J. Physiol. (1970), **206**, *pp.* 591–604
With 1 plate and 5 text-figures
Printed in Great Britain

SPECIES AND TISSUE VARIATION IN EXTRANEURONAL AND NEURONAL ACCUMULATION OF NORADRENALINE

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(*Received 7 July* 1969)

SUMMARY

1. The ability of arterial and non-arterial smooth muscle in five tissues (vas deferens, heart, bladder, colon, spleen) in four species (mouse, rabbit, rat, guinea-pig) to accumulate and retain noradrenaline (NA) was measured in thin tissue slices exposed to NA for 30 min, then washed in cold saline solution for 30 min. NA accumulation was assessed histochemically by measuring the fluorescence brightness of the tissue with the Leitz MPV microphotometer. In addition, similar measurements were made on smooth muscle in the cat spleen, on cardiac muscle and on the terminal adrenergic nerves.

2. In general, arterial smooth muscle had a greater capacity to accumulate and retain NA than non-arterial smooth muscle, but there was a great species and organ variability. The ability to accumulate and retain NA was best developed in the mouse, followed by the rabbit, rat and guinea-pig in that order. Among organs the artery to the vas and the coronary arteries showed the greatest retention. Among non-arterial smooth muscle the mouse vas and the rabbit colon were notable.

3. Cardiac muscle accumulates NA during exposure to the amine but, unlike smooth muscle, cannot retain it when washed with NA-free solution.

4. Terminal adrenergic nerves in different tissues show some variability in fluorescence intensity, and this is increased after exposure to NA. This may indicate a variable capacity of these cells to accumulate and retain NA.

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INTRODUCTION

The preceding paper (Gillespie, Hamilton & Hosie, 1970) describes two types of uptake of noradrenaline (NA) in smooth muscle in the cat spleen. In arterial smooth muscle NA is present in the cytoplasm and, judging from the fluorescence brightness, in high concentration. In trabecular smooth muscle the NA fluorescence is confined to the cell perimeter and is less intense. Previous work on perfused isolated arteries from rabbits had shown that arterial smooth muscle in this species also accumulated large amounts of intracellular NA (Avakian & Gillespie, 1968). These experiments left an impression that arterial smooth muscle in general has a well developed mechanism for accumulating NA intracellularly, and that this property is, in general, absent from non-arterial smooth muscle, which is capable of surface binding only. However, one or two observations on the spleen of the rat and the mouse suggested that the arteries in this species did not accumulate NA (D. N. H. Hamilton, unpublished). We have, therefore, investigated the ability of arterial and non-arterial smooth muscle in five organs, the vas deferens, the heart (coronary arteries), the bladder, the colon and the spleen, in four species, the mouse, rabbit, rat and guinea-pig, to accumulate NA. In addition, some results from the cat spleen and the central artery of the rabbit ear were available from earlier experiments.

METHODS

Animals, with the exception of the cats, which were anaesthetized with chloralose (60 mg/kg), were stunned and bled and the appropriate pieces of tissue quickly removed and cut into slices with a thickness of about 1 mm. Hollow organs (bladder, colon, but not the vas) were cut open and pinned out flat on dental wax to ensure free access of the solution to the tissue. In the heart it was arranged that the slice cut through a coronary artery near its origin from the aorta. Two slices were taken

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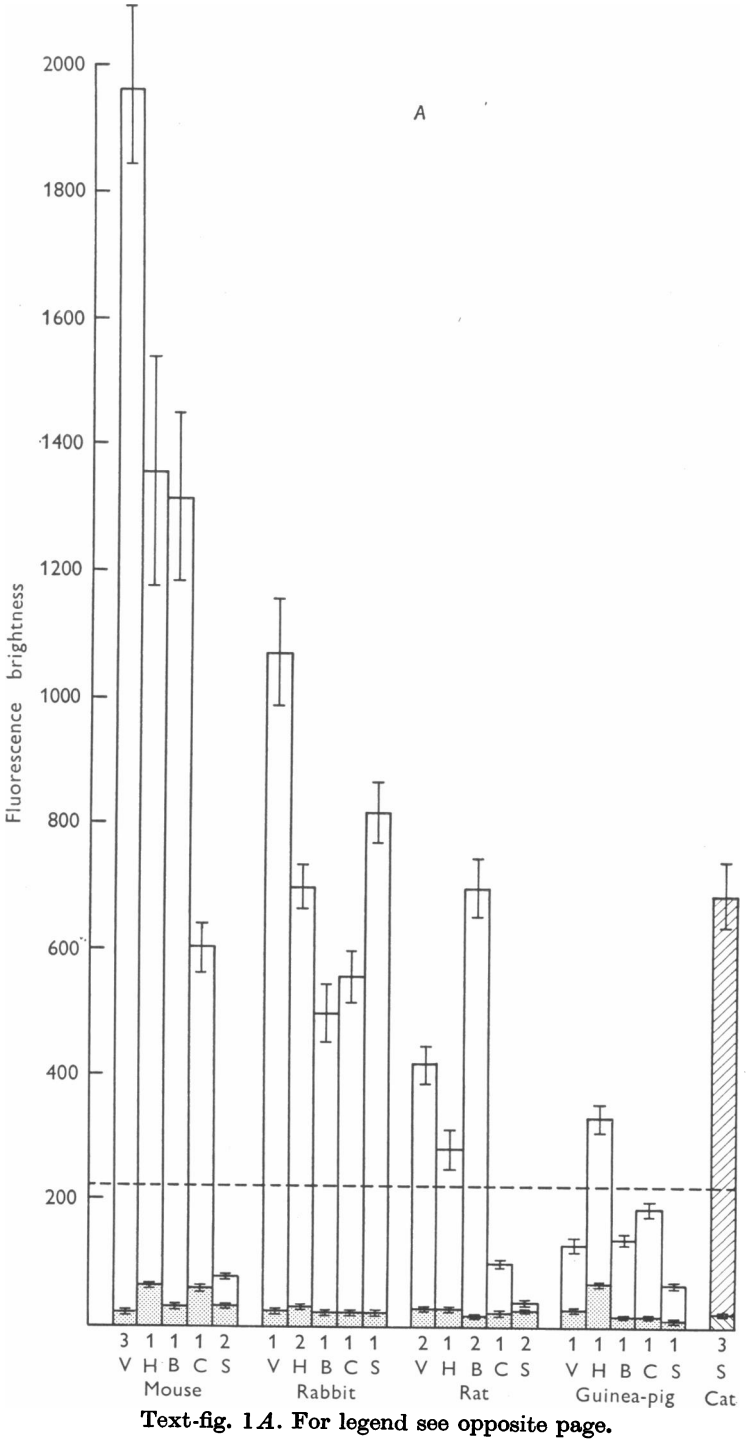
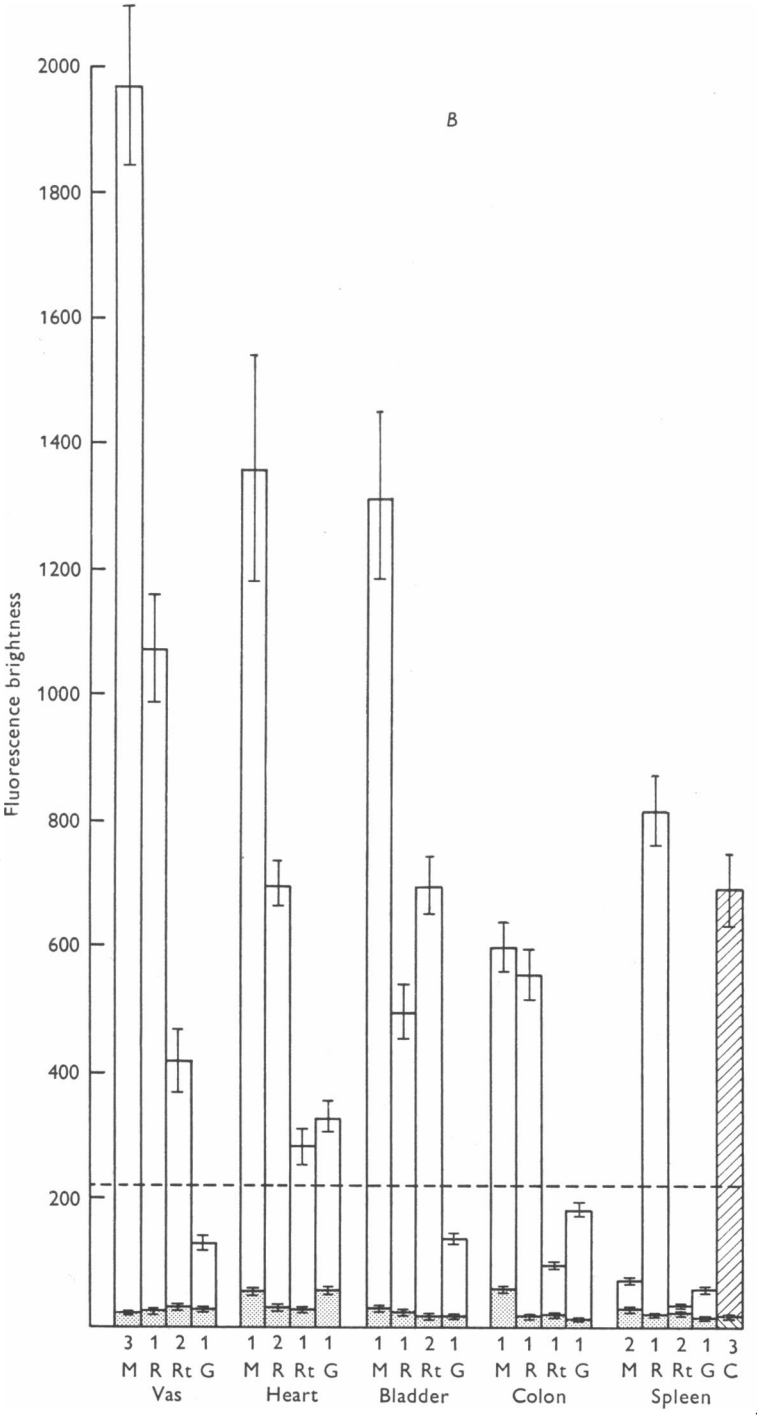
Legend for Fig. 1 *A* and Fig. 1 *B*

Text-fig. 1. The fluorescence brightness of arterial smooth muscle in various tissues and species after exposure for 30 min to NA in a concentration of 3×10^{-4} g/ml. and 30 min wash in NA-free solution. In *A* the results are grouped according to species, and in *B* according to tissue. In this and subsequent Figures the stippled columns are the control fluorescence before exposure to NA and the horizontal dashed line the residual fluorescence of collagen after washing. The numbers under each column are the numbers of animals, the vertical bars represent ± S.E. Values of smooth muscle fluorescence equal to or less than this probably indicate the absence of any special ability to retain NA. The hatched column is the fluorescence in cat spleen from other experimental work in which the concentration of NA was 5×10^{-4} g/ml. V, vas; H, heart; B, bladder; C, colon; S, spleen; M, mouse; R, rabbit; Rt, rat; G, guinea-pig and C, cat.



Text-fig. 1*A*. For legend see opposite page.



Text-fig. 1*B*. For legend see page 592.

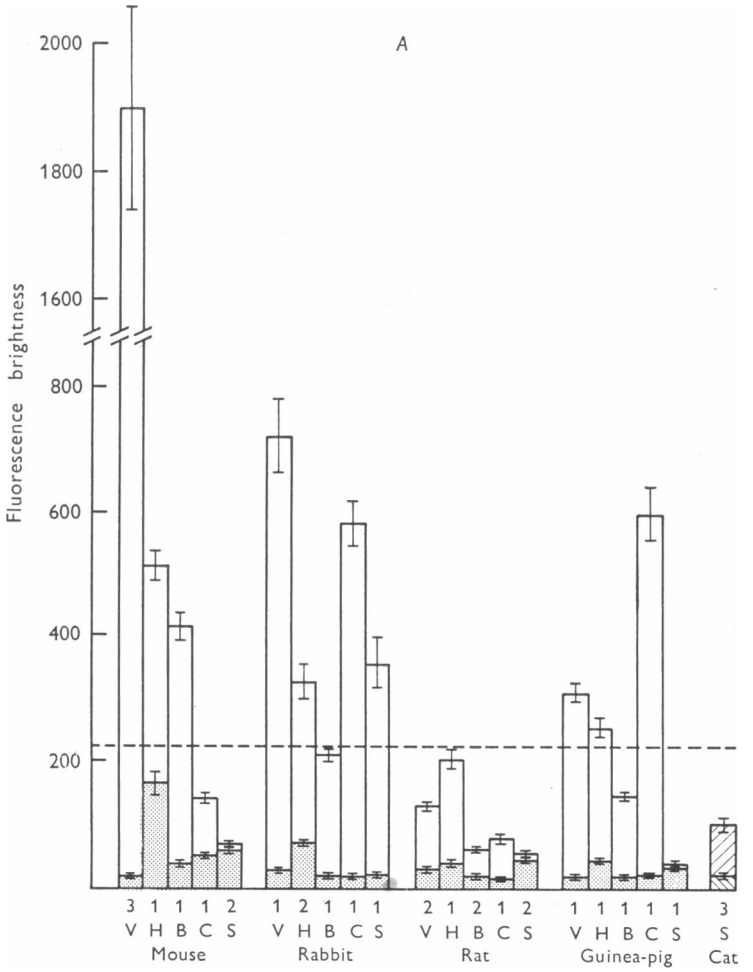
from each tissue and one placed in 25 ml. McEwen's solution as control, and the other in 25 ml. McEwen's solution containing NA in a concentration of 3×10^{-4} g/ml. of the base. Both solutions were kept at 37° C and were stirred and oxygenated with 95 % O_2 + 5 % CO_2. Ascorbic acid (5×10^{-5} g/ml.) was added to each solution to reduce the oxidation of the NA. The tissues were left in these solutions for 30 min to allow penetration of NA throughout the slice. They were then removed and washed for 30 min in three changes of cold McEwen's solution at 0·5° C. At this temperature collagen and other surface-binding tissues quickly lose their NA, but smooth muscle, which accumulates NA in its cytoplasm, does so only slowly (Gillespie *et al.* 1970). At the end of this time, the control and the NA-soaked tissue were removed, frozen in liquid N_2-cooled isopentane, freeze-dried and exposed to formaldehyde vapour at 80° C for 1 hr in a modified form (Gillespie & Kirpekar, 1966) of the fluorescence technique introduced by Hillarp & Falck. The tissue slices, after mounting in paraffin wax and sectioning at 6 μ, were examined with the × 100 oil immersion objective of the Leitz fluorescence microscope with a BG12 3 mm filter in the exciting light pathway (an HbO 200 mercury vapour or an XO 150 Xenon lamp), a Leitz 53 secondary filter and a high-power dark-field condenser. Fluorescence brightness was measured with the Leitz MPV microphotometer over an area of 0·7 $\mu \times 0{\cdot}7$ μ, an area sufficiently small to allow individual nerve varicosities to be measured. Ten readings were taken for each tissue. In comparing the fluorescence intensity of different tissues, some difficulty was found through fluctuations in the lamp intensity. This was detected and compensated for by measuring the intensity of a quinine standard slide at the beginning and end of each experiment. The quinine standard was a 10^{-3} g/ml. solution of quinine sulphate in 0·01 % H_2SO_4 sealed in a slide with a fine photo-etched silver grid (EMI, 7 μ grid). An average of 10 grids was measured.

RESULTS

Arterial smooth muscle. The ability of arterial smooth muscle in different animals and different organs to accumulate and retain NA is shown in Text-fig. 1. The dashed line represents the mean residual fluorescence retained in collagen after 30 min washing. Any values for smooth muscle lower than this probably indicate the absence of any specific ability to accumulate and retain NA. It is clear that the ability to retain NA is a graded phenomenon, and from Text-fig. 1*A* that there is a great difference between species, and from Text-fig. 1*B* an almost equally great difference between tissues. In general, the arteries in the mouse exceed those in any other species in their ability to retain NA, and the rabbit, rat and guinea-pig possess this property to a progressively diminishing degree. Text-fig. 1*B* shows that among tissues, the arteries of the vas deferens show the greatest fluorescence, followed by those of the heart and bladder and then by the colon and spleen. Within these generalities, there are great individual variations. For example, the arteries of the mouse spleen are an outstanding contradiction in having practically no ability to retain NA. The spleen is also unusual (Text-fig. 1*B*) in that in different species the ability to retain NA is an all-or-none phenomenon, the mouse, rat and guinea-pig cannot retain NA whereas in the cat and rabbit this ability is well developed.

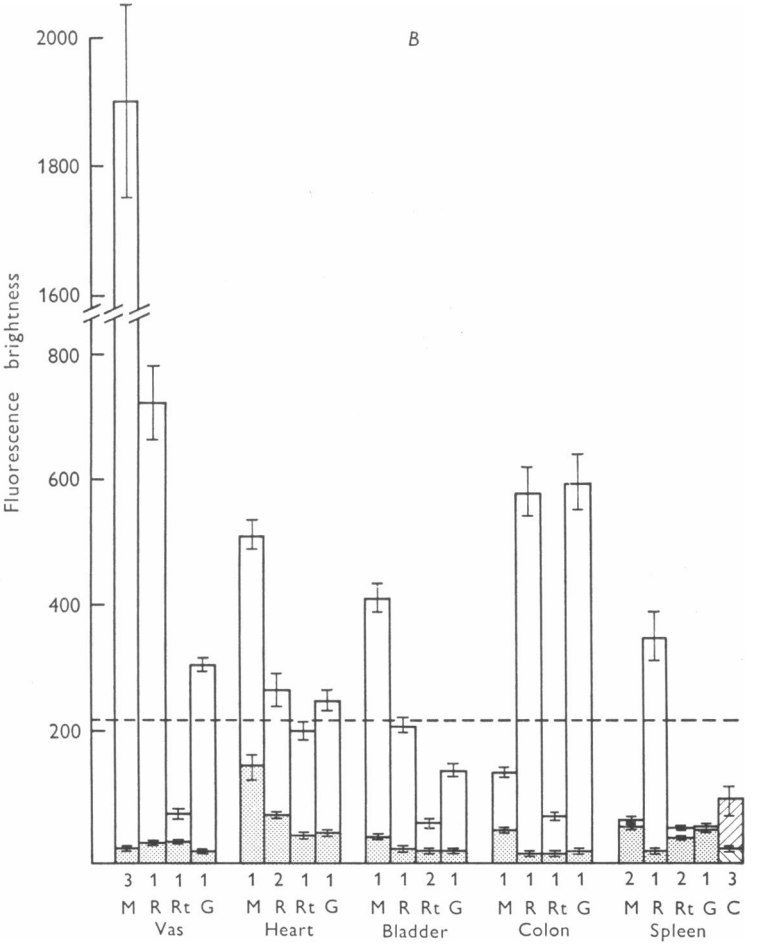
These variations in retention of NA probably represent true differences between the smooth muscle cells and are not the result of differences in the local environment such as pH or anoxia, since the thin tissue slices used should allow near diffusional equilibrium between the tissue and the external medium.

Non-arterial smooth muscle and cardiac muscle. Text-fig. 2 shows the ability of different organs in different species to accumulate and retain



Text-fig. 2. The fluorescence brightness of non-arterial smooth muscle from various tissues and species after exposure for 30 min to NA in a concentration of 3×10^{-4} g/ml. The stippled columns represent the control values and the dotted line the residual fluorescence on collagen as in Fig. 1. In *A* the results have been grouped according to species and in *B* according to organ. Abbreviations as in Text-fig. 1.

NA. Clearly our original concept that non-arterial smooth muscle lacked this ability was wrong; the smooth muscle of several tissues, particularly the vasa deferentia, can retain NA, but comparing Text-fig. 2 with Text-fig. 1 it is true that in general non-arterial smooth muscle has a less well developed ability to accumulate NA than arterial smooth muscle. Again there is a marked species and organ variation. In general, where arterial

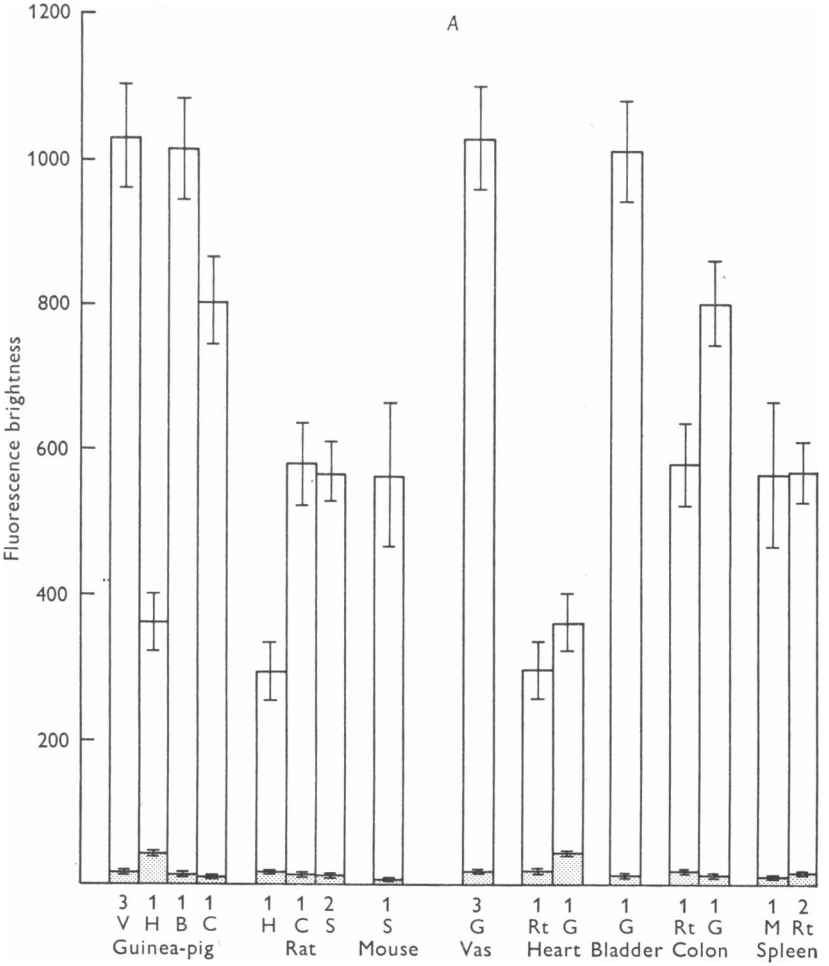


Text-fig. 2*B*. For legend see opposite page.

smooth muscle accumulates NA, the smooth muscle of the organ containing those arteries will also accumulate the amine. Once again, there are outstanding exceptions, particularly the bladder of the rat and the spleen of the cat, where the arterial smooth muscle's ability to retain NA is well developed but the general musculature of the tissue has no such

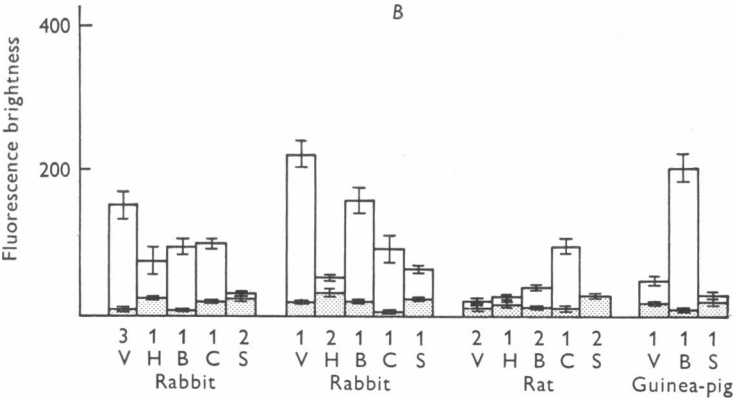
ability. Text-fig. 2 also shows that in no species, with the possible exception of the guinea-pig and the mouse, does cardiac muscle have the ability to accumulate NA and retain it when washed for 30 min with cold saline. If the tissue is examined without washing, then uptake into cardiac muscle cells is demonstrable.

Plate 1 shows microphotographs of smooth muscles which accumulate and retain NA compared with those which do not.



Text-fig. 3. *A*. The fluorescence brightness of collagen exposed for 30 min to NA in a concentration of 3×10^{-4} g/ml. *B*. The residual fluorescence after 30 min washing with NA-free solution. The stippled columns are the control values before exposure to NA. The results have been grouped according to both species and tissue; the symbols are the same as in Text-fig. 1. The highest value for residual fluorescence after washing (the rabbit vas deferens) is used in Text-figs. 1 and 2.

Collagen and adrenergic nerves. The ability of collagen to bind NA and the ease with which this binding is reversed by washing has already been reported (Avakian & Gillespie, 1968; Gillespie *et al.* 1970). The results shown in Text-fig. 3 confirm both observations. The fluorescence of collagen in cardiac muscle has been included in Text-fig. 3*A*, but in this tissue the collagen occurs in fine unsupported strands around the blood vessels and some of the collagen measured may be less than the thickness of the sections. The accuracy of the measurements on this tissue, as a consequence, is doubtful. The mean fluorescence brightness of collagen in

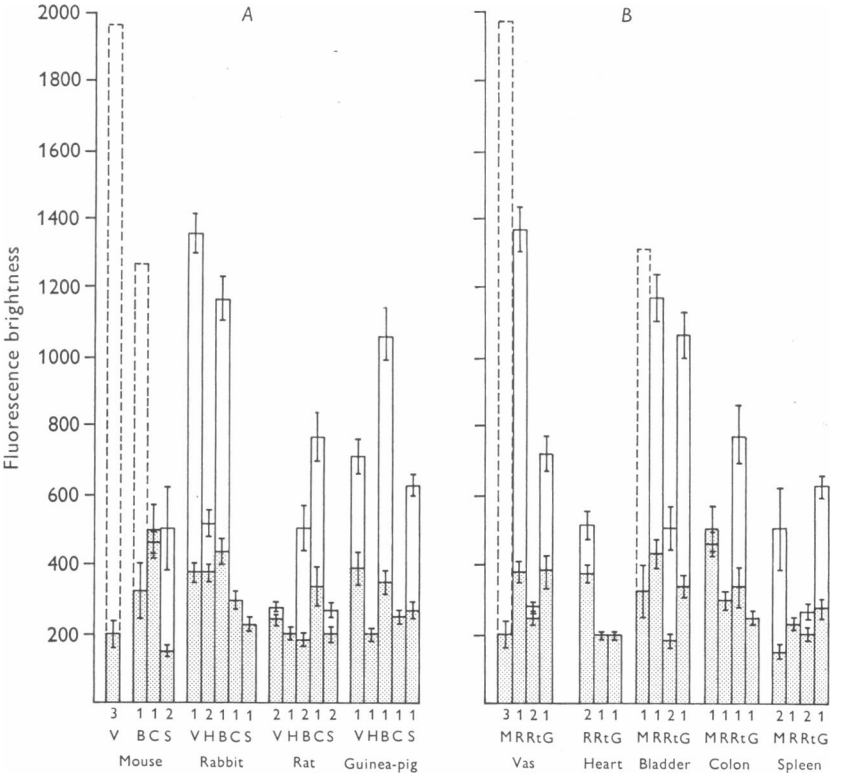


Text-fig. 3*B*. For legend see opposite page.

other tissues varies, but the variation is less than that of any other tissue element. It is not possible to say whether this variation represents a basic difference in the collagen filaments or differences in the size, grouping and cross-linking of these filaments into the collagen fibres which are measured with the light microscope.

The increase in fluorescence brightness of the adrenergic varicosities in the various tissues after exposure to NA are shown in Text-fig. 4. The brightness of the varicosities in the control tissue is high, reflecting the high endogenous NA content. The mean brightness varies from one tissue and species to another, but statistical analysis by Student's *t* test shows no significant difference at the 0·05 level. The variability within each mean, however, is considerable, and indicates a variation in brightness from one nerve varicosity to another, this in spite of the fact that our technique of sampling nerve varicosities is such as to minimize variability. For all other tissues, random samples are taken, but this is difficult to maintain with adrenergic varicosities, since in each high-power field several are visible. There is an unavoidable subjective tendency to select the largest or brightest of these for measurement. To standardize conditions from one tissue to another the brightest varicosity in each field was selected. Such a

selection would be expected to reduce any existing variation. After infusing NA, all nerve fibres increased in fluorescence but the degree of increase varied so that there were now statistically significant differences between the means for different tissues. In certain instances, for example the mouse vas deferens, the adrenergic fibres become indistinguishable



Text-fig. 4. The fluorescence brightness of adrenergic nerve varicosities before (stippled columns) and after exposure to NA in a concentration of 3×10^{-4} g/ml. for 30 min. In *A* the results are arranged according to species, and in *B* according to the tissue; the symbols are the same as in Text-fig. 1. There is a variation in fluorescence brightness in different tissues which is increased after exposure to NA. The values for the mouse vas and bladder are estimates, since the nerve varicosities are masked by the smooth muscle uptake (see text).

from the smooth muscle cells around them and could not, therefore, be measured. We have indicated these experiments in Text-fig. 4 by using the measured fluorescence of the smooth muscle, on the assumption that the adrenergic varicosity fluorescence should be close to this. After exposure to NA, not only do the adrenergic fibres become brighter but they also

become less varicose, because the axons between varicosities, and even some distance from the terminal region, also take up NA. It is possible that part of the measured variability in nerve fibre fluorescence after NA was due to measuring both varicose, intervaricose and even pre-terminal regions of the axon.

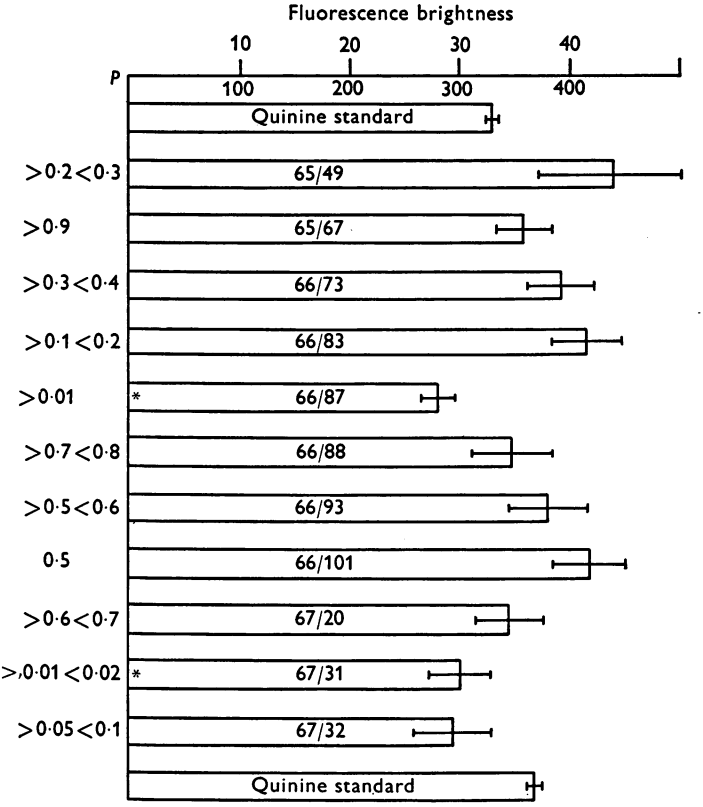
DISCUSSION

In trying to decide whether the intracellular uptake of NA is of physiological interest, two questions, in order of importance, are: first, does the mechanism ever operate under physiological conditions, and second, if it does, what, if any, are the consequences? Based on indirect evidence, we have argued that the conditions for uptake into smooth muscle could exist in the vicinity of adrenergic varicosities (Avakian & Gillespie, 1968; Gillespie *et al.* 1970). The present experiments were undertaken in the hope that the pattern of distribution of the uptake mechanism would suggest its function. In particular, if uptake had been a property peculiar to arterial smooth muscle, it could be argued that the mechanism is related to the unusual anatomical arrangement of arterial innervation with varicose nerve fibres confined to the outer surface of the muscle mass, and therefore some distance from most cells. In these circumstances, muscle uptake which could serve as a secondary store of transmitter would be useful. The capricious distribution of smooth muscle uptake in arteries, both between species and between arteries in different organs of the same species, makes such a hypothesis difficult to sustain.

The removal of NA from the extracellular space is believed to be accomplished mainly by uptake into adrenergic nerves. In tissues with a sparse innervation, particularly where the innervation is confined to one region of the muscle, the development of a smooth muscle uptake mechanism could be a useful evolution. Such a suggestion, that extraneuronal uptake serves as an inactivation mechanism, has been made (Costa, Boullin, Hammer, Vogel & Brodie, 1966).The distribution of the uptake mechanisms, however, provides little support. Certainly arteries, colon and bladder have a rather sparse and localized innervation, but the mouse vas deferens is one of the most densely and diffusely innervated tissues, yet its smooth muscle is one of the most effective in accumulating NA. It may be that the action of intracellular catecholamines is not to be looked for in those familiar effects which follow an action on surface membrane receptors, and that less familiar metabolic or trophic effects should be considered.

Whatever the physiological function of smooth muscle uptake, it is likely that it will play a role in certain experimental situations. For example, the two-stage disappearance of NA from mouse blood has been attributed to uptake into nerves (Axelrod, Weil-Malherbe & Tomchick,

1959), but in view of the highly developed smooth muscle uptake in the mouse and the advantageous position of arterial smooth muscle, it is possible that this tissue also contributes to uptake and release. A situation in which differential retention of NA by smooth and cardiac muscle may explain the findings is the cardiovascular shock which develops after



Text-fig. 5. Measurements of fluorescence brightness of adrenergic nerve varicosities in eleven cat spleens accumulated over a 3-year period but re-measured together. The intensity of the quinine standard used to correct for fluctuations in lamp intensity is also given. The variations in nerve fluorescence are not significantly different from one another except for those experiments indicated by an asterisk.

infusion of large quantities of NA (Gillespie & Muir, 1967). When the infusion is stopped, there is sudden collapse and death. The loss of the adrenergic drive to the heart because of the cardiac muscle's inability to retain NA while the peripheral resistance remains high might explain the sudden cardiovascular collapse.

The fluorescence brightness of adrenergic nerve varicosities varied between tissues and species, and surprisingly this variability was increased, not diminished, after exposure to NA. If the initial variation was due to varying degrees of depletion of the NA stores, refilling these should cause increasing uniformity of brightness. The possibility that the variability is an artefact due to technical factors such as variation in the microscope lamp intensity, or in the completeness of the histochemical reaction, is unlikely, since re-measurement of the fluorescence intensity of adrenergic varicosities in eleven cat spleens originally examined at various times over a 3-year period showed a much smaller variation (Text-fig. 5) and the means by Student's *t* test were not significantly different from one another with the exception of two experiments. It may be that adrenergic nerves in different locations have a different capacity to store NA but other possibilities, such as variation in the size (depth for fluorescence purposes) of varicosity, or in the rate of enzyme destruction of transmitter inside the varicosity or measurements made on intervaricose axons, would first have to be excluded. An incidental finding of these measurements is the remarkable stability of the NA fluorophor when the tissues are stored in complete darkness. The fluorescence brightness of the 3-year-old blocks was as great as that of the recent tissue.

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EXPLANATION OF PLATE

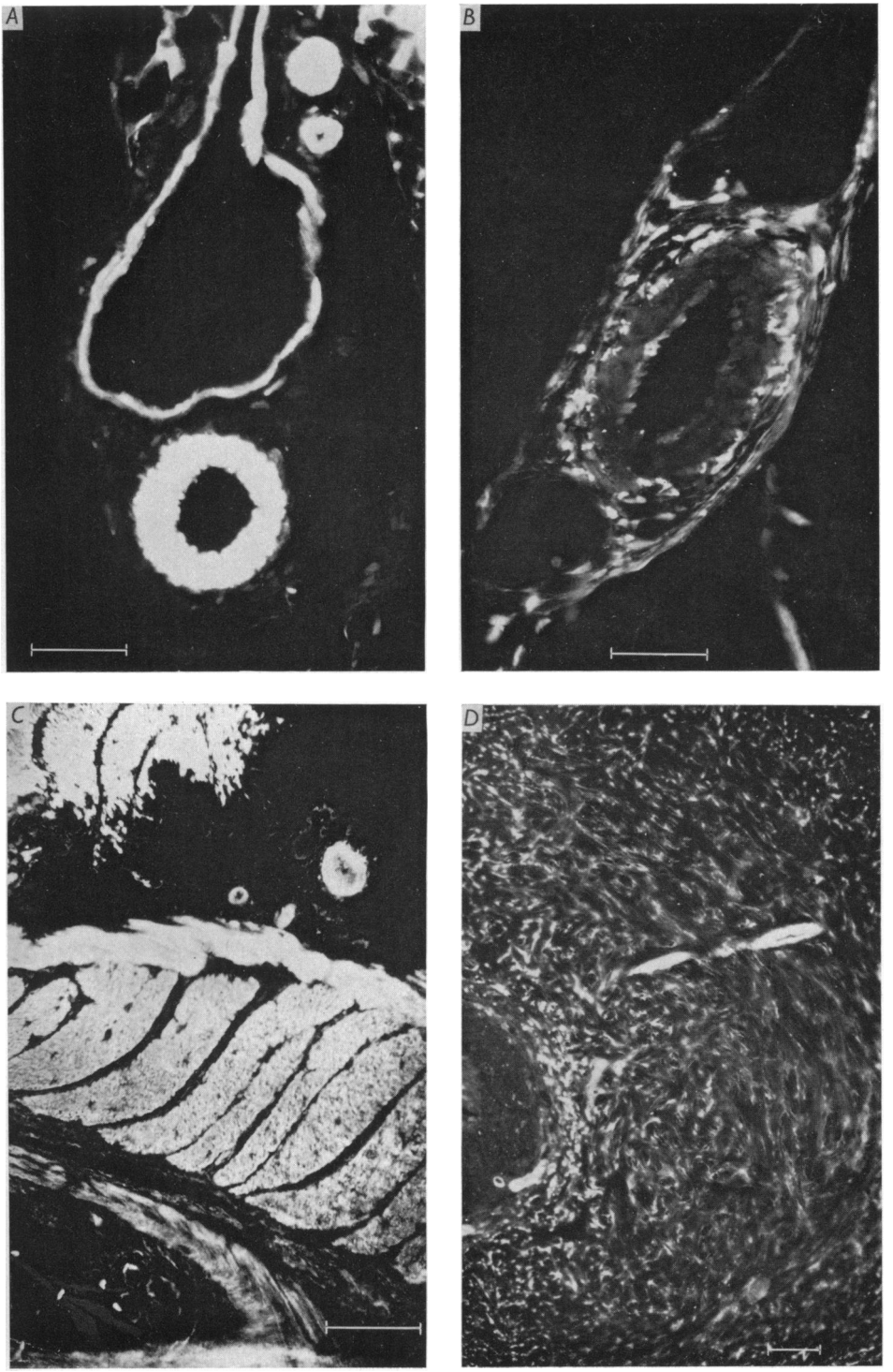
Examples of the range in fluorescence brightness of arterial and non-arterial smooth muscle after exposure to NA in a concentration of 3×10^{-4} g/ml. for 30 min, followed by 30 min wash.

A. A small artery and vein on the surface of the mouse vas deferens showing intense uptake. Calibration, 20 μ.

B. A small artery on the surface of the guinea-pig vas deferens showing almost no uptake. Calibration, 20 μ.

C. T.S. of the rabbit colon showing NA uptake in the external longitudinal and internal circular muscle. Calibration, 50 μ.

D. T.S. of the rat vas deferens with no uptake into the smooth muscle cells between the nerve varicosities. Calibration, 20 μ.



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