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Noradrenaline uptake by non-innervated smooth muscle

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Summary

1. Uptake of noradrenaline (NA) into the non-innervated smooth muscle cells of the human umbilical artery and the chick amnion has been studied with the fluorescence histochemical technique for localizing monoamines. Comparison has been made with uptake into sympathetically innervated smooth muscle of the rabbit ear artery.

2. Accumulation of NA within non-innervated smooth muscle cells is observed histochemically after exposure to much lower concentrations of NA (10^{-7} g/ml) than in sympathetically innervated smooth muscle cells, where accumulation occurs with NA (10^{-5} g/ml).

3. In contrast to innervated smooth muscle, uptake of NA (10^{-4} g/ml) by non-innervated smooth muscle is characterized by lack of inhibition by phenoxybenzamine, normetanephrine and cold, although some inhibition is apparent at lower NA concentrations. Retention of NA during prolonged washing in NA-free Krebs demonstrates that it is strongly bound within the non-innervated smooth muscle cells, particularly in the nucleus.

4. After inhibition of catechol-*O*-methyl transferase, the accumulation of NA in innervated smooth muscle closely resembles that in non-innervated smooth muscle.

Introduction

Uptake of noradrenaline (NA) by smooth muscle has been studied histochemically in a wide range of tissues (Gillespie & Muir, 1970). However, the precise physiological role of this mechanism, and the relationships between neuronal uptake, enzyme degradation and muscle uptake have not yet been clarified.

The extraneuronal accumulation of NA has many of the properties of $Uptake_2$ (Gillespie, Hamilton & Hosie, 1970) which occurs when the rat heart is perfused with high concentrations of NA (Iversen, 1965) and results in the accumulation of NA within cardiac muscle cells (Ehinger & Sporrong, 1968; Farnebo & Malmfors, 1969). Wide variation is observed in the characteristics of NA uptake by both cardiac and smooth muscle in different tissues and species (Gillespie & Muir, 1970; Jarrott, 1970).

The early observations that uptake was greatest in remotely innervated arterial smooth muscle (Gillespie & Hamilton, 1967) led to the suggestion that smooth muscle uptake may be a mechanism which facilitates or prolongs the effector response of cells not in direct contact with nerves (Avakian & Gillespie, 1968; Gillespie, 1968). This now appears unlikely, however, as the wide variation in

the ability of smooth muscle to accumulate NA shows no correlation with innervation density (Gillespie & Muir, 1970).

It has been further suggested that $Uptake_2$ may be an extraneuronal mechanism for NA inactivation by intracellular catechol-*O*-methyl transferase (COMT) (Eisenfeld, Axelrod & Krakoff, 1967a ; Eisenfeld, Landsberg & Axelrod, 1967b ; Iversen, 1968 ; Lightman & Iversen, 1969). This mechanism probably operates at all concentrations of exogenous NA, but accumulation is only evident when uptake is greater than metabolism and may not be demonstrable unless metabolism is inhibited (Lightman & Iversen, 1969). Variations in the activity of degradative enzymes have been suggested by Lightman & Iversen (1969) as an explanation for the species and tissue differences in the uptake of NA by smooth muscle observed by Gillespie & Muir (1970). This may not be valid, as Jarrott (1970) was unable to show a correlation between the extraneuronal metabolism of NA by perfused hearts and the activity of degradative enzymes in heart homogenates of a number of vertebrate species.

In this investigation, the uptake of NA into non-innervated smooth muscle cells of both the chick amnion and the human umbilical artery is studied histochemically with the Falck-Hillarp technique for monoamines.

Methods

Chick amnion

Amnions of 8–10 day old chick embryos were dissected out and incubated in Krebs Ringer solution (bubbled with 95% O_2, 5% CO_2) at 37° C for 1 hour. All drugs were added to the incubation solution. After incubation the tissue was washed in Krebs, and then prepared for the histochemical localization of NA with the Falck-Hillarp technique. The amnions were stretched over glass, quenched in propane (cooled in liquid N_2), then freeze dried at −35° C and 10^{-3} Torr (1 Torr≙1.333 mbar) for 36 hours. After exposure to formaldehyde vapour for 1 h at 80° C and 70% relative humidity, they were infiltrated with paraffin oil before examination for NA fluorescence. Transverse sections (6 μm) were prepared by embedding in Paraplast (m.p. 56°–57° C). Fluorescence brightness was measured using exposure times of the Leitz Orthomat automatic camera. The areas selected for these comparative fluorescence intensity measurements contained sheets of smooth muscle cells which covered the whole field. The reciprocal of the mean exposure time for five areas in each experimental situation was used for plotting the graphs in Fig. 5.

Human umbilical artery and rabbit ear artery

The arteries were dissected free of surrounding tissue, cut into small lengths (approx. 5 mm) and incubated in Krebs Ringer as above. After freeze-drying and formaldehyde treatment, the pieces were embedded in Paraplast and sectioned (7 μm) for examination of fluorescence.

Pharmacology

Contractions of the chick amnion were recorded using a Statham tension transducer while mounted in an organ bath in Krebs Ringer bubbled with 95% O_2, 5% CO_2. Acetylcholine (2×10^{-6} g/ml) was added directly to the bath. Stimuli

were delivered from a Grass stimulator as single pulses (strength 20 V, duration 2 ms) using Burn-Rand platinum electrodes.

Drugs

Solutions of the following drugs were added directly to the incubation medium: noradrenaline bitartrate (Levophed-Winthrop); phenoxybenzamine HCl (Dibenzyline; Smith, Kline & French); propranolol HCl (Inderal, I.C.I.), normetanephrine (Calbiochem); metaraminol (Aramine 1%; Merck, Sharp & Dohme); nialamide (Niamid; Pfizer); catechol (B.D.H.).

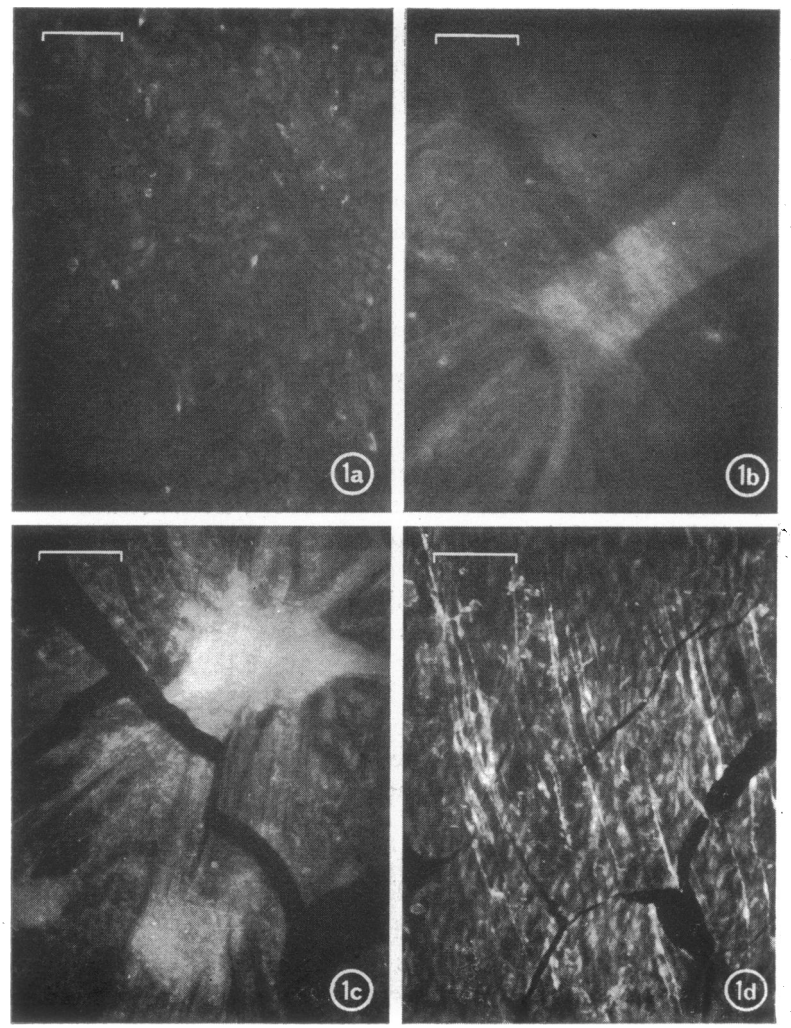

FIG. 1. Development of fluorescence within smooth muscle cells of the chick amnion after incubation for 1 h at 37° C. (a), Control. Note slightly autofluorescent background. (b), NA (10^{-7} g/ml). A faint specific fluorescence is visible within the rosettes of smooth muscle. (c), NA (10^{-6} g/ml). The rosettes show increased fluorescence, with the background fluorescence slightly higher. Note the cracks in the thin membrane, resulting from freeze-drying. (d), NA (10^{-5} g/ml). Individual smooth muscle cells easily recognizable. Uptake by ectodermal cells is becoming evident, increasing the background fluorescence. Freeze-dried whole-mounts treated with hot formaldehyde vapour for 1 hour. Calibration 50 μm.

Results

Chick amnion

The muscle cells of the 8–10 day old amnion are irregularly distributed over a supporting sheet of ectodermal cells and connective tissue. They can be seen as scattered isolated cells (Figs. 2c, 3c), in single layered sheets of cells (Fig. 4a, c) or interweaving in cross-shaped formations (rosettes) (Fig. 1b, c).

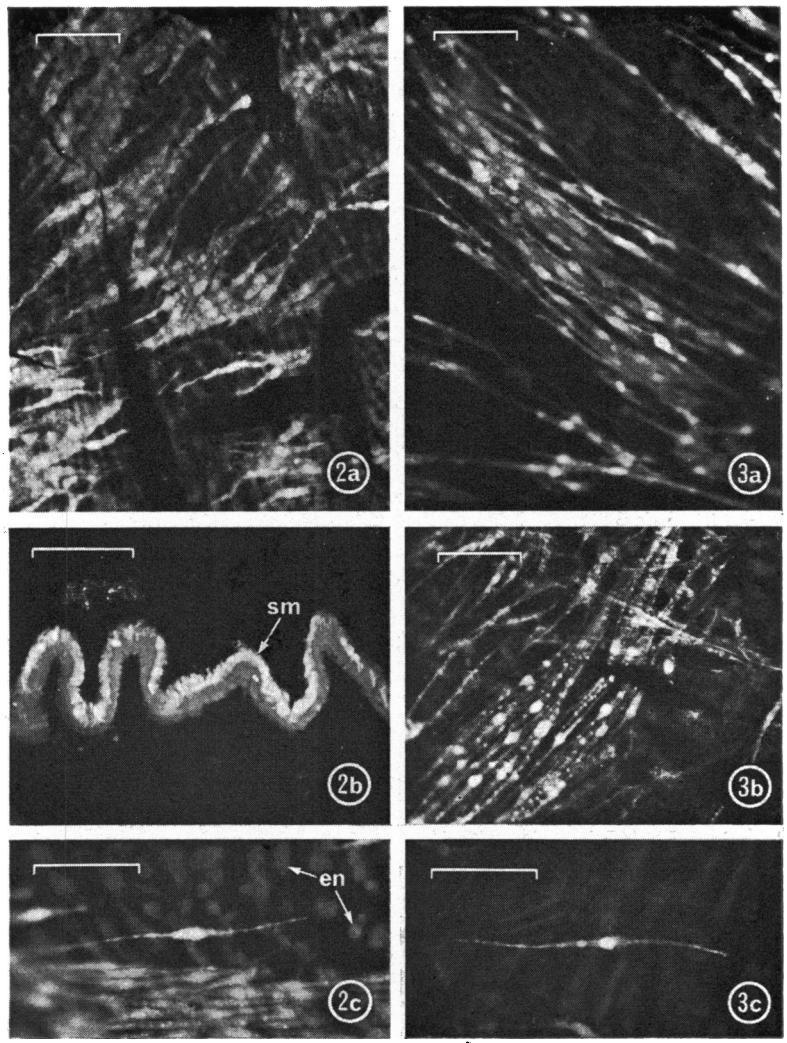

FIG. 2. Whole mounts of chick amnion. Fluorescence of smooth muscle cells after incubation in 10^{-4} g/ml NA for 1 h at 37° C. (a), Whole mount. (b), transverse section. Note the brightly fluorescent smooth muscle layer (sm). (c), Isolated cell, illustrating the typical distribution of NA. Note the fluorescence of ectodermal cell nuclei (en). Preparations treated with hot formaldehyde vapour for 1 h after freeze-drying. Calibration 50 μm.

FIG. 3. Whole mounts of chick amnion. Retention of NA while rinsing in NA-free Krebs, after incubation in 10^{-4} g/ml NA. (a), Forty minute rinse. The fluorescence intensity within the smooth muscle cells is slightly reduced, with the nuclear region becoming more prominent (cf. 10 min wash, Fig. 2a). (b), Sixty minute rinse. Residual fluorescence of NA is seen within the nucleus and localized areas of the cytoplasm. (c), Isolated cell exhibiting typical localized retention after 60 minutes. Whole mounts treated in hot formaldehyde vapour for 1 h after freeze-drying. Calibration 50 μm.

Uptake of NA

In control tissue, autofluorescence was low, and the muscle cells were invisible (Fig. 1a). After incubation in 10^{-7} g/ml NA for 1 h, the muscle cells were just visible due to a faint general fluorescence of the entire cell (Fig. 1b). With increasing concentration of NA, the fluorescence of the cells increased (Figs. 1c, d, 2, 5a). The fluorescence of the ectodermal cells and connective tissue was also high at 10^{-4} g/ml. At all concentrations from 10^{-5} g/ml, the nuclei of the ectodermal cells were fluorescent (Fig. 2c), although fluorescence was always higher in the associated muscle cells and varied more markedly with concentration.

Fluorescence due to NA appeared to be distributed throughout the cytoplasm and nucleus of the smooth muscle cells in the whole mount. This was confirmed

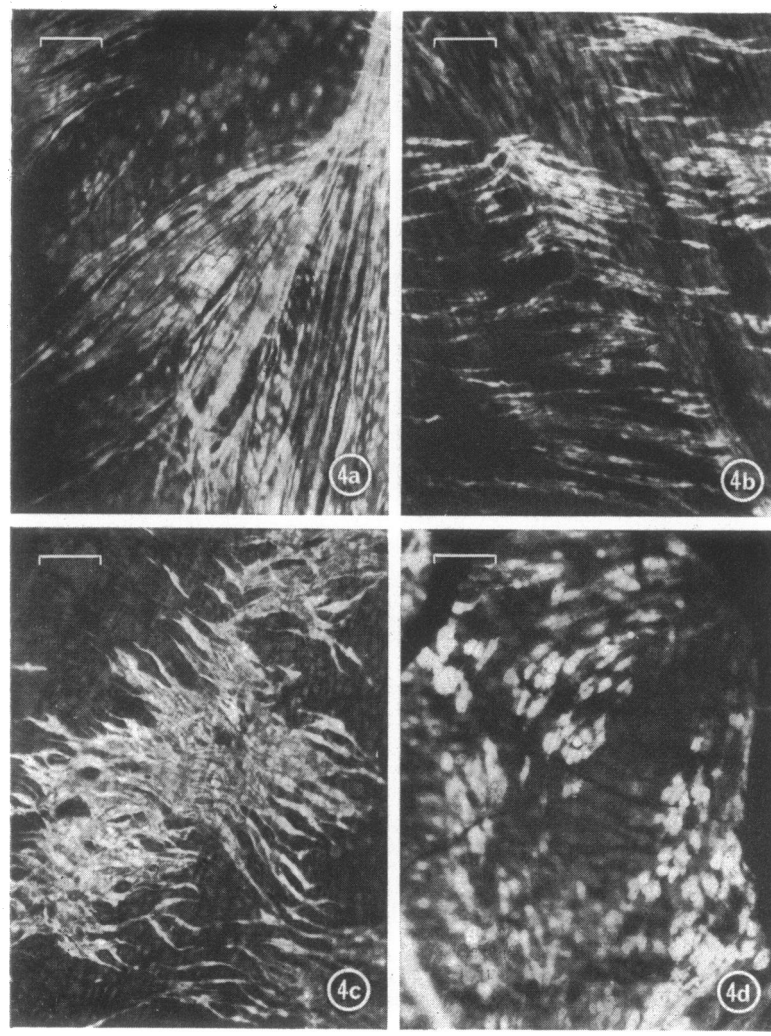

FIG. 4. Whole mounts of chick amnion incubated in NA (10^{-4} g/ml). (a), Normal uptake. (b), Uptake in the presence of NMN (10^{-4} g/ml). (c), Uptake in the presence of PBA (5×10^{-5} g/ml) and after preincubation in PBA. (d), Uptake at 2° C. Formaldehyde vapour treatment for 1 h at 80° C after freeze-drying. Calibration 50 μm.

by observation of transverse sections (Fig. 2b). Frequently the fluorescence of the nuclear region appeared brighter than the peripheral cytoplasm (Fig. 2a), particularly in isolated smooth muscle cells (Fig. 2c).

Retention of NA

After incubation of NA (10^{-4} g/ml) for 1 h, the tissue was washed in several changes of Krebs Ringer at 37° C for periods from 1 min to 1 hour. Fluorescence declined slowly with washing (Fig. 5b), with a residual fluorescence remaining in the nucleus and localized areas of the cytoplasm after washing for 1 h (Fig. 3a,b,c).

After this treatment, the amnion was still capable of response to drugs and electrical stimulation, indicating that this result is not due to the death of the smooth muscle cells.

Effect of drugs on uptake

Enzyme inhibitors. Preincubation for 30 min in the monoamine oxidase (MAO) inhibitor, nialamide (5×10^{-5} g/ml), and the COMT inhibitor, catechol (2×10^{-6} g/ml), before addition of NA (10^{-6} and 10^{-7} g/ml) did not affect the uptake of NA into smooth muscle cells.

Receptor blocking agents. The α-adrenoceptor blocking agent, phenoxybenzamine (PBA—5×10^{-5} g/ml), when added to the incubation solution 30 mins before addition of NA, reduced fluorescence to control values with NA concentrations of less than 10^{-4} g/ml (Fig. 5a). At higher concentrations, however, there was no significant reduction in fluorescence. On the contrary, fluorescence was often slightly increased after PBA treatment (Figs. 4c, 5). The β-adrenoceptor blocking agent propranolol (5×10^{-5} g/ml), did not affect the uptake of NA.

NA uptake inhibitors. Neither cocaine (2×10^{-4}M) nor metaraminol (2×10^{-4}M) reduced NA uptake. Both drugs, like PBA, even slightly increased the fluorescence

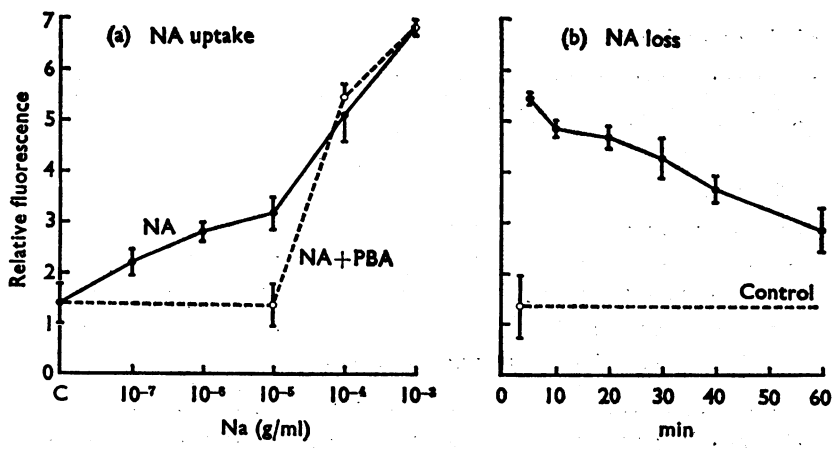

FIG. 5. Fluorescence of chick amnion smooth muscle cells following incubation at 37° C. (a), Increasing concentrations of NA (——). The threshold of uptake is increased to 10^{-5} g/ml NA in the presence of 5×10^{-5} g/ml phenoxybenzamine, PBA (– – –). (b), Effect of prolonged washing in NA-free Krebs after uptake of NA (10^{-4} g/ml for 1 h). Each point is the mean of five observations and the vertical lines=1 S.E.

of the muscle cells. Normetanephrine (NMN—10^{-4} g/ml) added to the incubation solution together with NA also reduced uptake at less than 10^{-4} g/ml NA, with only slight reduction in fluorescence at higher concentrations of NA (Fig. 4b), compared with normal NA uptake (Fig. 4a).

Effect of cold

Incubation at 2° C reduced fluorescence at low NA concentration (less than 10^{-4} g/ml). There was only slight reduction in fluorescence at NA 10^{-4} g/ml, although cells often appeared shorter and thicker than normal (Fig. 4d), making it difficult to determine differences in uptake.

Human umbilical artery

There has been a long controversy over the presence of nerves in the umbilical cord (ten Berge, 1962; Jacobson & Chapler, 1967; Ehinger, Gennser, Owman, Persson & Sjöberg, 1968). However, there has been no indication of the presence of an adrenergic innervation using the very sensitive Falck fluorescence method (Ehinger *et al.*, 1968; Walker & McLean, 1971). This gives reasonable assurance that the smooth muscle of the umbilical arteries is at least not innervated adrenergically.

Uptake of NA

Uptake into smooth muscle cells in the wall of the arteries followed a similar pattern, in general, to the uptake of NA in the chick amnion muscle. In the control tissue, fluorescence levels were high, making small increases in fluorescence at low NA concentrations difficult to evaluate. However, since an increase in fluorescence is quite evident at NA 10^{-6} g/ml, it is probable that the threshold for uptake by these smooth muscle cells is approximately 10^{-7} g/ml, as in the amnion smooth muscle.

Effect of drugs and cold

Uptake of NA (10^{-4} g/ml) was not significantly reduced by NMN (10^{-4} g/ml), PBA (5×10^{-5} g/ml) or cold (2° C).

Enzyme inhibitors

Preincubation in nialamide (5×10^{-5} g/ml) and catechol (2×10^{-6} g/ml) for 30 mins before addition of 10^{-6} or 10^{-7} g/ml of NA did not affect the uptake of NA into smooth muscle cells.

Rabbit ear artery

The rabbit ear artery showed uptake characteristics consistent with those reported by Avakian & Gillespie (1968) for innervated arterial smooth muscle. The uptake threshold for NA was 10^{-5} g/ml and the uptake was inhibited by PBA (5×10^{-5} g/ml), NMN (10^{-4} g/ml) and cold (2° C). In addition, inhibition of both COMT and MAO lowered the threshold for uptake to 10^{-7} g/ml (compare with Lightman & Iversen, 1969).

Discussion

The characteristics of NA uptake as observed in the chick amnion and human umbilical artery differ in many respects from those described for innervated smooth muscle (Gillespie *et al.*, 1970).

Inhibition by PBA, NMN and cold is used as the criterion for the innervated muscle uptake mechanism (Gillespie *et al.*, 1970; Iversen, 1968), but non-innervated muscle uptake is not affected by these agents except at low NA concentrations. In addition, accumulation of NA is observed histochemically after exposure to 10^{-7} g/ml of NA, which is 100 times lower than the concentration at which accumulation occurs in sympathetically innervated smooth muscle. This accumulated NA is retained for much longer periods in NA-free medium.

The low concentration at which accumulation of NA occurs within these non-innervated smooth muscle cells corresponds to the threshold for $Uptake_2$ after inhibition of both COMT and MAO (Lightman & Iversen, 1969). Increased NA uptake has also been reported in both the rat heart (Eisenfeld *et al.*, 1967b) and the rat femoral artery (Doležel, 1966) following COMT inhibition, but Gillespie (1968) was unable to observe any change in uptake by the cat spleen after COMT and MAO inhibition. In our study, COMT and MAO inhibition resulted in a lowered uptake threshold of accumulation for smooth muscle cells of the rabbit ear artery but did not affect accumulation by the non-innervated smooth muscle cells. This suggests that absence of transmitter-inactivating enzymes in non-innervated muscle cells may explain several of the characteristics of uptake in these tissues, especially at low NA concentration. This hypothesis is at present being tested by enzyme assay of MAO and COMT in the chick amnion and the human umbilical artery. In particular, if levels of COMT are low in non-innervated cells, a lack of locally metabolized NMN may be responsible for a more efficient uptake mechanism at low NA concentrations, since NMN might normally regulate the entry of NA into effector cells by its inhibitory action (Eisenfeld *et al.*, 1967b). Absence of degradative enzymes might also be responsible for the observed nuclear uptake of NA in the chick amnion. This explanation is supported by the observation that nuclear uptake occurs in endothelial cells, pericytes in brain tissue and reserpinized neurones, but only after MAO inhibition (Falck & Owman, 1966). In contrast, nuclear uptake was not observed in innervated smooth muscle cells (Avakian & Gillespie, 1968).

Both PBA and NMN are potent inhibitors of $Uptake_2$ (Iversen, 1965, 1967) and smooth muscle uptake (Gillespie, 1968), but this inhibition is not complete at high concentrations of NA (Avakian & Gillespie, 1968; Hamilton, 1968). This may be due to competitive inhibition as suggested for PBA by Iversen & Langer (1969). The inhibitory effect of PBA or NMN would appear to be greater in cells which were able to metabolize the reduced amounts of NA entering, so that accumulation would not be apparent until higher concentrations of NA flooded the cell. In the non-innervated smooth muscle cells, inhibition of uptake by PBA or NMN is evident at low NA concentrations, but lack of inhibition at higher concentrations may also be due to the absence of intracellular degradative enzymes.

The persistence of fluorescence in the muscle cells of the amnion and the umbilical artery during prolonged washing suggests that NA may be strongly

bound within these cells and is further evidence that intracellular enzymes are not present in active form in these cells. There is little indication of similar binding in other smooth muscle cells (Gillespie *et al.*, 1970), where the rapid loss of fluorescence may also be assisted by intracellular metabolism of NA (Lightman & Iversen, 1969).

The ineffectiveness of cold in preventing uptake suggests that the uptake mechanism does not involve active transport across the membrane. Inhibition by cold led Gillespie (1968) to propose that uptake into smooth muscle involved an energy-demanding pump, possibly involving a specific carrier because of the effect of drugs such as PBA and NMN (Gillespie *et al.*, 1970). Alternatively, Clarke, Jones & Linley (1969) suggested that it may be tissue changes at low temperatures which create a diffusion barrier preventing the NA from reaching the smooth muscle. The uptake mechanism in the non-innervated cells examined in our study does not appear to be influenced by diffusion barriers. While the diffusion barrier is negligible in the chick amnion where most cells are exposed at the surface of the membrane, uptake still takes place in the cold within the thick muscle wall of the umbilical artery, in marked contrast to the uptake in the wall of the rabbit ear artery which is inhibited by cold. In the absence of intracellular binding of NA, passive diffusion across the membrane could not account for the marked accumulation of NA within smooth muscle (Gillespie, 1968). Eisenfeld *et al.* (1967a, b) have, however, suggested that $Uptake_2$ may occur by facilitated diffusion alone. The binding of NA would reduce the effective concentration of NA within the cytoplasm, thus allowing further diffusion to occur (compare with Bozler, 1961) and for accumulation to become apparent within the cytoplasm even at this low temperature. This binding mechanism could also account for the NA accumulation at low concentrations.

It is not yet clear whether uptake of NA by smooth muscle has any physiological significance. It was originally suggested that uptake may involve α-adrenoceptors because the uptake could be blocked by PBA (Gillespie & Hamilton, 1966). This now appears unlikely, due to the lack of correlation between the pharmacological response to PBA and its inhibition of uptake (Gillespie, 1968) and because so many other drugs without antagonist activity act as uptake inhibitors (Iversen, 1967; Eisenfeld *et al.*, 1967a). Neither does it seem to be a mechanism which facilitates or prolongs the response of smooth muscle not directly innervated (Gillespie & Muir, 1970). However, it is possible that smooth muscle uptake plays a role in transmitter inactivation similar to that proposed for $Uptake_2$ (Eisenfeld *et al.*, 1967a, b; Iversen, 1968; Lightman & Iversen, 1969).

NA uptake by smooth muscle persists after sympathetic denervation of the spleen (Gillespie *et al.*, 1970) and extraneuronal metabolism of the rat heart is unaffected by immunosympathectomy (Eisenfeld *et al.*, 1967a). This indicates that the absence of nerves in these experimental situations does not alter the uptake characteristics of the muscle. However, we have shown that NA uptake characteristics of smooth muscle, which is not normally innervated, are different from those of innervated muscle. These differences in uptake characteristics in the two situations are currently being investigated in relation to COMT and MAO levels. It is possible that non-innervated smooth muscle uptake is an unspecialized diffusion process which may be masked in innervated smooth muscle.

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