C.1

TROPHIC INTERACTIONS BETWEEN RAT THIGH BLOOD VESSELS AND THEIR INNERVATION

By

NANCY LYNN SCHINDELHAUER

Hons. B.Sc., McMaster University, 1983

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Anatomy, The University of British Columbia)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1986

78

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of ANATOMY

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date APRIL 30/86

ABSTRACT

Much less work has been done on the denervation of smooth muscle compared with the extensive studies carried out on skeletal muscle. It was thought that denervation of smooth muscle produced few alterations in its morphology or physiology, especially since many blood vessels have virtually no innervation, and therefore, they can survive without it. Simple severing or excising a section of nerve trunk is sufficient to denervate skeletal muscle, but this does not apply to smooth muscle. Therefore, denervation methods for smooth muscle have included those with widespread effects such as chemical and immunological sympathectomies, and superior cervical ganglionectomy. In this study, I have developed a a semi-permanent, localized denervation method for rat thigh vessels and have used this method to study the trophic interactions between blood vessels and their innervation.

Female Wistar rats were denervated at 1-3 or 12 days of age, and examined at 30, 60, 90 and 120 days of age. The femoral nerve, which carries the vasomotor innervation to the thigh vessels, was severed in the thigh and brought inside the abdominal cavity. This method was necessary since preliminary experiments showed rapid re-innervation of the vessels if any part of the proximal root remained in the thigh. Inside the abdominal cavity, the nerve was slipped into a plastic tube and heat sealed. The tubing further inhibited re-innervation by preventing collateral sprouting from the proximal stump. Samples of the distal nerve stump, the proximal nerve stump, and from the femoral vein and saphenous artery were taken. In every animal, the contralateral side acted as a control.

The distal and proximal nerve stumps showed marked evidence of

degeneration. Fluorescence microscopy (specific for catecholamines) showed a significant decrease in the number of fluorescing dots around both the artery and vein. The presence of some fluorescencing dots around the denervated vessels may be from the nerves that were seen re-innervating the vessels at the time of sampling. These nerves came from aberrant areas. Arteries sampled at 90 days showed a significant decrease in the cross-sectional area of the tunica media on the denervated side.

The denervated femoral vein, in situ, was seen to be grossly dilated compared to the control side. Measurements of the luminal perimeter of sections of the vein showed that the denervated vein had a luminal area up to three times that of the controls. The difference in wall thickness in the femoral vein was not significant at the $p<0.05$ level. My results indicate that adrenergic nerves may not only have a trophic influence on arteries, but may influence veins as well. Therefore, in this study, trophic influences of nerves on blood vessels have been suggested by denervation causing 1) a thinner arterial wall and by producing 2) a reproducible dilation of the femoral vein. Also, trophic influences of blood vessels on nerves is suggested by the presence of re-innervation from aberrant areas.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I sincerely thank Dr. M. E. Todd for her support and encouragement, especially when I lacked self-confidence. It was her positive comments that kept me going. The assistance provided by Mrs. Connie Leung and Miss Fanny Chu was greatly appreciated. A special thanks goes to Dr. A. W. Vogl for two reasons: 1) his advice on sectioning and 2) for his mere presence in the Anatomy Building late in the evenings. I would also like to thank Fred McConnell and Bernie Cox who helped me with many technical difficulties. Special appreciation goes to Dr. J. Weinberg, to Mrs. Hella Prochaska and especially to my very dear friend, Mrs. Sharon Bezio. Also, I thank my parents who encouraged me to continue my education.

INTRODUCTION

Introductory Statement

Nerves have a profound effect on the development and maintenance of muscle. This has been shown as early as the 1800s by studying alterations in human skeletal muscle histology caused by diseases which lead to the destruction of motor neurons or by lesions of the peripheral nerves (Pellegrino and Franzini, 1963; Sarnat, 1983). From biopsies, the time at which the alteration in human muscle takes place is not always known; therefore, laboratory animals have been used to obtain a better correlation between the time of denervation and the onset of such alterations as atrophy. Cross-union experiments, which arose in the 1960s, took advantage of the normal condition seen in many mammalian laboratory animals which was that certain skeletal muscles are composed almost entirely of fibres of one type (Sarnat, 1983). These experiments, by producing changes in fully mature adult muscle, have increased our insight into the pathologic reactions of denervated muscle because they have shown that a muscle's innervation determines, not only its development and maintenance, but also the physiologic, metabolic, histochemical and ultrastructural properties of individual muscle fibres (Sarnat, 1983). As a result, these additional alterations were looked for in future denervation studies.

Since human diseases of skeletal muscle have been the impetus for studying the effects of denervation on muscle, much less work has been done on denervation studies involving cardiac and smooth muscle. In fact, it was generally believed that denervation of smooth muscle *in vivo* produced few morphological or physiological changes (Chamley and Campbell, 1975). Another factor which may have deterred denervation

studies on smooth muscle is the problems with the denervation method. While severing or removing a portion of the nerve is a common method for skeletal muscle, denervation which selectively destroys the sympathetic innervation has been problematic. Nevertheless, we are becoming aware that these nerves do appear to have a trophic influence on smooth muscle.

In Stedman's Medical Dictionary, "trophic" is defined as "relating to or dependent upon nutrition." Nerves and their target tissues are known to exhibit this "nutritive" dependency. The exact nature of neurotrophic interactions is still unknown; however, nerves can influence their effector tissues by its impulse activity and by any neurotrophic factors the nerve cell may produce. Neurotrophic interactions, therefore, have been defined by Gutman (1976) as the "long-term maintenance regulation not mediated by nerve impulses." That is, diffusable substances which are non-impulse related are thought to be the neurotrophic factors. Drachman (1976), on the other hand, believes that:

> " a definition of "neurotrophic" must be both broad and non-prejudicial: broad in the sense that it includes all the relationships by which nerve cells and target cells alter each other's anatomy, chemistry or physiology, and non-prejudicial in the sense that it must neither presuppose nor exclude any particular mechanism of trophic interaction. ... Thus, we may define as "neurotrophic" any long-term relationship in which nerve cells and target cells interact so as to influence the structure or function of either member of the pair."

Therefore, Drachman's definition of "neurotrophic" can include the effects of impulse activity.

Even though the exact nature of the trophic interaction is still unknown, the biochemistry, physiology and morphology of target tissues are indeed dependent on their nerve supply. These parameters have been looked at extensively in skeletal muscle and less so in cardiac and smooth muscles.

The Effects of Innervation on Skeletal Muscle

In skeletal muscle, binding studies have allowed the biochemical examination of the density of cholinergic receptors in mouse and rat muscles (Thesleff, 1974). In normally innervated hemidiaphragm muscles of the rat and mouse, the density of cholinergic receptors at the junctional site is two thousand times greater than at extrajunctional sites (Thesleff, 1974). Following denervation of these muscles, the receptor density at the junctional site is maintained, but the extrajunctional density is increased by 20-200 times.

As a result of this increase, one observes supersensitivity of the muscle membrane. This physiological parameter was seen in a 14-day denervated muscle fibre of the cat's tenuissimus muscle where a constant pulse of acetylcholine produced depolarization at each point of the membrane (Thesleff, 1974). In the innervated fibre, depolarizations to acetylcholine were produced only at the end-plate region. Luco and Eyaguirre (1955) found that the onset of hypersensitivity to acetylcholine in the cat tenuissmus muscle occurred earlier, and the onset of "spontaneous" fibrillary potentials occurred sooner when the nerve was severed at a locus closer to the muscle (that is, when the length of the distal nerve stump was shorter).

In cultured triceps muscle of the newt, Triturus viridescens, the presence of a sensory ganglion prevented the loss of muscle cholinesterase activity (Lentz, 1974). After three weeks in culture, the cholinesterase activity declined unless the muscle was cultured with the ganglion explant. Boiling of the ganglion for one minute abolished this maintenance effect. The effects of the ganglion were seen whether the ganglion was placed on the muscle surface or whether it was placed in a separate chamber separated from the muscle by a Millipore filter.

Hoffman and Thesleff (1972) studied the trophic influence of nerve on the physiology of the rat extensor digitorum longus muscle. By injecting cholchicine into the sciatic nerve 2cm proximal to this muscle, the organization of microtubules was destroyed, thereby preventing the proximo-distal movement of macromolecules and organelles. Measurements of the frequency and amplitude of the miniature end-plate potentials and of the single twitch and tetanic twitch tensions on the colchicine treated side did not differ from the corresponding measurements on the contralateral control side. Therefore, colchicine had no effect on nerve conduction. On the fourth day, after the colchicine injection, extrajunctional acetylcholine sensitivity and tetrodotoxin-resistant action potentials were exhibited by the muscle (tetrodotoxin generally blocks action potential generation). These effects gradually subsided after the fourth day. When ß-bungarotoxin (a neurotoxin that blocks spontaneous neurotransmitter release) was subcutaneously injected into the anterolateral region of the right hind leg (thereby paralysing it), or when the extensor digitorum longus was denervated by sectioning the deep peroneal nerve close to the knee, extrajunctional sensitivity and tetrodotoxin-resistant action potentials were observed. However, when these latter two methods were used, the extrajunctional acetylcholine sensitivity and tetrodotoxin-resistant action potentials developed earlier and remained for 3 days longer than in the colchicine experiments (Hoffman and Thesleff, 1972). That is, in comparing these two methods, the denervation changes observed following colchicine treatment were of a shorter duration and were less pronounced. Since the consequences of nerve severing or of using ß-bungarotoxin cannot be reproduced totally by using colchicine, Hoffman and Thesleff (1972) suggested that not only are trophic substances released by the nerve, but also neuromuscular

transmission and resulting muscle activity are contributing trophic influences. This is the same point that Drachman (1974) expressed in his definition of "neurotrophic".

Denervation methods often have been used to study the dependency of skeletal muscle morphology on its innervation. Lentz (1974) found that by severing the brachial plexus in the newt, the junctional folds on the muscle surface decreased in height and eventually flattened out once the traces of the axoplasm disappeared (21 days). The remaining Schwann cells soon withdrew into the extracellular space leaving the neuromuscular junction unidentifiable. He also found the same phenomenon occurring in cultured newt skeletal muscle, only in this case, the changes occurred more rapidly than in vivo. Since the cultured muscle maintained its junctional folds in the presence of a sensory ganglion, even though its own axon had degenerated, this suggests that perhaps the maintenance of the neuromuscular junction might be dependent on a diffusable neurotrophic factor.

Ultrastructural examinations and morphometric analyses have been carried out on denervated mammalian muscle (Pellegrino and Franzini, 1963; Gauthier and Dunn, 1973; Engel and Stonnington, 1974;). The most obvious change they saw was a decrease in the size of the muscle fibre. During the first 3 weeks following denervation, morphometric analysis of the rat soleus (red) and the rat gastrocnemius (white) muscles showed an 80% decrease in the transverse mean fibre area (Engel and Stonnington, 1974). At the same time, the mean transverse myofibrillar area decreased proportionately to, or at a slightly greater rate than the fibre area. Two weeks post-denervation, Pellegrino and Franzini (1963) noted a distinct reduction in the number of myofibrils in the rat soleus and gastrocnemius. They suggested that this decrease in myofibrils accounted

for a large proportion of the weight loss measured in these muscles. At 2 weeks, the denervated muscle weighed only two-thirds that of controls and by 4 weeks, it weighed only four-ninths that of controls. Engel and Stonnington (1974) noted that in the soleus and gastrocnemius, myofibrillar atrophy initially began at the periphery of muscle fibres, and after a month, spread to the interior of the muscle. In contrast, Pellegrino and Franzini (1963) saw peripheral and interior myofibrillar atrophy in the soleus (red fibers) but only peripheral atrophy in the gastrocnemius (white fibers). Initially during the atrophy process, the Z-lines lost their straight line configuration across the fibrils, becoming bent and "smeared" in appearance (Pellegrino and Franzini, 1963; Gauthier and Dunn, 1973). Red fibres have a wider Z-line than white fibres (Gauthier and Dunn, 1973); however, 2 weeks after denervation, the difference in width of the Z-line between red and white fibres became less apparent (Engel and Stonnington, 1974). Gauthier and Dunn (1973) contradict this. They maintain that differences in width remained even 14 days after denervation. One might expect their differing observations to be due to differences in the length of the distal stump. That is, one might expect Gauthier and Dunn (1973) to have a much longer distal stump than Pellegrino and Franzini (1963); however, this was not the case. Gauthier and Dunn (1973) severed the sciatic nerve close to its contact with the muscle, whereas Pellegrino and Franzini (1963) severed the sciatic nerve high in the thigh. Since both studies used adult albino rats, the differences may have occurred because Gauthier and Dunn (1973) studied the semitendinosus muscle, and Pellegrino and Franzini (1963) studied the soleus and gastrocnemius muscles. In their 60 day study, Pellegrino and Franzini (1963) found that eventually the Z-lines no longer showed any filamentous structure. They also discovered that a

single fibril may show regions of disorganization at several points along its length with normal sarcomeres in between. One month post-denervation, peripheral filaments of myofibrils were found in the interfibrillar spaces where it was assumed they were enzymatically destroyed by the lysosomes seen between the fibrils (Pellegrino and Franzini, 1963).

While fiber size and myofibrils decrease in size, there also occurs an absolute and relative increase in mitochondrial mass (Engel and Stonnington, 1974); however, absolute mitochondrial mass decreases after a week and becomes proportionate to fibre size (Engel and Stonnington, 1974; Pellegrino and Franzini, 1963). Mitochondria, which normally are relatively uniformly distributed, tended to aggregate into small clusters, and change from being elongate in the transverse plane to being virtually parallel to the long-axis of the myofibril after denervation (Engel and Stonnington, 1974).

The trend seen for mitochondrial mass is the same for sarcotubular element concentration, only the decrease in sarcotubular elements (sarcoplasmic reticulum and transverse tubules) seen after the first week is less than the decrease seen in contractile elements; therefore, as a net result, the concentration of sarcotubular elements increases (Engel and Stonnington, 1974). Morphologically, the spatial arrangement of the sarcotubular elements becomes increasingly irregular, and sometimes the elements possess focal dilations (Engel and Stonnington, 1974). After one month of denervation, parallel arrays of tubular profiles representing proliferating components of the sarcoplasmic reticulum are seen (Engel and Stonnington, 1974).

Morphological changes in other skeletal muscle organelles have also been noted. Lysosomes are present soon after denervation (Gauthier and

Dunn, 1973; Pellegrino and Franzini, 1963), and as the rate of degeneration in the fibers increases, lysosomes become larger and heavily loaded with material (Pellegrino and Franzini, 1963). Lipofuscin granules and small autophagic vesicles were seen one week post-denervation by Engel and Stonnington (1974). Sometimes, centrally-located nuclei are found (Engel and Stonnington, 1974; Pellegrino and Franzini, 1963), and prominent Golgi networks are seen with moderate frequency in the denervated muscle (Engel and Stonnington, 1974). Gauthier and Dunn (1973) discovered an increase in subsarcolemmal sarcoplasmic ribosomes in denervated muscle, and they hypothesized that this increase in protein synthesising material could produce many new acetylcholine receptors that might account for the increase in acetylcholine sensitivity of the skeletal muscle membrane.

The Effects of Cardiac Muscle on Nerves

Thus, denervation studies have shown that skeletal muscle is dependent on its innervation to maintain its integrity. Muscles also appear to affect their nerves. Experiments using the supernatant from homogenized, denervated rat cardiac muscle showed that the effector muscle tissue can affect the survival of neurons (Kanakis et al., 1985). Adult rats were denervated using an injection of 6-hydroxydopamine (6-OHDA) which produces a chemical sypathectomy. The denervated cardiac muscle was tested to see how effective 6-OHDA was at denervating this muscle. The activity of tyrosine hydroxylase, an enzyme involved in the rate-limiting step in the biosynthesis of noradrenaline, was used to assess this denervation. Although it was found that denervation of the heart was not complete, it was indeed effective since treatment with 6-OHDA caused a significant reduction in tyrosine hydroxylase. Hearts were removed 4

days after injection and then homogenised. The heart extracts were then used to assess their ability to promote the survival of dissociated 12-day old chick lumbar sympathetic ganglia. Sympathetic neuronal survival was significantly increased using the extracts of denervated heart when compared to the normal, control heart extracts. Extracts from the control and denervated hearts were then run through gels containing antibodies to Nerve Growth Factor (NGF). In normal tissue, NGF occurs in very low amounts. There was no significant difference in the effects on sympathetic neuronal survival between the treated and untreated control extracts. However, the effect of the anti-NGF-treated extract from denervated hearts on sympathetic neuronal survival was significantly decreased when compared to the untreated, denervated heart extract. This showed that NGF increases in denervated tissue and that NGF is an important factor for the survival of sympathetic nerves. It was very interesting to note that in their experiments, Kanakis et al. (1985) found that the anti-NGF-treated extracts from denervated hearts still promoted sympathetic neuronal survival better than untreated and anti-NGF-treated control extracts, although the difference was only significant at the 0.05 level with the treated control extract. This demonstrated that components (other than NGF) which have the ability to enhance the survival of cultured sympathetic neurons may also be increased after denervation.

As shown, many denervation studies on skeletal muscle have been carried out. Changes in the biochemistry resulting from denervation have been seen as an increase in the density of extrajunctional acetylcholine receptors. Supersensitivity of the muscle membrane and the presence of tetrodotoxin-resistant action potentials indicated changes in the physiology of the denervated muscle. Morphological changes include

decreases in the junctional folds, decrease in size of muscle fibre, reduction of myofibrils and changes in the organelles. Also, with the work involving the denervated cardiac muscle extract, we can see that the effector tissue has a trophic influence on the nerve.

The Effects of Innervation on Smooth Muscle

There has been a tremendous amount of research carried out on the role of cholinergic innervation on skeletal muscle maintenance; an interest has also developed in the trophic interactions between smooth muscle and its adrenergic innervation. However, since not as much is known in this particular area, many preliminary hypotheses of trophic influences on smooth muscle have been derived by analogy to skeletal muscle. Nevertheless, skeletal muscle is very different from smooth muscle, both in its ultrastructure and its innervation; thus, the analogies that have been made may not be correct.

In vitro experiments, designed to study the trophic influences of sympathetic nerves on smooth muscle, have been carried out on smooth muscle from the guinea-pig vas deferens (Chamley and Campbell, 1975). Single cell suspensions of these cells were allowed to settle on collagen-coated glass coverslips, and then they were exposed to various substances. Afer 8 days in culture, smooth muscle cells in control cultures had undergone intense proliferation and ultrastructurally, they looked dedifferentiated. That is, they resembled embryonic smooth muscle. After 8 days in culture in the presence of sympathetic ganglion extract, dibutyryl cyclic AMP or theophylline, smooth muscle proliferation was prevented and the cells were maintained in their differentiated state. Spinal cord extract, liver extract or noradrenaline resulted in smooth muscle cells that were intermediate in

appearance between those just described and the dedifferentiated smooth muscle cells of the control cultures. However, after 8 days in culture in the presence of dibutyryl cyclic AMP plus theophylline (a smooth muscle relaxant which increases intracellular cyclic AMP) smooth muscle cells appeared very well-differentiated. In fact, they possessed few organelles, a characteristic of adult in vivo guinea-pig vas deferens. Chamley and Campbell (1975) suggested that the trophic substance or substances may act by selectively stimulating the enzyme adenyl cyclase (which converts ATP to cyclic AMP), a suggestion further exemplified by the addition of the substance, theophylline.

Smooth muscles from different areas of the body respond differently to similar experimental treatments. For instance, smooth muscle from young rabbit thoracic aorta and ear artery were cultured and treated by Chamley and Campbell (1976) in similar ways as they treated the guinea-pig vas deferens smooth muscle just described. After 1-2 days in culture, the vascular smooth muscle consisted of 2 types of smooth muscle cells distinguished on the basis of morphology. They included (1) a 'differentiated' cell type, which resembled the cells of normal in vivo rabbit media and only a small number underwent division, and (2) an 'undifferentiated' cell type, which underwent frequent division and whose cytoplasm was filled with protein synthesizing machinery. The 'differentiated' cells maintained their morphology for only 4 days before changing to resemble the 'undifferentiated' cell type. In the presence of a sympathetic chain homogenate, the 'differentiated' smooth muscle cell type maintained its differentiated appearance for at least 6 days. The presence of spinal cord extract or noradrenaline made no difference to the cultured medial smooth muscle cells; that is, these treated cultures did not differ from the control cultures. In contrast, these

same substances did affect the cultured guinea-pig vas deferens smooth muscle cells somewhat, producing a cell intermediate in appearance between the 'differentiated' and the 'dedifferentiated' types.

Smooth muscle from different areas of the body also may reflect their differences by their ability to attract regenerating neurons. In normal skin, nonspecific cholinesterase and acetylcholinesterase activities are observed in erector pili muscles and their nerves; however, fluorescent varicose adrenergic nerves were also also seen in the erector pili muscles (Waris, 1978). In rat skin autographs, fluorescence microscopic techniques specific for catecholamines showed that the erector pili muscles were not re-innervated at the end of a 20 week period, but blood vessels in this graft were partially innervated at 16 and 20 weeks after transplantation (Waris, 1978). Hence, trophic interactions between one type of smooth muscle and its sympathetic innervation should be compared with other studies that have used the same type of smooth muscle, and, in studies of the circulatory system, from the same part of the vascular tree.

That vessels from different parts of the circulatory system may reflect differences in their ability to attract regenerating neurons has been shown by transplantation experiments and tissue cultures of smooth muscle. Transplantation of the rat femoral artery into the anterior chamber of an eye of a host rat did not induce irideal nerve sprouting whereas the tail artery did (Todd, 1986). Also, smooth muscle cultures containing medial cells from rabbit thoracic aorta or ear artery illustrated a difference in their interaction with rabbit sympathic ganglion explants (Chamley and Campbell, 1976). Nerve fibers growing from the explants formed longer-lasting associations (up to 8 days) with the smooth muscle cells from the ear artery than they did with cells from

the thoracic aorta (1-2 hours). Therefore, results from one area of the vascular tree may not always be extrapolated to predict the results in another part of the vascular tree, even within the same animal.

Most of the in vivo work involving neurotrophic influences on vascular smooth muscle has been done on the rabbit ear artery by Bevan and her co-workers. In one study, Bevan (1975) denervated the left rabbit ear artery by completely removing the left superior cervical ganglion in 4 week old rabbits. Two weeks postoperatively, a decrease in the uptake of ^{3}H-Tdr by the vascular smooth muscle cells in the denervated left ear artery was measured by scintillation counting and by autoradiography. Denervated smooth muscle also appeared to have fewer mitotic figures. These results seem to indicate that denervation inhibited the proliferation of the medial smooth muscle cells.

Bevan and Tsuru (1979) later postulated that since proliferative growth decreased upon denervation, this might create a smaller blood vessel which would be incapable of producing the maximum force of contraction. Rabbits of 9-11 weeks of age underwent superior cervical ganglionectomy. Eight weeks after the ganglionectomy, there was a noticeable decrease in the wall thickness and in the weight of the rabbit ear artery. The maximum force and the maximum tension were markedly decreased and it was postulated that the results were due to a loss of smooth muscle mass and to a qualitative change in the contractile machinery, respectively. Supersensitivity to norepinephrine was also a consequence of denervation. An increased elastic modulus (stiffer wall) was evident as well. This may reflect changes in the elastic tissue. Denervation in this case affected proliferation and sensitivity of the smooth muscle cells which, in turn, affected the architecture and mechanics of the blood vessel.

Bevan and Tsuru (1981) repeated the above procedure on three different age groups to see the effect it may have on developing arteries. The three groups were (1) a growing group (3-4 weeks old), (2) a young adult group (9-11 weeks old) and (3) a mature group (16-20 weeks). When compared with their controls, significant decreases in cross sectional area of the media were seen in the first two denervated age groups. In the third group (mature animals), there was no significant decrease in the cross sectional area of the media after denervation.

These studies on development carried out by Bevan and Tsuru (1981) correlate with those carried out by Rusterholz and Mueller (1982). Rusterholz and Mueller (1982) used the method of a unilateral superior cervical ganglionectomy on rabbits to evaluate the possible chronic influence that the sympathetic nerves might have on vascular resistance and to see if the results were age-dependent. They studied denervation in three separate groups which they termed (1) growing acute denervation (rabbits denervated at 4 weeks and studied approximately 9 days later), (2) growing chronic denervation (rabbits denervated at 4 weeks and studied 9 weeks later) and (3) adult chronic (rabbits that were denervated at 16 weeks and studied 10 weeks later). Changes in vascular resistance were seen as changes in flow-perfusion pressure curves. Perfusion pressure was measured in maximally dilated vessels and this pressure measures the resistance of the vessels. Results from the vascular bed in denervated ears and the contralateral innervated ears were compared. A comparison between the denervated and innervated ears in the growing acute denervated group (ie. those studied only 9 days after denervation) showed no difference in the perfusion pressure. The perfusion pressure of the denervated ear of the growing chronic

denervated group (ie. those studied 9 weeks after denervation) was significantly lower than that from its contralateral control side. Therefore, it seems that 9 days was an insufficient length of time after denervation for any noticeable changes to occur. No differences in the perfusion pressure curves were seen between the denervated ear and the contralateral innervated ear from the adult chronic denervated group. Thus, denervation does produce a decrease in vascular resistance in developing vessels but not in mature vessels and this decrease is observed only after a substantial length of time following the ganglionectomy. Rusterholz and Mueller (1982) believe their results are compatible with the idea of the existence of an interaction between sympathetic nerves and blood vessels. Although the mechanism of this interaction is unknown, they proposed that the decrease in vessel resistance was probably not exclusively the result of smooth muscle atrophy but also may have involved other factors such as an alteration in elastin/collagen ratio or an in alteration smooth muscle configuration. Bevan's report (1983) of an increase in arteriovenous anastomoses in the denervated rabbit ear artery may also be responsible for the observed decrease in perfusion pressure.

Morphological studies on denervated blood vessels have been carried out by Branco et al. (1984). They looked at the dog saphenous vein and the rabbit ear artery and found the wall of the denervated saphenous vein was thicker. Ultrastructurally, the smooth muscle cells of the denervated saphenous vein had the appearance of dedifferentiated cells containing all the organelles characteristic of active protein synthesis. Their findings were similar in the denervated saphenous artery except the alterations in the artery were restricted to the 2-3 smooth muscle layers closest to the adventitia. In both the artery and

vein, the alterations were reversible.

Therefore, denervation studies on smooth muscle, particularly vascular smooth muscle, are not as extensive as in skeletal muscle. Nevertheless, changes in vascular smooth muscle, as a consequence of denervation do occur. These changes include increased supersenstivity to norepinephrine, decreases in maximum force and maximum tension, a decrease in smooth muscle cell proliferation and a reversal to a dedifferentiated ultrastructural appearance.

Methods of Sympathetic Denervation

To study the denervation effects on the rabbit ear artery, Bevan and Tsuru (1975; 1979; 1981), Branco et al. (1984) and Rusterholz and Mueller (1982) used the method of unilateral sympathetic ganglionectomy. In the case of studying the rabbit ear artery, this involves the removal of the entire superior cervical ganglion. Consequently, innervation to one half of the head is lost.

Chemical sympathectomy involved the use of 6-hydroxydopamine (6-OHDA) as the denervating factor (Finch et al.,1973). Finch et al. (1973) noted that administration of 6-OHDA into adult rats produced a selective, temporary destruction of adrenergic nerve terminals. Regeneration of the adrenergic nerve terminals in blood vessels was very fast and within just a few days after injection of 6-OHDA, and almost complete functional recovery was seen. Finch et al. (1973) were therefore interested in seeing if 6-OHDA injected into newborn rats would produce a complete and permanent destruction of the adrenergic nerves supplying the vascular system. In their study they compared the effects of administering 6-OHDA into two different age groups of rats: one group was treated for the first 14 days directly after birth and looked at 8 weeks later and the

other group, the adult group, was given two injections, 7 days apart and looked on the next day after the last injection. To determine the effectiveness of denervation produced by 6-OHDA, stimulation of the entire sympathetic outflow was carried out on pithed rats which were previously adrenalectomized. The steel pithing rod was used as an electrode to stimulate spinal nerve roots. Sympathetic outflow was stimulated with supramaximal voltage and increasing frequencies. A rise in blood pressure with increasing stimulation frequency was seen in the control and newborn-treated animals although the newborn-treated pithed group was markedly lower (37 mmHg) than the controls (120 mmHg). No rise in blood pressure was seen in the adult-treated pithed group at any stimulation frequency. Since a small rise in blood pressure was observed in this latter age group in unpithed preparations, it was possible that in the pithed rat, the steel rod used for stimulation did not excite all sympathetic nerves (Finch et al., 1973). They also found a depletion in norepinephrine levels in the newborn treated group, however, the percent depletion varied with the tissue type (eg. mesentery vascular bed had 50-60% of the norepinephrine content left whereas norepinephrine in cardiac muscle was depleted to less than 5% of control levels). Since the levels of norepinephrine did not increase up to an age of 16 weeks, Finch et al. (1973) considered the denervation to be permanent as a consequence of the destruction of cell bodies in the sympathetic ganglia by 6-OHDA.

Using anaesthetized rats, Finch et al. (1973) compared the extent of vasoconstriction of isolated renal artery preparations between the two groups. Periarterial nerve stimulation was carried out to observe the vasoconstrictor responses. Vasoconstrictor responses of the rats treated at birth did not differ from those of the controls. The adult-treated

rats showed reduced vasoconstrictor responses. Even though Finch et al. (1973) showed in their study that chemical sympathectomy is a successful method of denervating certain organs such as the heart, it did not prove to be very promising as a means of achieving vascular denervation in younger rats. In newborn-treated rats, this method produced a permanent but incomplete denervation whereas in the adult it produced a virtually complete but non-permanent denervation.

Immunological sympathectomy is an alternative method of denervation. Histology, response to electrical stimulation of lumbar sympathetic ganglia, increased sensitivity to norepinephrine and results of chemical stimulation of the sympathetic ganglia with 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) are methods that have been used to determine the success of denervation (Brody,1964; Levi-Montalcini and Angeletti, 1966). DMPP is a white crystalline substance that is soluble in water and is not intended for therapeutic use (Chen et al., 1951). When injected intravenously into animals, it stimulates the sympathetic ganglia by acting at the nicotinic-like receptors of the postganglionic synaptic membrane (Szekere, 1980). This excites the post-synaptic neuron thereby causing an increase in arterial blood pressure and tachycardia.

Immunological denervation has been achieved by injecting antiserum to Nerve Growth Factor into rats and mice immediately after birth. Initiating the injections immediately after birth is essential to produce the most extensive denervation (Levi-Montalcini and Angeletti, 1966). Denervation has been proven to be permanent by carrying out histological studies on mice two years after they were treated at birth with the antiserum (Levi-Montalcini and Angeletti, 1966). This method of denervation was shown to successfully abolish vasomotor function since neither vasoconstrictor nor vasodilation occurred when the lumbar chains

of the immunized rat were electrically stimulated (Brody, 1964). Levi-Montalcini and Angeletti (1966) found that the average resting blood pressure in treated rats was only 70 mmHg compared to 100mmHg in controls. Blood pressure seems to be proportional to the degree of sympathetic innervation. Another change found by Levi-Montalcini and Angeletti (1966) with this type of denervation was a severe decrease (but not total abolition) in the cell population of various ganglia - superior cervical (rats:residual cell population was 10-15% of controls), celiac, stellate and thoracic chain ganglia. However, their results from pharmacological and physiological testing (ie. reactivity of the vascular system to chemical or electrical stimulation) suggest that the residual cell populations have very little, if any, functional activity.

The view that blood pressure values are proportional to the degree of sympathetic innervation was also supported by Gerova et al. (1974) who found that the maximum diameter reduction (compared to resting diameter as a percent) of the densely innervated femoral arteries in puppies, was much higher than than that found in adult dogs, whose innervation was less dense relatively.

In their examination of the responses of an isolated vascular bed to sympathetic neurotransmitters and sympathetic nerve stimulation in order to determine the functions of vascular smooth muscle and its innervation in newborn dogs, Boatman et al. (1965) used mongrel puppies aged 1 day and 1, 2, 4, and 8 weeks and adult dogs. They found that the vasoconstriction of the blood vessels in the hind limbs of dogs which was induced by nerve stimulation increased with age. They attributed this rise to the increase in functional maturity of the adrenergic vasomotor function. It was also noted that this onset and development of induced vasoconstriction coincided in age with the onset and development of the

systemic blood pressure. This age-related increase in blood pressure was also demonstrated on rats by Lais et al. (1977) as well as by Gerrity and Cliff (1975). These observations on blood pressure relate directly to the increase in catecholamine fluorescence, to the presence of dense-cored vesicles and to the number of nerve processes in rat arteries as they matured (Todd, 1980; Todd and Tokito, 1981).

Problems with Denervation Methods

From these studies, it is evident that sympathetic innervation can affect vascular smooth muscle both structurally and functionally, and thereby may alter the structure and function of the entire blood vessel. The in vivo methods of denervation used so far have included surgical ganglionectomy (Bevan and Tsuru, 1979; Bevan and Tsuru, 1981; Branco et al., 1984), chemical (Finch et al., 1973) and immunological (Levi-Montalcini and Angeletti, 1966) sympathectomies.

Surgical sympathectomy involves the removal of an entire sympathetic ganglion such as the superior cervical ganglion. This procedure eliminates the innervation to one half of the head.

Chemical sympathectomy involves using 6-hydroxydopamine (6-OHDA) which destroys adrenergic nerve terminals. This destruction is selective, but permanent if administered to newborns, and complete, but non-permanent if given to adults (Finch et al.,1973). Kanakis et al. (1985) found that in adult rats it produced an effective but incomplete denervation of the heart.

To produce an immunosympathectomy, injections of antiserum to Nerve Growth Factor are given intravenously. If given immediately after birth, it produces the most extensive and permanent denervation (Levi-Montalcini and Angeletti, 1966). Thus, only when these three denervaton procedures

are carried out on very young animals, are they the most permanent and do they produce the most noticeable changes in blood vessels (Levi-Montalcini and Angeletti, 1966; Finch et al., 1973; Bevan and Tsuru, 1979; 1981).

These methods of denervation eliminate innervation from a large area in the body of the animal, that is, the denervation is not localized. These widespread denervations may produce unwanted, possibly toxic, effects which may have contributed to the results obtained by these past investigators.

To avoid these aberrant effects, a localized denervation method is necessary. This was attempted by Todd (1986) on rat thigh blood vessels, but was unsuccessful as a result of rapid regeneration. Severing, removing or repositioning the femoral nerve are not successful methods since re-innervation of the saphenous and superficial and epigastric arteries occurs in under 15 days (Todd, 1986). Rusterholz and Mueller (1982) found that denervation of young rabbit ear arteries produced a decrease in perfusion pressure 9 weeks post-denervation, but not at 9 days post-denervation. Hence, not only is a localized denervation method required, but also a permanent or semi-permanent denervation method since a sufficient post-operative length of time must past before changes are seen in the vessels.

Thesis Topic

The aim of my study, therefore, was two-fold. First, I wanted to develop a way of studying denervation effects on blood vessels under the most normal conditions possible, meaning, <u>in vivo</u>, leaving all of the nerves intact except for those innervating the vessels under investigation, and without having any side effects produced by

circulating drugs or antibodies. Therefore, it was necessary to develop a technique that would keep the blood vessels denervated for as long as possible. Second, I wanted to test the hypothesis that blood vessels and their adrenergic innervation do exert trophic influences over each other. To achieve my objectives, I developed a novel method of denervating blood vessels in the rat thigh and measured changes in vessel wall thicknesses and luminal perimeters.

MATERIALS AND METHODS

Animals

Female Wistar rats from an inbred colony maintained in the Department of Anatomy were denervated at 3 or 12 days of age. The mothers were fed Purina Lab Chow and water ad libitum. The pups were weaned at one month of age at which time they were fed the same diet as their mothers. They were housed in pairs in hanging cages in a controlled environment having a 12 hour dark, 12 hour light cycle. The denervated blood vessels were sampled when the pups were 30, 60, 90 or 120 days of age. A total of 18 animals was used for the light and electron microscopic analysis and 8 animals were sampled for fluorescence microscopy

Denervation Procedure

The animals were anaesthetized in a glass desiccator in which absorbent cotton was moistened with anhydrous ether (Fisher Scientific Ltd.). Anaesthesia was maintained during the denervation procedure by using a nose cap containing absorbent cotton moistened with ether. The anaesthetized animals were placed on a small plexiglass table, dorsal side down. Their fore- and hindlimbs were loosely pinned down under elastic bands and their abdomens were disinfected with Savlon (Ayerst Laboratories).

A small vertical skin incision was made in the right thigh and the right femoral, superficial epigastric and saphenous arteries and femoral nerve exposed (Figure 1a). The femoral nerve was traced back to the inguinal ligament. The femoral nerve was mobilized and separated from

the femoral artery and skeletal muscle adjacent to the inguinal ligament. A length of black suture silk (8.0, Deknatel) was tied around the femoral nerve in two places, as close to the inguinal ligament as possible, and then again more distally (Figure 1b). The femoral nerve was severed just distal to the second knot (Figure 2a). The portion of femoral nerve distal to the cut was separated from the femoral artery and severed where the saphenous artery branches off the femoral artery (Figure 2a). Therefore, the length of femoral nerve from the second knot to the branch point was removed and discarded.

Inside the abdominal cavity, the femoral nerve runs cranially in the posterior wall of the abdominal cavity. Therefore, a tiny, ventral, paramedial incision was made in the abdominal wall and the femoral nerve inside the abdominal cavity was found. By gently pulling on the nerve here, the portion of the femoral nerve that was out in the thigh was brought inside the abdominal cavity. The black suture silk that was tied around the nerve was threaded through a length (approximately 1 cm) of polyethylene tubing (PE-10, Clay Adams). The tube was slid over the nerve and the distal end was melted using a cautery gun (Figure 2b). The melted plastic sealed the tube and also secured the black suture silk when it hardened, thereby securing the nerve inside the tube. The encased nerve was tucked back inside the abdominal cavity and the abdominal wall was sutured closed using 6.0 Deknatel suture silk. The skin incision was clamped with 7.5 mm wound clips (Medicon) and cleaned of blood using Savlon. After regaining conciousness from the anaesthetic, the pups were sprinkled lightly with baby powder and returned to their mother. Vick's Vapo rub was inserted into the mothers' nostrils when the pups were returned to them. Both, the Vick's Vapo rub and the baby powder were used to prevent cannibalism by the mothers.

Tissue Sampling and Processing

At the time of sampling, the animal's length, weight and tail cuff blood pressure were recorded. A programmed electro-sphygmomanometer (Narco Bio-Systems, Inc.), connected to a polygraph, was used for tail cuff pressure recordings. Following this, the animals were anaesthetized in the same manner as for denervation and then perfusion fixed using the following procedure (Todd et al., 1983). The ventral neck and abdominal regions were shaved and the areas were sponged with Savlon. A midline incision was made in the ventral neck region. The left common carotid artery was found and cleared of its enveloping connective tissue. The vagus nerve was then carefully separated from the common carotid artery. The artery was ligated in two places using 4.0 Deknatel suture silk. The first ligature was tied tightly and as cranially as possible, and a second loose knot was made with this same ligature (Figure 3). The second ligature was tied approximately 1 cm caudal to the first . The latter knot was not tightened until the cannula was inserted, serving to hold it in place. A small arterial haemostat was clamped immediately proximal to the position of the second ligature. The cannula was a length of PE-50 polyethylene tubing (Clay Adams) bevelled at both ends, filled with a 1% heparin solution in saline and clamped at one end with a pair of hemostats. A tiny incision was made in the ventral wall of the artery and the cannula was inserted through the loose knot of the first ligature and then into the lumen of the artery. Once inside the lumen of the artery, the cannula was gently pushed caudally past the region of the second ligature. At this point, the second ligature and the second knot of the first ligature were securely tightened, and the small arterial hemostat was released.

The hemostats were removed from the cannula to see if blood were

being pumped through the cannula without being blocked. The cannula was then refilled by injecting heparin and then connected through a T-tube to a syringe perfusion pump (Sage Instruments) and a pressure transducer (Statham Transducer, Gould). The pressure transducer was connected to a polygraph (Grass Instruments Co.) for blood pressure recordings, and was calibrated using a mercury manometer.

The systolic, diastolic and mean blood pressures were recorded via the intra-arterial cannula (Figure 4) and then the perfusion was started. A 3% glutaraldehyde/2% paraformaldehyde solution (pH 7.3) made up in glucose-Krebs solution (Palaty, 1971) was perfused at the animal's mean blood pressure which was maintained with the continuously variable perfusion pump. Once the perfusion was started, the tip of the tail was cut off and the skin on the ventral abdomen was cut and reflected back to expose the thigh vessels. The right and left superficial epigastric veins were cut. Clear fixative was seen passing out of these veins and the tail shortly after perfusion started. Over a period of approximately 20 minutes, a total of 30-50 ml of fixative was perfused through each animal. A continuous recording was made of the perfusion pressure (Figure 4).

In all animals, the right and left saphenous arteries were removed. In some animals, samples of the right and left saphenous and femoral nerves and veins were taken. Therefore, 18 samples of the right and left saphenous arteries and 6 samples of right and left femoral veins were removed. Also, femoral nerve samples were taken from inside the abdominal cavity. For the right femoral nerve, they were taken adjacent to the proximal end of the tube. The left nerve was sampled from an equivalent area.

Fixation: All tissues were processed the same way. They were fixed

in the 3% glutaraldehyde/2% paraformaldehyde solution for a total of 1.5hr at room temperature (including perfusion time), followed by 0.5hr at 0°C. Since higher temperatures of this fixative promote the rate of penetration and maintain labile structures such as microtubules, and since artifacts due to polymerization of glutaraldehyde are minimized at lower temperatures, this sequence of aldehyde fixation acts as a compromise of the effects that occur at higher and lower temperatures. This was followed by two-5 minute washes in glucose-Krebs solution at 0°C and two-5 minute washes in 0.1M cacodylate buffer at 0°C. After washing, the tissues were fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1.5 hr at 0°C. Following this, were four-5 minute washes in 0.1M cacodylate buffer at 0°C and one-5 minute wash in distilled water at 0°C in preparation for en bloc staining in saturated aqueous uranyl acetate (1 hr at 0°C). Before dehydration, the tissues were washed again in distilled water for 5 minutes at 0°C.

Dehydration: The tissues were put through an acetone dehydration sequence: 50%, 0°C, 5 min.; 75%, 0°C, 10 min.; 90%, 0°C, 10 min.; 100%, 0°C, 10 min.; 100%, room temperature, 5 min.; 100% room temperature, 5 min.

Infiltration: Infiltration of the tissues using increasing ratios of Mollenhauer's (1961) embedding mixture (25 Epon 812 : 15 Araldite 502, Electron Microscopy Sciences): acetone solutions was done at room temperature. Tissues remained in the solutions of the ratios 1:3 and 1:2 for 30 min.. They were transferred to a 1:1 solution and the vials were put on an electric rotator (Labtronix Equipment) overnight. The next day, tissues were exposed to solutions of 2:1 followed by 3:1 ratios, 60 minutes in each. The tissues were then transferred to clean vials containing pure Epon-Araldite embedding mixture and placed in a vacuum

for approximately 1 hr. Following this, they were placed in pure, fresh embedding mixture again and put on the electric rotator overnight. In the morning, the embedding mixture was exchanged for fresh, and the open vials were placed in a vacuum oven until the length of time the tissue spent in pure Epon-Araldite embedding mixture totalled 24 hours.

The tissues were embedded individually in freshly made, pure Mollenhauer's embedding mixture. The vessels were placed in rubber molds and oriented so that they would be at right angles to the plane of sectioning. The resin was polymerized at 60°C for 48 hours and the blocks were further cured for 2 or more days at room temperature.

Sectioning

The vessels were aligned perpendicular to the knife edge so that complete transverse sections could be cut for light microscopy. Thick sections (0.5µm) for light microscopy were cut on a Reichert OmU3 ultramicrotome with a glass knife. The sections were placed on a glass slide and heat fixed at a high temperature on a Sybron Thermolyne hot plate. They were stained with a 1:1 mixture of 1% azur II and 1% toluidine blue made up in 1% borax (Pease, D. C., 1964; Humphrey and Prittman, 1974). The stain was filtered each day, just prior to use. Enough stain to cover the sections was placed on each slide with a dropper and left to dry on the hot plate (approximately 85°C). The stain was rinsed off the slides with distilled water and slides were returned to the hot plate to dry. The slides were coverslipped using Histoclad (Clay Adams) as a mounting medium. Thick sections of the blood vessel and nerve tissue samples were cut from the 60 and 90 day old age groups only.

Thin sections of one femoral nerve stump from the 60 day old age group were cut. All thin sections were cut on a Reichert OmU3

ultramicrotome using a glass knife. Sections were collected on 200 and 300 mesh, rhubidium coated copper grids and stained for 15 minutes in 1% aqueous uranyl acetate (the rhubidium coat on the grids lessens the effect of surface tension at the time the grid is breaking through the water's surface as the sections are being collected). After a thorough washing in distilled water, sections were stained with lead citrate for 15 minutes and then washed again.

Fluorescence Microscopy

A quick and consistent method for fluorescence of monoaminergic neurons and their axonal varicosities is the sucrose-potassium phosphate-glyoxylic protocol as modified by De la Torre (1980). This method of fluorescence microscopy was used to determine if norepinephrine were present at the adventitial-medial borders in the denervated arteries and veins. Animals for fluorescence were terminated at 30, 60, 90 or 120 days. Animals were anaesthetized using sodium pentobarbitone (0.06g/ml) made up in 0.9% saline solution. The dosage given was 1ml/kg body weight. A 1% aqueous solution of Trypan blue injected into the left femoral vein at a dose of 15mg/kg was left to circulate for one hour. Trypan blue makes elastic tissue of the artery fluoresce red. This helps to differentiate the elastic tissue from the fluorescing norepinephrine-containing nerve varicosities (McInnes, 1977). Without trypan blue, elastic tissue and adrenergic nerve varicosities have almost the same blue-green fluorescence. Evans blue can also be used (De la Lande and Waterson, 1968); however, Todd (1980) stated that trypan blue was the most effective.

After one hour, the right and left saphenous and femoral arteries and saphenous and femoral veins were excised. Each vessel was immediately embedded with in Tissue Tek II O.C.T. Compound (Miles Laboratories, Inc.)

and frozen (-30°C). The vessels were embedded perpendicular to the cork stub so that complete transverse sections could be cut. The vessels were cryostat sectioned at -30°C and at a thickness of 18μm.

Sections were picked up with a glass coverslip and exposed for 3 seconds to a pH 7.4 sucrose-potassium phosphate-glyoxylic acid solution (De la Torre, 1980). The sections were put under an airstream until the sucrose-potassium phosphate-glyoxylic acid solution had completely dried (30-45 min.). A drop of non-drying Type A immersion oil (R.P. Cargille Laboraties, Inc.) was placed on each coverslip and the coverslips were put into a 90°C oven for 3.5 min.. The coverslips were mounted onto cleaned glass slides and the edges were sealed with melted dental wax. In almost all cases, there were four slides per vessel. For each vessel, the slides were numbered from 1 to 4, representing the order of the first to the last sections cut.

ANALYSIS

Light Microscopy and Morphometrics

Light microscopic photographs were taken of all the right and left saphenous arteries in the 60 and 90 day age groups on a Leitz-Wetzlar Orthoplan light microscope at 10X (on 35mm film). The right and left femoral veins that were sampled in these age groups were photographed at 6.3X. Montages of the veins were assembled. Three complete transverse sections from each arterial and venous vessel were photographed. Black and white photographs were taken using 35mm Kodak Technical Pan (black and white) 2415 film. The film was developed using Kodak HC110 developer in a dilution of 1:15 for 7 minutes. The developer was discarded and the film was washed in distilled water containing a few drops of Kodak Stop Bath for 1 minute. The film was fixed for 5 minutes. Prints were made on Agfa-Gevaert Rapitone paper, using either grades 3 or 4 at final

magnifications of 145-320X for the veins and 220-570X for the arteries. The prints were developed using a Rapidoprint electric print processor using the Agfa-Gevaert Rapidoprint activator and fixer.

From each photograph, the entire cross-sectional area and the luminal area of the arteries were traced on an Apple II digitizing Tablet. The morphometric programme permitted the luminal area to be subtracted from the entire cross-sectional area to calculate the area of the tunica media (the area containing the smooth muscles cells). Therefore, from the photographs of the denervated and control arteries, the medial area was calculated. The montages of the veins required tracing on a larger Talos CYBERGRAPH digitizing board interfaced with the University mainframe AMDIAHL 471/V8 computer. Only the luminal cross-sectional area was traced since the microscopic magnification for these photographs was too low to determine the outer limits of the tunica media.

In order to measure wall thickness of the veins, four points from one of the montages of each vein were randomly chosen. These points were photographed on a Leiz-Wetzlar Orthoplan light microscope at 40X using 35mm Kodak Technical Pan black and white 2415 film. The film and prints were developed as described above. The higher magnification (40X) was necessary to identify the limits of the tunica media. The midpoint of each photograph was located, and at that point, the thickness of the tunica media was measured (in mm) using a ruler placed perpendicular to the endothelium. The four thicknesses of the tunica media for each vein were averaged together to produce the average medial thickness (in μm).

Electron Microscopy

The thin sections of a femoral nerve trunk were photographed on a Philips 300 electron microscope using 35mm film. The film was developed for 3 minutes in full strength D19 developer followed by a wash in

distilled water. The film was fixed for 5 minutes then washed with filtered water for 45 minutes at 70°F.

Fluorescence Microscopy

The cryostat sections stained for fluorescence were examined with a Zeiss fluorescence microscope using a Zeiss ultraviolet filter H365 01 (FT 395, LP 397) that permitted wavelengths in the upper 400nm region to pass. Beginning in the upper left corner, each coverslip was examined thoroughly to find the transverse sections of the saphenous arteries or femoral veins. Using a 40X Plan Neofluor objective, the number of fluorescing areas per section found at the advential-medial border of the vessel was counted. Counts were made for the right and left saphenous arteries and femoral veins from 8 animals. From each animal, 14-35 sections of the saphenous artery were counted and 10-25 sections of the femoral vein were counted. The number of sections counted for each vessel represented the number of sections cut minus the few that were unsuitable due to their being folded or blurred by a bubble in the mounting medium (immersion oil).

Black and white photographs and coloured slides were taken of some of these vessels using 35mm Kodak Tri X black and white film and 35mm Kodak Ektachrome colour slide film (ASA 400). The black and white film was developed in Acufine developer for 5 minutes. After a rinse with distilled water, the film was fixed for 5 minutes followed by a 1 hour wash in filtered water. Prints were developed in the same manner as for light microscopy. The colour film was developed commercially.

Statistics

The measurements of the tunica media of the saphenous arteries and femoral veins and of the lumen perimeter of the veins for both the denervated and control sides were compared using a paired Student's

t-test. Results are shown as mean $\pm$ SEM. The counts of the fluorescent areas from the saphenous arteries and femoral veins are compared using three different analyses of variance (ANOVAs). ANOVAs were run on the other data as well except for the measurements of the medial thickness of the femoral vein.

RESULTS

Sampling for Light Microscopy

Three animals per group were sampled for light microscopy at 60 and 90 days of age, thus making a total of 12 animals (Tables I and II). At the time of sampling, the animals were checked for gross clinical symptoms of denervation: dragging of the right foot when the animal walked; incomplete extension of the right leg when the animal is suspended by its tail.

Inside two animals, milky white scar tissue had to be removed from the denervated area to locate the positions of the femoral, the superficial epigastric and saphenous arteries. In the remaining animals, a more transparent connective tissue blanketed the vessels; therefore, the vessels were clearly visible. There were no indications of infection in any of the animals.

Generally, on the denervated side, there never appeared to be a healthy, glistening white saphenous nerve. Instead, the remnant of the nerve was so translucent that it was not visible or it was very thin and yellowish. The translucent nerve remnants had no form and were probably just connective tissue. This was indeed the case when these samples were examined under the light microscope.

In four animals, re-innervation was noted and in two of these four cases, the re-innervation pathways were the same. That is, in both cases, a very pale, translucent nerve, seemingly coming from the surrounding skeletal muscle of the thigh about half way along the femoral artery, coursed diagonally across the thigh in a caudal direction to reach the superficial epigastric artery a bit distally to its branch point from the femoral artery. One of these animals was denervated at 1-3 days and sampled at 90 days and the other animal was denervated at 12

days and sampled at 60 days. In the third animal, re-innervation appeared to have come from the abdominal wall, and in the last animal, a nerve was found running parallel with the inguinal ligament, although its origin and destination were undetermined.

Out of the six animals that were denervated at 1-3 days of age, only one had its tubed femoral nerve intact at the time of sampling (60 days of age). Because the nerves are so delicate at this stage (1-3 days), the nerve in three animals tore when pulling it into the tube, therefore, three of the six animals had no tube at all. Despite this however, a large segment of the femoral nerve was removed from within the abdominal cavity in these three cases. Tubes from the remaining two animals were present, but were unattached to the rest of the femoral nerve and were found lying in a mass of adipose tissue inside the abdominal cavity. In these two cases, the femoral nerve stump was found and it had a transparent yellowish appearance. One of these femoral nerve stumps seemed to be leading into the underlying skeletal muscle.

All six animals denervated at 12 days of age had their tubes still attached to the femoral nerve. The tubes were encased in connective tissue and no nerves appeared to be growing out of the melted end of the tube. The portion of the femoral nerve just proximal to, as well as the portion within the tube was thin, pale and yellowish compared to the larger, glistening white femoral nerve on the control side.

It was not until sampling the remaining six animals in the experiment that a change in the denervated femoral vein was noticed. The femoral vein showed a dilation in the area of the branch points of the superficial epigastric and saphenous veins. This dilation continued proximally, gradually tapering back down to its normal diameter at approximately one centimeter from its branch points. A small segment at

the branch points of the superficial epigastric and saphenous veins was slightly dilated as well. No anomalies of the femoral or saphenous arteries were apparent at the gross level.

LIGHT MICROSCOPIC APPEARANCE

Saphenous Artery

There was no remarkable difference in the light microscopic appearance between the denervated and control saphenous arteries (Figure 5). Both had a conspicuous endothelium with its many endothelial cell nuclei bulging into the lumen. A prominent internal elastic lamina, a well developed tunica media and a thin external elastic lamina were characteristic of their histological appearance. The internal elastic lamina was consistently thicker (one-half to two thirds thicker) than the external elastic lamina in both the denervated and control vessels. In some of the denervated arteries sampled at 60 days, the internal elastic lamina appeared to be slightly thicker than the internal elastic lamina in other denervated or control arteries. Small breaks in the internal and external elastic laminae in control and denervated arteries were apparent (Figure 5). In one control artery, the connective tissue from the media appeared to be continuous with connective tissue of the adventitia through one of the breaks in the external elastic lamina.

There were no differences in the tunica media between the denervated and control sides at the light microscopic level. Both had longitudinal and cross-sectional profiles of smooth muscle cells. Consequently, the profiles were of many different shapes and sizes. Some of the longitudinal profiles were long slender spindle shapes or long, thin and flat. Cross-sectional profiles included square, triangular, round, oval and polygonal shapes. Each cell was clearly separated from its neighbours by a thin unstained area, thereby emphasizing the highly

irregular cell boundaries. In both the denervated and control arteries, the profiles of the smooth muscle nuclei were also of various shapes and sizes because of the different planes of section at which these cells were cut. However, the shape of nuclear profiles did not necessarily conform to the shape of the cell profile, and, occasionally, the nuclei were eccentrically located. The nuclei were very euchromatic, there were none that were picnotic.

The adventitia, in both cases, appeared the same. From visual assessment,the adventitia of the denervated artery did not appear to contain more or fewer connective tissue cells than the adventitia of the control arteries.

Femoral Vein

Although the lumen of the denervated veins was dilated, their walls did not differ in their light microscopic appearance from those of the control veins (Figure 6). The definitive layers so characteristic of blood vessels were not easily identifiable in the veins (Figure 6). In a cross-section, an endothelium and adventitia were always present around the entire vein, but distinctive elastic laminae and media were not. Connective tissue occupied most of the area under the endothelium, and it was dotted with small groups of or individual smooth muscle cells. It was difficult to always positively identify the isolated cells as smooth muscle cells at this level of microscopy; some may have been fibroblasts.

Nerve

Figures 7(a) and 8(a) clearly show the light microscopic appearance of normal saphenous and femoral nerve tissue taken respectively from the contralateral control side. The multiplicity of myelin sheaths are packed tightly together, each one surrounded by a thin layer of endoneurium. The profiles of myelin sheaths ranged from large to very

small. Schwann cell and fibroblastic nuclei, small blood vessels, and unmyelinated nerve profiles are interspersed amongst the myelinated axons.

Distal Stump of Saphenous Nerve from the Denervated Side

Four samples of the distal stump (that part distal to the point of the segment removed) of the saphenous nerve were thick sectioned and stained, and the light microscopic appearance of one of these samples is seen in Figure 7(b). The nerve bundle is surrounded by a thick perineurium encapsulating a highly compact mass of cells and connective tissue. Some of the cells were large and pale staining while others were thin and more fibroblast-like. The cells are so closely packed together that cells boundaries are unidentifiable. The other samples had very similar appearances. In one of these sections, a macrophage was seen, and in another, there were still myelinated axons present, however, these were very small, were few in number and were surrounded by a thick endoneurium. All of these samples were well vascularized.

Abdominal Cavity Nerve from the Denervated Side

Thick sections from one tube showed the light microscopic appearance of the encased nerve (data not shown). A very thick capsule of circularly arranged, highly cellular dense connective tissue surrounded a core of denser connective tissue. This core was also very cellular, well vascularized and contained macrophages.

The three samples of that portion of the femoral nerve just proximal to the tube had slightly different light microscopic appearances. Figure 8(b) illustrates the appearance of one such sample. Here, a thick, circularly arranged coat of connective tissue surrounded an inner core of connective tissue. The connective tissue core consisted of many nuclei

belonging to cells whose boundaries were not distinguishable. Some nuclei were flat and fusiform, whereas others were square or round. The core was well vascularized and in the very centre were tiny profiles of myelinated axons (Figure 8, insert), much smaller than those from the control nerve. The number of these axons varied with each sample. For example, the sample in Figure 8(b) had only a few whereas the other two samples had more. Even though two of the samples had many profiles of myelinated axons, the number was not comparable to the control side. Moreover, all the profiles were very small and they were surrounded by a very thick endoneurium. Although a positive identification of the cells in the core cannot be made at this level of microscopy, perhaps they were a mixture of fibroblasts and Schwann cells.

Electron Microscopy of the Abdominal Cavity Femoral Nerve

The electron microscopic appearance of one sample was examined (Figure 9). The bulk of the tissue consisted of collagen fibres scattered amongst a few fibroblasts. The tissue also exhibited signs of degeneration. Occasionally, myelinated and unmyelinated nerve profiles were seen (Figure 9, insert), but only in the very centre of the nerve's core.

FLUORESCENCE MICROSCOPY

Altogether, eight animals were sampled for fluorescence, thus, 2 animals were sampled at 30, 60, 90 and 120 days of age. All exhibited positive clinical symptoms of denervation at the time of sampling.

Gross Morphology of the Denervated Area

In half of the rats sampled, a dilation of the femoral vein was noted. Also in four of the animals, the denervated saphenous and superficial epigastric arteries and veins branched from the femoral

artery in very close proximity to each other. In some cases, they branched off side by side, and in others, the superficial epigastric artery branched from the saphenous artery rather than from the femoral artery. On the control side, in all cases except for one, the branch points of the saphenous and superficial epigastric arteries and veins were approximately 0.5 cm apart.

Even though most of the animals sampled had the tube still attached to the femoral nerve, re-innervation of the saphenous artery was seen in half of the animals. The re-innervating nerves were very pale and translucent, one being similar in appearance to an empty arteriole. Re-innervation came from aberrant areas such as the abdominal wall, the skeletal muscle underneath the vessels, and from within the scar tissue blanketing the area. All led to the branch point of the saphenous artery.

In two animals, sprouting of the femoral nerve inside the abdominal cavity was visible. In one of these animals, the tube had detached, and in the other animal, sprouting of the femoral nerve was proximal to it entering the tube. I was unable to determine the destinations of these axonal sprouts because the contents of the abdominal cavity were blue from the trypan blue.

Description of the Sections under the Fluorescence Microscope

Figures 10 and 11 show the appearance of the denervated and control vessels of the saphenous artery and saphenous vein, respectively. After injection with trypan blue, the external elastic lamina always fluoresced in the red range, however, the internal elastic lamina did not fluoresce red as consistently. Although specific adrenergic fluorescence was concentrated at the adventitial-medial border, occasionally it was seen in the adventitia. In some of the veins, the fluorescent nerves extended beyond the adventitial border towards the endothelium. The fluorescent

areas varied in size from very tiny to large dots. Sometimes, the smaller dots had a "beads on a string" appearance. Generally, fluorescent dots were relatively uniform in their distribution around the circumference of the control arteries, whereas the veins had patches of fluorescent dots irregularly spaced around their circumference.

The identity of a re-innervating adrenergic nerve that had the appearance of an arteriole was verified by fluorescence. Cross sections of this nerve contained many small, pale fluorescent dots.

ANALYSIS

Morphometrics

Medial Area of the Saphenous Artery

From light micrographs, morphometrics were carried out on the saphenous artery and femoral vein. The raw data as well as the means are listed in Tables I and II. For the arteries, a Student's t-test was carried out on the data from each table separately. When the arteries were sampled at 60 days, the results of the t-tests show that there was a tendency for a thinner media, but this was not significant at the $p<0.05$ level (Table I). However, if each sample is looked at individually, the measurements do show that the area of the tunica media was decreased in four of the six animals. The remaining two animals show a very slight increase in the media of the denervated arteries; therefore there was virtually no change in the media of these two samples. Animals sampled at 90 days showed a significant decrease in the wall on the denervated side (Table II).

A two-factor and a three-factor ANOVA were carried out on the data from Tables I and II. The two-factor ANOVA of age of denervation (1-3 or 12 days) versus condition (denervated or control) was carried out separately on the 60 and the 90 day old groups. This ANOVA verified the

t-test results, but it also showed that the age at which the animals were denervated was not significant, and that there was no interaction between the two variables.

The three-factor ANOVA included, in addition to the two factors just mentioned, the age at time of sampling. From this, sampling age did make a significant difference (P=0.024).

Figure 12 summarizes the data from Tables I and II as per cent decreases in the media, where the total mean area of the control side represents 100%. The graph shows that both sampling age groups show a 15% decrease.

Vein Perimeter and Wall Thickness

From light microscopic cross-sections of the femoral vein, the luminal perimeter and the wall thickness were measured for each of the denervated and contralateral control veins. The raw measurements of the luminal perimeter and their means are listed in Table III. Both a Student's t-test and an ANOVA were carried out on these means, and both tests confirmed that the increase in luminal perimeter of the denervated veins was significant. The ANOVA also showed that denervating the femoral vein at either 3 or 12 days had no significance, and there was no interaction between the two factors tested, these being, age at denervation and condition (denervated or control). The percent increase is shown in Figure 13.

The results of measuring the wall thickness of the denervated and control femoral veins are recorded in Table IV. The t-test results indicate that the difference in wall thickness between the denervated and control femoral veins is not significant. Also, the raw data shows that the wall thicknesses from both sides were quite variable.

Fluorescence Counts

The number of fluorescing dots found around the denervated and control saphenous arteries and femoral veins was counted. The counts are listed in Table V. Two, two-factor and one, three-factor ANOVAs were carried out on the data. The two, two-factor ANOVAS showed that there was a significant decrease in the number of fluorescent dots both for the artery and for the vein. The age at denervation was not significant for denervating the femoral vein.

The second two-factor ANOVA (condition versus age of sampling) was designed to see if the sampling age had any effect. That is, by ignoring the effect of condition (denervated or control), the ANOVA showed that the effect of age of sampling on the fluorescence counts was significant for the arteries ($P=0.019$) and for the veins ($P=0.048$). The additional information gained from the three-factor ANOVA confirms a point which may be self-evident; that is, there was a significant difference in fluoresence between the two blood vessel types.

DISCUSSION

In this study, I have developed a denervation technique to study the trophic influences between nerves and blood vessels in the rat thigh. This technique was designed to be as localized and as permanent as possible for the following reasons: (1) sympathetic ganglionectomy and chemical and immunological sympathectomies destroy the adrenergic innervation to such a large area of the body that it is important to determine whether the denervation effects seen with these methods are due solely to the absence of innervation on the blood vessel or whether other denervated structures had changed and thereby contributed to the results, and (2) simple crushing, severing or repositioning the nerve are unsuitable methods for denervating blood vessels since re-innervation occurs within a few days (Todd, 1986) - a period of time which may be too short for denervation changes to occur. After determining the success of this technique, I then used the method to determine whether depriving blood vessels of their vasomotor innervation significantly alters their architecture. In this study, the area of the tunica media of the rat saphenous artery decreased and the femoral vein was dilated. The experiments also reconfirmed that it is extremely difficult to prevent aberrant re-innervation of the thigh vessels.

In previous studies, the success of denervation on blood vessels was determined by a variety of ways. Supersensitivity of the smooth muscle to exogenously applied norepinephrine, stimulation of the entire sympathetic outflow using a steel pithing rod as the electrode (6-hydroxydopamine denervation), histological studies, and chemical and electrical stimulation of specific ganglia (immunological sympathectomy) are just a few of these ways (Levi-Montalcini and Angeletti, 1966; Finch

et al., 1973; Bevan and Tsuru, 1979; 1981). Although the femoral nerve was torn in 3 out of the 6 animals that I denervated at 1-3 days of age, I still regarded them as being successfully denervated. The nerves tore while pushing the tube on; therefore, the resulting proximal stump was very short (ie. close to the sympathetic chain) in the 3 day old animal. It has been reported that the closer the injury is to the cell body, the greater the damage to the cell body (Gabella, 1976; Barr and Kiernan, 1983). Thus, I assumed that the nerves torn in the 3 day old animals tore close enough to their nerve cell bodies to most likely cause cell death. Since (1) atrophy and degeneration of cell bodies can persist months after axotomy (Gabella, 1976), (2) destruction of adrenergic cell bodies abolishes vasomotor control, and (3) cell bodies in newborn animals are more susceptible to denervation techniques (Levi-Montalcini and Angeletti, 1966; Finch et al., 1973), I assume in my experiment that the three animals who had the torn femoral nerve were successfully denervated, even at time of sampling.

For all animals, those with or without a tube, the degree of success of my denervation technique on the saphenous artery and femoral vein was determined. The methods used for this included showing that the proximal and distal stumps of the femoral nerve had a degenerated appearance using light and electron microscopy (electron microscopy was carried out only on the proximal nerve stump). In addition, evidence for denervation was obtained by examining the gross morphology of the thigh for the presence of regenerating nerves, by light microscopic examination of the adventitia of the vessels, and by a fluorescence technique specific for catecholamines. The light microscopic appearance of the distal and proximal stumps from my study showed definite signs of degeneration.

When a peripheral nerve is cleanly transected, proper axonal

regeneration requires appositioning the two cut ends and suturing through the epineurium (Barr and Kiernan, 1983). This surgical repair is not necessary if the axons have been transected by a crush because the connective tissues in the nerve remain intact (Barr and Kiernan, 1983). Axonal transection, therefore, produces permanent degeneration of the distal adrenergic fibres and in temporary degeneration of the cell body (Burnstock and Costa, 1975). Distal to the site of injury, the detached portion of the peripheral nerve undergoes Wallerian degeneration whereas the proximal portion undergoes the axon reaction which is best displayed in the cell bodies as chromatolysis (Barr and Kiernan, 1983). Wallerian degeneration is characterized by the axon initially becoming swollen and then breaking up into fragments. Accompanying these axonal changes are changes in the myelin sheath. It is broken into short ellipsoidal segments and then gradually disintegrates. The Schwann cells multiply, filling the cylindrical area enclosed by the endoneurium. The remnants of motor neurons (the axon and its myelin sheath) and the axons of unmyelinated fibres are phagocytosed, with the distal stump being composed of columns of Schwann cells (bands of von Bungner). The content of norepinephrine disappears anywhere from 18 to 48 hours after damage to the nerve (Burnstock and Costa, 1975); which may explain why terminal nerve transmission is not immediately stopped following damage (Gabella, 1976). These results vary between organs and species. Blood vessels of the rabbit ear still responded to nerve stimulation three days post-severing (Gabella, 1976). The general structure of sympathetic axons appears normal for several days (Burnstock and Costa, 1975), and the length of time for degeneration is probably directly proportional to the axon length (Burnstock and Costa, 1975; Gabella, 1976). That is, a short distal stump degenerates faster than a longer one. Since

application of colchine produces degeneration of a nerve distal to its application, this suggests that transport of substances is vital for the survival of the axons (Burnstock and Costa, 1975). Therefore, the light microscopic appearance of the distal portion of the saphenous nerve in my study indicates that it was probably devoid of adrenergic fibres, and the cells that were there were most likely Schwann cells arranged in the bands of von Bungner. The distal stump of the saphenous nerve closely resembled the distal stump of the cat tibial nerve described by Pellegrino and Spencer (1985). Seven weeks post-denervation, the cat tibial stump is virtually devoid of myelin debris, and 89% of the nuclei in a cross-section represent bundles of Schwann cells which are separated by a lot of collagen and elastin fibres, scattered fibroblasts and patent blood vessels. In addition, Pellegrino and Spencer (1985) found that seven weeks post-denervation the uptake of ^{3}H-thymidine into the first 9.5cm of the distal tibial nerve stump is linear over a 3-hour period and does not differ along the length of the nerve whereas if this 7 week denervated distal stump is joined end-to-end to a newly severed proximal stump of the peroneal nerve, an increase in ^{3}H-thymidine uptake is seen within the first 6cm of the coapted distal stump. Beyond these first 6cm, the ^{3}H-thymidine uptake is similar to that along the entire 9.5cm length of the 7 week denervated (non-joined) distal stump. Pellegrino and Spencer (1985) found that the first 2-6.5cm distal to the site where the nerves were joined is an area of axon-Schwann cell contact. This suggests the presence of axons is mitogenic thereby stimulating the myelination process. The results of Pellegrino and Spencer (1985) also suggest that in my study, the Schwann cells remained as bundles. That is, since direct re-innervation of the distal saphenous nerve stump by its proximal stump was prevented by encasing the proximal end of the

femoral nerve in a tube, the Schwann cells in the distal stump had no stimulus to divide and form myelin,

Changes in the proximal portion of a severed nerve may vary depending on the type of neuron; therefore, some neurons may totally disappear whereas others may not be significantly altered (Barr and Kiernan, 1983). Large motor neurons supplying skeletal muscle exhibit the cytological details of the classical axon reaction, the description of which follows. The most significant alteration in the severed axon occurs immediately adjacent to the cut. The remainder of the axon is not altered appreciably. Coarse clumping of Nissl substance can appear in the cell body as soon as 6 hours after section. The nucleus becomes eccentrically located (Barr and Kiernan, 1983; Gabella, 1976) and flattens out, later on becoming indented (Gabella, 1976); this process reaches a maximum at 10-20 days after injury (Barr and Kiernan, 1983). Organelles become somewhat disorganized (Barr and Kiernan, 1983). At the end of 6 weeks, 40-50% of the cells may still show signs of chromatolysis (Gabella, 1976), or some may persist for months (Barr and Kiernan, 1983; Gabella, 1976).

In my study, the proximal end of the femoral nerve was encased in a polyethylene tube which may have contributed to the increased spread of degeneration along the length of this nerve. A similar appearance to my observations was seen in the nerve repair studies by Colin et al. (1984). In their study, a 5-7mm portion of rat tibial nerve was excised from one side. The rats were divided into 2 groups. To induce the formation of a fibrovascular sheath in one group, all rats had their proximal and distal nerve stumps connected by a silicone rod whereas, in the other group, the 2 ends were left separated. Four weeks later, all the silicone rods were removed, leaving a fibrovascular sheath behind.

These rats were divided into 3 subgroups, and further experimental treatment was carried out on 2 of these subgroups while the third acted as a control. The same protocol was applied to the group without the rod inserted, that is, the unsheathed group. The animals were left for 3 months. One of the experimental treatments carried out on a subgroup involved connecting the two free ends of the nerves with a collagen tube. The results were interesting since both the sheathed and unsheathed collagen-tube-encased nerves exhibited the thick fibrosed epineurium which I found on my plastic tube-encased femoral nerve. However, the cores of the nerves in the study by Colin et al. (1984) had a normal histological appearance. Therefore, the collagen tube may have induced the thick, fibrosed epineurium but it also facilitated the normal regeneration of the nerve by guiding the axonal sprouts into the distal segment. The core of the femoral nerve in my study resembled most that of the unsheathed control group in Colin et al.'s study. That is, the core had a fibrosed internal milieu with a few diffuse mini-fasicles, the axons of which were small in diameter with thin myelin sheaths. From these comparisons, it can be seen that the proximal and distal stumps in my study definitely had a degenerated appearance.

By examining the gross morphology of the thigh area, I was able to see very thin, translucent, unmyelinated nerves re-innervating the saphenous and superficial epigastric arteries in some of the denervated animals. Although I did not see any nerves in the adventitia of the denervated vessels with light microscopy, the fluorescence technique did reveal the presence of some catecholamines at the adventitial-medial border. These results indicate that re-innervation did occur.

As in humans (Williams and Warwick, 1975), the femoral nerve of the rat carries the adrenergic innervation to the saphenous and femoral

arteries and veins (Todd, 1986). Therefore, adrenergic regulation of the vasomotor activity of these vessels ceases by severing the femoral nerve. However, after denervation, vascular smooth muscle exhibits automaticity which is the ability of a blood vessel to maintain a basal tone without a coordinating nerve supply (Page and McCubbin, 1965). Under artificial conditions in restricted areas (in hamster cheek pouch or web of frog's foot) two phenomena have been observed after denervation. These include the maintenance of a basal tone and rhythmic changes in tone (Page and McCubbin, 1965). Page and McCubbin (1965) state that certain blood vessels, such as those that supply the skin, are much more dependent on the nervous system for coordination than blood vessels supplying other areas. Denervation of the blood vessels of the brain, heart, liver and kidneys does not appear to alter the organs' blood supply, suggesting that these blood vessels depend on other controlling factors such as myogenic activity or the local chemical environment (Page and McCubbin, 1965). If the dependency of a blood vessel on its neural coordination may reflect that vessels' ability to stimulate its re-innervation, then the occurrence of re-innervation in my study and in Todd's (1986) experiments suggest that the blood vessels in the rat thigh are highly dependent on adrenergic control. Since the femoral nerve does not appear to be the source of these re-innervating fibres, the axonal sprouting from aberrant areas in the vicinity of the denervated vessels suggests that vascular smooth muscle cells may have a widespread and powerful mechanism which prevents permanent denervation from occurring. Being so widespread, this mechanism might be a diffusible chemotrophic or chemotactic factor.

Nerve density around a blood vessel may be directly proportional to a vessel's ability to induce sprouting of neighbouring nerves. This is

supported by transplantation experiments carried out by Todd (1986). In her study, segments of the densely innervated rat tail artery and the rat femoral artery, which has virtually no innervation, were transplanted separately into the anterior eye chamber of a host rat. Only the tail artery was capable of inducing iridial nerve sprouting. Although these results suggest it is the blood vessel itself that is the trophic inducer, Politis et al. (1982) provides evidence that the transected distal nerve stump contains diffusible factors which can attract regenerating axons.

Re-innervation of blood vessels from aberrant areas is also seen in denervation experiments where the entire superior cervical ganglion is removed (R. D. Bevan, personal communication with M. E. Todd; Kobayashi et al., 1983). Bevan states that re-innervation of the rabbit ear vessels occurs between 6-8 weeks in the denervated rabbit whereas Kobayashi et al. (1983) report nerves regenerating between 4-6 weeks and reach a maximum between 9 and 12 months in denervated Wistar rats. However, the maximum number of regenerating nerves is approximately half of the normal number.

In other cases, nerve regeneration after denervation has been reported to occur in under 15 days (Todd, 1986; Dyck and Hopkins, 1972). Todd (1986) tried four different methods of surgically denervating the saphenous and superficial epigastric arteries. They included severing the femoral nerve, removal of a segment of the femoral nerve, cauterizing the proximal stump after a segment was removed and repositioning the severed femoral nerve by suturing it to the abdominal wall. In all of these cases, re-innervation occurred within 15 days. Axonal sprouts were seen 5-15 days after crushing the cervical sympathetic trunk by Dyck and Hopkins (1972). Since the re-innervating fibres in these two studies

sprouted from the severed or crushed nerve, rather than from aberrant areas as in studies involving more drastic methods of denervation, this may account for the short regeneration time.

Another method I used to analyze the degree of success of my denervation procedure was fluorescence. The presence of catecholamines in some of the denervated vessels suggested that these vessels had been re-innervated. In arteries, adrenergic nerve varicosities occur at the adventitial-medial border (Burnstock and Costa, 1975; Todd, 1980) rather than penetrating into the media as they do in veins. Since resolution of the light microscope is limited, the fluorescing dots are more likely to represent cluster of axonal varicosities rather than individual varicosities. However, the "beads on a string" appearance is characteristic of the series of adrenergic varicosities of a single nerve ending (Ham and Cormack, 1979) and has been shown by Burnstock and Costa (1975) using fluorescence.

A rapid and consistent histofluorescence method specific for the visualization of catecholamines was modified by De la Torre (1980). Since he has standardized this method, a very consistent intensity of monoaminergic neurons and their axonal varicosities from one section to the next has been achieved (De la Torre, 1980). Therefore, differences in counts should truly reflect differences in catecholamines rather than inconsistencies produced by the method. By injecting trypan blue into the animal before sampling, any confusion between the fluorescence of the external elastic lamina and that of nerve varicosities is eliminated, thereby ensuring that only the dots produced by the fluorescence of catecholamines were counted. The significant decrease in fluorescence obtained in this study, represented a decrease in the amount of norepinephrine. Whether this represented an absence or a decrease in

unmyelinated nerve terminials awaits further investigation.

The fluorescence data indicate that some nerves were present around the denervated vessels. These counts probably represent the norepinephrine content of the re-innervating nerves. As previously discussed, re-innervation did occur sometimes. The re-innervating nerves had a very translucent gross morphology; therefore, the actual number present probably exceeded that viewed. This would account for the fluorescence seen in all the vessels sampled whereas by gross observation, re-innervation was only reported in half of the animals. In spite of how well the surgical technique prevents the proximal end of a severed nerve from sprouting and re-innervating its target blood vessel, it seems that their re-innervation by adrenergic nerves cannot be prevented.

Once the degree of success of my denervation technique was established, morphological and morphometrical analyses were carried out on the saphenous artery and femoral vein samples. Changes in the morphology of arterial walls has been recorded in only a few other denervation studies (Branco et al., 1984; Todd, 1986). The external elastic lamina of the surgically denervated superficial epigastric artery becomes irregular and broken as seen ultrastructurally by Todd (1986). In the present study, it appeared from light microscopic examination that the internal and external elastic laminae may have been more broken on the denervated side compared to the contralateral controls; however, the breaks were not counted and compared statistically. Although the internal elastic lamina looked somewhat thicker under the light microscope and in preliminary electron microscopic examinations, further ultrastructural investigation and measurements of the internal elastic lamina is needed to determine this conclusively.

Branco et al. (1984) showed that the smooth muscle cells in the denervated dog saphenous vein and the denervated rabbit ear artery appear dedifferentiated with characteristics of active protein synthesis. That is, they are larger, have larger euchromatic nuclei with prominent nucleoli and the cytoplasm is rich in ribosomes and has well developed rough endoplasmic reticulum. Although all the smooth muscle cells in the venous wall show these morphological changes, only 2-3 smooth muscle cell layers adjacent to the adventitial-medial junction in the rabbit ear artery showed these changes. The increases in the diameter of smooth muscle regresses towards normal values after 120 days in the vein and 35 days in the artery (Branco et al., 1984). The dedifferentiated appearance of smooth muscle cells was seen in vitro by Chamley and Campbell (1976) and this dedifferentiation was prevented for a few extra days by the presence of sympathetic ganglion extract. Contrary to the findings of Branco et al. (1984), other investigations involving the denervation of the rabbit ear artery via superior cervical sympathectomy have suggested that denervation creates a thinner tunica media (Bevan and Tsuru, 1979; Bevan and Tsuru, 1981; Bevan et al., 1983). Branco et al. (1984) sampled the rabbit ear artery 15 and 35 days post-denervation whereas Bevan and Tsuru (1981) sampled theirs 8 weeks later which is well past the time where Branco et al. (1984) saw regression of the artery back to normal; therefore, it may be that the difference in the sampling age is what produced these opposing observations. Todd (1986) saw no structural difference in the media at either the light or electron microscopic level. A change in the media was not obvious by light microscopic examination in my study either. My preliminary electron microscopic investigations of the denervated media also did not show any apparent differences from the contralateral control side although

fibroblasts and fibroblastic processes abutted the adventitial-medial border of the denervated vessels. This observation may have been the result of the lack of nerves in the area since Branco et al. (1984) found that fibroblasts were more numerous in the denervated vessels and showed characteristcs of synthetically active cells.

Although a decrease in the tunica media of the denervated saphenous artery did not appear markedly thinner by light microscopic observation, actual measurements of the media demonstrated a decrease. Decreases in the wall thickness of the denervated rabbit ear artery have been shown by Bevan and Tsuru (1979), and decreases in weights of denervated middle and posterior rabbit cerebral arteries was shown by Bevan et al. (1983). Bevan and Tsuru (1979) suggested that denervation creates a smaller wall. This appears to be true in my study as well. Contrary to these findings, however, are the results of Branco et al. (1984) who found an increase in wall thickness in the denervated rabbit ear artery.

In my study, a preliminary electron microscopic investigation was carried out. No change in the ultrastructure of the media between the denervated and control saphenous arteries was apparent. This suggests that the decrease in the media may have been the consequence of changes in the extracellular matrix (Rusterholz and Mueller, 1982). More extensive electron microscopic studies of this denervated smooth muscle should be carried out to investigate this possibility. Small changes in cell size (volume) that are not distinguishable by qualitative examination might also contribute. For example, if each medial smooth muscle cell had a slight decrease in size or change in shape, the additive effects of these individual decreases may possibly produce a measureable decrease in the media. Morphometric analysis, such as that developed by Todd (1983) for vascular smooth muscle, should also be

carried out to see if cell size actually contributes to the medial decrease.

Differences in measurements were expected between the two ages of denervation (1-3 days and 12 days). Todd (1980) reported that neurotransmitter in nerves did not appear around the sapheous artery until 3 days of age, as determined by the presence of fluorescence in the developing innervation. With this in mind, the intent of my study was to denervate the artery before the neurotransmitter appeared and had any influence over the developing artery. Also, two peaks in the number of nerves per unit area were seen by Todd (1980), one at 5 days of age and the other at 12-15 days of age. The greater the density of the adrenergic plexuses, the greater the potential for neurogenic muscular tone (Bevan and Su, 1973). Perhaps, then, a greater nerve density also might mean a greater trophic influence on the smooth muscle cells. Hence, the denervation at the two ages, in this study. However, the statistics proved that there was no difference in denervating at 1-3 or 12 days of age. This may be a question of whether or not the neurons are functional before 12 days of age. Thus, even though there may be a greater density of nerves at 12 days, their effect on the smooth muscle would be no different than that at 3 days if the nerves have not fully developed, that is if they are not generating an action potential and transmitting, and not producing any trophic factors. Ultrastructural nerve profiles very different in appearance from profiles of mature nerves are seen at 11 days (Todd and Tokito, 1981). Even though adrenergic terminals have been identified in the hind limb of the dog by one week of age (Dolezel et al., 1974), Boatman et al., (1965) have reported that vascular adrenergic innervation in the hind limb is nonfunctional until after two weeks of age.

Even though I could find no abnormality in the gross morphology of the saphenous artery itself, other alterations in the gross arrangement of the denervated vessels were noticed. When sampling during the fluorescence procedure, the estimated distance of 0.5cm between the superior epigastric and saphenous branch points from the femoral artery and vein was noticeably decreased on the denervated side only. Also, a dilation in the denervated femoral vein at the area of its branches was very obvious. Such gross structural changes have not been mentioned in the literature; however, Bevan (1984) does mention an increase in arteriovenous anastomoses in the denervated rabbit ear artery. These changes in the pattern may be secondary effects to a local alteration in blood pressure resulting from denervation.

The results from the denervated vein, where the vessel became grossly dilated, may be due to structural changes in the wall for two reasons. First, other studies (Bevan and Tsuru, 1979; 1981) and this one have indicated that wall thickness decreases following denervation. Since denervation causes a decrease in the mitotic index of the smooth muscle cells from the media of the rabbit ear artery (Bevan and Tsuru, 1975) this suggests that denervation may indeed cause a thinner wall. Also, maximum tension (force/cross-sectional area) was less in the denervated rabbit ear artery; therefore, Bevan and Tsuru (1979) attributed this decrease to a qualitative change in the contractile machinery. Therefore, changes in smooth muscle mass, in smooth muscle number and in the contractile machinery may produce a weaker wall resulting in the dilated veins reported here.

Secondly, changes in the connective tissue fibers (elastin and collagen) may affect the resistance of the blood vessel wall. Aneurysms and varicose veins are clinical examples of dilations in blood vessels.

Although aneurysms are mainly associated with the arterial side of the circulatory system, aneurysms in veins, such as the portal vein, do exist (Ohnishi et al., 1984). The etiologies of these two clinical examples may involve changes in the connective tissue fibre components of blood vessel walls (Grobety et al., 1977; Niebes et al., 1977; Crissman, 1984; Dobrin et al., 1984). Rusterholz and Mueller (1982) suggested that the decrease in perfusion pressure (representing vascular resistance) of the denervated rabbit ear vascular bed may not be exclusively due to a decrease in smooth muscle mass, but may be the result of alterations in the collagen or elastin. Therefore, the importance of collagen and elastin in the blood vessel wall should not be overlooked. Fibroblasts synthesize collagen and elastin as do smooth muscle cells; however, the fibroblasts are only found in the adventitia of blood vessels. Also, when Chamley and Campbell (1975) cultured smooth muscle cells from the guinea pig vas deferens, they described the ultrastructure of fibroblasts present in the culture after 1-2 days in culture. However, in their study, they did not follow through on the appearance of the fibroblasts; therefore, any effect that norepinephrine, sympathetic ganglion extract and cyclic AMP might have had on these cells was not mentioned.

Dobrin et al. (1984) have looked at the importance of these two types of fibres in canine and human arteries. Treatment of canine common carotid arteries and human external, internal and common iliac arteries with elastase produced dilation of these vessels, with a decrease in compliance seen at higher pressures. The integrity of these elastase-treated vessels was always maintained. With collagenase treatment, the vessels were less dilated than the elastase-treated vessels, although they were significantly different from the controls. Also, the collagenase-treated vessels leaked uncontrollably and

eventually ruptured. Thus, collagen is needed to maintain the integrity of the vessel whereas elastin is needed to maintain the normal shape of the wall, but not the integrity. The normal shape of the denervated femoral vein in this study was significantly dilated, but it did not rupture or leak. The fact that the femoral vein did not rupture or leak does not prove that collagen was not changed at all in amount; however, the fact that the normal shape of the vessel was changed suggests that the elastin was affected somehow. For instance, the normal content of elastin might not have been attained during the development of the denervated vein thereby disturbing the elastic/collagen ratio. An alteration in the elastic/ccllagen ratio as a consequence of denervation was suggested by Rusterholz and Mueller (1982). Changes in the structure of the elastic fibres themselves or in their orientation may have been affected by the denervation procedure.

The three-dimensional network of elastic fibres in canine saphenous veins has been studied by Crissman (1984). In normal saphenous vein, he found that the internal elastic lamina, the media and the external elastic lamina each had their own unique elastic fibre organization. The elastic fibres from each of the three layers merged with the adjacent layer, forming a continuous network through the entire thickness of the wall. The single layer of elastic fibres in the internal elastic lamina consisting of large longitudinally oriented (at a slight angle from the true longitudinal) branching fibres intersected by finer fibres is thought to distribute stress around and along the longitudinal surface. The media also had two sets of fibres, both larger than those of internal elastic lamina, but arranged somewhat the same. However, the longitudinal thicker fibres were angled tangentially, either directed externally or internally, traversing different levels of the media and

did not form stratified layers of fibres. This organization is thought to distribute tension throughout the venous wall. The external elastic lamina was formed by several parallel layers of wide ribbons of closely opposing thick elastic fibres. Ribbons within the same level as well as those in adjacent levels were interconnected by the thick and thin elastic fibres. It was suggested that the arrangement and thickness of this layer would increase its rigidity thereby maintaining the shape of the vein and keeping the lumen open when external pressures were exerted on the venous wall by external organs. Thus, Crissman (1984) believes that the architecture of the elastic network would contribute to vascular integrity and flexibility as well as aid in the distribution of stress throughout the venous wall. With this in mind, it is possible that denervation may have disrupted the elastic architecture in the wall of the rat femoral vein in my study. Since Todd (1986) found ultrastructural changes in the external elastic lamina, this layer may be the one most affected.

Changes in the collagen content and other connective components were seen in varicose veins (Grobety et al., 1977; Niebes et al., 1977). Light microscopic studies carried out on human saphenous varicose veins showed a marked increase of interstitial staining with Alcian blue and Toluidine blue as well as an increase in PAS positive material (Grobety et al., 1977), which widely separated the bundles of smooth muscle. Electron microscopy confirmed the increased interstitial space and also showed the loss of normal organization and structure of the interstitial connective tissue (Grobety et al., 1977). These histochemical results coincided with the biochemical results obtained by Niebes et al. (1977). They found that the insoluble collagen content was significantly less in the varicose veins, but the total amount of glycosaminoglycans and

glycoproteins was significantly greater. Since no variations were in the smooth muscle or energetic metabolism (Niebes et al., 1977), the combined histological and biochemical findings indicate that the main abnormalities of varicose veins are in the connective tissue components. It appears from past studies that connective tissue components play an important role in maintaining the shape of the blood vessel wall. If the collagen component is changed by lack of innervation, then the wall may weaken and a dilation such as that seen in the femoral vein in this study may occur.

Not many denervation studies have been done on veins. Denervation of the rat portal vein (Aprigliano, 1983) produced supersensitivity of the smooth muscle cell membrane to norepinephrine. Morphological studies of the denervated dog saphenous vein were carried out by Branco et al. (1984). They found that the wall thickness of the denervated saphenous vein in the dog was greater than that of the controls. In their study, this thicker wall persisted in the vein even at 120 days post-denervation. The smooth muscle cells in the denervated dog saphenous vein had a dedifferentiated appearance, a characteristic of which was larger smooth muscle cells. Since I found a decrease in medial thickness of the saphenous artery, I assumed that, in my study, the media of the veins would be thinner too, especially since the vein was so dilated. However, this was not the case. Some cross-sections of the dilated rat femoral vein showed that some areas of the wall were very thin and tenuous. Although, when four randomly chosen points were measured and averaged together, the wall of the denervated femoral vein in my study was not thinner than that of the control side. In fact, the total mean wall thickness of the denervated femoral vein tended to be higher than the mean of the control vein. This difference was not

significant at the $P<0.05$ level (Table IV). It is possible, therefore, that the dilation may not be the result of a decrease in the elastin content as implied by the work of Dobrin et al. (1984), but rather a change in the architecture of the elastic fibres (Crissman, 1984).

No other morphological differences between the denervated rat femoral vein and its contralateral control side were seen at the light microscopic level in this present study. The explanation given for the lack of difference between denervating the saphenous artery at the two different age groups (that is, at 1-3 or 12 days) probably applies to the femoral vein.

CONCLUSIONS

The method of surgical denervation in this study is as successful as superior cervical ganglionectomy, although my method is far more localized. Although re-innervation from aberrant areas does occur, my method of denervation does produce alterations in the blood vessels. The decreases in the medial area of the artery do persist long after denervation (78-87 days); however, whether the decrease is due to a reduction in smooth muscle cell size or a reduction in the paracellular matrix remains to be determined. Also, this denervation technique leads to a reproducible dilation of the femoral vein, the mechanism by which this dilation occurs is unknown.

TABLE I: Changes in the Area of the Tunica Media of the Saphenous Artery when Sampled at 60 Days of Age.

Age at Denervation (Days old)	Age Sampled At (Days old)	Area of the Tunica Media of the Saphenous Artery			
		Control Side (μ^2)	Mean	Denervated Side (μ^2)	Mean
1-3	60	34481.6		24534.7	
		34859.5	34559.4	24054.2	24248.3
		34337.2		24155.9	
	60	41183.1		40783.2	
		40846.5	40902.2	41534.0	41305.4
		40676.9		41599.0	
	60	37182.5		34244.1	
		37633.1	37379.1	34720.0	34426.6
		37321.7		34315.0	
12	60	36068.1		19305.9	
		36361.7	36479.1	19842.9	19664.5
		37007.6		19844.6	
	60	25176.9		26254.4	
		25125.3	24936.2	26180.6	26158.5
		24506.3		26040.5	
	60	47077.6		42412.7	
		46928.9	46878.7	42360.2	44180.9
		46629.6		47769.8	
	TOTAL MEAN		36855.8		31664.0
	±SEM		±2969.8		±4027.2
	P=0.10				
	n=6				

TABLE II: Changes in the Area of the Tunica Media of the Saphenous Artery When Sampled at 90 Days of Age

Age at Denervation (Days old)	Age Sampled At (Days old)	Area of the Tunica Media of the Saphenous Artery			
		Control Side (μ^2)	Mean	Denervated Side (μ^2)	Mean
1-3	90	41634.3		34345.0	
		42159.2	41905.8	35579.2	34906.5
		41924.0		34795.3	
	90	42103.4		35665.2	
		41638.5	41714.4	36092.9	35861.6
		41401.2		35826.8	
	90	41926.8		45722.4	
		42529.4	42564.5	46054.6	45831.7
		43237.4		45718.2	
12	90	44045.5		42659.4	
		44412.5	44384.7	43784.8	43132.7
		45182.8		43069.5	
	90	43897.9		43017.1	
		48085.9		34849.4	
		47745.3	47713.1	34287.4	33698.0
		47627.3		32486.1	
	90	47393.8		33269.2	
		50364.2		37405.1	
		50675.9	50568.2	38171.1	37455.3
		50664.5		36789.6	
	TOTAL MEAN		44808.4		38481.0
	±SEM		±1470.0		±4883.4

P=0.05

n=6

TABLE III: Comparison of the Perimeters of the Denervated and Control Femoral Vein

Age at Denervation (Days old)	Age Sampled At (Days old)	Perimeter of Femoral Vein			
		Control Side (μ^2)	Mean	Denervated Side (μ^2)	Mean
12	60	3808		6895	
		3796	3797	6878	6874
		3792		6850	
	60	3317		4461	
		3327	3324	4427	4439
		3328		4430	
1-3	90	4425		7206	
		4273	4237	7268	7224
		4213		7198	
	90	2809		4844	
		2709	2793	4909	4888
		2862		4910	
12	90	4512		6751	
		4488	4490	6776	6757
		4471		6743	
	90	4318		6801	
		4216	4289	6766	6817
		4333		6885	
	TOTAL MEAN		3822		6167
	±SEM		±267		±483

P=0.0005

n=6

TABLE IV: Comparison of Wall Thickness between the Denervated and Control Femoral Vein.

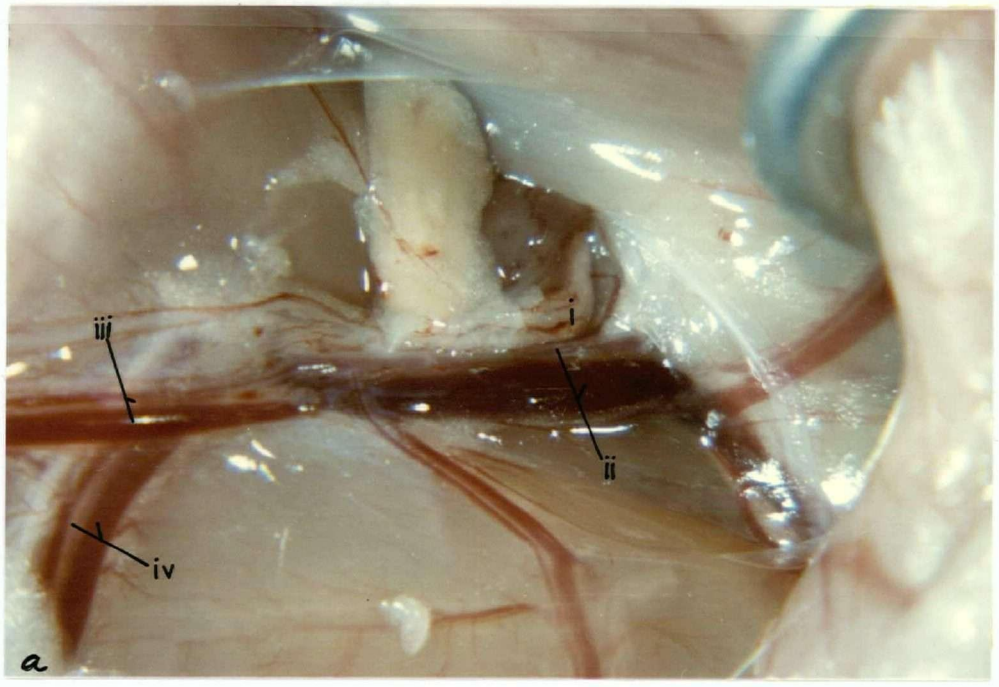
Age at Denervation (days old)	Age at Sampling (days old)	Mean Thickness of Femoral Vein Wall	
		Control Side (μ)	Denervated Side (μ)
12	60	6.02	5.37
12	60	4.60	5.93
1-3	90	7.67	13.57
1-3	90	2.58	3.56
12	90	2.86	4.14
12	90	5.93	3.20
	TOTAL MEAN ±SEM	4.94 ±0.81	5.96 ±1.58
	T-TEST RESULTS n=6	P=0.25	

TABLE V: Number of Fluorescing Areas Counted from the Control and Denervated Saphenous Arteries and Femoral Veins

Age at *DN (days old)	Age Sampled At (days old)	Number of Sections: Arteries	+Average Number per Saphenous Artery		Number of Sections: Veins	+Average Number per Femoral Vein	
			Control Side	Denervated Side		Control Side	Denervated Side
3	30	10	15	5	-	-	-
12	30	14	28	3	10	48	0
12	60	32	40	2	19	220	27
12	60	32	49	25	13	105	54
3	90	25	75	27	15	169	62
3	90	36	63	18	30	168	91
12	120	45	68	6	25	101	3
12	120	36	113	30	31	105	26
		ANOVA Results	P=0.008 n=8			P=0.004 n=7	

*DN = denervation
+ refers to the average number of fluorescing areas.

Figure 1: Denervation Procedure. (a) and (b) show the rat thigh with the skin reflected. The (i) femoral nerve, (ii) the femoral artery and vein, (iii) the superficial epigastric artery and vein and (iv) the saphenous artery and vein can be seen. In (b), black suture silk has been tied around the femoral nerve in two places.


iii
i
ii
iv
a

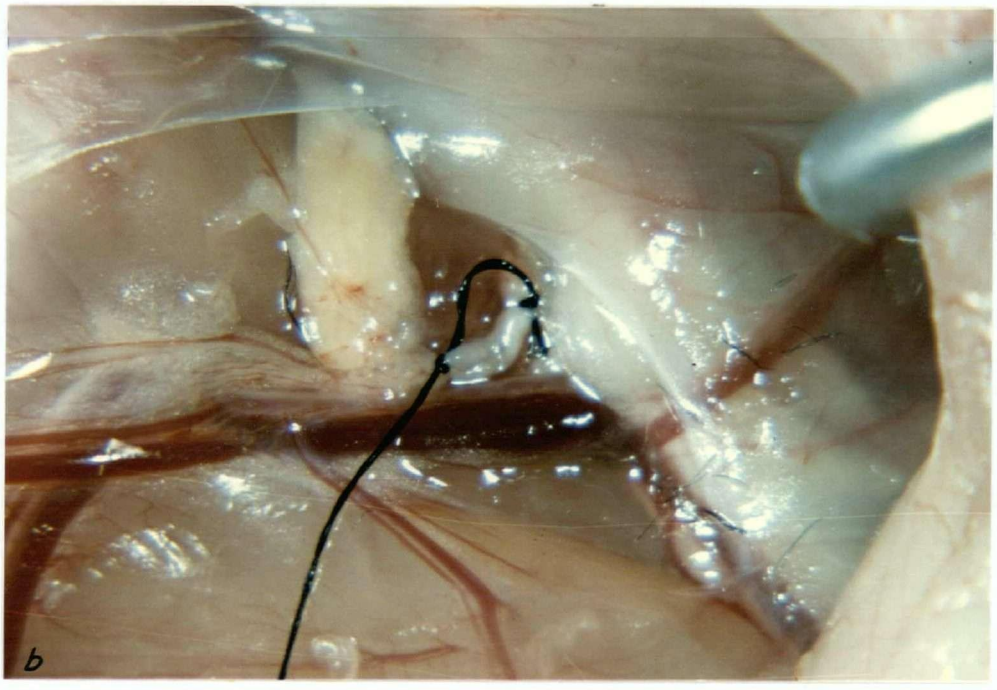
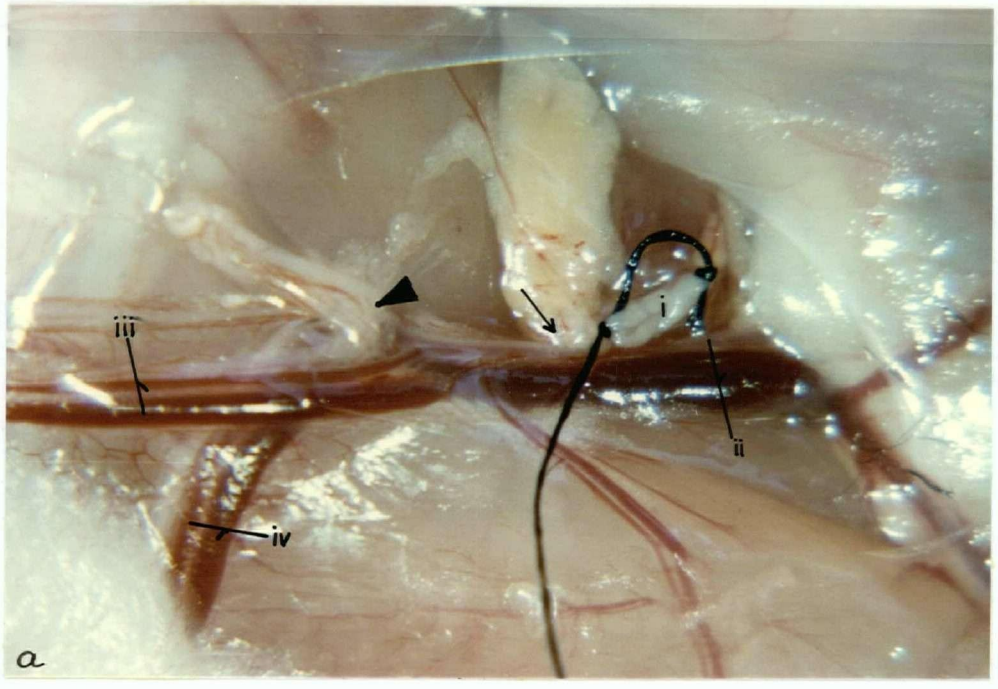

b

Figure 2: Denervation Procedure continued. (a) In the thigh, the (i) femoral nerve is severed just distal to the second knot (arrow). The distal portion of the femoral nerve is separated from the (ii) femoral artery and vein up to the branch points of the (iii) superficial epigastric and (iv) saphenous arteries and veins. The nerve is then severed again at the branch points (arrowhead). (b) Inside the abdominal cavity, the femoral nerve is threaded through the tube and the end is heat sealed (arrowhead) The asterisk indicates the abdominal wall.


i
ii
iii
iv
a

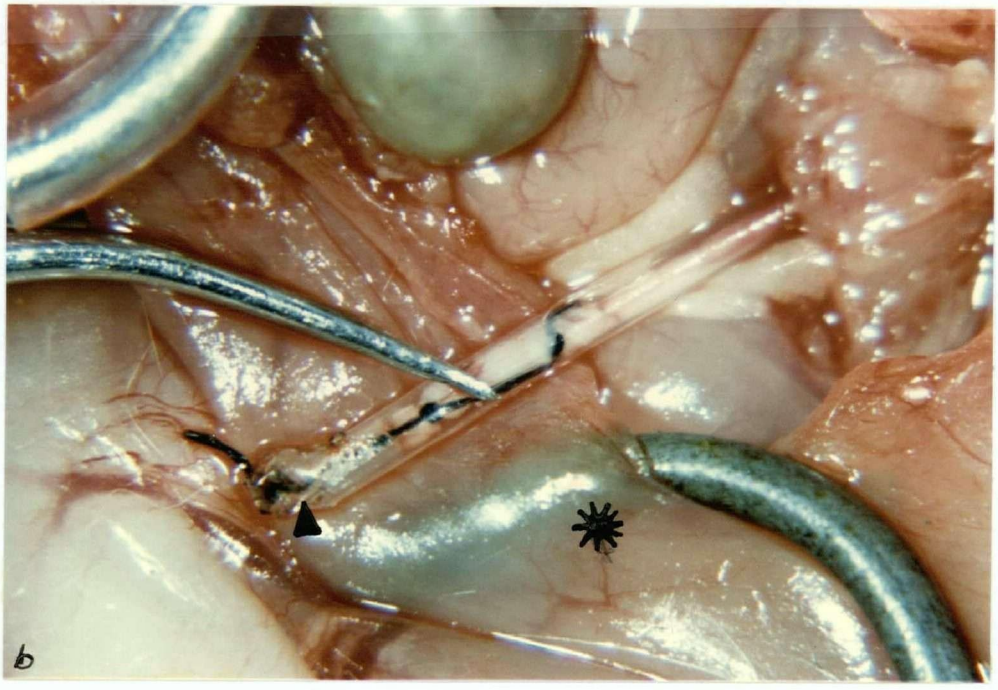

b

Figure 3: Diagram showing the cannulation procedure. The carotid artery is tied off cranially with suture silk (i) and the cannula is inserted through the loose knot (ii) and then into a small incision in the carotid artery. Once the cannula is inside the artery, then the small arterial clamp (iii) is released so the cannula can be pushed in more caudally. The second knot (iv), the one most caudally, and the loose, more cranial knot (ii) are tightened.

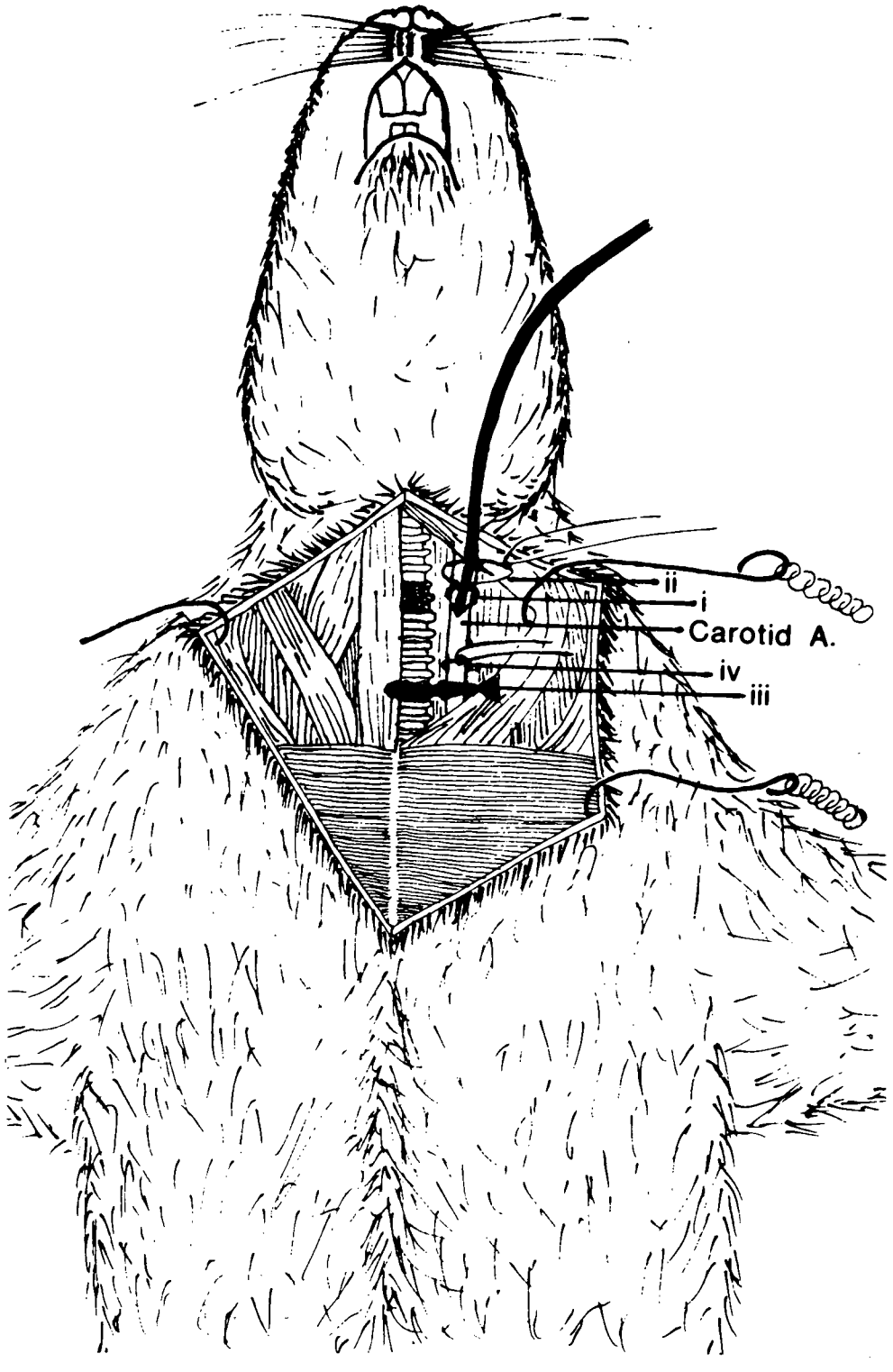

ii
i
Carotid A.
iv
iii

Figure 4: Perfusion Pressure Recording. The (a) mean, (b) systolic and (c) diastolic blood pressures are recorded. (d) Perfusion is carried out at the mean blood pressure.

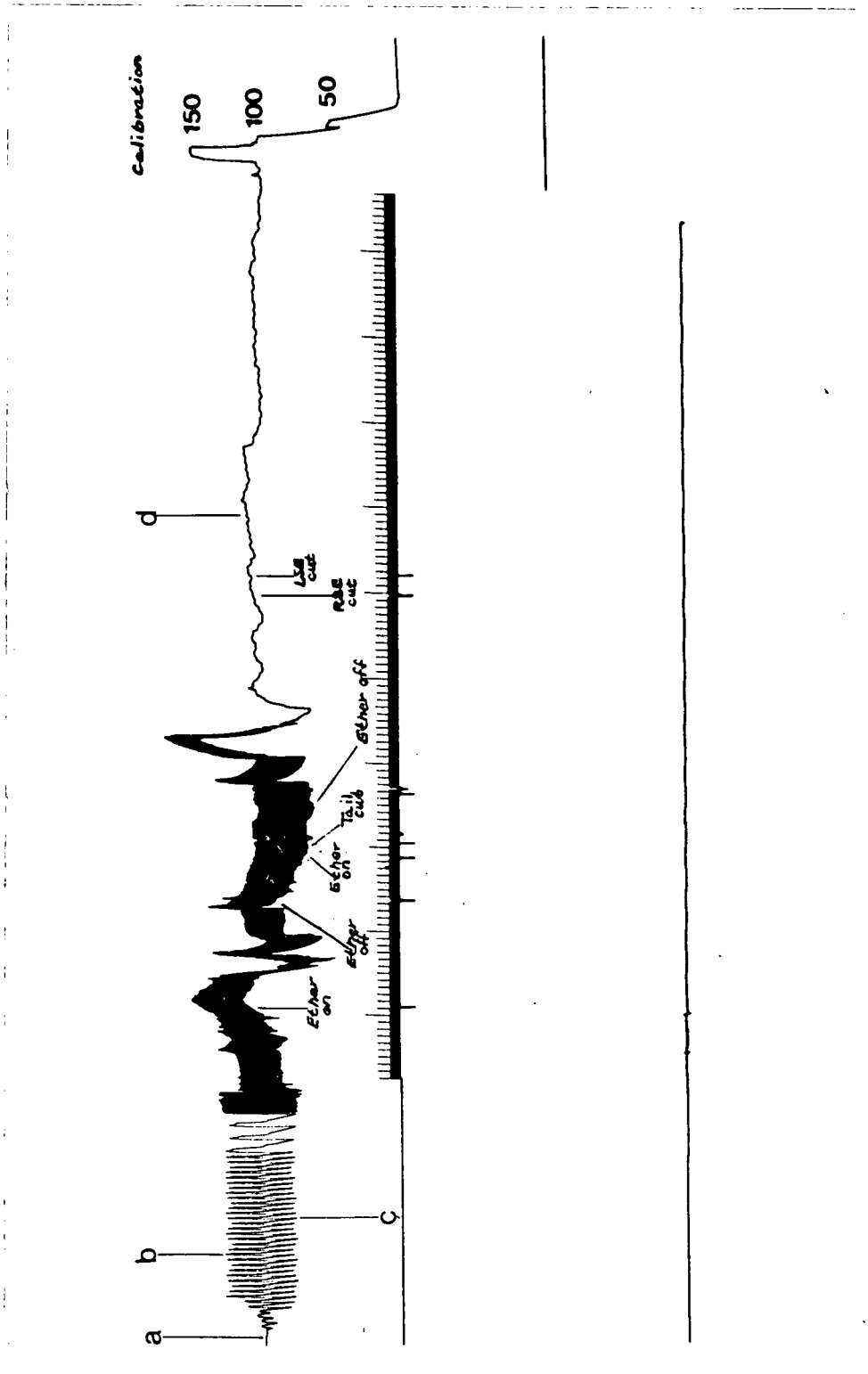
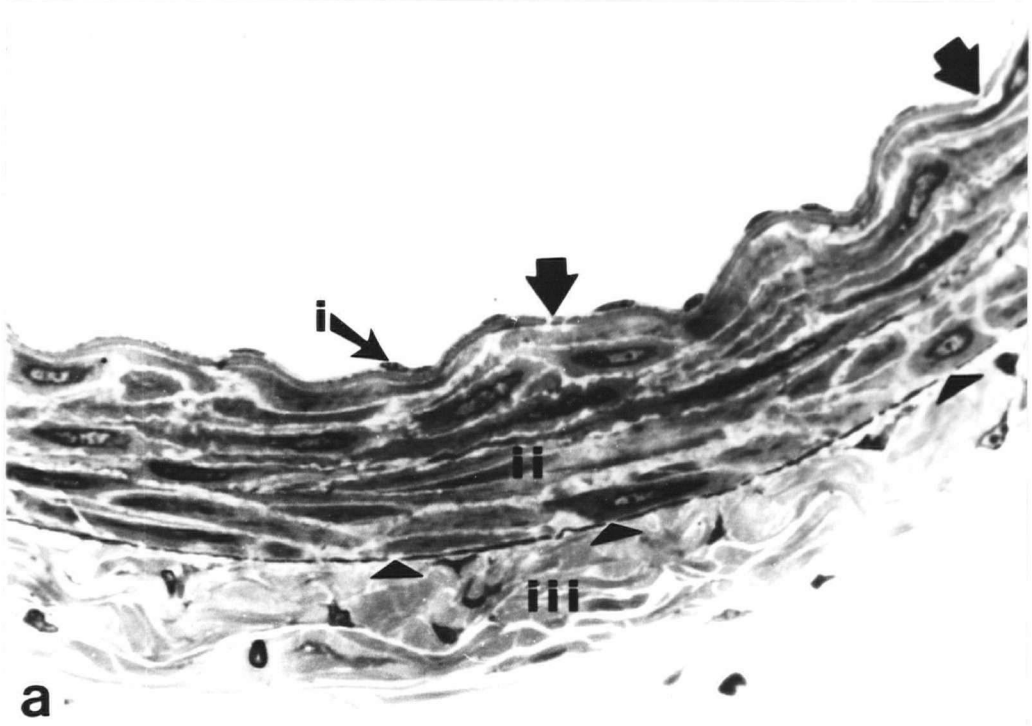
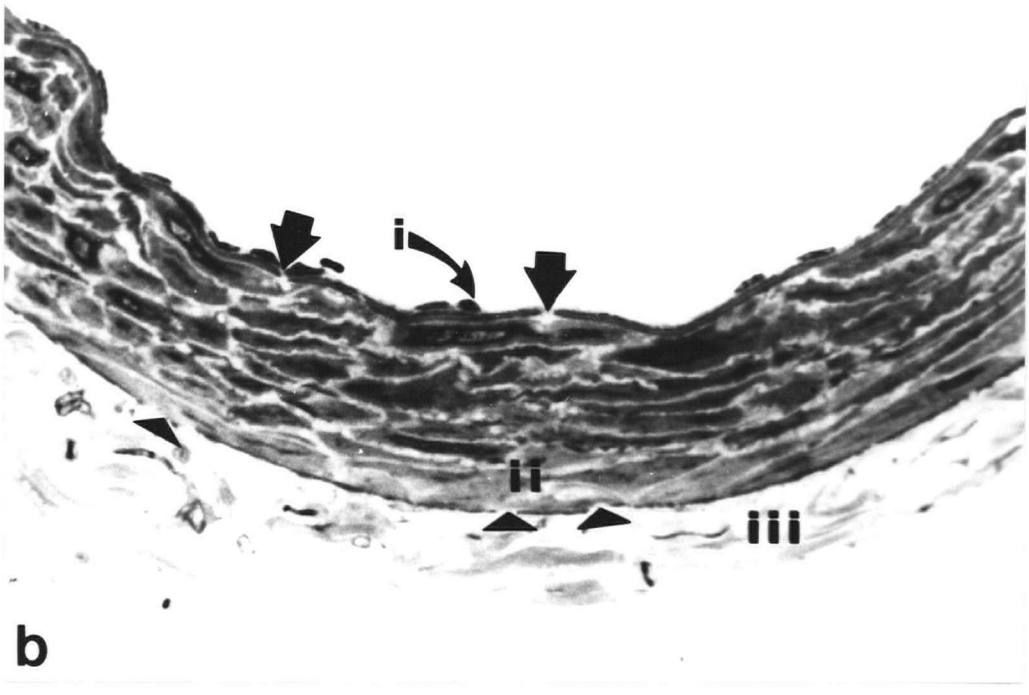

Calibration
150
100
50
d
Ether off
Tail cut
Ether on
Ether off
Ether on
c
b
a

Figure 5: Light micrographs of (a) control and (b) denervated saphenous artery walls. The three tunics are well defined: (i) tunica interna, (ii) tunica media and (iii) tunica externa. The breaks in the thicker internal elastic lamina (arrows) and thinner internal elastic lamina (arrowheads) are seen in both (a) and (b). There is no remarkable difference between the denervated and its contralateral control side. These samples were taken from an animal denervated at 3 days and sampled at 60 days of age. Magnification for (a) and (b) is 670X.



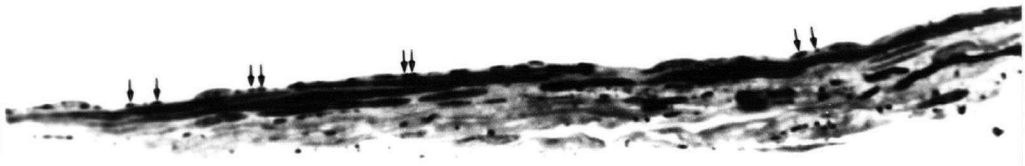
a



b

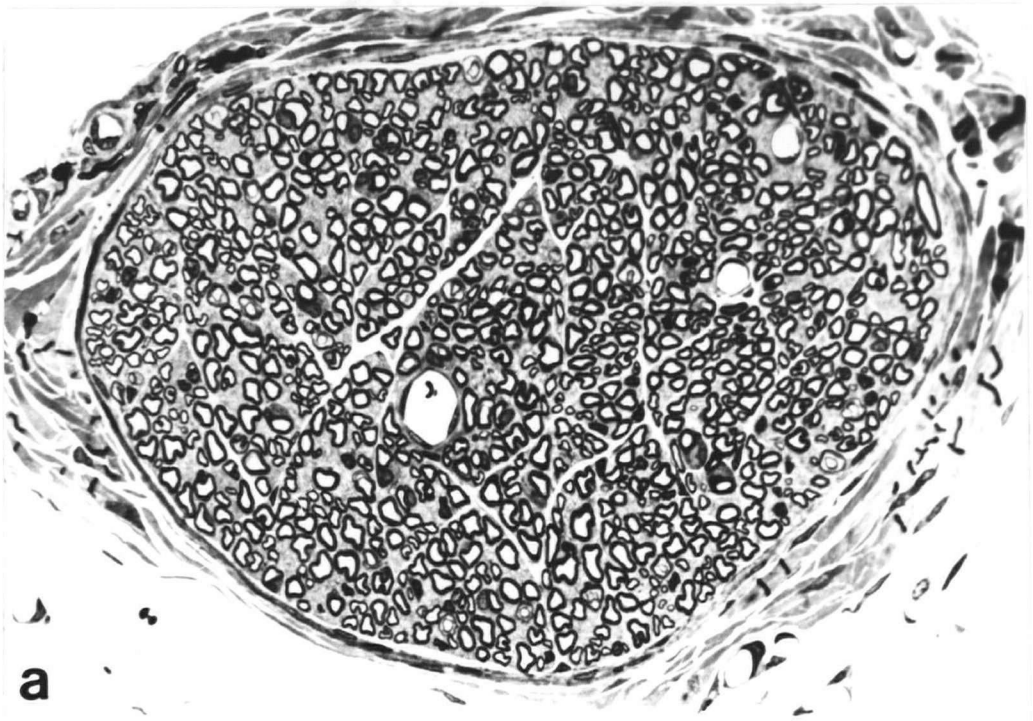
Figure 6: Light micrographs of the (a) control and (b) denervated femoral veins. The three tunics so characteristic of blood vessels are not so easily identifiable in these veins. In (b), the endothelium has a granular appearance (arrows). Magnification for (a) and (b) is 690X.

a



b

Figure 7: Light micrographs of the (a) normal saphenous nerve taken from the control side and (b) the distal stump from the denervated side. These tissue samples came from an animal denervated at 3 days and sampled at 60 days, and were taken adjacent to the saphenous artery. The overall histological appearance has changed, and there is a marked decrease in size following denervation. Magnification for (a) is 415X and for (b) it is 660X.


a

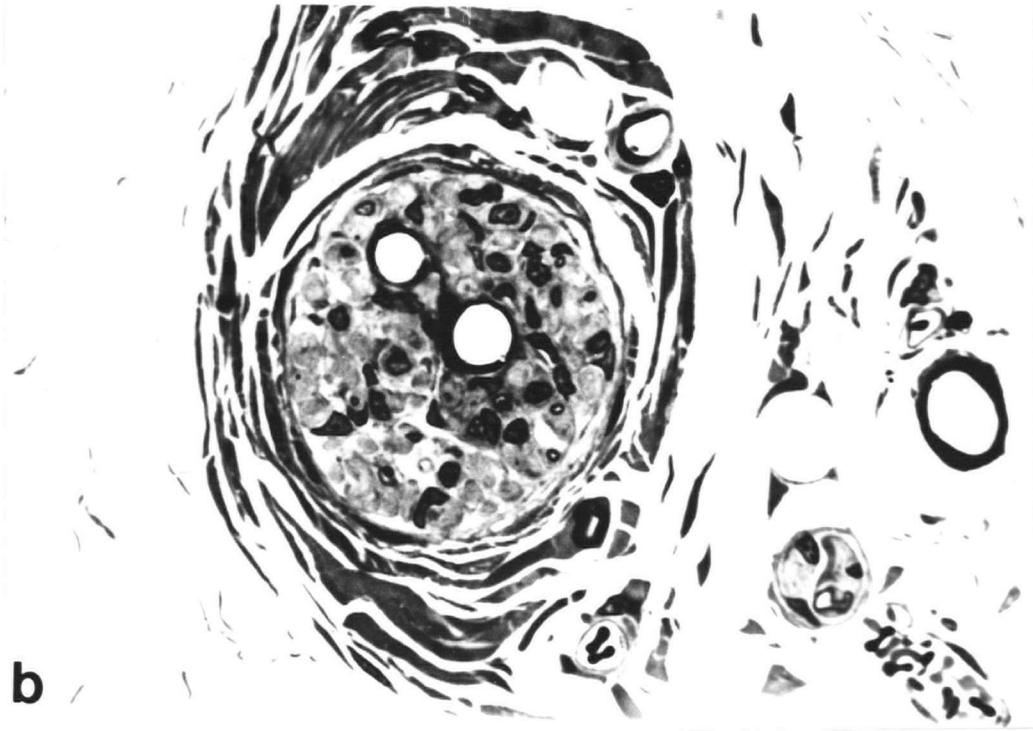

b

Figure 8: Light micrographs of femoral nerves from (a) control (125X) and (b) denervated sides (125X). Samples were taken from within the abdominal cavity. Sample (b) was taken just proximal to the tube and therefore represents the proximal nerve stump. There is a marked decrease in size and a noticeable change in the histology of the nerve that is tubed. The insert (1050X) shows an enlarged portion of the core with few very small myelinated axons remaining. Sample (a) was taken from a corresponding area. Samples were taken from an animal denervated at 3 days and sampled at 60 days.

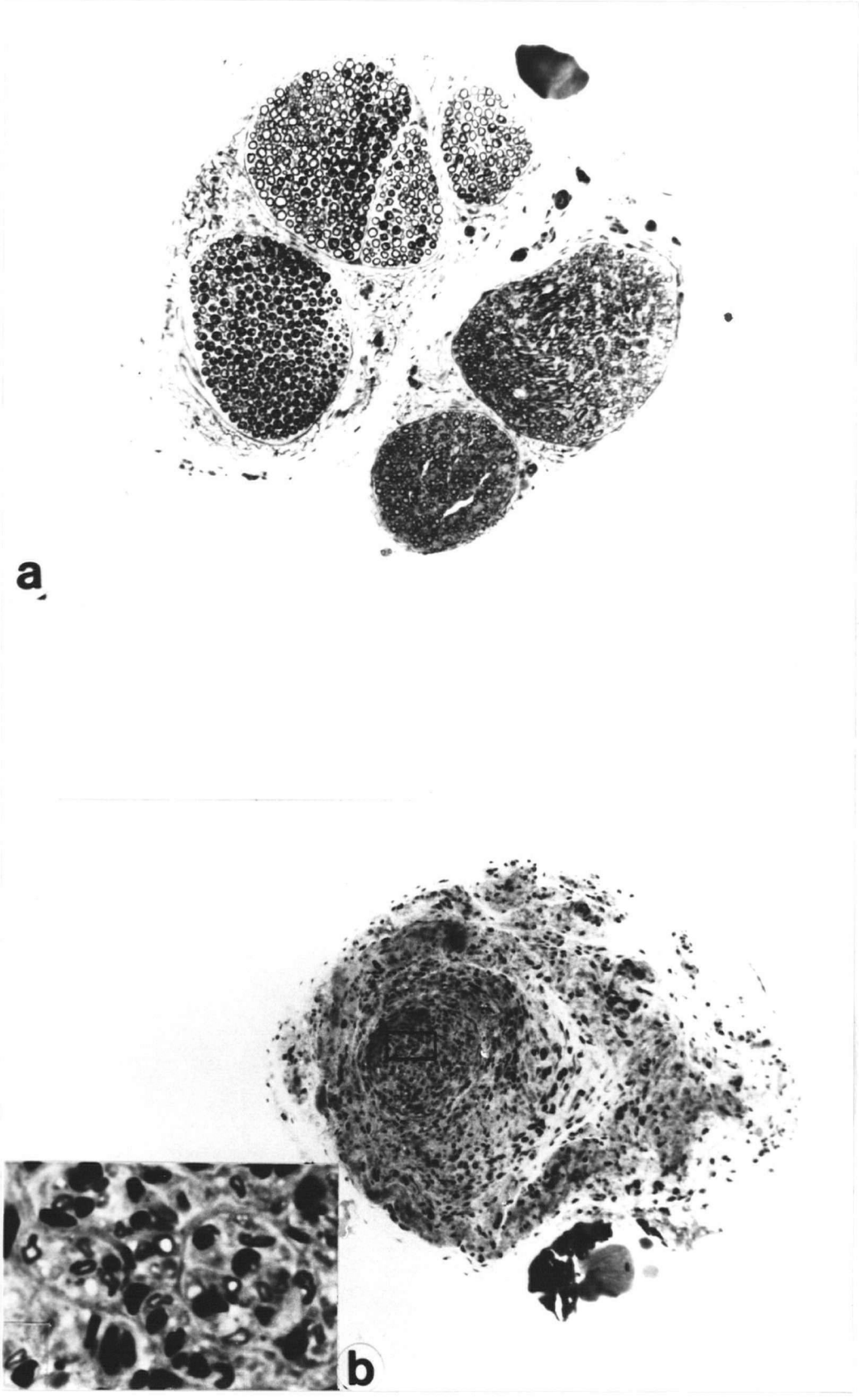

a
b

Figure 9: Ultrastructurally, the femoral nerve (same sample as seen in Figure 8(b)) is composed mainly of collagen (5610X). Fibroblasts (arrows) can be seen in the midst of all the collagen. Membrane-lined areas of degeneration (arrowheads) are present. A capillary is located in the centre of the micrograph. The insert illustrates a small myelinated nerve (asterisk) and an unmyelinated nerve (arrow) observed in the core of this sample (16030X).

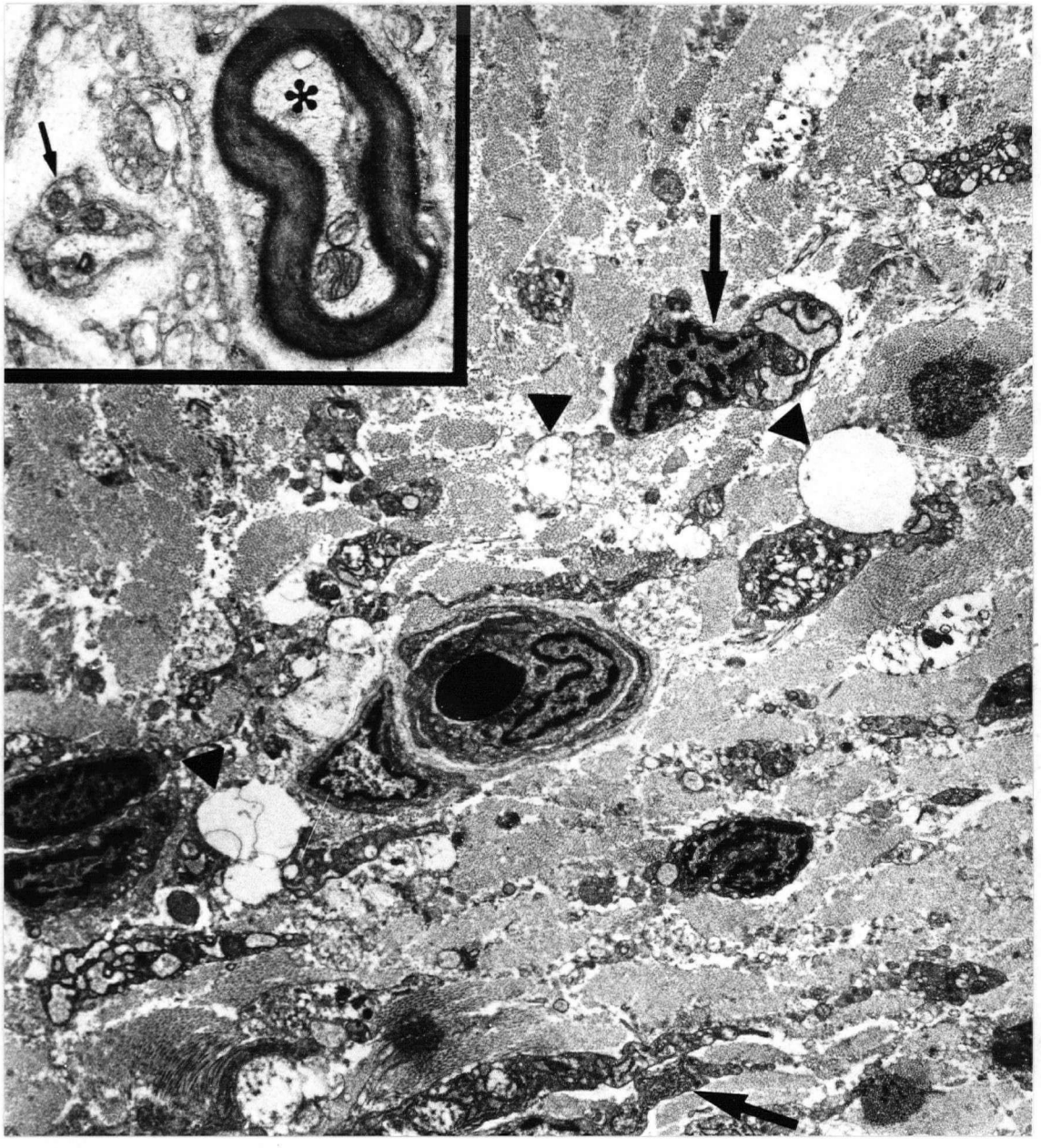
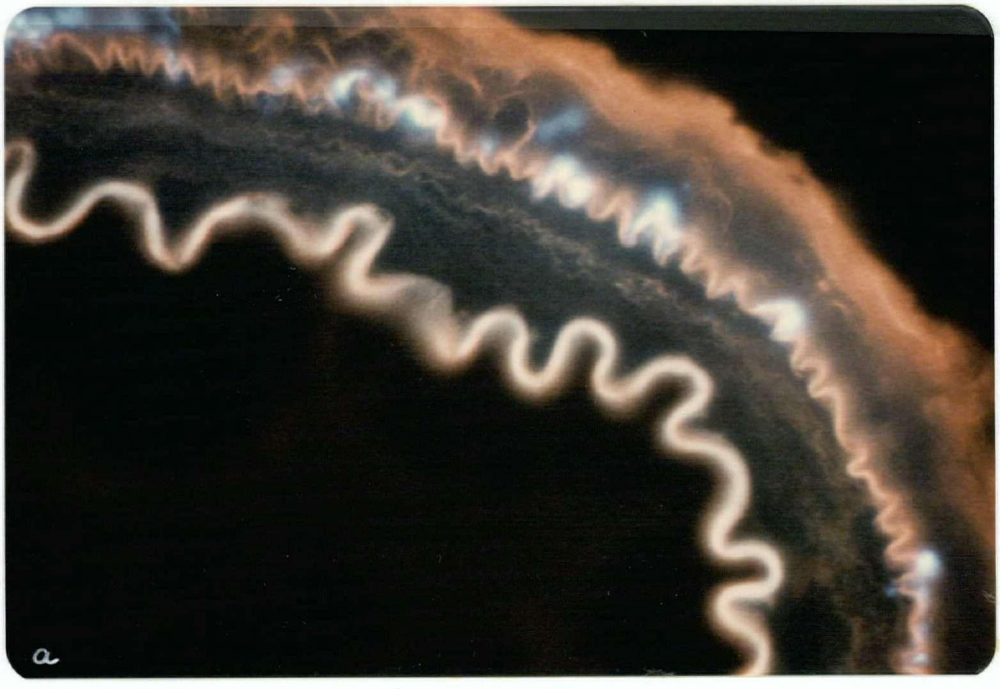
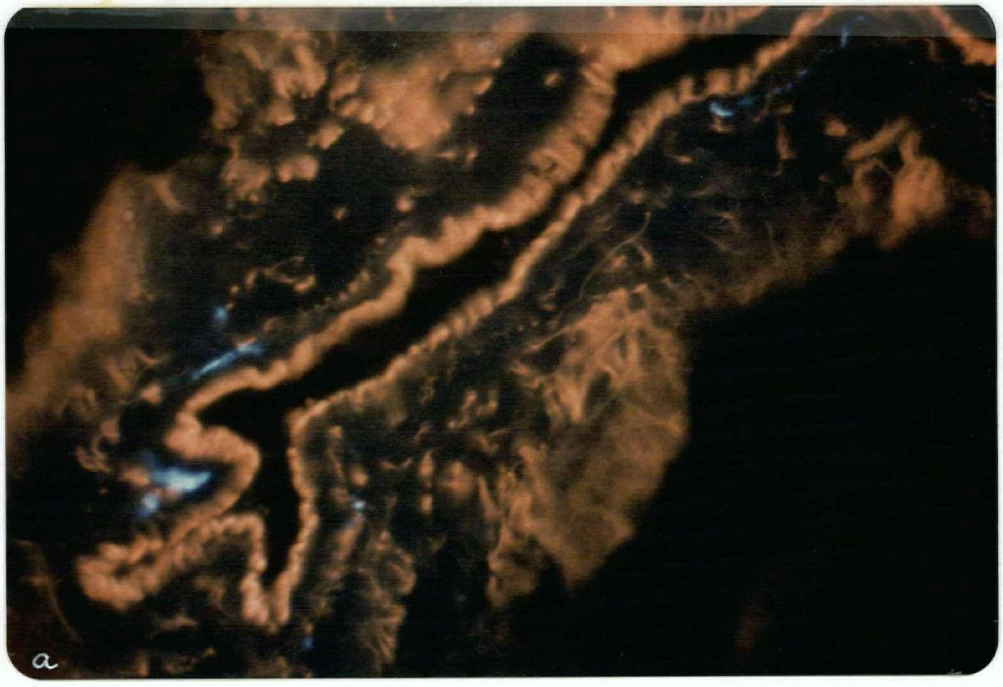


Figure 10: Fluorescence microscopy of control and denervated saphenous arteries. Trypan blue causes the elastic tissue to fluoresce in the red range. (a) Catecholamine fluorescence from the adrenergic terminals is seen at the adventitial-medial border of the control saphenous artery. (b) Virtually no fluorescence of catecholamines is seen in the denervated artery.


a


b

Figure 11: Fluorescence microscopy of control and denervated femoral veins. Dense innervation is seen in the (a) control versus the (b) denervated saphenous vein.


a

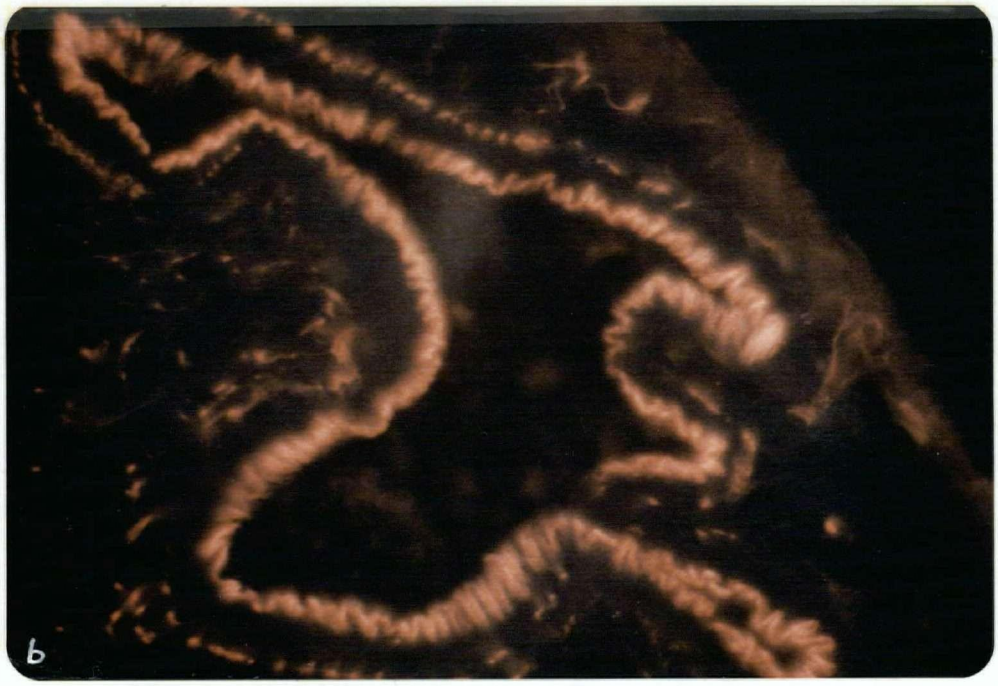

b

Figure 12: Per cent decrease in medial area of denervated saphenous arteries from animals sampled at 60 and 90 days of age. The control side represents 100%.

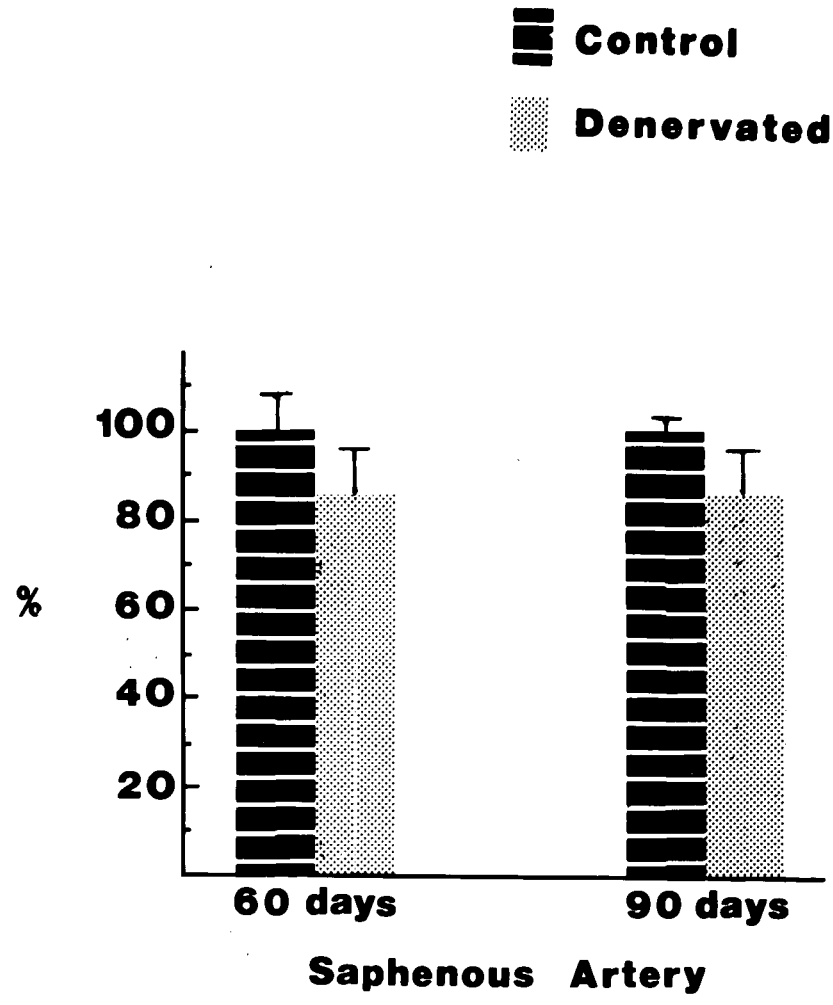
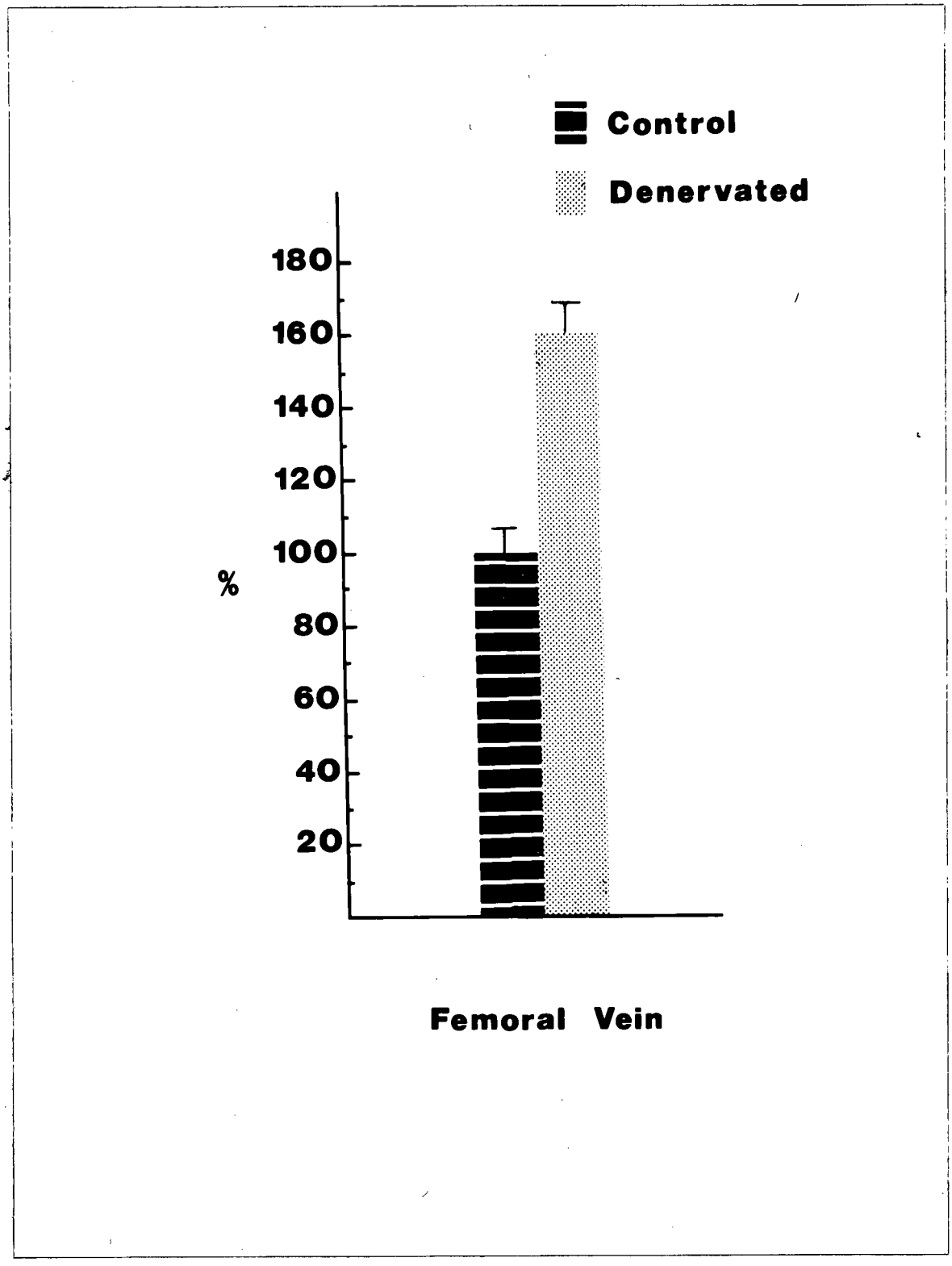

Control
Denervated
100
80
%
60
40
20
60 days
90 days
Saphenous Artery

Figure 13: Per cent increase in luminal perimeter of denervated femoral veins. The control side represents 100%.


Control
Denervated
180
160
140
120
100
80
60
40
20
%
Femoral Vein

BIBLIOGRAPHY

Aprigliano, O. (1983) Neural Influences and Norepinephrine Sensitivity in the Rat Portal Vein. Federation Proc. 42:257-262.

Barr, M.L. and Kiernan, J.A. (1983) The Human Nervous System. An Anatomical Viewpoint. 4th Ed. Harper and Row, Publishers, Philadelphia.

Bevan, J.A. and Su, C. (1973) Sympathetic Mechanisms in Blood Vessels: Nerve and Muscle Relationships, Annual Review of Pharmacology. 13:269-285.

Bevan, R.D. (1984) Trophic Effects of Peripheral Adrenergic Nerves on Vascular Structure. Hypertension 6[Suppl. III]:III-9 - III-26.

Bevan, R.D. (1975) Effect of Sympathetic Denervation on Smooth Muscle Cell Proliferation in the Growing Ear Artery. Circ. Res. 37:14-19.

Bevan, R.D., Tsuru, H and Bevan, J.A. (1983) Cerebral Artery Mass in the Rabbit is Reduced by Chronic Sympathetic Denervation. Stroke 14(3):393-396.

Bevan, R.D. and Tsuru, H. (1979) Long-Term Denervation of Vascular Smooth Muscle causes not only Functional but Structural Change. Blood Vessels 16:109-112.

Bevan, R.D. and Tsuru, H. (1981) Functional and Structural Changes in the Rabbit Ear Artery after Sympathetic Denervation. Circ. Res. 49:478-485.

Boatman, D.L., Shaffer, R.A., Dixon, R.L. and Brody, M.J. (1965) Function of Vascular Smooth Muscle and its Sympathetic Innervation in the Newborn Dog. J. of Clinical Investigations 44:241-246.

Branco, D., Albino Teixeira, A., Azevedo, I., and Osswald, W. (1984) Structural and Functional Alterations caused at the Extraneuronal Level by Sympathetic Denervation of Blood Vessels. Naunyn-Schmiedeberg's Arch. Pharmacol.326:302-312

Brody, M.J. (1964) Cardiovascular Responses Following Immunological Sympathectomy. Circ. Res. 15:161-167.

Burnstock, G. and Costa, M. (1975) Adrenergic Neurons. Chapman & Hall,

Ltd., London.

Chamley, J.H. and Campbell, G.R. (1975) Trophic Influences of Sympathetic Nerves and Cyclic AMP on Differentiation and Proliferation of Isolated Smooth Muscle Cells in Culture. Cell Tissue Research 161:407-510.

Chamley, J.H. and Campbell, G.R. (1976) Tissue Culture: Interaction Between Sympathetic Nerves and Vascular Smooth Muscle. In Vascular Neuroeffector Mechanisms, edited by J.A. Bevan, G. Burnstock, B. Johansson, R.A. Maxwell and O.A. Nedergaard. Basil, Karger, pp 10-18.

Chen, G., Portman, R. and Wickel, A. (1951) Pharmacology of 1,1-dimethyl-4-phenyl piperazinium iodide, a Ganglionic Stimulating Agent. J. Pharmacology 103:330

Colin, W. and Donoff, R.B. (1984) Nerve Regeneration Through Collagen Tubes. J. Dent. Res. 63(7):987-993.

Crissman, R.S. (1984) The Three-Dimensional Configuration of the Elastic Fibre Network in Canine Saphenous Vein. Blood Vessels 21:156-170.

De la Lande, I.S. and Waterson, J.G. (1968) Modification of Autofluorescence in the Formaldehyde-treated Rabbit Ear Artery by Evans Blue. J. Histochem., Cytochem. 16:281-282.

De la Torre, J.C. (1980) An Improved Approach to Histofluorescence using the SPG Method for Tissue Monoamines. J. Neurosci. Methods 3:1-5.

Dobrin, P.B., Baker, W.H. and Gley, W.C. (1984) Elastolytic and Collagenolytic Studies of Arteries. Arch. Surg. 119:405-409.

Dolezel, S., Gerova, M. and Gero, J. (1974) Postnatal development of the sympathetic innervation in skeletal muscles of the dog. Physiol. Bohemoslov. 23:138-139.

Drachman, D.B. (1974) Trophic Actions of the Neuron: An Introduction. In Trophic Functions of the Neuron. edited by D.B. Drachman, Annals New York Acad. Sci. 228:3-5.

Dyck, P.J. and Hopkins, A.P. (1972) Electron Microscopic Observations of Degeneration and Regeneration of Unmyelinated Fibres. Brain 95:223-234.

Engel, A.G. and Stonnington, H.H. (1974) Morphological Effects of Denervation of Muscle. A Qualitative Ultrastructural Study. In Trophic Functions of the Neuron, edited by D.B. Drachman, Annals New York Acad. Sci. 228:68-88.

Finch, L., Haeusler, G. and Thoenen, H. (1973) A Comparison of the Effects of Chemical Sympathectomy by 6-Hydroxydopamine in Newborn and Adult Rats. Br. J. Pharm. 47:249-260.

Gabella, G. (1976) Structure of the Autonomic Nervous System. Chapman and Hall, Ltd., London.

Gauthier, G.F. and Dunn, R.A. (1973) Ultrastructural and Cytochemical Features of Mammalian Skeletal Muscle Fibers Following Denervation. J. Cell Sci. 12:525-547.

Gerova, M., Gero, J., Dolesel, S. and Konecny, M. (1974) Postnatal Development of Sympathetic Control in Canine Fermoral Artery. Physiologia Bohemoslovaca 23:289-295.

Gerrity, R.G. and Cliff, W.J. (1975) The Aortic Tunica Media of the Developing Rat. Laboratory Investigation 32:585-600.

Grobety, J. and Bouvier, C.A. (1977) Studies on Normal and Varicose Human Saphenous Veins I: Structural Differences, Histochemical and Electron Microscope Investigations. 9th Europ. Conf. Microcirculation, Antwerp, 1976. Bibl. Anat. 16:298-300.

Gutman, E. (1976) Neurotrophic Relations. Annu. Rev. Physiol. 38:177-216.

Ham, A.W. and Cormack, D.H. (1979) Histology 8th Ed., J.B. Lippincott Company Philadelphia and Toronto. p. 575.

Hayat, M.A. (1970) Principles and Techniques of Electron Microscopy: Biological Applications Vol. 1. Van Nostrand Reinhold Company, New York. Hoffman, W.W. and Thesleff, S. (1972) Studies on the Trophic Influence of Nerve on Skeletal Muscle. Eur. J. Pharmac. 20:256-260.

Humphrey, C. and Prittman, F. (1974) A Simple Methylene blue-Azure II Basic Fuchsin for Epoxy-Embedded Tissue Sections. Stain Tech. 49:9-14.

Illustrated Stedman's Medical Dictionary, 24th Ed. (1982) Williams and Wilkins, Baltimore, MD.

Kanakis, S.J., Hill, C.E., Hendry, I.A. and Watters, D.J. (1985) Sympathetic Neuronal Survival Factors Change after Denervation. Dev. Brain Res. 20:197-202.

Kobayashi, S., Tsukahara, S., Tsuji, T., Sugita, K. and Nagata, T. (1983) Histochemical Studies on Regeneration of Aminergic Nerves in Rat Cerebral Artery after Superior Cervical Ganglionectomy Histochemistry 77:57-62.

Lais, L.T., Rios, L.L., Boutelle, S., DiBona, G.F. and Brody, M.J. (1977) Arterial Pressure Development in Neonatal and Young Spontaneously Hypertensive Rats. Blood Vessels 14:277-284.

Lentz, T.L. (1974) Neurotrophic Regulation at the Neuromuscular Junction. Ann. New York Acad. Sci. 228:323-337.

Levi-Montalcini, R. and Angeletti, P.U. (1966) Immunosympathectomy. Pharmac. Rev. 18:619-628.

Luco, J.V. and Eyzaguirre, C. (1955) Fibrillation and Hypersensitivity to Ach in Denervated Muscle. Effect of Length of Degenerating Nerve Fibres. J. Neurophysiol. 18:65-73.

McInnes, A. (1977) Modification of the Falck-Hillarp Technique with Intravital Trypan blue to Differentiate Elastic Fibres from Noradrenergic Endings. J. Physiol. 268:22P-23P.

Mollenhauer, H.H. (1964) Plastic Embedding Mixture for use in Electron Microscopy. Stain Tech. 39:111-114.

Niebes, P., Engels, E. and Jegerlehner, M. (1977) Studies on Normal Varicose Human Saphenous Veins II: Differences in the Compostion of Collagen and Glycosaminoglycans. 9th Europ. Conf. Microcirculation, Antwerp, 1976. Bibl. Anat. 16:301-303.

Ohnishi, K., Nakayama, T., Saito, M., Nomura, F., Koen, H., Tamaru, J., Iwasaki, I. and Okuda, K. (1984) Aneurysm of the Intrahepatic Branch of the Portal Vein. Gastroenterology 86:169-173.

Page, I.H. and McCubbin, J.W. (1965) Chapter 61: The Physiology of Arterial Hypertension. In Handbook of Physiology. Section 2, Circulation. Vol. II, American Physiological Society, Wash., DC.

Palaty, V. (1971) Distribution of Magnesium in the Arterial Wall.

J.Physiol. 218:353-368.

Pease, D.C. (1964) Histological Techniques for Electron Microscopy. 2nd Ed. Academic Press Inc., New York and London.

Pellegrino, C. and Franzini, C. (1963) An Electron Microscope Study of Denervation Atrophy in Red and White Skeletal Muscle Fibers. J. Cell Biol. 17:327-349.

Pellegrino, R.G. and Spencer, P.S. (1985) Schwann Cell Mitosis in Response to Regenerating Peripheral Axons in vivo. Brain Res. 341:16-25.

Politis, M.J., Ederle, K. and Spencer, P.S. (1982) Tropism Nerve Regeneration in vivo. Attraction of Regenerating Axons by Diffusible Factors Derived from Cells in Distal Stumps of Transected Peripheral Nerves. Brain Res. 253:1-12.

Rusterholz, D.B. and Mueller, S.M. (1982) Sympathetic Nerves Exert a Chronic Influence on the Intact Vasculature that is Age Related. Ann. Neurol. 11:365-371.

Sarnat, H.B. (1983) Ch. 3. Denervation and Reinnervation of Muscle. In Muscle Pathology and Histochemistry. American Society of Clinical Pathologists Press, Chicago. pp. 35-43.

Szekere, L. (ed.) (1980) Adrenergic Activators and Inhibitors. Handbook of Experimental Pharmacology 54:I, Springer-Verlag, Berlin.

Thesleff, S. (1974) Physiological Effects of Denervation of Muscle. In Trophic Functions of the Neuron. edited by D.B. Drachman. Ann. New York Acad. Sci. 228:89-104.

Todd, M.E. (1980) Development of Adrenergic Innervation in Rat Peripheral Vessels: A Fluorescence Microscopic Study. J. Anat. 131:121-133.

Todd, M.E., Laye, C.G. and Osborne, D.N. (1983) The Dimensional Characteristics of Smooth Muscle in Rat Blood Vessels: A Computer-Assisted Analysis. Circ. Res. 53:319-331.

Todd, M.E. and Tokito, M.K. (1981) An Ultrstructural Investigation of Developing Vasomotor Innervation in Rat Peripheral Vessels. Amer. J. Anat. 160:195-212.

Todd, M.E. (1986) Trophic Interactions Between Rat Nerves and Blood Vessels in Denervated Peripheral Arteries and in Anterior Eye Chamber Transplants. Circ. Res. 58: In Press.

Waris, T. (1978) Re-inneration of Free Skin Autografts in the Rat. Scand. J. Plast. Reconstr. Surg. 12:85-93.

Williams, P.L. and Warwick, R. (1975) Functional Neuroanatomy of Man. Philadelphia, W.B. Saunders.