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Development of adrenergic innervation in rat peripheral vessels: a fluorescence microscopic study

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INTRODUCTION

Autonomic vasomotor control is an important element in regulating the flow and distribution of blood. Many vessels have adrenergic innervation only (Devine, 1978), but in the vessels of the brain and skeletal muscle other neurotransmitters are released in addition (Devine, 1978; Lee, 1975; Su & Lee, 1975, 1976). The higher the density of adrenergic varicosities with small granular storage vesicles, adjacent to or within the tunica media, the more neurotransmitter will reach the smooth muscle cells (Bevan & Su, 1973; Su & Lee, 1976; Uehara, Campbell & Burnstock, 1976).

There are conflicting reports concerning the density of the vascular innervation at various levels (Somlyo & Somlyo, 1968; Burnstock, Gannon & Iwayama, 1970; Somlyo, 1975). There may be considerable differences along the course of a vessel as well as in the same vessel taken from different species (Burnstock *et al.* 1970; Su & Lee, 1976; Osborne-Pellegrin, 1978). Furness (1971, 1973), investigating intestinal vessels, found that arteries were densely innervated down to vessels the size of arterioles only. Venules and small veins were not innervated but on larger vessels the density of innervation increased as the veins increased in size.

Little attention has been devoted to the development of vascular innervation (Burnstock & Costa, 1975). DeChamplain, Malmfors, Olson & Sachs (1970), in their studies of various other tissues in prenatal and postnatal rats, suggested that the formol-fluorescence technique could demonstrate all or nearly all adrenergic nerves even in early development. In the case of the mouse vas deferens there is apparently a delay between the appearance of nerves and the responsiveness of the smooth muscle (Furness, McLean & Burnstock, 1970). However, a good correlation is found between the postnatal development of innervation in rat portal vein and its functional response (Ljung *et al.* 1975; Ljung & Stage, 1975). There is evidence of a close correlation between adult density of innervation and functional requirements in vessels (Bevan *et al.* 1974).

The present report compares the development of catecholamine stores in selected rat peripheral vessels from birth to maturity. Since whole mount preparations commonly used for fluorescence microscopy do not permit accurate evaluation of the relative densities of nerve varicosities related to smooth muscle cells, the comparisons were carried out in paraffin sections.

MATERIALS AND METHODS

Sixty eight male Wistar rats 1–30 days old were anaesthetized with sodium pentobarbitone (30 mg/kg), and segments of the femoral vessels and their branches, the superficial epigastric and saphenous vessels, and the main tail artery were frozen in isopentane previously cooled in liquid nitrogen (Björklund, Falck & Owman, 1972). The vessels were excised in such a way that the same area of vessel was examined in each experiment, that is, the initial portion of the femoral artery, and the superficial epigastric and saphenous arteries immediately after they branched. The accompanying veins were included with each specimen. The sections of tail artery came from the beginning of the hairless portion of the tail. The tissues were transferred to the stage of an Edwards Pearse Tissue Drier for not less than 22 hours at −40 °C. The vessels were then subjected to formaldehyde vapour over paraformaldehyde (80 % relative humidity) at 80 °C for 75 minutes and then vacuum embedded in paraffin wax. The blocks were sectioned at 8 μm and cross sections from three areas, approximately 250 μm apart, were examined and photographed with a Leitz Orthoplan microscope equipped with an HBO/200 high pressure mercury lamp. A 3 mm and a 5 mm BG12 excitation filter, dark field condenser, and 530 nm barrier filter gave the best contrast for photography.

In the arteries, the cross sectional area of the vessel rather than the circumference gave the most reproducible results, and this was calculated by enlarging the negative and following the internal and external outlines of the vessel with a planimeter. The planimeter reading was converted to the actual vessel cross sectional area. The number of discrete fluorescent dots at the tunica media-adventitial border was counted. The number of nerves/cm^2 was calculated. All counts and measurements from each tracing were made in triplicate and averaged.

The question of whether or not the fluorescence was due to the stored catecholamine was tested by means of reserpine pre-treatment (Palatý & Todd, 1978), and by the sodium borohydride specificity test (Corrodi, Hillarp & Jonsson, 1964). A Philips 300 electron microscope was used for ultrastructural examination of vessels from control and reserpinized animals, after potassium permanganate fixation and routine processing (Bloom & Crayton, 1972). In addition, tests were done with Evans blue and trypan blue to distinguish elastic tissue from catecholamine fluorescence (De la Lande & Waterson, 1968; McInnes, 1977). Animals were injected, 1 hour before tissue samples were removed, with dye in Krebs solution to give a final concentration of 45–100 mg/kg.

RESULTS

There was virtually no fluorescence in the 1 and 2 day old animals. After 30 days there were no further changes in the relationships of the parameters studied, and the period from 3 to 30 days was studied intensively (52 animals).

The three muscular arteries (tail, superficial epigastric, saphenous) were similar in size in young animals, whereas by 30 days the cross sectional area of the superficial epigastric was approximately half that of the other two; its growth rate slowed down by 20 days while that of the other two continued undiminished (Fig. 1). The developing innervation is illustrated in the saphenous artery (Fig. 2), and the other two followed the same pattern. The external elastic lamina indicated the position of the nerves in relation to the tunica media-adventitial border and had an appearance

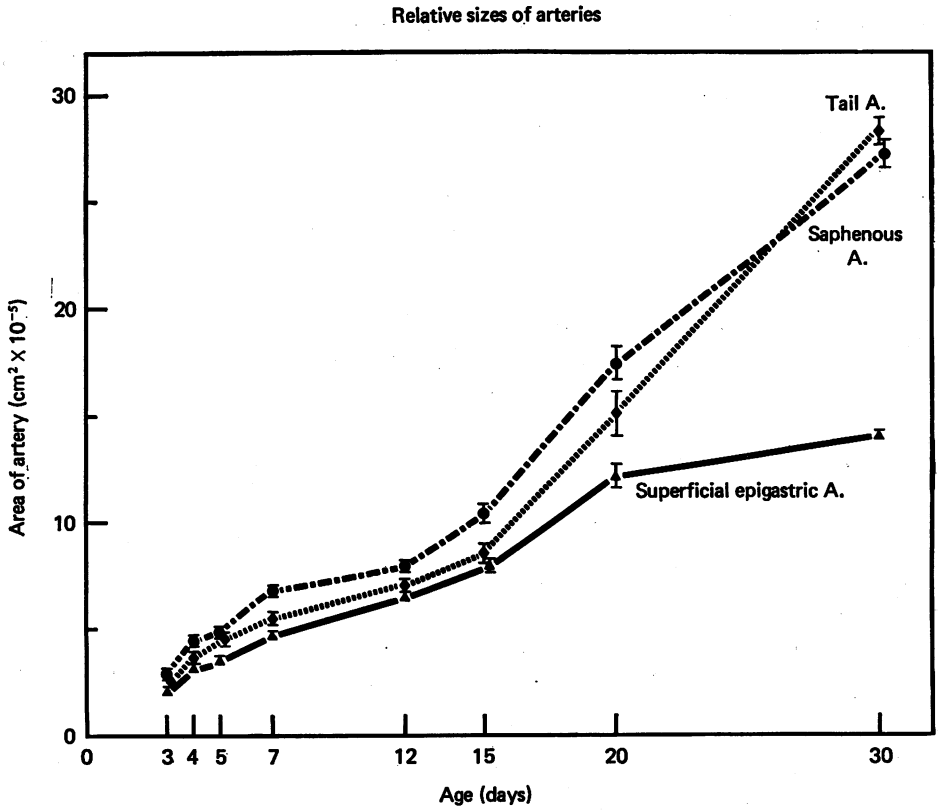


Fig. 1. Comparison of cross sectional areas of superficial epigastric, saphenous and tail arteries in growing Wistar rats.

different from the catecholamine fluorescence. In the animals injected with dye, the trypan blue (100 mg/kg) proved to be the most effective in causing the elastic tissue to fluoresce in the red range with the catecholamine continuing to fluoresce in the yellow–green range. These results confirmed that only nerves at the tunica media-adventitial border had been counted. There were a number of other areas with intense fluorescence, such as in main nerve trunks and in mast cells. The lumen of the arteries was also clearly defined by the non-specific fluorescence of the internal elastic lamina.

The development of fluorescent nerves was similar in the saphenous and superficial epigastric arteries. In contrast, the tail artery had approximately twice as much total fluorescence by 30 days as the other two, and it maintained this lead to maturity (Fig. 3). However, its fluorescence did not surpass that of the other two vessels until after 12 days of age (Fig. 4).

When the nerves per unit area of vessel were examined (Fig. 5), more specific information was obtained. Again, the saphenous and superficial epigastric arteries followed a similar pattern, with a peak in nerves per unit area approximately three times that of the tail artery at 5 days of age. A second peak occurred in all three vessels between 12 and 15 days, after which the artery wall grew faster than the rate of increase in numbers of reactive nerves, yielding a fall in numbers of nerves per unit area. The final order in terms of decreasing density of nerves was tail, superficial epigastric and saphenous arteries.

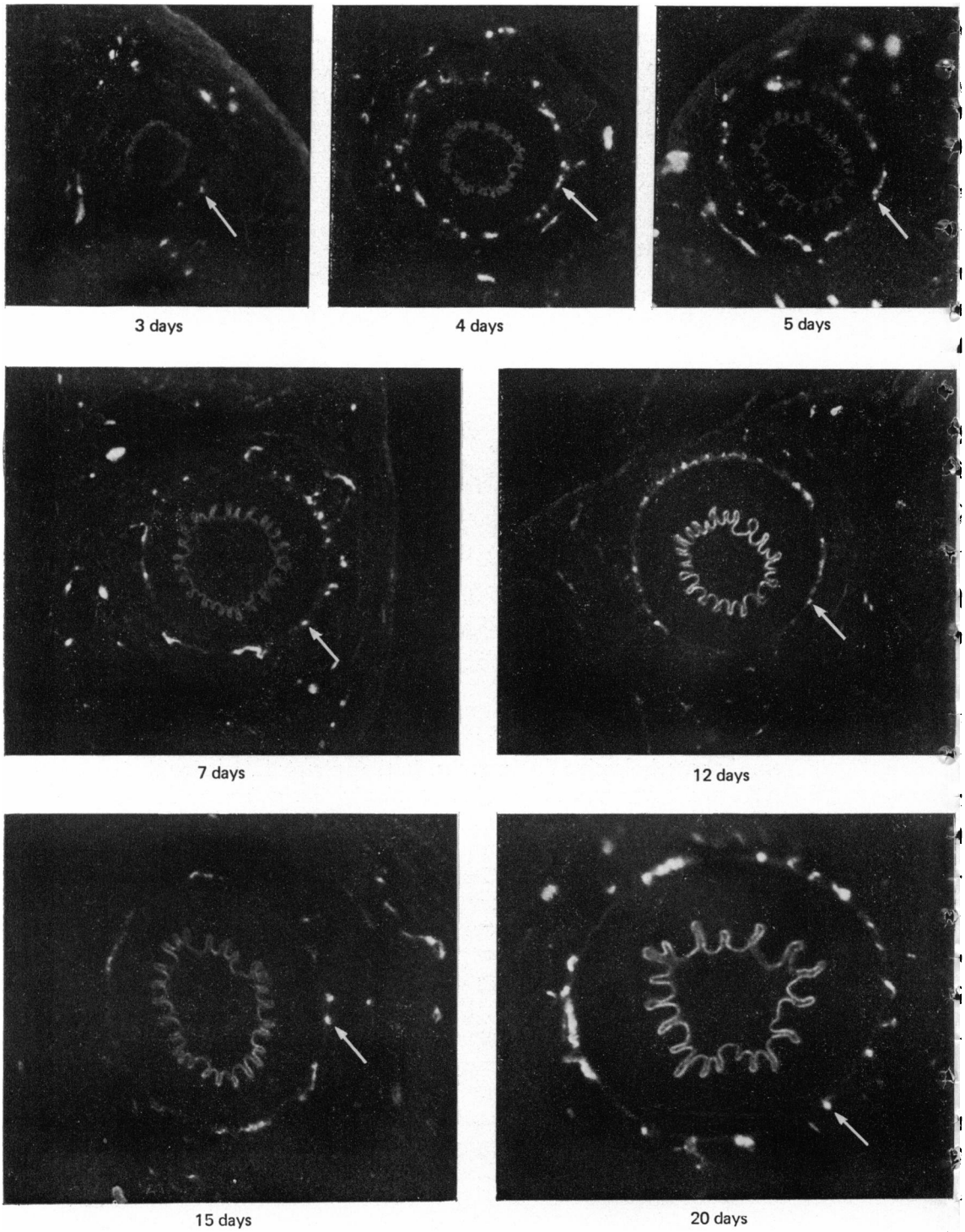


Fig. 2. Fluorescence microscopy of 8 μm sections of the saphenous artery. This illustrates the developing stores of catecholamine in the nerves adjacent to the external elastic lamina (arrows). These were the only nerves included in the calculations although other nerves, nerve trunks and mast cells in the area also fluoresce. The internal elastic lamina was prominent in all the vessels. $\times$ 275.

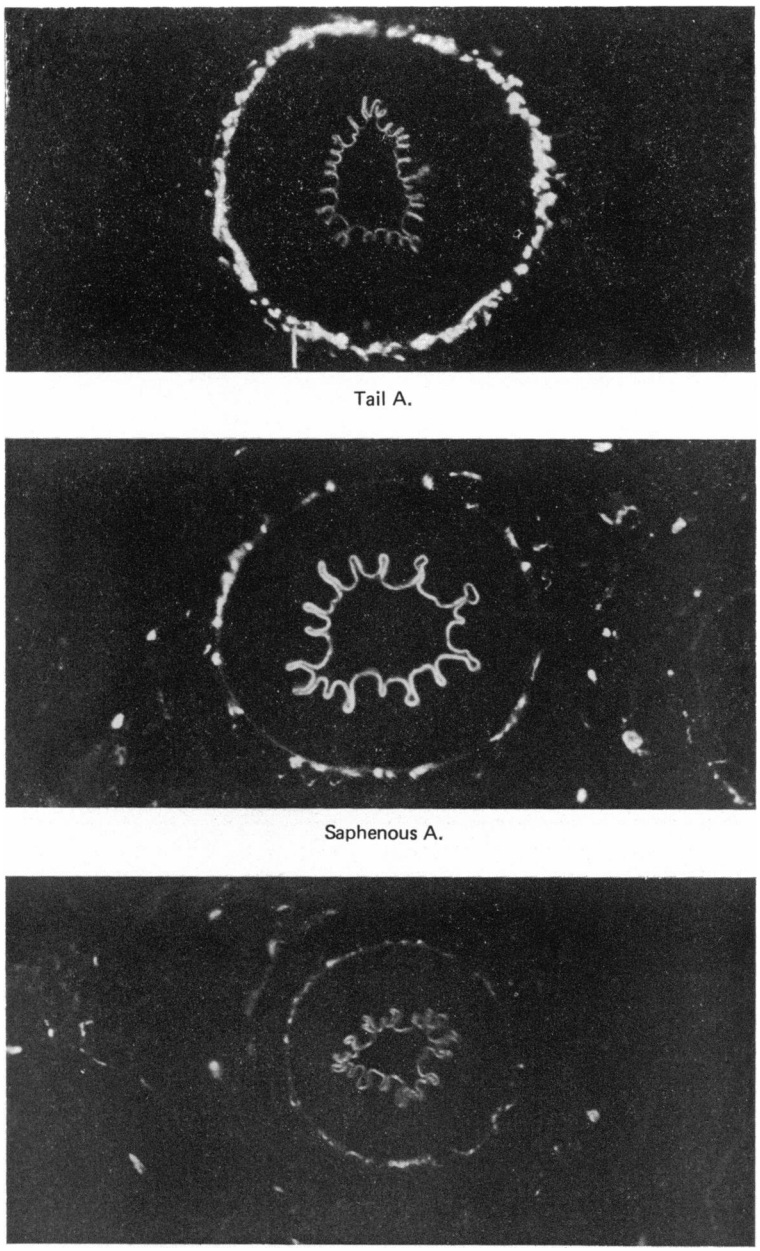


Fig. 3. Comparison of the innervation of the three muscular arteries at 30 days. ×250.

Results from the femoral artery were quite different (Fig. 6). The number of nerves was virtually constant throughout the period studied and, therefore, the number per unit area decreased progressively.

In the veins, the nerves with stored catecholamine were initially adjacent to the outermost smooth muscle cells, similar to their usual position in the arteries. With subsequent development, they penetrated the tunica media and seemed to run

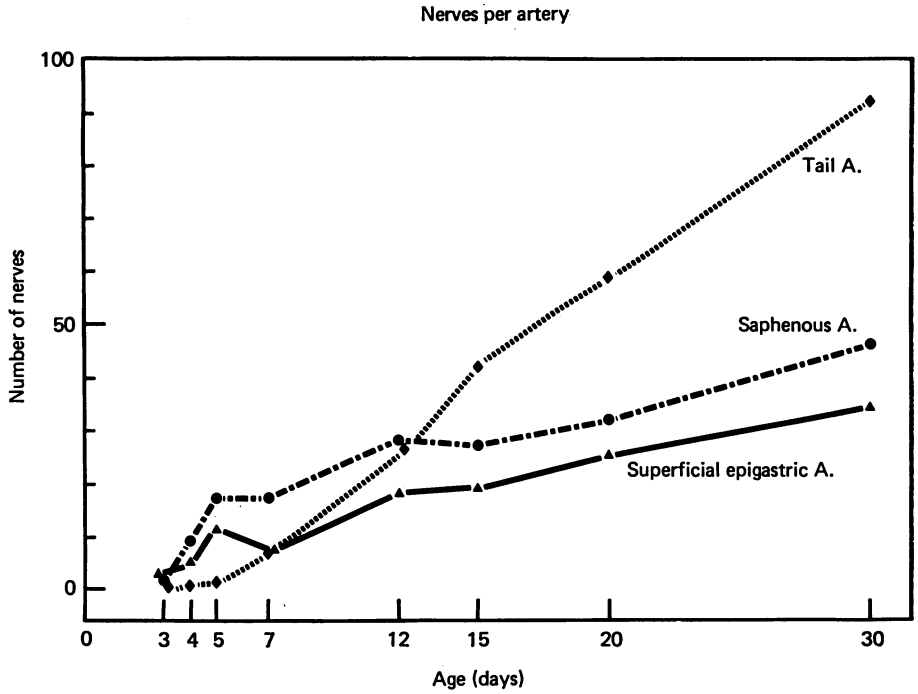


Fig. 4. Numbers of discrete fluorescing areas ('nerves') in 8 μm sections of the three muscular arteries.

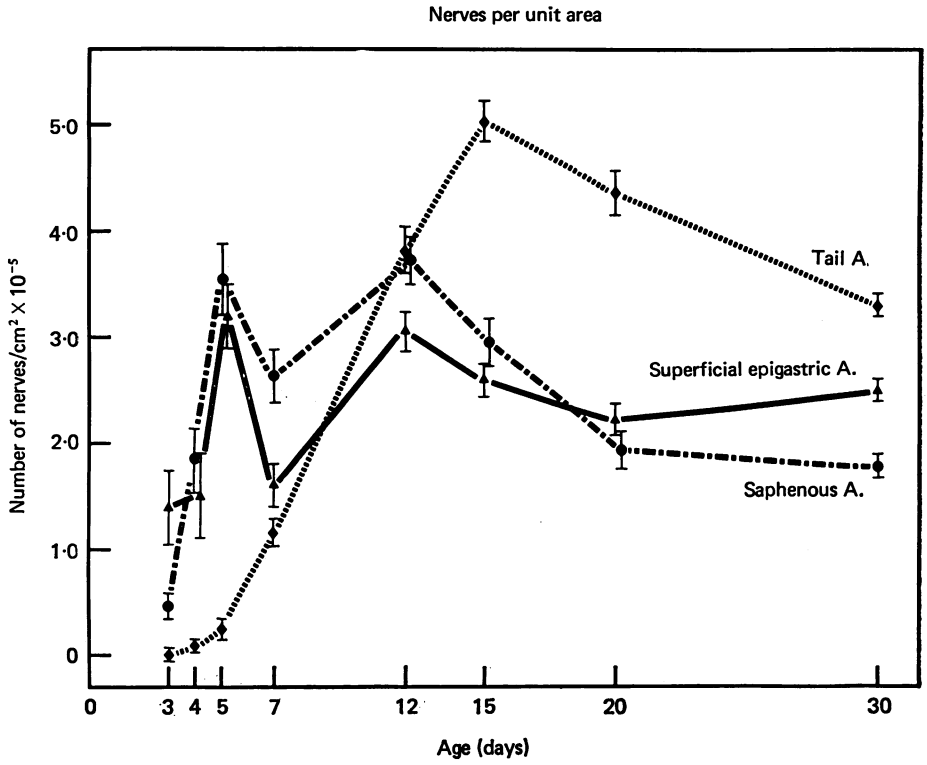


Fig. 5. Innervation density of the three muscular arteries.

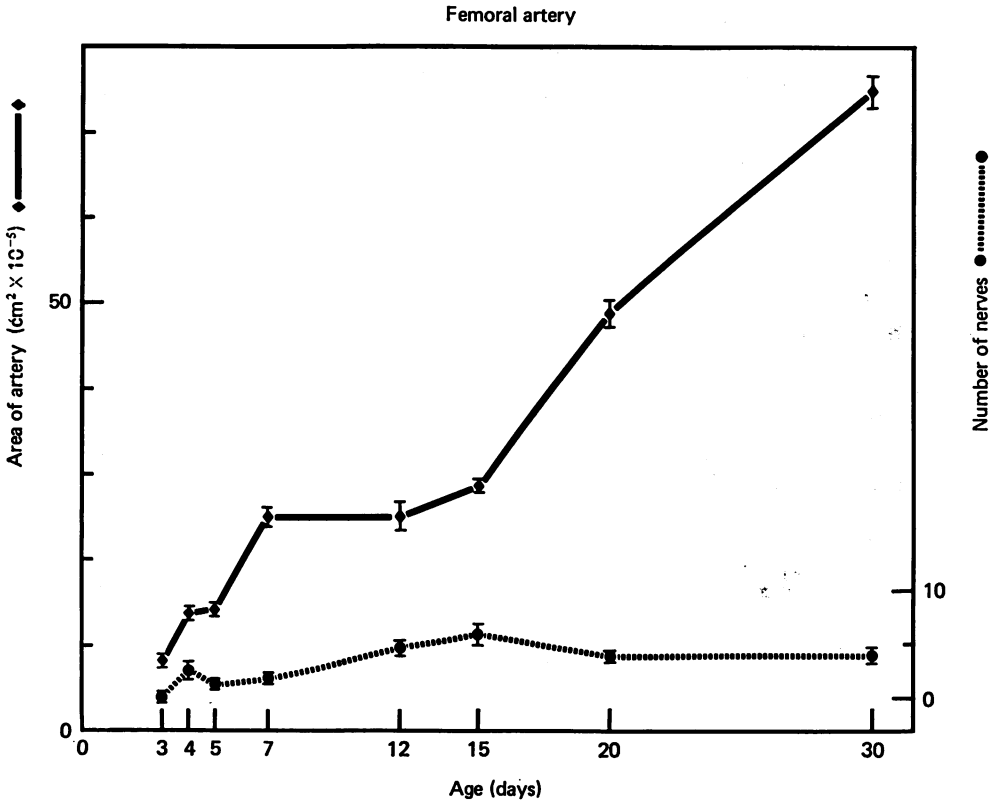


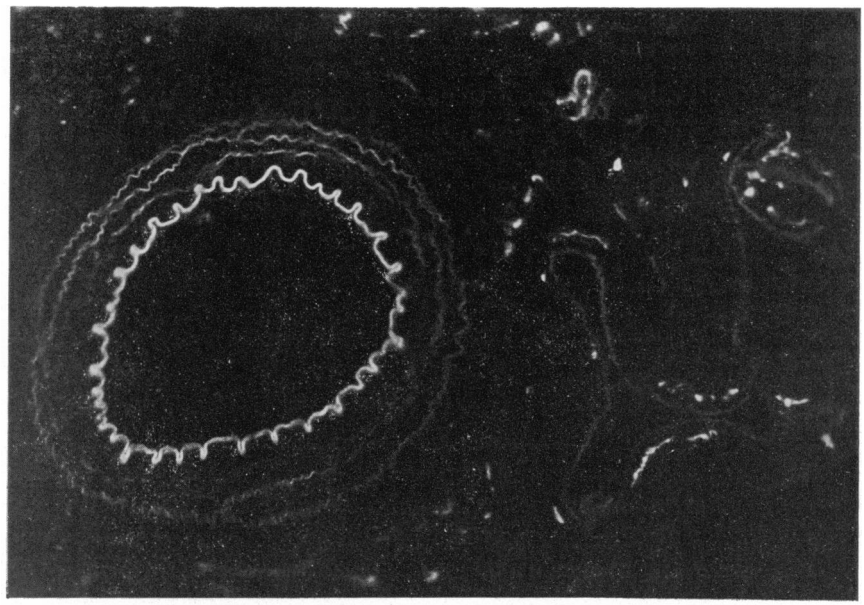
Fig. 6. Cross sectional area and nerve fibre numbers in the femoral artery.

mainly concentrically, so that long strips of fluorescence resulted. The veins accompanying the tail, superficial epigastric and saphenous arteries developed considerably less stored neurotransmitters than did the arteries. However, the femoral vein had considerably more catecholamine than did its companion artery (Fig. 7).

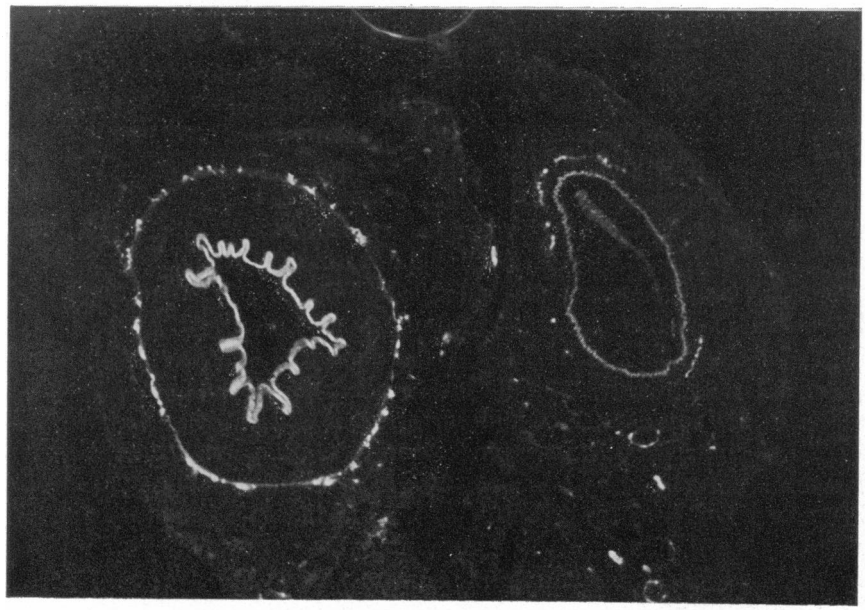
The ultrastructural examination of thin sections confirmed that the fluorescent dots in the tunica media-adventitial border area corresponded in size to the size of nerves (Fig. 8). There was no evidence of any other structure or material other than the small granular vesicles in the varicosities that would account for the fluorescence. Reserpinized animals demonstrated neither catecholamine fluorescence nor dense cores in the vesicles. The sodium borohydride method confirmed that the fluorescence was formaldehyde-induced and due to the catecholamine.

DISCUSSION

The histochemical fluorescence method used in the present investigation has been regarded by other investigators as extremely sensitive in demonstrating the storage sites of catecholamines and other biogenic amines (De Champlain *et al.* 1970; Van Orden, 1975). The specificity tests undertaken in this study suggest that this method demonstrated developing adrenergic innervation of vessels, with noradrenaline being the neurotransmitter. Since variation in the innervation along the length of arteries is well documented, the same segment of each vessel was examined at the different ages studied to ensure comparable results.



Femoral A. and V.



Saphenous A. and V.

Fig. 7. The innervation of the saphenous and femoral vessels at 30 days. The greater amount of elastic tissue in the femoral versus the saphenous artery is indicated by the non-specific fluorescence. × 175.

In the arteries examined here the nerves were restricted to the tunica media-adventitial border. The femoral artery, the most elastic of the four studied, had very little stored catecholamine. Elastic arteries are considered to be low in adrenergic nerves when compared with muscular arteries (Su & Lee, 1976). This vessel is the

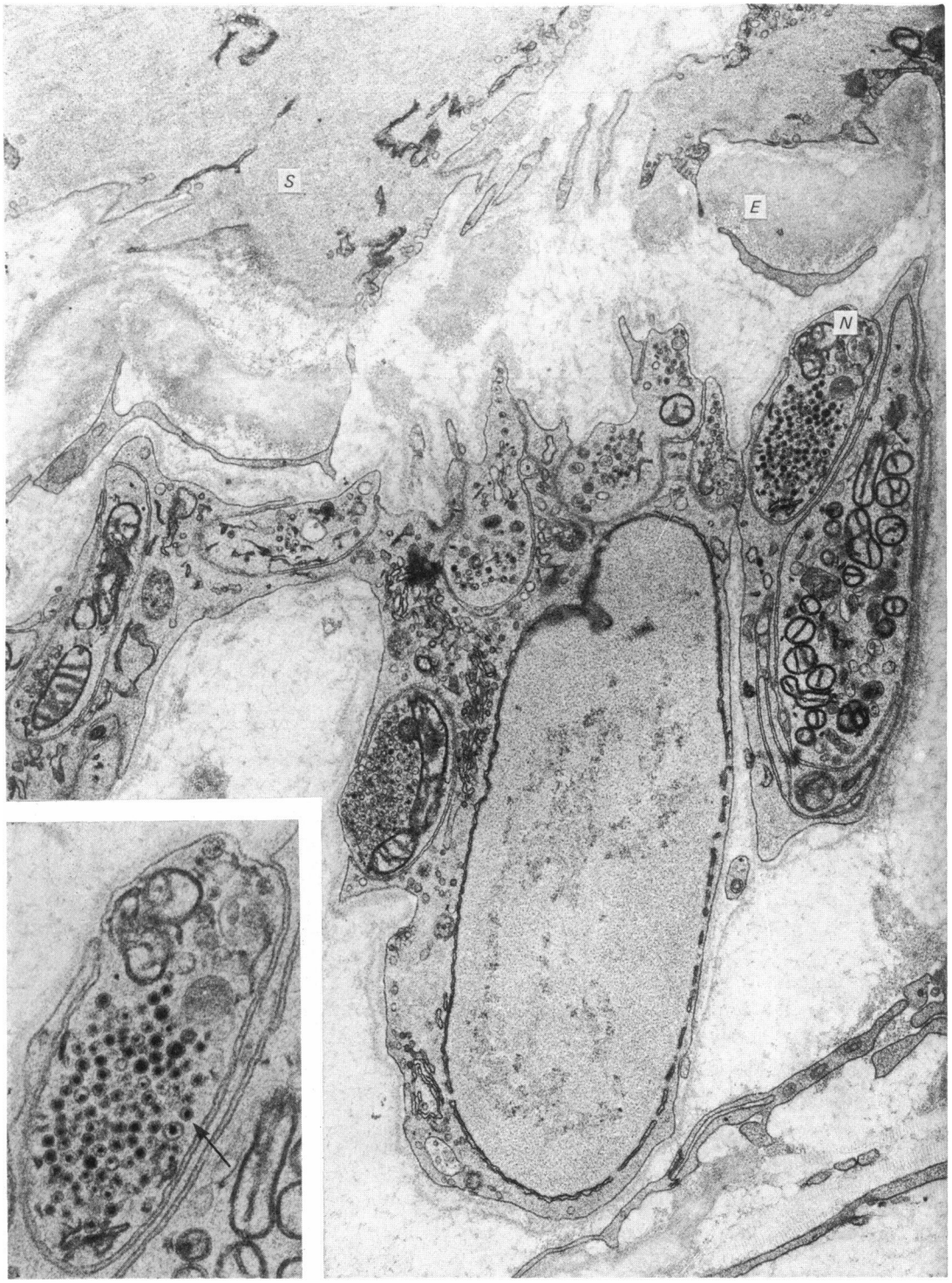


Fig. 8. Unmyelinated nerve fibres at the tunica adventitia–tunica media junction of the adult tail artery. The related Schwann cell is sectioned through its nucleus. *E*, External elastic lamina; *S*, smooth muscle cell. The nerve varicosity (*N*) is in part not enclosed by the Schwann cell, and it is enlarged in the insert, demonstrating that the majority of vesicles are small with dense cores (arrow), typical of adrenergic innervation. × 18000 and × 37500.

Table 1. *Total numbers of arterial sections examined quantitatively for formol-fluorescence*

Number of rats	Days	Numbers of sections			
		Tail	Superficial epigastric	Saphenous	Femoral
3	3	8	17	19	12
5	4	10	19	24	22
5	5	18	30	26	29
7	7	19	30	35	27
8	12	31	57	59	54
8	15	25	45	54	61
7	20	26	37	39	44
9	30	36	51	45	51

main distributing artery to the hind limb, and the results would suggest that flow control is in its branches. The branch points themselves may be particularly heavily innervated, since nerve terminals can be demonstrated within the tunica media only in the proximal end of the rabbit saphenous artery (Bevan & Purdy, 1973).

In contrast to the condition found in arteries, nerves usually penetrate the tunica media of veins (Shepherd & Vanhoutte, 1975). In the present study, the veins accompanying the tail, superficial epigastric and saphenous arteries demonstrated considerably less innervation than did the arteries. Mellander & Johansson's (1968) review of vascular control emphasized that the capacitance vessels, mainly the voluminous venous section, contain about 80 % of the regional blood volume. Nerve mediated smooth muscle contraction could, therefore, produce marked regional haemodynamic shifts, influencing venous return and cardiac output. The response to nerve stimulation of both isolated veins and those in the organism correlates well with the density of innervation (Shepherd & Vanhoutte, 1975). The veins may play a much larger part than is realized in vascular control, and the femoral vein had relatively much greater innervation than did the femoral artery. The outward flow then, in this particular vascular bed, appears to be controlled by the muscular arteries, and the venous return by large veins. This suggests that the larger the vein the greater the control, since the veins accompanying the muscular arteries studied here had much less innervation than the femoral vein had. Smaller veins, such as those in skeletal muscle, are described as having very few adrenergic nerves (Fuxe & Sedvall, 1965), and nerves are absent from visceral venules and small veins (Furness, 1971). It is noteworthy that the rabbit femoral vein lacks both fluorescence and response (Bevan *et al.* 1974).

Reports on development of adrenergic innervation in other locations suggest that the accumulation of stored catecholamine occurs mainly in the postnatal period (De Champlain *et al.* 1970; Furness *et al.* 1970; Burnstock & Costa, 1975; Borchard, 1978), and this is supported by the present findings. There was very little stored catecholamine present in the first few days after birth. In the rat portal vein, nerve varicosities were detected by the end of the first week, which corresponds to the first responses to transmural stimulation (Ljung *et al.* 1975; Ljung & Stage, 1975). In contrast to this, adrenergic innervation of chick vessels was found with fluorescence histochemical methods as early as the 14th day, prior to hatching (Rickenbacher & Ruflin, 1974). Transmural stimulation of fetal sheep carotid artery elicited

contraction due to excitation of adrenergic neurons; neurotransmitter in this case must therefore have been present before birth (Su, Bevan, Assali & Brinkman, 1977). With all the differences within vessels and between vessels in mammals, it can indeed be expected that there should be also considerable differences between classes of vertebrates. It is possible that the overall degree of immaturity at birth is linked to the stage of development of the sympathetic nervous system.

The three muscular arteries studied are remarkably similar in the early neonatal period. At the light microscopic level, the surrounding tissues distinguished them from each other. The superficial epigastric vessels were embedded in connective tissue, the saphenous vessels were lying on skeletal muscle, and the tail artery had a number of smaller veins accompanying it rather than a single large one. Yet, as they differentiated, they developed totally different characteristics. When the catecholamine stores per unit area were compared, the superficial epigastric artery, a cutaneous vessel, had an intermediate density of innervation and, when mature, was approximately half the size of the other two vessels. The saphenous artery, a main branch to the hind limb and foot, had somewhat less innervation and, although it was approximately the same size as the tail artery, it was greatly surpassed by the density of innervation of the tail artery. The only other artery described with a similar intensity of innervation is the rabbit ear artery (Burnstock *et al.* 1970; Somlyo, 1975). Such intense innervation probably has some special functional significance, and it may be postulated that in the interests of temperature regulations non-fur covered appendages should have the possibility of marked vasoconstriction. In fact, in the rabbit ear artery, the response of the vessel to catecholamines and other agents is dependent on the temperature at which the rabbit is kept prior to experimentation (Nedergaard, 1976).

SUMMARY

The postnatal development of adrenergic innervation was followed in peripheral blood vessels of Wistar rats. The femoral vessels and their branches, the superficial epigastric and saphenous vessels, and the tail artery, were investigated from birth to maturity. The proximal ends of the vessels were studied with fluorescence microscopy after the catecholamine was converted to a fluorophore by hot formaldehyde vapour, and ultrastructural morphology confirmed that the nerve varicosities mainly contained small vesicles with dense cores, typical of adrenergic innervation. Further confirmation was obtained with reserpine pre-treatment, the sodium borohydride specificity test, and experiments to alter the non-specific fluorescence of elastin.

The nerves in the arteries were immediately adjacent to the external elastic lamina, and they retained this position throughout postnatal development. Of the three muscular arteries, the development of innervation was earlier and more intense in the saphenous and superficial epigastric arteries than in the tail artery. However, the tail artery surpassed the other two both in the total number of nerves and in the density of innervation per unit area beyond 12 days of age, and maintained this lead to maturity. The superficial epigastric artery had the smallest total number of nerves but had a greater density of innervation than the saphenous. The femoral artery did not develop any appreciable innervation. The femoral vein demonstrated the greatest amount of fluorescence of any of the veins, the others having considerably less innervation than their companion arteries.

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