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NORADRENALINE AND MOTOR TRANSMISSION IN THE VAS DEFERENS OF THE MOUSE

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1 A comprehensive investigation of the innervation of the vas deferens of the mouse was made using pharmacological, histochemical and electronmicroscopical techniques.

2 Guanethidine inhibited the response of the vas to transmural stimulation and potentiated the response to noradrenaline (NA). Phentolamine abolished responses to NA and to transmural stimulation.

3 After chemical sympathectomy degenerative changes were seen in presumptive noradrenergic axons; histochemical fluorescence due to catecholamines was absent. The vas failed to respond to transmural stimulation, and a 10-fold increase in sensitivity of the vas to exogenous NA was observed.

4 NA is shown to diffuse slowly through this tissue whose muscle cells are densely packed. This is discussed in relation to the apparent 'insensitivity' of the vas to exogenous NA.

5 A cholinergic component was identified histochemically which did not contribute significantly to the motor response of the vas as chemical sympathectomy abolished completely the motor response elicited by transmural stimulation.

6 It is concluded that NA is the motor transmitter for the smooth muscle of the vas deferens of the mouse.

Introduction

The vasa of rat, guinea-pig and mouse have a high content of noradrenaline (NA) (Sjöstrand, 1965) and have been shown to contain presumptive noradrenergic neurones (Richardson, 1962; Burnstock & Robinson, 1967; Yamauchi & Burnstock, 1969). However, relatively high concentrations of exogenous NA are required to induce contractions of vasa of guinea-pig (Birmingham & Wilson, 1963; Ambache & Zar, 1971) rat (Graham, Al Katib & Spriggs, 1968; Birmingham, 1970) and mouse (Holman, 1970). The difficulty in obtaining responses to exogenous NA of equal magnitude to those of nerve stimulation and the ability of NA to inhibit responses evoked by nerve stimulation has led to Ambache & Zar (1971) challenging the concept that NA acts as the motor transmitter substance in the vas of guinea-pig. In addition, α-adrenoceptor blocking drugs have been shown to block the effects of exogenous NA without affecting responses to nerve stimulation (Bentley & Smith, 1967; Ambache & Zar, 1971).

The present study was undertaken to establish the neurotransmitter responsible for the motor response of the longitudinal muscle of the vas deferens of mouse by the use of histological, histochemical and pharmacological techniques.

Methods

Mature male albino mice, T.O. strain, weighing 25-35 g and over four weeks old were injected intravenously with either 6-hydroxydopamine (6-OHDA) 1 mmol/kg or vehicle. The vehicle for 6-OHDA was 0.2 mg ascorbic acid/ml 0.9% w/v NaCl solution (saline). Twenty-four hours later the mice were killed by cervical dislocation and both vasa deferentia removed.

Single organs were suspended in Huković's solution (1961), gassed with 5% CO_2 in O_2 at 32° C. The tissue was subjected to 300 mg applied tension and allowed to equilibrate for 30-45 minutes. Isometric contractions were monitored on a Devices two-channel pen recorder via a 2 oz dynamometer UFI strain gauge. Transmural stimulation (Birmingham & Wilson, 1963) of vas suspended between two parallel platinum wire electrodes and immersed in a 50 ml bath was by

means of an SRI 6053 stimulator delivering rectangular pulses of 0.3 ms duration at frequencies of 20 Hz and of supramaximal voltage every 3.25 minutes. An oscilloscope was used to confirm the uniformity and reproducibility of the stimulation parameters under experimental conditions with the electrodes totally immersed in the bathing fluid.

In experiments not involving transmural stimulation, tissues were suspended in 10 ml baths. NA or acetylcholine (ACh) was left in contact with the tissue for 30 s every three minutes. Bathing fluid was replaced at 1.5 min intervals.

An analysis of covariance (Snedecor, 1956) was undertaken to ascertain whether the regression lines of log dose-response curves for NA or ACh in normal vasa were parallel or coincident to those obtained in 6-OHDA pretreated vasa.

Catecholamine histofluorescence

Cryostat-cut 18 μm sections of frozen vasa were dried and exposed to moist formaldehyde vapour under strictly controlled conditions, after the method of Spriggs, Lever, Rees & Graham (1966). The sections were viewed with a Zeiss fluorescence microscope.

Electron Microscopy

Routine investigation. Approximately 2 mm of tissue were removed from the epididymal end of the vas and cut into 1 mm cubes of tissue which were immersed in 1% osmium tetroxide buffered to pH 7.5 with veronal acetate for 1 h at 4° C, dehydrated and subsequently embedded in araldite. Fine sections were stained with lead citrate (Reynolds, 1963) and viewed with a Phillips 300 electron microscope.

Demonstration of acetylcholinesterase (AChE). Specimens were fixed in glutaraldehyde and incubated for 4 h at pH 5.5 with acetylthiocholine in the presence of 2×10^{-4} M ethopropazine (a pseudocholinesterase inhibitor) after the method of Lewis & Shute (1966). If physostigmine, a non-specific anticholinesterase, was included in the incubating medium instead of ethopropazine no AChE reaction product was seen in the tissue.

Extracellular diffusion of [^{3}H]-noradrenaline or [^{14}C]-sorbitol

Male mice used in [^{3}H]-noradrenaline determinations were pretreated with phenoxybenzamine (1.2×10^{-4} mol/kg intravenously) 30 min before they were killed (Gillespie, Hamilton & Hosie, 1970) and the vasa excised. Mice used for the D-sorbitol-[1-^{14}C] determinations received no pretreatment.

Incubation media

The stripped vasa were placed immediately in ice cold Krebs-Ringer bicarbonate buffer, pH 7.4 (Sachs, 1970) for [^{3}H]-noradrenaline determination ([^{3}H]-NA, specific activity = 7.3 Ci/mmol) and unlabelled noradrenaline was added to the buffer to make a final concentration of 1.22×10^{-4} M. Desmethylimipramine (DMI) (9.4×10^{-7} M) and ascorbic acid (0.2 mg/ml) were included in the incubating medium. For D-sorbitol-[1-^{14}C] determination unlabelled sorbitol was added to the incubating buffer together with D-sorbitol-[1-^{14}C] (specific activity = 3 mCi/mmol) in order to obtain a concentration of 2.7×10^{-6} M (Morgan, Henderson, Regan & Park, 1961).

In vitro *incubation*

Two vasa were placed in each test tube containing 1 ml of incubation medium and incubated in a metabolic shaker at 32° C. The medium was pre-gassed with 5% CO_2 in O_2; each test tube was stoppered with a tight fitting rubber bung and re-gassed for 10 s after 30 min incubation (Sachs, 1970). Vasa were removed after 0.5, 1, 5, 10, 30 and 60 min from the incubation medium.

Determination of total radioactivity by liquid scintillation

One vas (approx. 20 mg) was placed in 1 ml digest medium (2N methanolic potassium hydroxide; Petroff, Patt & Nair, 1965) and placed in an oven at 60° C overnight. Formic acid (0.1 ml) was added to 10 ml of scintillation medium (toluene, 700 ml; ethoxyethanol, 300 ml; PPO, 4 g; POPOP, 0.1 g; Hall & Cocking, 1965) before 0.2 ml aliquots of sample were added for counting.

The total radioactivity was measured in a Unilux II (Nuclear-Chicago), liquid scintillation counter. All counts were corrected for errors due to quenching and counting efficiency, and are expressed per mg wet weight of vas.

Drugs used were: acetylcholine chloride (Sigma), acetylthiocholine chloride (Sigma), desmethylimipramine hydrochloride (Geigy), ethopropazine hydrochloride (May & Baker), guanethidine sulphate (Ciba), 6-hydroxydopamine hydrochloride (Ralph N. Emanuel), (−)-noradrenaline bitartrate (Koch-Light), (±)-[^{3}H]-noradrenaline hydrochloride (7.3 Ci/mmol) (Radiochemical Centre, Amersham, England), phenoxybenzamine hydrochloride (S.K.F.), phentolamine mesylate

(Ciba), physostigmine salicylate (B.D.H.), sorbitol (B.D.H.), D-sorbitol-[1-^{14}C] (3mCi/mmol) (Radiochemical Centre, Amersham, England). Doses refer to the base.

Results

Responses of normal vasa to noradrenaline, acetylcholine or transmural stimulation

The vas responded to NA with an initial rapid contraction which reached a maximum usually within 5 seconds. The relationship between the tension developed in the vas and the log concentration of NA in the bath was linear for the dose range 1.2×10^{-7} M to 1.2×10^{-4} M (correlation coefficient, $r = 0.93$; $P < 0.001$) (Figure 1). The mean maximum tension developed in response to NA in 12 vasa was 0.90 ± 0.28 g which was 62.5% of the mean maximum tension developed during transmural stimulation (1.44 ± 0.08 g).

Responses to ACh, although often irregular and exhibiting several peaks of tension, showed a linear relationship to the log bath concentration of ACh in the range 1×10^{-6} to 1×10^{-3}M ($r = 0.92$; $P < 0.001$) (Figure 2). The mean maximum tension induced by ACh in eight vasa was 0.48 ± 0.040 g, equivalent to 33.7% of the mean maximum tension produced in response to transmural stimulation.

The vas contracted in response to transmural stimulation with 0.3 ms rectangular pulses of supramaximal voltage applied at 20 Hz for 10 s every 3.25 minutes. In the presence of guanethidine (2.0×10^{-6} M) the response of the vas to transmural stimulation was abolished, that to NA (6×10^{-6} M) potentiated and that to ACh (2×10^{-4} M) unmodified. Phentolamine (10^{-7} M) inhibited the response of the vas to NA and in higher concentration (5.3×10^{-5} M) abolished completely responses to NA and to transmural stimulation. Responses to ACh were unaffected by phentolamine.

Vasa from 6-hydroxydopamine pretreated mice

Transmural stimulation with 0.3 ms pulses failed to elicit contractions from the vasa of 6-OHDA-treated mice. With maximum voltage from the stimulator (13 V across immersed electrodes) the mean increase in tension from six denervated vasa was 1.2 ± 0.8% of that elicited from control vasa ($P < 0.05$).

The log dose-response curve for NA in 6-OHDA pretreated mice was linear in the dose range 3×10^{-8} M to 6×10^{-6} M ($r = 0.88$; $P < 0.001$) and the slope was not significantly different

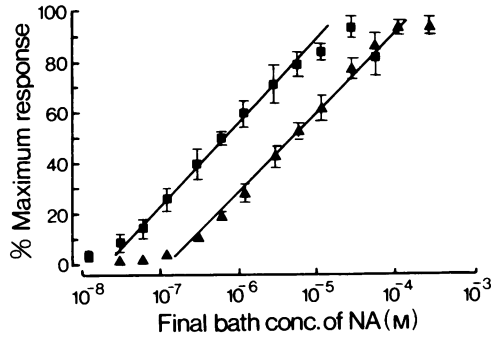

Figure 1 Log dose-response curves for noradrenaline (NA) in normal (▲) and chemically sympathectomized (■) vasa of mouse. Each point is the mean from at least nine vasa. Vertical bars show s.e. mean. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 1.2×10^{-7} M to 1.2×10^{-4} M for NA in normal vasa and 3×10^{-8} M to 1.2×10^{-5} M for NA in chemically sympathectomized vasa.

($P > 0.05$) from that obtained in vasa from untreated animals. The shift to the left of the curve for NA in treated vasa was significant ($P < 0.01$) (Figure 1).

A linear log dose-response curve was obtained for ACh in chemically sympathectomized vasa in the dose range 1×10^{-7} M to 1×10^{-3} M ($r = 0.94$; $P < 0.001$) which was neither parallel nor coincident to the regression line for ACh in untreated vasa ($P < 0.01$) (Figure 2). The chemically sympathectomized vasa did respond to lower doses of ACh (10^{-7} M). However, a similar maximal response was attained in both sympathectomized and untreated vasa with 10^{-3} M ACh (Figure 2).

The response of the vas to NA or ACh was qualitatively changed after 6-OHDA pretreatment of the mice. The response to NA reached several peaks of tension during the period of drug contact. The response to ACh was more prolonged in denervated vas preparations than in untreated vasa.

Electron Microscopy

The muscle cells in the mouse vas deferens occur in tightly-packed bundles, the interval between adjacent sarcolemmae being in the order of 100 nm. Large bundles of unmyelinated axons (8.3 ± 0.6 axons per bundle) embedded in Schwann cell processes were found in the extracellular spaces between muscle bundles. Smaller bundles of axons (5.6 ± 0.2 axons) were found in the extracellular spaces within muscle

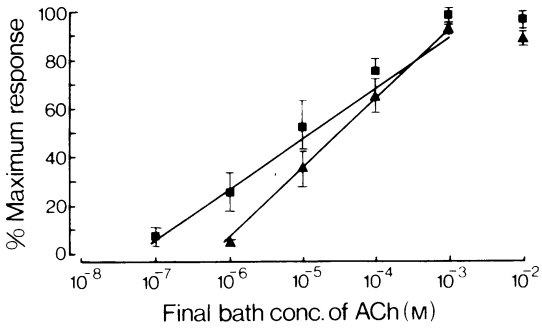

Figure 2 Log dose-response curves for acetylcholine (ACh) in normal (▲) and chemically sympathectomized (■) vasa of mouse. Each point is the mean from eight vasa. Vertical bars show s.e. mean. The lines are drawn through co-ordinates calculated by the method of least squares analysis in the dose range 1×10^{-6} to 1×10^{-3} M for ACh in normal vasa and 1×10^{-7} to 1×10^{-3} M for ACh in chemically sympathectomized vasa.

bundles. The Schwann cell sheath around the axons of the smaller axon bundles was often incomplete, leaving the axolemmae naked in parts.

The number of axons occurring singly and in bundles of two or three axons exceeded the number of muscle cells in any one plane of section. A mean of 82 axons were observed in a 7225 μm^2 grid space.

Axons partially denuded of Schwann cell investment and occurring in bundles of four or more were situated further from a muscle membrane than axons occurring in bundles of two or three axons. The distribution of the latter was similar to that of axons occurring singly (Figure 3). The proportion of axons totally or partially devoid of Schwann cell processes, situated within 100 nm of a muscle membrane, constitute over 70% of axons occurring singly, 60% of axons in bundles of two or three and under 40% of axons in bundles of four or more (Figure 3).

Ultimately some axons emerge from their Schwann cell sheaths to pass singly into extracellular spaces between muscle cells. These axons exhibit alternate constrictions and dilatations along their lengths. Axons are often embedded in the surface of the muscle cell and occasionally are completely surrounded by muscle cell processes (Figure 4).

The percentage of axons completely surrounded by smooth muscle constituted 1.2% of all axons investigated, 5.8% of the single axons investigated and 8.6% of axons lying less than 20 nm from a muscle membrane. These nerves contained small dense core vesicles and were devoid of Schwann cell processes. The subcellular space between the axon and the muscle membrane was between 10-20 nm. No morphological characteristic of the sarcolemma was apparent in relation to the embedded axon. However, an elongated sac of endoplasmic reticulum was found adjacent to

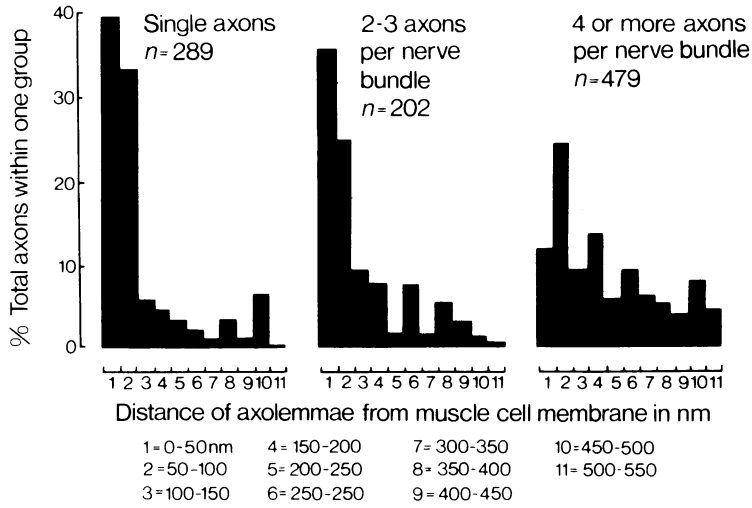

Figure 3 The distribution of axons in the mouse vas deferens. Axons are grouped as occurring singly, in bundles of two to three axons or in bundles of four or more axons and classified according to the distance of their axolemmae from the nearest muscle cell membrane. *n* = the number of axons investigated per group.

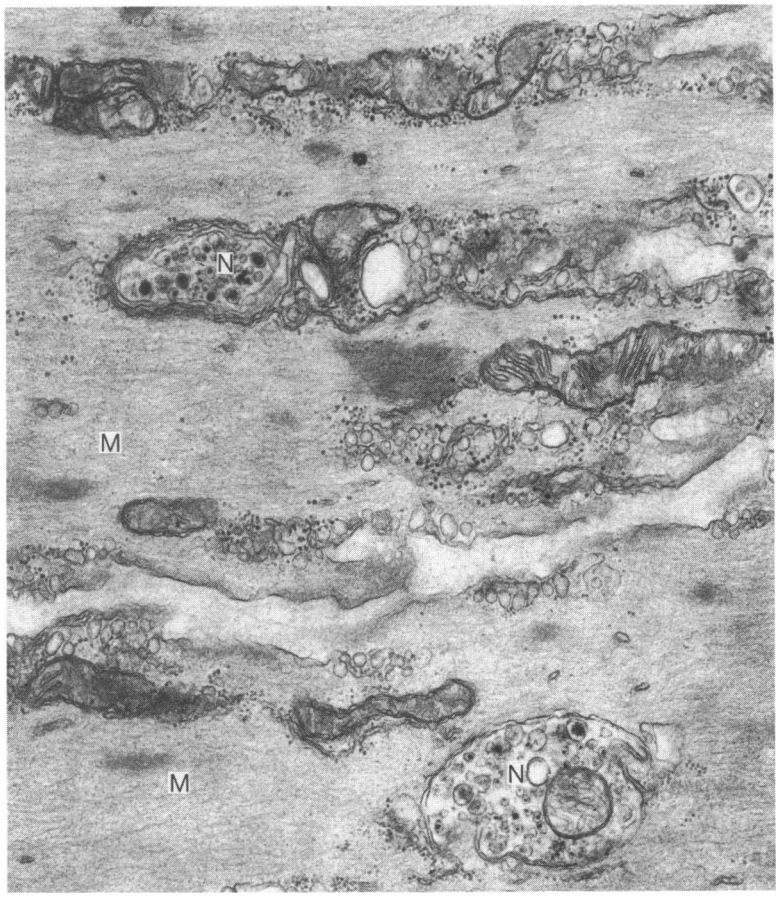

Figure 4 An electronmicrograph illustrating two profiles of noradrenergic axons (N) embedded in the cytoplasm of muscle cells (M) of vas taken from a mouse pretreated 1 h previously with 6-hydroxydopamine 0.2 mmol/kg. The interval separating axolemma from sarcolemma is less than 20 nm. Osmium tetroxide fixation.

the muscle membrane which was found adjacent to 78% of embedded axons. Of the 528 electronmicrographs of osmicated tissue studied, 145 elongated endoplasmic reticular sacs were found adjacent to axons and 200 were observed on muscle membranes remote from axons. These structures were found near 15.6% of single axons, 22.3% of axons in bundles of two or three axons and near 11.5% of axons in bundles of four or more axons.

In glutaraldehyde post-osmicated tissue 85% of axons contained small dense core vesicles (25-60 nm). Of the axon profiles, 22% contained small agranular as well as granular vesicles; 7.5% of axons contained small agranular vesicles only and had AChE reaction product on their axolemmae (Figure 5). In 6-OHDA pretreated vasa the majority of axons (81% of total axons) showed degenerative changes, 5% only having small dense core vesicles (Figure 5). A χ^2 test indicated that there was no significant difference ($P > 0.05$) in the population of AChE positive axons containing agranular vesicles between normal and sympathectomized vasa.

Catecholamine histofluorescence studies

The smooth muscle of the vas was densely innervated with a network of nerves exhibiting the bright green-yellow fluorescence characteristic for catecholamines. Twenty-four hours after an intravenous injection of 6-OHDA no specific fluorescence was observed in the vas.

Extracellular diffusion studies

The diffusion of [^{14}C]-sorbitol or [^{3}H]-NA into

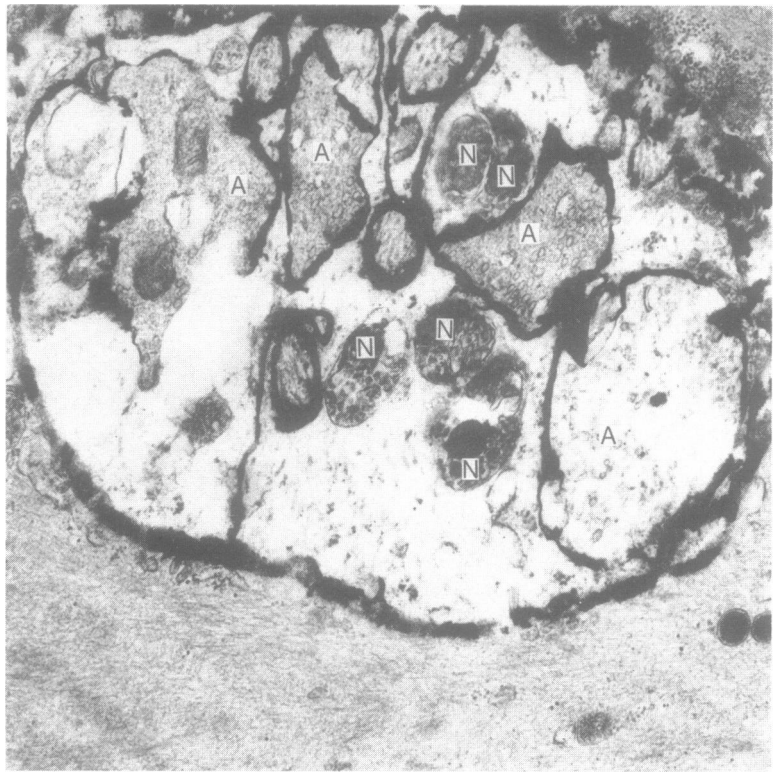

Figure 5 Electronmicrograph showing axons in smooth muscle layer of vas taken from mouse treated 24 h previously with 6-hydroxydopamine 1 mmol/kg, fixed with glutaraldehyde and incubated with acetylthiocholine. Acetylcholinesterase reaction product is seen on the axolemmae of axons which contain small electrontranslucent vesicles (A). These axons are presumptive cholinergic axons. The dense osmophilic masses in axons (N) are characteristic of axonal degeneration.

the extracellular fluid of the vas is expressed as the % accumulation ratio:

$$\frac{\text{d/min [}^{14}\text{C]-sorbitol (or [}^{3}\text{H]-NA) per mg vasa}}{\text{d/min [}^{14}\text{C]-sorbitol (or [}^{3}\text{H]-NA) per }\mu\text{l medium}} \times 100$$

The accumulation of [^{14}C]-sorbitol or [^{3}H]-NA by the vas was similar up to 10 min incubation. Between 10 and 60 min the accumulation of [^{3}H]-NA exceeded that of [^{14}C]-sorbitol (Figure 6).

Discussion

Intravenous administration of 6-OHDA in the mouse resulted in a chemical sympathectomy of the vas deferens. Electron microscopy of these vasa showed presumptive noradrenergic neurones exhibiting degenerative changes similar to those seen after surgical sympathectomy (cf. Van Orden, Bensch, Langer & Trendelenburg, 1967; Lever, Spriggs & Graham, 1968). Histochemical fluorescence due to catecholamines was absent, and the noradrenaline content of the vas was decreased to below 20% of control values (Jones & Spriggs, unpublished observations). Concomitantly neurotransmission was completely suppressed and a ten-fold increase in sensitivity to exogenous NA was observed. These findings together with evidence that guanethidine, an adrenergic neurone blocking drug or phentolamine, an α-adrenoceptor blocking agent, completely block neurotransmission in the vas is sufficient to establish the motor innervation to the vas deferens of the mouse as noradrenergic in nature.

Presumptive cholinergic axons, identified histologically as axons containing agranular vesicles and having an electron dense AChE

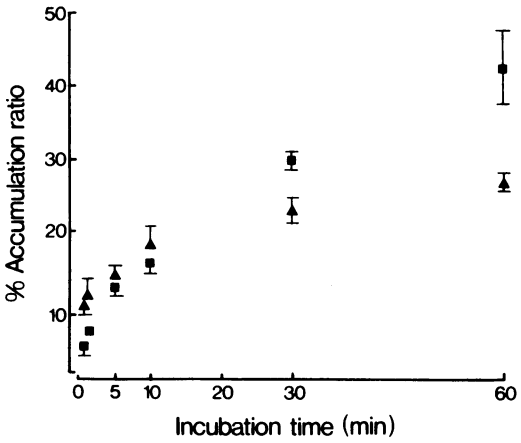

Figure 6 The diffusion of [^{14}C]-sorbitol (▲) or [^{3}H]-noradrenaline (■) through the extracellular spaces of the vas deferens of the mouse. Each point is the mean for eight preparations.

$$\% \text{ accumulation ratio} = \frac{\text{d/min per mg vasa}}{\text{d/min per } \mu\text{l medium}} \times 100$$

reaction product associated with their axolemmae, constituted less than 10% of the total axon population. In sympathectomized vasa no difference was detected in their appearance or in the number of such axons observed ($P > 0.05$). The contribution of these axons to the functional contraction of the vas is considered negligible as, after chemical sympathectomy with 6-OHDA, the vas failed to respond to nerve stimulation.

The response of the vas to NA and ACh is qualitatively and quantitatively changed by 6-OHDA pretreatment of the mice. The ten-fold increase in sensitivity to NA of the vas of the mouse is similar to the 16-fold increase in sensitivity of the rat vas reported by Birmingham, Paterson & Wojcicki (1970) who used a surgical method for denervation. In agreement also with the work of Birmingham *et al.* (1970) is the increase in sensitivity of the vas to low doses of ACh.

The increase in sensitivity to NA may be attributed to the destruction of the presynaptic neuronal uptake mechanism for noradrenaline following denervation resulting in more NA being available to α-receptors (Birmingham *et al.*, 1970; Ozawa & Sugawara, 1970). However, post-synaptic changes occurring secondarily to denervation cannot be precluded because of the non-specific nature of the supersensitivity and the qualitative changes which also occurred. Cocaine, an inhibitor of uptake$_1$ mechanism, has been shown to be without effect on responses of guinea-pig vasa to ACh or histamine whereas denervation produced a prolonged contraction to each stimulant (Westfall, McClure & Fleming, 1972).

Folkow & Häggendal (1970) estimated that 400 molecules of NA were released per varicosity per impulse assuming that most of tissue NA is present in the vesicles and that the majority of varicosities discharge transmitter when an impulse arrives. This amount of noradrenaline is equivalent to 6×10^{-6} M NA evenly distributed in a junction gap having a size of 1-2 μm^2 and a width of 100 nm. Applying this calculation to the present experiments in which trains of 200 stimuli were used over 10 s, the concentration of NA in the synaptic cleft could reach 1.2×10^{-3} M. We also found that a maximum response of the vas was achieved with a train of 60 stimuli over 2 s, affording a calculated concentration of 7.2×10^{-4} M NA in the synaptic cleft. These estimates presumably err on the high side as no allowances have been made for diffusion or neuronal reuptake of NA; they may, therefore, be considered compatible with the concentration of added NA (1.2×10^{-4} M) which elicited a maximum response of the vas. However, although the vas appears insensitive to NA this does not explain the failure of exogenous NA (even in concentrations up to 1×10^{-3} M) to produce a contraction of similar magnitude to that achieved by transmural stimulation; this may be a consequence of the morphological structure of the vas and the physiological properties of the smooth muscle cells.

The time taken for sorbitol to reach equilibration in tissue is considered to be indicative of the time required for substances to diffuse through the extracellular spaces and Morgan *et al.* (1961) have shown that sorbitol does not penetrate into cardiac muscle cells. In the vas of mouse, sorbitol reaches equilibration in the extracellular fluid between 30 and 60 min after starting incubation. The sorbitol space was 26.8 ± 1.1% after 60 min incubation. This value is comparable with the sorbitol space (22.8% ± 1.1) determined for the atria of mouse (Sachs, 1970). However, equilibration of sorbitol in atrial tissue took between 10 and 30 min incubation in contrast to between 30 and 60 min incubation in vasa. Although the extracellular spaces of both tissues are similar in volume the longer equilibration time for sorbitol in the vas is probably due to the tight packing of the muscle cells in this tissue. The taenia coli of guinea-pig has a larger sorbitol space (40%) which equilibrates faster (5 to 10 min, Goodford & Leach, 1966) than that of vas or atrium of mouse.

In vasa in which both uptake processes for NA into nerve and muscle were inhibited [^{3}H]-NA was accumulated up to 60 min after starting the

incubation. The dose of phenoxybenzamine injected into mice 30 min before they were killed has been shown to inhibit the accumulation of NA at all concentrations by smooth muscle, endothelium and nerves of the spleen of cat (Gillespie *et al.*, 1970). DMI has been shown to block the uptake$_1$ mechanism for noradrenaline into nerves (Iversen, 1967).

The persistent accumulation of [^{3}H]-NA after 30 min incubation may indicate that [^{3}H]-NA is taken up by collagen (Gillespie *et al.*, 1970) and other extraneuronal and extramuscular sites whose uptake is not blocked by phenoxybenzamine or DMI. Alternatively, [^{3}H]-NA, having saturated the extracellular spaces may enter nerves and muscles by passive diffusion or via any remaining small fraction of uptake$_1$ and uptake$_2$ mechanisms which remain unblocked.

Full equilibration of the extracellular space of the vas takes 30-60 minutes. The maximum response to noradrenaline (1.2×10^{-4} M) occurs within 30 s of exposure. If the accumulation ratio of noradrenaline in 30 s is expressed as a percentage of the equilibrated sorbitol space then it would appear that in 30 s exogenously administered noradrenaline has diffused through only 15% of the extracellular space, indicating a failure to reach all muscle cell membranes. Furness & Burnstock (1969), using electrophysiological techniques, have reported the inability of the muscle cells of vas of mouse to propagate actively an initiated spike potential although some stimulation may be transmitted passively and in a decremental manner to other cells surrounding the stimulated cell. In contrast to stimulation induced by exogenous NA, transmural stimulation presumably activates all synapses, and every muscle cell in vas of mouse possesses one to four possible neuromuscular synapses, ensuring a maximum contraction of the musculature.

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