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Factors affecting the extraneuronal inactivation of noradrenaline in cardiac and smooth muscle

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Summary

1. The relation between density of adrenergic innervation, noradrenaline (NA) accumulation (as seen with the fluorescence histochemical method) in tissues incubated in a high concentration of NA, and monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) activities, were examined in a wide range of tissues from different species.

2. Evidence was obtained to support the proposal that accumulation of NA in the non-innervated smooth muscle of the human umbilical artery and chick amnion is associated with very low activities of COMT within muscle cells.

3. The wide variation in tissue accumulation of NA in adrenergically innervated muscles was confirmed. For example, in the rabbit atrium and rat vas deferens, there was high NA accumulation in vascular smooth muscle but not in other muscle cells. In the mouse vas deferens there appeared to be preferential NA accumulation in the outer longitudinal muscle in comparison with the circular muscle. In the ventricle of the rat and mouse individual muscle cells showed different degrees of accumulation of NA. Many unidentified fluorescent cells were revealed in the submucosa of the guinea-pig ureter following loading with NA. The highest activities of COMT were found in the rat vas deferens and the lowest in the rabbit vascular tissues; the highest activity of MAO was found in the guinea-pig ileum, and the lowest in the rat aorta.

4. No simple relation between tissue activities of MAO and COMT and degree of NA accumulation was found. Possible directions for further investigation of the problem are discussed.

Introduction

Studies of noradrenaline (NA) uptake in the rat heart demonstrated the existence of two uptake mechanisms, $Uptake_1$ and $Uptake_2$ (Iversen, 1967). Both mechanisms were initially believed to be associated with adrenergic nerves, and it was not until later that $Uptake_2$ was shown to be extraneuronal (Eisenfeld, Axelrod & Krakoff, 1967; Eisenfeld, Landsberg & Axelrod, 1967; Malmfors, 1967; Ehinger & Sporrong, 1968). NA accumulation occurs in smooth muscle following exposure to high concentrations of NA (Gillespie, Hamilton & Hosie, 1970), and it seems probable that this process is similar or identical to $Uptake_2$ (Iversen, 1971). Accumulation of NA in cells is an indication of uptake into the cells and failure of intracellular metabolizing enzymes to keep pace, but the factors influencing tissue variation in NA accumulation have not been identified.

In the rat, two NA-metabolizing enzyme systems, catechol-*O*-methyl transferase (COMT) and monoamine oxidase (MAO), play an important role in controlling the accumulation of unchanged NA in cardiac muscle cells (Lightman & Iversen, 1969). However, Jarrott (1970) found no correlation between MAO and COMT activities and extraneuronal uptake differences in hearts of several species. In a recent histochemical study, Burnstock, McLean & Wright (1971) suggested that high NA accumulation in non-innervated smooth muscle might be associated with low intracellular MAO and COMT activity. In the present study, MAO and COMT activities, extraneuronal accumulation characteristics, and the adrenergic innervation density of a number of tissues from different species have been determined, in an attempt to clarify the basis of tissue and species differences in extraneuronal NA accumulation.

Methods

Tissues investigated were mouse heart, vas deferens; rat heart, vas deferens, thoracic aorta; rabbit atrium, thoracic aorta, ear artery, inferior vena cava; guinea-pig ileum, lung, taenia coli, ureter; human umbilical artery; chick amnion.

Enzyme assays

The animals were killed by a blow on the head. Amnions of 11 day chick embryos were dissected from eggs incubated at 38° C. Human umbilical cord was obtained from a nearby hospital and transported to the laboratory in ice-cold Krebs-Henseleit solution and dissected within 3 hours of delivery. All tissues were dissected into ice-cold Krebs-Henseleit solution, samples were taken for fluorescence histochemistry and NA accumulation studies, and the remainder quickly blotted, weighed and homogenized in 10 volumes of ice-cold 0·005 M potassium phosphate buffer (pH 7·0) with all-glass hand-driven tissue grinders. The vascular tissues and the guinea-pig ureter were first pulverized in a stainless steel crucible under liquid nitrogen.

MAO activities were determined on the whole tissue homogenates. For the COMT assay the homogenates were centrifuged at 100,000 *g* and 2° C for 60 minutes. The supernatant was dialyzed overnight at 2° C against 500 ml of the pH 7·0 phosphate buffer.

Monoamine oxidase assay

This was based on the radiochemical assay of McCaman, McCaman, Hunt & Smith (1965), with ^{14}C-tyramine as the substrate. The incubation volume was increased according to Jarrott (1971a) and the concentration of tyramine in the reaction mixture was optimal at 2×10^{-3}M. The reaction was carried out at 30° C in oxygen-filled tubes containing: 50 μl of 0·2 M potassium phosphate buffer (pH 7·2) containing 4×10^{-3}M ^{14}C-tyramine (specific activity 0·27 Ci/mole), 25 μl of water and 25 μl of tissue homogenate. For blanks, 25 μl of water was substituted for the tissue homogenate. Each assay was performed in triplicate. The period of incubation was 25 min except with the guinea-pig ileum homogenate, which was incubated for 5 min since with this tissue the reaction was linear only up to 10 minutes. The reaction was stopped with 10 μl of 3 M HCl and the deaminated product extracted into 0·7 ml of ethyl acetate. A 0·5 ml portion of

the ethyl acetate layer was washed with 0·1 ml of 3 M HCl in a clean tube and after centrifugation, 0·3 ml of the ethyl acetate layer was transferred to a counting vial containing 10 ml of scintillation solution of the following composition: 5·5 g of 2,5-diphenyloxazole (PPO), 0·1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and 33 ml of Triton-X per litre of toluene. The radioactivity was counted in a Packard liquid scintillation counter and corrections for counting efficiency were made by recounting representative vials after the addition of an internal standard (^{14}C-n-hexadecane).

Under the conditions described, the reaction proceeded linearly with incubation time and enzyme concentration. The enzyme activity was calculated in nmoles of product formed per hour per mg of whole homogenate protein.

Catechol-O-methyl transferase assay

A modification of the radiochemical assay of McCaman (1965) was used in which the incubation and solvent extraction volumes were increased according to Jarrott (1971b). The concentrations of *S*-adenosylmethionine (1×10^{-4}M) and 3,4-dihydroxybenzoic acid (1×10^{-3}M) were optimal. The reaction mixture contained 25 μl of dialyzed tissue supernatant, 50 μl of 0·16 M potassium phosphate buffer (pH 7·8) and 25 μl of substrate solution containing 4×10^{-3}M 3,4-dihydroxybenzoic acid, 4×10^{-4}M ^{3}H-*S*-adenosylmethionine (specific activity 11·3 Ci/mole) and 2×10^{-2}M $MgCl_2$. For blanks 25 μl of water was substituted for the tissue supernatant. The reaction was carried out at 38° C and the period of incubation was 25 minutes. Each assay was performed in triplicate. After incubation, 10 μl of 3 M HCl was added to the tubes and the radioactive product extracted into 0·5 ml of ethyl acetate. The tubes were centrifuged and 300 μl of the ethyl acetate layer was added to a counting vial with 10 ml of scintillation solution. The radioactivity was counted in a Packard liquid scintillation counter and corrections for counting efficiency were made with ^{3}H-n-hexadecane as an internal standard.

Under the conditions described the reaction proceeded linearly with incubation time and enzyme concentration. The enzyme activity was calculated in nmoles of product formed per hour per mg of protein in the supernatant.

Protein assay

The supernatant and whole homogenate protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Fluorescence histochemistry

Tissue pieces were quenched in propane cooled by liquid nitrogen, and freeze-dried for 36 hours. The pieces were then incubated with formaldehyde for 1 h at 80° C and 70% humidity, and embedded in Paraplast plastic wax. Sections, 10 μm thick were cut, mounted in paraffin oil and examined under a Leitz fluorescence microscope. Micrographs were obtained with the Leitz Orthomat automatic camera and Kodak Tri-X film.

Noradrenaline accumulation

Small pieces of tissue were incubated at 37° C in Krebs Ringer solution gassed with carbogen (95% O_2, 5% CO_2). In some experiments the rat heart was perfused with Krebs Ringer solution by the Langendorff technique. For these experiments the rats were injected with 500 units of heparin i.p. 1 h before they were killed.

In studies of NA accumulation, tissues were equilibrated in Krebs Ringer for 10 min at 37° C, NA (Levophed, Winthrop) was added to give a final concentration of 50 μg/ml and the incubation continued for 15 minutes. The incubation solution contained ascorbic acid (20 μg/ml). The tissue pieces were then rinsed in NA-free Ringer for 2 min at 37° C. The degree of NA accumulation was measured using the exposure times of an Orthomat automatic camera. Although the numerical values obtained were subject to considerable variation due to changes in the intensity of the lamp and the atmospheric conditions, the position of each tissue on a *relative* scale did not vary. Hence, this relative order of NA accumulation is quoted using a graded plus scale (Table 1).

Radiochemicals

These were obtained from the Radiochemical Centre, Amersham. *S*-adenosyl-L-methionine (methyl-^{3}H) (specific activity 7·2 Ci/mole) was supplied in H_2SO_4 (pH 2·2–3·5) at a radioactive concentration of 500 μCi/ml. This was stored at −30° C and diluted with non-radioactive *S*-adenosylmethionine as required to the specific activity stated.

Tyramine-1-^{14}C-hydrochloride (specific activity 44 Ci/mole) was supplied as a freeze-dried solid. This was dissolved in distilled water to give a radioactive

TABLE 1. *Density of adrenergic innervation, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) activities and extraneuronal accumulation of noradrenaline (NA) in tissues from six different species*

Tissue	*COMT activity (nmoles/h)/mg protein	*MAO activity (nmoles/h)/mg protein	†Adrenergic innervation	‡Extraneuronal accumulation of NA
Rat vas deferens	58·5±5·8(5)	94·8±5·1(3)	+++++	—
Rat ventricle	11·1±1·6(3)	153·3±25·8(3)	+++	+++
Rat aorta	15·1±4·1(3)	14·5±3·1(3)	—	++
Mouse ventricle	30·0±2·8(5)	120·2±8·0(3)	+++	+++
Mouse vas deferens	43·1±2·7(5)	198·3±15·2(2)	+++++	++++
Rabbit atrium	2·5±0·2(4)	70·6±13·7(3)	++++	++
Rabbit aorta	1·7±0·2(4)	68·9±22·0(3)	+	+++
Rabbit ear artery	1·7±1·2(4)	76·0±15·0(2)	++	+++
Rabbit inferior vena cava	2·6±2·6(3)	19·5±3·3(3)	++	+++
Guinea-pig ileum	13·2±1·8(3)	1,539·9±150·8(3)	++	+
Guinea-pig lung	6·2±0·6(3)	186·9±33·5(3)	+	+
Guinea-pig taenia	3·2±0·4(3)	209·5±22·6(3)	++	+
Guinea-pig ureter	22·1±1·2(5)	95·0±11·3(4)	+	+++
Human umbilical artery	1·0±0·5(4)	21·0±0·6(3)	—	++++
Chick amnion	0·3±0·2(4)	30·0±3·0(3)	—	++++

* Results expressed as mean±S.E. Numbers of determinations in parentheses, COMT activity was measured in tissue supernatant, MAO activity was measured in whole homogenate. † Density of adrenergic innervation has been estimated according to the number of adrenergic nerve bundles per unit area of tissue as demonstrated by the fluorescence histochemical technique. This is only semiquantitative since the innervation density varies in different areas of some tissues and there is wide variation in the size of the nerve bundles. ‡ Accumulation of NA was measured in terms of the intensity of fluorescence in tissue sections (or whole mounts of chick amnion and rabbit inferior vena cava) after loading with NA.

concentration of 50 μCi/ml. The stock solution was stored at −30° C and diluted with non-radioactive tyramine as required to the specific activity stated.

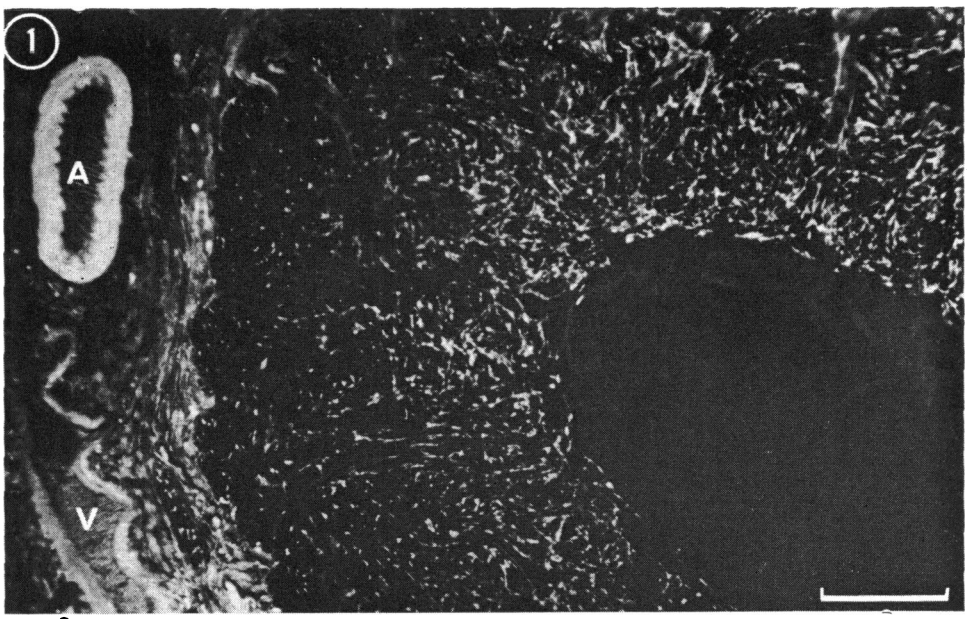

FIG. 1. Rat vas deferens, T.S., after incubation in noradrenaline 50 μg/ml for 15 minutes. Note the fluorescence of the vascular smooth muscle of the artery (A) and the vein (V) in contrast to the smooth muscle layers of the vas deferens which do not accumulate NA except in the numerous adrenergic nerves. Calibration 100 μm. Freeze-dried tissue incubated in formaldehyde vapour for 1 h at 80° C.

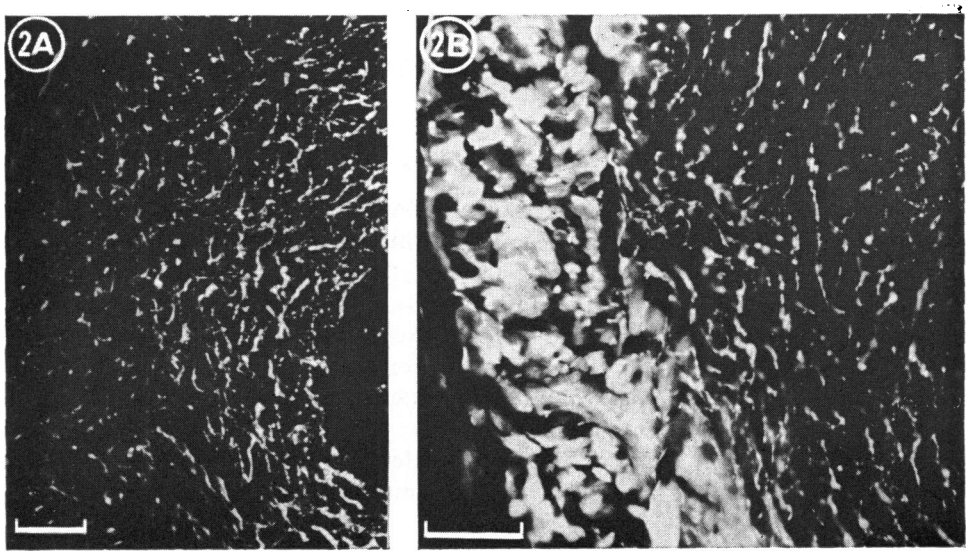

FIG. 2. Mouse vas deferens, T.S. A, Control, showing fluorescent nerves in the circular and longitudinal smooth muscle layers. B, Noradrenaline (NA) accumulation after incubation in NA 50 μg/ml for 15 minutes. Note NA accumulation in the longitudinal muscle coat only. Calibration 50 μm. Freeze-dried tissues treated with formaldehyde vapour for 1 h at 80° C.

Results

The density of adrenergic innervation, MAO and COMT activities and the extraneuronal accumulation of NA were examined in tissues from six different species. The results are summarized in Table 1.

Rat tissues

The three tissues, vas deferens, heart, and aorta, showed a wide variation in all the factors studied. The vas deferens had an extremely dense adrenergic innervation consisting of very fine varicose fibres. The smooth muscle of the vas deferens showed no NA accumulation, in contrast to the high NA accumulation in adjacent arteries (Fig. 1). The COMT activity in the rat vas deferens was the highest of all the tissues studied.

The rat ventricle had moderately dense adrenergic innervation, and showed moderate accumulation of NA, although the ability of individual cardiac muscle cells to accumulate NA varied greatly. The MAO activity of the rat ventricle was very high. In the ventricle retention of NA during prolonged washout was very low in comparison with other tissues.

The rat aorta had no detectable adrenergic innervation, and the MAO activity was the lowest encountered, although COMT activity was moderate. NA accumulation was low.

Mouse tissues

The ventricle of the mouse showed similar innervation density and NA accumulation to that of the rat. Enzyme activities were slightly different, there being more COMT and less MAO in the mouse heart. The vas deferens, while showing a dense adrenergic innervation, had an extremely high NA accumulation in the outer longitudinal muscle of the organ only, although different cells appeared to accumulate NA to different degrees (Fig. 2A, B). Both MAO and COMT activities were high.

Rabbit tissues

The rabbit tissues studied showed low COMT activity; MAO activity was moderate in all tissues, except the vena cava, where it was low. NA accumulation did not occur in the cardiac muscle of the rabbit atrium but was significant in the coronary vascular smooth muscle (Fig. 3A, B). There was no evidence of an adrenergic innervation of the rabbit aorta except in rare cases when isolated fluorescent nerves were seen (Fig. 3C). In contrast, the rabbit ear artery was densely innervated in the typical arterial pattern, the fluorescent nerves being located at the medial-adventitial junction (Fig. 3E). NA accumulation in both vessels was high (Fig. 3D, F). The rabbit inferior vena cava showed a typical adrenergic nerve plexus and high NA accumulation, but MAO activity was extremely low.

Guinea-pig tissues

The ileum, taenia coli and lung were characterized by low COMT activity, but there was high MAO activity, especially in the ileum. In these three tissues,

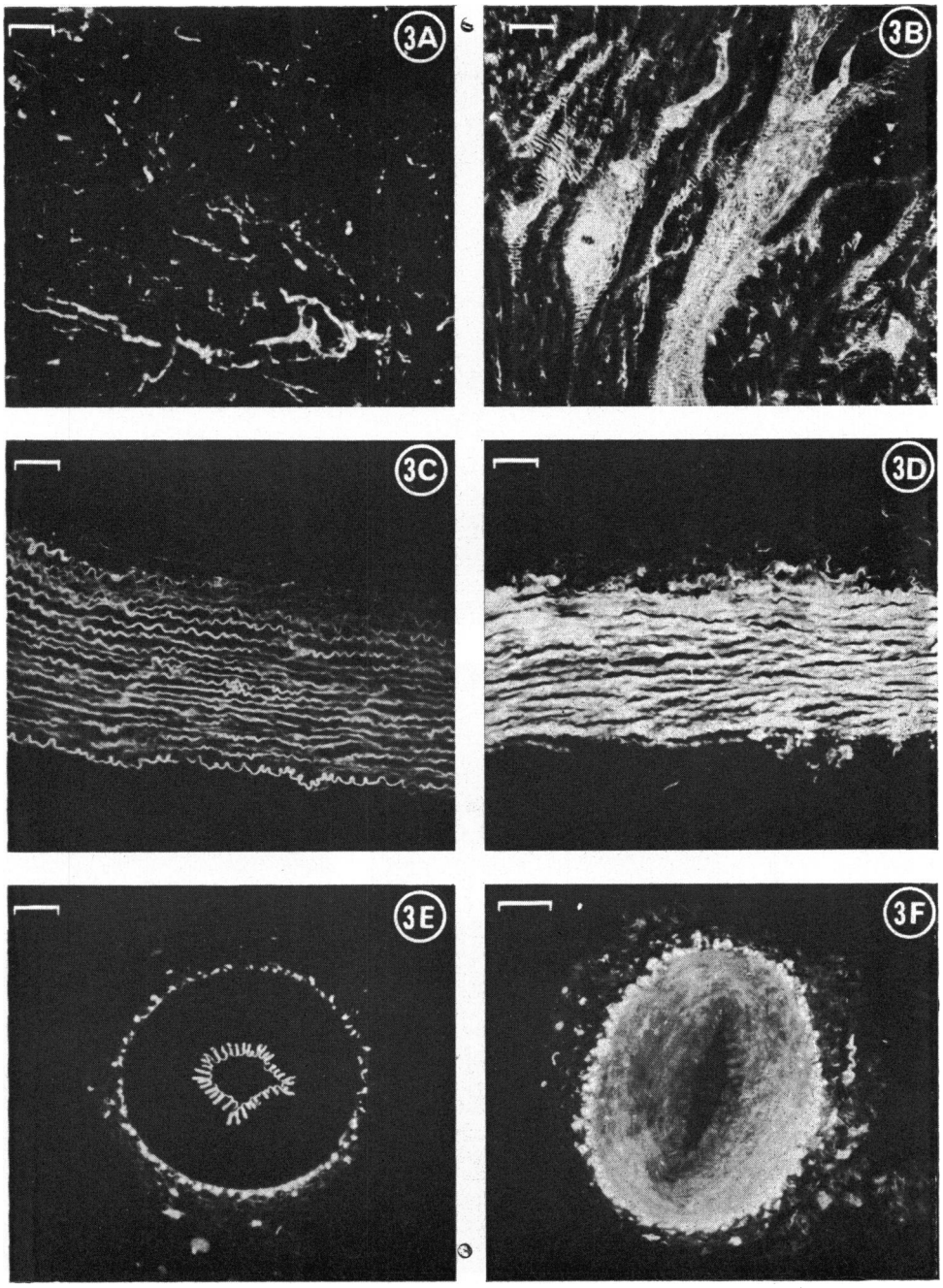

FIG. 3. Rabbit tissues. A, Section through control rabbit atrium, showing adrenergic innervation pattern. B, Rabbit atrium after incubation in noradrenaline (NA) 50 μg/ml for 15 minutes. Note the fluorescence of vascular smooth muscle and connective tissue fibres. C, Control rabbit aorta. T.S. Note autofluorescence of elastic laminae. D, Rabbit aorta, after incubation in NA 50 μg/ml for 15 minutes. Note accumulation of NA in smooth muscle bundles between the laminae. E, Control rabbit ear artery. T.S. F, Rabbit ear artery after incubation in NA 50 μg/ml for 15 minutes. Freeze-dried tissues incubated in formaldehyde vapour for 1 h at 80° C. Calibration 50 μm.

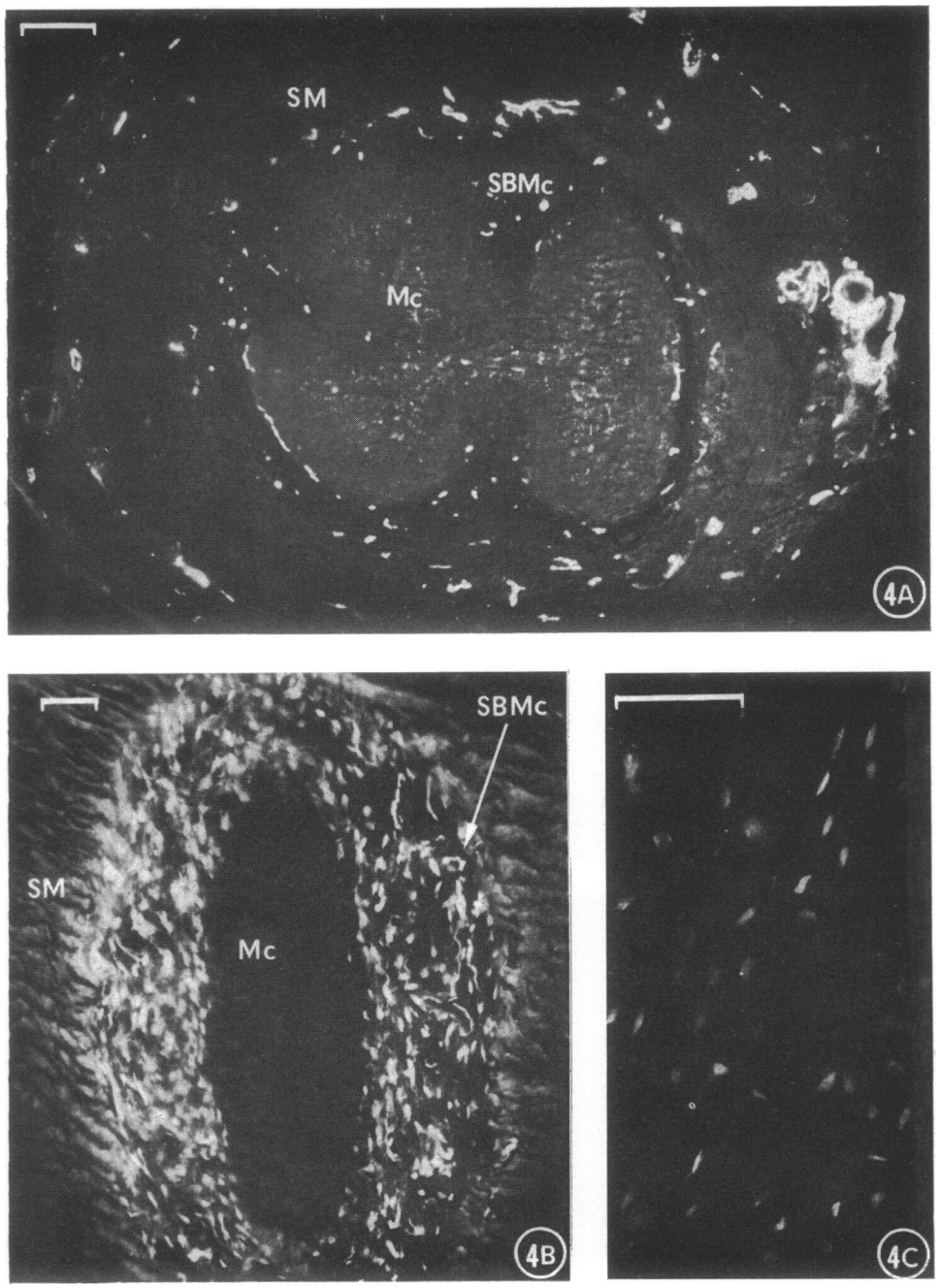

FIG. 4. Guinea-pig ureter. A, Control transverse section showing fluorescent nerves in the submucosa (SBMc), adventitia and associated blood vessels. Note the low density of innervation of the smooth muscle layers (SM). (Mc-mucosa.) B, Oblique section showing accumulation of noradrenaline (NA) after incubation in NA 50 μg/ml for 15 minutes. Note increased fluorescence of the smooth muscle cells (SM), and also the presence of fluorescent cells in the submucosa (SBMc). (Mc-mucosa.) C, High power detail of submucosal cells which have accumulated NA after exposure to NA 50 μg/ml. Freeze-dried tissue incubated in formaldehyde vapour for 1 h at 80° C. Calibration 50 μm.

smooth muscle constituted only a fraction of the total tissue, and smooth muscle accumulation of NA was low. In the lung, the bronchial smooth muscle was the only tissue which showed a high NA accumulation. The guinea-pig ureter showed high MAO and COMT activities, and moderate NA accumulation in the smooth muscle. Connective tissue cells in the submucosa also accumulated NA (Fig. 4A, B, C). The innervation density of all these tissues was low, except in the region of the myenteric plexus in the ileum and taenia coli.

Non-innervated smooth muscle

Both human umbilical artery and chick amnion showed very low COMT and low MAO activity. No fluorescent nerves were seen and NA accumulation was high.

Discussion

The present experiments were undertaken to examine whether high extraneuronal accumulation of NA in non-innervated smooth muscle is associated with low activities of COMT and MAO in the tissue, and also to test the hypothesis that enzyme activities in smooth muscle are related to the adrenergic innervation density. In confirmation of the suggestion put forward by Burnstock *et al.* (1971) enzyme activities were found to be very low in the non-innervated smooth muscle of the chick amnion and human umbilical artery. However, no consistent pattern was found in any of the other tissues. The results described here confirm the observation of Gillespie & Muir (1970) that there is a wide variation between tissues and between species in the extraneuronal uptake of NA.

The most densely innervated tissues, the mouse and rat vasa deferentia, and rabbit atrium, showed no obvious pattern of enzyme activity in relation to NA accumulation. High values for MAO activity were not always associated with dense adrenergic innervation since the densely-innervated rat vas deferens showed the same MAO activity as the guinea-pig ureter, where the adrenergic nerves to the muscle are few. The very high MAO activity in the guinea-pig gut is probably due to the high MAO activity in ganglion cells of non-adrenergic nerves in the myenteric plexus (Blaschko, 1952; Furness & Costa, 1972).

The degree of NA accumulation did not appear to be related to COMT activity. Although the very high COMT activity in the rat vas deferens might be responsible for the absence of NA accumulation in the smooth muscle, both COMT activity and NA accumulation in the mouse vas deferens were exceptionally high. The activity of COMT in the four rabbit tissues were surprisingly low in view of the report by Kalsner & Nickerson (1969) that COMT is the major pathway for NA inactivation in the rabbit aorta.

The enzyme activities were calculated using total tissue protein measurements. In those tissues where smooth muscle constitutes only a fraction of the total protein, for instance in the aorta, the ileum and the lung, the calculated activities might not reflect the true enzyme content of the smooth muscle component. In addition, results obtained *in vitro* might not represent the situation *in vivo*, where enzyme activities might be limited by submaximal concentrations of substrates. Furthermore, in the rat, mouse and rabbit hearts local differences in NA accumulation were detected histochemically, but enzyme activities were calculated for the tissue

as a whole. In the rat vas deferens and the rabbit atrium in particular, the vascular smooth muscle showed high NA accumulation, but there was no NA accumulation in the rest of the tissue.

It is clear that more information is required before the relation between enzyme activities, density of innervation and NA accumulation is fully understood. For example, it would be an advantage to know the rate of uptake of NA and the rate of production of NA metabolites. Denervation studies must also be carried out to determine what proportion of the total COMT and MAO activity is intraneuronal. For instance, in the cat nictitating membrane (Jarrott & Langer, 1971) and the rat vas deferens (Jarrott & Iversen, 1971), denervation studies have indicated that a significant proportion of COMT in these tissues is intraneuronal. Finally, since NA accumulation characteristics appear to vary widely in individual cells within the same tissue, it seems that measurements of NA uptake and metabolism at the cellular level may be necessary before meaningful correlations can be made.

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