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A histochemical study of extraneuronal accumulation of noradrenaline in the guinea-pig trachea

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Summary

1. Accumulation of noradrenaline in extraneuronal tissue of the guinea-pig trachea has been studied by the use of the fluorescence histochemical technique of Falck and Hillarp.

2. After incubation in solutions of noradrenaline, fluorescence developed in cellular structures (tracheal smooth muscle, vascular smooth muscle and endothelium, fibroblasts and chondroblasts), in the intercellular matrix of the cartilage and throughout the loose connective tissue of the adventitia and submucosa.

3. The effect of various experimental procedures on the development of this fluorescence has been examined i.e. incubation with noradrenaline at reduced temperature (0° C), removal of fluorescence by washing with Krebs solution at 37° C or 0° C, incubation with phenoxybenzamine (10^{-4}M) or metanephrine (10^{-4}M).

4. From these observations it has been concluded that noradrenaline accumulates in the trachea:

(a) in cellular structures where it is firmly bound, i.e. not easily removed by washing at 0° C, and where the accumulation is prevented by phenoxybenzamine, metanephrine or cold.

(b) in the intercellular matrix of the cartilage where it is also firmly bound, but where the accumulation is not prevented by phenoxybenzamine, metanephrine or cold. This probably represents binding to sulphated mucopolysaccharides.

(c) in the adventitia and submucosa where it is loosely bound and easily removed by washing.

5. Some implications of these findings in pharmacological experiments with guinea-pig trachea are discussed.

Introduction

A number of histochemical studies have shown that, after exposure of a wide range of tissues to noradrenaline, specific fluorescence can be localized extraneuronally. This has been demonstrated in the smooth muscle of various tissues e.g. spleen, colon, vas deferens, and arteries (Avakian & Gillespie, 1968 ; Gillespie, Hamilton & Hosie, 1970 ; Gillespie & Muir, 1970) and in cardiac muscle, fibroblasts and endothelial cells (Farnebo & Malmfors, 1969 ; Jacobowitz & Brus, 1971). The possibility that noradrenaline and other catecholamines might be accumulated

extraneuronally in the guinea-pig isolated trachea was suggested by Chahl & O'Donnell (1967) when attempting to explain the position of dose-response lines obtained using high concentrations of these amines.

In the experiments to be described in this paper in which the fluorescence histochemical technique of Falck & Hillarp was used, noradrenaline has been visualized in extraneuronal sites in guinea-pig trachea as a green-yellow fluorescence. The use of various procedures, i.e. incubation at reduced temperature, measurement of rate of loss of fluorescence, effects of drugs which are known to affect extraneuronal uptake in other tissues (phenoxybenzamine and metanephrine), has enabled us to conclude that extraneuronal noradrenaline is both firmly and loosely bound to tracheal structures and that this binding is of 3 distinct types: (1) loosely-bound noradrenaline associated with the extracellular structures of loose connective tissue (2) firmly-bound noradrenaline associated with the intercellular matrix of the cartilage and (3) firmly-bound noradrenaline associated with cellular structures such as smooth muscle and fibroblasts.

A preliminary account of this work was communicated to the Fifth International Congress on Pharmacology, San Francisco, July 23–28, 1972.

Methods

Tracheae were removed from adult female guinea-pigs, weighing 350–550 g, which had been killed by a blow on the head. Extraneous tissue was carefully cleared and each trachea was cut into 5 rings. In all experiments tracheal rings were first washed for 30 min in Krebs solution at 37° C oxygenated with a mixture of 95% O_2 and 5% CO_2. Tissues were then incubated for 15 min either in further Krebs solution or in Krebs solution containing the appropriate concentration of noradrenaline and 0·1 mg/ml ascorbic acid at either 37° C or at 0° C. The solutions were kept stirred and oxygenated by 95% O_2 and 5% CO_2. In some experiments, following the noradrenaline incubation, rings were then rinsed briefly in Krebs solution at 37° C for 5–10 s (referred to as dipped). In other experiments rings were washed for 30 min in 2 changes, each of 10 ml, of oxygenated Krebs solution maintained at 0° C. These two types of experimental procedure enabled a distinction to be made between noradrenaline which was firmly bound and that which was loosely bound to tissue structures. In experiments where the rate of loss of fluorescence was studied, rings were washed for 2, 10, or 30 min in Krebs solution at either 37° C or 0° C after incubation with noradrenaline at 37° C. In most of these experiments the 10 ml of Krebs washing solution was changed every 5 min during the 30 min period. In experiments where the tissues were exposed to phenoxybenzamine (10^{-4}M) or metanephrine (10^{-4}M), these drugs were included in the Krebs solution during the whole experiment, i.e. during pre-incubation washing, the incubation with noradrenaline and subsequent washing.

After these various procedures the tracheal rings were prepared for the histochemical localization by noradrenaline by means of the fluorescence technique introduced by Falck and Hillarp (Falck, 1962). The rings were rapidly frozen in isopentane cooled with liquid nitrogen, freeze-dried and exposed to formaldehyde vapour at 80° C and 70% relative humidity for 1 hour. They were then infiltrated and embedded in Paraffin Embedding Compound (Will Scientific Incorporation, U.S.A.). Cross sections (10 μm) through the trachea were examined with a Leitz Ortholux fluorescence microscope with a BG12 3 mm excitation filter, a Leitz

530 nm barrier filter and a dark field condenser (Tiyoda). Photographs were taken with the Leitz Orthomat camera on Kodak Tri-X film and all films were developed and printed under constant conditions so that photographs could be compared. In some experiments a photometric measurement of the degree of fluorescence was obtained by the method of Gillespie & Hamilton (1966). The areas selected for comparative fluorescence intensity measurements were chosen so as not to include any visible adrenergic nerves. They contained only smooth muscle cells, intercellular matrix of the body of the cartilage, epithelial cells or an area of adventitia containing mainly connective tissue elements i.e. without visible blood vessels, glands or nerves. The area of tissue to be measured was enlarged (X312·5) so as to fill the small central region in the microscope eye-piece. The detail setting was used on the camera and the exposure time then obtained from the automatic exposure meter by the use of a stop-watch. The reciprocal of the exposure time (in s) multiplied by a constant factor of 100 was used as an indication, in arbitrary units, of fluorescence brightness. Fluorescence brightness of each structure was estimated by measuring it in five different sections mounted on the same slide. The mean and standard error of these five readings were calculated and the means compared by Student's *t* test. The light microscope was used to examine sections of tracheae after staining with toluidine blue (Padawer, 1959).

The following drugs were used: 3,4-dimethoxy-5-hydroxybenzoic acid (Regis Chemical Company), (±)-metanephrine hydrochloride (Calbiochem), nialamide (Pfizer), (−)-noradrenaline bitartrate (Sigma), phenoxybenzamine hydrochloride (Smith, Kline and French). The composition of the Krebs solution was (per litre) NaCl 6·6 g, KCl 0·35 g, $CaCl_2$ 0·28 g, K_2HPO_4 0·162 g, $MgSO_4 \cdot 7H_2O$, 0·297 g, $NaHCO_3$ 2·1 g, dextrose 2·08 g.

Results

The accumulation of noradrenaline fluorescence and its loss on washing

After incubation of tracheal rings in concentrations of noradrenaline of 5×10^{-5}M, 5×10^{-4}M or 5×10^{-3}M, specific, green-yellow fluorescence was seen in various extraneuronal sites i.e. tracheal and vascular smooth muscle, chondroblasts and intercellular matrix of the cartilage, and the adventitia and submucosa. The quantitative method was too insensitive to show a very significant increase in fluorescence at the concentration of 5×10^{-5}M noradrenaline (Fig. 1), but it was subjectively convincing. No extraneuronal fluorescence was observed after incubation of rings in either 5×10^{-7}M or 5×10^{-6}M noradrenaline and rings incubated in these concentrations were indistinguishable from the controls. The only sites which exhibited specific fluorescence in these tissues were the adrenergic nerves, the location of which have been previously described by O'Donnell & Saar (1972).

Tracheal smooth muscle

The intensity of the fluorescence brightness which developed in tracheal smooth muscle increased as the concentration of noradrenaline was increased (Figure 1). The fluorescence was located throughout the smooth muscle and was only slightly reduced when tissues were washed for 30 min in cold (0° C) Krebs solution after the noradrenaline incubation (Figure 2). If the washing in Krebs solution was carried out at 37° C the fluorescence brightness was rapidly decreased (Figures 1, 2).

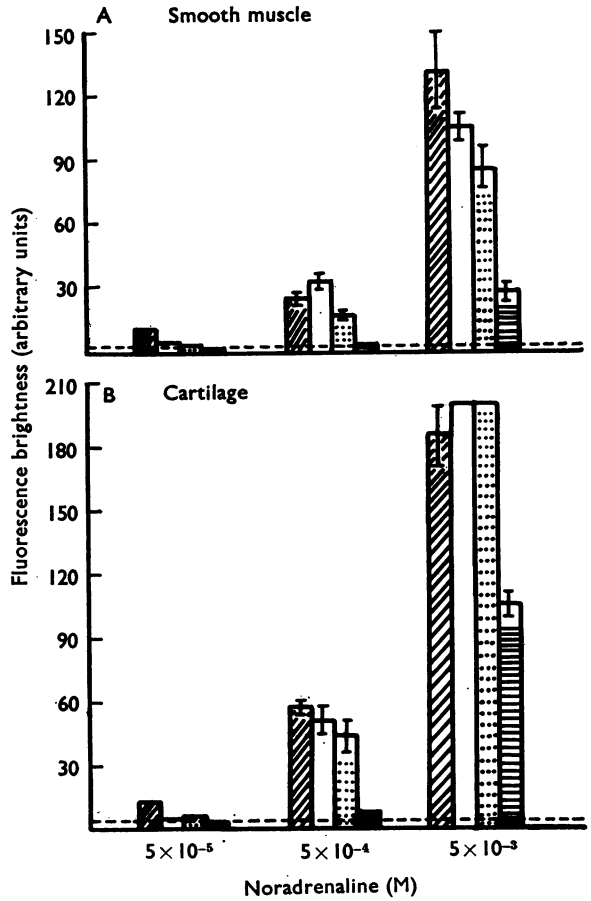

FIG. 1. The fluorescence brightness in arbitrary units in A. smooth muscle and B. intercellular matrix of the body of the cartilage of tracheal rings which were incubated in 3 different concentrations of noradrenaline and then dipped in Krebs solution (▩) or washed in 10 ml Krebs solution at 37° C for 2 (□) or 10 min (⁝⁝⁝) or in 2×10 ml for 30 min (▤). The horizontal dashed line is the fluorescence brightness measured in the smooth muscle and cartilage of a tracheal ring which had not been incubated in noradrenaline. The brightness was dependent on the concentration of noradrenaline. Fluorescence was cleared gradually with increasing time of washing but the extent of clearance was dependent on the concentration of noradrenaline. The vertical bars represent 1 standard error of the mean. Standard errors which were $<1{\cdot}5$ are not shown.

Tracheal cartilage

Fluorescence developed in the intercellular matrix of the cartilage, in chondroblasts but not in chondrocytes (Figure 3). The brightness in the intercellular matrix increased as the concentration of noradrenaline in the incubation medium increased (Figure 1). Washing in cold Krebs solution after incubation in noradrenaline cleared much more fluorescence from the intercellular matrix of the tip of the cartilage than from the body (cf. Figs. 5A and 5B) and revealed the bright fluorescent band in the capsule region around chondrocytes (Figure 3). The brightly fluorescent chondroblasts, which retained their fluorescence despite the washing procedure, also became clearly visible in the tip of the cartilage (Figure 5A). The fluorescence remaining in the intercellular matrix after washing at 0° C appeared to correspond

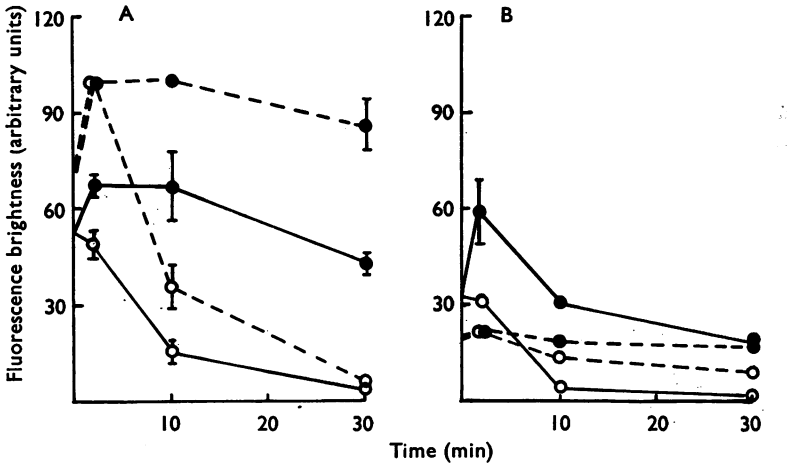

FIG. 2. The effect of time of washing in Krebs solution at 37° C or 0° C on fluorescence brightness, measured in arbitrary units, in A. tracheal smooth muscle (full lines) and intercellular matrix of the body of the cartilage (dashed lines) and B. adventitia (full lines) and epithelium (dashed lines). Rings were incubated in 5×10^{-4}M noradrenaline and washed in Krebs solution for 2 min (10 ml), 10 min (2×10 ml) or 30 min (6×10 ml) at 37° C (○) or 0° C (●). Vertical bars represent 1 standard error of the mean of 5 readings. Standard errors <1 have been omitted.

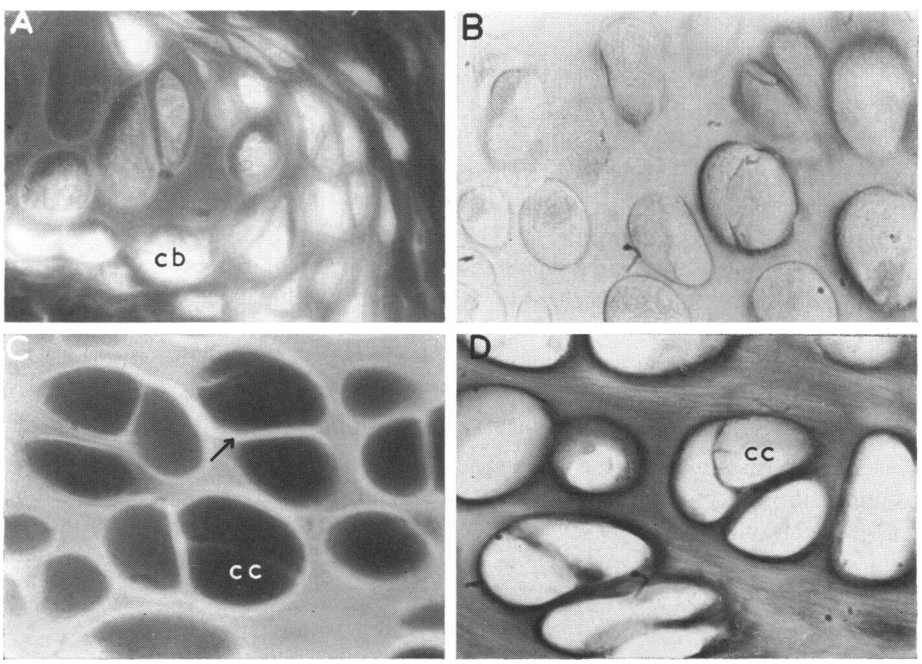

FIG. 3. Distribution of noradrenaline fluorescence and chondromucin in the tip (A and B) and in the body (C and D) of the cartilage. In A and C, a tracheal ring was incubated in 5×10^{-4}M noradrenaline and washed for 30 min in Krebs solution at 0° C. In B and D, a similar section was stained with toluidine blue. Sections show fluorescence in chondroblasts (cb) and in the capsule around chondrocytes (↗) but not in chondrocytes (cc). The purple staining seen after toluidine blue corresponded to noradrenaline fluorescence both in location and density (×1250).

in both location and brightness to the metachromatic staining of chondromucin seen after tolidine blue (Figure 3). The dark stain was concentrated in the capsule region around the chondrocytes and was darker in the body of the cartilage than in the tip.

Figure 2 compares the fluorescence brightness remaining in the intercellular matrix of the body of the cartilage of tracheal rings after washing in Krebs solution at either 0° C or 37° C. Washing at 0° C had little effect on the brightness in this region whereas there was a progressive loss of brightness after washing in Krebs solution at 37° C. However, 30 min of washing at 37° C only completely removed fluorescence from this region after incubation in a lower (5×10^{-5}M) concentration of noradrenaline. When higher concentrations (5×10^{-4}M or 5×10^{-3}M) were used, residual fluorescence was seen in both the intercellular matrix and in the chondroblasts.

Other tissue structures

In rings incubated in noradrenaline and then dipped in Krebs solution, fluorescence was seen diffusely distributed in the submucosa and adventitia, the brightness of the fluorescence being dependent upon the concentration of noradrenaline used. Fluorescence was not seen in the muco-serous glands which therefore appeared as dark areas on a brightly fluorescent background. A patchy and irregular fluorescence was occasionally seen in the tracheal epithelium but only after incubation in 5×10^{-3}M noradrenaline.

In rings incubated in noradrenaline and then washed for 30 min in cold Krebs solution, the noradrenaline which was loosely bound to the loose connective tissue of the submucosa and adventitia was removed. This revealed brightly fluorescent connective tissue cells, probably fibroblasts, and fluorescent smooth muscle and endothelial cells of blood vessels which had previously been obscured by the bright fluorescence of the loose connective tissue. Only after incubation of rings in the highest concentration of noradrenaline used (5×10^{-3}M) were the cellular structures not clearly visible because of incomplete clearance by the washing of bright fluorescence from other parts of the loose connective tissue. Staining of tracheal sections with toluidine blue revealed only 1 or 2 mast cells per section (mainly in the loose connective tissue of the submucosa and adventitia but occasionally in the mucosa) indicating that some of the fluorescent cells observed in these regions could be mast cells which had taken up noradrenaline.

In tracheal rings which were washed in Krebs solution at 37° C the fluorescence was more rapidly removed from the intercellular elements than after washing at 0° C (Fig. 2) and 30 min of washing at 37° C cleared fluorescence from all parts of the submucosa andadven titia including the cellular structures i.e. fibroblasts, vascular smooth muscle and endothelium.

Effects of phenoxybenzamine, metanephrine and cold on noradrenaline fluorescence

Tracheal rings were incubated with 5×10^{-4}M noradrenaline at 0° C instead of at 37° C or were incubated in the presence of phenoxybenzamine (10^{-4}M) or metanephrine (10^{-4}M) and were subsequently dipped or washed at 0° C. After these procedures, no fluorescence was seen in tracheal smooth muscle, in vascular smooth muscle and endothelial cells, in connective tissue cells or in

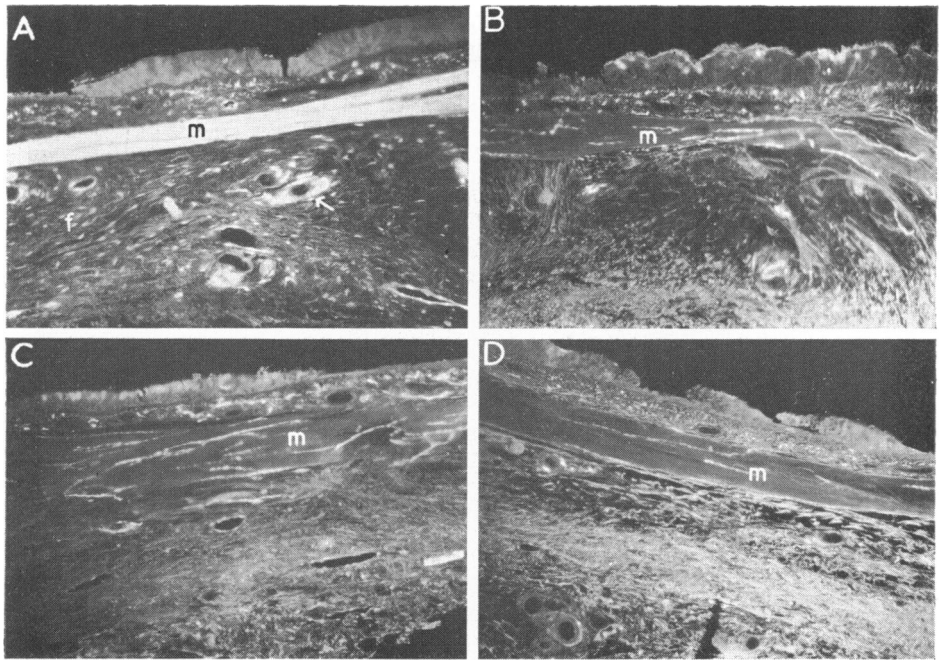

FIG. 4. The effects of phenoxybenzamine, metanephrine and cold on the accumulation of noradrenaline fluorescence in the tracheal smooth muscle. All rings were incubated in noradrenaline (5×10^{-4}M) and then washed for 30 min in Krebs solution at 0° C. Rings were incubated (A) only in noradrenaline, (B) in the presence of phenoxybenzamine (10^{-4}M), (C) in the presence of metanephrine (10^{-4}M) and (D) at 0° C instead of 37° C. In (B) (C) and (D) fluorescence is not seen in tracheal smooth muscle (m), fibroblasts (f) or vascular endothelium and smooth muscle (↗). (×125).

chondroblasts (Figure 4). Fluorescence in the intercellular matrix of the cartilage (Fig. 5) and in the adventitia and submucosa was not affected, but in the intercellular matrix of the tip of the cartilage it was enhanced (Figure 5). Measurements of fluorescence brightness of the tracheal smooth muscle and cartilage after these various treatments are compared in Table 1 and confirm the visual observations.

Inhibition of monoamine oxidase and catechol-0-methyl transferase

Tracheal rings from both untreated and nialamide-pretreated animals were incubated in various concentrations of noradrenaline (5×10^{-7}M, 5×10^{-6}M, 5×10^{-5}M, 5×10^{-4}M). The fluorescence brightness was greater in rings taken from nialamide-pretreated animals than from untreated animals. This increase in brightness was confined to the tracheal smooth muscle, fibroblasts and possibly to vascular endothelium. It was best observed after incubation in 5×10^{-5}M noradrenaline. However, the location of the sites which showed an increase in brightness was dependent on the washing procedure employed. When rings were incubated in noradrenaline and then only dipped, the increase in brightness was detected only in the tracheal smooth muscle. When the noradrenaline incubation was followed by a 30 min wash in cold Krebs solution an increase in fluorescence of other structures, such as fibroblasts and chondroblasts, was revealed.

Inhibition of catechol-*O*-methyl transferase with 10^{-5}M or 5×10^{-4}M 3,4,-dimethoxy-5-hydroxybenzoic acid, had no detectable effect on the intensity of extra-neuronal fluorescence resulting from incubation in 5×10^{-6}M, 5×10^{-5}M or 5×10^{-4}M noradrenaline.

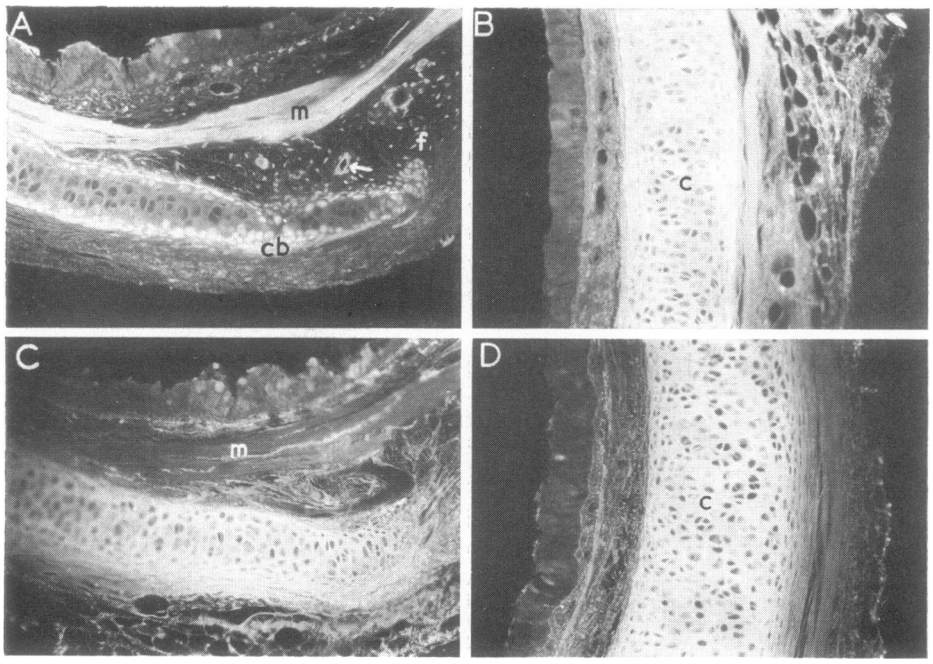

FIG. 5. Comparison of the effects of incubation of tracheal rings with 5×10^{-4}M noradrenaline at 37° C (A and B) and 0° C (C and D). Rings were then washed in Krebs solution at 0° C for 30 min. Incubation with noradrenaline at 0° C did not prevent accumulation of fluorescence in the intercellular matrix of the body of the cartilage (c) (compare B with D) but it did prevent accumulation of fluorescence in smooth muscle (m), fibroblasts (f) and vascular endothelium and smooth muscle (↗) (compare A with C). Fluorescence in chondroblasts (cb) at the tip of the cartilage (see A) was prevented by incubation at 0° C whereas fluorescence in the intercellular matrix of this tip region was enhanced (see B). Similar findings were seen in the presence of phenoxybenzamine (10^{-4}M) or metanephrine (10^{-4}M) but are not illustrated (×125).

TABLE 1. *The effects of phenoxybenzamine (PHB), metanephrine (MN) and cold (incubation at 0°C) on fluorescence brightness in tracheal smooth muscle and cartilage after incubation in noradrenaline ($5\times10^{-4}M$)*

	Fluorescence brightness (arbitrary units) after incubation in 5×10^{-4}M noradrenaline	
	Smooth Muscle	Cartilage
Inc. at 37°C	42·8±4·7	56·7±4·1
Inc. at 0°C	6·9±0·7**	48·0±5·6
Inc. with MN (10^{-4}M)	3·4±0·3**	86·7±8·2*
Inc. with PHB (10^{-4}M)	3·6±0·3**	60·7±7·0

All tracheal rings were washed in Krebs solution for 30 min at 0°C after the incubation. The individual values used were obtained by multiplying the reciprocal of the exposure time by 100. The values in the table are the mean± standard error of 5 readings from 5 different sections of trachea. * Significantly different from value at 37°C ($0{\cdot}01>P>0{\cdot}001$). ** Significantly different from value at 37°C ($P<0{\cdot}001$).

Discussion

Extraneuronal fluorescence of noradrenaline has been revealed in tracheal rings incubated in concentrations of noradrenaline of 5×10^{-5}M and above. Part of this noradrenaline fluorescence, mainly that in the submucosa and adventitia, was removed by washing in Krebs solution for 30 min at 0° C. This washing procedure was described by Gillespie & Muir (1970) and allows discrimination between noradrenaline which is loosely bound, e.g. to collagen and elastic tissue, and noradrenaline which is firmly retained by the tissue. In trachea firmly retained noradrenaline was seen in tracheal smooth muscle, vascular smooth muscle and endothelium, fibroblasts of connective tissue and in the chondroblasts and the intercellular matrix of the cartilage.

The characteristics of the noradrenaline accumulation by the smooth muscle (both vascular and tracheal) in the guinea-pig were similar to those described for other adrenergically innervated preparations (Gillespie, 1968 ; Avakian & Gillespie, 1968 ; Gillespie *et al.*, 1970) in that the accumulation was inhibited by phenoxybenzamine, metanephrine and cold. Use of these procedures to inhibit fluorescence provides some indirect evidence that accumulation at these sites might involve a transmembrane transport of noradrenaline into the cell (Gillespie, 1968). No direct evidence for this has been obtained in this study. However, the appearance of the fluorescence in tracheal muscle, in particular its distribution throughout the smooth muscle cells when these were cut in either longitudinal or transverse planes, suggested that the fluorescence was not solely due to surface binding to the basement membrane of the smooth muscle cells.

Fluorescence in connective tissue fibroblasts and in chondroblasts of trachea was also inhibited by phenoxybenzamine, metanephrine and cold. The accumulation of noradrenaline by fibroblasts has been described in rat and guinea-pig hearts and was also inhibited by these procedures (Farnebo & Malmfors, 1969 ; Jacobowitz & Brus, 1971) suggesting that noradrenaline may be taken into these cells. In the trachea it is possible that some of the fluorescent cells seen in the connective tissue could have been mast cells which had taken up noradrenaline. The presence of mast cells in the guinea-pig trachea is rarely described although guinea-pig lung is believed to have a high mast cell count (Eilbeck & Smith, 1967). The toluidine blue stain used for staining cartilage also stains mast cells but only a few mast cells were ever seen in the tracheal sections. Since guinea-pig mast cells are extremely fragile (Taichman, 1970), it is possible that they were disrupted during the procedures preparative to staining.

Inhibition of monoamine oxidase by pretreatment of the guinea-pigs with nialamide appeared to increase the brightness of extraneuronal noradrenaline fluorescence in tracheal smooth muscle and fibroblasts. However, no change in brightness was detected in preliminary experiments where catechol-*O*-methyl transferase was inhibited. This may indicate the presence of monoamine oxidase in the trachea, possibly located extraneuronally as well as in nerves. Further experiments are required to establish the location, activity and possible role for monoamine oxidase in relation to extraneuronal accumulation of noradrenaline in this tissue and to clarify whether catechol-*O*-methyl transferase plays any role in the metabolism of catecholamines in this tissue.

The difference in behaviour between the chondroblasts of cartilage, which accumulated noradrenaline, and the chondrocytes, which did not, may reflect the

metabolic activity of the cells. The immature chondroblasts are actively metabolizing and dividing cells whereas the mature chondrocytes are less metabolically active. This difference may be of significance if preparations of tracheae are being used which contain a high proportion of chondroblasts, e.g. from very young animals, since a greater amount of noradrenaline may accumulate in the cartilage.

Noradrenaline fluorescence in the intercellular matrix of the cartilage was not removed by washing in Krebs solution at 0° C. In this respect it resembled the firmly-retained accumulation in cellular sites, namely smooth muscle, chondroblasts and fibroblasts. Whereas accumulation in the cellular sites was inhibited by phenoxybenzamine, metanephrine and cold, that in the intercellular matrix of cartilage was not and thus in this respect it was similar to accumulation at other extracellular sites, e.g. collagen and elastic tissue (Avakian & Gillespie, 1968; Gillespie, 1968; Gillespie *et al.*, 1970). However, the fluorescence in the intercellular matrix of the cartilage was not readily removed by washing in Krebs solution at 37° C, a procedure which easily clears other extracellular fluorescence. The difference between the characteristics of the extraneuronal accumulation of noradrenaline in the cartilage and in the loose connective tissue of trachea might be due to the type of mucopolysaccharide present in the ground substance. In the cartilage there are large amounts of the sulphated mucopolysaccharide, chondromucin. Since hyaline cartilage was not present in tissues previously studied histochemically for extraneuronal accumulation of noradrenaline it was thus of interest to locate, by staining, the sulphated mucopolysaccharide of tracheal cartilage. The dark staining corresponded to the sites in the cartilage where bright fluorescence had remained after washing at 0° C. Staining was particularly dark in the capsule region around the chondrocytes, where chondromucin is concentrated (Leeson & Leeson, 1970), and this corresponded to the location of particularly bright fluorescence of noradrenaline. Similarly, there was less chondromucin staining in the intercellular matrix at the tip of the cartilage than the body and this corresponded to less bright noradrenaline fluorescence at the tip than in the body of the cartilage. It is possible that this could result from a more favourable surface area to volume ratio or smaller diffusion distance in the tip region allowing easier clearance of fluorescence. However, the findings suggest that the firmly-retained extracellular accumulation of noradrenaline in tracheal cartilage may involve binding to the sulphuric (HSO^{3-}) radical of chondromucin and that the bond is firmer than that with the hyaluronic acid of loose connective tissue.

The results of this work, whilst not providing any information on a possible physiological role for extraneuronal uptake in the guinea-pig trachea, may have certain implications in pharmacological experiments on this tissue. The present study has demonstrated that, at concentrations of noradrenaline of 5×10^{-5}M and greater, various sites in the trachea exhibit fluorescence as a result of accumulation of noradrenaline. It is possible that extraneuronal accumulation occurs in this tissue at even lower concentrations of noradrenaline and that the failure to detect it is due to lack of sensitivity of the fluorescence histochemical technique and/or metabolism of noradrenaline, subsequent to its accumulation, by degradative enzymes present in extraneuronal tissue. Although a concentration of 5×10^{-5}M noradrenaline is relatively high compared with normal physiological conditions,

such concentrations are often reached in pharmacological experiments on isolated tissues, e.g. when dose-response lines in the presence of β-adrenoceptor blocking drugs are obtained. If noradrenaline is accumulated extraneuronally the concentration of amine reaching the receptor sites will be reduced and this could alter the position and/or slope of the dose-response lines. This possibility should be taken into account in the interpretation of dose-response lines for noradrenaline on this preparation. This could also apply to other sympathomimetic amines, e.g. adrenaline and isoprenaline, which have a high affinity for $Uptake_2$ in other tissues (Iversen, 1965; Callingham & Burgen, 1966), and in addition may also have an affinity for the additional uptake (by tracheal cartilage) which does not have the same characteristics of $Uptake_2$.

Our findings may also help in the interpretation of results obtained with [^{3}H]-noradrenaline uptake into this tissue. Foster & O'Donnell (1972) found that the noradrenaline uptake into trachea which was firmly retained after washing for 30 min in Krebs solution at 37° C, showed properties characteristic of a membrane active transport system similar to those described by Iversen (1963) for $Uptake_1$ in the rat heart. This uptake in trachea was assumed to be into nerves. It obeyed saturation kinetics of the Michaelis-Menten type but only over a 25 fold concentration range. Above a concentration of noradrenaline of 5×10^{-7}M the kinetics were not obeyed in that the plot of S/v against S (where S is concentration of noradrenaline and v is the initial rate of uptake) appeared to become horizontal. The washing time of 30 min used was assumed to clear the extracellular space and extraneuronally-bound noradrenaline. Less than 30 min washing would appear to be adequate in heart preparations (Jonsson & Sachs, 1971) but, the retention properties of trachea for noradrenaline differ from those of heart and the present histochemical results have suggested that 30 min washing, even at 37° C, might not completely clear extraneuronally located noradrenaline from the cartilage of the trachea, particularly from the chondroblasts and intercellular matrix in the body of the cartilage. Thus this firmly-bound noradrenaline, sequestered by the tracheal cartilage, could affect the estimated initial rate of uptake of noradrenaline into nerves and more than 30 min of washing may be necessary to remove it.

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