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Hypoxia-sensitive Neuro-epithelial Bodies Intrapulmonary Secretory Neuroreceptors, Modulated by the CNS

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Summary. In an attempt to further our knowledge about the structure and function of the recently identified intrapulmonary Neuro-epithelial Bodies (NEB's) (Lauweryns *et al.*, 1972a, 1972b), lungs of 84 neonatal rabbits and 6 neonatal mice were studied along four different lines of investigation. Several routine and silver staining methods, Falck's fluorescent amine technic and histochemical and electron microscopical techniques were performed.

1. In order to test the probable chemoreceptor function of the NEB's, animals were exposed to hypoxia. Under such circumstances, the corpuscular cells of the NEB's secrete their dense-cored, serotonin-containing vesicles at their basal vascular pole. 2. After reserpine pretreatment, the NEB's of otherwise normal animals reveal a distinct amine depletion, the corpuscular cells exhibiting a decreased yellow fluorescence and ultrastructurally a clearing up of their dense-cored vesicles. 3. Studied on serial sections with the electron microscope, various types of morphologically afferent-like and efferent-like nerve endings, making contact as well with the corpuscular cells as amongst themselves have been detailed. 4. Cytochemically the corpuscular cells react positively with alpha-glycerophosphate dehydrogenase, acetylcholinesterase and Solcia's lead hematoxylin stain for endocrine cells producing polypeptides and amines.

It is proposed that the NEB's provide an intrapulmonary, hypoxia-sensitive neuro(chemo-) receptor system in addition to the well established central and peripheral (e.g. carotid body) chemoreceptors. They contain and secrete serotonin and probably also related amines or peptides, which could influence the pulmonary vasoconstrictor response. According to classic morphologic criteria, they possess a dual innervation, both afferent and efferent.

Various other possible functions of the NEB's in normal and diseased lungs are briefly proposed.

Key words: Lung (mammals) — Chemoreceptors — Neuro-epithelial Bodies (NEB's) — Hypoxia — Light, Fluorescence, Electron microscopy, Histochemistry — Respiratory mucosa (innervation).

Introduction

The possibility that intrapulmonary air chemoreceptors, in addition to the well known central and peripheral (e.g. carotid body) chemoreceptors play a role in the regulation of the lungs has been an intriguing but unanswered problem for the past years (Comroe, 1964; Dejours, 1962; Fishman, 1960). Normal intrapulmonary air chemoreceptors had not been identified histologically by 1971

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(Ichinose *et al.*, 1971), even though physiologic evidence of their presence was available (Dawes *et al.*, 1954). It was indeed well established that hypoxia causes a pulmonary vasoconstriction with the aid of an intrapulmonary receptor (Daly *et al.*, 1966; Laros, 1971). A major influence of the central nervous system (CNS), the arterial pH and lactic acid on this system was excluded (Fishman, 1964; Hauge, 1969; Lloyd, 1966, 1967, 1968; Naeye, 1965), while serotonin could be mediating it (Sjoredsma, 1959).

We have recently identified in the mammalian lung (including man) (Lauweryns *et al.*, 1972a, 1972b, 1973), the occurrence throughout the intrapulmonary airways of intramucosal corpuscles or so-called "Neuro-epithelial Bodies" (NEB's) composed of argyrophil, argentaffin, yellow fluorescent, ultrastructurally granulated and innervated epithelial cellular organs. We proposed this innervation to be probably afferent as well as efferent, though we were only able to demonstrate the efferent type. We postulated them to be intrapulmonary chemo-, stretch-, baro- or tactile neuroreceptor organs modulated by the central nervous system which exhibit local secretory activities. We proved indeed that these corpuscles contained serotonin, probably amongst other related amines or peptide substances.

Several aspects about the structure and function of these NEB's remaining thus unsolved, the present study was undertaken (1) to investigate their reaction to hypoxia as related to their probable chemoreceptor function, (2) to test the influence of an amine-depleting agent, reserpine, upon their normal fluorescence and ultrastructure, (3) to study furthermore the fine structural nature of their innervation, and of (4) some common cytochemical properties (alpha-GPD: alpha-glycerophosphate dehydrogenase; AChE: acetylcholinesterase) of cells producing polypeptide hormones (Pearse, 1968).

Material and Methods

In this study the lungs of 84 neonatal rabbits and 6 neonatal mice have been investigated. They were killed by a lethal dose of intraperitoneally injected sodium pentobarbital (Nembutal®), with the exception of the animals submitted to hypoxic air which were decapitated within their airlocked cages.

1. *Hypoxic Experiments.* Eighteen neonatal term rabbits (1–2 days old) have been aerated, two by two, within an airlocked cage for a variable time interval under various concentrations of oxygen, as indicated in table 1. The oxygen concentration was continuously monitored and kept constant with a Beckmann Oxygen Analyzer 7.700 through controlled inlets of oxygen and nitrogen.

For light microscopy the tissues were immediately fixed in Bouin's fluid, embedded in paraffin, serially sectioned and stained with the usual techniques. Argyrophilia was detected according to Bodian's silver proteinate technique as modified by Van Campenhout (1951) and Grimelius' silver nitrate technique (1968), and argentaffinity according to Fontana-Masson's technique. Tissues were also investigated with the histochemical fluorescent amine technique according to Falck *et al.* (1965). They were quenched in liquid nitrogen and lyophilization performed for four days at temperatures increasing from –80° C to +30° C. Afterwards the tissues were condensed for one hour with paraformaldehyde vapor with a relative humidity of 47% at 80° C. After embedding in paraffin, 7–10 μ sections were cut and mounted in Entelan to be investigated with a Zeiss fluorescence microscope. (HBO 200 W/4 bulb, BG 12, UG 1 activating filters, 47/53 barrier filters). When NEB's occurred, photographs were taken of the same area by UV light alone, combined UV light and phase contrast, phase contrast alone and plane light alone. These proved to be very valuable for the study of the fluorescence, emitted by the NEB's.

Table 1. Number of neonatal rabbits submitted to various degrees of hypoxia during different periods of time

Number	% O_2 in N_2	Duration (in minutes)
2	5	2′
2	5	10′
2	5	20′
2	10	2′
2	10	10′
2	10	20′
2	15	2′
2	15	10′
2	15	20′

For electron microscopy, biopsies were immediately fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer pH 7.2) for 2 hours at 4°C, followed by a postfixation in OsO_4 1% (0.1 M phosphate buffer pH 7.2) during 1 hour at 4°C. After dehydration, the tissues were embedded in Epon and 1 μ sections cut and stained with toluidine blue. As the NEB's are distinctly visible on such sections, the blocks were carefully trimmed and a correlated electron microscopical investigation (EM Philips 300) carried out on the immediately adjacent ultrathin sections (200–600 Å) which had been stained with uranyl acetate and lead citrate (Reynolds, 1963).

2. *Reserpine Pretreatment.* 6 neonatal term rabbits received two intramuscular injections of 5 mg/kg reserpine (Serpasil®) with a 24 hours interval and were killed one day after the second injection.

These lungs were studied with the same techniques as performed on the hypoxic animals (see item 1).

3. *Fine Structural Nature of the Innervation.* The material included the 18 lungs of the animals submitted to hypoxia (see item 1) and of 36 normal and untreated neonatal term rabbits (1–21 days old) which had served as controls for all experiments.

The method applied consisted of an electron microscopical investigation, carried out as mentioned above (see item 1), including numerous ribbons of serial sections. In two instances, NEB's were totally cut into sections and all sections which could be obtained, studied to trace the fine structure of the nerves throughout the corpuscles.

4. *Further Histochemical Studies.* For the light microscopical demonstration of acetylcholinesterase, lung biopsies of 6 neonatal term mice and of 6 neonatal term rabbits were quickly frozen in CO_2 ice and cut with a freezing microtome. The 15 micron thick sections were dried for 3 minutes at room temperature by an air current and incubated for AChE according to the technique of El-Badawi *et al.* (1967) using iso-OMPA (8.10^{-6} M) (tetra-isopropylpyrophosphoramide) as an inhibitor for non specific esterases.

The electron microscopical demonstration of AChE was carried out on lungs of another 6 neonatal term rabbits, according to Bloom *et al.* (1966). One hundred micron sections were cut with a Smith and Farquhar tissue chopper, preincubated in iso-OMPA (8.10^{-4} M), incubated according to Barnett (1962), postfixed in 1% OsO_4 (1 hour, 4°C, cacodylate-buffer 0.05 M pH 7.2), dehydrated and Epon embedded. The ultrathin sections were stained with uranyl acetate only.

Another group of histochemical investigations included lung biopsies of another 6 neonatal term rabbits treated with the light optical alpha-glycerophosphate dehydrogenase technique (alpha-GPD) of Pearse (1961) to which Menadion was added according to Wattenberg *et al.* (1960).

Finally, lung biopsies of another 6 neonatal term rabbits were stained with Solcia's *et al.* lead-hematoxylin stain for endocrine cells which produce polypeptides and amines (1969).

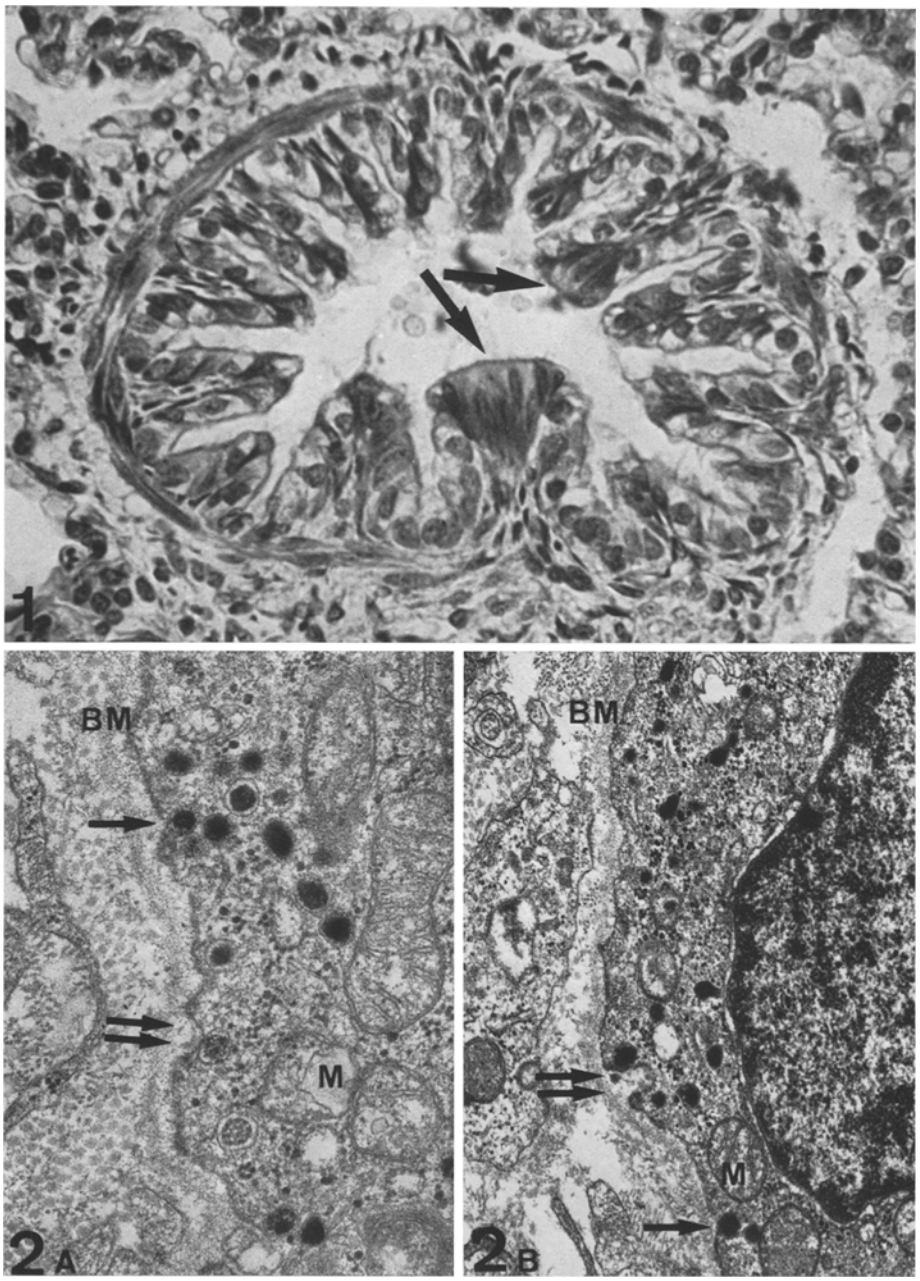

Fig. 1. Neuro-epithelial Bodies (arrow) intercalated within the bronchiolar mucosa, stained with Solcia's *et al.* lead haematoxylin stain and revealing a dark-blue to purple cytoplasmic reaction at the level of the corpuscular cells; neonatal rabbit lung; 533×

Figs. 2 and 3. Both figures illustrate various stages of the exocytosis cycle of DCV's of the granulated cells of the NEB's under hypoxic conditions; they illustrate each time a part of the basal area of the granulated cell cytoplasm (to the right of each figure), their basement

Observations

1. Effects of Hypoxia. Light optically, no difference was observed between the NEB's of the hypoxic and the control animals. In both instances the Neuro-epithelial Bodies (Fig. 1) appear as intramucosal corpuscles, composed of more or less parallely oriented, non-ciliated cylindrical cells which contain oval nuclei and a rather dark cytoplasm and reach from the basement membrane to the airway lumen. They display a selective and prominent cytoplasmic argyrophilia, a less pronounced argentaffinity and an intense yellow fluorescence with Falck's technique.

Ultrastructurally however marked differences are observed between the NEB's of the hypoxic and the control animals. While the ultrastructure of the latter conforms to our earlier observations (Lauweryns *et al.*, 1972b, 1973), electron microscopy revealed in all animals exposed to hypoxia, a distinct and pronounced exocytosis of the dense-cored vesicles (DCV's) of the corpuscular cells at the level of the adepithelial basement membrane (Figs. 2, 3, 5). This phenomenon is only exceptionally observed in the control and normal (Lauweryns *et al.*, 1972b, 1973) animals. All classic morphological phases of the exocytosis cycle (Hubbard, 1971) are seen; besides the usual and filled or granulated DCV's dispersed throughout the cytoplasm (preferentially the subnuclear areas) of the epithelial cells of the NEB's, numerous DCV's concentrate in a much larger number than in the control animals in the vicinity of the basal cell membrane; next DCV's are observed whose membranes become fused with the cell membrane itself (Figs. 2, A, B). Consecutively they open at the level of the basal membrane with an extrusion and exocytosis of their contents into the space between the basal cell membrane itself and the basement membrane. At this stage of the secretory cell process, the vesicle may still be observed to contain a small dense core which may have become fragmented (Figs. 2B, 3A) or the vesicle may appear entirely empty (Fig. 3B). Finally, we observed areas of the basal epithelial cell cytoplasm which contained vesicles both empty and smaller than the classic DCV's and which are not seen in the normal state. They probably correspond to so-called "refilling" vesicles (Hubbard, 1971) (Fig. 3A). Besides this exocytosis, hypoxia treated animals reveal occasionally a slight and focal mitochondrial lysis and a pronounced development of the Golgi complex, which is located above the cell nucleus and forms small DCV's and many empty small cisternae (Fig. 4).

2. Effects of Reserpine Pretreatment. Light optically no difference was observed after applying routine and silver staining techniques between the NEB's of the experimental and control animals. Using Falck's histochemical fluorescent amine

membrane (*BM*) and the immediately adjoining subepithelial extracellular space; *M* mitochondrion of corpuscular cell; neonatal rabbit lungs; glutaraldehyde-fixation with post-osmification

Fig. 2. A. DCV's contacting and fusing with the basal cell membrane (arrow); exocytosis of a practically empty appearing DCV, though it still contains a fragment of its dense core (double arrow); another corpuscular DCV contacts the cell membrane at the same place; 5% O_2, 10′; 43890×. B. DCV fusing with the basal cell membrane (arrow); Exocytosis (double arrow) of a DCV which still contains small fragments of its dense core; 5% O_2, 10′; 28066×

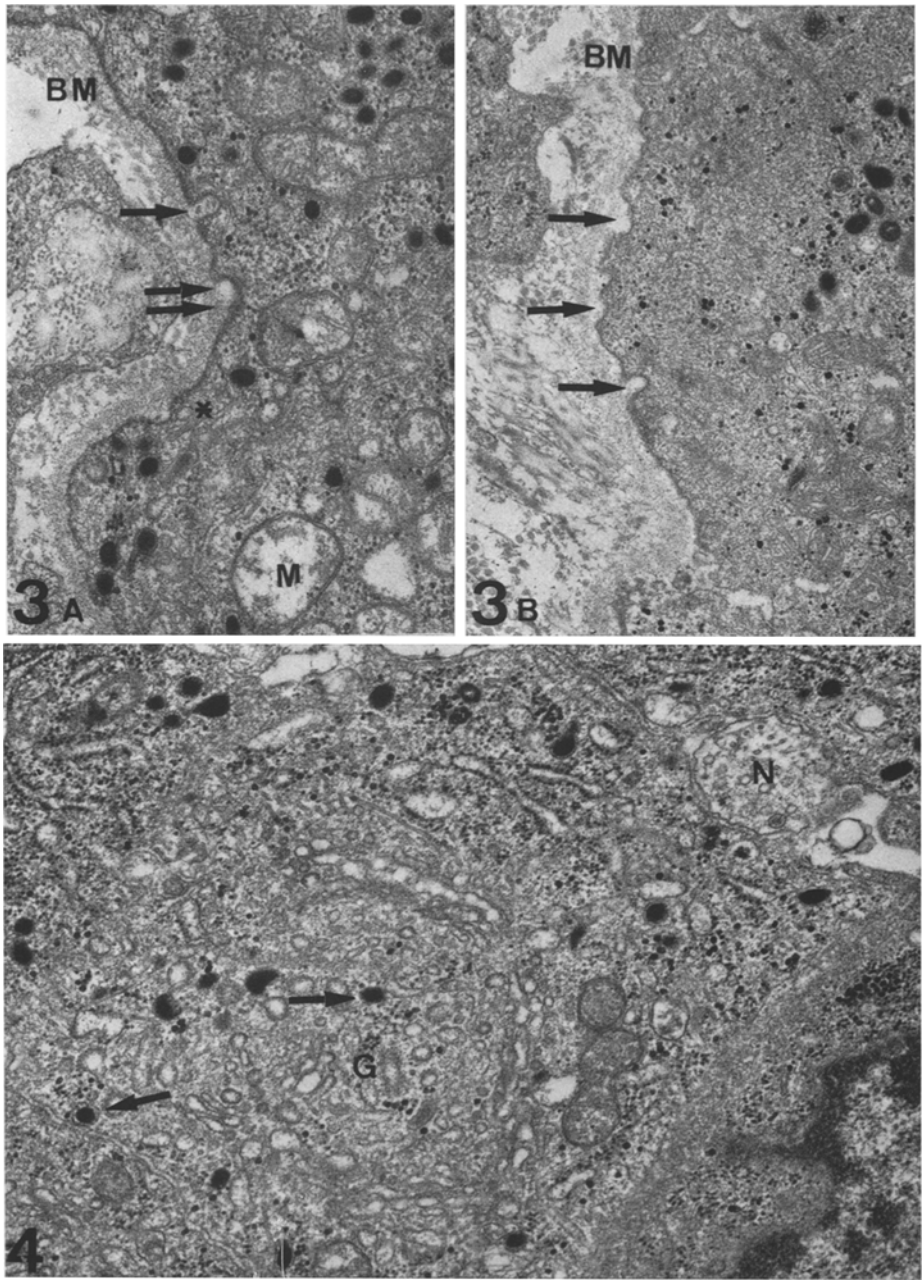

Fig. 3. A. Exocytosis of DCV's which still contain some fragments of their dense core (arrow) or which have become empty (double arrow); so-called "refilling vesicles" (*); 5% O_2; 10′; 31920.× B. Remarkably undulating basal cell membrane due to the exocytosis in the extracellular space of several DCV's, which are already empty (arrrows); the basal part of the granulated cell cytoplasm has lost most of its DCV's; this is seen after short hypoxia (15% O_2, 2′); 27132×

Fig. 4. Very active golgi zone (*G*) forming DCV's (arrow) after exposure to hypoxia; unmyelinated nerve fibre (*N*) close to the corpuscular cell; neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 5% O_2, 10′; 29087×

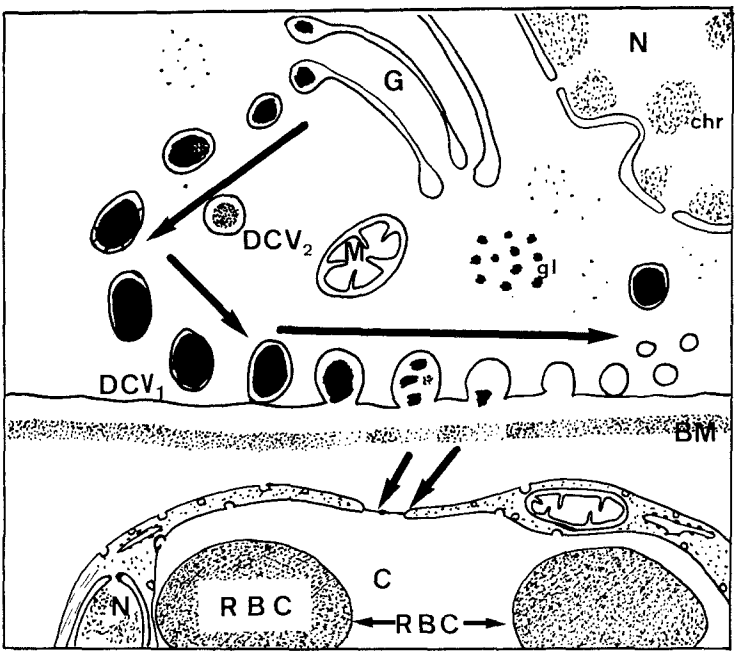

Fig. 5. Scheme illustrating the different stages observed during the presumed exocytosis cycle of the DCV's of the granulated cells of NEB's submitted to hypoxia; DCV_1 dense-cored vesicle of the first type; DCV_2 dense-cored vesicle of the second type; *BM* basement membrane; *C* capillary; *chr* chromatin; *G* Golgi system; *gl* glycogen granules; *M* mitochondria; *N* nucleus; *RBC* red blood cells; *rer* rough endoplasmic reticulum; *ser* smooth endoplasmic reticulum

technique, the NEB's of the reserpine pretreated animals, exhibited a markedly decreased cytoplasmic fluorescence—as compared to the control animals—, though in both groups the spectrum of the emitted fluorescence remained yellow (Fig. 6A, B).

Electron microscopy (Fig. 7) confirmed the amine depletion of the NEB's in the experimental series. In these animals the dense core of the corpuscular DCV's had totally cleared up in about one third of the vesicle population, leaving only a small peripheral ring or a half moon-like amount of material; in numerous other instances it became fragmented into dark-grey granules of about 30 Å. Only a few DCV's remained normal. These phenomena were not observed neither in the control group nor in our earlier observations of normal animals.

3. Fine Structural Observations of the Innervation of the NEB's (Fig. 14). Studied on serial sections, the ultrastructure of the innervation of the NEB's appears much more complex than reported in our original observations (Lauweryns *et al.*, 1972b, 1973) on single ultrathin sections. Numerous unmyelinated nerve fibres (Fig. 8) are present within the NEB's. These fibres which have no Schwann cell envelopment, are characterized by an electron-lucent axoplasm and several beaded enlargments or varicosities, which contain many characteristic small vesicles, lipofuchsine pigments, neurofibrils, neurotubuli and mitochondria. As they exhibit a variegated appearance and form numerous and

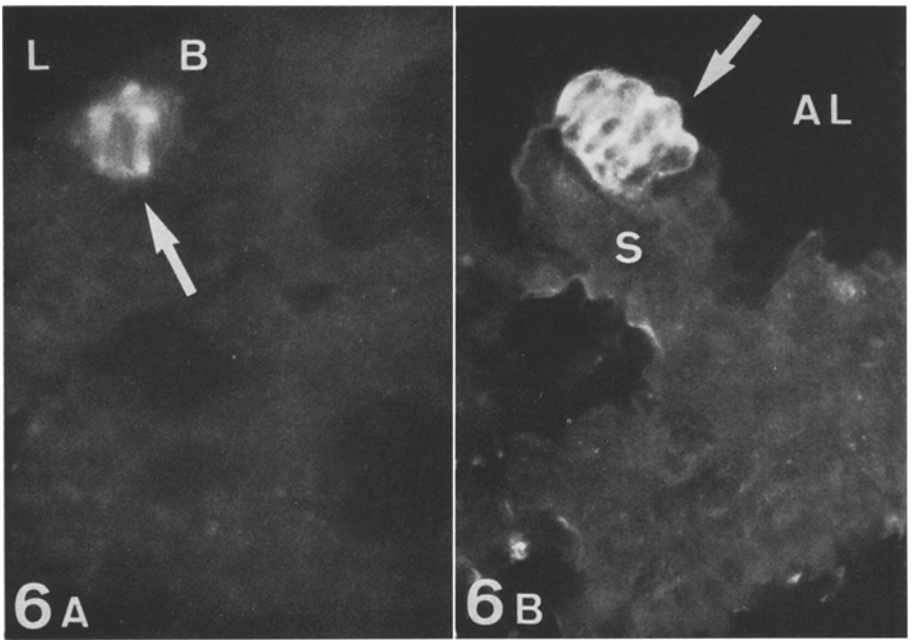

Fig. 6. A. NEB (arrow) occurring within the bronchiolar mucosa and only weakly fluorescent after reserpine pretreatment; the rest of the lung parenchyma is in focus hence clearly indicating that the poor fluorescence of the NEB is due to the reserpine pretreatment and not to bad focussing; bronchiolar lumen (*L*); bronchiolar mucosa (*B*); neonatal rabbit lung; 767×
B. NEB (arrow) of untreated control animal revealing its usual and intense cytoplasmic fluorescence; alveolar lumen (*AL*); interalveolar septum (*S*); 767×

various contacts, the description will be itemized as follows for the sake of comprehensiveness: the ultrastructural characteristics of (a) the different types of nerve endings in contact with corpuscular cells, (b) the junctional contact between these nerve endings and the corpuscular cells and (c) the synaptical contacts between nerve endings within a Neuro-epithelial Body.

a) As regards the ultrastructure of the *different types of nerve endings* in contact with the corpuscular cells, two major types do emerge. The first type is constituted by a nerve fibre which forms on its course numerous varicose nerve endings as well "en bouton" as "en passant", which are in direct cytoplasmic continuity and of two variants (type 1a, type 1b). The first variant (type 1a) (Fig. 9) is characterized by an accumulation of mitochondria whose cristae are usually longitudinally oriented. Amongst them some small and agranular synaptic vesicles (300–500 Å diameter) occasionally occur. Larger dense-cored vesicles (800–900 Å) are exceptionally seen as well. In only one instance (Fig. 13), was such a terminal "bouton" filled with mitochondria observed in close proximity to the bronchial lumen, which it did however not contact. It remained indeed covered by the very thin cytoplasmic extensions of the two adjoining corpuscular cells between which it was sandwiched. The second variant (type 1b) (Fig. 10) of

varicosity is on the other hand packed with numerous empty synaptic vesicles (300–500 Å diameter), while the large dense-cored vesicles (800–900 Å diameter) and the mitochondria occur only occasionally.

According to the classic morphological criteria, these varicosities are of the afferent-like (type 1a) and efferent-like (type 1b) kind. Both types are however demonstrated on serial sections to occur along the same nerve fibre and to be connected by cytoplasmic continuities (Fig. 10).

The second type (type 2) (Fig. 11) ist the one reported in our earlier observations (Lauweryns *et al.*, 1972b, 1973). It includes varicose nerve endings which are filled with a practically homogeneous population of small and agranular synaptic vesicles (300–500 Å diameter); some large dense-cored vesicles (800–900 Å diameter) occur occasionally. As far as the nerve fibres on which such nerve endings were present could be followed on serial sections, they never revealed varicosities filled with mitochondria. According to the usual morphologic criteria, this second type of nerve ending is of the efferent-like type.

b) As regards the *junctional contacts* between these nerve endings and the corpuscular cells, both the afferent-like (type 1a) (Fig. 12) and efferent-like (type 1b, type 2) (Fig. 11) nerve varicosities reveal synaptical end formations, making a "direct contact" with the granulated cells. Being localized in intimate relationship to each other, both the granulated cell membrane and the respective nerve membrane exhibit thickenings or synaptosomes.

In some instances, an exocytosis of some corpuscular DCV's has been observed at the site of such a synaptical contact between an afferent-like type (type 1a) of nerve ending and a granulated epithelial cell (Fig. 12).

c) Finally and as regards the occurrence of *synaptical contacts* between the various nerve endings themselves within a Neuro-epithelial Body, a direct synaptical contact is observed between the nerve endings characterized by the occurrence of numerous small and agranular synaptic vesicles (type 2) and those filled mainly with mitochondria (type 1a). At such sites characteristic synaptolemmal membrane thickenings and densifications are seen (Fig. 13).

As mentioned earlier, the type 1a and type 1b nerve endings are in direct cytoplasmic continuity along the same (type 1) nerve fibre (Fig. 10).

4. Further Histochemical Observations. Applying the light optical AChE technique of El-Badawi *et al.* (1967), a positive red-brown staining reaction is obtained as well throughout the cell cytoplasm of the NEB's as in their nerve endings. These stem from the subepithelial, bronchial and bronchiolar nerve plexus (Lauweryns *et al.*, 1972a, 1972b, 1973) which is also AChE positive (Fig. 15A).

With Bloom's ultrastructural cytochemical technique, the corpuscular cells of the NEB's reveal a distinct reaction, a very fine, well contrasted and dense AChE-positive precipitate being observed exclusively in the halo of the DCV's of the first type, their core remaining unstained (Fig. 15B). These DCV's cover about 70% of the entire population, their shape is rather pleomorphic and the halo around the sometimes excentrically located dense core rather small (Lauweryns *et al.*, 1972b, 1973). No reactions occurred neither within the DCV's of the second type which are more circular and form an obvious halo, nor elsewhere in the cytoplasm of the corpuscular cells.

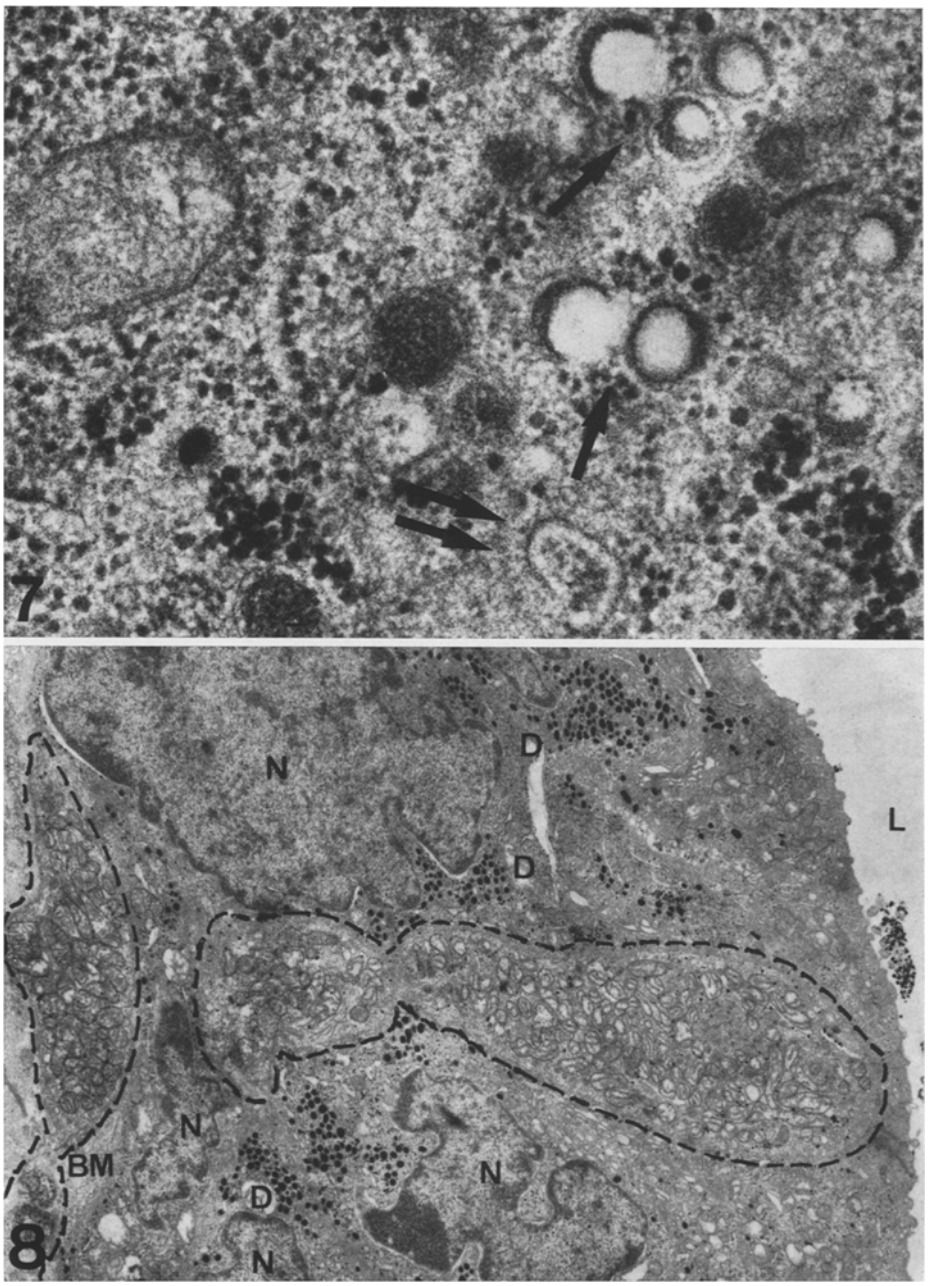

Fig. 7. Cytoplasm of a corpuscular cell of a NEB revealing the depletion of the DCV's after reserpine pretreatment; many DCV's are practically entirely cleared up, revealing only at their periphery a small half moon-like dense ring (arrow); in other DCV's, the core is fragmented into dark grey granules (double arrow); neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 92568×

Applying the alpha-GPD technique, a black precipitate occurs in the mitochondria of the conducting airway epithelial cells. At the level of the NEB's this staining reaction is much more pronounced, revealing in addition to the mitochondrial reaction, a diffuse and massive cytoplasmic precipitate (Fig. 16).

Using Solcia's *et al.* lead hematoxylin stain for endocrine cells which produce polypeptides and amines, the corpuscular cells of the NEB's reveal a positive and selective dark-blue to purple reaction, the bronchial and bronchiolar epithelium remaining otherwise unstained (Fig. 1).

Discussion

1. Effects of Hypoxia. One of the most important results obtained in these series of observations is without doubt the ultrastructural demonstration of a hypoxia-induced secretory release of the dense-cored, serotonin containing vesicles at the basal pole of the corpuscular cells of the NEB's. As the apical pole of the corpuscular cells immediately contacts the airway lumen and its contents on the one hand, and as a fenestrated blood capillary is closely apposed to their basal or vascular pole on the other hand (Lauweryns *et al.*, 1972b, 1973), it appears logical that the NEB's are chemoreceptor organs with a local intrapulmonary secretory activity, one of the substances released within the blood stream of the lungs being serotonin. This identifies the previously unelucidated, intrinsic morphological mechanism explaining the occurrence of a hypoxia induced pulmonary vasoconstriction (Comroe, 1964; Dejours, 1962; Fishman, 1960) which is in itself not markedly influenced by the nervous system, blood pH and lactic acid, but mediated by humoral substances (Daly *et al.*, 1966; Hauge, 1969; Laros, 1971; Lloyd, 1968; Naeye, 1965), e.g. serotonin (Sjoredsma, 1959). Niden *et al.* (1960) have demonstrated that serotonin injected into the pulmonary circulation causes an increase in the oxygen saturation of the pulmonary venous blood. As most of the intrapulmonary bronchial capillary and venous blood is drained off via the pulmonary circulation (Lauweryns, 1962, 1964, 1968, 1971), it may well be that the serotonin secreted by the NEB's during hypoxia causes a vasoconstrictor response with blood shunting from the poor to the better oxygenated and ventilated portions of the lung, providing besides the central and peripheral (e.g. carotid body) chemoreceptors a third or locally inbuild intrapulmonary chemoreceptor system which finely adjusts the ventilation to perfusion ($\dot{V}/\dot{Q}$) ratios.

2. Effects of Reserpine Pretreatment. After reserpine pretreatment the NEB's of otherwise normal animals exhibit a decreased but constantly yellow fluorescence with Falck's technique; ultrastructurally their DCV's are cleared up and even fragmented. As reserpine is known to be an amine- and also a serotonin-depleting

Fig. 8. Low power electron micrograph of part of a NEB in the bronchiolar mucosa of a normal neonatal rabbit lung exhibiting a panoramic view on the course of an unmyelinated nerve fibre (encirculed with a dotted line) between the corpuscular cells; the nerve fibre may be followed from under the basement membrane (*BM*) to the immediate vicinity of the airway lumen (*L*); nucleus (*N*) of a granulated cell; dense-cored vesicles (*D*); glutaraldehyde-fixation with post-osmification; 10944×

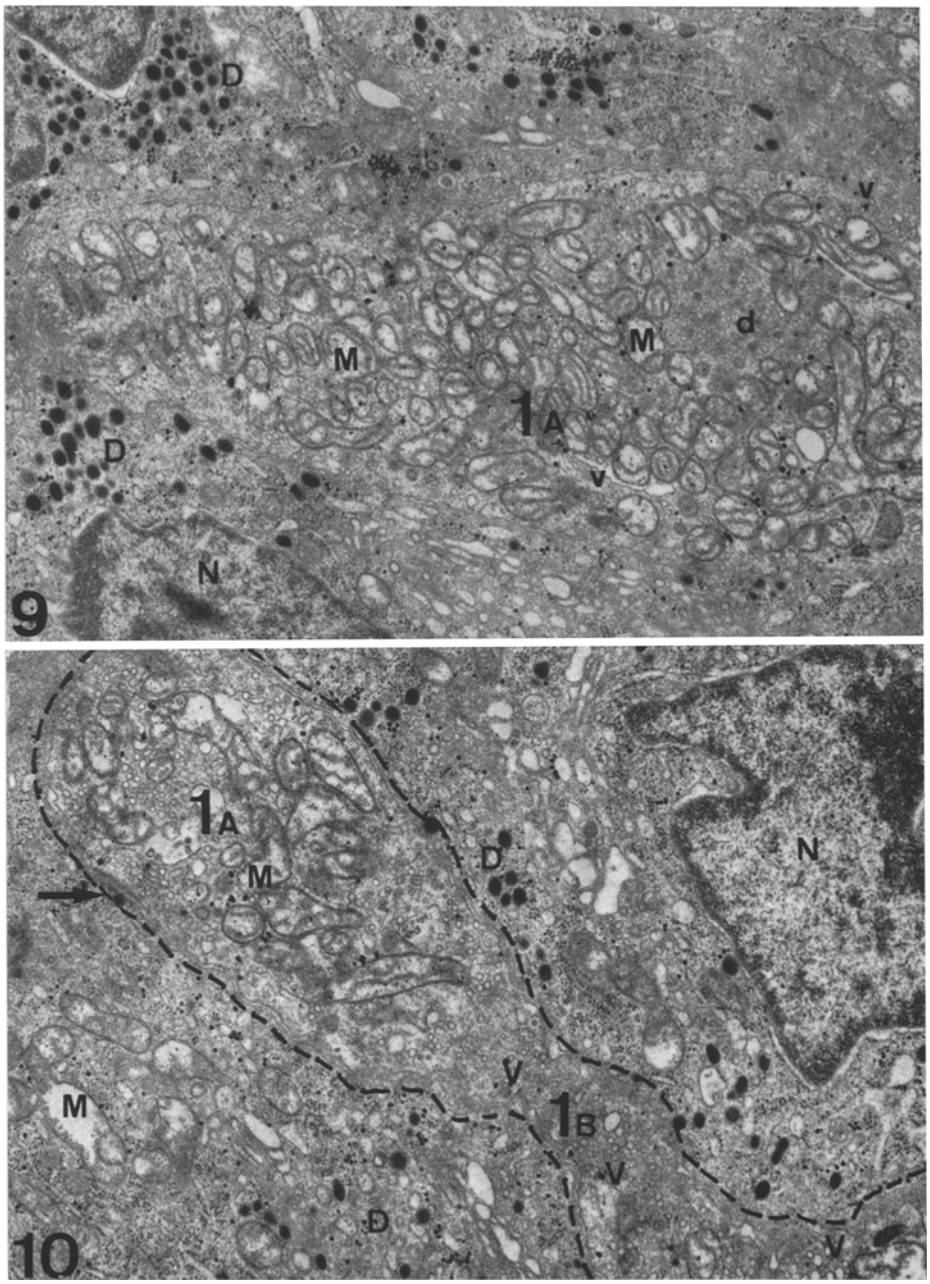

Fig. 9. Nerve varicosity filled up mainly with mitochondria (*M*); it also contains some small and agranular synaptical vesicles (*v*) and some rare dense-cored synaptical vesicles (*d*); these nerve endings correspond to type 1.a (*1A*); nucleus (*N*) and dense-cored vesicles (*D*) of a corpuscular cell; neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 17807×
Fig. 10. Nerve fibre (encircled with a dotted line) illustrating the cytoplasmic continuity between its so-called type 1.b part (*1B*), which is packed with numerous empty synaptical vesicles (*V*), and a type 1.a varicosity (*1A*) which is characterized by an accumulation of mitochondria (*M*); synaptical contact (with the formation of synaptosomes) (arrow) between the afferent-like type (type 1.a) nerve ending and a granulated epithelial cell; nucleus (*N*) and dense-cored vesicles (*D*) of a corpuscular cell; neonatal rabbit lung; glutaraldehyde fixation with postosmification; 20235×

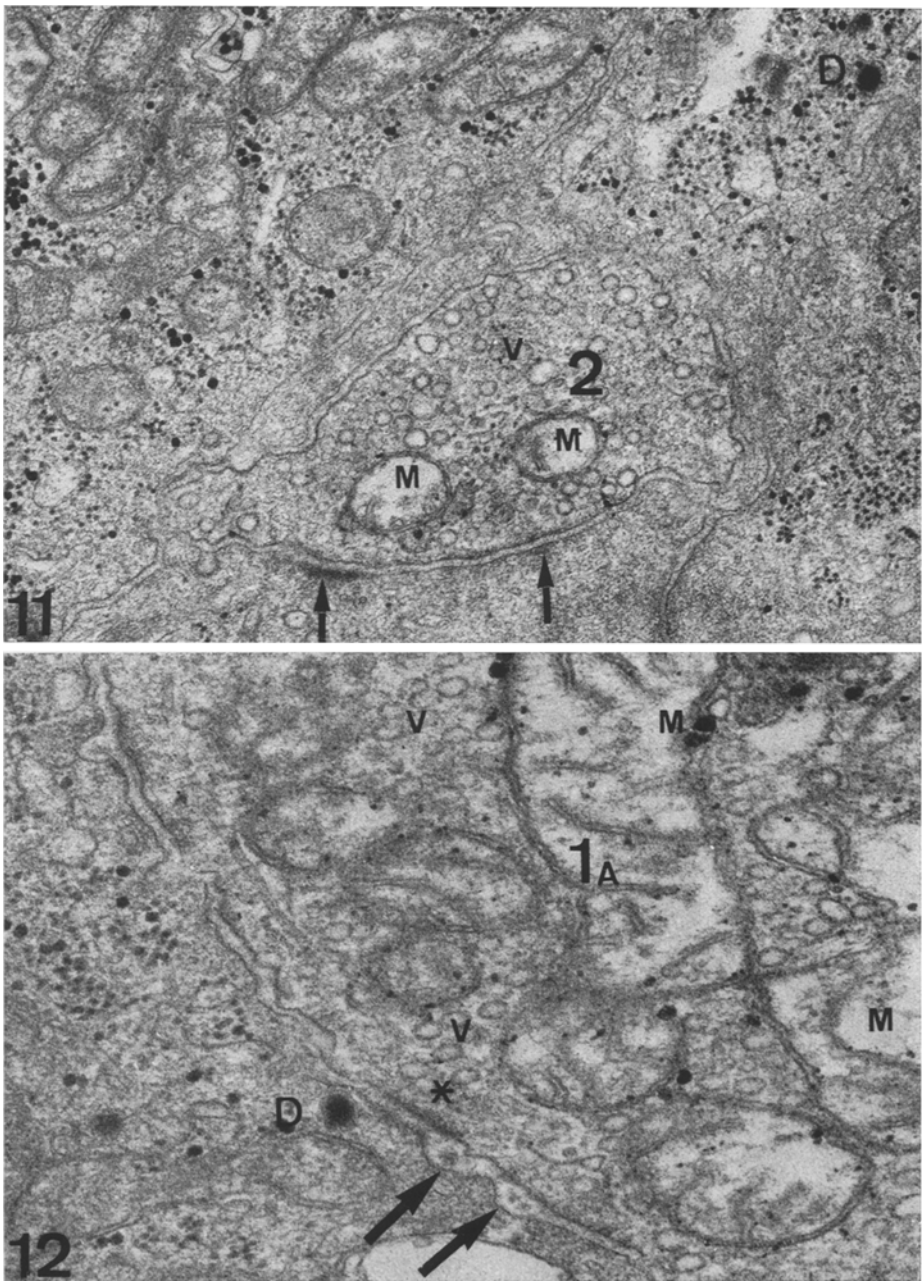

Fig. 11. Nerve ending corresponding to the type 2 (*2*) practically filled with small and agranular synaptic vesicles (*V*); mitochondria (*M*); synaptolemmal densifications (arrow) at the synaptical contact place of this efferent-like nerve ending with a corpuscular cell; dense-cored vesicle (*D*) of corpuscular cell; neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 42865

Fig. 12. Synaptical contact between a nerve varicosity of type 1.a (*1A*) with a corpuscular cell; observe the exocytosis of DCV's at the nerve membrane (arrow) and the formation of synaptolemmal densifications at this site (*); dense-cored vesicle (*D*) of corpuscular cell; *M* mitochondrion; *V* empty vesicles; neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 65436×

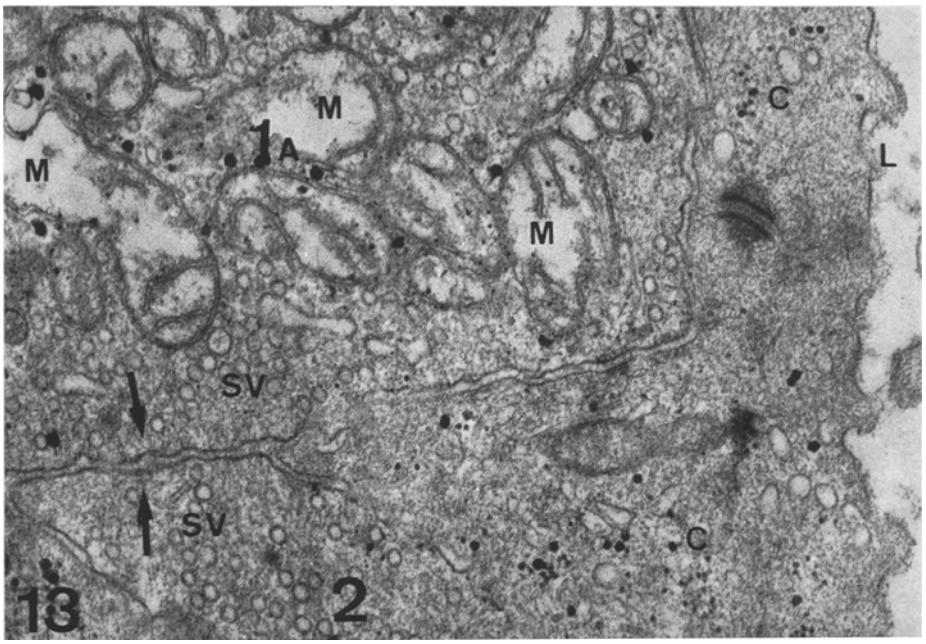

Fig. 13. Synaptical contact between an afferent-like type 1.a nerve varicosity (*1A*) packed with mitochondria (*M*), and a type 2 varicosity (*2*) filled with a homogeneous population of small and agranular synaptic vesicles (*SV*); formation of synaptosomes (arrow); the type 1.a ending lies in close proximity of the airway lumen (*L*), though remaining covered by the cytoplasmic extensions of two adjoining corpuscular cells (*C*); neonatal rabbit lung; glutaraldehyde-fixation with postosmification; 45486×

agent (Pletscher *et al.*, 1955), this furthermore confirms our earlier studies (Lauweryns *et al.*, 1972b, 1973) in which we proposed that the corpuscular cells of the NEB's produce serotonin and other related amine and peptide substances.

It has moreover been established (Barer, 1966) that reserpine pretreatment prevents the pulmonary hypoxic vasoconstrictor response. This harmonizes with our first series of experiments (in which a hypoxia-induced secretory release of the DCV's was observed), as the corpuscular cells are already depleted of their DCV's under the effects of reserpine.

3. The Innervation of the NEB's. The NEB's appear on serial sections to be heavily innervated by various types of nerve endings which end upon the corpuscular cells and form synapses amongst themselves.

On the one hand varicosities occur along the same nerve fibre which are of the afferent-like type (type 1a) and efferent-like (type 1b) kind. They are indeed filled up mainly and respectively with numerous mitochondria (type 1a) or empty synaptic vesicles (type 1b). Analogous pictures have been described recently in the carotid body by Verna (1973) who interpreted them as various morphological aspects occurring along one and the same nerve "of afferent type". Similar pictures are also seen in the (probably) afferent nerve endings of the neuromuscular spindle (Merrillees, 1960; Robertson, 1960), of the corpuscles of Meissner

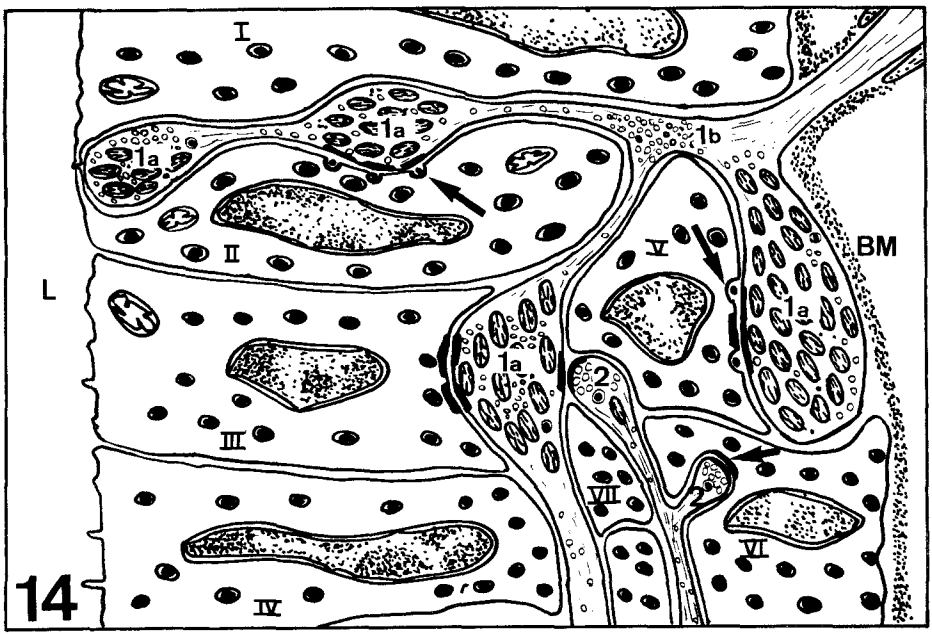

Fig. 14. Schema illustrating the innervation of a NEB in a normal neonatal rabbit lung; *L* lumen of the airway; *BM* basement membrane; *1A* nerve varicosities of the afferent-like type 1.a; *1B* nerve varicosity of the efferent-like type 1.b; *2* nerve varicosity of the efferent-like type 2; a type 1.a nerve ending makes contact with the corpuscular cells with the formation of synaptolemmal densifications (at cells labeled II and III); exocytosis (arrows) of some DCV's at the place of a synaptical contact between a type 1.a nerve ending and the corpuscular cells (cells II and V); a type 2 nerve ending makes a synaptical contact with a corpuscular NEB cell (cell VI) and with a type 1.a nerve varicosity (between cells III, V and VII); the type 1.a and type 1.b varicosities are in direct cytoplasmic continuity along the same nerve fibre, which enters the basement membrane from the corium

(Cauna *et al.*, 1960) and of the taste buds of the tongue (Gray *et al.*, 1965; Scalzi, 1967). In the absence of any currently available physiologic data concerning the function of the NEB's, we propose to interpret our observations along with the above-mentioned studies: the nerve fibres on which type 1a and type 1b varicosities occur, are probably afferent nerves; this is furthermore suggested by the exocytosis of DCV's by the corpuscular cells at the place of a junctional contact between such nerves and the granulated cells.

On the other hand and confirming our earlier studies (Lauweryns *et al.*, 1972b, 1973), varicose nerve endings filled with a practically homogeneous population of small and agranular synaptic vesicles (type 2) are again observed. They also establish a "direct contact" with the corpuscular cells. Their ultrastructural aspect as well as the positive staining reaction of the unmyelinated nerve endings for acetylcholinesterase, are classically interpreted as occurring in cholinergic efferent nerve endings (Burnstock *et al.*, 1971; Gray *et al.*, 1966; Grillo, 1966).

Drawing upon these morphological observations—and as originally suggested (Lauweryns *et al.*, 1972b, 1973)—it thus appears probably that the corpuscular cells of the NEB's are innervated both by afferent (type 1a) and efferent (type 2)

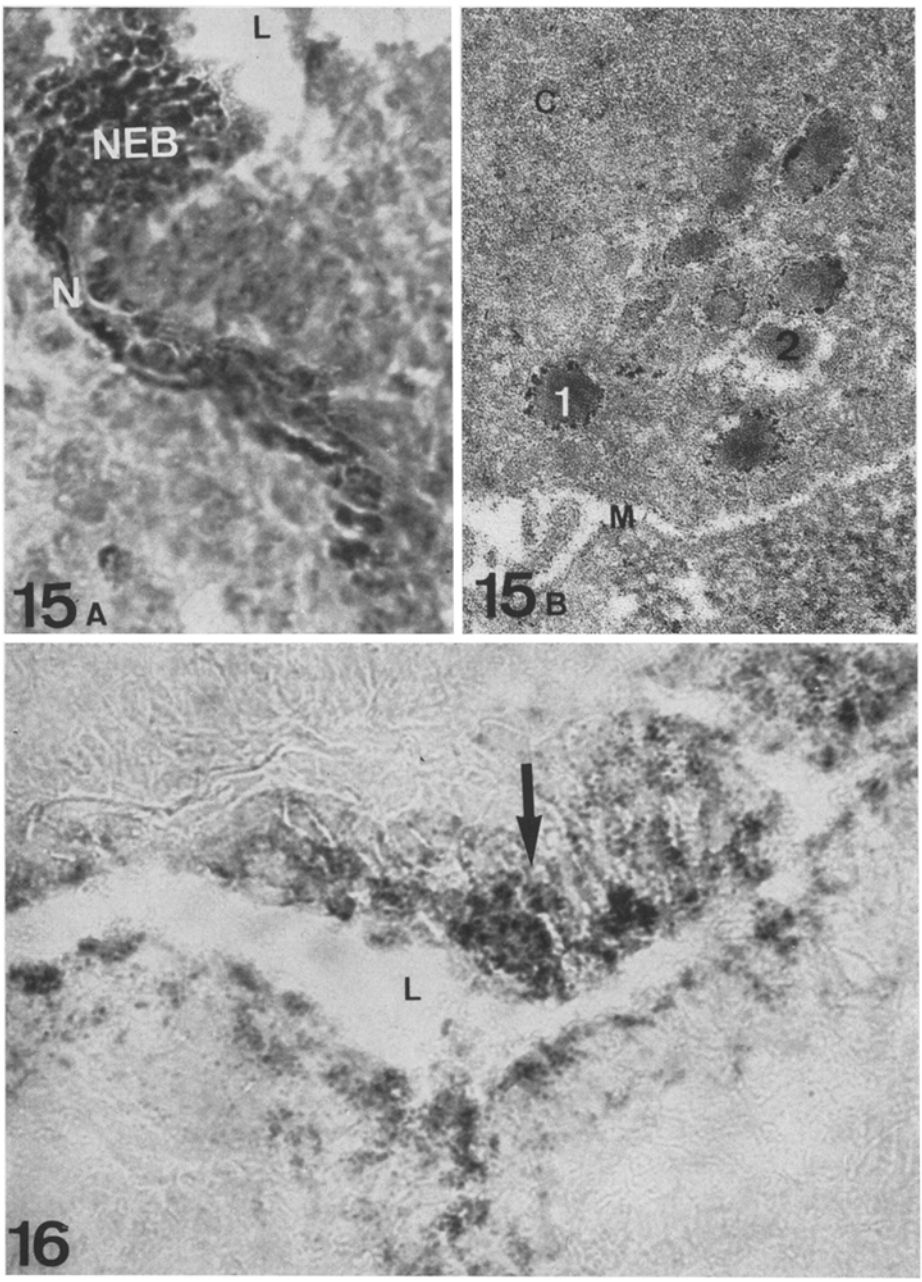

Fig. 15. A. NEB within the bronchiolar mucosa (NEB) of a neonatal rabbit lung, displaying a pronounced staining reaction after the AChE-staining methods of El-Badawi *et al.*; also the nerve fibre (*N*) ending upon the NEB is positively stained; *L* bronchiolar lumen; 715× B. Part of the cytoplasm of a corpuscular cell revealing a black and dense reaction product for AChE, localized exclusively in the halo of DCV's of the first type (*1*); no reaction is seen at the level of the cell membrane (*M*), the cytoplasm (*C*) of the corpuscular cell or the halo of DCV's of the second type (*2*); neonatal rabbit lung; AChE-technique of Bloom *et al.*; 97 812×

nerve endings. Finally the recent studies of Hung *et al.*, (1973) should be mentioned. These authors have investigated the ultrastructure of nerves and associated cell in bronchiolar epithelium of the mouse lung, observing however only an afferent type of innervation.

A direct synaptical contact is moreover observed between these presumed afferent (type 1 a) and efferent (type 2) nerve fibres. Similar pictures were again observed by Verna (1973) in the carotid body of the rabbit and interpreted by this author as the morphological expression of some kind of "negative feedback" mechanism by which the afferent or receptor nerves should be modulated efferently by the central nervous system. This interpretation is of course hypothetical as concerns the NEB's, as a physiological demonstration of such a modulation is lacking.

Summarizing, it appears reasonable to accept that the NEB's possess a dual innervation, both afferent and efferent. The afferent nerve endings could be influenced (stimulated ?) by the DCV's released at a place of junctional contact through exocytosis by the corpuscular cells. Amongst their various possible functions, these cells appear already to be secretory, hypoxia-sensitive chemoreceptor cells. The efferent nerve endings make junctional contacts as well with the corpuscular cells as with the afferent nerve endings. They appear as fibres modulating from the central nervous system onwards (by a negative or depressing feed-back mechanism ?) the activity of the corpuscular cells and the afferent nerve endings.

4. Further Histochemical Studies. Although the corpuscular appearance and obvious innervation of the NEB's are characteristics establishing a separate morphological entity, they also exhibit several features of the bronchial and bronchiolar AFG (for Argyrophil, Fluorescent and ultrastructurally Granulated) cells (Lauweryns *et al.*, 1969, 1970; Rosan *et al.*, 1971) which we have identified histochemically and ultrastructurally in the human infant lung and which we have supposed to be related to the growing list of presumably peptide-secreting APUD cells (Pearse, 1968). Like APUD cells, the cells of the NEB's reveal a positive reaction for alpha-GPD, AChE, serotonin and Solcia's *et al.* stain for endocrine cells; however, several ultrastructural features do not correspond. Unlike NEB's, APUD cells are characterized by numerous cytoplasmic fibrils, high levels of smooth endoplasmic reticulum in vesicular form and a different ultrastructural localization of the AChE. Moreover, the APUD-like cells of the rat larynx (Ewen *et al.*, 1972) contain no DCV's but intracytoplasmic dense granules, with a diameter up to 20.000 Å, the DCV's of the NEB's measuring between 1340 Å (type 1) and 980 Å(type 2).

The so-called "Brush cells" form another recently identified cell type in the rat trachea by Luciano *et al.* (1968) and in the rat alveoli by Meyrick *et al.* (1968). In contradistinction to the corpuscular cells of the NEB's, the Brush cells are arranged either alone or in pairs; they are characterized by a "brush border" of

Fig. 16. NEB in the bronchiolar mucosa of a neonatal rabbit lung stained with the alpha-GPD technique (Pearse, 1961); a slight positivity occurs throughout the entire mucosa; heavy reaction at the level of the NEB (arrow); *L* lumen of bronchiole; 663×

numerous narrowly placed and very regular microvilli, the axial filaments of which continue inside the cell cytoplasm without ending in a terminal web. Such a "brush border" is never seen in NEB's, the luminal cell membrane of the corpuscular cells being flat to slightly undulated with only an occasional microvillus. Even after a glutaraldehyde fixation with postosmification, "Brush cells" do not contain intracytoplasmic DCV's.

Two types of morphologically and morphometrically different (Lauweryns *et al.*, 1972b, 1973) DCV's occur within the corpuscular cells of the NEBs': the first type reveals a positive AChE deposit within its halo, while the second type remains negative. This as well as the positive staining reaction using Solcia's *et al.* stain (1969) for endocrine cells which produce polypeptides and amines, indicates that the granulated cells probably produce substances other than serotonin, whose metabolism may be combined with a variety of cellular activities in the amine and peptide spectrum. Hence we propose that the NEB's probably have various local secretory functions, modulating not only vasomotion but also other bronchial and bronchiolar functions such as mucosal secretion, smooth muscle tone or the integration of the activities of the pulmonary unit lobules (Lauweryns, 1970; Lauweryns *et al.*, 1971). Their occurrence as secretory chemoreceptors in the relatively hypoxic (not hypoxemic!) fetal lung (Lauweryns *et al.*, 1972b, 1973) could help to explain the active pulmonary vasoconstriction during fetal life (Isabel *et al.*, 1972; Assali *et al.*, 1964), the rapid pulmonary vasodilation upon aeration of the lung with room air (Rudolph *et al.*, 1961) and even the pulmonary hypoperfusion in the respiratory distress syndrome associated with hypoxia (Chu *et al.*, 1965; Lauweryns, 1961; 1966). They could influence the occurrence of bronchial asthma or of CNS-induced pulmonary edema. They could also generate bradykinin or allied substances within the pulmonary circulation, substances which are produced by oat-cell carcinomas and bronchial carcinoids, both of which have several ultrastructural features of the NEB's (Bensch *et al.*, 1965).

Many additional biochemical, physiologic and pharmacologic investigations will be needed to further elucidate the functions of the NEB's, hereby identified as a hypoxia-sensitive and locally secretory chemoreceptor system, which appears dually (both afferently and efferently) innervated. Its corpuscular cells are depleted by reserpine and are AChE- and alpha-GPD positive.

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