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Uptake kinetics and ion requirements for extraneuronal uptake of noradrenaline by arterial smooth muscle and collagen

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Summary

1. The ionic requirements for noradrenaline uptake into vascular smooth muscle cells were studied by perfusing rabbit isolated ear arteries with noradrenaline (10^{-3}M) either in Krebs solution or in Krebs solution modified by altering the concentration of one or more ion. Noradrenaline uptake was measured by quantitative microphotometry.

2. Some uptake into smooth muscle continued in isotonic sucrose in the absence of all ions. Omission of Na^+ from the Krebs solution partially inhibited uptake as did high (100 mM) K^+. Omission of K^+, Ca^{++} or Mg^{++} had no effect on uptake. Lithium was able completely to substitute for Na^+.

3. Alteration in ion concentration did not affect the binding of noradrenaline to collagen.

4. The kinetics of uptake of noradrenaline into smooth muscle were analysed and found to be saturable with a Km of $4{\cdot}9 \times 10^{-4}$M.

5. It is concluded that the ionic requirements of the transport mechanism for the uptake of noradrenaline by vascular smooth muscle show a relatively low specificity.

Introduction

The ionic requirements for neuronal uptake of noradrenaline into sympathetic nerve terminals and brain synaptosomes have been studied in some detail (Iversen & Kravitz, 1966; Gillis & Paton, 1967; Bogdanski & Brodie, 1969; Horst, Kopin & Ramey, 1968; Kirpekar & Wakade, 1968; Colburn, Goodwin, Murphy, Bunney & Davis, 1968; Sugrue & Shore, 1969; Bogdanski, Blaszkowski & Tissari, 1970; White & Keen, 1970). There is an absolute requirement for sodium in the extracellular medium and uptake is enhanced by physiological concentrations (6 mM) of potassium. Higher concentrations of potassium partially inhibit uptake. Other ions, calcium and magnesium, for example, which are of importance in the neuronal release of transmitter appear to play a negligible part in neuronal uptake (Colburn, *et al.*, 1968; Keen & Bogdanski, 1970).

In addition to uptake into nerves, noradrenaline is also taken up into a variety of effector cells and also bound to collagen (Gillespie, Hamilton & Hosie, 1970). This extraneuronal uptake is probably identical with the process described by Iversen (1965) in the heart as $Uptake_2$. In contrast to neuronal uptake the ionic

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requirements of this process have not been reported except for one observation that high potassium concentration inhibits uptake (Gillespie, 1968 ; Hamilton, 1968). The kinetics of extraneuronal uptake have been studied usually on perfused whole organs such as the heart or on tissue slices. Because of the number of tissue components that may be involved in this uptake it is possible that the results represent an average of several elements. Quantitative microphotometry has been used to measure the noradrenaline uptake of single smooth muscle cells (Avakian & Gillespie, 1968) and the technique has been used in the present investigations to obtain answers to two questions. First, what are the ionic requirements for noradrenaline uptake and secondly, what are the kinetics of uptake into a single cell type, the smooth muscle cell of the rabbit ear artery. A preliminary account of some of these results has been published (Gillespie & Towart, 1972).

Methods

Rabbits of either sex in the weight range 1·8–2·8 kg were anaesthetized with intravenous pentobarbitone (approximately 60 mg/kg) and the central artery of each ear was cannulated and isolated. The larger side branches were tied off to ensure uniform flow through the whole length (3–5 cm) of artery used. The two arteries were set up in separate compartments of a bath containing Krebs solution at 37° C. Watson–Marlow constant output roller pumps (model MHRE) were used to perfuse each artery with Krebs solution at a rate of 5–8 ml/min, which gave a resting perfusion pressure of 30–50 mmHg. The perfusion pressure was measured with Bell and Howell type 4-327-L221 pressure transducers and recorded on a Devices M2 twin channel recorder. The perfusate reservoirs were kept at or below room temperature and before reaching the arteries the perfusion fluid was passed through heat exchange coils of rigid, fine bore polythene tubing immersed in the water jacket surrounding the artery bath. This water jacket was normally kept at 37° ($\pm 1°$) C, but in some experiments was cooled to 1–2° C.

Both arteries were first perfused with Krebs solution for 30 minutes. Where the ionic composition of the solution was to be altered the artery under test was then perfused with the altered solution for 20 min while perfusion of the control artery with Krebs solution was continued. The arteries were next perfused with noradrenaline solutions of the required concentration, made up as appropriate with Krebs solution or with the altered solution. Short lengths (2–3 mm) were cut from the ends of the arteries before and exactly 1, 2, 5, 10, 20, 40 and, sometimes, 80 min after beginning the perfusion with noradrenaline. These pieces of artery were frozen in liquid-nitrogen cooled isopentane, freeze-dried in a Pearse-Speedivac drier overnight and exposed to formaldehyde vapour at 80° C for 1 h (Gillespie & Kirpekar, 1966). They were then wax embedded and sections, cut at 6 μm and mounted in liquid paraffin, were examined or their fluorescence measured within 12 hours.

Fluorescence brightness was measured with a Leitz Ortholux microscope fitted with an MPV microphotometer and a Ploem incident light illuminator. The latter uses a dichroic mirror which allows the exciting light to be reflected into the optical axis towards the object but transmits the longer-wavelength emitted light back up to the eyepiece or microphotometer. Thus incident illumination (in which the objective lens doubles as condenser in order to eliminate variations due to inaccurate condenser focussing) is feasible with little loss of sensitivity. The exciting light was

the 405 nM line of a HBO 100 mercury vapour lamp isolated by a Leitz 405 nM narrow band filter. A x100 oil immersion lens was used with the variable diaphragm in the MPV microphotometer set to measure an area of 0·7 μm × 0·7 μm. This was small enough to measure fluorescence inside smooth muscle cells with minimal glare from the cell membrane. Variations in excitation intensity arising from changes in the mercury lamp were corrected for by frequent measurement of a fluorescence standard consisting of a fine silver grid (EMI 7 μm) embedded in fluorescent plastic. The fluorescence of the noradrenaline fluorophore fades rapidly so that each section was scanned once only and each measurement was taken at least two high power fields away from the previous reading. Ten readings were taken for each tissue.

The background fluorescence of the untreated control artery was found to be very low with the incident light illumination described above. A mean value of 1·3 ± 0·08 units of brightness was subtracted from all measurements to give the increase in brightness due to the accumulated noradrenaline.

Solutions and Drugs

Krebs bicarbonate solution was used with the following composition (mM): NaCl, 119 ; KCl, 4·8 ; $CaCl_2$, 2·5 ; KH_2PO_4, 1·2 ; $MgSO_4$, 1·4 ; $NaHCO_3$, 25 ; glucose 0·2% w/v. This, and all altered solutions with a bicarbonate buffer system, was bubbled with 95% O_2+5% CO_2. Sucrose solutions were bubbled with 100% O_2. The disodium salt of ethylene diamine tetra-acetic acid (EDTA), 5 μg/ml (or K_2 EDTA in Na^+-free solutions) was routinely added as an antioxidant.

Sodium was replaced either by sucrose or choline. Where choline was used a sodium-free solution required the replacement of both NaCl and $NaHCO_3$ by choline chloride and choline bicarbonate respectively. In Na^+-reduced solutions, choline salts were substituted for Na^+ salts. When lithium was substituted for Na^+, LiCl and $LiHCO_3$ were used to replace the Na^+ salts.

The concentrations of Na^+ were confirmed by flame photometry. When either choline or lithium was used to replace all sodium, never more than 0·2 mM Na^+ was measured in the 'Na^+-free' solution.

K^+-free solutions were made by replacing KCl and KH_2PO_4 with NaCl and NaH_2PO_4 respectively ; increases in K^+ concentration were made by the addition of solid K_2SO_4 to the Krebs solution.

NaCl and Na_2SO_4 were used to replace $CaCl_2$ and $MgSO_4$ respectively when Ca^{++} or Mg^{++} were omitted.

(−)-Noradrenaline bitartrate was obtained from Koch-Light.

Results

Noradrenaline accumulation by smooth muscle in normal ionic environment

Perfusion of the isolated rabbit ear artery with noradrenaline, 10^{-3}M, in normal Krebs at 37° C resulted in the increase of fluorescence of the smooth muscle cells with time shown in Figure 1. The increase in brightness was rapid at first but levelled off to reach equilibrium after about 20 min perfusion.

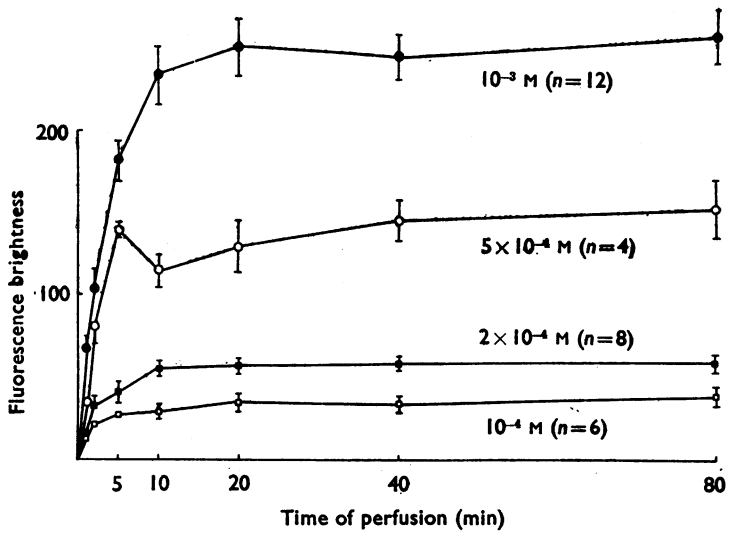

FIG. 1. The accumulation of noradrenaline in rabbit ear arteries perfused with noradrenaline of various concentrations in Krebs saline. The molar concentration of noradrenaline is given beside each curve. In this and in subsequent figures the background fluorescence of untreated smooth muscle has been subtracted to give the increase in brightness due to accumulated noradrenaline. Fluorescence brightness is given in arbitrary units. n=number of arteries and the vertical lines ±S.E.M.

The effect of altering the concentration of the noradrenaline perfused was next examined and Fig. 1 shows the result. The accumulation was clearly concentration-dependent and each curve reached equilibrium in about the same time.

Cold is known to inhibit the uptake of noradrenaline into arterial smooth muscle (Gillespie, *et al.*, 1970). The extent of this inhibition is shown in Figure 2. The test arteries were perfused for 20 min with Krebs solution at 1–2° C and then

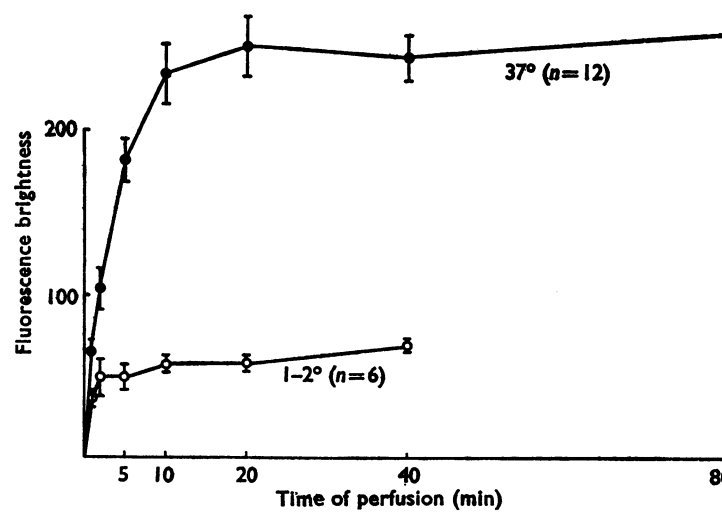

FIG. 2. Effect of cold on the accumulation of noradrenaline by the smooth muscle of rabbit ear arteries perfused with noradrenaline, 10^{-3}M, at 1–2° C or at 37° C. From 2 min onwards all differences between the two curves are statistically significant ($P<0.01$).

perfused with noradrenaline, 10^{-3}M at 1–2° C. At this temperature the accumulation of noradrenaline by the arterial smooth muscle was inhibited by approximately 70%.

Noradrenaline accumulation in an altered ionic environment

Figure 3 shows the result of replacing all ions by sucrose. The test arteries were perfused for 20 min with isotonic sucrose and then perfused with 10^{-3}M, noradrenaline in sucrose. The replacement of all ions inhibited the accumulation of noradrenaline by smooth muscle and the inhibition was equal to that produced by cold.

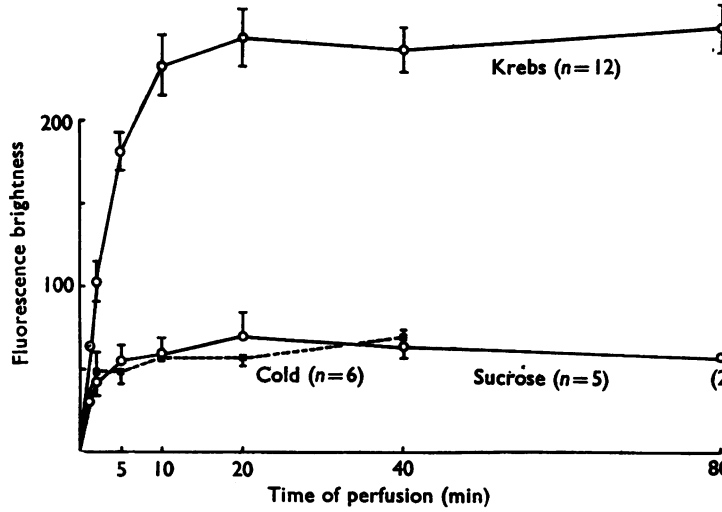

FIG. 3. The accumulation of noradrenaline in the smooth muscle of rabbit ear arteries perfused with noradrenaline (10^{-3}M) in isotonic sucrose solution at 37° C. The accumulation of noradrenaline in Krebs solution at 37° C and at 1–2° C is shown for comparison. From 1 min onwards the differences between the points in sucrose solution and Krebs solution at 37° C are highly significant ($P<0.01$). There is no statistically significant difference between the values in sucrose solution at 37° C and those in Krebs solution at 1–2° C.

The effect of sodium replacement

Figure 4 shows the effect of reducing the sodium concentration. The test arteries were perfused for 20 min with the low sodium solution and then perfused with noradrenaline 10^{-3}M, in the same solution. Reduction of the sodium concentration from the normal value (144 mM) to 25 mM caused a small but significant inhibition of accumulation of noradrenaline in the smooth muscle. Further reduction to 2·5 mM caused further inhibition. However, the complete removal of sodium had no further effect and the accumulation of noradrenaline in arteries perfused with sodium-free or 2·5 mM sodium was not significantly different. This level of accumulation was, however, significantly greater than the level of accumulation of noradrenaline by smooth muscle either in sucrose solution or at 1–2° C.

The ability of lithium to replace sodium was investigated and the results are shown in Figure 5. Arteries were perfused for 20 min with Krebs solution in which all sodium had been replaced by lithium and then perfused with noradrenaline 10^{-3}M, in the same solution. The level of accumulation of noradrenaline in smooth

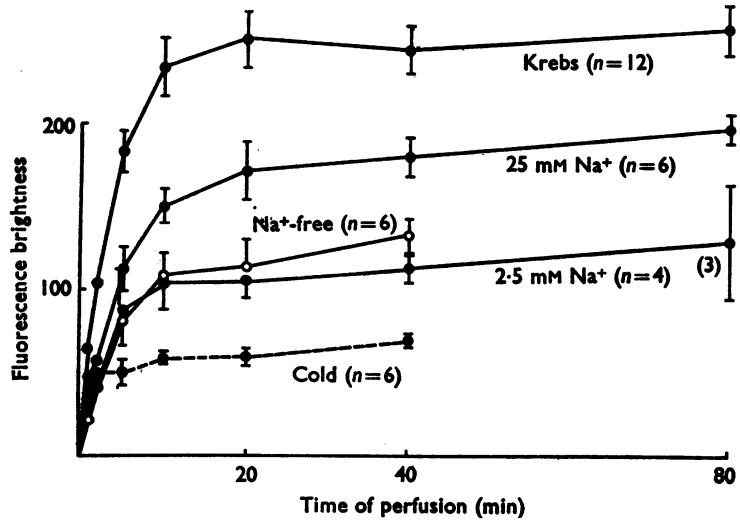

FIG. 4. Effect of increasing replacement of sodium by choline on the accumulation of noradrenaline by the smooth muscle of rabbit ear arteries perfused with noradrenaline, 10^{-3}M. The accumulation in Krebs solution (144 mM Na+) at 37° C and 1–2° C is shown for comparison. There is no significant difference between the accumulation of noradrenaline in Na+-free solution and in 2·5 mM Na+ solution, but the accumulations in Na+-free, 2·5 mM and 25 mM are all significantly different from the control at all times ofter 1 minute. At 40 min the *P* values are all less than 0·005.

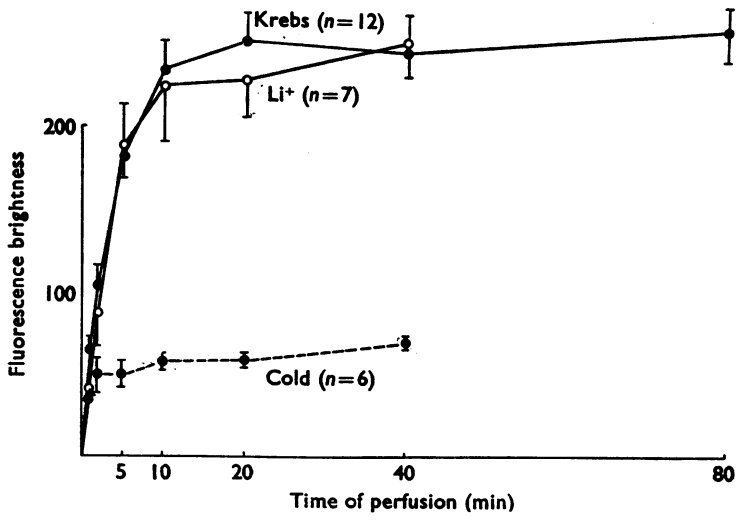

FIG. 5. Effect of complete replacement of sodium by lithium on the accumulation of noradrenaline by the smooth muscle of rabbit ear arteries perfused with noradrenaline, 10^{-3}M. The accumulation in Krebs solution at 1–2° C is shown for comparison. The accumulation of noradrenaline in the lithium solution is not significantly different from control.

muscle was not significantly different from accumulation in the physiological sodium concentration of 144 mM.

The effect of calcium, magnesium or potassium replacement

The effect of omission of calcium, magnesium or potassium is shown in Figure 6. The arteries were perfused for 20 min with the calcium-, magnesium- or potassium-

free Krebs solution and then perfused with noradrenaline 10^{-3}M in the same solution. The levels of noradrenaline accumulated by the smooth muscle in calcium-, magnesium- or potassium-free solution were not significantly different from control.

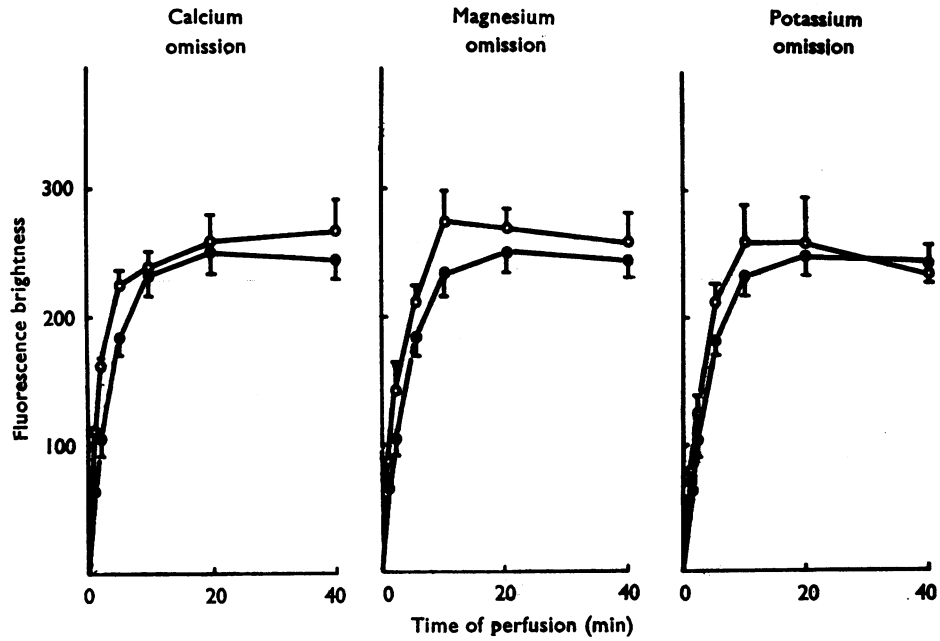

FIG. 6. Effect of calcium, magnesium, or potassium omission (upper curves, $n=3$) on the accumulation of noradrenaline by the smooth muscle of sets of arteries perfused with noradrenaline (10^{-3}M) compared with its accumulation in Krebs solution (lower curves, $n=12$). None of the observed differences is statistically significant.

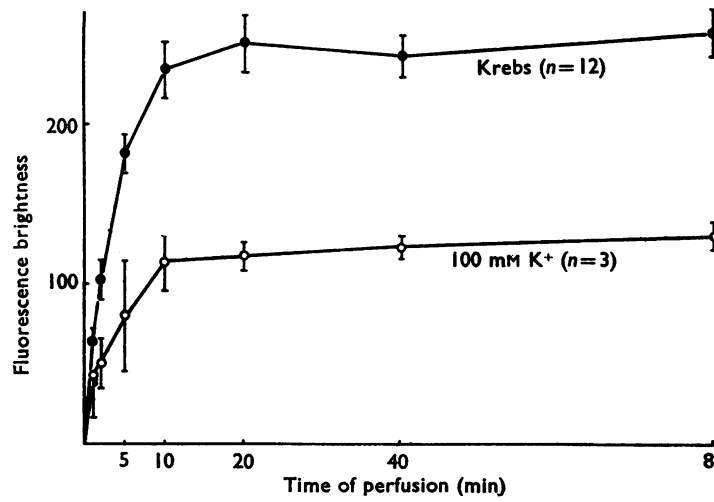

FIG. 7. Effect of increased potassium concentration on the accumulation of noradrenaline by the smooth muscle of rabbit ear arteries perfused with noradrenaline (10^{-3}M). The high [K+] was achieved by the addition of solid K_2SO_4 to Krebs solution. In high K+ the noradrenaline accumulation at 80 min is significantly different from control ($P<0.0025$).

The effect of increased potassium concentration

The ability of high concentrations of K^+ to inhibit noradrenaline uptake has previously been reported from this laboratory (Gillespie, 1968; Hamilton, 1968). The inability of K^+ removal (which reduces neuronal uptake of noradrenaline) to influence the uptake into smooth muscle prompted us to re-examine the effect of high K^+ (100 mM). Arteries perfused with high K^+ solution constricted, as shown by the development of a vasopressor response. Perfusion was continued for 20 min and then changed to noradrenaline 10^{-3}M in the same solution. The result is shown in Figure 7. The depolarized smooth muscle accumulated noradrenaline only to about 50% of control, an effect about the same as that produced by Na^+-free solution.

Kinetics of noradrenaline accumulation by smooth muscle

Kinetic analysis of the accumulation of noradrenaline by arterial smooth muscle was performed by the method of Iversen (1965). The curve for accumulation against time for each concentration (Fig. 1) was drawn on large graph paper and the gradient (i.e. rate of accumulation) measured at various times by drawing a tangent to the curve. These values were then plotted against time to examine the behaviour with time of the rate of accumulation of noradrenaline by the smooth muscle (Fig. 8) and were found to give a reasonable straight line for each concentration examined. Extrapolation to time $t=0$ of the line of best fit (obtained by

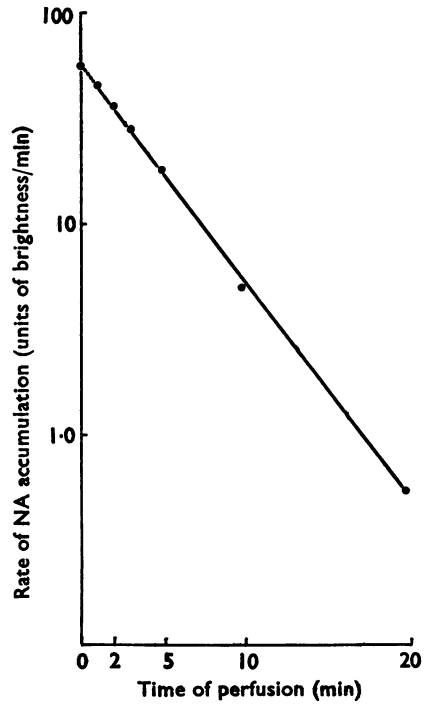

FIG. 8. The rate of noradrenaline (NA) accumulation in the smooth muscle of rabbit ear arteries plotted against time during perfusion with noradrenaline (10^{-3}M). The line of best fit has been determined by regression analysis and when extrapolated to $t=0$, gives an initial rate of accumulation of 56 units of brightness/minute.

regression analysis) gave the initial rate of accumulation (V) for each concentration (S). Plotting these values against concentration in the form S/V vs S (Dixon & Webb, 1958) gave straight lines showing that the uptake process obeyed Michaelis–Menten type kinetics (Figure 9). Extrapolation of the line to the abscissa gave a K_m value of $4{\cdot}9 \times 10^{-4}$M.

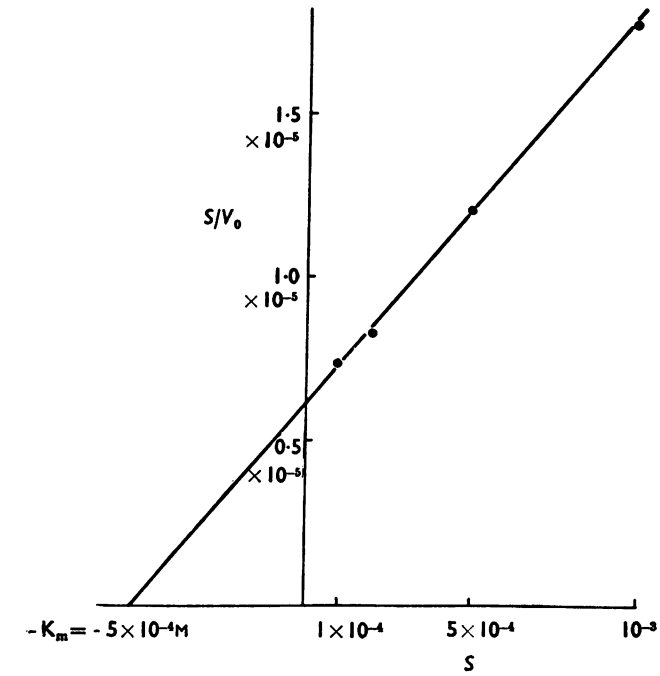

FIG. 9. Michaelis–Menten analysis of the initial rate of noradrenaline accumulation (V_0) as a function of substrate concentration (S) plotted as S/V_0 (ordinates) against S (abscissae). Intercept on abscissa $= -K_m$.

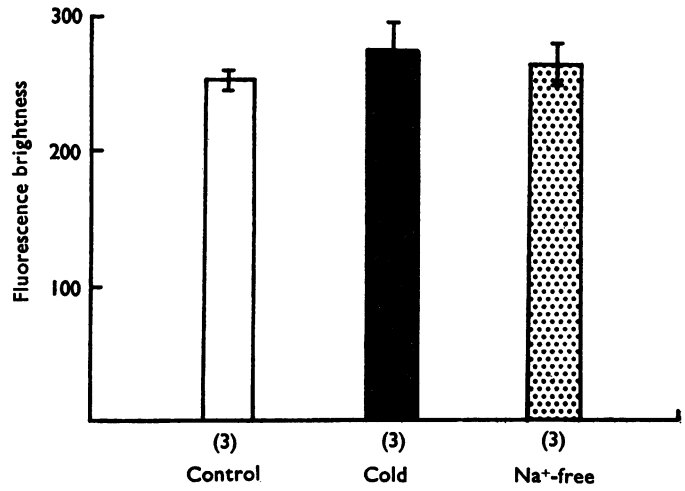

FIG. 10. A histogram of the binding of noradrenaline to collagen after 40 min perfusion with noradrenaline (10^{-3}M) in Krebs at 37° C (open column) and at 2° C (hatched column) and in Na^+-free solution (stippled column). The number of animals is shown beneath each column. There is no significant difference between the three values.

Accumulation of noradrenaline by collagen

Collagen is unaffected by cold or by drugs which block uptake into smooth muscle (Gillespie & Hamilton, 1966). It was, therefore, of interest to see whether sodium replacement would affect noradrenaline binding to collagen.

The brightness of the collagen of arteries perfused wih noradrenaline, 10^{-3}M, in Krebs solution at 1–2° C and in Na^+-free solution at 37° C was, therefore, measured. The measurements were made after 40 min noradrenaline perfusion when uptake equilibrium had been reached. The results are shown in Figure 10. In neither case was the brightness significantly different from the collagen of control arteries perfused with noradrenaline in normal Krebs solution at 37° C.

Discussion

Both the semilogarithmic plot of rates of noradrenaline uptake against time at any given noradrenaline concentration and the plot of S/V against S of initial rates of uptake at various concentrations are reasonably straight lines. These results suggest not only that uptake into vascular smooth muscle obeys saturation kinetics but also that the technique of quantitative microphotometry can be used for this kind of investigation. Ritzén (1966) has produced experimental evidence in model systems that concentration quenching can diminish fluorescence from catecholamines at concentrations of 10 mg/g, and higher concentrations at the upper level of those found physiologically in adrenergic nerve varicosities. We have found no evidence for similar quenching in the present experiments.

The ionic requirements for noradrenaline uptake into smooth muscle show similarities and some striking dissimilarities from those required for neuronal uptake. Similarities are the partial dependence on sodium, the partial inhibition by high potassium concentrations and the lack of effect of the divalent cations calcium and magnesium. A striking dissimilarity is the ability of lithium to substitute fully for sodium. We were also unable to detect any stimulating effect on uptake of low, physiological concentrations of potassium. This latter may be a technical problem. Within the muscle mass it may be difficult to control the ion concentrations so that leakage of K^+ in particular is able to maintain a low but functionally important concentration of K^+ in the vicinity of the cell membrane. Certainly most studies demonstrating the stimulating effect of K^+ on noradrenaline uptake has been either on isolated synaptosomes or on thin tissue slices. Experiments on a whole organ, the perfused spleen, failed to reveal this effect of K^+ (Kirpekar & Wakade, 1968).

Many cellular uptake processes, particularly those for sugars and amino acids, are dependent on Na^+ in the external medium (Crane, 1965; Kipnis & Parrish, 1965). A particular feature of these Na^+-coupled uptakes is the high specificity for sodium; no other ion tested can fully replace Na^+ (Schultz & Curran, 1970). This high specificity applies to the neuronal uptake of noradrenaline where lithium, tris, choline, rubidium, caesium and potassium are all ineffective substitutes (Horst *et al.*, 1968; Bogdanski & Brodie, 1969; Paton, 1971). Lithium has been particularly studied and only one study on synaptosomes shows that it can substitute to a small extent for Na^+ (Baldessarini & Yorke, 1970). Against this background the ability of lithium to substitute completely for sodium in the extra-neuronal

uptake of noradrenaline is unexpected and suggests that the mechanism involved in noradrenaline transport into smooth muscle may be different from that into nerve.

Because of the similarity in ion requirements, inhibition by metabolic inhibitors and by ouabain, neuronal uptake of noradrenaline is increasingly considered to be an active transport process similar in mechanism to the active transport of glucose and amino acids. In the absence of information on membrane potential, the free concentration of noradrenaline in the cytoplasm and its degree of ionization, it is not possible to be certain that transport is against an electrochemical gradient as would be required for active transport. However, the high tissue medium ratios observed and the inability of high depolarizing concentrations of K^+ to abolish uptake make it likely that active transport is involved. The need to postulate such a process is less for extraneuronal uptake. The tissue/medium ratios in comparison are low, a maximum of 2 or 3 (Gillespie, 1968), so that relatively little intracellular binding together with the braking effect of the membrane potential would be sufficient to achieve an equilibrium without active energy expenditure. On the other hand, the ability of cold, of drugs such as phenoxybenzamine, normetanephrine (Avakian & Gillespie, 1968; Gillespie *et al.*, 1970) and ouabain (Gillespie, 1968) and now of low sodium or isotonic sucrose to reduce or abolish uptake suggests the intervention of some specific mechanism, presumably a carrier, in the uptake process. When this mechanism is blocked exposure times as long as 40–80 min result in little or no noradrenaline accumulated in the cells. The inhibiting effect of low or absent Na^+ may represent a requirement for simultaneous binding of this ion and the transported amine. The inhibition observed by high K^+ may be the result of competition between the two ions for the carrier as suggested by Bogdanski & Brodie (1969) for neuronal uptake but might also be due to the abolition of the normal membrane potential. We have not investigated whether a simultaneous increase in the external sodium concentration would abolish the inhibitory effect of high potassium—an observation which led Bogdanski & Brodie to dismiss an effect on membrane potential as contributing to the inhibitory effect of high potassium and neuronal uptake. Complete removal of sodium does not abolish uptake into the smooth muscle cell nor does it produce as great inhibition of uptake as either isotonic sucrose or cold which again suggests that some other ions in the sodium-free Krebs solution can partially replace sodium and that the sodium specificity of this transport process is not high. Indeed, it is possible that the inhibition in the absence of sodium is due to the loss of intracellular K^+ and the consequent depolarization of the cell resulting from the inactivity of the sodium pump. The noradrenaline uptake at 1–2° C is the same as that occurring in isotonic sucrose, suggesting that some uptake, approximately 25% of that in Krebs solution, continues. Our method may exaggerate this uptake. Noradrenaline binding to the basement membrane of the cell is unaffected by cold or by changes in ions (Gillespie *et al.*, 1970) and though these membranes are not within the area measured by the photometer some glare from them may be deflected into the optical axis and artificially raise the background level. We do not think this contributes significantly to the fluorescence but rather believe the residual uptake in sucrose-free solution another indication of the lesser ion dependence of the uptake mechanism in this tissue.

The distinction between uptake into smooth muscle and binding to collagen previously reported is extended by the present observation. Collagen binding is not

only unaffected by cold, by normetanephrine and by phenoxybenzamine but is now shown to be unaffected by the removal of Na^+. The lesser degree of binding to the basement membrane around and between cells has similar properties.

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