THE AUTONOMIC NERVE SUPPLY OF BONE*

AN EXPERIMENTAL STUDY OF THE
INTRAOSSEOUS ADRENERGIC NERVI VASORUM IN THE RABBIT

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The anatomy of the autonomic sympathetic vasomotor nerve supply of bone was studied in rabbits by methods of histochemistry, and fluorescent and electron microscopy. Our observations show that the intraosseous vessels are richly supplied by adrenergic nerves. The large primary nerves are located on or about the surface of the vessel; the medium sized secondary nerves spiral around the long axis of vessels lying more deeply in the tunica adventitia; and the fine tertiary nerves form a rich plexus at the outer area of the tunica media. The tertiary nerves have various structures which probably contain neurotransmitter substance—that is, noradrenaline—and function as neuro-vasomuscular synapses. The sympathetic nerve supply of bone originates from the appropriate ganglion, and in the case of the tibial diaphysis it descends through the sciatic nerve and thereafter mainly through the medial popliteal nerve and enters the bone alongside the nutrient artery.

It is now generally accepted that bone is richly supplied with myelinated and unmyelinated nerves (Ottolenghi 1901; Hurrell 1937; Miller and Kasahara 1963; Sherman 1963), a concept which is most commonly invoked to explain the phenomenon of bone pain (Sherman and McFarland 1965) and to support the theory of a neural component in the control of bone blood flow (Drinker and Drinker 1916). But despite its far-reaching clinical and physiological implications, we lack a clear understanding or definition of this nerve supply. The physiological basis of the vasomotor effect on bone of various extraosseous nerves is well established (Shim 1968), but not so its morphological or anatomical correlations. This is in marked contrast to other organs, and in particular the intracranial vessels (Peerless, Yasargil and Kendall 1972), where increasing interest in the anatomy, physiology, biochemistry and pharmacology of local blood flow has resulted in a simultaneous and correlative acquisition of knowledge from both the anatomical and physiological standpoints.

This deficiency in our understanding of bone prompted us to embark on an investigation of the autonomic nerve supply of bone, with particular reference to its blood supply. Accordingly, we undertook a study of the anatomy, both in microstructure and in ultrastructure, of the intraosseous nervi vasorum, both adrenergic and cholinergic.

The purpose of this paper is to establish a sound morphological basis to the role of the sympathetic division of the autonomic nervous system in the control of bone blood flow, and the text will therefore deal solely with the adrenergic or purely sympathetic intraosseous nerves. Our observations regarding the cholinergic nerves will appear in a future publication, after further investigation. By this means we hope to complement our current body of knowledge regarding the physiology of blood flow in bone, and also to proffer some insight into the mechanism of bone pain.

MATERIAL AND METHOD

The intraosseous extent of the nutrient diaphysial artery of the tibia formed the basic experimental model, and was studied on both sides in forty New Zealand white rabbits, each weighing 2 to 3 kilograms. Under intravenous Nembutal anaesthesia this vessel was carefully removed from both tibiae after unroofing the cortex of the shaft, and then micro-dissected and cleared of marrow under stereomicroscopic control.

Histochemical preparation—The technique developed by Falk, Hillarp, Thieme and Torp (1962) and subsequently modified by Malmsors (1965) was used. Each blood vessel was carefully spread and whole-mounted on a glass slide. The initial microdissection in each case was conducted under cold Tyrode’s solution adjusted to a pH of 7.4. The whole-mounts were then exposed to anhydrous phosphorus pentoxide under vacuum for two hours at room temperature, and then transferred to a separate reaction vessel under conditions of low humidity where each was exposed to paraformaldehyde gas at 80 degrees Celsius for one hour.

In this reaction noradrenaline becomes embedded in situ in a dry protein layer, and is then converted to a dihydroxyquinoline or tri-hydroxyquinoline compound which fluoresces intensely in ultra-violet light (maximally at a range of 470–480 m). This highly sensitive technique renders structures that contain noradrenaline selectively visible under ultra-violet light. The vessels in this study were examined by a Zeiss Ultraphoton II fluorescence microscope.

Electron microscopy—In preparation for electron microscopy the hind limbs of the living anaesthetised animal were infused with 2-5 per cent glutaraldehyde, after preliminary clearance of blood with a bolus of physiological saline. The infusion was carried out through the aorta proximal to its abdominal bifurcation, and the inferior vena cava was allowed to bleed.

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freely into the peritoneal cavity. The tibial diaphysial vessels were then removed and cleared of marrow as described above. Each vessel was then further exposed to glutaraldehyde by immersion for a minimum of two hours, and post-fixed in Palade's osmium fixative, also for a minimum of two hours. Selected segments were then dehydrated in alcohol, embedded in epon, and stained with uranyl acetate and lead citrate. Ultra-thin sections were prepared from the segments, and were examined with a Philips 200 electron microscope.

A large nerve entered bone alongside the nutrient artery. It lay just outside or within the outer limits of the tunica adventitia, and dividing accordingly, it accompanied the major branches of the nutrient vessel inside bone (Fig. 1). This primary nerve contained many fluorescent fibres, and repeatedly gave off branches to the vessel wall. As it came to accompany the small vessels inside bone, and following its many divisions and branches, this nerve became successively smaller and contained fewer sympathetic fibres.

The next and indeed the most striking feature was an immensely rich network of small fibres which looped around the vessel wall perpendicular to its long axis (Fig. 2). This system lay deep within the vessel wall, but was consistently separated from the internal elastic lamina by a distance of fairly uniform depth. On transverse section these fibres were again separated from the elastic lamina by a uniform distance, and in fact for the most part they lay at the junction of the tunica media with the tunica adventitia. None was found within the tunica media itself, and these nerves were therefore considered terminal or tertiary in type.

The density of the fine network of tertiary fibres as seen within the vessel wall was related to the size of the vessel, and to its proximity to the nutrient portal of entry into bone. As the vessel divided and became smaller, the network became less complex and the fibres more obliquely oriented. These fibres, however, retained a characteristic morphological appearance throughout, as each fibre expanded at intervals to give a beaded or varicose appearance, each varicosity corresponding not only to a segment of increased size, but of more intense fluorescence as well (Fig. 3).

Within the walls of the smaller vessels, the varicose fibres mingled with a system of longitudinally oriented nerves. These latter nerves had been observed on the more proximal vessels, but with difficulty, as they were partly obscured by the density of the varicose network. These nerves were typically larger, non-varicose, spiralled around the vessel wall in a helical fashion, and in both size and position they were obviously intermediate to the larger primary and smaller tertiary nerves already described. Along with the primary nerves, this secondary type was responsible through a series of many branches for both the formation and maintenance of the finer varicose plexus.

The origin and course of the sympathetic fibres were studied. Transperitoneal sympathectomy at the mid-lumbar level uniformly failed to affect the microstructure and organisation of the nervi vasorum of the tibial nutrient vessels, when these vessels were examined two weeks later. A lower sympathectomy was obviously indicated, but rendered difficult in terms of

RESULTS

Microstructure and organisation of the adrenergic nerves

It soon became obvious that the blood vessels inside bone were richly endowed with sympathetic fibres. Even some of the smallest vessels that had been preserved by the whole-mount technique, branches down to 12 microns in diameter that had remained attached to the parent stem, were accompanied by fluorescent strands. Initially the sympathetic fibres seemed to run parallel, perpendicular and oblique to the long axis of the vessels, and furthermore appeared to vary considerably in size and shape. However, on more detailed and repeated analysis of many specimens this random spatial and morphological configuration receded, and was replaced by a pattern of organisation that will now be described.
primary nerve type. The unmyelinated components typically contained many small axons, each ensheathed by a common Schwann cell plasmidum. In contrast, the myelinated axons were large and single and each axon was surrounded by a separate multi-layer of electron-dense lipoprotein, this density attributable to the affinity of myelin for osmium tetroxide, which had been used as a post-fixative.

Smaller secondary nerves were next seen, lying more deeply within the vessel wall. These nerves were unmyelinated, were devoid of perineurium, and for the most part lay within the outer half of the tunica adventitia, separated from the outermost smooth muscle cell of the tunica media by a number of interposed fibroblasts. Each contained a variable number of small axons sharing a common Schwann cell coat, and in configuration they were similar to the unmyelinated components of the large primary nerve.

Many other nerves of varying size and axon content were observed throughout the adventitia, but a characteristic third type was found within its inner half. These very small nerves usually retained a thin covering of Schwann cell cytoplasm and contained one or very few axons. They closely approximated the outer plasma membrane of the outermost smooth muscle cell by a distance which in most instances had a lower limit of 3,000 Å (Fig. 4). In a few instances the neuromuscular relationship was even more intimate, with fusion of the

surgical accuracy, morbidity, and mortality by the rich regional vascular anatomy. The upper limb was therefore used, and in a series of animals unilateral stellate ganglionectomy was carried out on the right side, and the nutrient vessels of the right and left radius were examined two weeks later. On the right side, the vessels were either totally devoid of fluorescent fibres, or almost so, in each case.

Similarly, in two small groups of animals the tibial nutrient vessels were examined two weeks after selective peripheral neurectomy. Unilateral sciatic neurectomy caused a total ipsilateral loss of fluorescent fibres in every case, while medial popliteal neurectomy caused a profound, but incomplete loss of fibres, chiefly of the small varicose type. There was furthermore a fall off in intensity of fluorescence in the remaining fibres.

Ultrasound
On electron microscopy the nervi vasorum presented a pattern of organisation that could be defined, and furthermore correlated to the earlier findings on fluorescent and light microscopy.

Firstly, a large nerve was commonly seen just within or outside the tunica adventitia. This nerve contained a varied number and proportion of myelinated and unmyelinated fibres, all enclosed by a common perineurium, the latter a characteristic feature of this

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**Fig. 3**
High magnification fluorescent micrograph of the tertiary plexus of sympathetic nervi vasorum. The varicose outline of each fibre is obvious, each varicosity corresponding not only to a segment of increased size, but of more intense fluorescence as well.

**Fig. 4**
Electron micrograph. A small tertiary nerve containing four axon profiles, with partially interposed Schwann cell cytoplasm, is seen separated from the plasma membrane of the outermost smooth muscle cell (M) by a distance of 3,000 Å.
contiguous basement membranes of nerve and muscle, and in one case the nerve lay within a trough on the surface of the muscle cell and formed a loose junction with its plasma membrane.

The axons within these small tertiary nerves clearly contained neurofilaments, neurotubules, and occasional mitochondria. In addition, these axons were commonly enlarged and contained many mitochondria and a collection of vesicles varying from 300 to 800 Å in diameter (Fig. 5). Such vesicles, in extraosseous locations, are considered synaptic in function by most authorities, and are responsible for chemical transmission of the nerve impulse to adjacent nerve, muscle or glandular cells, by virtue of their content of noradrenaline if adrenergic in type (Grillo 1966; Devine and Simpson 1968; Bloom and Crayton 1972).

In an effort to substantiate this view, one animal was given 5-hydroxydopamine (SHDA), 50 mg/kg, by the intraperitoneal route four hours before death. It was felt that two of the properties of this derivative of dopamine could be used to advantage at this stage of the investigation. Firstly, 5-HDA is electron-dense in high concentration, and therefore visible by electron microscopy; and secondly, it is known to accumulate within the synaptic vesicles of sympathetic fibres only (Tranzer and Thoenen 1967). Examination of serial sections of the intraosseous vessels of this animal revealed that the nervi vasorum had an ultrastructural form and organisation identical to those of the other animals. In distinctinction, however, most synaptic axonal enlargements now contained small dense-cored vesicles due to accumulation of 5-HDA (Fig. 6). By this means, it was demonstrated clearly that the sympathetic system actively participated in the neuromuscular synaptic arrangements seen at this level.

DISCUSSION

Many investigators have demonstrated the evidence on which is based the concept of a neural influence in the control of bone blood flow. Drinker and Drinker (1916) cannulated the tibial nutrient vessels in dogs and demonstrated a decrease of bone blood flow by stimulation of the nerves to bone. Shim and Patterson (1967) reproduced this effect by stimulation of the sciatic nerve, and other authors (Herzig and Root 1959; Azuma 1964) have achieved similar results using more indirect means. An increase of 15 to 110 per cent in bone blood flow was demonstrated by Trotman and Kelly (1963) and Yu, Shim and Hawk (1972), after lumbar sympathectomy in dogs, and Shim, Copp and Patterson (1966) found that blood flow was increased by up to 45 per cent in the bones below the knee by sciatic nerve section in rabbits. It is therefore reasonable to accept that extraosseous vasomotor nerves exert a considerable influence on the rate of blood flow to or within bone, at least in the experimental situation. The rank of this neural component in the hierarchy of other controlling factors, particularly hormonal and metabolic, is however uncertain, and it seems likely there is a delicately balanced...
interaction between all three in this regard. From the morphological standpoint Gros (1846), who is generally attributed with the first description of nerves in bone, described how nerves entered bone alongside the nutrient artery and remained closely associated with the blood vessels within. Variot and Remy (1880) were the first to suggest that two nerve types, myelinated and unmyelinated, could be distinguished in bone, and de Castro (1930) and Ottolenghi (1901), with increasing clarity described how the nonmyelinated components ramified within the tunica adventitia.

Overall, and in marked contrast to other organ systems, there has been a slow and somewhat meagre acquisition of knowledge, and, so far, a failure to achieve a unified and definitive description of the nerve supply of bone as an organ, and as a tissue. This is largely attributable to the nature of bone itself, because deossification damages the finer features of its nerve supply. In addition, the techniques of investigation until now have been largely confined to the silver cyanide and methylene blue methods, both notoriously capricious and unreliable in demonstrating more than a fraction of the nerves in the field under examination.

Our method was designed to achieve the maximum specificity and sensitivity, and to this end both fluorescent histochemistry and electron microscopy were used. The reliability of both methods in extraosseous locations is well proven. Furthermore, to avoid the effects of deossification, the blood vessels were simply removed from bone before processing.

The concept of chemical synaptic transmission of nerve impulses is now settled. The discovery by Falk et al. (1962) that noradrenaline may be converted to intensely fluorescent products by formaldehyde gas treatment, subsequently applied and modified by Malmfors (1965) rendered this neurotransmitter directly visible under the microscope. Noradrenaline is the principal transmitter substance in the post-ganglionic sympathetic nerve, and strong evidence has been presented by Dahlström and Hågglund (1966) to suggest that it is formed in the cell body and transported down the axon within transmitter granules to be stored within terminal-like axon enlargements which appear as intensely fluorescent varicosities on fluorescent microscopy. Overall, as Norberg (1967) described, the structure and organisation of the sympathetic plexus within the blood vessel wall, the concentration of noradrenaline in the varicosities, the reaction of the varicose terminals to nerve section or stimulation, and their response to certain drugs leave little doubt that each varicosity is a structure specialised for the storage and release of noradrenergic transmitter.

Electron microscopy (Grillo 1966; Nielson, Owman and Sporrong 1971; Bloom and Crayton 1972), applied to blood vessels of many tissue beds has also served to support the storage-release hypothesis, and has revealed the varicosities to form "en-passage" or non-terminal synaptic junctions with the smooth muscle effector cells. The use of 5-hydroxydopamine has been particularly rewarding, as shown by Tranzer and Thoenen (1967). This compound seemingly displaces noradrenaline from the sympathetic terminals and on account of its high reactivity with osmium tetroxide caused opacification of the synaptic vesicles within sympathetic terminals. Along with electron microscopy this substance has served to establish the small granular or dense-cored vesicles within the axonal expansions as the site of noradrenaline storage. Supplementary evidence for this view is based on cell fractionation studies, electronmicroscopic autoradiography, and ultrastructural studies following drug manipulation or pre-treatment (Devine and Simpson 1968).

The express purpose of this paper is to establish a sound morphological basis to the role of the autonomic nervous system in the control of bone blood flow. The accumulated evidence and conclusions concerning the histochemical and electron microscopic localisation of the autonomic nervous system in organ systems apart from bone, as they relate directly to our investigation, have been outlined above. Judging by this body of information, it seems that the results of this study satisfy the purpose of this paper, and justify a report at this time.

In the upper limb, the sympathetic fibres destined for bone were demonstrated to take origin from the appropriate sympathetic ganglion. In the lower limb, selective peripheral neurctomy revealed that the sympathetic nerves, with reference to the tibia, descended in the sciatic nerve, and thereafter principally in the medial popliteal nerve, and entered bone alongside the nutrient vessels (Fig. 7).

Inside bone, the microstructure and organisation as seen on fluorescent microscopy could be considered in three parts (Fig. 8). Large primary nerves, associated with the outer limits of the tunica adventitia, accompanied the nutrient artery and its branches. Smaller secondary nerves spiralled around the vessel axis in a helical fashion lying more deeply within the vessel wall, and in conjunction with the primary trunks, through a complex system of branches, formed and maintained the tertiary network which was arranged as a rich plexiform system of varicose fibres at the junction of the tunicae adventitia and media. Each varicosity in the noradrenergic preparation corresponded with a widened segment of more intense fluorescence, amply indicating the increased concentration of noradrenaline at that point. The tertiary, or terminal effector network itself was ideally situated therefore to affect the tone of the tunica media, and therefore the diameter of the vessel lumen, by the release of neurotransmitter.

Similarly the ultrastructural organisation could be considered in three parts (Fig. 9). Again a large primary nerve was seen associated with the outer reaches of the tunica adventitia, and this nerve contained a varied
A proportion of myelinated and unmyelinated nerves. Smaller secondary nerves were located within the adventitia, but confined to its outer half. These did not contain synaptic-like arrangements and were unmyelinated. These may well account for the secondary, small, but non-varicose preterminal fibres visualised by histochemical methods. Finally, very fine tertiary nerves were seen within the inner half of the tunica adventitia. These contained one or very few axons, typically of two distinct types. Firstly, small axons were seen which contained a variable number of neurofilaments, neuritubules and occasional but single mitochondria. Second, and in considerable contrast, the axons became expanded apparently to accommodate a large number of loosely packed mitochondria and vesicles of both the small and large variety. Both these axonal structures closely approximated the outer smooth muscle cell, at which point the expanded axons assumed the morphological features of a neuromuscular synapse. It is tempting to assume that the small and large (or expanded) components of the tertiary nerves correspond with the inter-varicose and varicose segments respectively of the tertiary fibres seen on fluorescent microscopy. Obviously the vesicles within the enlarged axons are synonymous with the synaptic vesicles that have been described in other locations. Pre-treatment with 5-HDA supported this point and clearly indicated the participation of the sympathetic system in the terminal effector plexus and the likely synonymity of this plexus with the sympathetic fluorescent tertiary meshwork seen in the noradrenergic histochemical preparation.

To our knowledge this study represents the first application to bone of recent advances in the field of neurological research. By these means we have traced the origin and course of the sympathetic fibres destined for bone, and in detail defined the microstructure, ultrastructure and three-layered organisation of the nervi vasorum inside bone. Furthermore, we believe that we have established a clear and reliable morphological correlate to the increasing volume of direct and indirect experimental evidence supporting the role of the sympathetic division of the autonomic nervous system in the control of bone blood flow.

Regarding the specific question of bone pain, and what role the intraosseous nervi vasorum may play in its perception and transmission, we have to rely on the evidence of other workers. There is no doubt that bone is richly supplied with pain sensitive fibres, and we may thus explain the pain associated with inflammatory, neoplastic and degenerative lesions in bone. Helal (1965), Phillips (1966), Trueta (1968), and Arnoldi, Linderholm and Müßbichler (1972) implicated vascular engorgement in the genesis of such pain in osteoarthritis, and Sherman and McFarland (1965) and

FIG. 7—Diagrammatic outline of the sympathetic nerves. The nerve fibres destined for bone take origin within the appropriate sympathetic ganglion, and with reference to the tibial diaphysis, descend in the sciatic nerve, thereafter principally in the tibial nerve, and then enter bone in large primary nerves which accompany the nutrient vessels. Figure 8—Diagrammatic outline of the microstructure and organisation of the intraosseous adrenergic nervi vasorum. At the top, a cross-section of the blood vessels showing lumen (L), endothelial lining and internal elastic lamina (I), tunica media (M), and tunica adventitia (A). A large primary nerve accompanies the nutrient artery and its many branches, located just within or outside the tunica adventitia. Smaller secondary nerves, lying within the adventitia, spiral around the vessel axis in a helical fashion. Very small and varicose tertiary nerves compose a rich terminal effector plexus located at the junction of the tunica media and adventitia. Within the wall of the smaller vessels, the secondary nerves mingle closely with the tertiary network which has now become less dense and more obliquely orientated. Figure 9—Diagrammatic outline of the ultrastructure and organisation seen by electron microscopy. Again a large primary nerve, in similar location, is seen accompanying the nutrient vessel. It contains a varied number and proportion of myelinated and unmyelinated nerve fibres and is limited by a thin perineurium. A smaller secondary nerve (magnified diagrammatically in this illustration) lying within the outer half of the tunica adventitia contains a number of axons, all sharing a common coat of Schwann cytoplasm. These nerves are unmyelinated and devoid of perineurium. Small tertiary nerves containing one or very few axons (again magnified), usually retaining a thin Schwann cell covering, are located more deeply at the junction of the tunica media with adventitia.
Schulman and Dorfman (1970) postulated such pain in osteoid osteoma. In this regard it is well to remember that blood vessels are sensitive structures, and that a significant pathway of this sensitivity, and therefore the sensitivity of bone, is by the perivascular autonomic route (Kjær 1950).

SUMMARY AND CONCLUSIONS
The purpose of this phase of a continuing investigation of the autonomic nerve supply of bone was to establish a sound morphological basis for the role of the sympathetic division of the autonomic nervous system in the intraosseous control of bone blood flow. To this end, the origin and course, the microstructure and organisation, and the ultrastructure of the intraosseous adrenergic nervi vasorum was studied in forty growing rabbits. We concluded that:

1. The blood vessels inside bone are richly endowed with adrenergic nervi vasorum.
2. The sympathetic fibres destined for bone take origin in the appropriate ganglion, and in the case of the tibial diaphysis descend in the sciatic nerve, thereafter principally in the tibial nerve, and then enter bone alongside the nutrient vessels.
3. Inside bone three sizes of adrenergic fibre are found in three successive locations: the large primary nerves just within or outside the vessel wall; the smaller secondary nerves which spiral around the vessel axis and lie more deeply in its wall; and the fine tertiary nerves that form a rich plexus at the junction of the tunica media and tunica adventitia.
4. The tertiary plexus of fine nerve fibres gains origin from the primary and secondary nerves, and individually each fibre has a varicose appearance. The location and morphology of these varicosities suggest they contain a high concentration of neurotransmitter, and probably function as non-terminal “en-passage” neuromuscular synapses.
5. The electron microscopic ultrastructure reveals that the large primary nerves surrounded by perineurium contain myelinated and unmyelinated fibres. The smaller unmyelinated secondary nerves lie within the outer part of the tunica adventitia and contain many small axons which are nonsynaptic in appearance and share a common Schwann cell covering. Finally the fine tertiary nerves containing one or very few axons lie within the inner adventitia and very closely approximate the outer smooth muscle cell of the tunica media.
6. The axons within the tertiary nerves periodically expand to accommodate a concentration of mitochondria and vesicles, and thereby take on a synaptic appearance. Therefore in morphology and location these expansions would appear synonymous with the varicose enlargements seen on fluorescent microscopy.
7. The selective uptake and concentration of 5-hydroxydopamine within the vesicles of certain axonal expansions testifies to the function of these vesicles as areas specialised for the storage of neurotransmitter.
8. The selective uptake of 5-hydroxydopamine further demonstrates the presence of the sympathetic system at this level, and establishes the dense-cored vesicles as the final common pathway of this system in its contribution to the neural control of bone blood flow.

REFERENCES


