J. Anat. (1982), **134**, 4, pp. 719–728
With 12 *figures*
Printed in Great Britain

Distribution of nerves in long bones as shown by silver impregnation

T. J. THURSTON

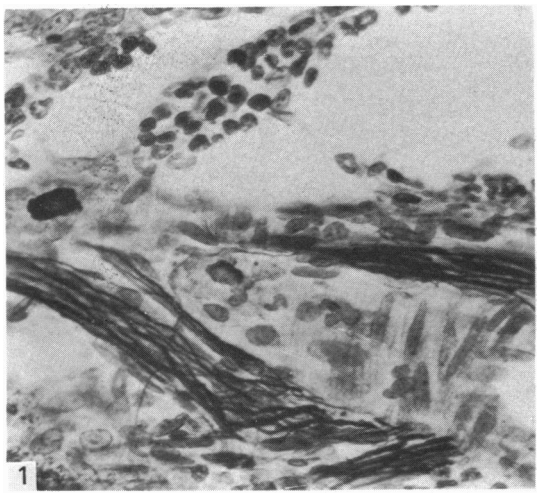
Department of Anatomy, Guy's Hospital Medical School, London SE1 9RT

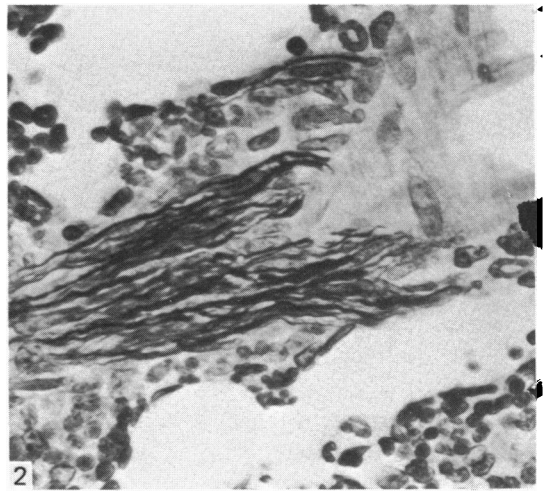
(*Accepted* 25 *June* 1981)

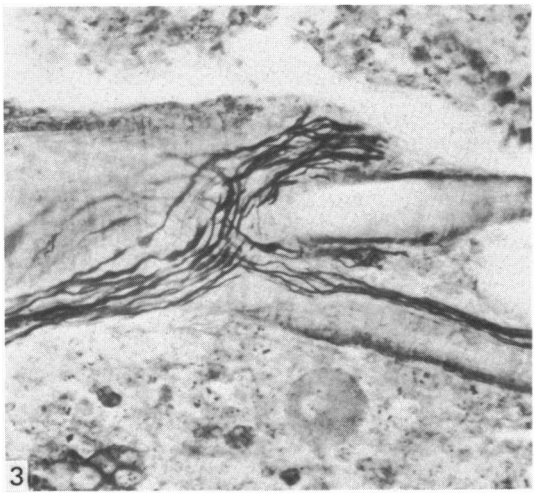
INTRODUCTION

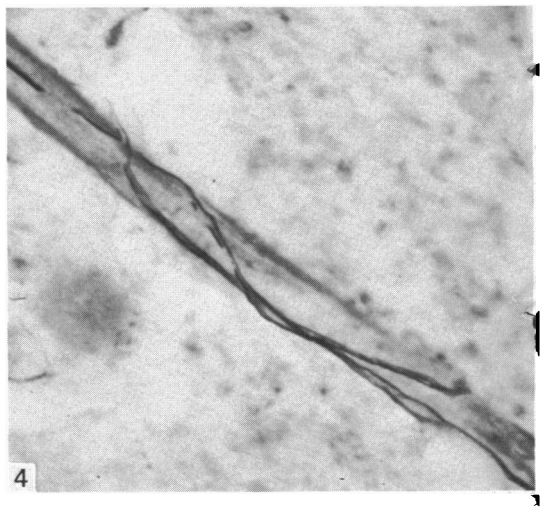
The results of previous investigators suggest that nerves enter a long bone through the nutrient foramen and smaller, epiphyseal foramina (Calvo, 1968). They may be myelinated or non-myelinated, and within the bone many are in bundles associated with the medullary arteries (Miller & Kasahara, 1963). Offsets from these form two distinct plexuses, one near the surface of the adventitia, and the other deeply within it (Duncan & Shim, 1977). Evidence of nerves continuing on to marrow capillaries is sparse. Venous vessels in bone are associated with straight or spiralling nerves (Calvo, 1968). Other nerves proceed independently from vessels into the marrow parenchyma, and may form a medullary network (De Castro, 1930; Calvo, 1968). The majority of Haversian canals contain non-myelinated fibres which are mostly in close relation to blood vessels (Cooper, Milgram & Robinson, 1966; Cooper, 1968). The presence of nerves in periosteum and joints is well authenticated, but nerves have never been convincingly demonstrated in articular cartilage.

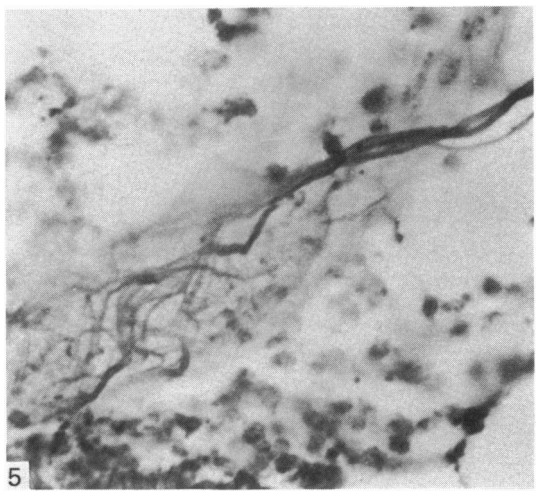
The methods used to date comprise principally dissection (Gros, 1846); electron microscopy (Cooper *et al.* 1966; Cooper, 1968); fluorescence microscopy; staining with haematoxylin and eosin, and with methylene blue; and silver impregnation. Electron microscopy is restricted to small, possibly unrepresentative fragments of tissue. The techniques of fluorescence microscopy (Duncan & Shim, 1977) cannot be used on decalcified material. Haematoxylin and eosin staining (Sherman, 1963; Calvo, 1968) gives little contrast between nervous and non-nervous tissue. Methylene blue staining (Ottolenghi, 1902), was 'consistently unsuccessful' (Miller & Kasahara, 1963) if the bone was decalcified. Silver impregnation has frequently been used in order to obtain an over-view of the nerve supply to a whole bone (de Castro, 1930; Hurrell, 1937). The many varieties of silver technique that have been described testify to the difficulty of obtaining reproducible results in studies of bone innervation. Recently Linder (1978) has devised a silver impregnation technique which, in his hands, is specific for nerve fibres in a wide variety of tissues. Bones, however, were not mentioned. It was therefore decided to apply Linder's technique to a study of bone innervation, both to test the reliability of the method, and to see the extent to which it could reveal the anatomy of the nerve supply of a long bone.

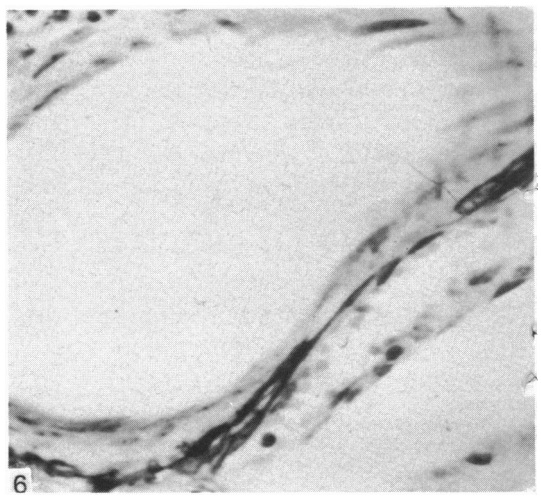

1


2


3


4


5


6

MATERIALS AND METHODS

The femora of eighteen Wistar rats, weight 200–250 g, and four adult greyhounds were used. They were fixed with 10% neutral buffered formalin solution, by perfusion in the case of the rats, by immersion for the dog bones (the dogs had been killed for other purposes). The bones were excised by cutting through the hip joint, and through the mid-shaft level of the tibiofibula. They were trimmed, and left in excess formalin for between 24 and 36 hours prior to decalcification. Either Gooding & Stewart's fluid, or 5 % nitric acid solution, or EDTA was used for decalcification. The end point of decalcification was determined by radiography, and because the sensitivity of this method is limited by the grain size of the Ilford X-ray film used, even after bones were observed to be radiolucent, all were left in EDTA for 48 hours. Dog femora were then cut into shorter pieces, and all specimens were washed for 24 hours in excess formalin to remove traces of decalcifying agents. They were then dehydrated in ethanol, 12 hours in each of 70 %, 90 % and two changes of 100 %. They were cleared in toluene for 3 hours or less, until clear. The bones were then impregnated under vacuum with six one hourly changes of Fibrowax (56 °C), blocked out, cooled to −4 °C, and sectioned at 10 μm longitudinally so that the whole femur, knee joint, and upper tibia were included in the sections. These were then mounted on glass, dried, and impregnated with silver strictly according to Linder (1978).

To test the specificity of the method, rat jugulo-omohyoid lymph nodes, known to contain reticulin fibres, and adrenal medulla, known to contain mixed nerve bundles, were prepared and sectioned in an identical manner to the bone specimens. Sections of these tissues, like the bone sections, were stained following the Linder procedure and also the silver impregnation technique described by Gordon & Sweet (Culling, 1974) as specific for reticulin fibres.

RESULTS

After silver impregnation by the Linder method, a bone shows nerves as black fibres running against a gold background of marrow cells, with yellow-brown blood vessels and pale grey bone cortex.

Marrow blood vessels

A large, and several lesser bundles of nerves entered the femur through the nutrient foramen. Within the nutrient canal, the nerves were closely associated with the nutrient artery, and ran a straight course just outside its adventitia (Fig. 1). Within the marrow cavity, arteries were associated with one or more nerve bundles which still lay just outside the adventitia (Fig. 2). Each bundle divided with the

Figs 1–6. All but Fig. 5 are made from rat specimens.

Fig. 1. Nerve bundles passing through the nutrient foramen of the femur, in close contact with the nutrient artery. × 400.

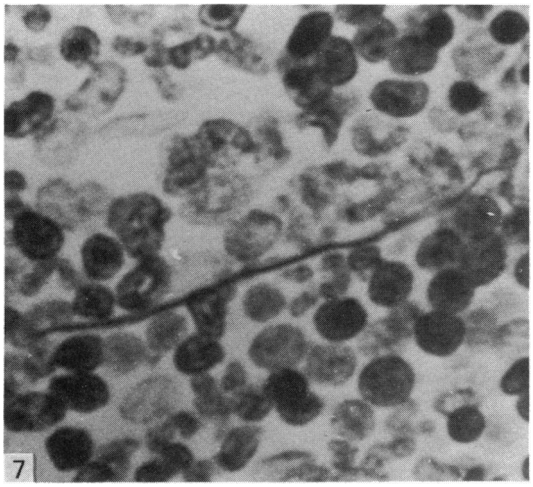
Fig. 2. A large nerve bundle running with a major branch of the nutrient artery. × 480.

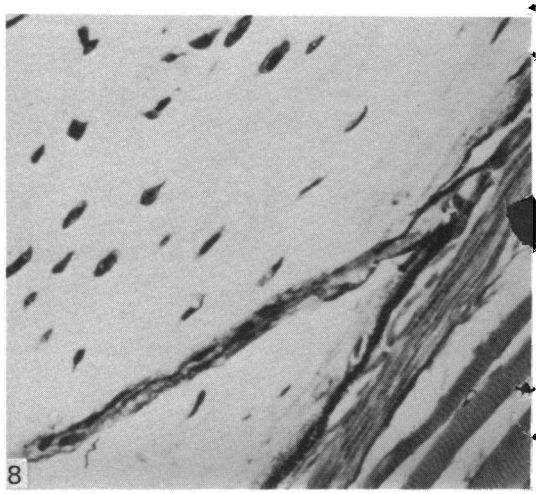
Fig. 3. A large nerve bundle dividing at the bifurcation of a medullary artery. × 450.

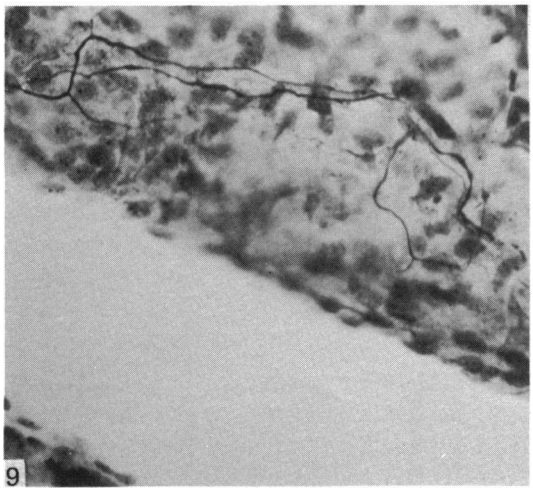
Fig. 4. Nerve fibres associated with a small, straight medullary artery. × 450.

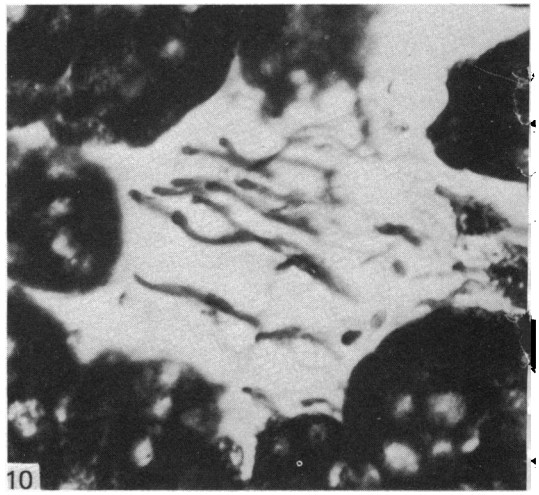
Fig. 5. A nerve network in association with a small artery in the bone marrow of a dog. × 600.

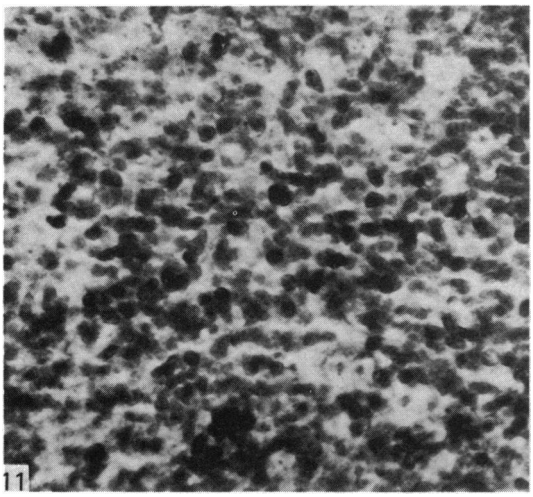
Fig. 6. Perivascular nerves in epiphyseal marrow. × 420.

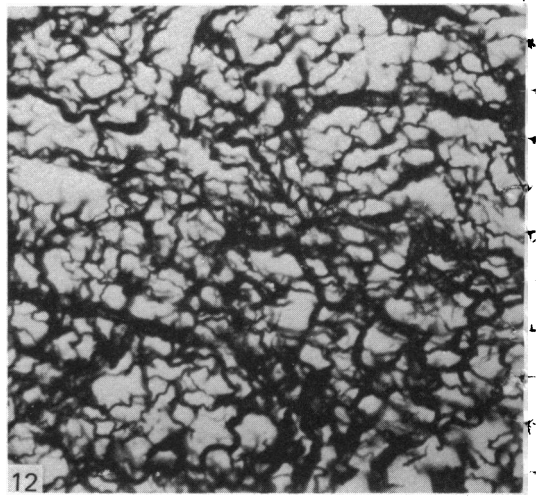

7


8


9


10


11


12

arteries (Fig. 3). The smaller medullary arteries were straight, and were associated with one or two nerve bundles, each containing up to four nerve fibres in close contact with the vessel wall (Fig. 4). In the dog, some spiralling nerves gave rise to a plexus around the vessel (Fig. 5). Medullary nerves were abundant in the vicinity of the nutrient foramen, but sparser in cancellous bone. Nerves in epiphyses were still generally perivascular in location (Fig. 6).

In both rat and dog material, no nerves were observed passing on to marrow capillaries. Nerve bundles and isolated nerve fibres were found in association with marrow venous sinuses. The bundles passed along the central venous sinus, and were traced on to the walls of smaller sinuses. Sometimes a nerve fibre ended in a bouton-like expansion in contact with the wall of a medullary venous vessel.

Marrow parenchyma

Some nerve fibres detached from the bundles on a blood vessel and ran independently through the marrow parenchyma, either in small bundles or as single fibres (Fig. 7). They sometimes branched as they proceeded, or formed an irregular plexus. No nerve endings on parenchymal cells were observed.

Growth cartilage

On the metaphyseal side of the growth cartilage there was very little evidence of nerves. Fibres were present in the metaphysis distant from the growth cartilage, but at no time could they be tracked to the near neighbourhood of the cartilage itself. In contrast, on the epiphyseal side of the growth cartilage, nerve fibres were present in the vascular tissue adjacent to the germinal zone of the epiphyseal plate. In this region, free nerve endings were present as were occasional bouton-like terminal expansions.

Cortex

Nerve fibres entered Haversian canals from both the narrow cavity and periosteum (Fig. 8). In the rat and dog, the fibres travelled close to the blood vessels, and some exhibited varicosities. No fibres were observed to leave the vascular canals to enter calcified bone matrix.

Periosteum

Many nerves passed from muscles into the underlying periosteum. In the outer, fibrous layer, nerves were frequently associated with blood vessels, branching around them and occasionally ending in contact with their wall. Nerve fibres were also present in the deeper, cellular layer. Single fibres or small nerve bundles took a winding course between the cells, independently of the vessels.

Figs 7–12. All are made from rat specimens.

Fig. 7. A nerve fibre running independently between marrow parenchymal cells. × 700.

Fig. 8. A nerve fibre entering a cortical vascular canal from the periosteum and travelling along it. × 300.

Fig. 9. Nerve fibres branching within the synovial membrane of the knee joint. × 340.

Fig. 10. A nerve bundle in adrenal medulla impregnated following Linder's technique. × 700.

Fig. 11. Lymph node impregnated following Linder's technique, showing no fibres. × 400.

Fig. 12. Lymph node impregnated following Gordon & Sweet's method, demonstrating branched, tortuous connective tissue fibres. × 500.

Joints

Some nerves in the capsule of the knee joint were associated with blood vessels. Others did not have this relationship and ended in expanded terminals. The synovial membrane was richly innervated in a plexiform fashion. Numerous varicose offsets radiated toward the synovial mesothelium (Fig. 9) and formed a subsynovial plexus. Nerve fibres were not observed to penetrate the articular cartilage or subchondral bone lamella.

In the longitudinal sections of a rat's hind limb examined here, the whole femur, upper tibia and patella were available for study. An incidental finding was that, in the patella, many more nerves were present in the subchondral areas of the marrow than were found in central patellar marrow regions. This is a direct reversal of the situation observed in the femoral and upper tibial condyles.

Control materials

Bone marrow impregnated by the Gordon & Sweet reticulin method showed no structures which approached the size and regularity of the nerve fibres demonstrated by the Linder method. Adrenal medulla stained following the Linder method showed structures similar to those seen in bone marrow (Fig. 10); but reticulin staining following Gordon & Sweet showed no fibres at all.

Lymph nodes impregnated by the Linder method revealed no fibres (Fig. 11); but the reticulin method demonstrated a vast network of connective tissue fibres in lymph nodes, which were more frequently branched, and more tortuous than fibres seen in bone (Fig. 12).

DISCUSSION

The results indicate that in materials prepared by the methods of this study, Linder impregnation is specific for nerves and does not reveal reticulin fibres. The silver impregnated structures seen in bone stained according to Linder do not resemble reticulin fibres, but do resemble mixed nerves as seen in the adrenal medulla.

Linder's technique involves the use of several complex solutions and careful temperature control. It is important for tissues to be fixed thoroughly and promptly. Small segments of femur fixed by immersion, and whole rat bones fixed by perfusion, subsequently show well defined nerves after impregnation with silver. Larger blocks of tissue fixed by immersion show poorly defined, interrupted nerves. The decalcifying agent used affects silver impregnation much less. Nitric acid, formic acid, and EDTA solutions were all used, but only in cases of excessive exposure to acids was there a reduction in silver uptake by the nerves. The need for carefully controlled fixation and decalcification is seen in the dog bones examined here, whose fixation (by immersion) and decalcification (rather prolonged) were outside the author's control. In these bones, the nerves had less affinity for silver and hence were less clearly observed.

The results show that nerves are abundant in periosteum and bone marrow. Most of the fibres are present in relation to blood vessels; in the marrow parenchyma they are relatively sparse. In some respects, the silver method used here is inadequate. Rat epiphyseal foramina are lined with osteoid which stains heavily with silver, preventing demonstration of nerves entering bone. Furthermore, any silver method produces refractive artefacts, which makes nerve endings difficult to observe. In this

study, reference is made to 'nerve terminals' only where such structures appear consistently at certain sites. In the cortex, few fibres were demonstrated, yet electron microscopy suggests that at least 60 % of vascular canals contain nerves (Milgram & Robinson, 1965).

The results show numerous nerve networks in intimate relation to marrow arteries in the dog, which were not present in the rat. Other workers have observed networks in the rabbit, cat, and dog (Ottolenghi, 1902; Kuntz & Richins, 1945; Calvo, 1968; Duncan & Shim, 1977), but not in smaller laboratory animals (Takase & Nomura, 1957), suggesting that a difference in pattern of medullary vessel innervation exists between species.

Experimental blood perfusion of an isolated tibia is directly affected by stimulation of its nerve supply, and the pattern of response is 'characteristically vasomotor' (Drinker & Drinker, 1916). Stimulation of the sympathetic nerves to the femur decreases marrow blood flow (Herzig & Root, 1959), whilst, following sympathectomy, there is an increased rate of blood flow in the marrow (Lowenstein, Pauporte, Richards & Davison, 1958; Brookes, 1974). It is therefore well established that some nerves in bone marrow subserve a vasomotor function. However, it would seem unlikely that nerves associated with capillaries in vascular canals control the blood flow in the cortex, since this is more readily achieved by the profusion of nerves on the medullary arteries, the source of the cortical blood flow (Brookes, 1971).

That the central nervous system has an effect on haemopoiesis has not been convincingly demonstrated (Grant & Root, 1952), although peripheral nerves do have a marked influence on the cellular output of marrow (Drinker & Drinker, 1916). Haemopoiesis could be affected either directly, by nerves to the medullary parenchyma, or indirectly, by the effect of vasomotor nerves on the medullary circulation. The distribution of medullary nerves seen here suggests that the nervous effect on haemopoiesis is likely to be indirect, mediated by vasomotor fibres. Miller & McCuskey (1973) point out that haemopoiesis in a developing bone begins when the marrow is morphologically mature, which suggests that a competent neurovascular network influences the medullary micro-environment and makes it favourable for haemopoiesis. Vasoconstrictor drugs like adrenaline (Herzig & Root, 1959), and sympathetic stimulation (Drinker & Drinker, 1916; Herzig & Root, 1959) reduce the rate of medullary blood flow and also affect the cellular output of the marrow. Vasoconstrictor drugs cause increased numbers of red blood cells to be found in the blood (Davis, 1942); sympathetic stimulation increases the number of circulating erythrocytes (Foa, 1943), and also promotes the release of neutrophils into the blood (De Pace & Webber, 1975). Conversely, sympathectomy, which increases the rate of medullary blood flow (Lowenstein *et al.* 1958; Brookes, 1974), and parasympathetic stimulation cause more erythroblasts to be seen in the marrow, as well as causing a mobilization of red cells and the appearance of reticulocytes in the peripheral circulation (Grant & Root, 1952). These observations suggest that the nervous influence on haemopoiesis is likely to be indirect, mediated by nerve fibres to the medullary blood vessels.

It is difficult to distinguish between vasomotor, muscular, and direct nervous influences on bone growth (Ascenzi & Bell, 1972). Various authors present evidence of a possible trophic nervous influence upon bone. Inman & Saunders (1944) have provided maps of the human limb showing zones to which pain is referred on stimulation of particular bones. Each zone corresponds to a specific spinal level

of sensory innervation, which they term a 'sclerotome'. In human thalidomide dysplasias, there is an exact correspondence between absence of bone and absence of 'sclerotome' (McCredie & Loewenthal, 1978). In 10–20 % of young children, a slow growth of leg bones persists following amputation. In rats, this overgrowth is significantly reduced by resection of the sciatic nerve 'when the trophic effect of nerve is absent' (Bunch, Deck & Romer, 1977). Other authors suggest a relation between bone growth and muscle activity. Atrophic changes in the thickness of the cortex and epiphyseal cartilages were observed in a growing humerus after brachial plexus excision; also, centres of ossification were smaller, and onset of ossification was delayed (Pottorf, 1916). These effects of denervation have been ascribed to loss of muscle activity (Pottorf, 1916; Howell, 1917; Tower, 1937). There is no difference between bony changes produced by denervation, and those produced by immobilization; the changes in bone vary with the degree of disuse (Allison & Brookes, 1921). Sectioning the lumbosacral plexus also causes a highly correlated (+0·79) loss of weight in bone and muscle, suggesting 'that bone atrophy in a paralysed limb is due entirely to loss of muscle power and tone, and not to loss of any specific neurotrophic influences' (Gillespie, 1954). It is possible that bone growth can be affected by vasomotor influences. In Hirschsprung's disease treated by left lumbar sympathectomy, the left leg may grow longer than the right (Harris, 1930). However, it is generally conceded that unilateral sympathectomy in normal animals does not result in increased bone growth (Bisgard, 1933). The findings of this study support the view that innervation of muscle probably has a greater influence on bone growth than direct neurotrophic or vasomotor effects: no nerves penetrate the growth cartilage, and there is only a sparse innervation of the small blood vessels adjacent to the germinal zone.

The afferent functions of nerves in bone probably include perception of pain, pressure and position. When a needle touches the periosteum, two types of pain are felt, a prick and a diffuse ache (Weddell & Harpman, 1940). Puncture of the bone marrow causes pain (Kuntz & Richins, 1945), as does a rapidly growing abscess, but not a slowly growing sarcoma (Sherman, 1963). Changes of intramedullary vascular pressure also cause pain (Helal, 1965). It is held by some that bone cortex is not sensitive to operative procedures (Sherman, 1963; Milgram & Robinson, 1965). Others, however, claim that osteotomy is painful when the bone cortex is cut (Ascenzi & Bell, 1972). In the present investigation, appropriate anatomical nerve pathways were found which could possibly subserve all these cases of pain perception. The periosteum contains many nerves, and the sensitivity of the marrow could be conveyed by parenchymal nerve fibres or those on vessels. If the cortex is sensitive to pain, this could be transmitted by some of the nerve fibres in the vascular canals.

The Pacinian corpuscles present in marrow, cortical bone (Milgram & Robinson, 1965), and other skeletal elements (Barnett, Davies & MacConaill, 1961) are probably concerned with detection of medullary pressure or the position and movement of bones and joints. Many more fibres in cortical bone are probably concerned with mechanoreceptive functions, contributing to the overall mechanisms of coordinated movement of the limbs and skeleton generally by monitoring stresses undergone by bone cortex during normal function.

SUMMARY

Rat and dog femora were impregnated with silver by Linder's (1978) method. Control staining suggested that the method is specific for nerve fibres in decalcified bone. The distribution of nerve fibres in bone marrow, bone cortex, cancellous bone, periosteum and joints is described. The anatomical findings are discussed in relation to the function of the nerves, with reference to pain, pressure and position sensation, bone growth, vasomotor effects, and haemopoiesis.

I would like to thank Dr M. Brookes of the Department of Anatomy, Guy's Hospital Medical School for the invaluable help and advice he has given throughout this work, and Mr K. Fitzpatrick of the Photographic Unit for his assistance in producing the photographs.

REFERENCES

ALLISON, H. & BROOKES, B. (1921). Bone atrophy: an experimental and clinical study of the changes in bone which result from non-use. *Surgery, Gynecology and Obstetrics* **33**, 250–255.

ASCENZI, A. & BELL, G. H. (1972). In *The Biochemistry and Physiology of Bone*, vol. 1 (ed. G. H. Bourne), pp. 341–345. New York: Academic Press.

BARNETT, C. H., DAVIES, D. V. & MACCONAILL, M. A. (1961). *Synovial Joints: Their Structure and Mechanics*, pp. 105–115. Edinburgh: Longmans.

BISGARD, J. D. (1933). Longitudinal bone growth, the influence of sympathetic denervation. *Annals of Surgery* **94**, 374–376.

BROOKES, M. (1971). *The Blood Supply of Bone*, pp. 118–122. London: Butterworths.

BROOKES, M. (1974). La circulation osseuse normal et pathologique. In *La Circulation Osseuse* (ed. J. Ficat & J. Arlet), pp. 5–13. Paris: INSERM.

BUNCH, W. H., DECK, J. D. & ROMER, J. (1977). The effect of denervation on bony overgrowth after below knee amputation in rats. *Clinical Orthopaedics and Related Research* **122**, 333–339.

CALVO, W. (1968). The innervation of bone marrow in laboratory animals. *American Journal of Anatomy* **123**, 315–328.

CASTRO, F. DE (1930). Quelques observations sur l'intervention du système nerveux autonome dans l'ossification. Innervation du tissu osseux, et de la moelle osseuse. *Travaux du Laboratoire de recherches biologiques de l'Université de Madrid* **26**, 215–244.

COOPER, R. R. (1968). Nerves in cortical bone. *Science* **160**, 327–328.

COOPER, R. R., MILGRAM, J. W. & ROBINSON, R. A. (1966). Morphology of the osteon. *Journal of Bone and Joint Surgery* **48A**, 1239–1271.

CULLING, C. F. A. (1974). *A Handbook of Histopathological Techniques*, pp. 427–428. London: Butterworths.

DAVIS, J. E. (1942). The production of experimental polycythæmia by the daily administration of epinephrine or posterior pituitary solution. *American Journal of Physiology* **137**, 699–702.

DE PACE, D. M. & WEBBER, R. H. (1975). Electrostimulation and morphologic study of the nerves to the bone marrow of the albino rat. *Acta anatomica* **93**, 1–18.

DRINKER, C. K. & DRINKER, K. R. (1916). A method for maintaining artificial circulation through the tibia of the dog, with a demonstration of the vasomotor control of the marrow vessels. *American Journal of Physiology* **40**, 514–521.

DUNCAN, C. P. & SHIM, S. S. (1977). The autonomic nerve supply of bone. *Journal of Bone and Joint Surgery* **59B**, 323–330.

FOA, P. P. (1943). Studies on the innervation of the bone marrow. *University Hospital Bulletin, Michigan* **9**, 9–21.

GILLESPIE, J. A. (1954). The nature of bone changes associated with nerve injuries and disuse. *Journal of Bone and Joint Surgery* **36B**, 464–474.

GRANT, W. C. & ROOT, W. S. (1952). The fundamental stimulus for erythropoiesis. *Physiological Reviews* **32**, 449–498.

GROS, M. (1846). La disposition des nerfs des os. *Bulletin de la Société anatomique de Paris* **21**, 369–372.

HARRIS, R. I. (1930). The effect of lumbar sympathectomy on the growth of legs shortened from anterior poliomyelitis. *Journal of Bone and Joint Surgery* **12**, 859–865.

HELAL, B. (1965). The pain in primary osteoarthritis of the knee. Its causes and treatment by osteotomy. *Postgraduate Medical Journal* **41**, 172–181.

HERZIG, E. & ROOT, W. S. (1959). The relation of the sympathetic nervous system to blood pressure of the bone marrow. *American Journal of Physiology* **196**, 1053–1056.

HOWELL, J. A. (1917). An experimental study of the effects of stress and strain on bone development. *Anatomical Record* **13**, 233–236.

HURRELL, D. J. (1937). The nerve supply of bone. *Journal of Anatomy* **72**, 54–61.

INMAN, V. & SAUNDERS, J. B. (1944). Referred pain from skeletal structures. *Journal of Nervous and Mental Disease* **99**, 660–674.

KUNTZ, A. & RICHINS, C. A. (1945). Innervation of bone marrow. *Journal of Comparative Neurology* **83**, 213–222.

LINDER, J. E. (1978). A simple and reliable method for the silver impregnation of nerves in paraffin sections of soft and mineralized tissues. *Journal of Anatomy* **127**, 543–551.

LOWENSTEIN, J. M., PAUPORTE, J., RICHARDS, V. & DAVISON, R. (1958). Effects of sympathectomy on blood turnover rates in muscle and bone. *Surgery* **43**, 768–773.

MCCREDIE, J. & LOEWENTHAL, J. (1978). The pathogenesis of congenital malformations. *American Journal of Surgery* **135**, 293–297.

MILGRAM, J. W. & ROBINSON, R. A. (1965). An electron microscopic demonstration of unmyelinated nerves in the Haversian canals of the adult dog. *Bulletin of the Johns Hopkins Hospital* **117**, 163–173.

MILLER, M. L. & MCCUSKEY, R. S. (1973). The innervation of bone marrow in the rabbit. *Scandinavian Journal of Haematology* **10**, 17–23.

MILLER, M. R. & KASAHARA, M. (1963). Observations on the innervation of human long bones. *Anatomical Record* **145**, 13–23.

OTTOLENGHI, D. (1902). Sur les nerfs de la moelle des os. *Archives italiennes de biologie* **37**, 73–80.

POTTORF, J. L. (1916). An experimental study of bone growth in the dog. *Anatomical Record* **10**, 234–241.

SHERMAN, M. S. (1963). The nerves of bone. *Journal of Bone and Joint Surgery* **45A**, 522–528.

TAKASE, B. & NOMURA, S. (1957). Studies on the innervation of the bone marrow. *Journal of Comparative Neurology* **108**, 421–437.

TOWER, S. S. (1937). Trophic control of non-nervous tissues by the nervous system. A study of muscle and bone innervated from an isolated and quiescent region of spinal cord. *Journal of Comparative Neurology* **67**, 241–267.

WEDDELL, G. & HARPMAN, J. A. (1940). The neurohistological basis for the sensation of pain provoked from deep fascia, tendon and periosteum. *Journal of Neurology, Neurosurgery and Psychiatry* **3**, 319–328.