). Two distinctively different endocrine-like cells in both lamb and armadillo were distinguished on the basis of their dense-core vesicle size and morphology. The dense-core vesicles in granulated cells designated

Type I were large (1700 Å in diameter) and had a homogenously dense central core. In Type II cells the DCVs were generally smaller, 1200 Å in diameter. In rabbit only one type of DCV was identified which tended to be more pleomorphic with a mean diameter of 1400 Å.

Neonatal and Adult

Ericson et al. (1972) reported the presence of endocrine-like cells in the tracheo-bronchial mucosa of the mouse. Fluorogenic amines were not demonstrated with the formaldehyde-induced fluorescent technique in the epithelium of control mice. However, after the administration of DOPA or 5-HTP, an intense formaldehyde-induced fluorescence selectively accumulated in cells. Subsequently, these same cells were found to be argyrophilic. The endocrine-like cells were most numerous in the epithelium of the trachea just below the cricoid cartilage. In the lower parts of the trachea and main bronchi the number of fluorescent cells decreased. In the bronchi, fluorescent cells were mainly in clusters (5 to 20 cells). The cells were triangular with their basal portions resting on the basement membrane.

Endocrine-like cells were found in the laryngeal epithelium of rats (Ewen et al., 1972) and adult guinea-pigs (Kirkeby et al., 1977). Ewen et al. (1972) were unable to demonstrate argyrophilic cells in the larynx, but did report that these cells were eosinophilic, PAS positive, and showed

a slight metachromasia with toluidine blue. The granules were intensely fluorescent in the animals treated with L-DOPA and L-5-HTP. Without pretreatment, only weakly fluorescent granules could be seen. Pretreatment of animals with Ro4-4602, an L-amino acid decarboxylase inhibitor, prevented the appearance of intense fluorescence in cells when the animal was subsequently injected with L-DOPA. In contrast, Kirkeby et al. (1977) was able to identify argyrophilic cells in both the lining epithelium and in the small glands of the guinea pig larynx. The cells were either in groups of up to 15 to 20 cells, or single. The cells were pyramidally shaped with their bases located on the basement membrane. Argyrophilic cells were not present in the superior portion of the larynx but were found mainly in the middle third.

Serotonin containing endocrine-like cells in the trachea and lung of adult rats were identified by fluorescent histochemistry as well as routine light microscopy (Sorokin et al., 1978; Hoyt et al., 1979; and Echt et al., 1980). Tissue segments were fixed with buffered formaldehyde and embedded in glycol methacrylate. Endogenous fluorogenic amines were not demonstrated in any tracheal epithelial cells. but were evident in L-DOPA treated animals. In control animals, however, fluorescence was present in a varity of cells known to contain serotonin (mast cell and neuroepithelial

bodies). Following administration of 5-HTP an intense yellow fluorescence was observed in scattered single tracheal epithelial cells. In control animals, fluorescence was not observed in plastic embedded sections of adrenal medulla nor thyroid "C" cells. Because of these results, it was suggested that this method selectively demonstrates the monoamine, 5-HT.

Complementary monoamine fluorescence techniques on plastic sections of trachea from control animals prior to additional histologic staining demonstrated that fluorescent cells were identical to those stained by PAS-lead hematoxylin (Sorokin et al., 1978).

The morphologic, morphometric, and histochemical characteristics of endocrine-like cells in the tracheal epithelium of adult rabbits were studied by Dey and Echt (1976), Dey (1979), and Dey et al. (1980). Endogenously fluorescent cells were found to be both ferric-ferricyanide and argyrophilic positive. Single, triangularly-shaped cells were located throughout the entire trachea. Microspectrofluorimetric analysis of the fluorescent cytoplasm in individual cells, and sodium borohydride treatment (Corrodi et al., 1964) demonstrated that the fluorescent cells contained serotonin. The ventral aspects of the tracheal epithelium contained significantly more fluorescent cells than the dorsal region. In addition, the ventral cranial region had significantly more cells than any other region. The number of fluorescent cells was not affected by

pretreatment with L-DOPA. However, pretreatment with reserpine resulted in a significant decrease in cell numbers when compared with control rabbits.

Neonatal and Adult Ultrastructure

The ultrastructure of the rat laryngeal granulated cells was also studied by Ewen et al. (1972). The laryngeal mucosa contained numerous cells with large, 5,000 to 20,000 Å in diameter, pleomorphic granules. These granules were densely osmiophilic and randomly dispersed throughout the cytoplasm, though mainly on the luminal side of the nucleus. The granulated cells seldom reached the luminal surface. Mitochondria and smooth endoplasmic reticulum were present in the cytoplasm of all granulated cells.

Tracheal epithelium from adult mice (Ericson et al., 1972) and adult rabbit (Cutz et al., 1975) contain numerous granulated cells. In adult mice the greatest number of endocrine-like cells were found in the upper trachea. The cells were variable in shape and rested on the basement membrane. The cytoplasm contained spherical secretory vesicles 800-1000 Å in diameter and the space surrounding the dense-core was 100-200 Å. The outer limiting membrane was 70-80 Å thick. In addition, the cytoplasm contained: microfibrillar material, smooth and rough endoplasmic reticulum, mitochondria, and Golgi complexes.

Storage of amine was suggested by the intense staining using the argentaffin silver method of cytoplasmic granules

in DOPA treated animals. Only a very weak argyrophilic reaction was observed. After administration of ^{3}H-DOPA or ^{3}H-5-HTP, autoradiographic silver grains selectively appeared over all tracheal endocrine-like cells. Cutz et al. (1975) also noted similar cytoplasmic organelles in adult rabbits. However, the DCV tended to be more pleomorphic and larger (1400 Å in diameter). The core was uniformly homogenous and the halo almost indistinguishable.

Studies of adult rat (Jeffery et al., 1973) and adult cat (Das et al., 1978, Hung et al., 1975) also demonstrated endocrine-like cells in the trachea. However, the endocrine-like cells were only rarely found in association with nerve fibers.

The ultrastructural studies of Jeffery et al. (1975) revealed the presence of dense-core secretory vesicles in the tracheal cells of adult rat. Tracheas were removed and fixed by immersion in 1% buffered glutaraldehyde and post-fixed in osmium. Occasionally, granulated cells with numerous dense-core, membrane bound vesicle (1300 Å in diameter) were observed. A significant number of globular leukocytes were also noted. This cell was characterized by the presence of very large, homogenous, electron dense granules (6,250 Å mean diameter). Cell projections were numerous and filiform. The granules of the globular leukocyte were significantly larger than the granules of the connective tissue mast cell. The concentration of globular leukocytes was greatest in the upper trachea.

Walsh and McLelland (1974), investigating the innervation of the tracheal epithelium of domestic chickens, noted the presence of endocrine-like cells. The granulated cells contained DCVs which were 1400 Å in diameter. These granules were surrounded by a clear area and followed by a single membrane. Two types of vesicles, agranular and granular, were present in the axon fibers. Evidence for synaptic contact between intraepithelial axons and epithelial cells was not observed.

Globular Leukocytes

Globular leukocytes are found within the epithelium of mucous surfaces and most typically within the intercellular spaces of the epithelium itself (Kent et al., 1966; Breeze et al., 1977). Kent et al. (1954) investigated the effects of corticotropin, cortisone, and adrenalectomy on the number of globular leukocytes in rat tracheal epithelium. Following administration of cortisone or corticotropin, a significant reduction in the number of globular leukocytes was observed. Adrenalectomy, however, exerted no consistent effect on the number of globular leukocytes in the tracheal epithelium. For each animal, the numbers of globular leukocytes per five histologic sections of trachea were counted and averaged. The sections were stained with Masson's trichrome technique or phosphotungstic acid hematoxylin and basic fuchsin. The

staining technique used for quantifying cell numbers was not identified by these investigators.

The effect of irradiation and hypophysectomy on globular leukocytes in rat intestine were investigated by Kent et al. (1956). After four days of whole body irradiation (650r) the number of globular leukocytes were reduced to 1-2% of their normal number. The effect appeared to be locally mediated due to the fact that lead shielding prevented any decrease in the number of globular leukocytes in the intestine. However, after 30 days of x-irradiation (800r) there was a significant decrease in the number of globular leukocytes in lead-shielded intestinal segments. Hypophysectomy prevented this decrease and, therefore, it was concluded that the decrease was not directly related to the x-irradiation but rather to stress. The number of globular leukocytes was also significantly decreased after hypophysectomy.

Kent et al. (1966) also investigated the histochemical and ultrastructural characteristics of globular leukocytes in the respiratory epithelium of bronchioles, bronchi, trachea, and larynges of adult rats. Numerous globular leukocytes were present in almost every intercellular space. The spherical cytoplasmic inclusions (globules) are acidophilic. These globules are phosphotungstic acid, hematoxylin and basic fuchsin positive. The globules also stain metachromatically with toluidine blue and azure.

The fine structure of the globular leukocyte was also investigated. Tissue segments were fixed in aldehyde followed by postfixation with osmium. The cytoplasmic matrix contained small amounts of rough endoplasmic reticulum, free ribosomes and a well-developed Golgi complex. The most characteristic feature was the presence of globules. No predilection in the distribution of these globules was noted. The globules were finely granular and electron dense. There were however variations, even in globules within the same cell. The globules were frequently bounded by a typical unit membrane. Rounded vacuoles were occasionally observed within a globule and the cell nucleus was frequently eccentrically located.

Most studies of the substance contained within these globules have been carried out on the gastrointestinal globular leukocyte. Murray et al. (1968) demonstrated a greenish fluorescence in the gastrointestinal globular leukocyte. It was suggested that a monoamine may be stored within these globules.

In summary, of all of the species studied, only the fetal (Cutz et al., 1975), infant (Echt et al., 1982), and adult rabbit trachea (Dey et al., 1976 and 1979) demonstrated endogenous fluorescent cells. Microspectrofluorimetric analysis of these fluorescent cells demonstrated the presence of serotonin (Dey et al., 1980). Following administration of 5-HTP Hoyt et al. (1979) also demonstrated

the presence of serotonin in single endocrine-like cells of the neonatal rabbit trachea. Following administration of L-DOPA or 5-HTP, an intense formaldehyde-induced fluorescence selectively accumulated in the extrapulmonary airways of all animal species at all ages studied (Erickson et al., 1972; Ewen et al., 1972; and Cutz et al., 1974). In addition, argyrophilic cells were also present in all animal extrapulmonary airways (Kirkeby et al., 1977), except for adult rat (Ewen et al., 1972). The morphology of the fluorescent and argyrophilic cells was similar. The cells were always observed singly along the basement membrane and were triangularly shaped with a thin apical cytoplasmic projection. Argentaffin positive cells were not observed in any animal species.

The fine structure of endocrine-like cells in extrapulmonary respiratory epithelium has also been investigated. The most characteristic feature in the cytoplasmic matrix is the presence of dense-core granules. These dense-core granules ranged from 800 to 1700 Å in diameter in most species studied (Cutz et al., 1974; Ericson et al., 1972). In adult rat (Ewen et al., 1972), the granules ranged from 5,000 to 20,000 Å in diameter. At the ultrastructural level the tracheal endocrine-like cells were also identified by their ability to accumulate radioactivity after administration of ^{3}H-DOPA and ^{3}H-5-HTP (Ericson et al., 1972). In addition to dense-core granules in the cytoplasmic

matrix, the cytoplasm contains moderate amounts of smooth and rough endoplasmic reticulum, small mitochondria, microfibrillar material, and a prominant Golgi complex (Cutz et al., 1974).

Endocrine-like cells of adult rat (Jeffery et al., 1973) and adult cat (Hung et al., 1975; and Das et al., 1978) were rarely found in association with nerve fibers. Evidence for synaptic contacts between intraepithelial axons and granulated epithelial cells have not been observed (Jeffery et al., 1973; Walsh et al., 1974; and Das et al., 1978).

Intrapulmonary Single, Endocrine-like Cells and Neuroepithelial Bodies in Animals

Fetal

Hage (1974) investigated the morphologic and histochemical characteristics of endocrine-like cells in lungs of fetal rabbit, mouse and guinea-pig. Injection of the mother or incubation of fetal lungs were performed with D- or L-DOPA, L-5-HTP, dopamine, pargyline, and Ro4-4602 (a decarboxylase inhibitor) in order to investigate the amine handling mechanisms of endocrine-like cells in respiratory epithelium.

In samples of fetal lung from control rabbits groups of 2 to 10 cells as well as single cells exhibited a weak yellow fluorescence. The cells were round to cylindrical

and occasionally reached the luminal surface of the epithelium. Injection or incubation with L-DOPA or L-5-HTP resulted in an increased fluorescence intensity. The number and distribution of fluorescent cells were similar to control animals. Following pretreatment with L-5-HTP the fluorophore was strictly localized to the cytoplasm. After pretreatment with L-DOPA, the florophore could be found over the nucleus. Fluorogenic amines were not demonstrated in the bronchial epithelium of untreated fetal mice or guinea-pigs. After administration of L-DOPA or L-5-HTP groups of fluorescent cells were observed in the mouse but only rarely in the guinea-pig.

No epithelial cells reacted to HCl-toluidine blue or lead-hematoxylin staining in any of the animal species examined. However, argyrophilic cells were demonstrated in fetal rabbit bronchi. No argyrophilia was observed in the bronchial epithelium of fetal mice or guinea-pigs. Intraepithelial nerve endings were not in contact with the fetal argyrophilic cells.

Clusters of fluorescent, argentaffin, and argyrophilic positive cells were observed by Lauweryns et al. (1974) in fetal rabbit lung. The cellular morphology was similar to that previously described by Lauweryns et al. (1972a) for human fetal lung. The neuroepithelial bodies were 15 to 25 mu high and 25 to 35 mu wide (at the base). The number of cells per NEB ranged between 10 and 30. Silver impregnation as well as formaldehyde-induced fluorescence

demonstrated numerous nerve fibers which were in close association with the NEB. However, no direct contacts between neuroepithelial bodies and axons were noted.

Cutz et al. (1974) reported the presence of amines along with some of the amine handling properties of endocrine-like cells in rat fetal lungs. With light microscopy the argyrophilic cells appeared singly as well as in clusters (3-6 cells). At all levels of the bronchial respiratory epithelium the single cells were triangular with the base resting on the basement membrane. In contrast, groups of endocrine-like cells were most numerous in large or medium sized bronchi. Serial sectioning revealed that the transition of single cells into groups did not occur. Only a few cells exhibited a slight metachromasia with HCl-toluidine blue staining. Control formaldehyde vapor treated lung segments demonstrated only a few weakly fluorescent cells. Following pretreatment (both in vivo and in vitro) with L-DOPA or 5-HTP, the number of cells and intensity of fluorescence increased. In samples treated with 5-HTP the color of fluorescence was yellow-green and with L-DOPA more green. The general size, shape and distribution of fluorescent cells were similar to the argyrophilic cells.

Morikawa et al. (1978) noted the occurrence of acetylcholinesterase (AChE) positive cells in the intrapulmonary airway epithelium of fetal rats. Neuroepithelial-like cells with lightly staining cytoplasmic

acetylcholinesterase positive granules were first noticed at 17 days of fetal life. Numerous AChE positive cells were identified throughout the tracheobronchial epithelium from 17 to 21 days of gestation.

Hung (1978, 1979a, and 1979b) investigated the innervation of pre and post-natal mouse trachea and lungs. Acetylcholinesterase positive cells were not observed in the epithelial lining. A few single nerve fibers were observed to innervate the neuroepithelial bodies. These nerves were neither acetylcholinesterase positive nor fluorescent. It was inferred from these results that these nerve fibers were sensory in function.

Lung explants (7 to 21 days) from fetal rabbits were examined by light and fluorescent microscopy to determine if endocrine-like cells could maintain their structural integrity (Sonstegard et al., 1979). Single endocrine-like cells and neuroepithelial bodies were identified by formaldehyde induced fluorescence and argyrophilia in the bronchial epithelium of control and cultured lung explants.

Fetal Ultrastructure

The ultrastructural appearance of neuroepithelial bodies in the pulmonary respiratory epithelium of rabbit fetuses was investigated by Lauweryns et al. (1972b). Lung segments were fixed by four different methods: (1) 2.5% phosphate buffered glutaraldehyde for two hours at 4°C

followed by post fixation in osmium for one hour at 4°C; (2) 1% osmium; (3) 3% glutaraldehyde for four hours at 4°C followed by a 2.5% solution of potassium-dichromate containing 1% sodium phosphate (GD technique); and (4) prefixed in 8% formaldehyde for 24 hours followed by the GD technique. The last technique described is a specific technique (Jaim-Etcheverry et al., 1968) for the demonstration of serotonin granules at the ultrastructural level. The cytoplasmic matrix of the endocrine-like cells contained: many free ribosomes; elongated mitochondria mostly in a supranuclear position; a well developed rough endoplasmic reticulum; a few glycogen particles; and many dense-core vesicles.

After glutaraldehyde fixation, two types of DCVs were observed within the same cell. The Type I granules represented 70% of the entire population of dense-core vesicles. These granules were pleomorphic (1340 A by 1021 Å), and usually no halo was observed between the outer limiting membrane and the dense-core. Within a single dense-core two subpopulations of course granules were described. The smaller granules were 30 Å in diameter and were suggested to consist of a carrier substance. Such a carrier substance has been demonstrated in the adrenal medulla and composed of ATP, proteins, lipids and calcium ions (Kirshner et al., 1967). The larger granule, 90 to 100 Å in diameter, was suggested to be composed of the active ingredient

(serotonin) bound to the carrier substance. The second type of DCV, which represented 30% of the vesicle population, was more circular (1121 A by 989 Å). An obvious halo of 150-200 Å was always present. The small granules within the DCV were 30 Å in diameter.

With both glutaraldehyde-dichromate and formalin-glutaraldehyde-dichromate fixations, the two populations of DCVs were again identified. The Type I granules were identifiable without any contrast staining. In contrast, the Type II granules were only visualized after uranyl acetate staining. According to Jaim-Etcheverry et al. (1968) only sites which contain serotonin will react to form an electron dense precipitate. After contrast staining with uranyl acetate the type II DCVs were also visualized. From these results, it was concluded that the Type I vesicles contain serotonin.

Ultrastructurally, several unmyelinated nerve fibers were observed within the epithelium and demonstrated a "direct contact" with the grouped endocrine-like cells. The nerve endings were separated from the granulated cells by a 200 Å gap. Local thickenings of both the axon and granulated cells were also noted. The axoplasm contained numerous small mitochondria and granular vesicles of about 500 Å in diameter.

Hage et al. (1974) reported the ultrastructural characteristics of endocrine-like cells in respiratory epithelium of fetal rabbit, guinea pig, and mouse. Lung segments were fixed by immersion in buffered glutaraldehyde or formaldehyde. Groups of columnar endocrine-like cells were most frequently observed. Desmosomes were occasionally noted. The cytoplasmic matrix was composed of: many free ribosomes; scant amounts of rough endoplasmic reticulum; mitochondria; a Golgi complex; and numerous dense-core vesicles.

The granulated cells of fetal rabbits were columnar and seldom reached the lumens of bronchi. A discontinuous membrane surrounded all of the secretory granules. The average secretory granule diameter of 1420 Å. The shape of endocrine-like cells of the mouse fetuses were columnar or polygonal. Occasionally, cells with their long axis parallel to the basement membrane were observed. Frequently, the luminal surface of the cell was covered by extensions from surrounding epithelial cells. Only rarely were they observed reaching the bronchial lumen. The secretory granules were: surrounded by a discontinuous membrane; uniformly round; and approximately 1070 Å in diameter. Only one cell could be demonstrated in one bronchial tubule from the lungs of guinea-pig fetuses.

The ultrastructural appearance of granulated cells in the respiratory epithelium of rat fetuses was studied by

Cutz et al. (1974). Granulated cells, singly or in groups, were present within the bronchial epithelium. Lung samples were fixed by immersion in phosphate buffered glutaraldehyde, post-fixed in osmium, and embedded in Epon. The single cells were triangular with the base close to the basement membrane. The apical portion of the cell nerve reached the bronchial lumen. Cytoplasmic extensions between adjacent epithelial cells were frequently observed. These endocrine-like cells contained intracytoplasmic granules (700 to 1200 Å in diameter) with a central dense-core surrounded by a single limiting membrane. The granules were randomly distributed throughout the cell. Other numerous cytoplasmic organelles were microfibrils, microtubules, and free ribosomes. Less conspicuous organelles were smooth and rough endoplasmic reticulum and Golgi apparatus.

The group endocrine-like cells bulged slightly into the lumen. These granulated cells were frequently covered by other epithelial cells but occasionally reached the lumen. The cytoplasmic organelles were similar to those of the single granulated cells. In contrast to the single cells, intraepithelial nerve endings were found in contact with the endocrine-like cells. The ultrastructural features of the intraepithelial nerve endings were: neurotubules, small mitochondria, and occasional groups of empty vesicles 400 to 500 Å in diameter.

Sonstegard et al. (1979) demonstrated that the ultrastructural characteristics of cells within the neuroepithelial bodies could be maintained in short-term organ cultures. Lung explants from fetal rabbits were maintained in organ culture for up to 22 days. Tissue segments were fixed in phosphate buffered glutaraldehyde, post-fixed in osmium, and embedded in Epon. The ultrastructure of NEB cells in organ cultures was different from that of uncultured controls. In lung explants the DCVs in NEB cells: appear more pleomorphic; are significantly larger; appear to be redistributed throughout the cytoplasm (from an infranuclear position to a supranuclear position); and a few have a clear space within the matrix.

Neonatal

The histological features of bronchial endocrine-like cells of the neonatal rat were investigated by Moosavi et al. (1973). Argyrophilic cells were present singly as well as in clusters. These cells were: cuboidal or columnar, located on the basement membrane, and usually exposed to the lumen of the airway.

Lauweryns et al. (1973a) reported the presence of argyrophilic, argentaffin, yellow fluorescent cells in the intrapulmonary epithelium of neonatal rabbits. Microspectrographically, the emission spectrum (maximum 540-550 nm) of the neuroepithelial bodies (Lauweryns et al., 1972b) corresponded to the emission spectrum of serotonin

in bovine albumin standards. In addition, the excitation spectra (460 nm) of the NEBs was identical to that of serotonin standards and colonic enterochromaffin cells.

Sorokin and Hoyt (1978) and Hoyt et al. (1979) investigated the staining characteristics of endocrine-like cells in tracheas and lungs of neonatal rabbits. Tissue segments were fixed in 6% formaldehyde and subsequently embedded in glycol methacrylate. Single cells as well as clusters of cells emitted a weak yellow fluorescence. This endogenous fluorescence was intensified following administration of 5-HTP (30 minutes prior to sacrifice). Tissue sections stained with PAS-lead hematoxylin demonstrated that the fluorescent cells corresponded exactly with the PAS-lead positive cells.

Utilizing the histochemical fluorescent method of Falck-Hillarp (1962), Wasans et al. (1979) demonstrated the presence of endogenous amine-containing cells in the primary and secondary bronchi of infant chickens. Numerous triangularly shaped, basally located, yellow, fluorescent cells were observed throughout the epithelial lining. These cells occurred singly as well as in clusters.

Neonatal Ultrastructure

Lauweryns and Cokelaire (1973b) investigated the ultrastructural characteristics of the intrapulmonary neuroepithelial bodies in neonatal rabbits and mice. Following reserpine pretreatment the cores of the DCVs were

cleared in about one third of the vesicle population, leaving only a half moon-like or a completely empty vesicle. Numerous unmyelinated nerve fibers were present within the NEBs. Two types of varicose nerve endings were reported to be in contact with the corpuscular cells. Type 1a (afferent-like nerve fibers) nerve endings were characterized by: many mitochondria; small agranular synaptic vesicles (300-500 Å diameter); and larger dense-core vesicles (800-900 Å). The second type (type 2) were like those previously described (Lauweryns et al., 1972b). These afferent-like varicose nerve endings were similar to the type 1a fibers except they never revealed varicosities filled with mitochondria. Afferent and efferent-like nerve endings revealed synaptical end formations making "direct contacts" with granulated cells. Occasionally, an exocytosis of corpuscular DCVs was observed at these areas of direct contact.

In rabbit NEBs, after applying the ultrastructural acetylcholinesterase (AChE) technique of Bloom et al. (1966). a very fine, dense AChE-positive precipitate was noted exclusively in the halos of the DCVs (type 1) while the cores remained unstained. The type 2 DCV, as well as other cytoplasmic organelles, remained unstained.

Cytochemically the cells of the NEBs revealed a positive reaction for acetylcholinesterase, alpha-glycerophosphate, and Solica's lead-hematoxylin stain for endocrine

cells producing peptides and amines. In view of the fluorescent, histochemical, and ultrastructural results it was concluded that NEBs contain serotonin and probably a related amine or polypeptide.

The possible occurrence of an additional substance other than serotonin in the corpuscular cells of neuroepithelial bodies of neonatal rabbits (Lauweryns et al., 1972b) was investigated using fluorescamine by Lauweryns and Liebens (1977a). Fluorescamine is a reagent reported to stain selectively cells containing and secreting polypeptides (Larsson et al., 1975). Unfixed lung sections freeze-dried and treated with fluorescamine demonstrated an intense fluorescamine-induced fluorescence in groups of cells. The average emission maximum for the control group, was 510-520 nm. Lung tissue fixed with gaseous formaldehyde and post-treated with fluorescamine also demonstrated an intense and selective fluorescamine induced fluorescence in clusters of cells. The average emission maximum was 550-560 nm (510-520 corrected). From these results it was suggested that neonatal rabbit neuropeithelial bodies probably contain a polypeptide substance.

Scanning and transmission electron microscopy were used to demonstrate the presence of bronchiolar neuroepithelial bodied in neonatal mouse lungs (Hung et al., 1974 and 1979). Lungs were fixed by immersion in phosphate buffered glutaraldehyde for two hours, post-fixed in osmium for one hour, and

embedded in Epon. Those lung segments to be used for scanning electron microscopy were fixed in glutaraldehyde, dehydrated and then critical point dried. Two cell types (Clara, and specialized granular) were observed to comprise the neuroepithelial bodies. They were most numerous at the bifurcation points of the bronchioles. The specialized granular cells contained many dense cored granules (800 to 1000 Å in diameter) surrounded by a clear area and followed by a unit membrane. These granules were located mainly in the basal half of the cytoplasm. Golgi apparatus, mitochondria, and rough endoplasmic reticulum were also present in the cytoplasm. Intraepithelial axons were found to originate from non-myelinated axons in the lamina propria. These axons penetrated the epithelial basal lamina and ramified among the granular cells and the Clara cells. No specific synaptic contacts were noted between the axons and any of the epithelial cells. Scanning electron microscopy demonstrated that the NEBs bulged into the bronchiolar lumen and that both cell types were in contact with the lumen. The boundaries of the NEBs were outlined by both the ciliated and Clara cells. Clara cells covered most of the surface of the NEB leaving only small oval areas for the exposed surfaces of the specialized granulated cells. Microvilli were noted to project from both Clara and granulated cells.

Intrapulmonary airways of chickens (3to 5 days old) also contain endocrine-like cells (Wasano et al., 1979). Distinctive granulated cells were identified in both the primary and secondary bronchi. These cells were located along the basement membrane and appeared as single cells or in groups (3-5 cells). Infrequently, a single cilium was observed projecting from the exposed surface of the cell into the lumen. Close to the luminal surface of the epithelium these granulated cells were joined to the adjacent epithelial cells by junctional complexes. The cytoplasm contained numerous dense-core vesicles whose average diameter was 1400 Å. The dense-core was followed by a clear area (140 Å) and surrounded by a unit membrane. At the ultrastructural level both the argentaffin and chromaffin reactions produced electron dense precipitates in the cytoplasm of the granulated cells. Three of the fifty four cells studied were closely associated with intraepithelial nerve endings. The interspace between nerve ending and the cell membrane was 200 Å. Membrane thickenings of both nerve ending and cell membrane, suggesting a synapse, were noted.

Adult

Two populations of biogenic amine-containing cells in the mouse lung were identified by Eaton and Fedde (1977). Mice were simultaneously perfused fixed via the circulatory and respiratory systems with a phosphate buffered

formaldehyde solution. Lung segments were removed and processed according to the amine histochemical method of Falck and Owman (1965). An intense yellow-green fluorescence was emitted from one population of cells. The second group of cells produced a bright yellow fluorescence. Microspectrofluorimetric analysis demonstrated a different emission wavelength in these two groups of fluorescent cells. Spectral analysis of the fluorophores was not reported and, therefore, their conclusion that two different amines were present was questionable.

Adult Ultrastructure

Nerves and their associated cells in the bronchiolar epithelium of mice (Hung et al., 1973) and in the primary and secondary bronchi of avian lung (Cook and King et al., 1969; and King et al., 1974) were studied with the electron microscope. Axons, devoid of Schwann cells, were noted between the epithelial cells in the basal zone of the epithelium. Circular or oval intraepithelial axons were frequently associated with epithelial cells which contained many dense-cored granules. The diameters of the epithelial cell dense-core granules ranged from 750 to 1300 Å. King et al. (1974) noted that the nerve endings were of the afferent type because they contained: many narrow mitochondria; a few granular vesicles 600-1000 Å in diameter;

and numerous agranular vesicles 300-1000 Å in diameter. In all three reports evidence of synaptic contacts between small clusters of granulated cells and unmyelinated nerve endings were observed. Hung et al. (1973) reported that the endocrine-like cells appeared in groups. These cell clusters were similar to those described as neuroepithelial bodies by Lauweryns et al. (1972b).

Summary

In summary, using the histochemical method of Falck and Owman (1965), endogenous fluorogenic amines were demonstrated in the intrapulmonary airways of fetal (Hage et al., 1974; Lauweryns et al., 1974), and infant rabbits (Lauweryns et al., 1973a. The number and intensity of fluorescent cells in fetal animals increased significantly in animals pretreated with L-DOPA or 5-HTP (Hage, 1974; Cutz et al., 1974). The fluorescent cells occurred singly (Hage et al., 1974) as well as in groups (Lauweryns et al., 1973a and 1973c). Endogenously fluorescent cells were also demonstrated in mouse lungs which were liquid fixed, freeze-dried, and exposed to formaldehyde vapor (Eaton et al., 1977). Two populations of fluorogenic amine-containing cells were demonstrated with this method. One population of cells emitted an intense yellow fluorescence while the other emitted a yellow-green fluorescence. Spectral analysis of the fluorophore was not done, and therefore their conclusion

that two different amines were present in mouse intrapulmonary airways was questionable.

In the intrapulmonary airways of all animal species and age groups studied argyrophilic cells were observed (Cutz et al., 1974 and Lauweryns et al., 1974 and 1976). Argentaffin positive cells were rarely present. However, Hage (1974) and Lauweryns et al. (1973b and 1974) reported the presence of argentaffin positive cells in fetal and infant rabbit lungs.

Several investigators (Mossavi et al., 1973; Palisano et al., 1980; Keith et al., 1981; and Redick et al., 1982) have attempted to quantify neuroendocrine cells in the intrapulmonary airways of animals at various stages of development. Despite the numerous efforts to quantify the number of neuroendocrine cells in fetal, neonatal, and adult lungs the results vary and are confusing at best. The general impression is that neuroendocrine cells increase in number until just prior to birth and then decrease (are not demonstrated using fluorescent microscopy) just prior to birth and then reappear following birth (Redick et al., 1982). After birth the number of neuroendocrine cells then decrease (Keith et al., 1981), remain constant (Palisano et al., 1980), or increase (Moosavi et al., 1973). The method of fixation, identification, quantification, as well as species differences have not been universally addressed.

Only a very detailed morphometric analysis (perhaps serial reconstruction) will provide an understanding of these neuroendocrine cells and their developmental pattern.

The ultrastructure of single (Hage 1974) and groups (Lauweryns et al., 1973b, 1974) of respiratory endocrine-like cells has been described in fetal, infant, and adult animal intrapulmonary airways. Single cells were most frequently triangular or oval with the base close to the basement membrane (Cutz et al., 1974). The thin apical projections seldom reached the lumen (Hage et al., 1974 and Cutz et al., 1974). Intraepithelial nerve fibers were frequently in close association with the single cells but no synaptic contacts were observed (Cutz et al., 1974). Nerve fibers were frequently observed to penetrate and ramify among the groups of granular cells (Hung et al., 1979).

Evidence of synaptic contacts between groups of granulated cells and unmyelinated nerve endings have been demonstrated (Lauweryns et al., 1972b; Hung, 1973; Cook et al., 1969; and King et al., 1974). Hung (1978 and 1979) demonstrated that these nerve fibers were neither acetyl-cholinesterase positive nor fluorescent. It was inferred from these results that the nerves were sensory. The morphology of these nerve fibers at the ultrastructural level suggested to Lauweryns et al. (1973b) that both afferent and efferent nerve fibers were in "direct contact" with the neuroepithelial bodies.

The most characteristic cytoplasmic feature of these endocrine-like cells is the presence of dense-core vesicles surrounded by a clear area and followed by a single unit membrane (Cutz et al., 1972; Hage, 1974; and Lauweryns et al., 1972b). The diameter of the dense-core vesicles ranged from 700 Å in the rat fetus (Cutz et al., 1972) to 1420 Å in fetal rabbits (Hage et al., 1974). Additional cytoplasmic organelles include: many free ribosomes (Hage et al., 1974); a well developed rough endoplasmic reticulum; many small mitochondria; and a Golgi complex (Lauweryns et al., 1972b).

Several studies have attempted to characterize the contents of these respiratory endocrine-like cells. Microspectrofluorimetric analysis of groups of endogenously fluorescent cells of neonatal rabbits (Lauweryns et al., 1973a, 1977b) demonstrated the presence of serotonin. Ultrastructurally, two populations of dense-core vesicles have been demonstrated in fetal and infant rabbit endocrine-like cells (Lauweryns et al., 1972b). Using formalin glutaraldehyde-dicromate fixation, a specific stain for serotonin (Jaim-Etcheverry et al., 1968), the DCV, formed an electron dense precipitate (Type I) and therefore contained serotonin (Lauweryns et al., 1972b). After contrast staining with uranyl acetate the Type II DCVs were visualized. This suggested that an additional substance was present within fetal and infant rabbit

neuroepithelial bodies. NEBs have also demonstrated a positive reaction for α-glycerophosphate dehydrogenase and acetylcholinesterase. This is indicative of polypeptide secreting cells (Lauweryns et al., 1973b). In addition, using fluorescamine, reported to be a specific stain for polypeptides, a distinct fluorescence in groups of endocrine-like cells (NEBs) was also observed in neonatal rabbits (Lauweryns et al., 1977a).

Sorokin et al. (1978) have also demonstrated groups of fluorescent cells in neonatal rabbit and hamster after liquid fixation. This technique is reportedly specific for serotonin containing cells (Hoyt et al., 1979). In summary, it appears that fetal and neonatal rabbit neuroepithelial bodies contain serotonin as well as an additional substance which is probably a polypeptide. At this time one cannot extrapolate these results to other animal species or to single occurring endocrine-like cells.

Physiologic and Pharmacologic Stimulation of Single Respiratory Endocrine-like Cells and Neuroepithelial Bodies

The endocrine-like cells of both the extrapulmonary (Cutz et al., 1975; Dey, 1979; Echt et al., 1980, 1982; and Friedenbach et al., 1980) and intrapulmonary (Lauweryns et al., 1972a; Cutz et al., 1974; and Hage, 1974) epithelium of various mammals, including man (Hage, 1973a; and Lauweryns et al., 1975, 1976), have been investigated with

Table 2.--Single (†)and groups (***) of endocrine-like cells which have been demonstrated in intrapulmonary airways in animals and of various ages.
Note: (?) indicates that the author did not specify if single or groups of endocrine-like cells were observed.

		INTRAPULMONARY AIRWAYS		
		Fetus	Neonate	Adult
Mammila	Hedgehog			Lauweryns et al. (72)***
	Man	Cutz et al. (72)†,*** Hage (71,72,73a,73b)†,*** Rousseau-Merck et al. (76)? Wharton et al. (78)†,*** Cutz et al. (81)†,***	Lauweryns et al. (69,70,72b,75)*** Rosan et al. (71,72)*** Hage (76)†,*** Wharton et al. (78)†,*** Becker et al. (80)† Cutz et al. (81)†,***	Benach et al. (65)? Gmelich et al. (67)*** Terzakis et al. (72)†,*** Hage (73,76,77)†,*** Lauweryns et al. (75)*** Cutz, et al. (81)†,***
	Monkey			Lauweryns et al. (72)***
	Mouse	Hage (74,76)†,*** Hung et al. (78,79,82)***	Lauweryns et al. (73b)*** Hung et al. (74,79,82)*** Wasano et al. (81)***	Hung (73)*** Eaton et al. (77)? Wasano (77)***
	Rat	Jeffery et al. (73)? Cutz et al. (74)†,*** Morikawa et al. (78)*** Carabba et al. (82)***	Mossavi et al. (73)†,*** Palisano et al. (80)†,***	Shul'ga (64)*** Edmonson et al. (80)†,***
	Hamster	Sarikas et al. (82)***	Sarikas et al. (82)***	Sorokin et al. (78)†,*** Hoyt et al. (79,82a,82b)†,*** Edmondson et al. (80)†,*** Linnoila et al. (81)***
	Guinea Pig	Hage (74,76)†,***		
	Rabbit	Lauweryns et al. (72b,73a)*** Hage (74)†,*** Sorokin et al. (78,82a,82b)†,*** Cutz et al. (78)*** Hernadez-Vasquez et al. (78a,78b)*** Sonstegard et al. (79)†,*** Hung (80)*** Redick et al. (82)***	Lauweryns et al. (72b,73a,73b,77a,78)*** Sorokin et al. (78)†,*** Hernadez-Vasquez et al. (78a,78b)*** Hoyt et al. (79)†,*** Keith et al. (81a,81b)†,***	Lauweryns et al. (72b,73a,73c)*** Hage (74)†,*** Taylor (77)†,***
	Cat		Sorokin et al. (78)†,***	Lauweryns et al. (72b)*** Hung (75) *** Das et al. (78)?
	Lion			Lauweryns et al. (72b)***
	Coney			Lauweryns et al. (72b)***
	Pig		Lauweryns et al. (72b)***	
Aves	Chicken		Wasano et al. (79)***	Cook et al. (69)*** King et al. (74)***
Reptilia	Tortoise			Rogers et al. (80)***
	Lizard			Rogers et al. (80)***
	Snake			Wasano et al. (76)***
	Newt			Goniakowska (80)***
	Tree Frog			Goniakowska (81)***
	Frog			Wasano et al. (78)***
	Toad			Rogers et al. (78,80)***

histological, fluorescent, histochemical, scanning and transmission electron microscopy, and microspectrofluorimetric techniques. Each technique has revealed the presence of endocrine-like cells throughout the airways. Despite the extensive morphologic characterization of these cells little is known about their functional roles.

The effects of various pharmacologic agents on the amine handling properties of endocrine-like cells were investigated by Ericson et al. (1972). The tracheal epithelium of untreated control mice contained no cells with formaldehyde-induced fluorescence. Thirty minutes after administration of L-DOPA, however, an intense green formaldehyde-induced fluorescence was observed in a large number of endocrine like cells. No fluorescence was visible after inhibition of monoamine breakdown by nialamide.

Pretreatment of the mice with an inhibitor of the decarboxylating enzyme, Ro4-4602, one hour prior to L-DOPA-injection resulted in only a very faint greenish fluorescence. These results suggested that the amino acid itself did not accumulate in the cells and therefore the fluorescent cells contained the decarboxylation product. This conclusion was also supported by the fact that administration of D-DOPA, a poor substrate for the decarboxylating enzyme, usually did not result in any visible fluorescence. A cellular accumulation of dopamine

did not occur, even when the amine was administered in high doses. Pretreatment of the animals with reserpine 4 hours prior to L-DOPA injection resulted in a lower fluorescence intensity than after L-DOPA injection alone. From these results it was concluded that the amine was stored in the cytoplasm by a reserpine-sensitive mechanism.

Moosavi et al. (1973) was one of the first to study lung endocrine-like cells and their possible physiological role during chronic hypoxia. Four day old rats and their mothers were placed in a pressurized oxygen chamber. The atmospheric pressure was gradually reduced to 380 mm Hg, stimulating an altitude of 18,000 feet. The animals were maintained at this pressure until their sacrifice at 20 hours to 1 month. Lung samples were removed and fixed for light and electron microscopy. Lung segments, studied by electron microscopy, were rapidly fixed in ice cold glutaraldehyde.

Silver staining revealed argyrophilic granules containing cells in lung epithelium. These cells occurred singly as well as in clusters. Electron microscopy of control animals demonstrated a number of endocrine-like cells with dense-core vesicles, 1200 to 1730 Å in diameter. The central dense-core was surrounded by a narrow, clear halo and limited externally by a single unit membrane. The remaining cytoplasm contained mitochondria, smooth and rough endoplasmic reticulum, central nucleus,

microfibrilis and a Golgi apparatus. Light microscopy of hypoxic animals, did not reveal any detectable histologic changes. Pronounced alterations were observed in the ultrastructure of dense-core secretory vesicles in endocrine-like cells from hypoxic animals. The peripheral halos were wider while the central cores were smaller. The diameters of the altered osmiophilic bodies ranged from 1270 to 2070 Å; some as large as 3730 Å. The ratio of the area of the central core to that of the whole granule was significantly decreased in the hypoxic rats. At four days of age, the number of endocrine-like cells per cm of bronchial epithelium during hypoxia was not significantly different from control animals. No other age groups were studied.

Age related differences were observed in control rats. There was a significant decrease in the number of endocrine-like cells with age (70 cells/cm at 7 days and 16 cells/cm at 31 days). The percentage of bronchi and bronchioles containing endocrine-like cells decreased from 89% at the age of 7 days to 45% at the age of 31 days.

It was noted that the fine structure of the respiratory endocrine-like cells was similar to that of the chief cell of the carotid body. Edwards et al. (1972) demonstrated that the chief cells of the carotid body responded, at the ultrastructural level, to chronic hypoxia and therefore were chemoreceptors. It was concluded that the ultrastructural changes in the respiratory endocrine-like cells of animals

exposed to hypoxia were very similar to those reported by Edwards et al. (1972) in the chief cells. Because of their similarity in structure and response to hypoxia it was suggested that the respiratory endocrine-like cell may serve as a chemoreceptor function.

Lauweryns and Cokelaire (1973b, 1973c) suggested at the ultrastructural level that the neuroepithelial bodies of rabbits released the contents of their dense-core vesicles at their basal vascular pole after exposure to hypoxia. Neonatal rabbits were exposed for 2,10 or 20 minutes to a low oxygen atmosphere (5,10 or 15%). Lung samples were removed and prepared for routine staining procedures or prepared for electron microscopy. Material for electron microscopy was fixed in phosphate buffered, 2.5% glutaraldehyde (pH 7.2) for 2 hours at 4°C, post-fixed in osmium (1 hour at 4°C), and embedded in Epon. Light microscopy of hypoxic animals did not reveal any detectable histologic changes of the neuroepithelial bodies. Ultrastructurally, however, numerous alterations were observed between the NEBs of control and hypoxic animals. All animals exposed to hypoxia revealed a distinct exocytosis of the corpuscular dense-core vesicles along the basement membrane. All classical morphologic phases of the exocytotic cycle (Hubbard, 1971) were noted in hypoxia exposed rabbits.

In contrast to the control animals, the hypoxia exposed rabbit dense-core granules were: more numerous in the basal area of the cell; apparently fused with the cell membrane itself; were fragmented; many were entirely empty; and some were smaller than the classically described DCV. In addition, Lauweryns noted occasional mitochondrial lysis and a prounounced development of the Golgi complex in hypoxia exposed animals. No attempt to quantitate differences in control and experimental animals was reported.

Neonatal rabbit neuroepithelial bodies were studied under various experimental conditions by Lauweryns et al. (1977b). The reaction of neuroepithelial bodies to hypoxia (10% oxygen for 20 minutes), hyperoxia (30% oxygen for 20 minutes), and hypercapnia (5% CO_2), were investigated by microspectrofluorimetric and morphometric methods. Lung samples were processed according to previously described techniques (Lauweryns, 1973b). The effect of several pharmacologic agents (nicotine, L-DOPA, 5-HTP, reserpine, and L-DOPA followed by hypoxia) on neuroepithelial bodies were also investigated. As defined previously (Lauweryns et al., 1972b) the dense-core vesicles (DCV), with a dense amorphous depot in their granules were considered DCV_1, while those without a dense depot in their core were classified as DCV_2.

Morphometric studies were carried out at the ultrastructural level to determine significant alterations in the shape and location of the dense-core secretory vesicles.

An increase in the number of DCVs contacting the basal cell membrane per unit length, in conjunction with a decrease in the infranuclear cytoplasmic volume percent occupied by the dense-core vesicles were being released from the cell.

Hypoxia and hypercapnia caused a significant decrease in the peak fluorescence intensity emitted by the NEB at the emission wavelength of serotonin (550 nm). No spectral changes were observed following hyperoxic exposure.

Morphometric measurements, of hypoxic rabbits, indicated a significant decrease of the infranuclear cytoplasmic volume percent occupied by the dense-core vesicles (DCV_1 and DCV_2) and an increase number of DCVs contacting the basal cell membrane per unit length, thus, suggesting a release of the dense-core vesicles. In hypercapnic animals, an increase number of DCVs contacting the basal cell membrane per unit length were noted. There was no decrease of the infranuclear cytoplasmic volume percent occupied by the dense-core vesicles (DCV_2). However, there was a significant decrease of the infranuclear cytoplasmic volume percent occupied by the dense-cored vesicles (DCV_1). Hypercapnia resulted in a fragmentation of the dense-core vesicles (a change from DCV_1 to DCV_2). The total number of DCVs contacting the basal cell membrane per unit length, of hyperoxia treated animals, were significantly decreased from controls. Despite this significant treatment effect, the authors concluded that hyperoxia did not significantly alter the ultrastructure

of the DCVs in the neuroepithelial bodies. However, it was suggested that the NEBs responded to hypoxia and hypercapnia as intrapulmonary chemoreceptors.

Nicotine, reserpine, 5-HTP, and L-DOPA all induced ultrastructural changes of DCVs. Nicotine was a strong stimulus for the exocytosis of DCVs, an increase in DCVs contacting the basal cell membrane per unit length, and a decrease in the infranuclear cytoplasmic volume percent occupied by the dense-core vesicles. Reserpine pretreatment had no effect on the infranuclear cytoplasmic volume percent occupied by the dense-core vesicles but there was a distinct depletion of DCV_1 altering their morphology to DCV_2. Ultrastructurally, the injection of 5-HTP and L-DOPA resulted in an increased electron density of the dense-core vesicles. The total volume percent occupied by the DCVs remained unchanged when compared to control animals. Microspectrofluorimetric analysis revealed a decreased fluorescence intensity following administration of nicotine or reserpine.

Sonstegard et al. (1979) studied the effects of reserpine and a Ca^{++} ionophore (A23187) on the ultrastructure of NEBs in lung explants cultured for seven days. The primary changes were in the dense-core granules. The Ca^{++} ionophore produced a coarsely granular and slightly less electron-dense DCV. The perigranular halo was also wider than control. Reserpine pretreatment produced clearing and fine granularity of the matrix. The electron-dense core

appeared more granular and the perigranular halo was larger in some DCVs. The effect of reserpine on NEBs was similar to that reported by Lauweryns et al. (1977b). The number of DCVs affected by reserpine was dose-related (1 to 10 μg/ml), but not with the Ca^{++} ionophore, A23187. By means of cross-circulation experiments, Lauweryns et al. (1978) investigated the response of neuroepithelial bodies to: (1) alveolar hypoxia and arterial normoxic blood; and (2) alveolar normoxia and arterial hypoxic blood. Previously described (Lauweryns et al., 1977b) techniques for analysis of the dense-core vesicles were used. Fluorescent and light microscopic techniques revealed no differences between the neuroepithelial bodies of either group. Lung neuroepithelial bodies from young rabbits which received hypoxemic blood did not exhibit, at the ultrastructural level, an increased exocytosis of their dense-core secretory vesicles. Rabbits exposed to normoxic atmospheres containing lowered pO_2 but receiving normoxic blood, did demonstrate significant ultrastructural differences from control animals. Morphometric analysis revealed a significant increase in the number of DCVs in contact with the basal cell membrane per unit lenth as well as a decreased infranuclear cytoplasmic volume percent occupied by the dense-core vesicles, suggesting exocytosis of these vesicles.

Keith and Will (1981) have also investigated the potential relationship between neuroendocrine cells and medial

thickness of pulmonary arteries in neonatal rabbits following acute and chronic hypoxia. New Leland White does were maintained in a hypobaric (520 mm Hg) chamber from the twentieth day of gestation. The neonates were born and raised in the hypobaric chamber until sacrifice (one, three or five days after birth). All hypoxic neonates were sacrificed within 15 minutes after the pressure chamber was normalized. Neonates that died during the course of the experiment were also included in the study. However, no information was provided regarding the time sequence between death and fixation. In addition, several 4 day old hypoxic neonates were allowed to recover from hypoxia for one day prior to sacrifice. Grimelius's silver method in conjunction with Miller's elastic VanGiesan's stain were used for morphometric analyses. Although the exact method of analysis was not defined, the authors stated that an average surface area of 1.95 cm^2 per animal was examined. From these experiments, it was concluded that hypoxia significantly increased the number of neuroendocrine cells and pulmonary artery medial thickness in 5-day old neonates. In addition, one and three day old rabbits that died during hypoxia exposure also had significantly higher numbers of neuroendocrine cells than the hypoxic survivors. This result is difficult to accept due to the lack of information regarding the determination of death and subsequent handling of the

lung tissue. Hypoxic rabbits four days postpartum that were returned to normoxic conditions for 24 hours prior to sacrifice demonstrated a significant decrease in the number of neuroendocrine cells and in medial thickness when compared to the 5 day hypoxic neonates.

Echt et al. (1980 and 1982) and Friedenbach et al. (1980) investigated the effects of age, hypoxic hypoxia, and carbon monoxide on endogenous serotonin containing cells in tracheal respiratory epithelium of young rabbits (3,10 and 28 days old). Echt et al. (1982) employed the histochemical fluorescent technique according to Falck (1962) to demonstrate the presence of amine containing cells. Rabbits were exposed for 6 hours to an atmosphere of 13% oxygen (alveolar and arterial hypoxia) or to a concentration of carbon monoxide which produced a decrease in arterial oxyhemoglobin saturation equivalent to 13 percent oxygen (tissue hypoxia). Exposure of animals to atmospheres containing lowered pO_2 results in an airway or arterial hypoxia. In contrast, exposure of animals to normoxic atmospheres containing carbon monoxide only produces a tissue hypoxia without affecting airway or arterial pO_2 (Roth and Rubin, 1976). Quantification of the fluorescent tracheal epithelial cells was accomplished by calculating the number of fluorescent cells per centimeter of epithelium. Compared to their respective controls, there was a significant decrease in the number of

fluorescent cells per cm of epithelium in 10 day old hypoxic hypoxia exposed rabbits. The overall effect of hypoxic hypoxia (all ages considered) demonstrated a significant decrease in the number of fluorescent cells compared to controls. When all control animals (3,10 and 28 day animals) were compared to all treated (carbon monoxide) age groups there was a significant decrease in the overall number of fluorescent cells. It was concluded that in rabbits the amine-containing epithelial cells of the trachea respond to tissue hypoxia and a decreased airway or arterial pO_2 is not necessary to elicit a response. These results were different than Lauweryns et al. (1978) regarding the response of intrapulmonary NEBs in rabbits. They demonstrated that NEBs in rabbit lungs respond directly to lowered oxygen content of inhaled air and not to decreased oxygen of pulmonary blood. Echt et al. (1982) also investigated the effect of age on the number of fluorescent cells. There was a significantly greater number of fluorescent epithelial cells in 28 day old compared to 3 day old rabbits.

Ultrastructural changes of tracheal, endocrine-like cells in young rabbits after 6 hours exposure to carbon monoxide was reported by Friedenbach et al. (1980). Animals were perfused via the vasculature at systolic pressures, with buffered aldehydes and routinely prepared for electron microscopy. The size and shape of the granules were not

altered after exposure. Infranuclear granules were smaller (1300 Å) than supranuclear granules (1410 Å). Those granules infranuclear were also more circular than supranuclear. When compared to controls, the number of granules per cell decreased from 38 to 26; the number of granules per unit cell area decreased from 45 to 34 per μm; the granule area decreased from 67 to 52%; and the percentage of granules infranuclear increased from 37 to 58%. It was concluded that these data supported a basal movement of granules which may be released from the cell following exposure to carbon monoxide.

In summary, the existence of intrapulmonary sensory receptors reactive to changes in the composition of gases in the lung has been postulated by several authors (Fishman, 1960; Dejours, 1962). Lauweryns and Purskens (1972a) identified intrapulmonary corpuscles (neuroepithelial bodies) in the bronchial and bronchiolar epithelium of human infants. It was concluded that these unique histologic structures were probably intrapulmonary chemo-, stretch-, and/or tactile neuroreceptor organs.

The influence of acute (Lauweryns et al., 1973b and 1973c) and chronic (Moosavi et al., 1973) hypoxia on fetal rat and neonatal rabbit suggested numerous ultrastructural alterations of NEBs cells in both instances. It was concluded from these results that the NEBs were chemoreceptors and did respond to alveolar oxygen content.

Lauweryns et al. (1977b) further investigated the possible chemoreceptor function of the NEBs. The reaction of neonatal rabbit neuroepithelial bodies to hypoxia, hyperoxia, and hypercapnia, were investigated by microspectrofluorimetric and morphometric methods. From the results obtained it was suggested that the cells of the NEBs responded to hypoxia and hypercapnia and thus were intrapulmonary chemoreceptors. Despite a significant decrease in the number of DCVs touching the basement cell membrane, it was concluded that hyperoxia did not significantly alter the ultrastructure of NEBs. By means of cross-circulation experiments Lauweryns et al. (1978) investigated the response of NEBs to alveolar hypoxia and/or arterial hypoxia. Since the NEBs responded directly to alveolar hyposiz, but not to arterial hypoxia it was concluded that NEBs were chemoreceptors which responded directly to the gaseous composition of the inhaled air.

The cells of neuroepithelial bodies have some resemblance to single endocrine-like cells. Both cell types are argyrophilic, fluorescent and ultrastructurally granulated. However, NEBs appear to be innervated (Lauweryns et al., 1973b) whereas single endocrine-like cells have not been reported to have any association with the nervous system (Cutz et al., 1975). All of the physiologic studies regarding the possible functional roles of intrapulmonary respiratory endocrine-like cells have

been evaluated from groups of cells (NEBs). The only physiologic investigations on the functional roles of single endocrine-like cells during hypoxia have been performed on the extrapulmonary airways of neonatal rabbits (Echt et al., 1980 and 1982; Friedenbach et al., 1980). Neonatal rabbits exposed to both hypoxic hypoxia or carbon monoxide-induced hypoxia demonstrated a significant decrease in the number of detectable tracheal fluorescent cells when compared to controls (Echt et al., 1980 and 1982). At the ultrastructural level Friedenbach et al. (1980) reported significant alterations of the cytoplasmic location and number of dense-core secretory vesicles after exposure to carbon monoxide.

CHAPTER III

MATERIALS AND METHODS

Animal Care

Adult rats, bred and maintained in a colony in the Anatomy Department at Michigan State University, were housed in cages (36 cm x 31 cm x 16 cm) in a relatively constant environment. The animals had access to food (Wayne Laboratory Blox) and water *ad libitum*.

Specimen Removal

Forty-eight adult male and female rats (150 to 450 g) were anesthetized with sodium pentobarbital (Nembutal, Abbott), 50 mg/Kg, i.p. The rat was placed in a supine position and a ventral transverse incision was made below the level of twelfth rib. The diaphragm was dissected away from its reflection on the anterior abdominal wall and followed by a midline incision through the sternum up to the level of the mandible. The larynx and trachea were exposed and a hemostat was clamped around the oropharynx just beyond the epiglottis. The trachea was freed by gently teasing it away from the esophagus and surrounding connective tissue. The larynx, trachea, lungs, and heart were then removed *en bloc*. The trachea was cut from the larynx at the cricoid

cartilage and from the mainstem bronchi of the carcina. A small piece of duodenum, approximately 1 cm below the level of the pyloric valve, was used as a positive control for the fluorescent procedures. The length of time from the first incision to tissue processing was about two minutes.

Fluorescence Histochemistry Freeze-Dried Formaldehyde Induced Histofluorescence

Freezing and Freeze-drying

The Falck-Hillarp (1962) fluorescence histochemical technique involves treatment of freeze-dried tissues with formaldehyde gas. The principles of freezing and freeze-drying have been described by VanOrden (1975), while the technical aspects of this procedure have been reviewed by Falck (1962), Falck et al. (1965), and Björklund et al. (1972).

Tracheal segments were placed on small pieces of aluminum foil and immersed in 2-methylbutane (Eastman) cooled by liquid nitrogen for quick freezing. The quick freezing prevented significant ice crystal formation and also preserved morphology. The temperature of the 2-methylbutane was near its melting point (-160°C) judged by the presence of frozen areas within the container. The tissue segments remained in the 2-methylbutane for about two minutes and then were transferred into liquid nitrogen for storage and subsequent freeze-drying.

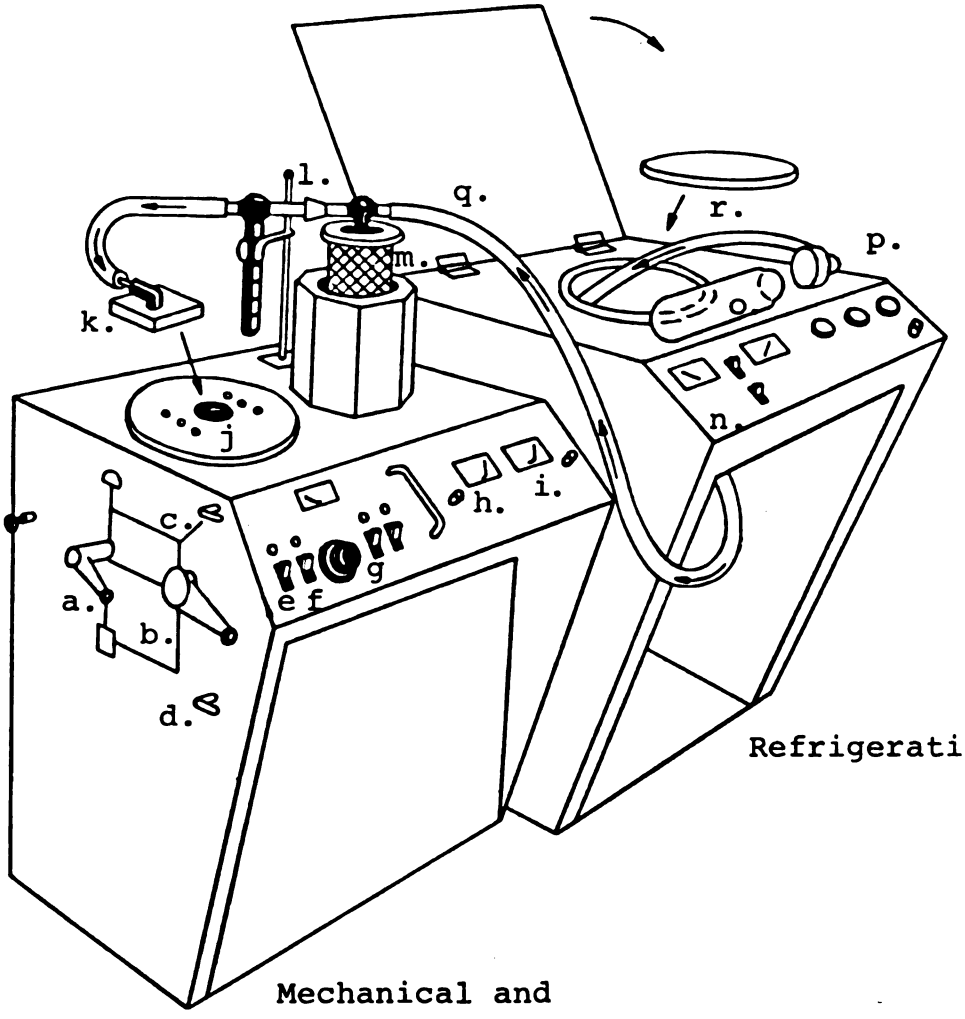
The freeze-dryer utilized in this study was constructed from existing laboratory equipment (Figure 1; Dey et al., 1980). Tissue segments were placed in a 2,500 ml flask (Virtis) which had been pre-cooled to -80°C by an acetone and dry ice slush. The flask, which contained phosphorous pentoxide (P_2O_5, Fisher) to enhance moisture absorption, was then connected to a vacuum system (Kinney) which generated a vacuum of 10^{-5} torr. The mechanical and oil diffusion pumps were protected from corrosive gases by cold and chemical traps.

Drying of the tissue segments was accomplished by sublimation (vaporization of a solid substance, water, to a gas without passing through a liquid state). The vacuum system reduced the energy necessary for vaporization while the cold trap in conjunction with the phosphorous pentoxide provided an effective vapor gradient for the removal of moisture from the tissue. To prevent ice crystal formation and still allow an adequate rate of sublimation the temperature of the tissue segments was maintained by a refrigeration unit (Edwards).

The flask was placed in the acetone and dry ice slush and maintained at - 80°C for one day (24 hours). During the second day, the dry ice was removed and the temperature was gradually raised to -30°C and maintained by the refrigerator. On the third day, the temperature was again raised, this time to room temperature (25°C). At the end of the

Figure 1.--Freeze-drying apparatus; (a) main valve; (b) roughing-backing valve; (c) air inlet valve; (d) pump air inlet valve; (e) mech. pump switch; (f) diff. pump switch; (g) main switch; (h) thermocouple gauge; (i) thermocouple gauge switch; (j) base plate opening; (k) adapter block; (l) cold trap; (m) N_2(l) trap; (n) refrigeration switch; (o) vacuum jar; (p) vacuum jar cap; (r) refrigeration chamber.

FREEZE-DRYING APPARATUS



Refrigeration Unit

Mechanical and
Diffusion Pumps

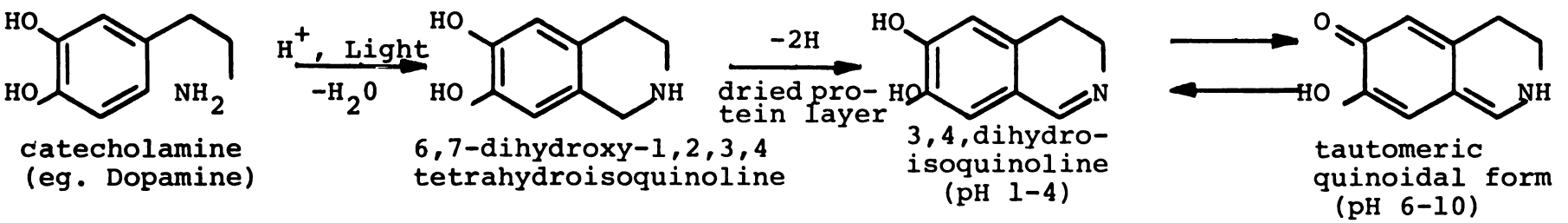
third day, the tissues were heated to 80°C for three hours in a water bath to prevent condensation of water, and then removed from the freeze-dryer for subsequent formaldehyde vapor treatment and/or embedding.

Formaldehyde Vapor Treatment

The fluorescence histochemical demonstration of catecholamines, tyramines, tryptamines and related substances depends upon condensation reactions with formaldehyde vapor leading to ring closure and the formation of fluorescent isoquinoline, quinoid or β-carboline (Figure 2). This reaction only takes place when the amines are associated with the tissue protein. The most important factors affecting the quality and intensity of formaldehyde gas induced fluorescence are; humidity, temperature, pH or acidity of the protein environment, monoamine concentration, and reaction time (Corrodi et al., 1967).

In order to obtain optimal fluorescence intensity 5 grams of paraformaldehyde powder (Electron Microscopy Sciences) was equilibrated for at least ten days in an atmosphere of 60% relatively humidity (Hamberger et al., 1965; Pearse, 1972, Appendix 27), attained from a 10 molar aquaeous solution of sulfuric acid. The sulfuric acid was removed and the closed container with paraformaldehyde powder was preheated to 80°C for one hour to generate an

Figure 2.--Picket-Spengler reaction of an amine with an aldehyde by condensation to form heterocyclic compounds which spontaneously oxidize yielding fluorescent dehydroisoquinoline or dihydro-β-carboline depending on whether the amine contains a catechol or indole ring structure respectively (Corrodi and Jonsson, 1967; and Björklund *et al.*, 1973).

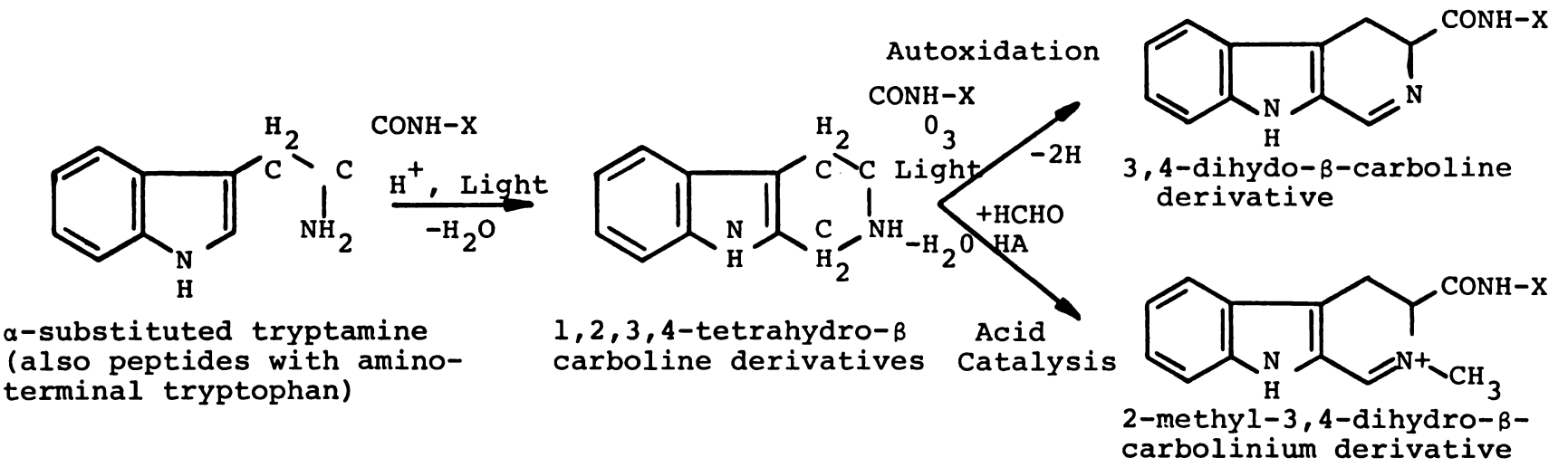


catecholamine (eg. Dopamine)

6,7-dihydroxy-1,2,3,4 tetrahydroisoquinoline

3,4,dihydro-isoquinoline (pH 1-4)

tautomeric quinoidal form (pH 6-10)



α-substituted tryptamine (also peptides with amino-terminal tryptophan)

1,2,3,4-tetrahydro-β carboline derivatives

Acid Catalysis

3,4-dihydo-β-carboline derivative

2-methyl-3,4-dihydro-β-carbolinium derivative

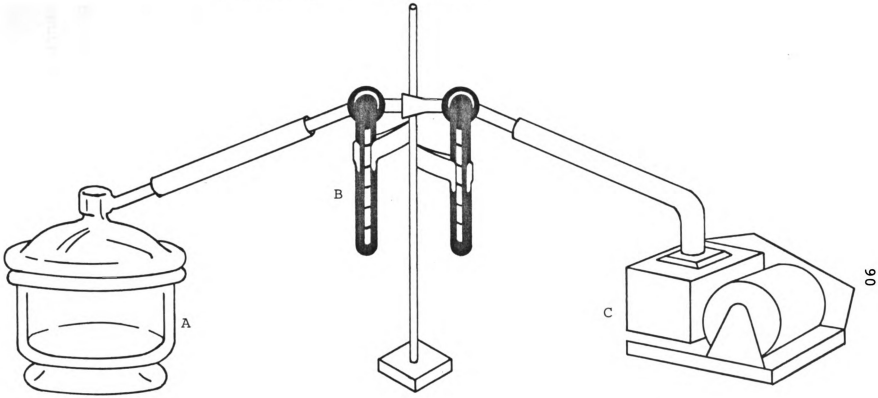
optimum formaldehyde vapor concentration. Freeze-dried tissue segments were then immediately removed from the freeze dryer, placed in the closed container with paraformaldehyde powder for 90 minutes and subsequently embedded in glycol methacrylate. Control tissues were not exposed to formaldehyde vapor prior to being embedded.

Plastic Embedding and Sectioning

The tissue segments were infiltrated and embedded in glycol methacrylate (Polysciences, Inc.) according to the method of Hoyt et al. (1979). Trachea, larynx, and small intestine were placed in small vials containing embedding solution A (which included catalyst). The vials were put in a dissicator and exposed to a mild vacuum (for 2 hours) containing on line chemical traps (Figure 3) and then allowed to stand overnight at atmospheric pressure. Tissue samples were then placed in the complete glycol methacrylate medium consisting of embedding solution A, solution B, plus accelerator and allowed to polymerize. Details of the embedding procedure appear in Appendix A. Sections (1-10 μm) were cut on an ultramicrotome (Sorvall JB-4) with glass knives, placed on a drop of water on acid cleaned glass slides, briefly dried on a warming tray at 40°C and mounted in Entellan (EM Laboratories, Inc.).

Figure 3.--Plastic embedding apparatus; (a) vacuum chamber; (b) chemical traps, and (c) vacuum pump.

PLASTIC EMBEDDING APPARATUS



Liquid Formaldehyde Induced Histofluorescence

Drug Treatment

Two groups of animals (four animals per group) were given a single intraperitoneal (i.p.) injection of 100 mg/Kg body weight of either L-DOPA (Sigma) or L-5-HTP (Sigma) twenty five to thirty minutes perior to sacrifice. The amine precursors were dissolved in distilled water and a total volume of 1 ml was delivered per animal. Four control animals received i.p. injections of distilled water only.

Surgical Procedure

Twenty five minutes after administration of the amine precursor the animals were anesthetized with sodium pentobarbital, 50 mg/Kg, i.p. The rats were then placed in a supine position and a midline incision was made at the level of the xiphoid process and extended up to the level of the first rib. The left ventricle was cannulated with an 18 gauge sterile needle and the animals were perfused at systolic pressures. Immediately following the cannulation of the left ventricle, the right atrium was incised to provide an outflow of the perfusion medium.

Fixation and Dehydration

The animals were initially perfused with a cold (4°C) buffered (0.1M phosphate, pH 7.2) solution of heparin (Nutritional Biochemicals Corporation) for 15 seconds.

Following the prewash the rats were perfused with a 6% formaldehyde (Ted Pella, Inc.) solution buffered at pH 7.2-7.3 with 0.1 M phosphate. The formaldehyde solution was always freshly prepared by dissolving paraformaldehyde powder to warm (90°C) phosphate buffer. The formaldehyde solution was immediately filtered and the pH corrected to 7.2 to 7.3 when necessary. The fixative solution was then cooled to 4°C for subsequent infusion via the left ventricle. The animals were perfused for 3 to 5 minutes until the vascular outflow was clear. Removal of the larynx, trachea, and duodenum was similar to that previously described for freeze-drying. The tissue segments were then immersed in fresh 6% formaldehyde solution for 48 hours at 4°C. Following fixation, the specimens were washed for two minutes in ice cold distilled water and then dehydrated in increasing concentrations of ethyl alcohol (25,50,75,95,100%) for ten minutes each at 4°C. The final dehydrating medium of 100% ethyl alcohol was allowed to come to room temperature over a 30 minute time interval.

Electron Microscopic Infiltration, Embedding and Sectioning

Tissue samples were infiltrated for one hour each in a 2:1, 1:1, 1:2 mixture of ethyl alcohol and Spurr's resin (Polysciences, Inc.). The final infiltration medium which consisted of pure plastic was allowed to infiltrate the tissues overnight at room temperature. Tissue segments

were then transferred to fresh Spurr's resin for embedding by polymerization in an oven for twelve hours at 65°C. After embedding in plastic, 1-6 mu thick sections were cut on a ultramicrotome (Sorvall Mt-2). Each section was then placed on a drop of water on a glass slide, dried on a warming tray at 55°C and then mounted with a coverslip in non-fluorescent immersion oil (R.P. Cargille Laboratories, Inc.).

Formaldehyde Vapor and HCl Treatment

Formaldehyde vapor was generated from paraformaldehyde powder as previously described. Sections (1-6 mu) were transferred to the closed vessel and exposed to formaldehyde vapor for one hour at 80°C. The sections were then immediately transferred to 2.5l jar which contained a small beaker with 60 ml of concentrated (37%) analytical grade hydrochloric acid (Mallincrodt Chemical Works). Sections were exposed for one hour to the HCl vapor generated at room temperature in the closed vessel.

Specificity of the Fluorophore

Although the histochemical fluorescence method of Falck and Hillarp (1962) for the demonstration of biogenic amines has a very high specificity, it is important to determine the specificity of the fluorophore. A number of histochemical criteria have been proposed (Corrodi and Jonsson, 1967) to determine if the observed fluorescence is due to the presence of a monoamine. The first criterion

demonstrated whether the fluorophore resulted from the formaldehyde vapor or was simply autofluorescence. Tracheal and gastrointestinal tissue segments that were freeze-dried but not formaldehyde vapor treated were compared to exposed tissue samples.

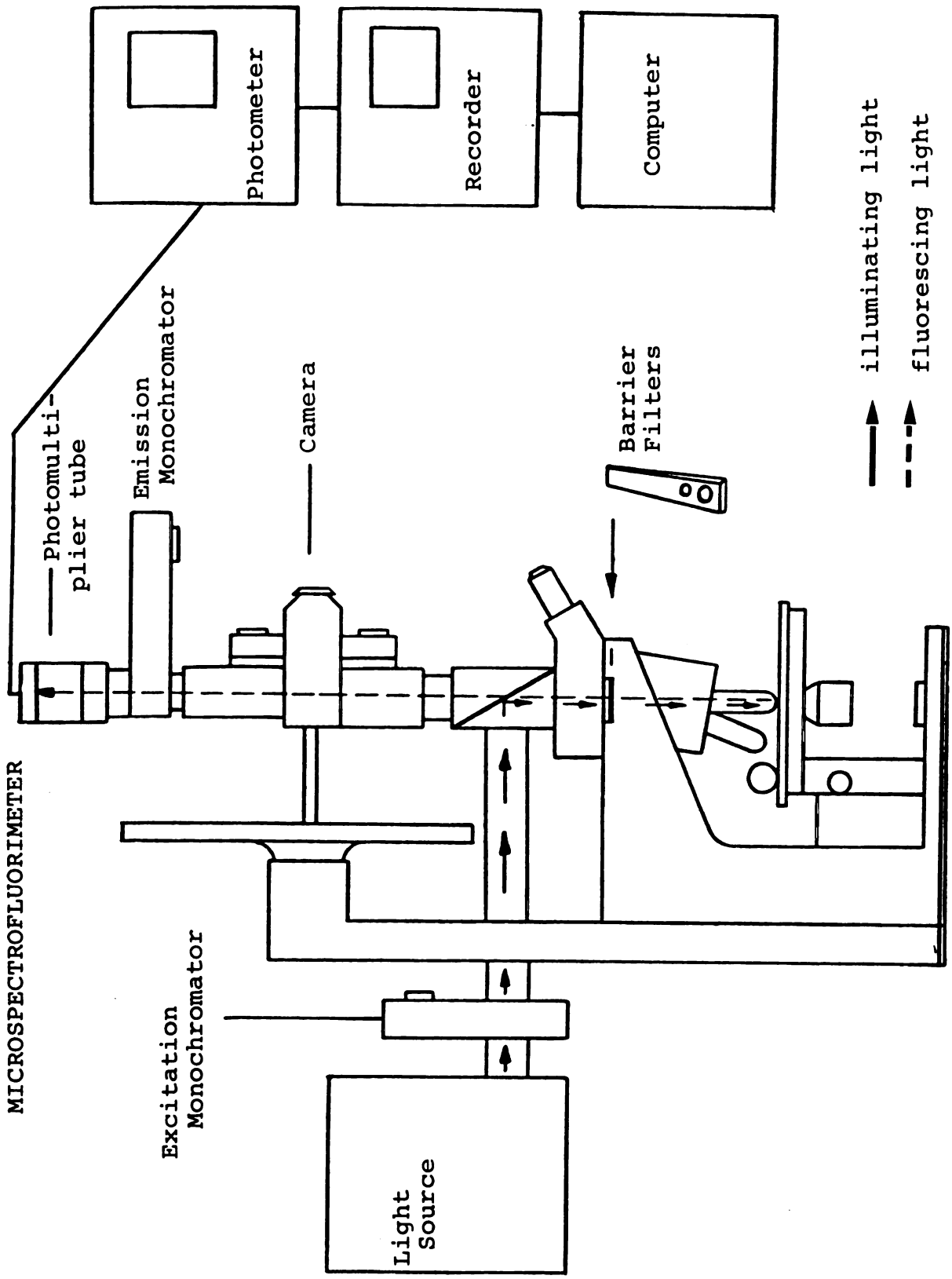
The second test determined if the fluorophore was sensitive to irradiation with ultraviolet light. Following exposure to ultraviolet light the fluorescent compounds of the monoamines demonstrate photodecomposition and loss of fluorescence intensity, whereas autofluorescent structures and proteins do not. This was accomplished by observing the fluorophore after exposure to ultraviolet light.

Corrodi et al. (1964) demonstrated that sodium borohydride in ethanol reduced monoamine fluorescent compounds. In the presence of sodium borohydride ($NaBH_4$) the carbolines are reduced to their corresponding nonfluorescent 1,2,3,4-tetrahydroderivatives. The proof of specificity however is the regeneration of fluorescence after $NaBH_4$ reduction. Fluorescent cells were first identified, then the tissue sections were exposed to 0.5% sodium borohydride (Sigma) in 80% ethanol, observed a second time, and finally treated again with formaldehyde HCl vapor and observed a third time.

Microspectrofluorimetric Analysis

Fluorescence excitation and emission spectra were measured with a microspectrofluorimeter (Figure 4) housed

Figure 4.--Microspectrofluorimeter; (a) illuminating light (⟶), and (b) fluorescing light (---→).


MICROSPECTROFLUORIMETER
Photomulti-
plier tube
Emission
Monochromator
Camera
Barrier
Filters
illuminating light
fluorescing light
Photometer
Recorder
Computer
Excitation
Monochromator
Light
Source

in the laboratory of Dr. Robert Dinerstein, Department of Physiology and Pharmacology, University of Chicago, Chicago, Illinois (Dinerstein et al., 1979). Fluorescence microscopy was carried out with a Leitz Orthoflux Fluorescent Microscope using light from a 200 watt mercury vapor lamp. Light from the lamp was passed through an excitation monochromator, objective, and glass coverslip to the tissue section. Fluorescent light was then directed through an emission monochromator and then emitted fluorescence intensity from each cell was recorded by photon counting. In all instances measurements were made in sections mounted in immersion oil. Both excitation and emission spectra were measured independently from the same cell. The emission curves were first determined by measuring light intensities at wavelengths from 500 to 600 nm in 5 nm steps with unfiltered light from the mercury vapor lamp. The excitation peak was calculated by measuring the intensities of emission peak while excitation wavelengths were increased from 350 to 500 nm in 5 nm steps. The excitation and emission spectra were directly recorded as quanta per unit of wavelength by an on line computer. The excitation and emission spectra were both corrected for the spectral sensitivity of the microspectrofluorimeter.

Histochemical Procedures

Freeze-dried, formaldehyde vapor-treated, tracheal and gastrointestinal segments (control) embedded in glycol

methacrylate were utilized to investigate the cytochemical staining characteristics of adult rat tracheal epithelium. Sections (1-6 mu) were placed on a drop of water on a glass slide, dried on a warming tray at 37°C, and subsequently stained by various histochemical procedures: methylene blue, ferric ferricyanide, alcian blue, periodic acid Schiff (PAS), PAS-lead hematoxylin; details of the histochemical procedures appear in Appendix A.

Lee's Methylene Blue-Basic Fuchsin Stain (Bennett et al., 1976)

Methylene blue, along with many other basic dyes, has been employed in the staining of endocrine tissues. Manocchio (1964) reported the metachromatic staining of pancreatic D cells with toluidine blue. It was concluded that toluidine blue reacted with carboxyl groups in the endocrine cell granules. Solcia et al. (1968) reported the selective staining of endocrine cells with basic dyes after acid hydrolysis. These investigators found that pancreatic A cells, which normally are not basophilic, showed a distinct metachromatic reaction. It was concluded that the mechanism of the metachromasia related to an unmasking of acid groups which were "blocked in fixed tissues".

Fujita et al. (1968) reported that aldehyde-fuchsin and pseudoisocyanin stained the pancreatic B-cell granules. It was concluded that these stains did not stain the insulin but rather the acidic protein of a large molecule, presumably a glycolipoprotein, contained within the granules.

Fujita et al. (1977) suggested that the masked metachromatic staining may have been due to the blocking of acidic groups in the hormone's carrier protein by basic amines and/or peptides. They believed that the carrier protein's acidic groups became exposed or unmasked after acidic hydration of the hormone and, are thus, available for staining by basic dyes. Sections were stained according to the method of Bennett et al. (1976) for the demonstration of endocrine cell granules.

Ferric Ferricyanide (Lillie and Burtner, 1953)

Plastic sections were stained in a solution of ferric chloride ($FeCl_3$) and potassium ferricyanide ($KFe(CN)_6$), for thirty minutes. The solution formed, ferric-ferricyanide, $[Fe_4^{+3}(Fe^{+3}(CN^{-1}_6)]$, is promptly reduced by various tissue components (phenols, indoles, etc.) to form ferric-ferrocyanide $[Fe_4^{+3}(Fe^{+2}(CN^{-1})_6)_3]$ which is Prussian blue. Weak reducing agents or low concentrations of strong reducing agents are thought to reduce ferric-ferricyanide to the intermediate product ferroso-ferric ferricyanide $[Fe_4^{+3}Fe^{+2}(Fe^{+3}(CN_6)_6]$, which is Prussian green. The ferric-ferricyanide solution is not stable and must be made up freshly every fifteen minutes to avoid precipitation on the tissue.

Alcian Blue (Luna, 1968)

Alcian blue is a highly selective stain for acid mucopolysaccharides. The basis of its staining activity is not fully understood but it has been suggested that the acidic groups of the carbohydrate may form salt linkages with the dye. Plastic sections were prepared for staining by a mordant with 3% acetic acid for 30 minutes. Sections were then placed in 1% alcian blue in 3% acetic acid for five hours at 60°C. The method described by Luna (1968) for paraffin sections was modified for plastic sections.

PAS-Lead Hematoxylin (Sorokin and Hoyt, 1978)

One of the most widely employed methods for carbohydrate staining is the periodic acid Schiff reaction. The periodic acid breaks carbon chains of polysaccharides containing 1,2 glycol groups and then oxidizes the broken ends into aldehyde groups. The exact mechanism by which Schiff's reagent colors the aldehyde groups is not well understood. Periodic acid-Schiff in combination with lead hematoxylin allows for the rapid identification of small-granule endocrine cell populations in the lung and gastrointestinal tract (Sorokin and Hoyt, 1978). Sections were immersed in periodic acid for 3 hours and then were stained with Schiff's reagent for 15 minutes. The sections were subsequently allowed to dry overnight and then stained with lead hematoxylin (1 to 3 hours). The mechanism for lead hematoxylin reactivity in tissues is not well understood.

Acid Phosphatase
(Higuchi et al., 1979)

Acid phosphatase appears to be associated almost exclusively with lysosomes (Humason, 1972). This enzyme exhibits optimal activity at a pH between 3.8 and 6.0 (H-mason, 1972). Azo dye methods are frequently used for the demonstration of acid and alkaline phosphatases, nonspecific esterases, β-glucuronidase, β-glucosaminidase and aminopeptidases (Lillie and Fullmer, 1976). Naphthol AS phosphates (substrate) are phosphate esters which release highly insoluble napthols following enzymatic hydrolysis. These napthols subsequently couple with diazonium salts (e.g. fast red violet) to form insoluble azo dyes at tissue sites of acid phosphatase activity.

Using a modification of Burstone's procedure, Higuchi et al. (1972) developed a method for demonstrating acid phosphatase in glycol methacrylate embedded tissue. Plastic embedded laryngeal and tracheal sections were incubated for twenty four hours, rinsed in running tap water, counter-stained with methyl green and mounted in Entellan.

Cryostat Histochemistry

The histochemical procedures described in this section were carried out on either cryostat sections (monoamine oxidase) or freeze-dried cryostat sections (histamine or tryptamine). Laryngeal and tracheal segments were quickly frozen as described previously. The frozen tissue segments

were rapidly transferred to an International Cryostat and allowed to equilbrate to -20°C thin sections fifteen microns thick were collected on glass slides without thawing. The sections were then transferred to a freeze-dryer and dried overnight (-30°C). The following day, the temperature was allowed to rise to 24°C and evacuation was continued for another 6 hours. The sections were then removed and immediately processed for the histochemical demonstration of histamine or tryptamine.

Monoamine Oxidase
(Glenner et al., 1957)

The histochemical demonstration of monoamine oxidase (MAO) by the use of tryptamine as substrate and tetrazolium salts as indicators was reported by Glenner et al. (1957). Tissue sites which contain monoamine oxidase deaminate tryptamine or serotonin and result in the formation of an aldehyde. The aldehyde formed subsequently reduces the tetrazolium to an insoluble colored formazan (Glenner et al., 1960). Fresh laryngeal, tracheal, and duodenal tissue sections (15 mu) were incubated in a solution containing tryptamine or serotonin and nitro-blue tetrazolium for 30 minutes at 37°C. Additional sections were also incubated in substrate free incubation medium for controls.

O-Phthalaldehyde Technique (OPT)
(Björklund et al., 1972)

Björklund et al. (1972) described a fluorescent histochemical method for the demonstration of histamine. A closed vessel (150 ml) containing 1g of o-phthalaldehyde (OPT) was placed in an oven at 100°C for 10 minutes to generate OPT vapors. The vessel was then transferred to room temperature and the freeze-dried tissue sections were placed in the vessel for 90 seconds. Laryngeal and tracheal sections were then removed and exposed to the steam of boiling water for 5 sec. Tissue sections were finally transferred to a 100°C oven, dried for 5 minutes, and mounted in Entellan.

Formaldehyde-Ozone Treatment
(Björklund et al., 1972)

Simultaneous exposure of freeze-dried cryostat sections to formaldehyde and ozone vapor was described by Björklund et al. (1972) for the fluorescent histochemical demonstration of tryptamine. In this procedure freeze-dried cryostat sections were placed in a 1 liter jar which contained 5g of paraformaldehyde and a Tesla coil which was attached to two metal electrodes. The jar was sealed, exposed to ozone vapor for thirty minutes, and finally transferred to an oven and heated at 80°C for 1 hour. Tissue sections were removed and immediately mounted in Entellan.

Combined Fluorescence and Light Microscopy

Freeze-dried formaldehyde vapor treated tracheas were used to determine if fluorescent cells were identical to cells seen by the staining techniques described above. Sections were first observed under the fluorescent microscope, photographed, and then were stained with methylene blue and rephotographed. Methylene blue staining was reversed by gently washing the sections in running water for up to ten minutes. Subsequently, the sections were stained with either ferric-ferricyanide, PAS-lead hematoxylin or alcian blue. Sections were always photographed without a coverslip.

Electron Microscopy

The investigation of tracheal tissue ultrastructure was carried out with the same tissue which had been fixed with 6% formaldehyde and embedded in Spurr's resin. After fixation, dehydration and embedding, 1 mu sections were treated with formaldehyde and HCl vapors as described earlier. When the fluorescent tracheal cells were distinctly visible in such sections, a correlated electron microscopic investigation was carried out. The blocks from which the sections were taken for fluorescence microscopy as described above were subsequently trimmed for thin sectioning with an Sorvall MT-2 ultramicrotome. The thin sections were stained with 1% alcoholic uranyl acetate and lead citrate (Reynolds, 1963) and examined with the electron microscope (EM Philips 200).

Microscopy and Photography

Fluorescence was observed and photographed with an Olympus BH-RFL epifluorescence microscope. The mounted section was observed by epifluorescence from a 200 watt mercury vapor lamp. A BG 12 excitation filter and a 470 nm barrier filter were used to visualize formaldehyde-induced, monoamine fluorescence in tracheal and gastrointestinal tissues. Photographs were recorded on Kodak Ektachrome Film (Daylight, ASA 400, DIN 27) and push processed one f stop. If the sections photographed were intended for subsequent staining and light microscopy, the appropriate horizontal and vertical coordinates were recorded from the microscope stage.

Light microscopy was observed and photographed by use of a Zeiss Universal microscope equipped with automatic exposure camera. Photographs were captured on Kodak Ektachrome Film (Daylight, ASA64, DIN19) with an 80B filter.

Statistical Analysis of Excitation Emission Maxima

The excitation and emission maxima of fluorescent cells in the rat trachea and duodenum were evaluated by a one-way analysis of variance (AOV), to determine if the spectral means were derived from the same or different populations. AOV assumptions of randomization, normality, equality of variances and independence were met. The critical region of rejection was set at $\alpha = .01$. If the AOV detected significantly different populations, a Duncan and Scheffé

a posteriori test were used to compare individual means. The critical region of rejection for the Duncan and Scheffé *a posteriori* test were set at $\alpha = .05$.

CHAPTER IV

RESULTS

Distribution of Fluorescent Tracheal Epithelial Cells

Application of the standard Falck-Hillarp technique followed by embedding in glycol methacrylate revealed the presence of numerous cells which were intensely fluorescent in the respiratory epithelial lining of the extrapulmonary airways (Figure 5). Yellow fluorescent mast cells and green fluorescent nerve fibers were evident in mucosal and submucosal connective tissues. Yellow fluorescent mast cells (Figure 7) and enterochromaffin cells were also seen in sections from duodenum and stomach. The fluorescent extrapulmonary cells in respiratory epithelium were most numerous in the larynx and upper trachea. However, they were seldom observed in the true vocal folds.

Liquid formaldehyde fixed tissues did not reveal any endogenous fluorescent epithelial cells in the larynx or trachea. Weakly fluorescent cells however, were present in the mucosa and submucosa of the extrapulmonary airways and gastrointestinal tract. In the epithelial lining of the duodenum numerous yellow fluorescent triangular cells with their base resting on the underlying basement membrane,

and thin apical processes projecting towards the lumen were present. Following formaldehyde vapor treatment, the intensity of fluorescence increased in mast cells and enterochromaffin cells. No visible endogenous fluorescence was observed in the epithelium of the extrapulmonary airways. Subsequent treatment of the plastic embedded section with fumes of concentrated HCl (37%) for one hour resulted in the demonstration of numerous intensely fluorescent cells in the epithelium of the larynx and trachea (Figure 6). The fluorescent intensity of respiratory mast cells as well as gastrointestinal mast and enterochromaffin cells (Figure 8) also increased. A summary of relative fluorescent intensities of the various cells studied is included in Table 3.

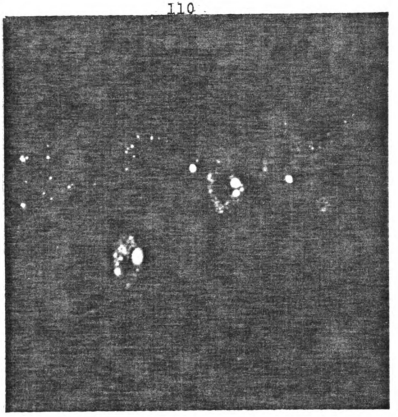
Pretreatment with the amino acid precursors L-DOPA or L-5-HTP did not result in the demonstration of any fluorescent cells in the tracheal epithelium fixed with liquid formaldehyde. Following exposure of the sections to HCl vapor the number, distribution, color, and intensity of the fluorescent cells appeared similar to control animals.

Morphology of the Fluorescent Epithelial Cells

In both freeze-dried (treated with formaldehyde vapor) and liquid formaldehyde fixed tissues (treated with formaldehyde and HCl vapor) the morphology of the fluorescent epithelial cells was similar. The diameter of the fluorescent epithelial cells ranged from 4 to 8 mu. The cells

Figure 5.--Freeze-dried formaldehyde vapor treated and glycol methacrylate embedded tracheal section (4 mu). Yellow fluorescent cells are present in the respiratory epithelium (2,000x).

Figure 6.--Liquid formaldehyde fixed, formaldehyde-HCl induced fluorescent tracheal epithelial cells (1 mu), embedded in Spurr's resin (1,000x).



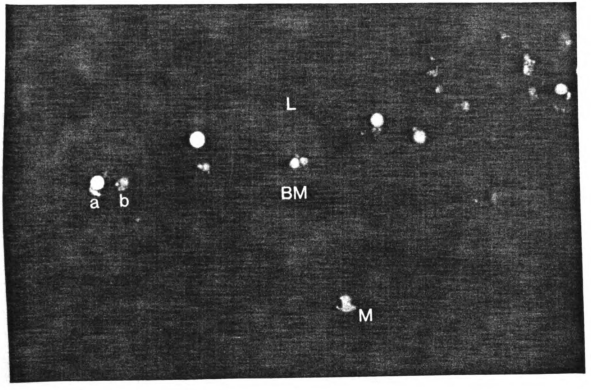
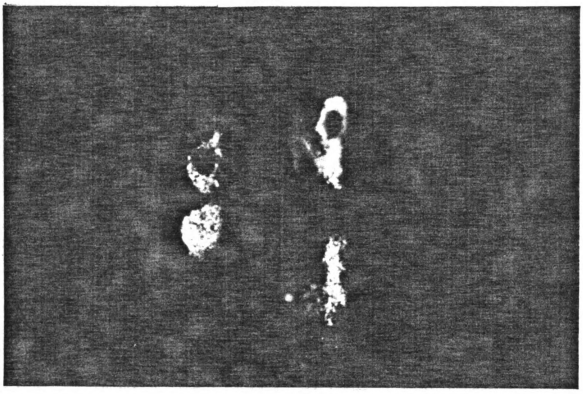

L
a
b
BM
M

Figure 7.--Freeze-dried, formaldehyde vapor treated, and glycol methacrylate embedded tracheal section (4 mu). Yellow fluorescent mast cells are present in the submucosa (2,000x).

Figure 8.--Liquid formaldehyde fixed, Spurr embedded, formaldehyde-HCl vapor treated duodenal section. Fluorescent enterochromaffin and mast cells are present in mucosa (1,000x).



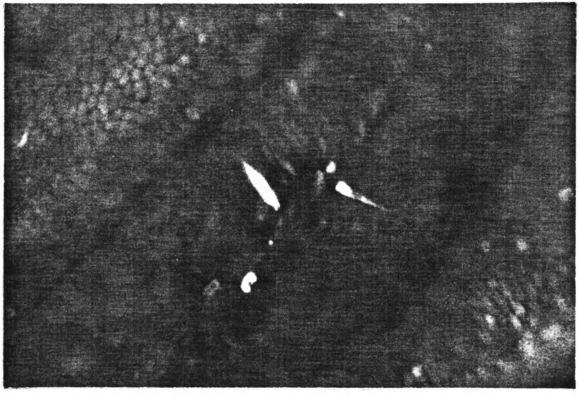


Table 3 .--Relative fluorescence intensity of cells in the respiratory and gastrointestinal tracts of adult rats after various histochemical procedures.

Treatment	Tracheal		Gastrointestinal	
	Epithelial Cell	Mast Cell	Mast Cell	Enterochromaffin
6% formaldehyde fixed tissues	-	+	+	++
Formaldehyde-vapor (1 hr, 80°C)	-	++	++	++
Concentrated HCl vapor (1 hr, 24°C)	+++	+++	+++	+++
Sodium Borohydride treatment (2 min.)	-	-	-	-
Re-exposure to formaldehyde vapor (1 hr, 80°C) and HCl vapor for (1 hr, 24°C)	+++	+++	+++	+++

Fluorescence intensity (-) = no fluorescence, (+) = slight fluorescence, (++) = moderate fluorescence, (+++) = strong fluorescence.

were characterized by conspicuous fluorescent cytoplasmic granules. The number of granules per cell varied from 1 to 15. The granules also varied in size in different cells and even within the same cell. The fluorescence was always confined to the granules within the cytoplasm. The granules appeared to be randomly distributed throughout the cytoplasm. The nucleus was seldom observed by fluorescence microscopy. Occasionally, a dark area was observed between the fluorescent granules which appeared to be the nucleus of the cell. Fluorescent cells were located at all levels of the epithelium, but most frequently they were located lightly apical to the more basal, non-fluorescing epithelial cells.

Microspectrofluorimetric Data

The fluorescence excitation and emission maxima of tracheal epithelial cells and gastrointestinal enterochromaffin cells fixed with liquid formaldehyde, embedded in plastic and treated with formaldehyde and HCl vapors are reported in Table 4. Spectral analyses of seven tracheal fluorescent cells from four animals demonstrated an average maximum excitation of 456 nm $\pm$ 5 and a maximum emission at 535 nm $\pm$ 13. The actual spectral curves from one representative is demonstrated in Figure 9. The average excitation and emission peaks of five gastrointestinal enterochromaffin cells were 425 nm $\pm$ 0 and 513 nm $\pm$ 3 respectively. An example of the actual spectral curve from one cell is presented in Figure 10.

Excitation and emission maxima of fluorescent tracheal epithelial cells and connective tissue mast cells which were prepared according to the standard Falck Hillarp method are reported in Table 5. Spectral analysis of three tracheal epithelial fluorescent cells demonstrated an average maximum emission of 493 nm ± 3. The excitation maximum was not demonstrated between 350 nm and 450 nm. The actual emission spectrum from one cell is demonstrated in Figure 11. In addition, the average excitation and emission maxima of three fluorescent tracheal connective tissue mast cells were 401 nm ± 11 and 515 nm ± 5 respectively. An example of the actual spectra from one cell is presented in Figure 12.

Table 4.--Spectral properties of tracheal (TRACHEAL) and duodenal (GI) fluorescent cells from formaldehyde-HCl treated sections. (Liquid formaldehyde fixed tissue embedded in Spurr's resin, 6 mu thick). Mean and standard deviation of the excitation and emission maxima are also presented.

	Excitation Peak (nm)	Emission Peak (nm)
Tracheal		
1	455	540
2	455	530
3	460	550
4	465	545
5	455	530
6	450	540
7	450	510
Mean	456 ± 5	535 ± 13
GI		
1	425	515
2	425	515
3	425	510
4	425	510
5	425	515
Mean	425 ± 0	513 ± 3

Table 5.--Spectral properties of fluorescent tracheal epithelial cells (EPITHELIAL) and fluorescent connective tissue mast cells (MAST) from freeze-dried paraffin embedded sections, 15 mu thick. Mean and standard deviation of the excitation and emission maxima are also presented.

Tracheal Fluorescent Cell	Excitation Peak (nm)	Emission Peak (nm)
EPITHELIAL		
1	---	490
2	---	495
3	---	495
Mean		493 ± 3
MAST		
1	415	515
2	395	520
3	395	510
Mean	401 ± 11	515 ± 5

Statistical Analysis of Excitation and Emission Maxima

The excitation and emission maxima of fluorescent cells in the rat tracheal epithelium and duodenum were evaluated by a one-way analysis of variance (AOV). All AOV assumptions of randomization, normality, equality of variances, and independence were met. One way analysis of variance indicated that excitation and emission maxima from the different cells evaluated were derived from different populations of fluorogenic substances at α=.01. In order to determine the source of significance, a Scheffe and Duncan *a posteriori* testswere calculated. The critical region of rejection for the *a posteriori* testswere set at α=.05.

Figure 9.--Excitation (---) and emission (——) curves of a fluorescent tracheal epithelial cell, liquid formaldehyde fixed, embedded in Spurr's resin, and treated with formaldehyde-HCl vapor.

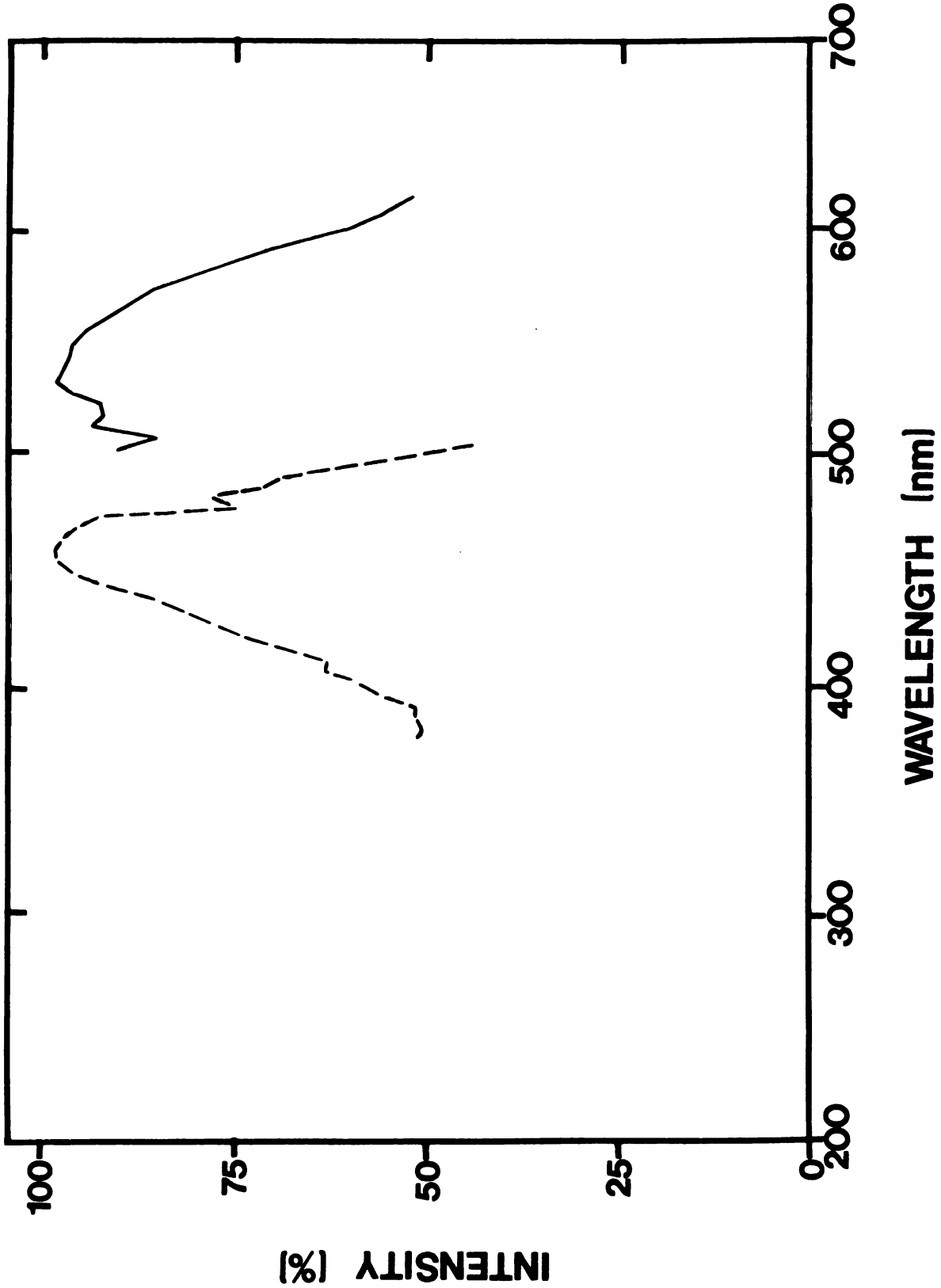

INTENSITY (%)
100
75
50
25
0
200
300
400
500
600
700
WAVELENGTH (nm)

Figure 10.--Excitation (---) and emission (——) curves of a liquid formaldehyde fixed, Spurr embedded, formaldehyde-HCl vapor induced fluorescent gastrointestinal (duodenum) cell.

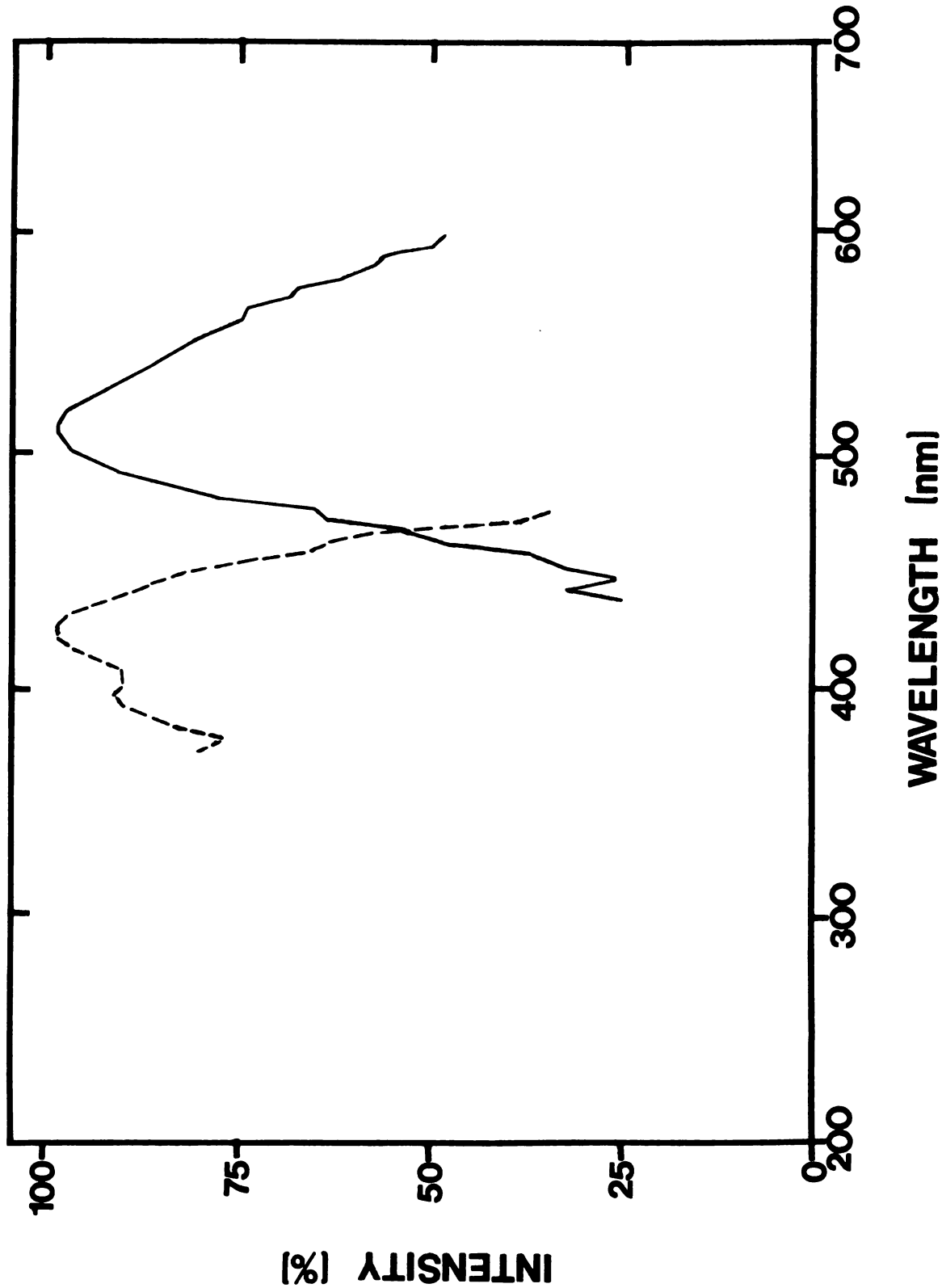

INTENSITY [%]
100
75
50
25
0
200
300
400
500
600
700
WAVELENGTH [nm]

Figure 11.--Excitation (---) and emission (——) curve of a fluorescent tracheal epithelial cell, freeze-dried and paraffin embedded.

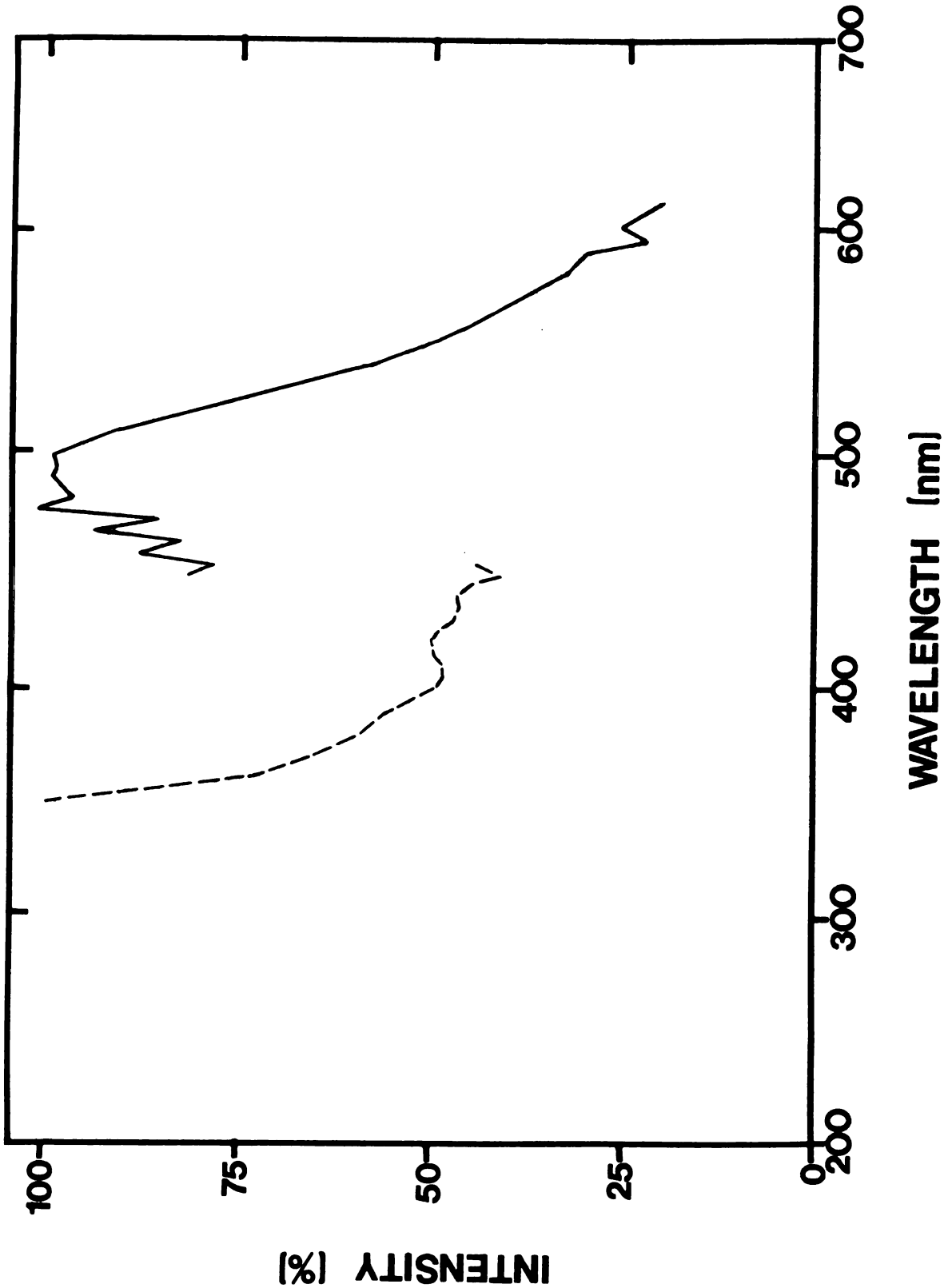
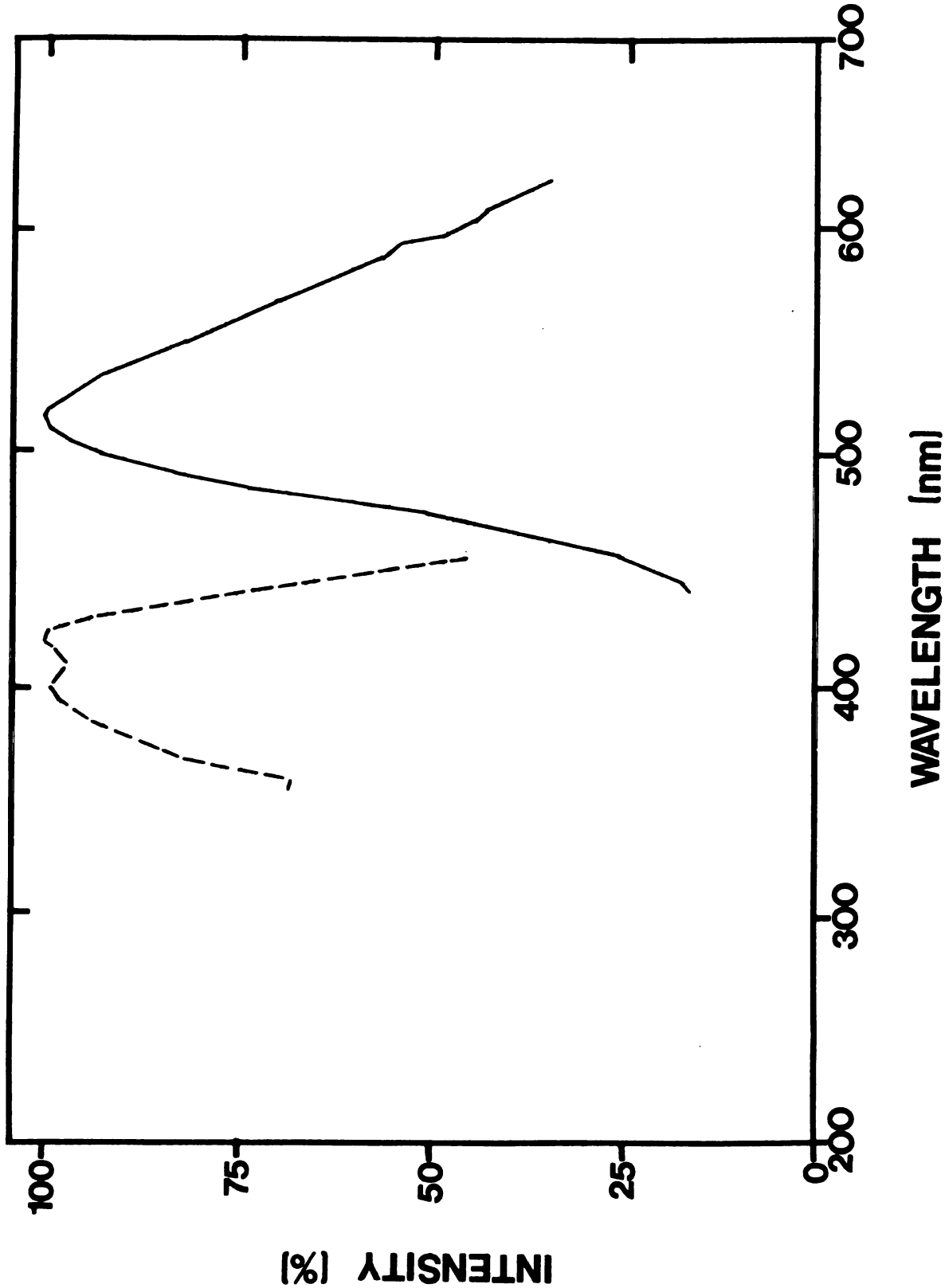

INTENSITY [%]
100
75
50
25
0
200
300
400
500
600
700
WAVELENGTH [nm]

Figure 12.--Excitation (----) and emission (———) curves of a fluorescent tracheal connective tissue mast cell, freeze-dried and paraffin embedded.


INTENSITY [%]
100
75
50
25
0
200
300
400
500
600
700
WAVELENGTH [nm]

The results from the Scheffé and the Duncan multiple comparisons tests were identical. They demonstrated a significant difference between the emission maxima of the tracheal epithelial cells when prepared by different methods. However, in cells known to contain serotonin (tracheal mast cells and duodenal enterochromaffin cells) the method of preparation had no effect on the emission maxima. In addition, regardless of the method of preparation a significant difference in the emission maxima was noted between tracheal epithelial cells and cells known to contain serotonin. Results of both multiple comparisons tests are summarized in Table 6. The excitation maxima of all the cells studied were significantly different from each other. However, excitation maxima from freeze-dried, formaldehyde vapor treated, paraffin embedded fluorescent epithelial cells were not recorded. Results of both multiple comparisons tests are presented in Table 7.

Specificity of the Fluorophore

Freeze-dried and liquid formaldehyde fixed tissues, embedded in glycol methacrylate and Spurr's resin were subjected to three histochemical tests to determine if the fluorophores were due to the presence of a monoamine. The first histochemical test on freeze-dried tissue demonstrated that the cellular fluorescence resulted from the formaldehyde vapor treatment. Fluorescent cells in respiratory and

Table 6.--Mean and standard error of emission maxima (in nm) of fluorescent cells in a 6 mu section from four cell types. The tissues were either fixed with liquid formaldehyde, embedded in plastic and treated with HCl vapor (LFPHCL) or freeze-dried and embedded in paraffin (FDP). The emission maxima were first analyzed with analysis of variance and when significance was noted the means were compared by Scheffé and Duncan a posteriori tests with α=.05.

Cell Type	Tracheal Epithelial Cell (LFPHCL) n=7	Tracheal Mast Cell (FDP) n=3	Gastrointestinal Enterochromaffin Cell (LFPHCL) n=5	Tracheal Epithelial Cell (FDP) n=3
Mean	537.8 ± 3.0	515.0 ± 2.8	513.0 ± 1.2	493.3 ± 1.6*

*Means connected by underlining are NOT significantly different.

Table 7.--Mean and standard error of excitation maxima (in nm) of fluorescent cells in a 6 mu section from three cell types. The tissues were either fixed with liquid formaldehyde, embedded in plastic and treated with HCl vapor (LFPHCL) or freeze-dried and embedded in paraffin (FDP). The excitation maxima were first analyzed with analysis of variance and when significance was noted the means were compared by Scheffé and Duncan a posteriori tests with α=.05.

Cell Type	Tracheal Epithelial Cell (LFPHCL) n=7	Gastrointestinal Enterochromaffin Cell (LFPHCL) n=5	Tracheal Mast Cell (FDP) n=3
Mean	455.7 ± 2.0	425.0 ± 0	401.6 ± 6.6*

*Means connected by underlining are NOT significantly different.

gastrointestional epithelia were absent in tissue samples not exposed to formaldehyde vapor.

The second test demonstrated that irradiation with ultraviolet light produced a marked photodecomposition and loss of fluorescent intensity in fluorescent cells in the laryngeal and tracheal respiratory epithelium. This was demonstrated in both freeze-dried and liquid formaldehyde-HCl vapor treated samples.

The third test investigated the reducing capabilities of sodium borohydride on cellular fluorescence. Freeze-dried and liquid formaldehyde fixed HCl vapor treated tracheal sections embedded in plastic were observed with fluorescence microscopy to establish that the fluorescent cells were present in the epithelium. The specific fluorescence disappeared completely within one or two minutes after the sections were treated with a 0.5% solution of sodium borohydride in 80% ethanol. Sections treated only with 80% ethanol did not cause a change in the appearance of the fluorophore. Following exposure to sodium borohydride, the cellular fluorescence was regenerated after renewed formaldehyde vapor or formaldehyde-HCl vapor treatment. A summary of the results are presented in Table 3.

Histochemical Results

Nearly all of the histochemical procedures described in the literature were accomplished with paraffin embedded sections. These results, however, concern the use of

histochemical techniques on tissue sections embedded in plastic. It has been reported (Bennett et al., 1976) that many of the conventional histologic staining methods used for paraffin can be applied to tissues embedded in glycol methacrylate. Freeze-dried, formaldehyde vapor treated plastic sections from control animals were used to investigate the histochemical characteristics of rat tracheal epithelium. A chart summarizing the staining reactions used appears in Table 8.

Methylene Blue

The methylene blue stain has been shown to be associated with the secretory granules of endocrine cells storing biogenic amines (Solcia et al., 1968; and Bennett et al., 1976). Methylene blue staining demonstrated numerous brilliant blue cells in the respiratory epithelial lining while leaving the remaining epithelial surface a pale blue. Mast cells, tracheal cartilage matrix, and nerve bundles also demonstrated a strong affinity for methylene blue. The staining of these various tissue components was reversed by washing the section in running tap water.

The question of whether methylene blue positive cells were the same as fluorescent cells in tracheal respiratory epithelium was answered by photographing a fluorescent section, subsequently staining with methylene blue, and then rephotographing the same area when viewed with ordinary light microscopy. The results of this procedure clearly

demonstrated that the methylene blue positive cells were identical to the fluorescent cells. The three fluorescent cells in the upper panel of Figure 13 were also methylene blue positive (lower panel).

Alcian Blue

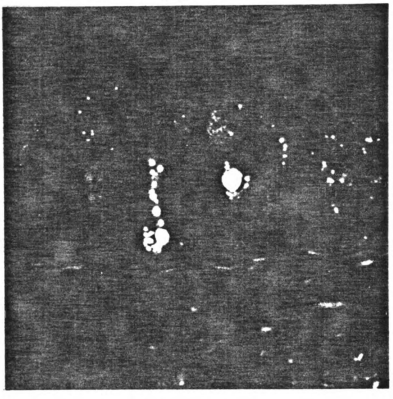
Alcian blue has been used to demonstrate the presence of acid mucopolysaccharides in tissue sections (Luna, 1968). Alcian blue stained tracheal epithelial cells, cartilage, mast cells, and some cells in the submucosal glands. The staining in the epithelium suggested two different cell populations. One population of cells appeared to stain a very faint blue. These cells were often in contact with the lumen and never appeared granular. The second population of cells stained much darker, seldom reached the lumen and always appeared to be very granular.

The results of restaining the same section with alcian blue after fluorescence microscopy demonstrated that the granular alcian blue positive cells were identical to fluorescent positive cells. The intensely fluorescent cell shown in the upper panel of Figure 14 is identical with the alcian blue positive cell in the lower panel. Several weakly fluorescent cells appearing in this figure were also intensely alcian blue positive.

Ferric-ferricyanide

Ferric-ferricyanide reacts with various tissue components which are capable of reducing ferric-ferricyanide to

Figure 13.--The same tracheal section (6mu) photographed first during fluorescence microscopy (upper panel), and then stained with methylene blue and rephotographed during ordinary light microscopy (lower panel) (2,000x).



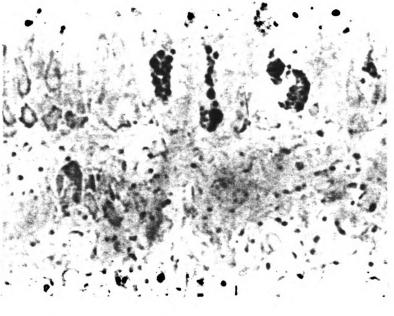
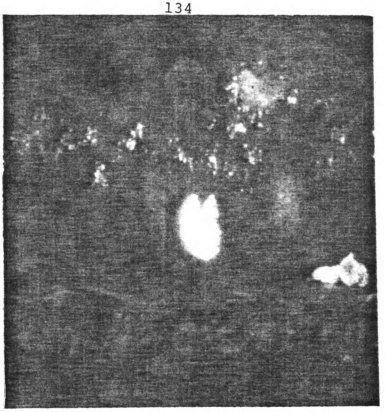


Figure 14.--The same tracheal section (6 mu) photographed first during fluorescence microscopy (upper panel), and then stained with alcian blue and rephotographed during ordinary light microscopy (lower panel) (2,000x).



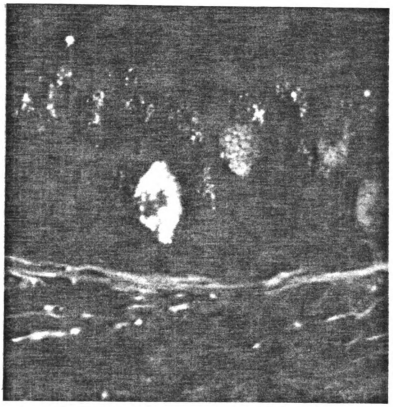
ferric-ferricyanide (Lillie and Burtner, 1953). This reaction is often associated with phenols or indoles (Lillie and Fullmer, 1976). One distinct ferric-ferricyanide positive cell can clearly be identified in the lower panel of Figure 15. Three faintly positive cells are also demonstrated in the same picture. The results of observing the same section after two different treatments is presented in Figure 15. The fluorescent epithelial cells demonstrated in the upper panel are also ferric ferricyanide positive (lower panel). This series of photographs demonstrated that the fluorescent and ferric-ferricyanide positive cells were identical.

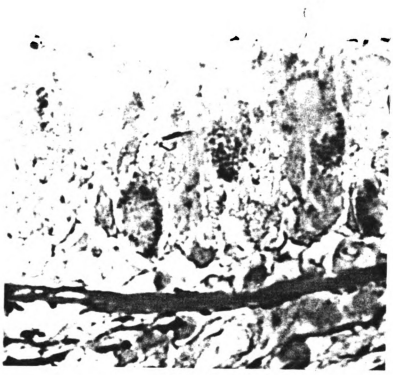
PAS-Lead Hematoxylin

The periodic acid Schiff (PAS) reaction has been associated with the presence of carbohydrates, neutral mucopolysaccharides, mucoproteins, and lipids (Sheehan, et al., 1973). PAS positive cells in the tracheal epithelium are demonstrated in Figure 17. Numerous PAS positive cells were observed to contact the luminal surface and a few positive cells were closer to the basement membrane.

The question of whether PAS positive cells were the same as fluorescent and methylene blue positive cells was answered by photographing fluorescent epithelial cells, staining the same tissue section with methylene blue, rephotographing during observations with ordinary light

Figure 15.--The same 6 mu tracheal section photographed first during fluorescence microscopy (upper panel) and then rephotographed by ordinary light microscopy after staining with ferric-ferricyanide (lower panel) (2,500x).





microscopy, subsequently washing the section with running tap water, and then restaining with PAS and photographing again. The results of methylene blue staining are demonstrated in Figure 16. The methylene blue positive cells in Figure 16 also were PAS positive (Figure 17). In addition, a second population of PAS positive but methylene blue negative cells was also demonstrated. Subsequent staining of the same section with lead hematoxylin resulted in a darkening of all of the PAS positive cells. Definitive selective staining of the fluorescent and methylene blue positive cells could not be demonstrated with lead hematoxylin.

Acid Phosphatase

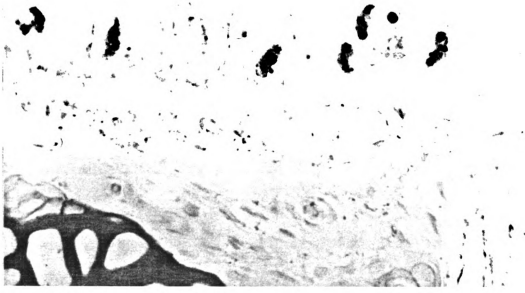
The acid phosphatase stains react almost exclusively with lysosomes (Humason, 1972). Azo dye granules were present in cells located in the lamina propria of the duodenum and the submucosa of the esophagus. However, acid phosphatase positive cells were not observed in the epithelial lining of the larynx, trachea, or duodenum.

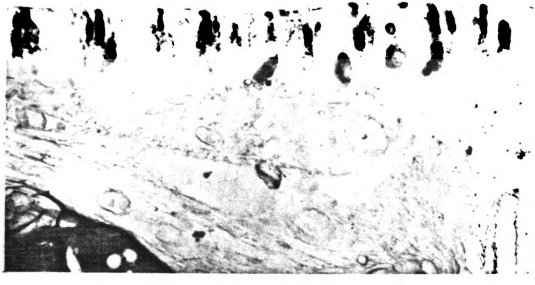
Monoamine Oxidase

Monoamine oxidase acts on long and short-chain monoamines Chayen et al. (1973). Glenner et al. (1960) suggested that monoamine oxidase activity could be demonstrated by the reduction of tetrazolium salts. In Glenner's scheme the product of monoamine oxidase, indolyl-3-acetyldehyde,

Figure 16.--A 6 mu tracheal section photographed after staining with methylene blue (1,000x).

Figure 17.--The same 6 mu tracheal section (Figure 16) after washing with tap water and restaining with PAS and photographing again (1,000x).





reacts spontaneously with nitro-blue tetrazolium to yield a colored formazan. A very intense monoamine oxidase activity was observed in fresh cryostat sections of larynx, trachea, and kidney epithelium incubated with serotonin. In contrast, sections that were incubated without the substrate serotonin, did not result in the formation of formazan granules.

O-Phthaladehyde and Formaldehyde-Ozone Reactions

The fluorescent histochemical demonstration of histamine or tryptamine was not observed in the laryngeal or tracheal epithelium of freeze-dried cryostat sections.

Electron Microscopy

One micron thick sections were cut from liquid formaldehyde fixed (6%) Spurr embedded material. Tracheal and gastrointestinal sections were then exposed to formaldehyde and HCl vapor and observed for cellular fluorescence. Numerous duodenal mast cells and enterochromaffin cells as well as tracheal mast cells and epithelial cells were distinctly fluorescent. Following identification of fluorescent epithelial cells, the blocks were carefully trimmed and a correlated ultrastructural investigation was carried out on immediately adjacent ultrathin sections. Two fluorescent cells shown in Figure 23 are also observed at the ultrastructural level in Figure 24.

Table 8.--Significance and location of selected histochemical reactions.

Stain	Substance Demonstrated	Tracheal Amine Containing Cell	Duodenal Enterochromaffin Cell	Goblet Cell
Formaldehyde Induced Fluorescence	Indole or Phenyl-Ethylamines	+	+	-
Methylene Blue	Endocrine Granules	+	-	-
Alcian Blue	Acid Mucopolysaccharides Endocrine Granules	+	-	-
Ferric-Ferricyanide	Reducing Substances	+	-	-
PAS	Carbohydrates Neutral Mucopolysaccharides	+	-	-
PAS Lead-Hematoxylin	Endocrine Granules	+?	+?	+?
Acid Phosphatase	Lysosomes	-	-	-

The ultastructural characteristics of these fluorescent cells in the trachea were very distinctive due to the presence of large homogeneous electron-dense cytoplasmic granules. Occasionally, there was evidence of a limiting membrane around the granules (Figure 22). The diameter of these cells ranged from 4 mu to 10 mu. Numerous elongated pseudopodia-like processes were frequently observed (Figure 22). These cells occurred at all levels of the respiratory epithelial lining. They were most frequently located close to the basement membrane, but occasionally were observed nearer to the airway lumen. However, these cells were never observed to contact the lumen (Figure 18,20 and 22). There were no junctional contacts noted between the plasma membranes of these cells and other epithelial cells. Junctional complexes (Figure 18,20 and 22) were present between other epithelial cells.

The nucleus was frequently located laterally within the cell (Figure 20 and 22). Occasionally it was centrally located (Figure 19). Heterochromatin was predominantly located peripherally within the nucleus. The cytoplasm of these cells contained moderate amounts of free ribosomes (Figure 19 and 22), small amounts of granular endoplasmic reticulum (Figure 19), and numerous characteristic large, homogeneous, electron dense granules with diameters of 3,000 Å to 4.4 mu. The number of granules per cell ranged from 1 to 36. The electron density of the large granules varied between cells as well as within the same cell. Most

frequently the granules were uniformly dense (Figure 19) but occasionally they were dense with rims of less electron dense matrix (Figure 18, 19 and 21) or were lucent (Figure 20 and 21). In addition, a few granules were opaque with areas of electron-dense figures within the granules (Figure 21).

The ultrastructural characteristics of a fluorescent duodenal epithelial cell is demonstrated in Figure 25. The cell shape, nucleus, and granule structure were very different from the fluorescent laryngeal and tracheal epithelial cells. The duodenal epithelial cells was similar to the previously described duodenal enterochromaffin cell.

Figure 18.--Globular leukocyte in tracheal respiratory epithelium of adult rat showing nucleus (N); large granules of uniform electron density, type I granules (1); or dense with small rims of less electron dense matrix, type II granules (2). Rough endoplasmic reticulum (arrowhead) appears around the type I granule. The cell does not extend to the tracheal lumen. Junctional complexes (arrows) were noted between other tracheal epithelial cells (E) lumen (L). Formaldehyde (6 percent), uranyl acetate, and lead citrate. 7,300X

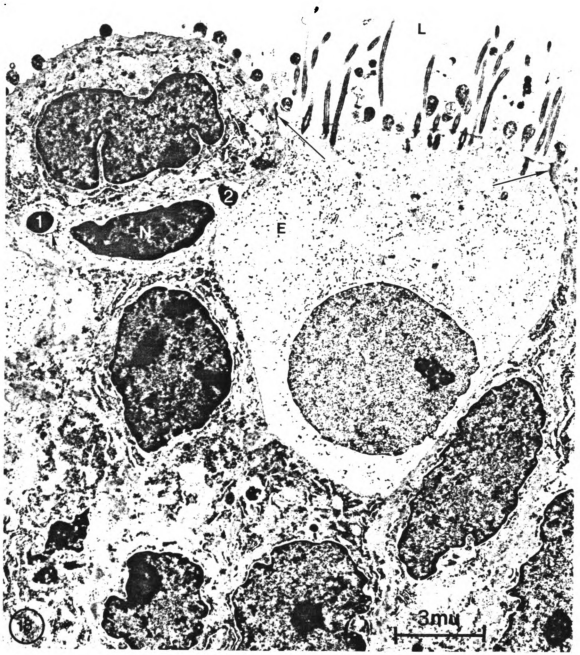

L
1
2
N
E
8mμ

Figure 19.--Globular leukocyte within the tracheal respiratory epithelium of an adult rat. Type I granules (1), Type II granules (2), nucleus (N), the cell membrane (arrow) and rough endoplasmic reticulum (R). Formaldehyde (6%), uranyl acetate and lead citrate. 16,600X

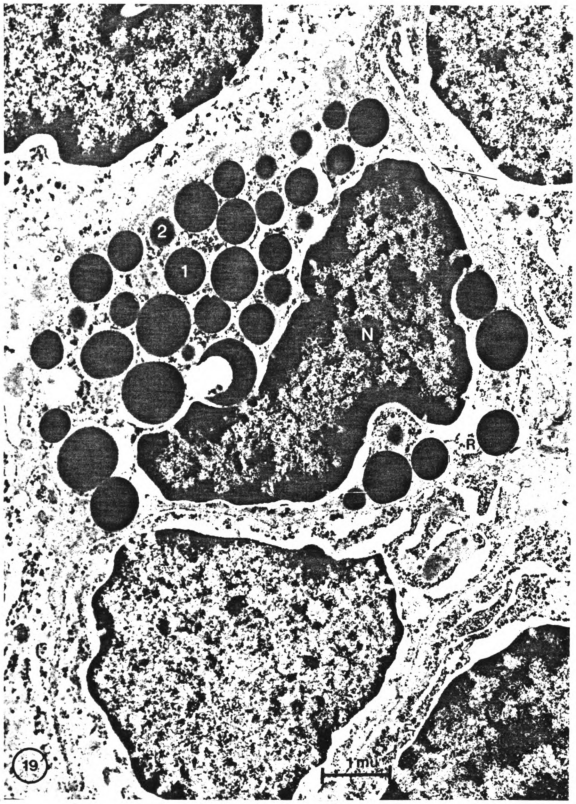

2
1
N
R
19
1 mu

Figure 20.--Globular leukocyte in the tracheal respiratory epithelium of adult rat. The cell cytoplasm contains granules of uniform density (1) and a Type III granule (3) with light and dark matrix areas. Nucleus (N) and epithelial junctional complexes (arrows) can be seen. Formaldehyde (6 percent), uranyl acetate, and lead citrate. 10,760X

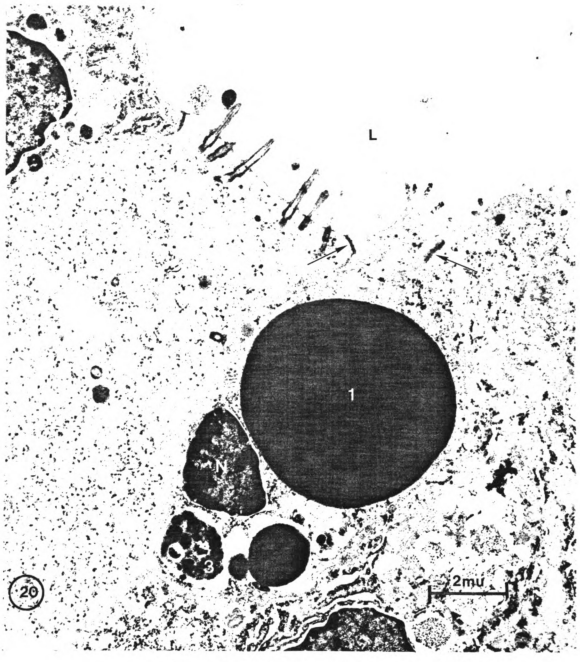

L
1
N
3
2mu
20

Figure 21.--Globular leukocyte in tracheal respiratory epithelium of adult rat showing a variety of granules: (1) electron dense matrix; (2) small rims of less electron-dense matrix surrounding a denser core; (3) light and dark matrix areas; and (4) light matrix with paracrystalline structures (arrow). Formaldehyde (6 percent), uranyl acetate, and lead citrate. 11,390X

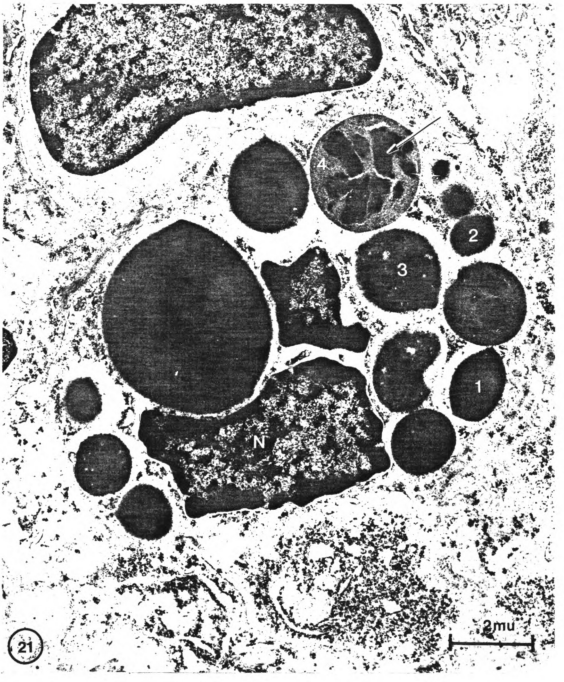

4
2
3
1
N
2mu
21

Figure 22.--Globular leukocyte in the tracheal epithelium of an adult rat showing granules of various sizes within the same cell. Nucleus (N), homogenous electron-dense granules (l), portion of a unit membrane around granule (arrow), junctional complexes (arrowheads), lumen (L), and ribosomes (R). Formaldehyde (6 percent), uranyl acetate, and lead citrate. 9,080X

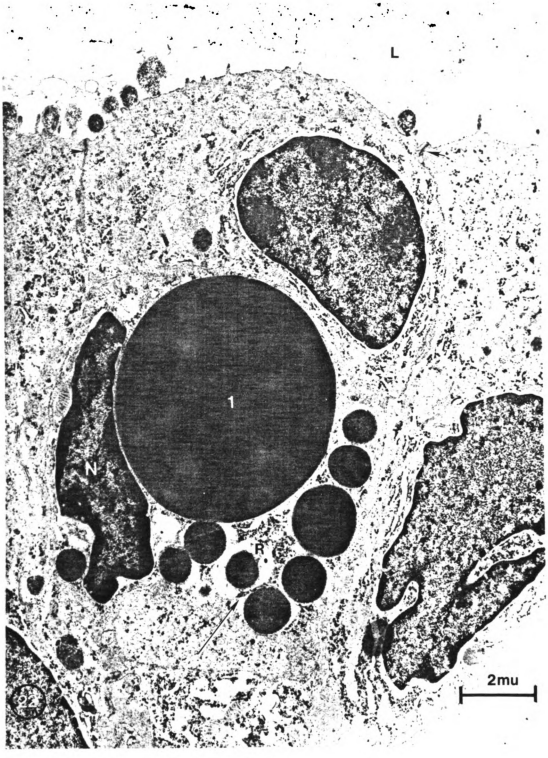
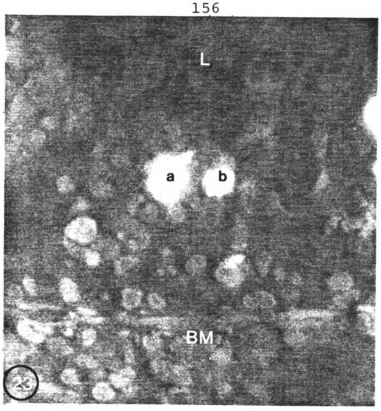

L
1
N
R
2mu

Figure 23. Liquid formaldehyde fixed (6 percent), Spurr embedded, HCl vapor treated tracheal section, 1 mu. The two fluorescent epithelial cells (a and b) are identical to the cells demonstrated at the ultrastructural level in Figure 24. Lumen (4), Basement membrane (BM). (2000X)

Figure 24.--Globular leukocyte within the tracheal epithelium of the adult rat. The cells observed at the ultrastructural level are identical to those observed at the light microscopic level. Formaldehyde (6 percent), uranyl acetate and lead citrate. 7,590X


L
a
b
BM

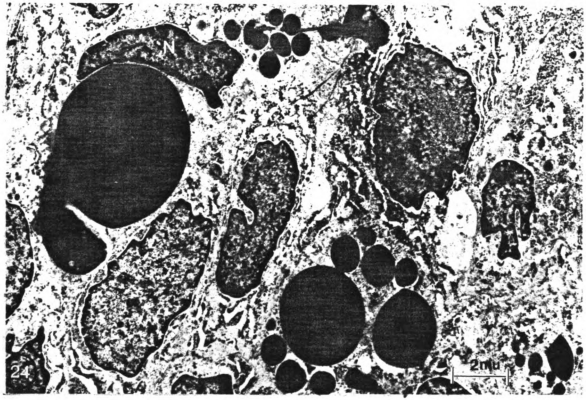
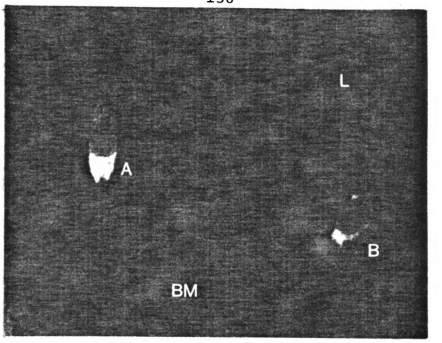
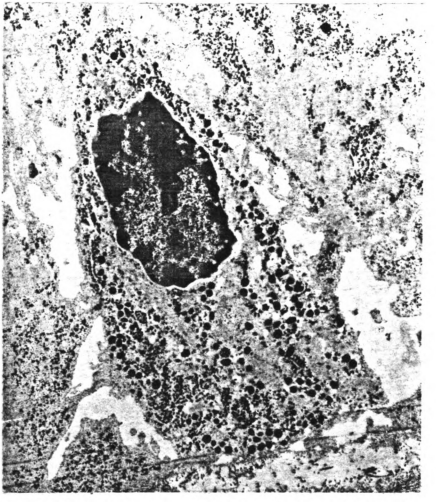

N
2
2mu

Figure 25.--Two fluorescent enterochromaffin cells (a and b) from liquid formaldehyde fixed (6%), Spurr embedded, HCl vapor treated duodenal section (upper panel), 1 mu thick (2000X). Lumen (L) and basement membrane (BM). The same enterochromaffin cell (6) at the ultrastructural level (lower panel). 6,400X


L
A
B
BM



CHAPTER V

DISCUSSION

This study has described morphologic and histochemical characteristics of a specific cell type in the laryngeal and tracheal respiratory epithelium of adult rats. The results of this study suggest that the globular leukocyte may be classified as a member of the paraneuron series of cells proposed by Fujita (1976). The essential characteristics of this concept are: (1) the cell must possess neurosecretory-like and/or synaptic vesicle-like granules; (2) the cell must produce substances identical with, or related to, neurosecretions or neurotransmitters; and (3) the cell must respond to stimuli and release its secretory product.

Secretory Granules of the Cell

The first characteristic of the paraneuron concept requires the presence of neurosecretion-like and/or synaptic vesicle-like granules.

The morphology of the fluorescent respiratory epithelial cell was similar in both freeze-dried and liquid formaldehyde fixed laryngeal and tracheal tissue sections. The cells were characterized by conspicuous fluorescent cytoplasmic granules. The granules appeared to be randomly distributed throughout

the cytoplasm. The fluorescent cells were located at all levels of the epithelium. However their most frequent location was slightly apical to the more basal, non-fluorescing epithelial cells. This description is very similar to cells described by Ewen, Bussolati, and Pearse (1972). In their study of adult rat laryngeal and tracheal respiratory epithelium, cells with very weak endogenous fluorescence were reported. It was demonstrated that these cells possessed amine-handling characteristics but not the enzymatic features of the APUD series of endocrine cells. They were unable to demonstrate argyrophilia, masked metachromasia and stainability of their intracytoplasmic granules with lead hematoxylin. At the ultrastructural level, numerous cells were described with large pleomorphic granules 5,000 to 20,000 Å in diameter. It was suggested that the fluorescent epithelial cells were identical to cells observed at the ultrastructural level. However, a direct correlation was never demonstrated.

The presence of fluorescent endocrine-like cells in the upper respiratory system has also been demonstrated by a number of investigators (Erickson et al., 1972; Cutz et al., 1975; Kirkeby et al., 1977; and Dey et al., 1981). The amine-handling properties of these respiratory endocrine-like cells were similar to those described by Ewen et al. (1972) however, their ultrastructural morphology was markedly different. Several investigators (Jeffery and Reid, 1975; and Breeze et al., 1977) have suggested that the

cell type investigated by Ewen et al. (1972) was a globular leukocyte. No direct correlation between fluorescence and ultrastructure had been attempted. In this investigation, intensely fluorescent respiratory epithelial cells have been identified and evaluated at the ultrastructural level. The ultrastructural characteristics were similar to the globular leukocyte previously described by Ewen et al. (1972), Kent (1966), and Jeffery et al. (1975). The procedures described in this report permit the simultaneous investigation of cellular amine handling properties as well as morphologic characterization at the ultrastructural level.

The globular leukocyte described in this study and in other studies (Ewen et al., 1972 and Jeffery et al., 1975) possessed the following characteristics at the ultrastructural level: large pleomorphic neurosecretion or synaptic vesicle-like granules and their cores were surrounded by a clear area and subsequently enclosed by unit membranes. From these characteristics, it appears as though the globular leukocyte may be classified morphologically as belonging to the paraneuron series of cells proposed by Fujita (1976).

The Content of the Cell

The second characteristic of the paraneuron concept requires the presence of substances identical with, or related to, neurosecretions of neurotransmitters. Thin section of freeze-dried, formaldehyde vapor-treated tissue

segments embedded in paraffin or plastic have demonstrated the presence of fluorogenic amines in adrenergic nerve terminals (Hökfelt, 1965). In addition, Partanen (1974) demonstrated numerous endogenously fluorescent cells in the hypophysis of human fetuses following freeze-drying and subsequent embedding in Epon. Following exposure to glacial acetic acid, a new population of hypophyseal fluorescent cells was observed as well as an increased in the fluorescence intensity in the cells already fluorescent. The fluorogenic substance was believed to be a peptide with NH_2-terminal tryptophan.

Eränkö (1952) demonstrated fluorogenic amines in liquid formaldehyde fixed tissue segments. In addition, fluorogenic amines have also been demonstrated in liquid formaldehyde fixed malignant melanoma cells, junctional nevus cells and normal melanocytes (Rost et al., 1969). The fluorescence excitation maxima were unaltered following exposure to hydrochloric acid vapor. The fluorescence was believed to be partly due to the presence of dihydroxyphenylalanine (DOPA), and mainly to a second substance which was not identified. Liquid formaldehyde fixation has also demonstrated fluorescent endogenous substances in antral and duodenal gastrin cells and pancreatic A_2-cells (Larsson et al., 1975a). In the same study, it was also demonstrated that liquid formaldehyde fixed, paraffin embedded tissue sections were sensitive to HCl vapor. It was suggested that the fluorogenic substance may be due to a peptide with

an NH_2-terminal tryptophan. Hoyt et al. (1979) demonstrated fluorescent cells in tissues from respiratory and gastrointestinal tracts fixed in liquid formaldehyde and embedded in glycol methacrylate. Fluorescent cells were not demonstrated in the adrenal medulla. In conjunction with several histochemical procedures, it was suggested that the fluorogenic cells contained serotonin.

The respiratory epithelial lining of the adult rat larynx and trachea contain numerous endogenously fluorescent cells following freeze-drying, formaldehyde vapor treatment and plastic embedding. In addition, endogenously fluorescent cells were observed following liquid formaldehyde fixation, plastic embedding and subsequent exposure to hydrochloric acid vapors. The morphologic and cytochemical characteristics were similar to globular leukocytes described in the respiratory and gastrointestinal epithelium of adult rats (Kent, 1966). The function and cytochemistry of this cell have not been identified.

Nature of the Fluorophore

The basis for the detection of biogenic β-phenylethylamines (catecholamines) and β-(3-indolyl) ethylamines in the Falck-Hillarp method is their transformation into intensely fluorescent dihydroisoquinoline or dihydro-β-carboline derivatives respectively (Corrodi and Jonsson, 1967). The first step in the reaction between the monoamines

and formaldehyde is a Picket-Spengler cyclization reaction. This results in the formation of a non-fluorescent tetrahydro-isoquinoline or a tetrahydro-β-carboline molecule. The fluorophore formation occurs during the second step of the reaction when the non-fluorescent tetrahydro derivatives are dehydrogenated to fluorescent 3,4-dihydro compounds (Corrodi and Jonsson, 1967). Björklund et al (1973) demonstrated that in the case of indolylethylamines the dehydrogenation reaction could proceed in either of two ways: through an autooxidation to 3,4-dihydro-β-carbolines, or through a second acid-catalyzed reaction with formaldehyde to yield 2-methyl-3,4-dihydro-β-carbolinium compounds

The specific nature of the cellular fluorophore in both freeze-dried and liquid formaldehyde fixed laryngeal, tracheal, and duodenal tissue segments embedded in glycol methacrylate was evaluated by several histochemical tests. The first test demonstrated that the fluorescence was the result of formaldehyde vapor. Fluorescent cells were not present in freeze-dried plastic embedded laryngeal and tracheal tissue segments that were not treated with formaldehyde vapor. This is considered a specific test for the demonstration of biogenic amines in tissue sections.

The second test demonstrated that the fluorophore was sensitive to ultraviolet light. Following exposure to ultraviolet light, fluorogenic amines demonstrate photo-decompensation but autofluorescent structures do not.

Freeze-dried and liquid formaldehyde laryngeal and tracheal fluorescent cells exposed to ultraviolet light demonstrated a distinct loss of fluorescent intensity.

Corrodi et al. (1964) demonstrated that fluorogenic monamine compounds could be reduced to their nonfluorescent derivatives following exposure to sodium borohydride. Reoxidation to fluorescent derivatives will occur following subsequent exposure to formaldehyde vapor. In this study, freeze-dried and liquid formaldehyde fixed laryngeal and tracheal fluorescent epithelial cells were quenched by sodium borohydride and subsequently regenerated after formaldehyde vapor treatment and formaldehyde vapor followed by HCl vapor respectively. These results suggest that the cellular fluorogenic substance is a biogenic amine.

The histofluorescence method of Falck and Hillarp (1962) permits the precise cellular demonstration of 3-hydroxylated β-phenylethyl amines (e.g., catecholamines) and β-indolyl-ethylamines (e.g. serotonin) (Corrodi and Jonsson, 1967; Jonsson, 1967). In addition, formaldehyde vapor forms weakly yellow fluorescence of substances such as tryptamine, tryptophan, and peptides with NH_2-terminal tryptophan which are chemically α-substituted tryptamines. (Björklund et al., 1968; Larsson, et al., 1975a, 1975b). In model experiments, it has also been demonstrated that histidine, tyrosine, indoleacetic acid and peptides lacking NH_2-terminal tryptophan did not fluoresce following formaldehyde vapor exposure (Larsson et al., 1975b). The weak fluorescence intensity of

tryptamines, tryptophan, as well as the nonfluorescent indoleacetic acid in model and tissue sections can be increased or induced to fluoresce by introduction of ozone or hydrochloric acid into the reaction vessel with formaldehyde vapor or following formaldehyde vapor treatment (Björklund et al., 1968, 1970, 1971; Larsson et al., 1975a, 1975b; and Partanen 1974, 1976). The increase in the fluorescence intensity of tryptamines, tryptophan and indoleacetic acid following exposure to ozone or hydrochloric acid following exposure to ozone or hydrochloric acid was also accompanied by a simultaneous decreased in the fluorescence intensity of catecholamines (Björklund et al., 1971).

In this study, a few scattered fluorescent cells were present in the tracheal and laryngeal respiratory epithelium of the adult rat when processed according to the method of Falck and Hillarp (1962). Fluorescent mast cells, known to contain serotonin (Ritzén, 1966), were present in the submucosae of the respiratory and gastrointestinal tracts. In addition, numerous duodenal enterochromaffin cells, also known to contain serotonin (Barter et al., 1955), were intensely fluorescent. Tissue segments that were fixed with liquid formaldehyde did not demonstrate any endogenous fluorescent cells in the respiratory epithelium. However, connective tissue mast cells in trachea and duodenum as well as enterochromaffin cells were fluorescent.

Numerous, intensely yellow fluorescent cells were observed in liquid formaldehyde fixed tissues following exposure of laryngeal and tracheal sections to fumes of concentrated HCl. The fluorescence intensity of mast cells as well as duodenal enterochromaffin cells also increased. Subjectively, it appeared that the number and intensity of freeze-dried and glycol methacrylate embedded laryngeal and tracheal epithelial cells did not appear to increase following exposure to the fumes of hydrochloric acid. The fluorescence intensity of mast cells (tracheal and gastrointestinal) and duodenal enterochromaffin cells was increased following exposure to HCl vapor.

Liquid formaldehyde fixed and freeze-dried tissue segments treated with formaldehyde vapor in the presence of ozone did not demonstrate an increase in the number or intensity of fluorescent epithelial cells. The presence of histamine in the respiratory epithelium was not demonstrated.

Microspectrofluorimetry

In the respiratory epithelial lining of the adult rat larynx and trachea, endogenously fluorescent cells have been demonstrated with excitation and emission maxima at 400 and 490 nm respectively (Ewen et al., 1972). This study confirms the presence of endogenously fluorescent cells in the larynx and trachea of adult rats. In this study, tissue prepared according to the standard Falck-Hillarp technique

and evaluated cytospectrofluorimetrically showed that the fluorescent epithelial cells had emission maxima at 493 nm ± 3. The excitation wavelengths were not recorded because they were below 350 nm. Wavelengths shorter than 350 nm require a quartz optical system rather than glass. These results were markedly different from those reported for catecholamines (410/480 nm) and for serotonin (395-410/520-540 nm) (Corrodi et al., 1967; Björklund et al., 1968). The emission peak was similar to that reported for tryptamine (Caspersson et al., 1966; and Björklund et al., 1968). A comparison of the excitation peaks was impossible due to the limitations of the microspectrofluorimeter. However, it was interesting to note that the excitation peak for tryptamine has been reported to be 360-370 nm (Björklund, et al., 1968). Spectral recordings from tracheal mast cell suggested the presence of serotonin (excitation/emission maxima: 401 nm ± 11/515 nm ± 5).

Microspectrofluorimetric analyses of freeze-dried, plastic embedded tissues have not been reported prior to this study. Spectral curves from liquid formaldehyde-fixed, HCl-treated paraffin embedded tissues have been reported to be similar to tissues prepared according to the standard freeze-dried method of Falck and Hillarp (Rost et al., 1969). In this study, laryngeal and tracheal segments prepared by liquid formaldehyde fixation, plastic embedding and exposed to the fumes of concentrated hydrochloric acid exhibited

excitation and emission spectra of 456 ± 5 nm and 535 ± 13 nm. Model experiments have demonstrated that the spectral characteristics of dopamine, after exposure to HCl, displayed a shift in the excitation maxima from 410 nm to 365 nm with a simultaneous decrease in fluorescence intensity (Björklund, et al., 1970; 1971). In addition, the excitation peak of both adrenalin and noradrenalin also show a shift to shorter wavelengths, 410 nm to 330 nm (Björklund et al., 1970; 1971) with a simultaneous decrease in fluorescence intensity following exposure to HCl vapor. Serotonin demonstrated either no change or only a slight decrease in the excitation maxima. Following exposure to HCl vapor, the excitation maxima of the fluorescent respiratory epithelial cells described in this study were similar to that for tryptophan, tryptamine, indoleacetic acid and tryptophan-containing peptides (Björklund et al., 1968; and Larsson et al., 1975b). The emission wavelength maxima were somewhat longer than many reported values. However, Ewen et al. (1972a) did report emission maxima of tryptamine models at 540 nm following exposure to acetic acid.

In summary, it has been demonstrated that the fluorophore in the laryngeal and tracheal respiratory epithelial cells of this study showed the criteria (Corrodi et al., 1967) for a specific amine fluorescence. The fluorophore: (1) was induced by exposure to formaldehyde vapor; (2) faded following irradiation with ultraviolet light; (3) was quenched by

sodium borohydride and subsequently regenerated following exposure to formaldehyde vapor and hydrochloric acid. In addition, the microspectrofluorimetric characteristics of the fluorophore were similar to those of tryptamine or a peptide with NH_2-terminal tryptophan as judged by the following criteria (Björklund et al., 1968); (1) fluorescence was present following the histochemical method of Falck and Hillarp; (2) the fluorescence emission maxima after conventional formaldehyde vapor were similar; (3) the excitation maxima were somewhere below 350 nm after conventional formaldehyde vapor treatment; (4) the fluorescence intensity was markedly increased following acidification; and (5) the excitation and emission spectra were similar following acidification. Amine content within these cells was also supported by the histochemical finding that ferric-ferricyanide positive cells were identical to the fluorescent cells. All of these findings strongly suggest that the fluorescent laryngeal and tracheal cell in respiratory epithelium contains a biogenic amine which may be tryptamine, a peptide with NH_2-terminal tryptophan, or a closely related β (3-indolyl) ethyalmine.

These results fulfill the second criterion of the paraneuron concept proposed by Fujita (1976) in that the globular leukocytes probably contain a neurotransmitter-like substance.

Stimulus-Secretion Coupling

The final criterion for a cell to be classified as a paraneuron requires that the cell respond to stimuli and release its contents. The globular leukocyte is a cell with a wide distribution in the digestive (Murray et al., 1968; and Miller et al., 1971, 1972) and respiratory (Kent, 1966) systems of animals. Globular leukocytes have also been observed in the respiratory system of humans (personal communication Sorokin and Hoyt, 1980). The functional significance of globular leukocytes in the respiratory system has not been studied extensively. However, Echt et al. (1980) have reported that rats exposed to carbon monoxide (250 ppm CO for 6 hours a day, 5 days per week from birth to 6 weeks of age) resulted in a significant decrease in the number of detectable fluorescent cells when compared to control animals. The fluorescent cell population responding to CO induced hypoxia may also have included globular leukocytes. This suggestion is based on the morphologic and cytochemical characteristics reported in this investigation. The function of the globular leukocyte in the gastrointestinal tract has also been postulated in relation to parasitic infections (Murray et al., 1968; and Miller et al., 1971, 1972). Following parasitic infection it has been shown quantitatively that subepithelial mast cells and epithelial globular leukocytes increase in number and probably release

their contents (biogenic amines) in response to an antigen (Jarrett et al., 1968). It was suggested that these biogenic amines may increase the permeability of the mucosa and allow circulating or locally produced antibodies access to antigen (Murray et al., 1968: Miller et al., 1971, 1972). Kirkman (1950), however, has demonstrated numerous globular leukocytes in the tracheobronchial epithelium of pathogen-free rats.

The physiologic role of globular leukocytes in the tracheal respiratory epithelium as a mediator of epithelial function remains speculative. Despite the fact that the exact stimulus secretion response has not been identified, it is apparent that the globular leukocyte does respond to physiologic stimuli. This would suggest that the final criterion is satisified and the globular leukocyte should be classified as a paraneuron.

Secondary Characteristics

In addition to the three essential characteristics previously defined, paraneurons also possess secondary characteristics to some degree (Fujita, 1976). The histochemical results in this study support the hypothesis that the globular leukocyte should be classified in the paraneuron series of cells. Furthermore, additional histochemical procedures were evaluated to further understand the biochemical nature of the globular leukocyte.

Histochemistry

In addition to the presence of an amine, the cytoplasmic granules also contain acid mucopolysaccharides. The presence of acid mucopolysaccharides in the granules was confirmed by staining with methylene blue and alcian blue. Bennett et al. (1976) demonstrated that methylene blue staining selectively displayed C-cells of the thyroid and endocrine cells in the intestinal and stomach epithelium. Fujita et al. (1968) suggested that basic dyes may stain a glycolipoprotein which is contained within the granules of endocrine cells. It was further suggested that the acidic glycolipoprotein acts as a carrier protein for the basic amines. The presence of acid mucopolysaccharides in globular leukocytes of the gastrointestinal tract has been previously reported by Murray et al. (1968) and Miller et al. (1972). It has been suggested that the globular leukocytes may arise from subepithelial mast cells (Murray et al., 1968; and Miller et al., 1972). However, the acid mucopolysaccharides content of the globular leukocyte is somewhat different from that of the connective tissue mast cell. The globular leukocyte stains metachromatically with basic dyes at pH 4 but not at pH 0.3 while the connective tissue mast cell stains metachromatically at pH 4 and at pH 0.3 (Cantin et al., 1972). These results could be accounted for by the fact that rat connective tissue mast cells contain heparin, a sulfated acid mucopolysaccharide, (Lillie and Fullmer, 1976) whereas

the metachromatic staining of the epithelial globular leukocyte may be due to the presence of a glycolipoprotein.

The periodic acid-Schiff (PAS) reaction is useful for localizing glycogen, glycoproteins and/or neutral mucopolysaccharides. The PAS procedure is a two step reaction: (1) 1,2-glycols are oxidized by periodic acid into aldehydes, and (2) the aldehydes formed are visualized by staining with Schiff's reagent. In this study, the fluorescent cells were also PAS-positive. It has previously been demonstrated that a fluorescent substance is stored with PAS-positive endocrine cells known to produce follicle stimulating hormone (FSH), luteinizing hormone (LH), thyrotrophic hormone (TSH), and melanocyte stimulating hormone (MSH) (Dahlström and Fuxe, 1966). Subsequently, Partanen (1974) suggested that the fluorogenic substance may be a peptide with NH_2-terminal tryptophan. Furthermore it was proposed that the peptide may be involved in the storage and/or release of protein hormones. The PAS-positive reaction demonstrated in the globular leukocytes was not observed in the connective tissue mast cell. This would again suggest significant biochemical alterations if the globular leukocytes were derived from the connective tissue mast cell.

The presence of the enzyme monoamine oxidase (MAO) in the laryngeal and tracheal respiratory epithelium of adult rats was demonstrated by the method of Glenner et al. (1957) in fresh cryostat sections. MAO oxidizes primary,

secondary and tertiary monoamines with the formation of either lower amines or aldehydes. It does not oxidize histamine, for which histaminase is necessary (Chayen et al., 1973). In control slides of kidney, a positive reaction product was demonstrated. In addition, tracheal and laryngeal sections that were incubated without the substrate serotonin also failed to give reactions for monoamine oxidase. These results demonstrated that monoamine oxidase was present in the respiratory epithelium of the larynx and trachea.

The enzyme acid phosphatase is associated almost exclusively with lysosomes (Humason, 1972). This enzyme was not present in the granules of the tracheal epithelial globular leukocyte. These results are identical to those reported by Kirkman (1950) in the globular leukocyte of the adult rat urinary tract. However, these observations are different than those reported by Kent (1952) in the globular leukocyte of sheep intestine. These differences may reflect species or functional differences of the globular leukocyte in the animals studied. These histochemical results suggest that significant biochemical alterations would be necessary for the globular leukocyte to arise from the connective tissue mast cell since the mast cell demonstrated intense acid phosphatase activity.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

In an attempt to provide a conceptual framework for the diffuse endocrine-like cell system, Pearse (1969) proposed the Amine Precursor Uptake and Decarboxylation Concept (APUD). This concept was based on embryological and histochemical characteristics. In order to extend this conceptual framework, Fujita (1976) proposed the paraneuron concept which was based on morphologic, embryologic, and physiologic characteristics. In 1979, Fujita and Kobayashi eliminated the embryologic characteristic from their paraneuron concept.

The first requirement for membership in the paraneuron series of cells is the presence of neurosecretion-like and/or synaptic vesicle-like granules. The present investigation demonstrated at the ultrastructural level that the fluorescent laryngeal and tracheal respiratory epithelial cells has previously been described by Kent (1966), Ewen et al. (1972), and Jeffery et al. (1975). These cells were irregularly shaped and were assumed to be identical to fluorescent cells in tissue prepared according to the

standard Falck-Hillarp technique. The ultrastructure of these large granulated cells was markedly different from other amine-containing cells of the tracheal epithelium (Ericson et al., 1972, and Cutz et al., 1975). This study demonstrated that the fluorescent tracheal epithelial cell was indeed a globular leukocyte. The granules in the globular leukocyte were similar in size and structure to connective tissue mast cells which are currently classified by Fujita and Kobayashi as belonging to the paraneuron series of cells. In view of the results of this study, it appears that the morphologic criteria of the paraneuron concept are satisfied by the laryngeal and tracheal gobular leukocyte.

The second essential criterion of the paraneuron concept requires that the cell produce a substance identical with, or related to neurosecretions or neurotransmitters. Amine containing cells in the laryngeal and tracheal epithelium of adult albino rats were investigated by morphological and histochemical methods. Endogenously fluorescent cells were present in the extrapulmonary airways following freeze-drying, formaldehyde vapor treatment and subsequent embedding in paraffin or plastic. In addition, endogenously fluorescent cells were present after liquid formaldehyde fixation, plastic embedding and subsequent exposure to the fumes of concentrated hydrochloric acid. The fluorogenic substance contained within the granules of the globular leukocyte demonstrated the requirements for a specific monoamine fluorescence in that: (1) it was induced

by exposure to formaldehyde vapor; (2) it faded following irradiation with ultraviolet light; (3) it was quenched by sodium borohydride and subsequently regenerated following exposure to formaldehyde vapor and hydrochloric acid. The characteristics of the fluorophore were similar to that of tryptamine, or a peptide with NH_2-terminal tryptophan as judged by the following criteria: (1) fluorescence was present following the histochemical method of Falck and Hillarp; (2) the fluorescence emission maxima after conventional formaldehyde vapor were similar; (3) the excitation maxima were somewhere below 350 nm after conventional formaldehyde vapor treatment; (4) the fluorescence intensity was markedly increased following acidification; and (5) the excitation and emission spectra were similar following acidification. All of these findings strongly suggest that the fluorescent laryngeal and tracheal cells in respiratory epithelium contains a biogenic amine with may be tryptamine, a closely related β (3-indolyl) ethylamine, or a peptide with NH_2-terminal tryptophan and therefore fulfills the second requirement of the paraneuron concept.

The third and final criterion necessary for a cell to belong to the paraneuron series requires that the cell respond to stimuli and release its contents. Although this requirement was not directly addressed in this investigation, it has been demonstrated in this lab (Echt et al., 1980) that the number of detectable fluorescent cells in rat

tracheal epithelium decreased significantly following chronic exposure to carbon monoxide. This would suggest the possibility of a stimulus-secretion response. Further investigation will be necessary to determine if this cell does indeed secrete its content in response to adequate stimuli.

Histochemical investigations provided additional information regarding the biochemical nature of the respiratory epithelial globular leukocyte. The fluorescent laryngeal and tracheal epithelial cells were correlated with histochemical reactions by photographing with fluorescent microscopy, staining by various histochemical procedures, and finally rephotographing the same section by light microscopy. The fluorescent cells were demonstrated to be methylene blue, alcian blue, peridic acid-Schiff, and ferric-ferricyanide positive. However, lysosomal enzymes were not demonstrated within the granules of the globular leukocyte. In addition, the enzyme monoamine oxidase was present in the epithelium of the adult rat trachea.

In conclusion it appears as if the globular leukocyte of the rat laryngeal and tracheal respiratory epithelium should be classified as a paraneuron cell because it contains neurosecretion-like granules, neurosecretion and/or neurotransmitter-like substance, and probably responds to stimuli with release of its secretory product.

Recommendations

1. Globular leukocytes should be isolated in vitro and the content of the cells evaluated by biochemical methods.

2. Additional microspectrofluorimetric recordings of tryptamine standards and related compounds should be undertaken.

3. The spectral properties of the globular leukocyte should be evaluated with a special quartz objective system to determine the excitation maxima of tissue prepared according to the standard Falck-Hillarp technique.

4. The amine-containing globular leukocyte should be examined in animals subjected to experimental conditions affecting respiratory function.

5. The origin of the globular leukocyte should be further investigated.

APPENDICES

APPENDIX A

HISTOCHEMICAL PROCEDURES

APPENDIX A_1

Lee's Methylene Blue-Basic Fuchsin (Bennett et al., 1976)

Stock Solutions

1. 4×10^{-3}M methylene blue

   .128g methylene blue

   100 ml distilled water

2. 4×10^{-3}M basic fuchsin

   .129g basic fuchsin

   100 ml distilled water

3. .2M phosphate buffer

Staining Solution

12 ml methylene blue

12 ml basic fuchsin

21 ml .2M phosphate buffer

15 ml 95% ethanol

Procedure

1. Filter solution after mixing
2. Immerse slides in stain for 10 to 15 seconds
3. Remove slides, dip briefly into distilled water, dry on a hotplate, and mount in Entellan.

APPENDIX A_2

Ferric-Ferricyanide (Lillie and Burtner, 1953)

Stock Solutions

1. 1% potassium ferricyanide

   1g potassium ferricyanide (Baker)

   100 ml distilled water

2. 1% ferric chloride

   1g ferric chloride

   100 ml distilled water

Staining Solution

10 ml 1% potassium ferricyanide

75 ml 1% ferric chloride

15 ml distilled water

Procedure

1. Immerse slides in staining solution for 30 minutes. Prepare fresh solution every 15 minutes to avoid precipitate.
2. Rinse in distilled water.
3. Dry slides at 37°C and mount in Entellan.

APPENDIX A_3

Alcian Blue (Luna, 1968)

Stock Solutions

1. 3% aqueous acetic acid

   3 ml glacial acetic acid

   97 ml distilled water

2. 1% alcian blue solution

   3 ml glacial acetic acid

   1g alcian blue

   97 ml distilled water

Procedure

1. Immerse slides in 3% acetic acid for 30 minutes.
2. Rinse in distilled water for 15 seconds.
3. Stain in 1% alcian blue solution for 5 to 8 hours at 60°C.
4. Rinse for 2 minutes in distilled water, dry overnight, and mount in Entellan.

APPENDIX A_4

PAS-Lead Hematoxylin
(Sorokin and Hoyt, 1978)

Stock Solutions

1. .5% periodic acid

   .5g periodic acid

   100 ml distilled water

2. Schiff's reagent

   2.0 gm basic fuchsin

   5.7 gm sodium metabisulfite

   255 ml distilled water

   45 ml in HCl

3. saturated aqueous ammonium acetate

   500 gm ammonium acetate

   500 ml distilled water

4. lead acetate solution

   5 gm lead nitrate

   100 ml distilled water

   100 ml saturated aqueous ammonium acetate

5. lead hematoxylin

   0.6 gm hematoxylin

   4.5 ml 95% ETOH

   210 ml distilled water

   30 ml stock 2.5% lead acetate solution

Staining Solution

1. .5% periodic acid
2. Schiff's reagent
   a. dissolve basic fuchsin in hot water (boiling)
   b. cool
   c. add HCl
   d. add bisulfite
   e. store in dark 48 hours
   f. add 5 gm activated charcoal and filter
3. Lead acetate solution
   a. dissolve 5 gm lead nitrate in 100 ml H_2O
   b. add 100 ml saturated aqueous ammonium acetate
   c. stir
   d. filter
   e. add 4 ml 40% formaldehyde
4. lead hematoxylin
   a. make fresh every hour to avoid precipitate
   b. dissolve hematoxylin (.6 gm) in ethanol (4.5 ml)
   c. add lead acetate (30 ml)
   d. add distilled water (30 ml)
   e. shake well and wait 30 minutes
   f. add remaining distilled water (180 ml)
   g. filter through 3 pieces of Whatman #4 filter paper
   h. filtrate should be colorless

Procedure

1. Immerse slides in periodic acid for 3 hours.
2. Rinse in tap water (10 minutes).
3. Stain in Schiff's reagent (15 minutes).
4. Rinse in running tap water (10 minutes).
5. Drain slides and dry (12 hours).
6. Stain in lead hematoxylin (1-3 hours).
7. Rinse thoroughly in running water (15 minutes).
8. Dehydrate and mount in Entellan.

APPENDIX A_5

Acid Phosphatase (Higuchi et al., 1979)

Stock Solution

1. 10% magnesium chloride

   1g magnesium chloride

   10 ml distilled water

Staining Solution

5 mg naphthol AS-BI (Sigma)

.5 ml dimethyl formamide

50 ml .1M acetate buffer (pH 5.2)

10 mg fast red violet LB salt

2 drops 10% magnesium chloride

Procedure

1. The staining solution was filtered and the sections were placed in the filtrate.
2. The sections were stained for 6 to 24 hours.
3. Remove slides, dip briefly into distilled water, dry on a hotplate (37°C), and mount in Entellan.

APPENDIX A_6

Monoamine Oxidase
(Glenner et al., 1957)

Staining Solution

37.5 mg serotonin

7.5 mg nitro blue tetrazolium

7.5 ml phosphate buffer (0.1M)

6 mg sodium sulfate

Procedure

1. Frozen sections (15 mj thick) were cut and attached to slides.
2. The sections are then immersed in preheated (37°C) substrate (pH 7.6) for 30 minutes.
3. The sections are then washed for 5 minutes in running water and mounted in glycerol gelatin.

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