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# Fine Structural and Cytochemical Study of the Innervation of Smooth Muscle in an Amphibian (*Bufo marinus*) Lung Before and After Denervation

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**Summary.** The innervation of the toad (*Bufo marinus*) lung was studied with transmission electron microscopy and fluorescence techniques, both before and after 12 or 20 days close vagosympathetic denervation. Four cytologically distinct types of neuronal processes were recognised, in relation to the visceral muscles of the lung. These were described as cholinergic, adrenergic, non-adrenergic/non-cholinergic (NANC) and sensory on the basis of the characteristics of their vesicular content and cytochemical reactions. An apparent efferent innervation of visceral smooth muscle was achieved by NANC (50%), cholinergic (25%) and adrenergic (25%) fibres. A few sensory fibres were also present. After denervation only NANC fibres persisted, showing that the cell bodies of these fibres were intrapulmonary. The vascular smooth muscle was supplied by cholinergic, adrenergic and sensory fibres. In the walls of the proximal branches of the pulmonary artery were fibres containing large dense-cored vesicles. These profiles, which were associated with the vasa vasorum, were similar to neurosecretory fibres. After denervation all neural profiles associated with the vasculature had degenerated. The observations suggest that vagal vasodepressor effects in the toad lung are mediated indirectly through relaxation of visceral muscle strands which in their contracted state compress vascular channels.

**Key words:** Lung – *Bufo marinus* – Innervation – Adrenergic nerves – Neurosecretion.

Several physiological and pharmacological studies of the innervation of the toad lung have been made in this laboratory (Wood and Burnstock, 1967; Campbell, 1971a, b; Campbell and Duxson, 1978; Holmgren and Campbell, 1978). In

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summary, the following conclusions about the innervation of the visceral and vascular pulmonary smooth muscle have been reached: The anterior sympathetic system provides adrenergic fibres which are vasodilator and which cause both contraction and relaxation of visceral muscle. The vagus nerve provides cholinergic fibres which are vasoconstrictor and non-adrenergic/non-cholinergic (NANC) fibres which cause relaxation of visceral muscle; the latter fibres cause vasodilatation but the effect may be due to mechanical interactions between visceral muscle and blood channels. The vagosympathetic trunk contains further cholinergic fibres, some but not all of which originate from the vagus (Duxson and Campbell, unpublished observations), which cause contraction of visceral muscle. While each vagosympathetic trunk mainly innervates the ipsilateral lung, there is some cross-over of fibres to the contralateral lung.

One attempt has been made to distinguish the fine structural detail of the innervation of the toad lung (Robinson et al., 1971). Although this work identified NANC fibres in the lung, the preservation techniques available at that time were inadequate to ensure the retention and localisation of biogenic amines at sufficient levels for visualisation with the electron microscope. Thus, it did not prove possible to identify adrenergic nerve fibres in the lung electron microscopically, even though the fibres had been demonstrated with formaldehyde-induced fluorescence (McLean and Burnstock, 1967). The chromate/dichromate methods recently developed by Tranzer and Richards (1976) are more reliable and more general in their application. This technique, coupled with the technique of close denervation of the lung, was used to identify adrenergic nerve terminals and to describe the pattern of innervation of both visceral and vascular muscle of the toad lung.

## Materials and Methods

### *Electron Microscopy*

Specimens (16) of the toad *Bufo marinus* were anaesthetised by immersion in 0.1% tricaine methane sulphonate (MS 222, Sandoz) and opened ventrally, exposing the lungs. The distal part of the left lung was pinned out on a wax sheet while the proximal part was still attached to the vascular supply of the animal. A clamp was applied to the proximal vessels and the glutaraldehyde-based fixative (see below) was injected into the lumen of the lung. The lung was then dissected free of its vascular connections and immersed in a bath of the same fixative before being cut open and pinned out, in order to expose all the tissue to the fixative. Suitable segments of tissue from the intrapulmonary branches of the pulmonary artery, vein, alveolar septa and lung wall were removed for subsequent processing from both proximal and distal regions.

### *Fixation*

Preservation of Biogenic Amines. Tranzer and Richards' (1976) fixative was used made up as follows: 0.1% glutaraldehyde, 0.4% EM grade formaldehyde buffered with 0.1 M sodium chromate/potassium dichromate at pH 7.3 at a temperature between 0–4° C. After 15 min the pieces were transferred to an incubating solution composed of 0.2 M sodium chromate/potassium dichromate at pH 6.2 for a further 18 h at between 0–4° C. The tissue was then post fixed in 2% $OsO_4$ buffered with 0.1 M sodium chromate/potassium dichromate at 0–4° C for 1 h at pH 7.3.

*Control.* The segments of tissue were fixed in a solution containing 2.5% glutaraldehyde, adjusted to pH 7.3 with Sorensen's phosphate buffer, at room temperature (about 20° C). After 1 h the pieces were washed in two changes of buffer and post fixed in 1% $OsO_4$ with the same buffer for 1 h at room temperature.

The tissue was washed in distilled water and block stained with a saturated aqueous solution of uranyl acetate.

Both control and test fixed pieces were dehydrated in a graded series of ethanol and embedded in Durcupan-Araldite. Thin sections were cut on a Huxley ultramicrotome with a diamond knife. Sections were stained with either uranyl acetate and lead citrate or lead citrate only and examined in a Jeol 100B transmission electron microscope (TEM).

*Fluorescence Microscopy*

Formaldehyde-induced fluorescence preparations were obtained by using the standard technique as detailed by Björklund et al. (1972) on air dried, stretched whole mounts of the pulmonary artery, vagosympathetic nerve trunk and lung.

Fluorescence preparations were also made by perfusing 3% glyoxylic acid in 0.1 M phosphate buffer at pH 7.2 through the ventricle for 5 min. After the lungs were dissected free, they were stretched over a glass slide and immersed for a further 30 min in the solution before being partially air dried and then heated at 100° C for 4 min.

Some of the preparations were intensified by injecting 15 mg/kg of α-methyl noradrenaline into the dorsal lymph sac 1 h prior to dissection. All preparations were examined on a Leitz Ortholux fluorescence microscope, using an incident radiation of 410 nm wavelength, and photographed on Kodak Tri-X film.

*Close Vagosympathetic Denervation*

Anaesthetised toads were laid on their sides and a lateral incision was made under the forelimb, revealing the dorsal surface of the proximal region of the lung. The connective tissue attaching the lungs to the stomach and pericardium was severed, exposing several branches of the vagosympathetic trunk entering the base of the lung. These small branches were cut away from the lung tissue and the vagosympathetic trunk was traced back to the oesophageal branches. Below this point, the pulmonary branch was ligated and severed to remove all extrinsic innervation to the lung via this source. Since crossing-over of both excitatory and inhibitory fibres to the contralateral lung has been demonstrated by Wood and Burnstock (1967) both lungs were denervated in this manner. After either 12 or 20 days the denervated lungs were processed for electron and fluorescence microscopy as outlined above.

## Results

A description of the internal anatomy of the toad lung has been given elsewhere (Smith and Campbell, 1977). That paper should be referred to for descriptions of the visceral smooth muscle bundles and blood vessels examined in the present work.

Five cytologically distinct types of neuronal processes were recognised in the lung. For the sake of convenience, these types will be named here and described, although the naming involves arguments presented in the Discussion.

*(1) Cholinergic.* The profiles contained small agranular vesicles (40 nm diameter) and large vesicles (80–100 nm) with electron-dense cores that were not reactive under chromate/dichromate fixation (Fig. 1).

*(2) Adrenergic.* The profiles contained small (40 nm) and large (80–100 nm) vesicles (Fig. 2). As a result of the procedure for preserving biogenic amines, both small and large vesicles contained electron-dense reaction products. The reaction products could be clearly observed without uranyl acetate staining. Indeed, lead citrate staining alone provided greater contrast than could be obtained using both lead and uranyl stains together.

*(3) Non-Adrenergic/Non-Cholinergic (NANC).* The profiles contained small agranular vesicles, with diameters of 25–60 nm, most of which were oblate rather than spherical. They also contained large vesicles (60–140 nm) with moderately electron-dense cores that did not react with the chromate/dichromate fixative. The large cored vesicles formed a significant component of the vesicle population in the profiles (Fig. 3).

*(4) Sensory.* The profiles contained numerous small mitochondria at the terminal level but were usually recognised as myelinated nerves at the pre-terminal level.

*(5)* A fifth type of neural process was found to which a name could not readily be given. The profiles contained 40 nm and 80 nm vesicles which developed electron-dense reaction products in their cores and numerous non-reactive dense-cored vesicles of 60–160 nm diameter (Fig. 4). These profiles were found in the vagosympathetic trunk (Fig. 4) and vasa vasorum (Fig. 12) and veins in the lung. Their function is not clear.

### *Innervation of Visceral Smooth Muscle*

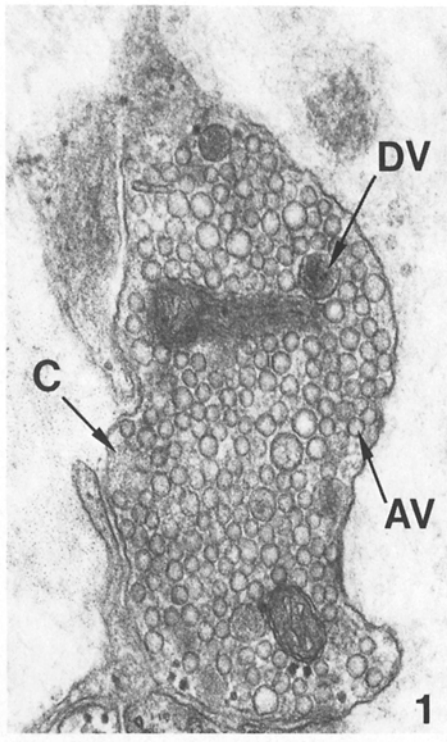
The large smooth muscle bundles lying in the alveolar septal margins and the smaller bundles lying in the lung wall and in the core of the septa contained a variety of neural profiles. About 50% of the unmyelinated profiles were identified as

**Fig. 1.** Profile of a nerve fibre (*C*) characterised by numerous agranular vesicles (*AV*), a few mitochondria and large, non-reactive, dense-cored vesicles (*DV*). This type of nerve profile is identified as cholinergic. All illustrations are of material which has been prepared according to the Tranzer and Richards technique and subsequently stained with lead citrate only. × 50,000

**Fig. 2.** Profile of a nerve fibre (*A*) characterised by numerous, reactive dense-cored vesicles of two sizes (*RDV*). This type of nerve profile is identified as adrenergic. × 50,000

**Fig. 3.** Profile of a nerve fibre (*NANC*) characterised by a moderate number of large, non-reactive, dense vesicles (*DV*) and spherical to oblate agranular vesicles (*AV*). This type of nerve profile is identified as non-adrenergic, non-cholinergic. × 50,000

**Fig. 4.** Unidentified profile (*N*) only observed in association with blood vessels and the vagosympathetic trunk. This type of process was characterised by both large, non-reactive, dense-cored vesicles (*DV*) and small and medium-sized reactive dense-cored vesicles (*RDV*). × 50,000


DV
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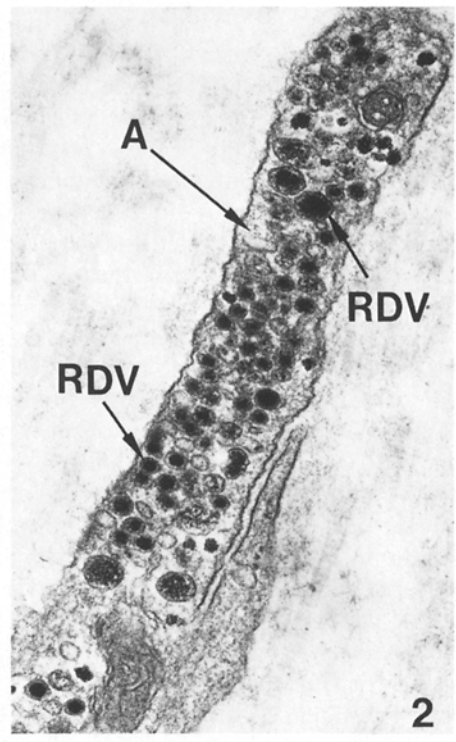
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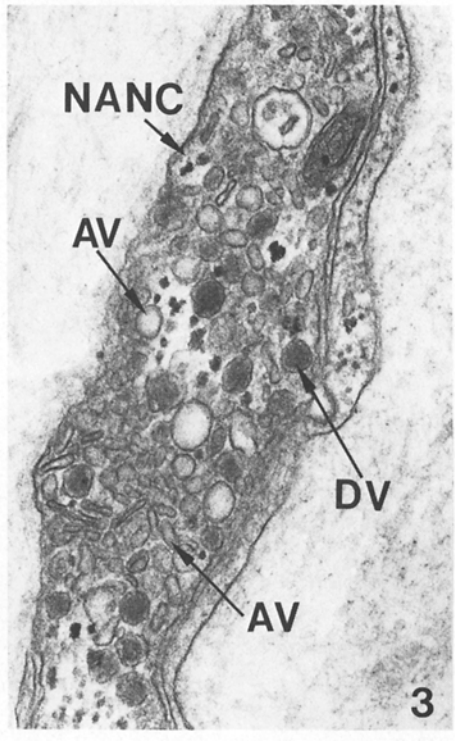
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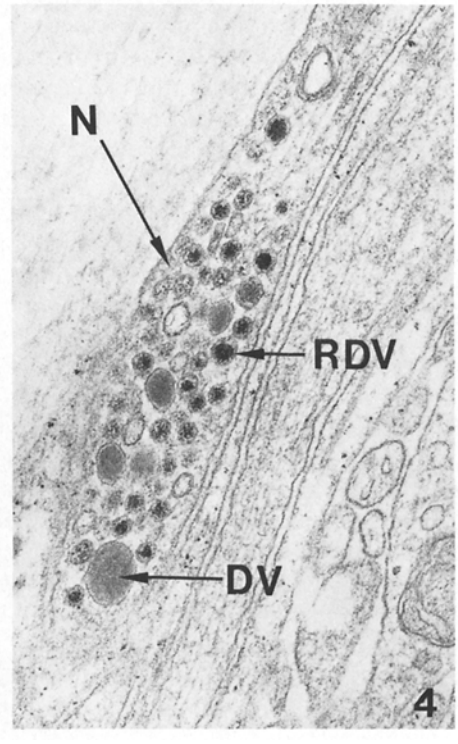
N
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NANC type, the remainder consisting of about 25% cholinergic and 25% adrenergic types. A small number of sensory nerve profiles were also identified.

Small branches of the vagosympathetic nerve, enclosed in perineural sheaths, were found in the connective tissue investing the muscle bundles at the septal margins. The branches contained both myelinated and unmyelinated nerve fibres. The unmyelinated fibres in these branches could be classified as either adrenergic or cholinergic on the basis of vesicle types and cytochemical reactivity. The branches eventually lost the perineural sheath and subdivided into smaller fascicles consisting of axons and Schwann cells only; at this level they were joined by NANC fibres (Figs. 5, 6).

These fascicles, containing adrenergic, cholinergic, NANC and sensory-type fibres, entered the muscle bundles, where they subdivided to form a terminal innervation consisting primarily of pairs of axons invested by Schwann cells (Fig. 7). Commonly the two axons in a pair were of different type, i.e. adrenergic and cholinergic, adrenergic and NANC or cholinergic and NANC. It was frequently observed that one axon of a pair entered into close apposition with a muscle cell while the other axon was prevented from achieving the same relationship at that level by the intervention of a Schwann cell process (Fig. 9). When the fibres were followed in serial sections, it was found that the second axon eventually entered into close contact with the same muscle cell (Fig. 10). Occasionally a close contact to the muscle was formed by both adjacent axons at the same level (Fig. 8). After making contact with a smooth muscle cell, the axons lost their Schwann cell sheath and diverged from each other to form further discrete close contacts with other muscle cells.

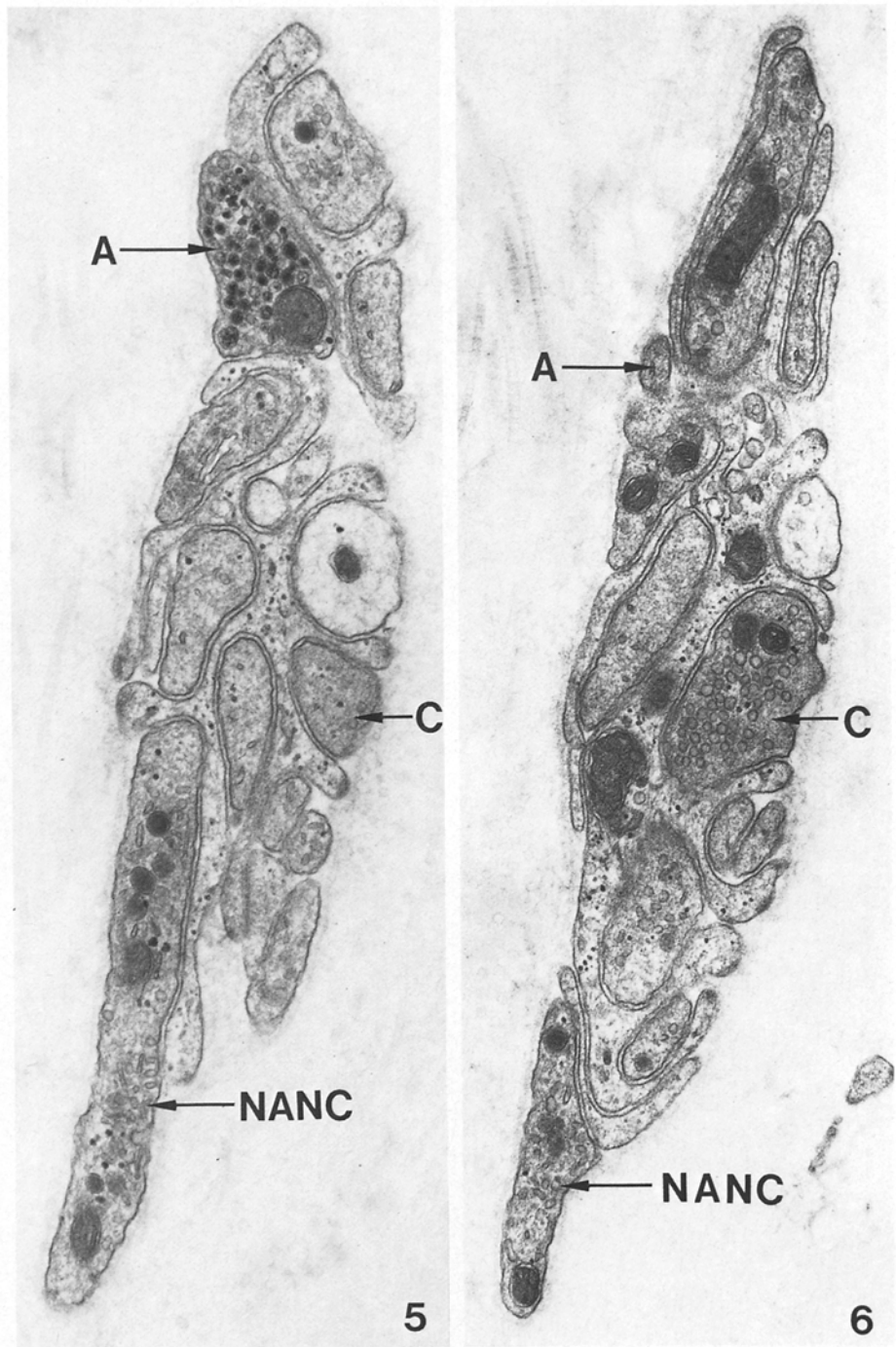
There seemed to be no difference in the innervation of muscle bundles from septal edges, from the lung wall or from the core of septa.

Twenty days after section of the pulmonary vagosympathetic branches close to the lung, no fibres which could be classified as adrenergic, cholinergic or sensory were found in the visceral muscle bundles. In partial confirmation of this, a brief fluorescence histochemical study of the denervated lungs did not reveal the typical adrenergic innervation of muscle bundles or of blood vessels. However, an innervation by NANC type fibres persisted and the density of these fibres in the muscle bundles appeared to be normal.

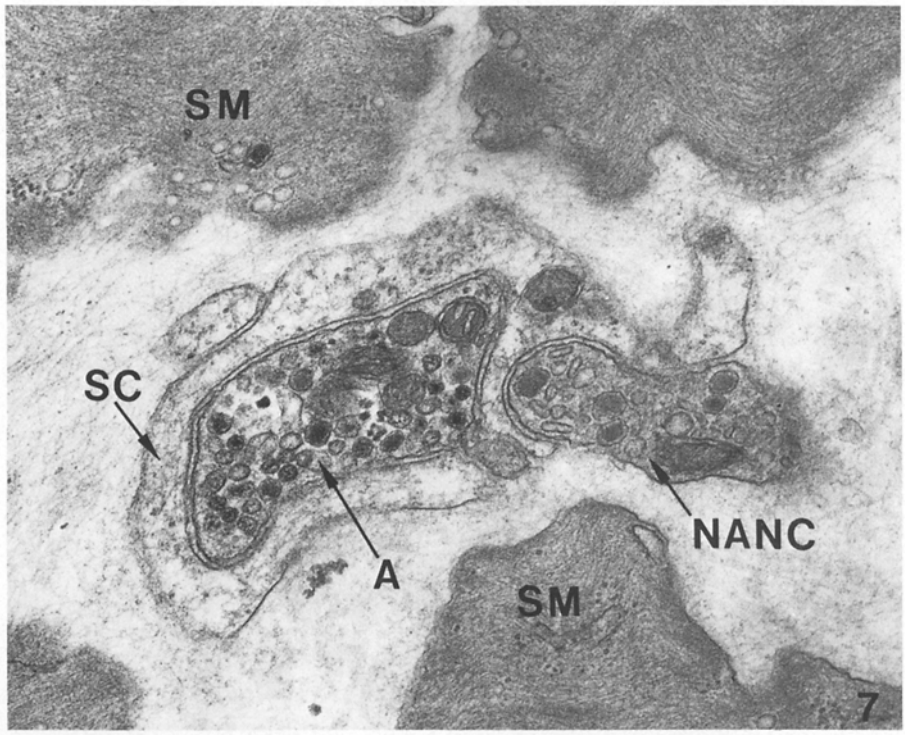
### *Innervation of the Vasculature*

Branches of the vagosympathetic nerve entered the proximal region of the lung parallel to divisions of the pulmonary artery. The nerve branches followed the arteries for 10–15 mm and supplied small nerve fascicles containing adrenergic, cholinergic and sensory fibres to the tunica adventitia of these vessels.

The orientation of smooth muscle cells in the branches of the pulmonary artery was essentially circular, with some tendency toward spiralling near the entry of the artery into the lung. The outer layer of muscle cells, four to five cells thick, was loosely arranged. The inner layer was of comparable thickness but the individual cells were closely apposed to each other at discrete sites, where septate junctional complexes were formed. The loose outer layer of muscle was provided with




**Figs. 5 and 6.** Semi-serial sections through medium sized branches of the vagosympathetic nerve below their junction with the intramural NANC nerve fibres. Cholinergic (*C*), adrenergic (*A*), and non-adrenergic non-cholinergic (*NANC*) profiles can be followed through the nerve branch. × 35,000



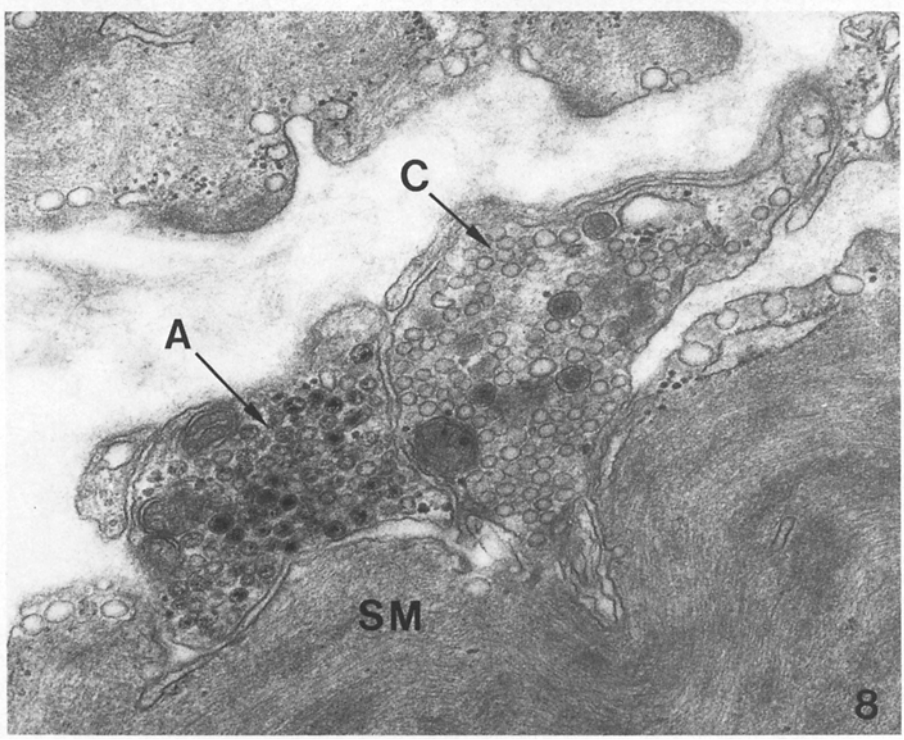
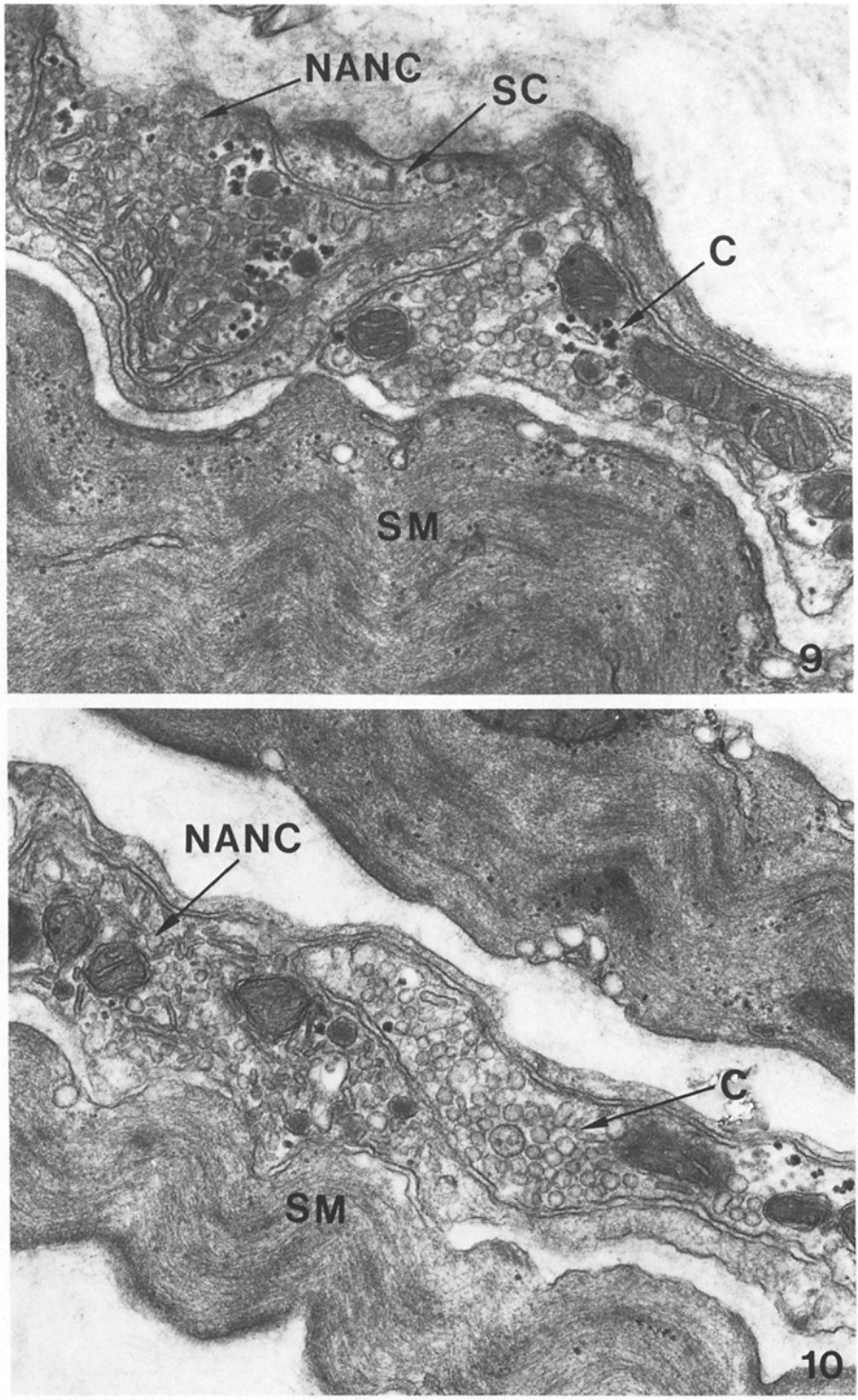
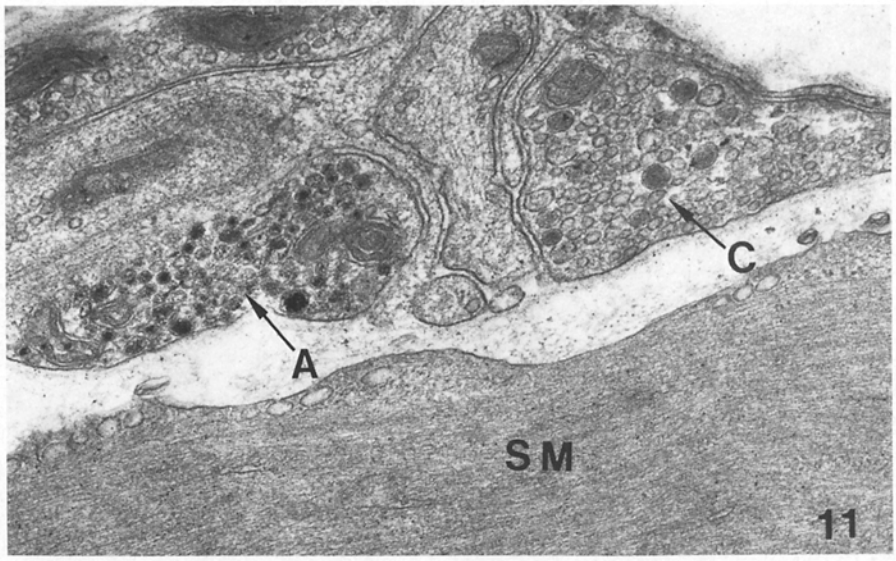




**Fig. 7.** Adrenergic (*A*) and non-adrenergic/non-cholinergic (*NANC*) fibers within the same Schwann cell sheath (*SC*) separated by a layer of collagen from the nearest muscle cells (*SM*). × 45,000

**Fig. 8.** Adrenergic (*A*) and cholinergic (*C*) axon pair simultaneously forming a close contact with a single smooth muscle cell (*SM*). × 45,000




**Figs. 9 and 10.** Semi-serial sections of an axon pair enclosed in the same Schwann cell sheath (*SC*) forming alternate close contacts with one muscle cell (*SM*); *NANC* non-adrenergic/non-cholinergic nerve profile; *C* cholinergic nerve profile. × 45,000



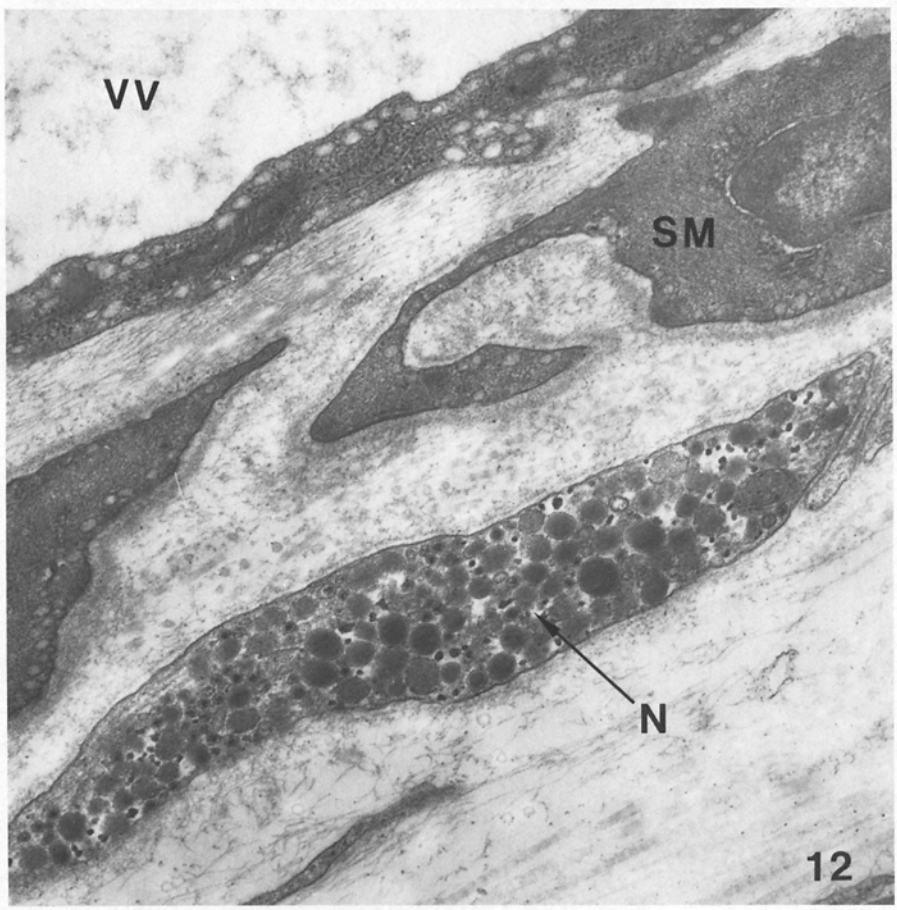




**Fig. 11.** Small nerve fascicle in wall of pulmonary artery. *A* adrenergic axon; *C* cholinergic axon; *SM* muscle cell. × 40,000

**Fig. 12.** Long, irregular profile (*N*) associated with medial smooth muscle cells (*SM*) of a vessel of the vasa vasorum (*VV*) in the tunica adventitia of a pulmonary arterial branch. × 35,000

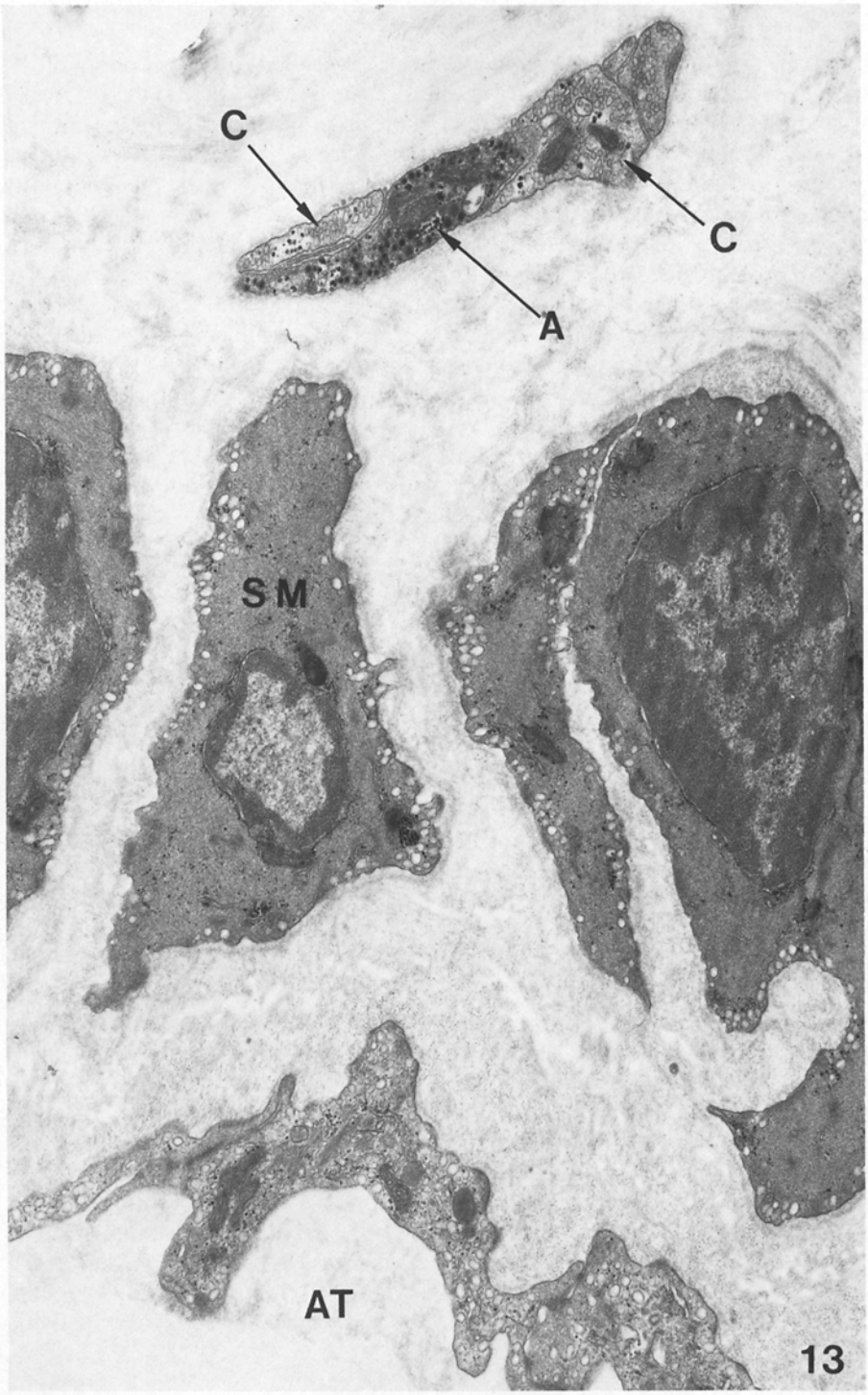



**Fig. 13.** Cholinergic (*C*) and adrenergic (*A*) axons associated with muscle cells (*SM*) of an arteriole (*AT*) lying in the wall of the alveolar septum. × 20,000

adrenergic and cholinergic nerve profiles which extended to the periphery of the inner layer but did not penetrate it. The gap between the axons and the surface of the smooth muscle cells was always of the same order of 200 nm or more (Fig. 11).

Near the entry of pulmonary arterial branches into the lung, the arterial tunica adventitia contained sparsely distributed vasa vasorum, consisting predominantly of small diameter vessels with a poorly developed muscular media. These vessels were not seen in a recent study of vascular casts of the lung (Smith and Campbell, 1976), presumably because the arteries involved were obscured from view by the overlying alveolar capillary meshwork. Running parallel with the vasa vasorum were long irregular profiles containing numerous 60–160 nm cored vesicles as well as 40 nm and 80 nm vesicles with cytochemically reactive cores (Fig. 12). Adrenergic and cholinergic type profiles were also associated with the vasa vasorum.

The media of the major intrapulmonary veins and arterioles consisted of a single layer of smooth muscle cells. On the outer surface of the medial layer of the veins were found nerve profiles of adrenergic and cholinergic types and also the long irregular profiles described above related to the arterial vasa vasorum. Only adrenergic and cholinergic nerve fibres were associated with the arterioles (Fig. 13).

Twelve days after section of the vagosympathetic nerve close to the lung, all nerve profiles associated with the vasculature had either disappeared or were undergoing degeneration.

## Discussion

Five types of axon profiles could be distinguished in the toad lung on the basis of their vesicular populations and their cytochemical reactivity. For convenience, four of the profile types were given names with functional connotations in the results section: adrenergic, cholinergic, non-adrenergic/non-cholinergic (NANC) and sensory. Justifications for the use of these names and a discussion of the fifth type follow.

The fibres termed cholinergic had a complement of small agranular vesicles and larger vesicles with moderately dense cores, lacking reactivity with the chromate/dichromate fixation procedure of Tranzer and Richards (1976). These features are typical of fibres which are elsewhere thought to be cholinergic (see Burnstock, 1972; Richardson, 1964). With earlier fixation techniques (Robinson et al., 1971) and with the control fixative used here, the cholinergic fibres could not be distinguished from adrenergic fibres. However, with the technique for demonstrating biogenic amines, about half of the fibres appearing as 'cholinergic' in control tissues showed cytochemical reaction products in both the small and the larger vesicles. These reacting profiles are therefore regarded as adrenergic. The identification is consistent with the evidence obtained by denervation, since both the cytochemically-reactive profiles and nerve fibres showing specific formaldehyde-induced fluorescence disappeared after close vagosympathetic denervation.

The fibres termed NANC, containing small agranular vesicles and large (60–140 nm) granular vesicles, were previously described in this tissue by Robinson et al. (1971). It was then known from nerve stimulation experiments that the lung is

innervated by vagal inhibitory fibres which are neither adrenergic nor cholinergic (Wood and Burnstock, 1967; Campbell, 1971a). Since the tissue also contained nerve fibres which could not be classified on their vesicular content as either adrenergic or cholinergic, Robinson et al. (1971) reasonably proposed that the anatomically distinct fibres and the physiologically distinct fibres were one and the same thing. Since the vagal inhibitory postganglionic neurones lie within the lung (Wood and Burnstock, 1967), they should remain intact after degenerative vagal section. Robinson et al. (1971) showed that the NANC profiles could indeed still be found in lungs after high section of the vagosympathetic trunk. One doubt remained; stimulation of the trunk outside the lung after high vagal section causes a cholinergic lung contraction (Wood and Burnstock, 1967) the fibres probably arising from the cell bodies which lie within the trunk (McLean and Burnstock, 1967). The persisting NANC profiles could then conceivably have been related to the cholinergic fibres. To overcome this difficulty, the vagosympathetic trunk was cut bilaterally immediately outside the lung in the present study. After this close denervation, the only nerve fibre profiles surviving in the lung were of the NANC type. It seems clear that the NANC fibres are the vagal inhibitory postganglionic fibres. Whether the inhibitory fibres are purinergic, as suggested by Robinson et al. (1971), remains to be seen. The lack of cytochemical reactivity in the present experiments confirms their non-adrenergic nature.

Unmyelinated profiles containing many small mitochondria were termed sensory because of their similarities to nerves identified as sensory in other tissues (see Burnstock, 1972). The only piece of internal evidence from this study supporting the idea that the fibres are sensory is the fact that the fibres seemed to be myelinated, with only a short unmyelinated terminal segment. Apart from sensory fibres, the only myelinated fibres which would be expected to appear in the lung are preganglionic fibres. Such fibres should be directed towards the postganglionic neurones, which are localised toward the root of the lung, and should not appear in more distal regions of the lung.

The fifth type of fibre observed was not named. The profiles were found related to arterial vasa vasorum and to the medial smooth muscle layer of major veins. The profiles contained small and moderately large vesicles, both with cytochemically reactive granules, and numerous non-reactive dense-cored large (60–160 nm) vesicles. Similarities can be noted between the large cored vesicles and the secretory vesicles of peptide secreting cells of, e.g. the neurohypophysis (Pooley, 1971). The presence of reaction products in the smaller vesicles indicates the storage, and probably the synthesis, of biogenic amines. The potential combination of peptide and amine storage is reminiscent of the series of cells, essentially endocrine in nature, which Pearse and his colleagues have termed 'APUD' (Pearse, 1976). The relationship of the fifth type of profile to vasa vasorum is also suggestive of a neurosecretory role. However, we have no opinion as to the hormone which might be secreted or the target organ which might be affected.

The findings presented here throw light on the difficult question of pulmonary vasodilator innervation. In an organ as highly vascularised as the lung, contraction and relaxation of visceral muscle can conceivably affect vascular resistance. Thus it did not prove possible to determine from physiological observations whether pulmonary vasodepressor responses to vagal stimulation were caused by a non-

adrenergic, non-cholinergic innervation of the vasculature or of the visceral muscle (Campbell, 1971b). A comparable problem arises over the adrenergic depressor responses caused by sympathetic stimulation. This study has shown that there are adrenergic nerve fibres closely related to all muscular segments of the pulmonary vasculature. A direct vasodilator function of the adrenergic fibres is implied. However, there were no NANC profiles closely associated with any part of the vasculature. It therefore appears that the vagal vasodepressor effects in the toad lung are mediated indirectly, resulting from the relaxation of visceral muscle strands which compress vascular channels in their contracted state.

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