J. Anat. (1970), **106**, 3, pp. 449–461
With 6 figures
Printed in Great Britain

Innervation of pulmonary and bronchial blood vessels of the dog

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(*Received* 23 *April* 1969)

The physiological evidence for the vasomotor control of pulmonary and bronchial blood vessels has been recently reviewed by Daly & Hebb (1966). Many of the experiments have been done on dogs and when this study was begun the only histological studies on the innervation of dog lung blood vessels were those of Larsell (1923) using methylene blue. In the present study histochemical methods for noradrenergic and cholinergic nerve fibres have been used to demonstrate the double motor innervation of lung blood vessels. The details of nerve–muscle relationships were studied with the electron microscope. A preliminary report of this work has appeared (Fillenz, 1966).

METHODS

The material for these studies consisted of lobes of lungs removed from anaesthetized dogs. The freshly removed lung lobes were studied in a variety of ways.

Silver staining. Lobes were perfused with 10 % formalin through the pulmonary artery and then stored for between 3 weeks and 12 months in Richardson's fixative. Frozen sections 75, 100 and 150 μm thick were cut, and these were stained by either Schofield's (1960) or Richardson's (1960) silver methods. In a few cases serial sections, 100 μm thick were examined using Richardson's method.

A few blocks of formalin-fixed lung were embedded in paraffin wax, cut at 10 μm and stained with haematoxylin and Van Gieson's stain.

Cholinesterase staining. Freshly removed lung tissue was cut into slices 3–5 mm thick and put into 10 % neutral formalin. After fixation periods of 1–2 h at 0 °C the tissue was stored in phosphate buffer, pH 6·0 at 0 °C, for up to 6 d before incubation. Such storage did not produce any diminution in staining.

Lewis's (1961) method was used with acetylthiocholine chloride as substrate and 10^{-4} M ethopropazine hydrochloride as inhibitor.

The technique of Karnovsky & Roots (1964) was used with and without ethopropazine. Incubation was carried out at pH 6·0 at room temperature, for periods of 15, 30, 45 and 60 min. The 100 μm frozen sections were counterstained with methyl green after incubation and before mounting on to gelatinized slides.

Fluorescence method of Hillarp & Falck. Slices of lung 3–5 mm thick were frozen in ethanol cooled with solid carbon dioxide. They were then freeze-dried for 24–48 h at −40 °C in a Pearce Speedivac tissue drier, brought to 20 °C and then removed from the tissue drier. After exposure to paraformaldehyde of 80 % r.h. at 76–80 °C for 1 h the pieces of tissue were embedded in paraffin wax. 10 μm sections were examined in a Zeiss photomicroscope with blue light fluorescence. Gevaert Scopix G film was used for photomicrography.

Electron microscopy. Small pieces of lung were fixed in ice cold 1 % osmium tetroxide in phosphate buffer at pH 7·4. They were embedded in Araldite, thin sections were cut on a Huxley microtome and after staining with lead hydroxide (Reynolds, 1963) examined in a Phillips 200 electron microscope.

In two dogs a lung lobe was perfused through the pulmonary artery with 2·5 % glutaraldehyde in 0·1 M phosphate buffer at pH 7·4. Pieces of tissue 1 mm³ were fixed for a further ½ h in glutaraldehyde, postfixed in 1 % OsO_4, embedded and cut as above. The sections were examined in a AEI electron microscope.

RESULTS

Schofield's silver stain showed large bundles of nerve fibres running with the branches of the bronchial vessels, but no nerve fibres were seen in the walls of pulmonary blood vessels.

Richardson's silver stain was capricious but when successful stained networks of nerve fibres accompanied by Schwann cell nuclei surrounding the branches of the pulmonary blood vessels, nerve fibres encircling the bronchial blood vessels and nerve fibres running among the smooth muscle bundles and glands of the bronchial wall. The large nerve bundles and the coarse fibres, so striking with Schofield's stain, were hardly stained at all with Richardson's stain.

Using frozen section 75–100 μm thick, and in many cases cut serially, it was possible to observe the relationship of the nerve fibres to blood vessels of various diameters. Pulmonary arteries, down to 30 μm in diameter, have smooth muscle cells in their media and bundles of fine nerve fibres accompanied by Schwann cell nuclei in their wall. The nerve fibres are arranged in a loose-meshed network, spiralling around the vessel, and are always found in a different focal plane from the muscle cells. The innervation is sparse with distances of 50–60 μm between meshes of the network. The nerve fibres often run at distances of 5–10 μm from the smooth muscle coat of the artery. Vessels 200–300 μm in diameter give off side branches 40–60 μm in diameter at right angles. The opening of these side branches is usually encircled by a fine bundle of nerve fibres (Fig. 1*a–c*). At a diameter of about 30 μm the pulmonary arterioles suddenly lose their smooth muscle coat and break up into the alveolar capillary network. No nerve fibres were ever seen in the walls of vessels which had lost their muscle coat, although the bundles of nerve fibres running at some distance from a pulmonary vessel were surrounded by lung parenchyma containing numerous alveolar capillaries.

Bronchial vessels down to 10 μm in diameter have fine bundles of nerve fibres spiralling around them.

Cholinesterase staining. The use of Lewis's method resulted in staining of bronchial smooth muscle, groups of ganglion cells and nerve bundles running in the walls of bronchi. There were no nerves stained in the walls of pulmonary blood vessels nor did the smooth muscle cells stain. On the other hand Karnovsky's method with acetylthiocholine as substrate resulted in staining of the same structures as Lewis's method but in addition a network of nerve fibres surrounding branches of the pulmonary artery was stained.

The use of 10^{-4} M ethopropazine hydrochloride suppressed most of the bronchial

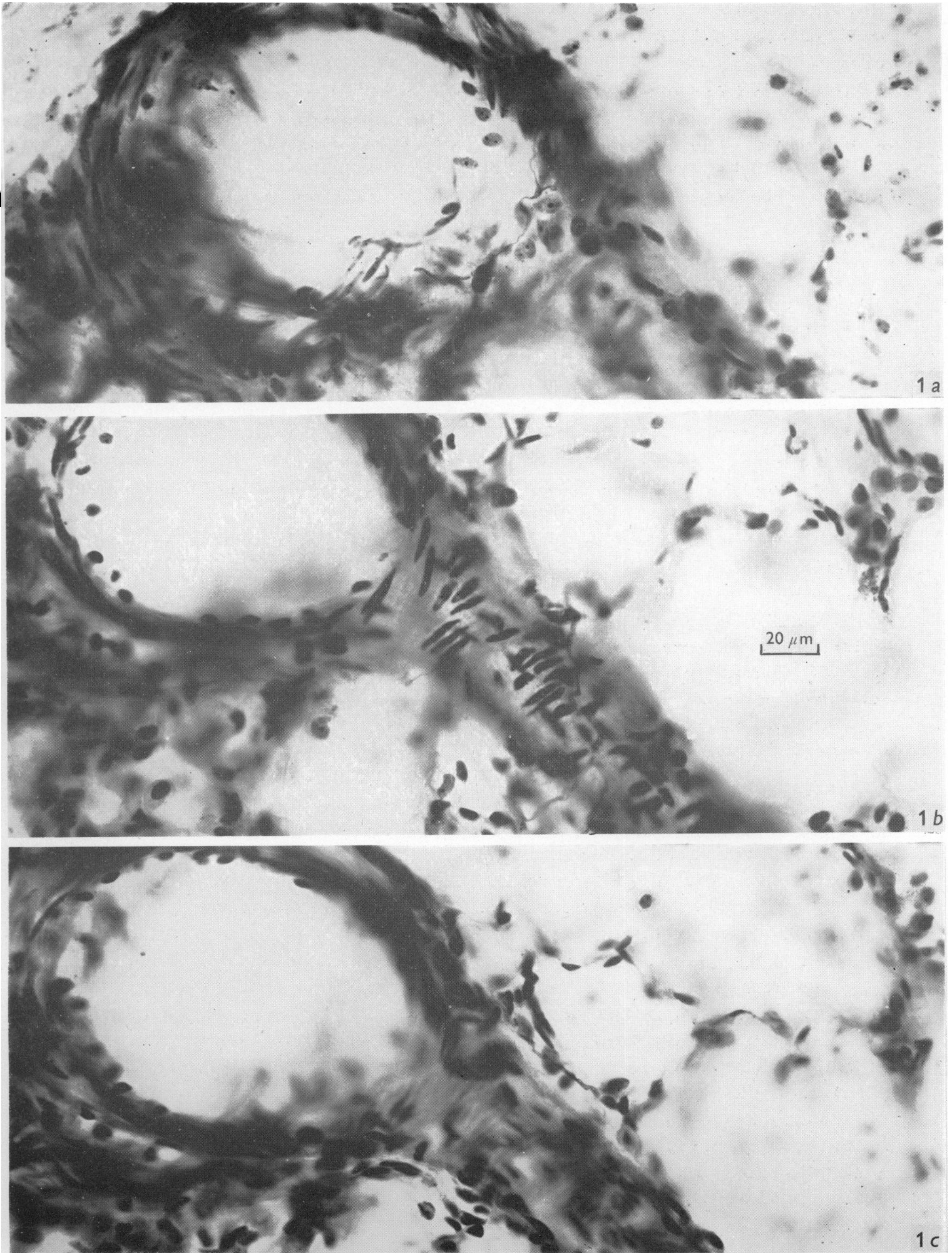

Fig. 1. Nerve bundle encircling origin of 45 μm side branch from a 100 μm pulmonary artery. *a*, *b*, *c* are at different focal depths. Richardson's silver method.

smooth muscle staining, and left a network of fine nerve fibres running among the bronchial smooth muscle, around the glands, the bronchial vessels and the branches (40–200 μm) of the pulmonary artery. The latter were identified by their course alongside bronchi and bronchioles. Pulmonary vessels running through the lung parenchyma unaccompanied by bronchioles were identified as veins and these did not have any nerve fibres containing acetylcholinesterase in their wall.

The network of nerve fibres surrounding the pulmonary arteries was less dense than that supplying the bronchial smooth muscle (Fig. 2). In order to compare the enzyme activity of the two sets of nerve fibres incubation periods of 15, 30, 45 and 60 min were tried. Faint staining appeared in both sets of nerve fibres after 15 min and intensified until it reached its maximum after 45–60 min. The nerve fibres supplying the pulmonary arteries always stained less deeply than those to the bronchial muscle. They form a network on the outer surface of the media and a fine bundle of acetylcholinesterase-containing fibres was often seen encircling the origin of a small side branch (30–40 μm) from a main vessel (200–300 μm). There were no ganglion cells or large nerve bundles related to pulmonary vessels.

Collections of acetylcholinesterase-positive ganglion cells and large nerve bundles run with bronchial blood vessels in the walls of bronchi and bronchioles. Fine nerve bundles containing acetylcholinesterase spiral around bronchial blood vessels down to 10 μm in diameter.

Fluorescence method. The method of Falck & Hillarp showed fluorescent nerves in the walls of airways and surrounding blood vessels. In addition to the specific fluorescence of the nerve fibres there was yellow autofluorescence in widely distributed cells and green autofluorescence in elastic tissue. The latter was found in the elastic laminae of the large pulmonary arteries, in the walls of medium-sized to small pulmonary veins, in a dense layer of fine elastic fibres underlying the epithelial lining of the airways, in the walls of the alveoli and under the pleura. The distinction between elastic fibres and fluorescent nerves is not easy to make when they are cut transversely and appear as dots. Although most of the fluorescent fibres in the lung parenchyma are elastin, it is not possible to exclude the presence of a few scattered nerve fibres.

Both pulmonary arteries (30–300 μm) and the larger veins showed fluorescent nerve fibres in their walls. The nerves were brightly fluorescent and coarse and did not usually appear clearly varicose (Fig. 3). They formed a loose network best seen in tangential sections of the blood vessel. In longitudinal sections through the blood vessels the meshes of the nerve net appeared as dots 50–100 μm apart. The large branches of the pulmonary artery have a musculo-elastic media, with a number of elastic laminae. In these vessels the fluorescent nerve fibres penetrate the outer third of the media. As the pulmonary arteries become smaller they have a purely muscular media and the nerve fibres run entirely outside the media. The thin-walled pulmonary veins which have elastic tissue among the smooth muscle cells of the media also have nerve fibres only at the medio-adventitial junction. Fluorescent nerve fibres were often seen encircling the opening of a side branch from a main vessel. No nerve fibres were seen in the walls of pulmonary vessels less than 30 μm in diameter.

The bronchial vessels are accompanied by large, dimly fluorescent nerve bundles and encircled by brightly fluorescent nerves. No fluorescent ganglion cells were seen.

All the fluorescent nerves showed a blue-green fluorescence, which faded very slowly over periods of many weeks and was fully developed after exposure to paraformaldehyde for only 1 h. These are the characteristics of a primary catecholamine.

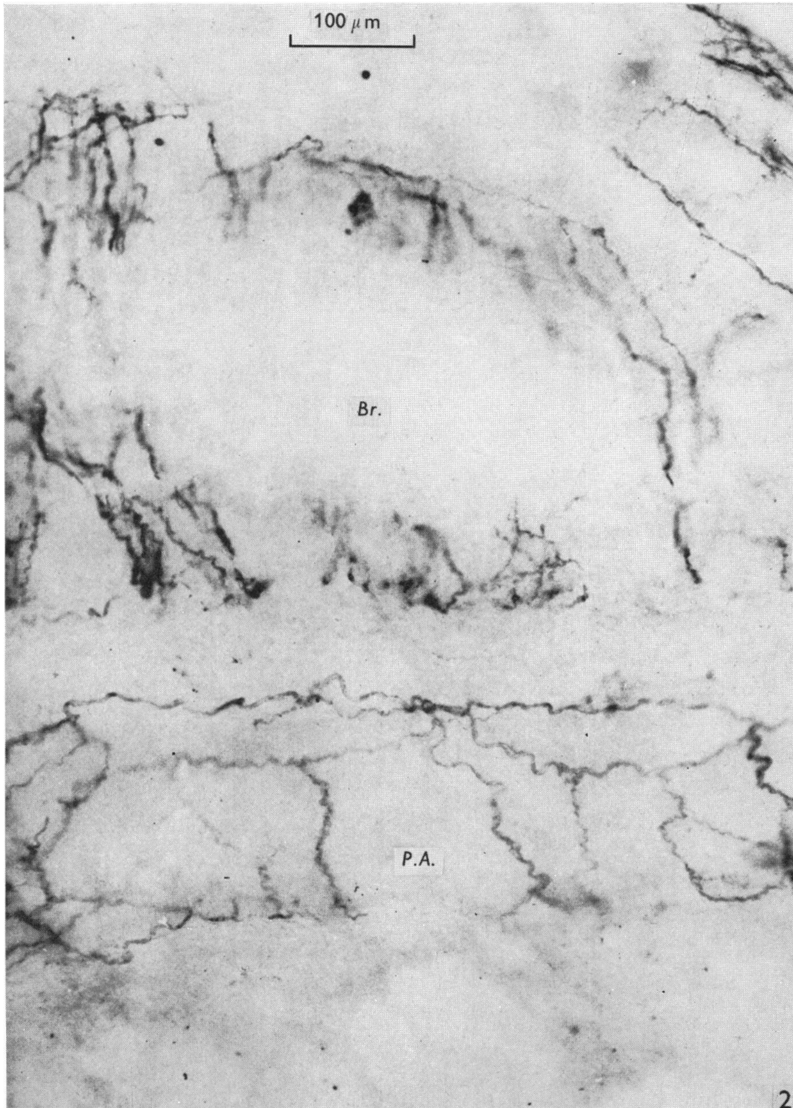

Fig. 2. Network of acetylcholinesterase-containing nerve fibres around branch of pulmonary artery accompanying a bronchiole. *Br.* bronchiole in tangential section. *P.A.* pulmonary artery. Karnovsky's cholinesterase method.

Electron microscopy. All blood vessels are difficult to fix and cut for electron-microscopy (Pease & Molinari, 1960), and the difficulties are even greater with pulmonary vessels which have very little tissue to support them: the blood vessel

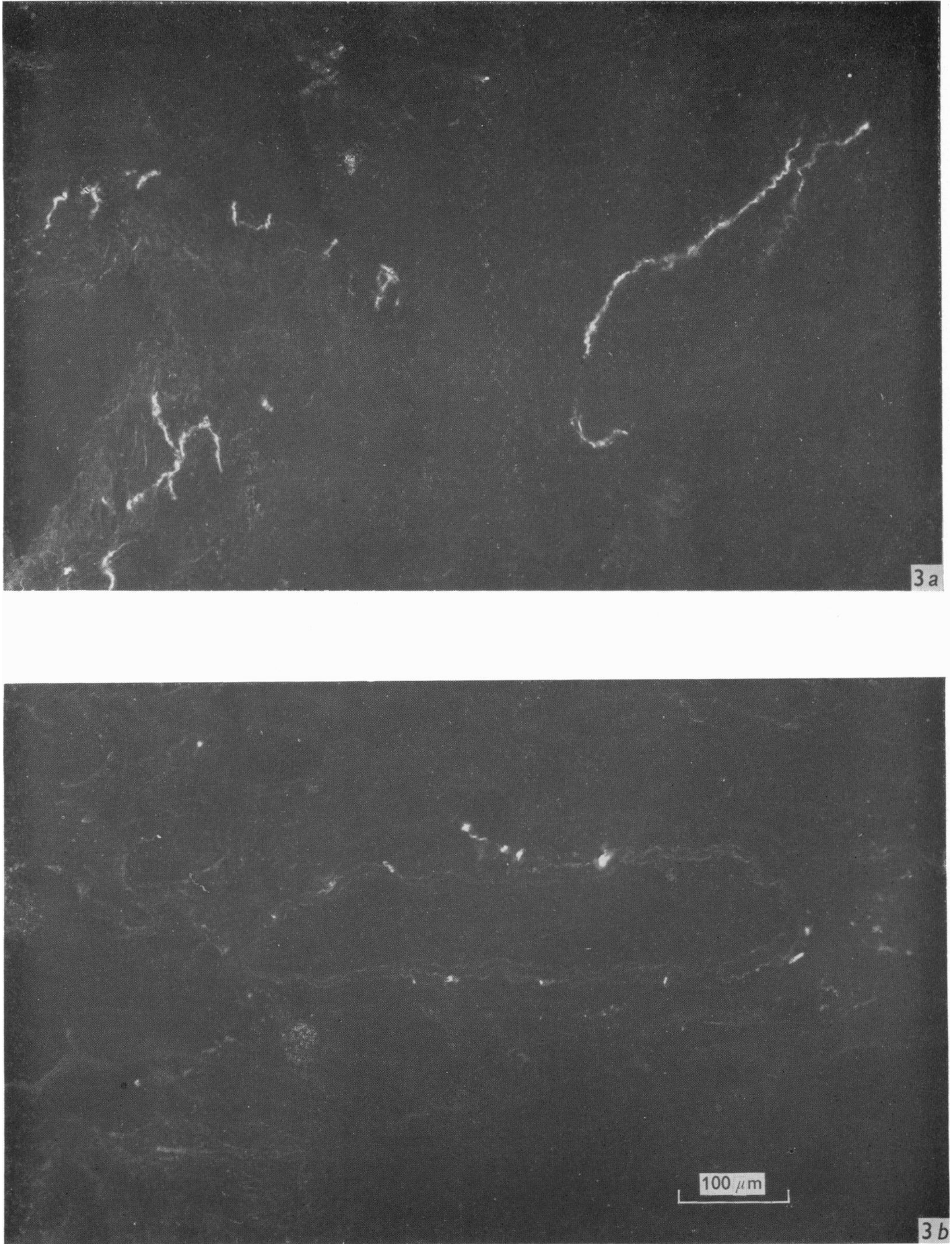

Fig. 3 (*a*) Tangential section of pulmonary artery surrounded by fluorescent nerve fibres. (*b*) Longitudinal section of 100 μm pulmonary artery with 37 μm side branch encircled by fluorescent nerve bundle. Fluorescent method of Falck & Hillarp.

wall tends to tear away from the surrounding lung parenchyma during fixation and the uneven density of the tissue tends to produce sections of uneven thickness. Also the sections tend to disintegrate in the electron beam. Twelve pulmonary vessels between 37 and 300 μm in diameter and four bronchial blood vessels between 9 and 30 μm in diameter were examined. Some of the sections were mounted on formvar grids and these were more resistant to the electron beam.

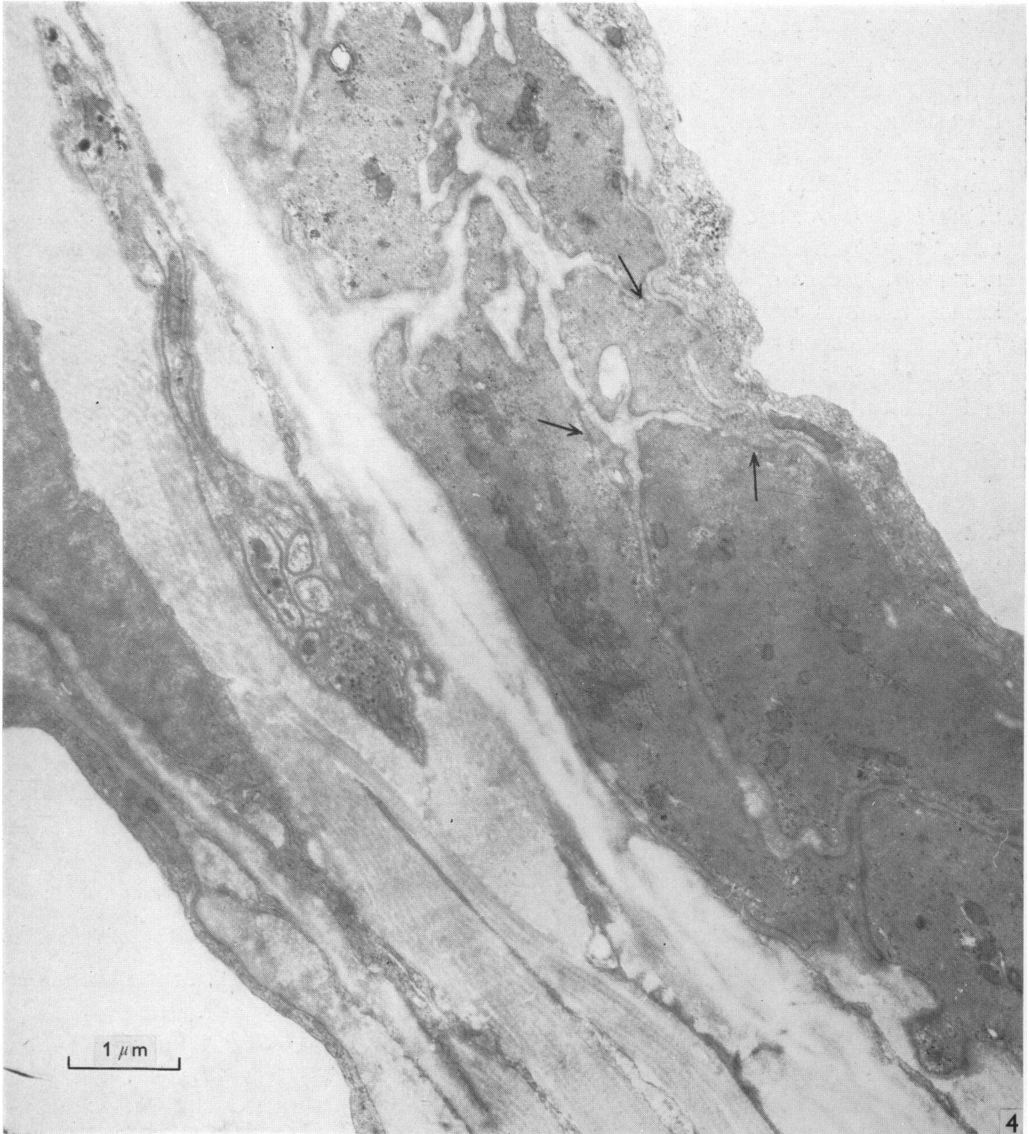

Fig. 4. Wall of pulmonary artery with axon bundle at distance of 1 μm from outer edge of smooth muscle cells. Close contacts between muscle cells at ↑. Glutaraldehyde fixation.

The tunica media of the pulmonary arteries and arterioles consists of one or more layers of tightly packed smooth muscle cells (Fig. 4). Each muscle cell is surrounded by a layer of basement membrane, but in places the intercellular gap between adjacent cells was reduced to 11 nm and the basement membranes disappeared. Close contacts between smooth muscle cells and processes of endothelial cells were occasionally seen. Bundles of non-myelinated nerve fibres enclosed by Schwann cells were found at the outer edge of the media. In one case such an axon bundle was followed for a distance of 42 μm along the edge of the tunica media in a montage. The axons varied in diameter and contents: lengths of axons containing little besides neurofilaments alternated with portions of axons containing mitochondria and vesicles. The vesicle-rich portions were sometimes partially denuded of Schwann cell cytoplasm; however, they did not necessarily lie on the side of the bundle facing the vessel wall. The axon bundles ran at distances of up to 4 μm from the outer edge of the smooth muscle cells, from which they were separated by the external elastic lamina and by processes of fibrocytes. Occasionally the external elastic lamina disappeared and vesicle-filled axons approached to within 150 nm of the smooth muscle cells. But more frequently a smooth muscle cell was seen to send out a process across the external elastic lamina towards a vesicle-filled axon partially denuded of Schwann cell cytoplasm. Fig. 5 shows such a process where the nerve–muscle gap is 100 nm; this gap is filled with basement membrane material. The muscle processes were usually rich in pinocytotic vesicles. The vesicle-filled axons were of two kinds: those containing uniformly-sized agranular vesicles 45–50 nm in diameter; and axons containing vesicles ranging from 50–100 nm in diameter, many of the larger vesicles having cores of variable electron density. The sections from glutaraldehyde-perfused lung lobes contained an additional group of vesicles, similar in size to the agranular vesicles, and with a small, very dense core.

Fig. 6 shows the wall of a pulmonary vein 42 μm in diameter. The smooth muscle cells are not tightly packed, but are separated by bundles of collagen fibres. The innervation is very sparse since many grid squares showing vessel wall contained no nerve fibre profiles. Some nerve bundles were, however, found in the adventitia. These were at distances of 1–1·5 μm from the smooth muscle cells and separated from them by collagen fibres and processes of fibrocytes. The axons contained both large and small dense-cored vesicles. The smooth muscle cells contained clusters of electron-dense granules 15–20 nm in diameter. Although the cells were separated by connective tissue there were numerous close contacts between processes of adjacent cells.

DISCUSSION

Pulmonary arteries have fewer smooth muscle cells in their media than bronchial arteries of corresponding diameter and the smallest vessels with a smooth muscle coat are 30 μm in diameter in the pulmonary circulation and 10 μm in the bronchial circulation. These vessels have been called arterioles in the present study.

Richardson's silver stain showed that all pulmonary and bronchial vessels containing smooth muscle cells in their wall are surrounded by a loose, large-meshed network of nerve fibres at the outer edge of the media. The presence of fine nerve bundles encircling the origin of pulmonary arterioles suggests that this may be an

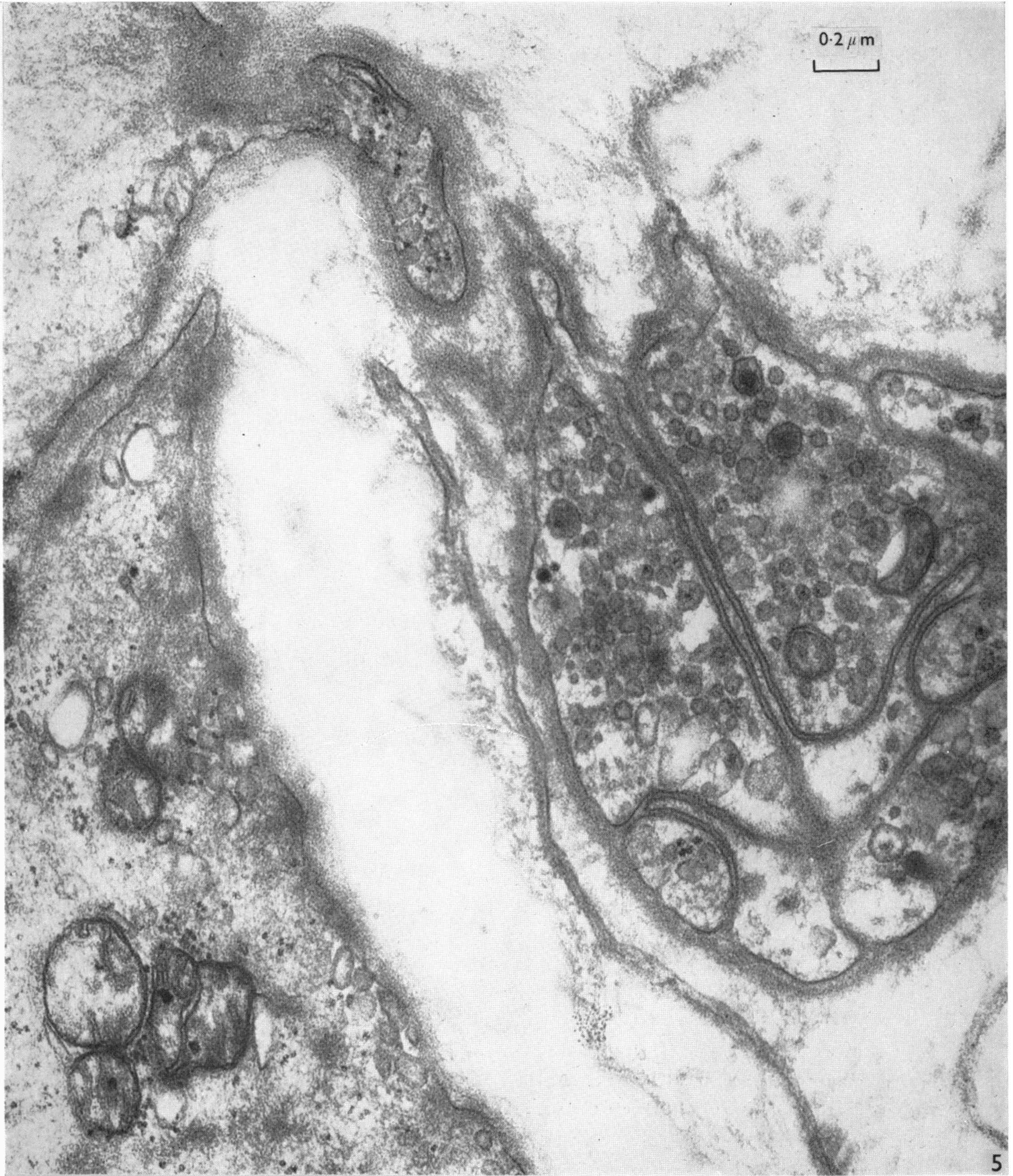

Fig. 5. Smooth muscle process approaching within 160 nm of axon bundle. Osmium fixation.

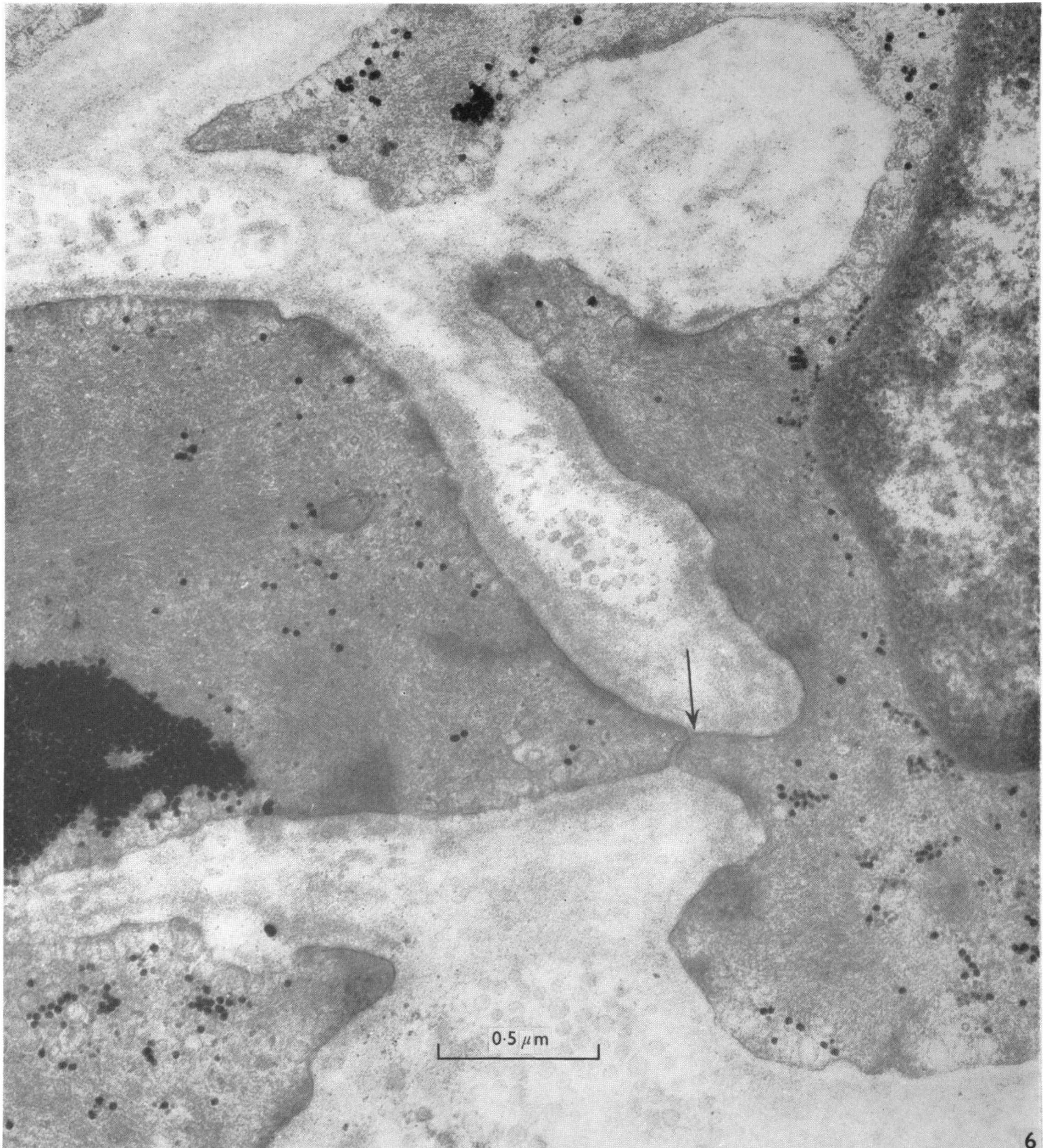

Fig. 6. Smooth muscle cells in wall of pulmonary vein are separated by collagen and make close contact at ↑. Glutaraldehyde fixation.

important point for controlling blood flow through portions of the alveolar capillary network.

Karnovsky's method showed acetylcholinesterase-containing nerve fibres in the wall of bronchial and pulmonary arteries but not pulmonary veins. The nerves to pulmonary arteries showed less enzyme activity than those to the bronchial vessels and bronchial muscle; their failure to show up with Lewis's method (Fillenz, 1966) is probably due to the greater inactivation of the enzyme at the low pH used in this method. Although long incubation periods with acetylthiocholine causes staining of noradrenergic sympathetic fibres in some species (Jakobowitz & Koelle, 1965), staining after incubation periods of 1 h has so far only been reported in cholinergic nerve fibres. The present results therefore imply that bronchial and pulmonary arteries in the dog receive cholinergic innervation whereas pulmonary veins do not. Hebb (1965) using Koelle's and Lewis's methods found some nerves containing acetylcholinesterase to both intrapulmonary arteries and veins in the dog.

The fluorescence method demonstrated the noradrenergic innervation of bronchial and pulmonary blood vessels. Whereas with cholinergic fibres the only difference between stem fibres and nerve terminals is their relationship to the effector cell, with fluorescent fibres there are other criteria. The stem fibres running in large bundles alongside the bronchial blood vessels were dimly and evenly fluorescent. The nerve fibres encircling the blood vessels were brilliantly fluorescent and uneven in diameter and brightness. Varicosities such as are seen in the iris (Malmfors & Sachs, 1965) and vas deferens (Sjöstrand, 1965) were not seen because the axons are never single but run in bundles.

The autonomic nerve terminal is a length of axon characterized by a high content of transmitter (Dahlström & Häggendal, 1966) and hence brilliant fluorescence; at the electron microscopic level such terminal fibres are found to contain accumulations of vesicles (Richardson, 1962). In many tissues such terminal fibres form close neuromuscular contacts (Richardson, 1962, 1964). The nerves encircling the pulmonary vessels had the characteristics of nerve terminals, but they ran at distances of up to 4 μm from the smooth muscle cells. Smooth muscle processes which come to within 1·0 μm of vesicle-filled axons may represent key points in the nervous control of the blood vessel wall. More distant smooth muscle cells could be reached by two possible routes: electrical spread across the close intercellular contacts, or diffusion of transmitter across the tissue spaces. Evidence for electrical spread has been obtained for a number of smooth muscle tissues (Tomita, 1967; Kuriyama, Osa & Toida, 1967*a*, *b*). That noradrenaline can diffuse across considerable distances is shown by its appearance in the venous blood of the spleen on nerve stimulation (Brown & Gillespie, 1957).

The present findings agree with other electron microscopic studies of blood vessel innervation (Pease & Molinari, 1960; Appenzeller, 1964; Brettschneider, 1964; Lever, Graham, Irvine & Chick, 1965; Simpson & Devine, 1966; Verity & Bevan, 1968), in showing that the innervation of blood vessels consists of bundles of vesicle-filled terminal fibres running at the outer edge of the media. Only Rhodin (1967) described axons among the smooth muscle cells of the media and forming close contacts with them; however, none of the axon profiles contained any vesicles, and are therefore not likely to be motor nerve endings.

SUMMARY

1. The innervation of pulmonary and bronchial blood vessels in the dog was studied with silver methods, histochemical methods for cholinergic and noradrenergic nerve fibres and electron microscopy.

2. Pulmonary and bronchial arteries are innervated by both cholinergic and noradrenergic fibres, whereas pulmonary veins have only a noradrenergic innervation.

3. The innervation of pulmonary blood vessels is sparse but the origin of pulmonary arterioles from larger arteries is usually encircled by noradrenergic and cholinergic nerve fibres.

4. Electron microscopy shows bundles of vesicle-filled terminal fibres running at distances of 0·15–4·0 nm from the outer edge of the smooth muscle cells. Occasionally smooth muscle cells send a process to within 100 nm of a vesicle-filled axon.

5. Axons contain either agranular vesicles of 45–55 nm in diameter, or two kinds of dense-cored vesicles, 45–55 and 85–100 nm in diameter.

6. There are numerous close intercellular contacts between adjacent smooth muscle cells.

I wish to thank Dr I. de B. Daly for helpful discussion and for reading the manuscript, and Mrs Carol McAllister for her skilled technical assistance.

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