Br. J. Pharmac. Chemother. (1968), **32**, 168–184.

UPTAKE OF NORADRENALINE BY ADRENERGIC NERVES, SMOOTH MUSCLE AND CONNECTIVE TISSUE IN ISOLATED PERFUSED ARTERIES AND ITS CORRELATION WITH THE VASOCONSTRICTOR RESPONSE

BY

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(*Received September* 14, 1967)

Perfusion of the cat spleen with blood containing large quantities of noradrenaline causes the smooth muscle cells, when exposed to formaldehyde vapour, to develop the fluorescence characteristic of this catecholamine (Gillespie & Hamilton, 1967). The development of fluorescence is paralleled by the retention of noradrenaline assayed biochemically (Gillespie, Hamilton & Hosie, 1967). The smooth muscle cells differed both in the degree to which they retained noradrenaline and in the site of retention; in trabecular and capsular smooth muscle the fluorescence was slight and confined to the periphery of the cells, whereas in the smooth muscle of arteries the fluorescence was intense and present within the cells. In the perfused spleen it was difficult to be sure that all smooth muscle cells were equally exposed to noradrenaline and in the present experiments with an isolated length of artery perfused with saline it was hoped to minimize this difficulty so that the relationship between the concentration of noradrenaline and the degree of fluorescence could be determined. In the experiments on the spleen, the α-receptor blocking agent phenoxybenzamine prevented the development of fluorescence of arterial smooth muscle (Gillespie & Hamilton, 1966), suggesting that α-receptors might be involved in the uptake. If this were so, one would expect phenoxybenzamine to interfere with the development of fluorescence and with the response in a parallel fashion. The vasoconstrictor response is easily measured in the perfused artery and it is therefore very suitable for this correlation.

A brief account of some of these results has previously been published (Avakian & Gillespie, 1967).

METHODS

The saline-perfused isolated artery preparation described by de la Lande & Rand (1965) was used. Rabbits of either sex in the weight range 1.8–2.8 kg were anaesthetized with intravenous pentobarbitone

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(approximately 60 mg/kg) and the central arteries of each ear isolated and cannulated. A length of 3–5 cm was used and the two arteries were set up in a Palmer isolated organ bath in separate vessels containing Krebs solution at 37° C and oxygenated with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution was as follows: NaCl, 118mM; KCl, 5.9mM; $CaCl_2$, 2.5mM; $MgSO_4$, 1.8mM; KH_2PO_4, 1.2 mM; glucose 0.2% (w/v). Each artery was perfused with Krebs solution by means of a Watson-Marlow constant output pump model MHRE. The flow rate (2–8 ml./min) was adjusted to give a mean perfusion pressure of about 80 mm Hg. The pressure was measured with Elema-Schönander pressure transducers and recorded on a Grass polygraph. To change the perfusing medium the pumps were stopped and the pump tubing quickly changed from one reservoir to the other. The dead space before the new medium reached the artery was 3.5 ml. and was usually cleared within 30 sec. The noradrenaline solutions were made up freshly each day in Krebs solution and stabilized with ascorbic acid 2×10^{-5} g/ml. Concentrations of noradrenaline refer to the base. All perfusion fluids, with or without noradrenaline, were oxygenated with 95% O_2 and 5% CO_2.

In cannulating the arteries it was necessary to use a cannula with a large internal diameter compared with the artery, otherwise the cannula rather than the artery contributed most of the peripheral resistance. This was not a problem with the central artery of the ear used in the present experiments, but it was in other experiments in which the femoral artery was used. A similar difficulty arose if the ligature tying the artery on to the cannula was so tight as to cause a constriction. To exclude these possibilities the artery was cut off close to the tip of the cannula at the end of each experiment; if the pressure fell to near zero and removal of the remaining stump plus the ligature produced little or no further fall the preparation was judged satisfactory.

This preparation is particularly suitable for the present study, because the same artery could be perfused with a succession of different concentrations of noradrenaline and, at each concentration, a few mm could be cut from the end of the artery and the fluorescence measured. A wash period of 1.5 min was allowed in most experiments before removing the end of the artery. The lengths of artery were immediately quenched in isopentane cooled in liquid nitrogen and freeze-dried in a Pearse Speedivac drier for 18–24 hr followed by exposure to formaldehyde gas at 80° C for 1 hr. The details of the Falck technique as used in this laboratory are given elsewhere (Gillespie & Kirpekar, 1966a). Sections were cut at 7μ, mounted in liquid paraffin and examined the same day with the X 100 oil immersion lens of the Leitz fluorescence microscope. A mercury vapour lamp (HBO 200) served as the light source with dark field illumination obtained with the Leitz high power cardioid condenser. A 3 mm BG 12 filter was used on the exciting light and a Leitz 53 barrier filter in the microscope tube. A photometric measurement of the degree of fluorescence was obtained in one of two ways. In the first method the Orthomat automatic camera was used to time the appropriate exposure, the reciprocal of this being a measure of brightness. A disadvantage of this method is that the diaphragm stop in front of the photo-multiplier is fixed in size and relatively large (7μ with the X 100 objective); as a result, small structures such as nerve endings or the internal elastic lamina fill it only partially. An allowance can be made for this, but it does reduce accuracy. The second method eliminated this. The Leitz MPV microspectrophotometer provides a variable diaphragm which can be made as small as 0.5 μ—smaller than the varicosities on the terminal adrenergic nerve fibre. Noradrenaline fluorescence fades rapidly with continued exposure to exciting light. As a result, we used a routine in which successive readings were at least two high power fields apart and all readings on a particular artery were made as rapidly as possible. These precautions seemed adequate, because ten readings were taken for each structure and there was no evidence of a progressive decline in these values with time.

Other drugs used were phentolamine and phenoxybenzamine. The doses of these refer to the salt.

RESULTS

Relationship between noradrenaline concentration and fluorescence

Perfusion of the isolated central artery of the rabbit ear resulted in the appearance of the green fluorescence characteristic of noradrenaline within the smooth muscle cells. The intracellular localization was suggested by the presence of fluorescence outlining

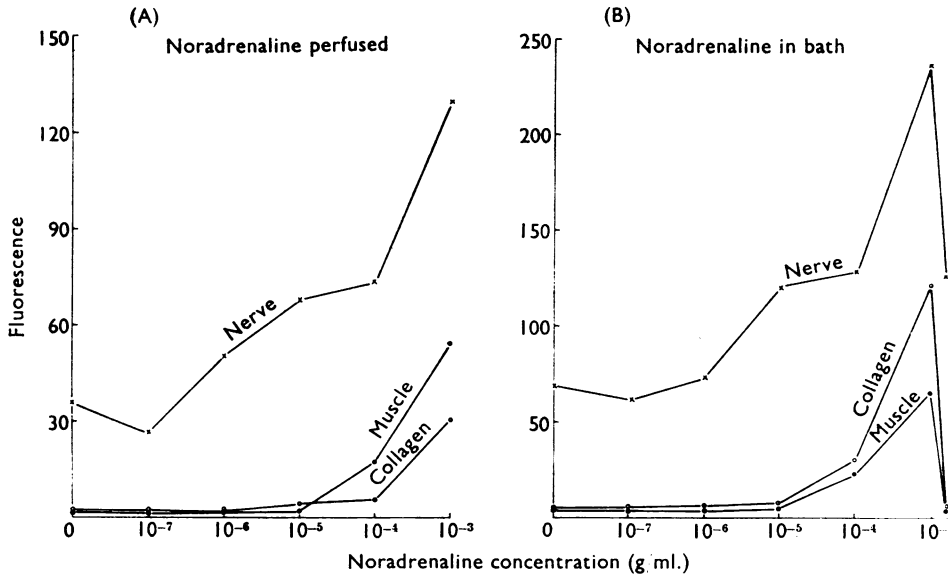

Fig. 1. Development of noradrenaline-fluorescence in nerve terminals, smooth muscle and collagen at increasing concentrations of noradrenaline. In (A) the noradrenaline was perfused through the arterial lumen, in (B) it was added to the external bath. The fluorescence of muscle and collagen disappears on returning to Krebs solution but some fluorescence remains in the nerve fibres. Fluorescence in this and subsequent figures is expressed in arbitrary units of brightness.

Standard errors

	(A)						(B)						
	Control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	Control	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	W
Nerve	8·2	3·1	8·7	3·6	3·9	8·4	3·9	4·9	4·9	10·2	8·2	2·2	10·7
Muscle	0·1	0·1	0·1	0·8	0·6	7·5	0·3	0·03	0·1	0·6	0·8	3·8	0·2
Collagen	0·3	0·1	0·2	0·3	0·8	5·1	0·6	0·3	0·2	0·7	4·5	8·3	1·0

some of the smooth muscle nuclei, and has been confirmed for arterial smooth muscle in the spleen by cutting very thin 1 μ sections from blocks embedded in Araldite. The degree of fluorescence was related in a characteristic way to the concentration of noradrenaline as shown in Fig. 1A. There was little or no increase in fluorescence below 10^{-5} g/ml., at 10^{-5} g/ml. there was sometimes a slight increase in fluorescence and at higher concentrations up to 10^{-3} g/ml. this rapidly increased. An almost identical dose-fluorescence curve was obtained for each of the six arteries from five animals. The fluorescence was reversible and even after the highest concentration of noradrenaline washing for 15 min reduced the fluorescence to the pre-infusion value. If noradrenaline is added to the bathing medium instead of to the perfusion fluid, an essentially similar result is obtained (Fig. 1B). The absolute value of fluorescence brightness varied from one experiment to the next, partly as a result of day-to-day variations in the intensity of the mercury vapour lamp. Where paired arteries from the same animal were perfused with noradrenaline at different concentrations and blocks taken from each, processed together and measured together, the fluorescence brightness of similar structures in the two arteries never differed by more than 20%. This was useful for the next group of experiments.

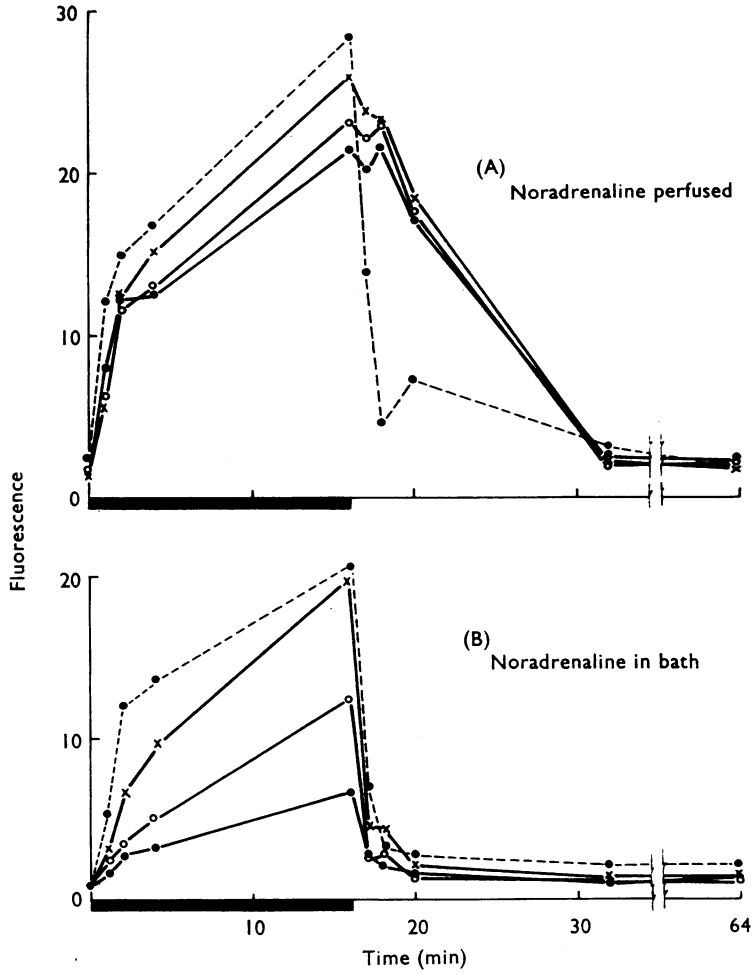

Fig. 2. Development with time of noradrenaline fluorescence in smooth muscle and collagen on exposure to noradrenaline 10^{-4} g/ml. and its decay on returning to Krebs solution. Measurements of smooth muscle fluorescence were made on inner, middle and outer layers of muscle in transverse sections of the artery. In (A) the noradrenaline was perfused through the artery; in (B) it was added to the external bath. The black rectangle represents the period (16 min) of exposure to noradrenaline. The rate of development of fluorescence on smooth muscle and collagen is similar but collagen loses its fluorescence faster on washing. The gradient of fluorescence through the muscle layer is greater when the noradrenaline is added outside. ●– – –●, Collagen; ●——● internal smooth muscle; ○——○, middle muscle; ×——×, external muscle.

Standard errors

(A)

Minutes	0	1	2	4	16	17	18	20	32	64
Internal muscle	0·04	0·6	1·0	2·6	0·9	1·9	1·2	1·2	0·07	0·2
Middle muscle	0·08	0·7	0·9	0·8	2·1	1·3	1·8	1·8	0·08	0·1
External muscle	0·07	0·5	1·3	0·8	3·0	1·4	2·1	1·2	0·1	0·3
Collagen	0·2	0·5	0·8	1·3	1·4	3·0	0·7	0·7	0·5	0·2

(B)

Internal muscle	0·06	0·26	0·1	0·23	0·5	0·17	0·9	0·38	0·05	0·38
Middle muscle	0·03	0·35	0·19	0·19	1·2	0·37	0·67	0·24	0·5	0·18
External muscle	0·03	0·67	0·5	1·3	1·9	8·47	0·1	0·14	0·1	0·08
Collagen	0·04	0·84	2·6	4·1	3·3	2·3	0·46	0·41	0·22	0·18

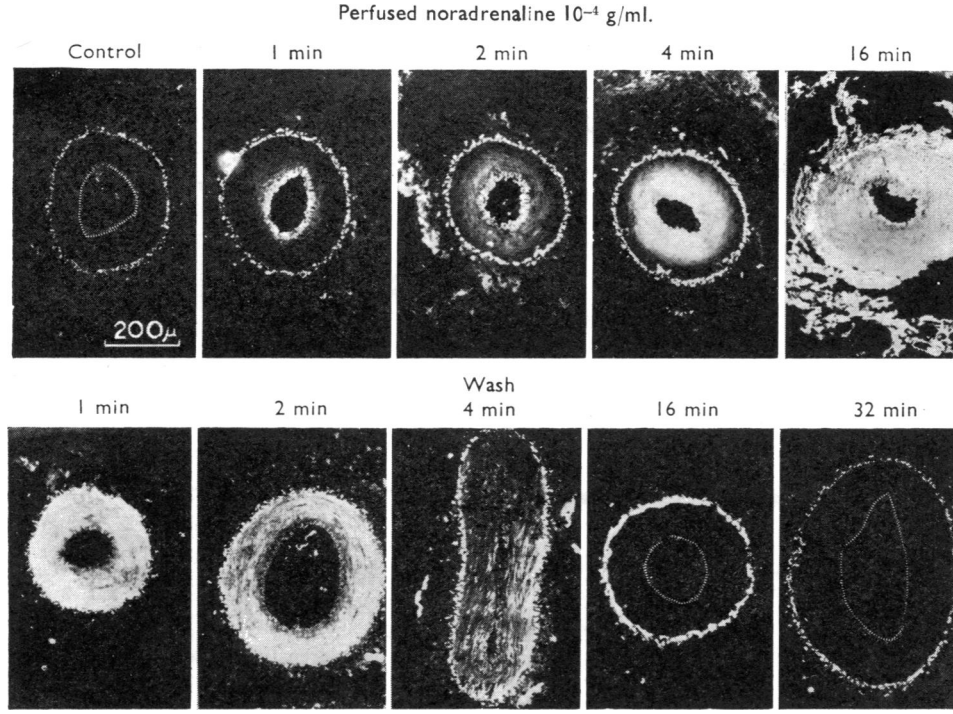

Plate 1. The top series of pictures shows the development of fluorescence with time on perfusing an artery with noradrenaline 10^{-4} g/ml. and the bottom series the loss of fluorescence on returning to Krebs solution. The arteries in the last panel in the upper row and the first in the lower row were so bright that an increase in exposure of 5 times for one and 4 times in the other was necessary to achieve any detail of the print. Calibration 200μ.

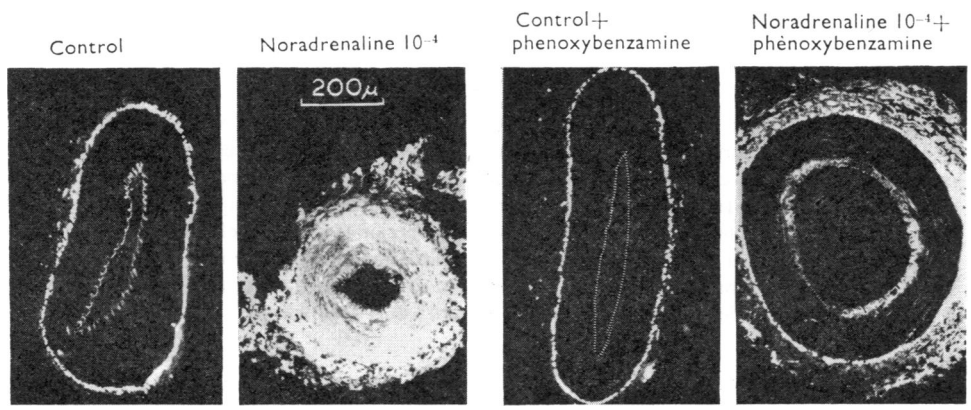

Plate 2. Effect of phenoxybenzamine on the fluorescence resulting from perfusion with noradrenaline 10^{-4} g/ml. for 16 min. The first panel shows a thin section of the artery before infusing noradrenaline, only the terminal nerve fibres at the outer border and the internal elastic lamina are fluorescing. The second panel shows the fluorescence of smooth muscle and collagen when perfused with noradrenaline 10^{-4} g/ml. The artery was then perfused with phenoxybenzamine 5×10^{-5} g/ml. in Krebs solution for 20 min, and the third panel shows the effect. There is a complete loss of the smooth muscle and collagen fluorescence but little or no change in the brightness of the adrenergic nerves. The infusion with noradrenaline 10^{-4} g/ml. was repeated in the presence of phenoxybenzamine and the last panel shows that this drug prevented the development of fluorescence in the smooth muscle but left that of the collagen in the tunica adventitia unaltered. Calibration 200μ.

Development and loss of fluorescence with time

The rate of development of fluorescence in arteries exposed to noradrenaline 10^{-4} g/ml. and the rate of loss of this fluorescence on washing with Krebs saline was examined in six pairs of arteries. In three of these the noradrenaline was in the bathing fluid and in three it was in the perfusion fluid. In most experiments both arteries were simultaneously perfused with noradrenaline and samples taken from each alternately. The rate of development of fluorescence was slow, and even after exposure for 16 min the maximum had not been reached (Fig. 2). There was no appreciable difference in rate between arteries exposed to noradrenaline in the perfusion fluid and those exposed to noradrenaline in the external medium (Fig. 2). When the noradrenaline was in the external medium, however, there was a very noticeable gradient of fluorescence, with the outermost layer of smooth muscle most fluorescent and that nearest to the lumen least fluorescent. When the noradrenaline was perfused through the artery the gradient was very much less. This difference can be seen in Fig. 2 and Plate 1, and may be caused by the bulk flow of fluid through the artery wall produced by the high hydrostatic pressure within the lumen supplementing diffusion. In one experiment there was an unexpected increase in the fluorescence in the first 2 min of the wash period. This was not an error in the measurements, because the increase was still present 6 months after the experiment, when a new set of sections were cut from the same blocks and the fluorescence measured with the more accurate MPV microspectrophotometer. The fluorescence of both the smooth muscle and the adrenergic nerve fibres increased, but not that of collagen.

The loss of fluorescence, as can be seen from Fig. 2, was more rapid than its development. The shape of the curve varied ; often there were two components, a rapid initial fall, then a slower decline, but the fast component was not always present. This difference in the shape of the curve was reflected in the half-time of loss, which varied from 1.0 to 8.0 min (Table 1). There was no correlation between the rate of loss of fluorescence and the source of the noradrenaline, either from the perfusion fluid or from the bathing fluid, neither was there correlation with the flow rate through the artery.

Fluorescence of other structures

Smooth muscle was not the only tissue to show an increase in fluorescence when perfused with noradrenaline. Three other structures—the terminal adrenergic nerve fibres, collagen and elastic tissue—also either increased their normal fluorescence or developed for the first time the green fluorescence characteristic of noradrenaline. The fluorescent brightness of these structures was also dependent on the concentration of noradrenaline as shown in Fig. 1. Terminal adrenergic fibres differed from smooth muscle in that they could take up noradrenaline at lower concentrations and also on washing the nerve fibres retained the noradrenaline much longer (Fig. 1B, Plate 1 and Table 1). By contrast, the dose-fluorescence curve for collagen and smooth muscle was very similar (Fig. 1). The development of fluorescence on the internal elastic lamina resembled that of collagen and smooth muscle, but the fluorescence was never so intense. As a result, at high concentrations of noradrenaline it usually appeared as a dark, less fluorescent band beside the intensely fluorescent smooth muscle. Collagen did differ from smooth muscle in two ways: first, in all experiments but one fluorescence was lost

more rapidly on washing (Plate 1): in the one exception the rates of loss were similar (Table 1 and Fig. 2B); second, the development of fluorescence on collagen was insensitive to phenoxybenzamine, which blocked the fluorescence of smooth muscle.

TABLE 1

RATE OF LOSS OF NORADRENALINE FLUORESCENCE FROM COLLAGEN, SMOOTH MUSCLE AND NERVE

In some experiments the noradrenaline was added to the perfusion fluid (inside), in others it was added to the bath (outside)

* No loss or too small a loss of fluorescence for calculation.

Expt.	Flow rate ml./min	Position of noradrenaline	Half-times (min) Collagen	Smooth muscle	Nerve
66/18	Not known	Inside	2·5	6·2	*
66/19	0·5	Inside	2·6	3·2	*
66/26	6	Inside	0·6	1·5	6·8
66/21	6·5	Outside	0·8	8·0	14
66/23	11	Outside	1·8	1·8	3·2
66/25	2	Outside	0·6	1·0	1·8

Action of phenoxybenzamine and propranolol

The action of phenoxybenzamine was examined in paired arteries from three animals; propranolol was used alone in one animal and a combination of the two drugs were used in arteries from a further two animals. Phenoxybenzamine in a concentration of 5×10^{-5} g/ml. almost completely abolished the noradrenaline fluorescence of smooth muscle (Plate 2). The effect at different concentrations of noradrenaline is shown in Fig. 3A and B. The increase in the noradrenaline fluorescence of the terminal nerve fibres, and particularly of smooth muscle, is reduced, whereas that of collagen and elastic tissue is little affected. The dose-response relationship for this fluorescence-inhibiting action of phenoxybenzamine was examined in the dose range 10^{-7} g/ml. to 5×10^{-5} g/ml. against a standard concentration of noradrenaline 10^{-4} g/ml. The results are shown in Fig. 4. The inhibiting effect of phenoxybenzamine on the noradrenaline fluorescence of smooth muscle was seen first at concentrations above 10^{-6} g/ml. and continued to increase until inhibition was almost complete at 5×10^{-5} g/ml. The noradrenaline fluorescence of collagen and elastic tissue was as high at the highest concentration of phenoxybenzamine as it was in the complete absence of this drug. Phenoxybenzamine did not abolish completely the ability of smooth muscle to develop fluorescence when exposed to very high concentrations of noradrenaline. If the development of fluorescence was the result of combination with the noradrenaline receptors, it was possible that the residual fluorescence was the result of combination with β-receptors unaffected by phenoxybenzamine. This possibility was investigated in two ways: first, by using propranolol together with phenoxybenzamine to see whether the residual fluorescence could be abolished, and second, by examining the effect of propranolol alone on the noradrenaline fluorescence. The results are shown in Figs. 5 and 6. The use of phenoxybenzamine with propranolol did not abolish the residual fluorescence with high concentrations of noradrenaline, and propranolol alone, even in concentrations so high (5×10^{-5} g/ml.) as to block non-specifically the vasoconstrictor response to noradrenaline had no effect on the fluorescence of nerve, muscle, collagen or elastic tissue.

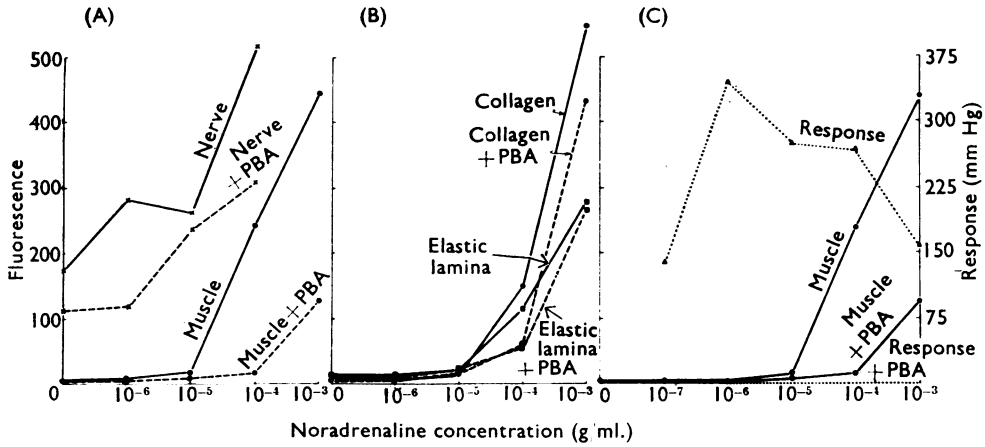

Fig. 3. The effect of phenoxybenzamine (5×10^{-5} g/ml.) on the development of fluorescence at increasing concentrations of noradrenaline and its effect on the vasopressor response. In each instance the heavy line represents the effect in the absence of phenoxybenzamine (PBA) and the interrupted line the effect in its presence. (A) shows the inhibiting effect of phenoxybenzamine on the fluorescence of nerve terminals and smooth muscle; (B) phenoxybenzamine has little effect on the fluorescence of elastic tissue or collagen; (C) phenoxybenzamine completely abolishes the response at all concentrations of noradrenaline but leaves a residual fluorescence. Fluorescence measured by the Leitz MPV microphotometer.

Standard errors

A

	Noradrenaline				
	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Nerves	9·6	19·6	11·0	2·8	Not distinguishable
Smooth muscle	0·2	0·5	1·0	11·5	28·2
Nerves + PBA	11·4	15·3	19·0	10·6	Not distinguishable
Smooth muscle + PBA	0·2	0·2	0·7	1·1	9·9

(B)

	Noradrenaline				
	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Collagen	1·6	0·7	1·2	8·1	35·8
Internal elastic lamina	1·0	1·8	0·8	10·1	16·7
Collagen + PBA	0·4	0·5	4·3	5·0	24·2
Internal elastic lamina + PBA	1·0	1·2	2·0	3·6	15·8

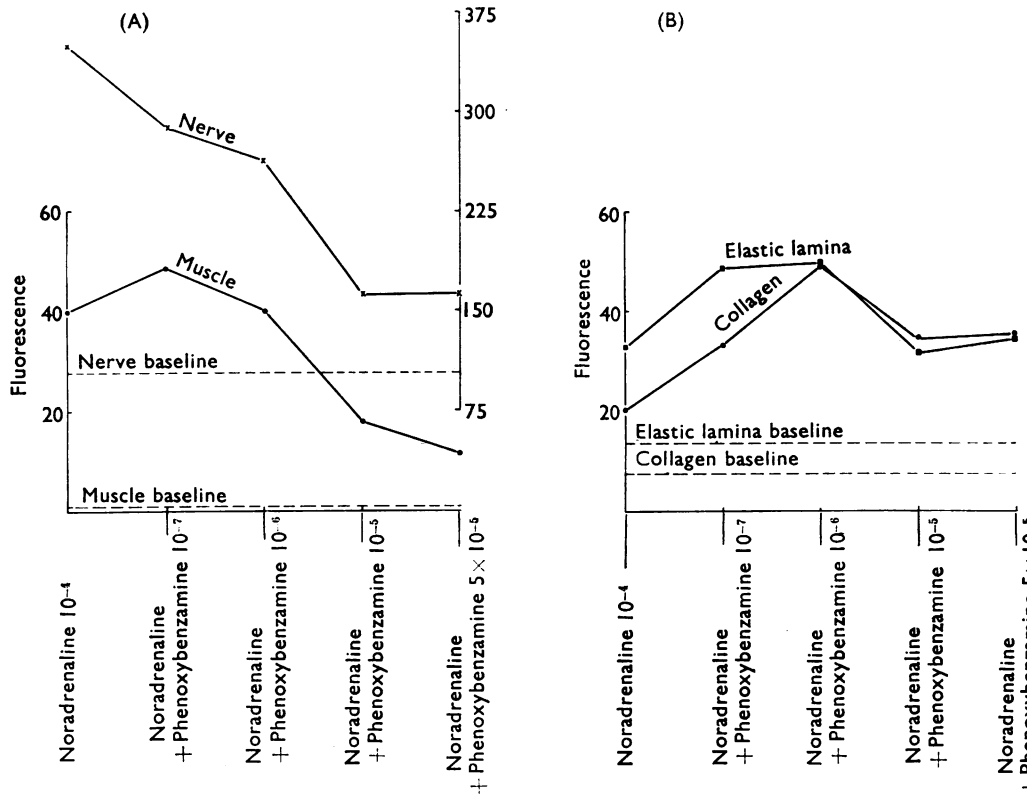

Fig. 4. Effect of increasing concentrations of phenoxybenzamine on the fluorescence produced by noradrenaline 10^{-4} g/ml. (A) The fluorescence of nerve is reduced by even small concentrations of phenoxybenzamine smooth muscle fluorescence only when the concentration of phenoxybenzamine exceeds 10^{-6} g/ml. (B) The increase in fluorescence of collagen and the internal elastic is as great at the highest concentration of phenoxybenzamine as it is with noradrenaline alone. In both (A) and (B) the interrupted lines represent the control fluorescence in the absence of perfused noradrenaline. Fluorescence measured by the Leitz MPV microphotometer.

Standard errors

	Noradrenaline 10^{-4}	Noradrenaline + phenoxybenzamine 10^{-7}	Noradrenaline + phenoxybenzamine 10^{-6}	Noradrenaline + phenoxybenzamine 10^{-5}	Noradrenaline + phenoxybenzamine 5×10^{-5}
			(A)		
Nerve	22·2	12·3	17·4	16·0	11·4
Muscle	3·3	4·6	2·5	1·1	0·7
			(B)		
Collagen	1·8	2·3	3·2	2·6	2·4
Elastic lamina	2·2	2·6	2·2	2·1	2·0

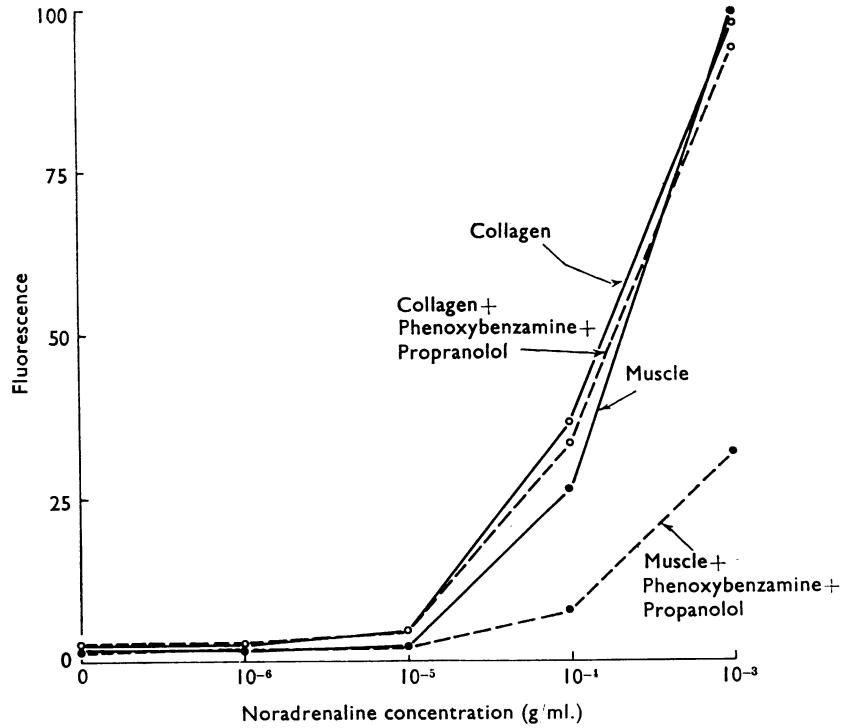

Fig. 5. Effect of phenoxybenzamine 5×10^{-5} g/ml. and propranolol 10^{-5} g/ml. on the development of fluorescence at different concentrations of noradrenaline. The addition of propranolol does not abolish the residual fluorescence of the smooth muscle at high concentration of noradrenaline. The combination of the two drugs has no effect on the fluorescence of collagen. Fluorescence measured by the Leitz MPV microphotometer.

Standard errors

	Noradrenaline					Noradrenaline + phenoxybenzamine + propanolol				
	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Muscle	0·14	0·05	0·17	3·6	11·1	0·33	0·05	0·13	0·16	6·2
Collagen	0·24	0·22	0·64	9·1	6·9	0·45	0·36	1·1	11·0	11·1

Relationship between smooth muscle uptake and the vasoconstrictor response

The action of phenoxybenzamine in blocking specifically the development of fluorescence in smooth muscle with no effect on the fluorescence of connective tissue suggested that α-receptors might be involved. Normally, maximal constriction of the artery was obtained at concentrations well below those at which fluorescence appeared, but this could mean that most of the receptor population is required for fluorescence whereas only a small fraction is sufficient for even a maximal response. If the α-receptors are involved, however, one would expect phenoxybenzamine to inhibit in a parallel fashion the development of fluorescence and the vasoconstrictor response, and perhaps to affect the fluorescence first if fluorescence involves a bigger proportion of the receptor pool. This point was examined in the experiments in the preceding section, and as Fig. 3C

shows, phenoxybenzamine was far more effective in abolishing the response to noradrenaline than in preventing the development of smooth muscle fluorescence. In other experiments low concentrations of phenoxybenzamine which had no effect on smooth muscle fluorescence almost completely abolished the response.

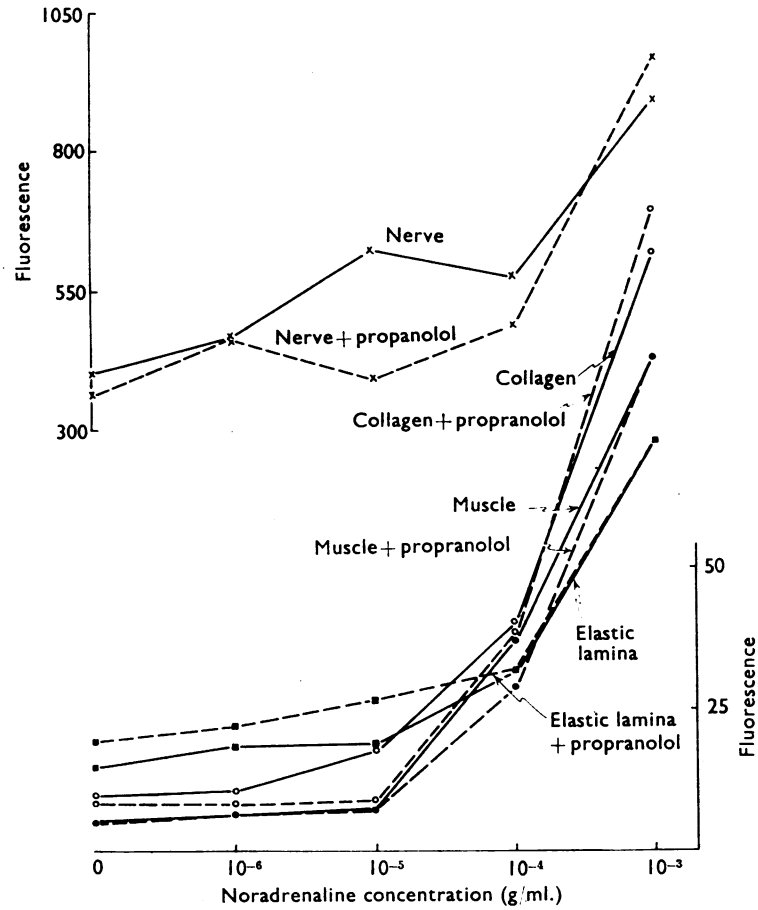

Fig. 6. Effect of propranolol (5×10^{-5} g/ml.) on the development of fluorescence at increasing concentrations of noradrenaline. The solid lines represent the fluorescence in the absence of propranolol, interrupted lines in its presence. The drug has no effect on the fluorescence of nerve, muscle, collagen or elastic tissue. Fluorescence measured with the Leitz MPV microphotometer.

Standard errors

		Noradrenaline			
	Control	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Nerve	3·5	41·3	66·7	36·2	39·5
Smooth muscle	0·2	0·2	0·4	3·7	13·0
Collagen	0·7	1·0	2·5	3·1	51·9
Internal elastic lamina	1·4	1·2	1·0	1·3	19·0
		+ propranolol 5×10^{-5}			
Nerve	25·2	30·3	25·3	51·2	46·1
Smooth muscle	0·2	0·2	0·3	0·7	20·9
Collagen	2·0	1·1	0·8	3·3	58·7
Internal elastic lamina	1·7	1·2	1·5	1·9	33·5

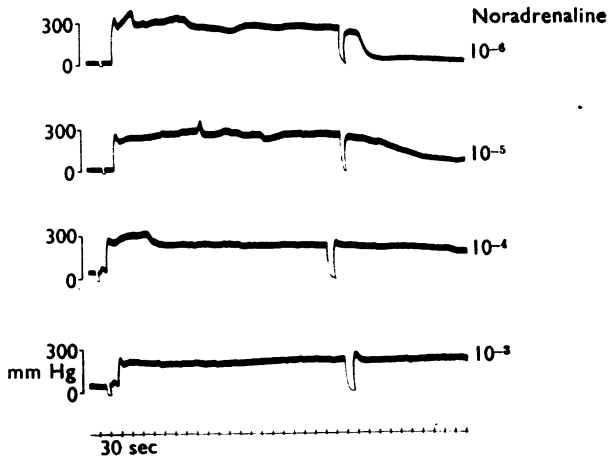

Fig. 7. Pressor response of isolated arteries to perfusion with noradrenaline of varying concentration. The concentration of noradrenaline is shown on the right of each trace. At concentrations up to 10^{-5} g/ml. the response declines rapidly on returning to Krebs solution but at 10^{-5} g/ml. and higher the decay of pressure becomes progressively slower. Time marker 30 sec.

In spite of this evidence that the α-receptors are not involved in the entry of noradrenaline into smooth muscle, there was indirect evidence that the noradrenaline taken up into the cells was producing an effect in prolonging the response. The constrictor response of arteries perfused with concentrations of noradrenaline up to 10^{-6} g/ml. quickly declines on returning to Krebs solution. At concentrations of 10^{-5} g/ml. and higher—that is, at those concentrations at which fluorescence appears in smooth muscle —the rate of decline of pressure slows down and at high concentrations becomes very slow. This point is illustrated in Fig. 7. If the rate of decline of pressure in sec/mm Hg is plotted against the concentration of noradrenaline, the relationship is very similar to that between the development of fluorescence and the concentrations of noradrenaline (Fig. 8). That the maintained elevated pressure is the result of a continuing action of noradrenaline is shown by the immediate fall in pressure when either phenoxybenzamine or phentolamine is injected, as is shown in Fig. 9.

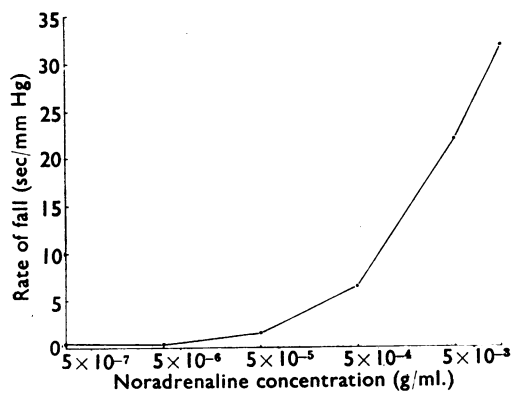

Fig. 8. Rate of decline of the pressure response following 10 min periods of infusion of noradrenaline plotted against the concentration of noradrenaline. The decline in pressure is expressed as sec/mm Hg.

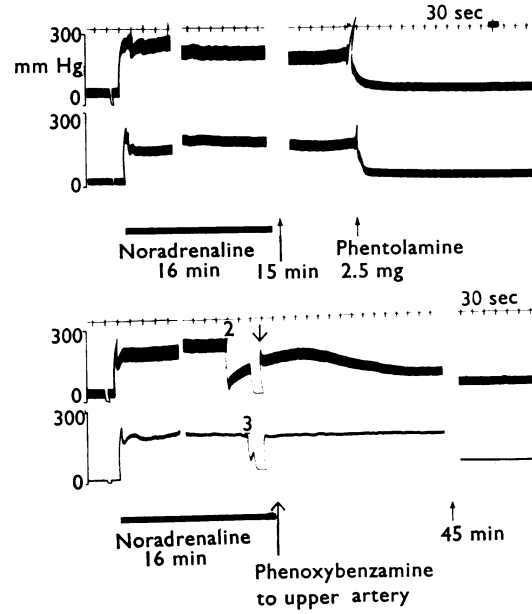

Fig. 9. Effect of α-blocking agents on the maintained response after a period of perfusion of arteries with noradrenaline (10^{-4} g/ml.). In the upper records the first panel shows the initial response and the middle panel the response at the end of the infusion period. In the last panel, 15 min after returning to Krebs solution, the response has hardly declined but is abolished by injecting phentolamine. The bottom series in another experiment shows in the first panel the initial response, and in the second panel the response at the end of the perfusion. The perfusion fluid in the upper artery of this pair was changed to Krebs + phenoxybenzamine 10^{-5} and the pressure fell to the pre-infusion level. The perfusion fluid in the lower artery was changed to Krebs alone and the pressure response was maintained into the wash period, but fell gradually so that in the final panel it was back to the pre-infusion level. The figures 2 and 3 and the corresponding artifacts indicate the removal of small portions of artery. The periods when the pumps were stopped can also be seen.

DISCUSSION

These results in the perfused isolated artery complement those we have obtained in the cat spleen (Gillespie & Hamilton, 1966 ; Gillespie, Hamilton & Hosie, 1967 ; Gillespie & Hamilton, 1967). Several tissues, namely terminal adrenergic nerve fibre, arterial smooth muscle, collagen and elastic tissue, can take up noradrenaline, though not all to a similar extent. Not all tissues develop fluorescence ; nerve bundles of the auricular nerve running alongside the artery showed little or no noradrenaline fluorescence. The nature of the uptake process varies. Smooth muscle and adrenergic nerve terminals resemble one another in that uptake is intracellular and involves transmembrane transport, that the noradrenaline once taken up is relatively difficult to wash away compared with that in connective tissue, and that uptake is blocked by phenoxybenzamine. Binding to collagen and elastic tissue, in contrast, is not intracellular ; phenoxybenzamine does not prevent such binding and the bound noradrenaline is easily lost on washing. The simplest explanation of these differences is to assume that binding to connective tissue is a surface phenomenon involving anionic sites on the fibres or on the mucopolysaccharide

ground substance but that some form of transport mechanism is responsible for the transfer of noradrenaline across the plasma membrane of smooth muscle and nerve terminals—a transport mechanism which is sensitive to phenoxybenzamine. Phenoxybenzamine is a drug which chemically is capable of reacting with a variety of sites and therefore potentially non-specific. Nonetheless, the absence of any blocking action on connective tissue binding suggests that the drug is blocking some specific carrier on smooth muscle and nerve terminals responsible for transport across the membrane. On smooth muscle the evidence, though indirect, is that this carrier is not the α-receptor. The residual fluorescence with high concentrations of noradrenaline in the presence of phenoxybenzamine may be caused by the non-specific entry of noradrenaline by diffusion.

There is insufficient evidence to say whether an energy-dependent active transport mechanism is present. Uptake into the arterial smooth muscle is very sensitive to temperature change (Gillespie & Hamilton, 1967), which is suggestive of active transport but is also consistent simply with chemical combination with a carrier. Active transport would be involved if it were certain that the noradrenaline within the cells was retained against an electrochemical gradient. In most experiments the smooth muscle fluorescence at high concentrations of noradrenaline exceeded that from the endogenous noradrenaline in the terminal nerve fibres in the control sections (Figs. 1, 3, 4 and 6). The concentration of noradrenaline or of dopamine in these terminal varicosities has recently been calculated (Norberg & Hamberger, 1964; Carlsson, Falck, Fuxe & Hillarp, 1964; Dahlström, Haggendal & Hökfelt, 1966; Anden, Fuxe, Hamberger & Hökfelt, 1966). The values vary from $1\text{–}3 \times 10^{-3}$ to 10^{-2} g/ml. It seems, therefore, quite possible that the concentration of noradrenaline in the smooth muscle cells is 10^{-2} g/ml. or more, an order of magnitude greater than the external concentration. The unknown factors are what fraction of this noradrenaline is diffusable, what proportion is ionized at the intracellular *p*H and what electrical potential is applied to these ions. No direct information is available. Noradrenaline, in causing contraction, depolarizes the muscle, so that at high concentrations the membrane potential gradient is not likely to be large. The extent of intracellular binding of noradrenaline is difficult to assess. Judged by the rate at which noradrenaline is lost, there is no binding comparable with that in the nerve fibres, and it seems unlikely that a high enough proportion could be bound to bring the concentration down to that in the external solution. In the absence of exact information on these variables, it is necessary to leave the question of active transport open.

Since Raab & Gigee (1955) reported uptake by the heart of large quantities of noradrenaline when this substance was injected intravenously, there has been an ever-growing literature on the mechanism involved. Most investigators, unlike Raab & Gigee, used very low concentrations of catecholamines which were within the physiological range. It was found that in these circumstances uptake was almost completely abolished after section and degeneration of the sympathetic nerves and also in immunosympathectomized animals (Hertting, Axelrod, Kopin & Whitby, 1961; Stromblad & Nickerson, 1961; Gillespie & Kirpekar, 1965; Iversen, 1965a, b). This process corresponds to the uptake of noradrenaline at low concentrations by the isolated rat heart. Iversen (1965a) called this "Uptake$_1$." At much higher concentrations he described another uptake process, "Uptake$_2$," which was capable of holding very large quantities of noradrenaline, and was presumably the mechanism involved in the experiments of Raab & Gigee. This

second uptake process was reduced in immunosympathectomized animals, and it was therefore suggested that it too was the result of uptake into nerves, perhaps into the cell bodies and pre-terminal fibres (Iversen, 1965b). Recent evidence has failed to find such a distinction between the various points of the adrenergic neurone and it has been suggested that extraneuronal sites, perhaps connective tissue, are responsible for "Uptake$_2$" (Malmfors, quoted in Hamberger, 1967). The experiments described here, and those with the perfused spleen in which the degree of fluorescence of smooth muscle and the retention of noradrenaline were shown to be related, suggest that uptake into effector cells may be an additional part of "Uptake$_2$". This is supported by the observation that in the cat spleen the uptake of noradrenaline into arterial smooth muscle is blocked by normetanephrine but little affected by Metaraminal (Gillespie & Hamilton, unpublished). Other sites at which extraneuronal uptake of noradrenaline has been found are the endothelial cells and pericytes of capillaries in the brain (Hamberger & Masuoka, 1965), and the parenchymal cells of the salivary glands (Hamberger, Norberg & Olson, 1967).

The most important question remains: does this uptake into smooth muscle take place under physiological conditions, and if so what is its significance? Fluorescence is seen only at concentrations of noradrenaline of 10^{-5} g/ml. and higher. Above this concentration the slope of the curve relating fluorescence to concentration is steep, suggesting that 10^{-5} g/ml. is a true threshold. Such concentrations never occur physiologically in the circulation, but may do so at the nerve endings. In the experiments reported by Gillespie & Kirpekar (1966b), short bursts of 200 stimuli to the cat splenic sympathetic nerves released sufficient noradrenaline to give concentrations in the venous blood as high as 5×10^{-7} g/ml. in normal cats and 1.2×10^{-6} g/ml. in cats treated with phenoxybenzamine. Concentrations of 10^{-6} g/ml. have also been obtained by Blakeley & Brown in similar experiments (personal communication, 1967). Considering the enormous dilution in extracellular fluid and blood which must occur before noradrenaline appears in the venous blood, a concentration at the site of release of 10^{-5} g/ml. or more seems probable. In the hope that localized uptake of noradrenaline in the vicinity of the nerve endings could be demonstrated during high frequency stimulation, lengths of artery were removed during stimulation at 30/sec and immediately frozen. Examination of sections treated by the Falck technique showed some local diffusion of transmitter with fluorescence of adjacent structures. These experiments are continuing and will be reported in full later. A similar local diffusion with nerve stimulation has been reported by others (Dearnaley, Fillenz & Geffen, 1966; Gillis, Schneider, Van Orden & Giarman, 1966), and reinforces the likelihood that extraneuronal uptake can occur. The function of this uptake will depend on whether noradrenaline inside effector cells can maintain a response; if so, uptake might be a way of producing a continuous effect from impulsive liberation of transmitter, if not, it might act to inactivate the transmitter (Costa, Boullin, Hammer, Vogel & Brodie, 1966). The correlation observed between fluorescence in smooth muscle and the prolongation of the vasoconstrictor response supports the first suggestion. The ability of phentolamine and phenoxybenzamine immediately to cut short the contraction suggests, however, that it is not the noradrenaline inside the cell which produces the effect. This is especially true for phenoxybenzamine, which has been shown to inhibit both uptake and loss from arterial muscle previously loaded with noradrenaline

(Gillespie & Hamilton, 1967). A more likely alternative is that noradrenaline leaking out of the cells acts on the α-receptors on the external surface.

SUMMARY

1. The development of noradrenaline-fluorescence in smooth muscle, collagen, elastic tissue and adrenergic nerve terminals of isolated arteries from the rabbit ear perfused with Krebs solution containing noradrenaline has been examined and correlated with the vasoconstrictor response.

2. All four tissues show a concentration-dependent binding of noradrenaline. The threshold for adrenergic nerves is 10^{-6} g/ml. or less; the other three tissues have a threshold at about 10^{-5} g/ml.

3. The development of fluorescence in adrenergic nerve terminals and in smooth muscle is inhibited by phenoxybenzamine in concentrations which have no effect on the fluorescence of collagen or elastic tissue. The β-blocking agent propranolol has no effect on the development of fluorescence in any of the tissues.

4. The fluorescence of collagen is quickly lost on washing compared with that in smooth muscle or nerve.

5. The development of fluorescence is related to the persistence of the vasoconstrictor response beyond the period of noradrenaline infusion.

6. It is suggested that both the smooth muscle cells and adrenergic terminals of these arteries possess a transport mechanism for the intracellular uptake of noradrenaline and that this effector cell uptake may be responsible for the uptake of noradrenaline at high concentrations ("Uptake$_2$").

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