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# THE UPTAKE KINETICS AND METABOLISM OF EXTRANEURONAL NORADRENALINE IN GUINEA-PIG TRACHEA AS STUDIED WITH QUANTITATIVE FLUORESCENCE MICROPHOTOMETRY

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**1** Extraneuronal uptake of noradrenaline and α-methylnoradrenaline into single cells of guinea-pig tracheal smooth muscle have been studied by means of quantitative fluorescence microphotometry.

**2** Fluorescence brightness due to accumulation of α-methylnoradrenaline was dose-dependent and was increased by the catechol-*O*-methyltransferase inhibitor drugs, tropolone or β-thujaplicin (200 μM) but not by 3,4-dimethoxy-5-hydroxybenzoic acid (200 μM).

**3** Fluorescence brightness due to accumulation of noradrenaline was increased if animals were pretreated with the monoamine oxidase inhibitor drug, nialamide.

**4** The study suggests that metabolism of amines by catechol-*O*-methyltransferase and monoamine oxidase can occur subsequent to extraneuronal uptake in guinea-pig tracheal smooth muscle.

**5** The uptake of noradrenaline into tracheal smooth muscle was concentration-dependent, saturable, and had a $K_m$ of 156 μM.

## Introduction

In a previous study (O'Donnell & Saar, 1973b) the fluorescence histochemical technique was used to study the localization and some of the properties of the extraneuronal accumulation of noradrenaline in guinea-pig trachea. In that study, it was not possible with the qualitative methods used to detect any effect of 3,4-dimethoxy-5-hydroxybenzoic acid (DHBA), used as an inhibitor of catechol-*O*-methyltransferase (COMT), on the intensity of fluorescence to noradrenaline even though Buckner, Birnbaum & O'Connor (1974) have reported that inhibition of COMT with tropolone potentiated responses to isoprenaline on guinea-pig trachea. In the present study quantitative microphotometry has been used to study in the smooth muscle of guinea-pig trachea (a) the effects of three COMT inhibitor drugs on the accumulation of α-methylnoradrenaline, which is not a substrate for monoamine oxidase (MAO), (b) the effects of the monoamine oxidase inhibitor drug, nialamide, on the accumulation of noradrenaline; and (c) the kinetics of the accumulation of noradrenaline.

## Methods

All guinea-pigs (280–350 g) were pretreated with 6-hydroxydopamine (6-OHDA, 50 mg/kg i.v., 24 h previously). Experiments on tracheal rings were carried out essentially as described by O'Donnell & Saar (1973b) with a 30 min washing period in Krebs solution at 0 to 2°C after an incubation for 15 min (unless indicated otherwise) in either α-methylnoradrenaline or noradrenaline. When COMT inhibitor drugs were used these were included in the Krebs solution throughout the experiment. Some animals were pretreated with nialamide (100 mg/kg, i.p., 4 h before the experiment).

The fluorescence histochemical technique used was that previously described (O'Donnell & Saar, 1973a) except that a Ploem incident light vertical illuminator was used. Fluorescence intensity was measured with a Leitz MPV microphotometer fitted to the Ortholux microscope. The exciting light was the 405 nm line of an HBO 100 W mercury vapour lamp isolated by a Leitz 3 mm BG12 excitation filter, a heat absorbing filter and a red suppression filter (BG 38). The light passed through the vertical illuminator containing a dichroic beam splitting mirror TK455 and built-in suppression filter K460. A K490 suppression filter was also placed in the filter slide. The output from the MPV was recorded on a digital voltmeter linked to an automatic printer.

A small area (1.2 μm × 1.2 μm) of a tracheal smooth muscle cell was measured with a × 100 oil immersion lens, the variable diaphragm present in the MPV microphotometer and × 12.5 eyepieces. The fluorescence standard used was a slice of uranium glass mounted on a microscope slide with a grid on

the reverse side. A known point on this standard was measured frequently during the experiment to correct for variations in excitation intensity due to fluctuations in the mercury lamp. This was achieved by altering the fine adjustment on the high voltage power supply to the photomultiplier so that a predetermined standard reading was obtained on the voltmeter.

All measurements were made blind i.e. the slides were coded and the experimental treatments not revealed until all measurements were completed. Three to 6 separate measurements (one measurement being the mean of the first 5 values from the printer) were made from each section (7 $\mu$m). The number of measurements depended upon the amount of smooth muscle available and the variations between readings. Care was taken not to measure any part of a section which had previously been exposed to the exciting light. The mean value of these measurements was taken as the result for that section. Between 3 and 5 sections were measured from each tissue to give a mean value for intensity for that tissue. Results quoted represent mean intensity values for tracheal tissue from 3 to 5 different animals. All fluorescence intensity values are expressed in arbitrary units either as actual intensity measured or, in the case of the kinetic experiments, as increase in intensity over that of the background fluorescence in a control tissue.

Krebs bicarbonate solution containing ascorbic acid (200 $\mu$g/ml) was used with the following composition (mM); NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7. Drugs used were:- 3,4-dimethoxy-5-hydroxy benzoic acid (Regis Chemical Company), 6-hydroxydopamine hydrochloride (Labkemi AB), $\alpha$-methylnoradrenaline (corbasil) hydrochloride (Sterling Winthrop), nialamide (Pfizer), (−)-noradrenaline bitartrate (Sigma), $\beta$-thujaplicin (Koch Light) and tropolone (Regis Chemical Company).

## Results

### *Accumulation of α-methylnoradrenaline*

Incubation of tracheal rings in $\alpha$-methylnoradrenaline resulted in a concentration-dependent increase in fluorescence intensity in the tracheal smooth muscle, both in the absence and in the presence of tropolone 200 $\mu$M (Table 1). At each concentration of $\alpha$-methylnoradrenaline the fluorescence intensity was greater in the presence of tropolone and this was statistically significant, except for 1 $\mu$M $\alpha$-methylnoradrenaline (Table 1). As a result of this experiment two concentrations of $\alpha$-methylnoradrenaline (10 $\mu$M and 100 $\mu$M) were selected for a second experiment in which the effects of three COMT inhibitor drugs were compared. The effects of DHBA, tropolone and $\beta$-thujaplicin on the fluorescence intensity in smooth muscle are summarized in Table 2. DHBA had no significant effect on the fluorescence produced by either concentration of $\alpha$-methylnoradrenaline whereas there was an increase in brightness with tropolone or $\beta$-thujaplicin but this was statistically significant in this experiment only for $\beta$-thujaplicin and the lower concentration of $\alpha$-methylnoradrenaline.

### *Accumulation of noradrenaline*

Fluorescence brightness due to accumulation of noradrenaline in smooth muscle was significantly greater in tracheae from nialamide-pretreated animals than in controls (Table 3).

All experiments relating to the kinetic analysis of the uptake of noradrenaline by tracheal smooth muscle were carried out on tissues taken from animals pretreated with 6-OHDA and nialamide and the Krebs solution contained tropolone 200 $\mu$M to inhibit COMT. Initially the uptake with time of noradrena-

**Table 1** Effect of tropolone (200 $\mu$M) on fluorescence brightness in guinea-pig tracheal smooth muscle resulting from incubation of tracheal rings from 4 animals in various concentrations of $\alpha$-methylnoradrenaline

| *Concentration α-methyl-noradrenaline (μM)* | *Fluorescence brightness (arbitrary units)* | |
|---|---|---|
| | *No tropolone* | *Tropolone* |
| 0 (Controls) | 95 ± 4.5 | 106 ± 3.1 |
| 0.1 | 91 ± 3.6 | 115 ± 3.5* |
| 1 | 111 ± 16.7 | 124 ± 9.5 |
| 10 | 167 ± 17.6 | 264 ± 25.9* |
| 100 | 972 ± 14.5 | 1785 ± 124.1** |

Values are means ± s.e. mean ($n$ = 4).
Significant increase in fluorescence brightness in the presence of tropolone (comparison of tropolone with no tropolone, paired $t$ test): *$0.05 > P > 0.01$; **$0.01 > P > 0.001$.

line 50 to 500 μM (as shown by increase in fluorescence brightness) was studied. The uptake increased rapidly at first and was approximately linear with time up to 8 min but equilibrium was not reached for these two concentrations during the 30 min experimental period which had been selected for these experiments. The time course of the uptake of 1 mM noradrenaline was followed over 120 min (replacing the noradrenaline in the bath fluid every 10 minutes). Uptake again was linear with time up to 8 min but equilibrium was now achieved at 20 minutes. Thus, in another experiment the initial rates of uptake of noradrenaline at four concentrations (100 μM, 300 μM, 1 mM and 3 mM) were estimated from the fluorescence brightness observed after a fixed incubation time of 5 minutes. A Michaelis-Menten analysis of the results gave a straight line from which the mean estimate of the $K_m$ for noradrenaline was 156 μM (Figure 1).

## Discussion

In a previous study (O'Donnell & Saar, 1973b), inhibition of COMT appeared to have no effect on the extraneuronal accumulation of noradrenaline in guinea-pig tracheal smooth muscle. In that study only one COMT inhibitor drug was examined, DHBA, which had been shown by Nikodejevic, Senoh, Daly & Creveling (1970) to be a COMT inhibitor. In the present study, the effects of DHBA have been re-examined using a quantitative fluorescence microscopic technique and in addition the effects of two other inhibitors of COMT have been examined viz. tropolone and β-thujaplicin (Belleau & Burba, 1963). Furthermore the accumulation of α-methylnoradrenaline was studied instead of that of noradrenaline. Since α-methylnoradrenaline is not a substrate for MAO (Ross, 1976) the effects of COMT inhibition could be studied without the added complication of achieving inhibition of all the MAO. The qualitative appearance of fluorescence in tracheal smooth muscle after α-methylnoradrenaline was similar to that previously described for noradrenaline (O'Donnell & Saar, 1973b) and the accumulation was concentration-dependent.

DHBA in a concentration of 200 μM had no effect on the fluorescence brighness due to α-methylnor-

**Table 2** Effect of catechol-*O*-methyltransferase (COMT) inhibitor drugs (200 μM) on fluorescence brightness in guinea-pig tracheal smooth muscle after incubation of tracheal rings from 4 animals in 10 μM or 100 μM α-methylnoradrenaline

| *COMT inhibitor* | *Fluorescence brightness (arbitrary units) after incubation in α-methylnoradrenaline (μM)* | | |
|---|---|---|---|
| | 0 | 10 | 100 |
| No COMT inhibitor | 33 ± 6.0 | 58 ± 2.7 | 343 ± 20.8 |
| Dimethoxyhydroxybenzoic acid | | 46 ± 4.5 | 322 ± 36.5 |
| Tropolone | | 91 ± 18.0 | 474 ± 51.6 |
| β-Thujaplicin | | 106 ± 8.3* | 683 ± 143.3 |

Values are means ± s.e. mean ($n = 4$).
* Significant increase in brightness over tissues with no COMT inhibitor (paired $t$ test, $0.05 > P > 0.01$).

**Table 3** Comparison of fluorescence brightness in guinea-pig tracheal smooth muscle after incubation of tracheal rings from 5 non-treated and 5 nialamide-pretreated animals in noradrenaline. Tropolone (200 μM) was present in the Krebs solution throughout

| | *Fluorescence brightness (arbitrary units) after incubation in noradrenaline (μM)* | | |
|---|---|---|---|
| | 0 | 100 | 500 |
| No nialamide | 0.79 ± 0.02 (3) | 51 ± 3.7 (5) | 105 ± 17.5 (5) |
| Nialamide | | 345 ± 29.2*** (5) | 730 ± 52.5*** (5) |

Values are means ± s.e. mean ($n = 5$ except for control rings (no noradrenaline) where only 3 rings were measured).
*** Significant increase in brightness in comparison with no nialamide animals (Students $t$ test, $P < 0.001$).

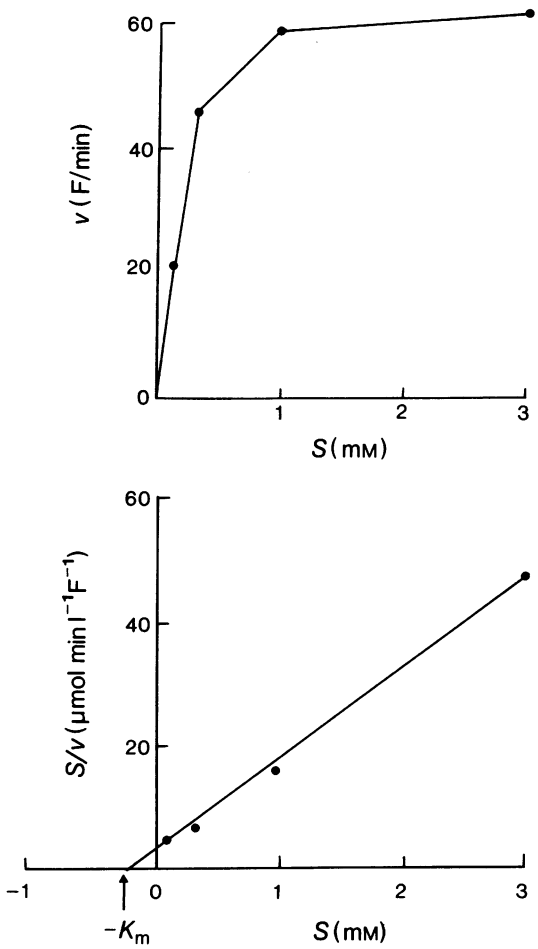


**Figure 1** Estimation of $K_m$ for uptake of noradrenaline by tracheal smooth muscle by use of a linear transformation of the Michaelis-Menten equation. F is fluorescence in arbitrary units, $S$ = concentration of noradrenaline, $v$ = initial rate of uptake (estimated from fluorescence brightness after 5 min incubation in noradrenaline). $K_m$ is found from the intercept on the abscissa scale and was 156 μM. Values are means from 5 animals. In this experiment animals were pretreated with 6-hydroxydopamine and nialamide, and tropolone 200 μM and ascorbic acid 400 μg/ml were present in the Krebs solution.

adrenaline. This confirmed the previous findings with this COMT inhibitor drug and noradrenaline (using tissues from nialamide-treated animals). However, an increase in fluorescence brightness could be detected with 200 μM of either tropolone or β-thujaplicin although this increase was not always statistically significant with the relatively small group of animals used. This suggests that COMT metabolism can occur subsequent to extraneuronal uptake in guinea-pig tracheal smooth muscle.

Gillespie (1975) expressed surprise that inhibition of MAO should be more effective in increasing the accumulation of noradrenaline in smooth muscle than inhibition of COMT. Thus the effects of nialamide on noradrenaline fluorescence were re-examined in the present study by quantitative fluorescence microscopy. The results obtained qualitatively in the previous study (O'Donnell & Saar, 1973b) were confirmed i.e. there was clearly an increase in noradrenaline fluorescence in the smooth muscle of tracheae from the treated animals. Tropolone was present during the experiments to inhibit breakdown by COMT. In another study pargyline, the MAO inhibitor drug, caused an increased extraneuronal accumulation of noradrenaline in cat spleen (Blakeley, Powis & Summers, 1973). It has been suggested that the enhancement of fluorescence in both these tissues might be unrelated to MAO inhibition (Gillespie, 1975). In the case of nialamide, this drug has been shown to be a weak inhibitor of neuronal uptake (Maxwell, Ferris & Burcsu, 1976) but in the present experiments all animals were pretreated with 6-OHDA. Thus noradrenaline could not have been diverted from neuronal to extraneuronal uptake. In some experiments in which guinea-pig tracheae were stained for MAO (Saar, unpublished results) staining was present in the smooth muscle cells. In other studies on smooth muscle, using fluorescence histochemistry, both MAO and COMT have been inhibited at the same time. In rabbit ear artery, Burnstock, McLean & Wright (1971) found that the concentration threshold at which accumulation (fluorescence) of noradrenaline occurred was reduced by inhibition of MAO and COMT whereas Gillespie (1975), using the same preparation, found that enzyme inhibition did not greatly alter the threshold nor markedly increase the accumulation of noradrenaline. Thus aspects of the relative importance of COMT and MAO in various smooth muscles remain to be clarified under standard conditions and taking into account species, particularly since it has been shown in heart muscle that MAO can play a more important role in metabolism subsequent to extraneuronal uptake in some species, e.g. rat, than in others, e.g. cat (Trendelenburg, 1976).

Kinetic analysis of the uptake of noradrenaline by guinea-pig trachea showed that it was concentration-dependent and that the uptake process was a saturable one, obeying Michaelis-Menten kinetics with a $K_m$ of 156 μM. This value is close to the value of 490 μM found by Gillespie & Towart (1973) using the same technique in the smooth muscle of the rabbit ear artery but without the presence of enzyme inhibitors. It is also close to the value of 260 μM obtained

for cultured bovine tracheal cells (Powis, 1973) and for rat heart of 252 $\mu$M using tritiated noradrenaline (Iversen, 1965). This indicates that an uptake process with a similar affinity for noradrenaline is being studied in each tissue. In addition, the results presented on tracheal muscle in this study support the suggestion of Gillespie (1975) that the technique of quantitative fluorescence microscopy can be applied to a kinetic analysis of the extraneuronal uptake process in single smooth muscle cells.

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