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The accumulation and metabolism of (—)-noradrenaline by cells in culture

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Summary

1. Cultured bovine embryonic tracheal cells, EbTr (NBL-4) possess a process for the intracellular accumulation of (−)-noradrenaline with the characteristics generally ascribed to extraneuronal uptake by cardiac and smooth muscle cells in the body. It has a K_m of $2{\cdot}6 \times 10^{-4}$M.

2. The accumulation process is inhibited competitively by normetanephrine, but only at relatively high concentrations, IC50$=2{\cdot}1 \times 10^{-4}$M. Inhibition also occurs with 17-β-oestradiol, IC50$=1{\cdot}3 \times 10^{-5}$M.

3. The noradrenaline metabolites, 3,4-dihydroxy-mandelic acid and 3,4-dihydroxy-phenylglycol potentiate accumulation and reduce intracellular levels of normetanephrine in a similar manner to known inhibitors of catechol-*O*-methyl transferase.

4. It is suggested that intracellular, rather than extracellular, normetanephrine may exert feedback inhibition upon noradrenaline accumulation by combining with the transport process at the inner surface of the cell membrane.

Introduction

The extraneuronal uptake of catecholamines has been described in cardiac muscle (Iversen, 1965a) and in smooth muscle from many tissues (Avakian & Gillespie, 1968 ; Gillespie & Muir, 1970 ; Burnstock, McLean & Wright, 1971). This process, commonly designated Uptake$_2$ (Iversen, 1965a), is characterized by a low affinity and a high maximum rate of uptake when compared with uptake into sympathetic neurones.

The present communication describes the accumulation of (−)-noradrenaline by cultured smooth muscle cells, investigates some of the kinetic parameters and studies the effects of drugs and of metabolites of noradrenaline on the accumulation process.

Methods

Cultured bovine embryonic tracheal cells EbTr (NBL-4) were obtained from Flow Laboratories, Irvine, as a suspension of 10^6 cells per ml, delivered and stored for not more than 24 h before use at 4° C. At least 95% of the cells have a morphology typical of smooth muscle cells and viability of 90% after 48 h at 4° C (Flow Laboratories). The cells (2×10^6) were suspended in 2·5 ml Hanks' balanced salt solution (Hanks & Wallace, 1949) containing drugs and inhibitors as required. ^{3}H-(−)-Noradrenaline (5·8 mCi/μmol, Radiochemical Centre, Amersham) diluted with unlabelled (−)-noradrenaline in 0·5 ml Hanks' solution was added to give a

concentration of 10^{-4} M, unless otherwise stated. The tubes were incubated at 37° C in a shaking water bath usually for 30 min and then the cells were collected by centrifugation for 5 min at 3,000 × *g*, at 4° C. The supernatant was decanted and the packed cells washed with 4 × 3 ml ice cold Hanks' solution before being lysed with 1 ml cold 0·01 N HCl. A sample (0·5 ml) of this lysate was taken for liquid scintillation counting, after being digested with 0·2 ml hyamine hydroxide, 1·0 M in methanol. Samples were corrected for quenching with an internal standard. All incubations were performed in at least triplicate and corrected for a zero time blank.

Aliquots of the original supernatant and of the lysate were taken and noradrenaline and metabolites separated by two dimensional thin layer chromatography on 80 μm cellulose plates (Macherey, Nagel MN 300 HR) according to the method of Fleming & Clark (1970). In our hands the lower limit for the detection of metabolites by this method is 0·02% of the total activity.

Metabolites have been classified as those resulting from the activity of *S*-anedosyl-methionine: catechol-*O*-methyl transferase (COMT) namely normetanephrine, those resulting from the activity of monoamine: oxygen oxidoreductase (deaminating) (MAO) namely 3,4-dihydroxy-mandelic acid and 3,4-dihydroxy-phenylglycol, and those due to the combined activity of COMT and MAO, 4-hydroxy-3-methoxy-mandelic acid and 4-hydroxy-3-methoxy-phenylglycol.

Drugs used were pargyline hydrochloride (Abbott Laboratories), 3,4-dimethoxy-5-hydroxy benzoic acid (Nipa Fine Chemicals), β-thujaplicin (Koch-Light), 17-β-

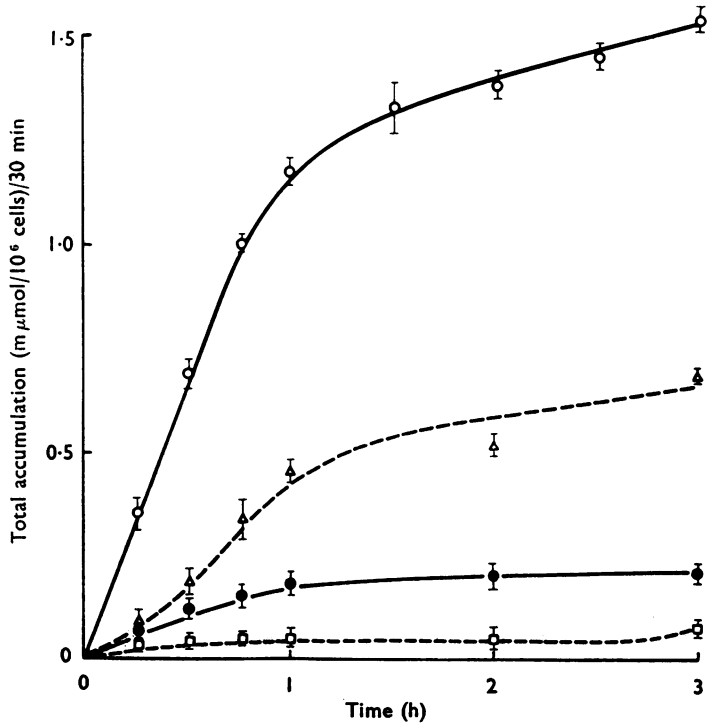

FIG. 1. Time course of the accumulation of (—)-noradrenaline and its subsequent metabolism by EbTr (NBL-4) cells in culture. ●, Accumulation at 4° C; ○, accumulation at 37° C. Metabolites are not detectable at 4° C. □, Monoamine oxidase metabolites at 37° C; △, catechol-*O*-methyl transferase metabolites at 37° C. Bars represent S.E.M., $n=4$.

oestradiol (Sigma Chemical Co.), desmethylimipramine hydrochloride (Geigy) and metaraminol bitartrate (Merck, Sharp & Dohme).

Results

Time-course of the accumulation of noradrenaline

The time-course of the accumulation of noradrenaline and its subsequent metabolism is shown in Figure 1. The major intracellular metabolite is normetanephrine which accounts for 64% of all intracellular metabolites after 30 min and 80% after 3 hours. The incubation medium itself contains negligible amounts of metabolites even after 3 hours.

In all further studies accumulation will refer to the intracellular accumulation of both noradrenaline and its metabolites over a 30 min period, unless otherwise stated.

Effect of metabolites upon the accumulation process

The effect of noradrenaline metabolites added to the incubation medium is shown in Figure 2. Both metabolites resulting from the activity of MAO potentiate accumulation, normetanephrine which results from the activity of COMT inhibits accumulation, whilst those metabolites resulting from the activity of both MAO and COMT have little effect.

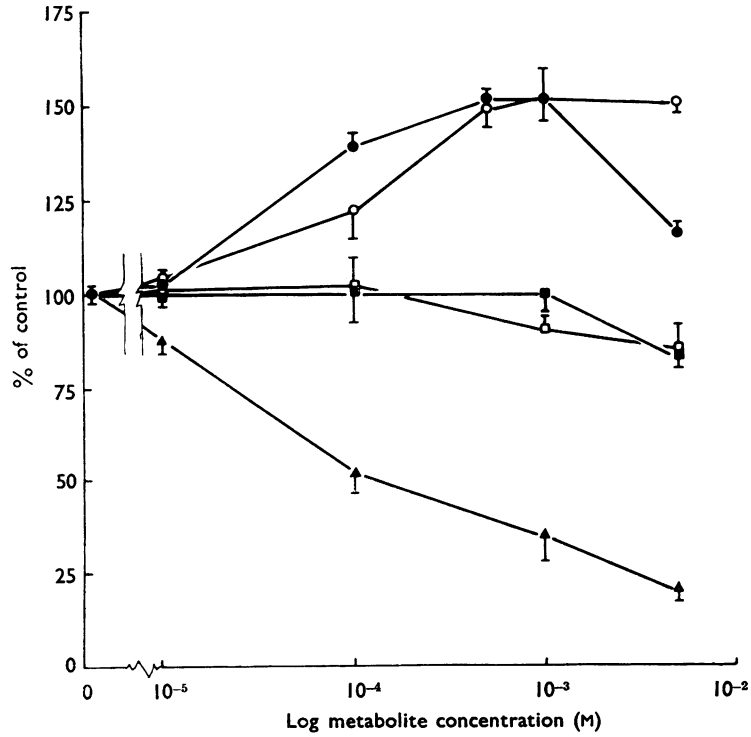

FIG. 2. Effects of metabolites upon the accumulation of (−)-noradrenaline by EbTr (NBL-4) cells in culture. ○, 3,4-Dihydroxy-mandelic acid; ●, 3,4-dihydroxy-phenylglycol; □, 4-hydroxy-3-methoxy-mandelic acid; ■, 4-hydroxy-3-methoxy-phenylglycol; ▲, normetanephrine. Each point represents the mean ± S.E.M. of 3 determinations.

Table 1 shows the change in the pattern of radioactively labelled metabolites in the presence of the exogenous metabolites. It appears that both 3,4-dihydroxy-mandelic acid and 3,4-dihydroxy-phenylglycol added to the incubation medium significantly depress the levels of intracellular normetanephrine, possibly by competitive inhibition of the enzyme COMT. Neither 4-hydroxy-3-methoxy-mandelic acid nor 4-hydroxy-3-methoxy-phenylglycol cause any change in the production of radioactively labelled metabolites.

TABLE 1. *Effect of exogenous metabolites on the production of radioactively labelled metabolites*

	Metabolites %		
	COMT	MAO	COMT-MAO
Control	25·9±2·6	4·6±0·9	9·8±2·9
MAO metabolites 10^{-3} M			
3,4-Dihydroxy-mandelic acid	2·7±1·9†	5·3±1·2	5·6±1·3
3,4-Dihydroxy-phenyl-glycol	11·1±2·1†	3·2±0·9	6·7±1·0
COMT metabolite 10^{-4} M			
Normetanephrine	22·6±3·1	6·4±1·5	5·7±1·4
MAO-COMT metabolites 10^{-3} M			
4-Hydroxy-3-methoxy-mandelic acid	23·8±3·1	3·8±1·1	8·2±2·1
4-Hydroxy-3-methoxy-phenylglycol	19·7±3·0	1·8±1·2	4·5±1·9

† $P<0·01$. Concentration of (−)-noradrenaline 10^{-4} M, incubation time 30 minutes. Results are the mean of three determinations ± S.E.M., expressed as a percentage of the total radioactivity accumulated.

Effect of drugs on the accumulation and metabolism of noradrenaline

The effects of various drugs upon the accumulation and subsequent metabolism of noradrenaline are shown in Table 2. Pargyline, an inhibitor of the enzyme MAO, gives rise to the expected decline in MAO metabolites and produces a 15% increase in accumulation. The COMT inhibitor 3,4-dimethoxy-5-hydroxy-benzoic acid (Nikodejevic, Senoh, Daly & Creveling, 1970) potentiates accumulation some 57%, whilst another reported inhibitor of COMT, β-thujaplicin (Belleau & Burba, 1963) has only a marginal effect. This is possibly related to the fact that β-thujaplicin fails to reduce the levels of intracellular normetanephrine unlike 3,4-dimethoxy-5-hydroxy-benzoic acid which produces an almost complete inhibition.

The classical inhibitors of extraneuronal uptake, normetanephrine (Burgen & Iversen, 1965) and 17-β-oestradiol (Iversen & Salt, 1970) both produce an inhibition of accumulation by the cells in culture, although only at higher concentrations than

TABLE 2. *Effect of drugs on the accumulation of noradrenaline and metabolites*

	Total accumulation	(pmol/10^6 cells)/30 min Metabolites		
		COMT	MAO	COMT-MAO
Control	695·8±20·9	180·2±18·1	32·0± 6·3	68·2±20·1
Pargyline 10^{-4} M	791·0±20·1†	215·1±29·7	3·1± 2·9*	0·8± 0·3*
3,4-Dimethoxy-5-hydroxy-benzoic acid 10^{-4} M	1,091·0±52·5†	1·1± 0·4†	42·2± 7·8	28·4±13·2
β-Thujaplicin 10^{-4} M	745·0±14·8	181·0±26·4	51·4± 3·7	61·8±20·7
Desmethylimipramine 10^{-4} M	485·0±25·8†	165·5±25·8	22·7± 6·4	37·8±20·4
Metaraminol 10^{-4} M	633·0±24·8	170·3±18·7	59·5±10·9	39·2±18·3
17-β-Oestradiol 10^{-5} M	379·0±15·3†	38·7±20·8†	30·9± 5·9	1·1± 0·7*

* $P<0·05$. † $P<0·01$. Concentration of (−)-noradrenaline 10^{-4} M, incubation time 30 minutes. Results represent the mean ± S.E.M. Uptake, $n=6$; metabolites, $n=3$ pooled lysates each from 2 determinations.

have previously been reported. The inhibitor of neuronal uptake, metaraminol (Burgen & Iversen, 1965) has no effect upon the accumulation process although desmethylimipramine, also an inhibitor of neuronal uptake (Iversen, 1965b) produces a 30% inhibition at 10^{-4}M. At 10^{-5}M it has no effect.

Kinetics of the accumulation process

Figure 3 shows the results of a kinetic study upon cellular accumulation. The Michaelis constant of (−)-noradrenaline for the accumulation process is $2{\cdot}62 \times 10^{-4}$M. Normetanephrine, as might be expected, shows the characteristics of a competitive inhibitor with K_i $1{\cdot}8 \times 10^{-4}$M, whilst 17-β-oestradiol shows a complex pattern of inhibition with an increasing potency at higher concentrations of noradrenaline. Both 3,4-dihydroxy-mandelic acid and 3,4-dimethoxy-5-hydroxy-benzoic acid potentiate the accumulation process by increasing the maximum rate of accumulation whilst having no effect on the affinity of noradrenaline for the accumulation process.

Cell weight and intracellular water

The wet weight and water content of $2{\cdot}5 \times 10^6$ cells harvested by centrifugation in the usual manner was determined gravimetrically before and after drying at 110° C for 24 hours. Polyethylene-1-2-^{14}C glycol (New England Nuclear Corp.) was used as a non-adsorbed extracellular fluid volume marker. The weight of 10^6 cells is $1{\cdot}87 \pm 0{\cdot}14$ mg and they contain $1{\cdot}65 \pm 0{\cdot}12$ mg intracellular fluid, $n=6$ in both instances.

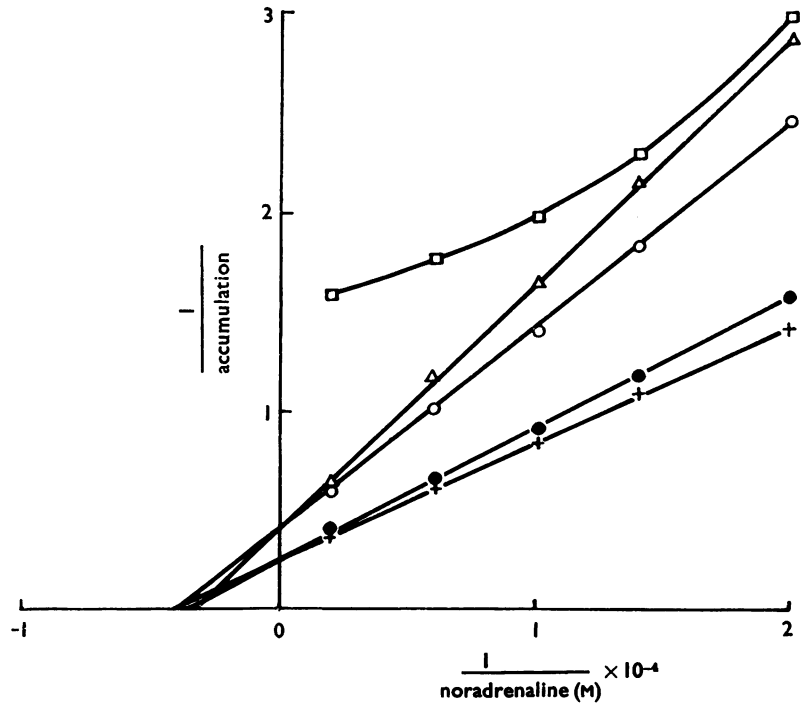

FIG. 3. Kinetics of the accumulation of (−)-noradrenaline and metabolites by EbTr (NBL-4) cells in culture. ○, Control; ●, 3,4-dihydroxy-mandelic acid, 10^{-3}M; +, 3,4-dimethoxy-5-hydroxy-benzoic acid, 10^{-4}M; △, normetanephrine, $1{\cdot}25 \times 10^{-5}$M; □, 17-$\beta$-oestradiol, 10^{-6}M.

Discussion

Isolated cells in culture appear capable of accumulating (−)-noradrenaline and this process possesses some of the characteristics generally ascribed to extraneuronal uptake of noradrenaline in the body (Table 3). (Iversen, 1965a ; Burgen & Iversen, 1965 ; Iversen & Salt, 1970).

TABLE 3. *Comparison of noradrenaline accumulation by cardiac muscle and by EbTr(NBL-4) cells in culture*

		Maximum accumulation	Inhibitors IC50	
	$Km \times 10^{-4}$ M	(nmol/min)/g	Normetanephrine	Oestradiol
Cardiac muscle	2·5[a]	100[a]	$4{\cdot}2 \times 10^{-6}$ M[b]	$1{\cdot}8 \times 10^{-6}$ M[c]
EbTr (NBL-4) cells	2·6	55	$2{\cdot}1 \times 10^{-4}$ M[d]	$1{\cdot}3 \times 10^{-5}$ M[e]

[a]—Iversen (1965a). [b]—Burgen & Iversen (1965). [c]—Iversen & Salt (1970). [d]—Measured directly from data in Fig. 2. [e]—Measured directly (data not shown).

Cultured bovine embryonic tracheal cells have a morphology typical of smooth muscle cells and Gillespie & Muir (1970) and Burnstock *et al.* (1971) have shown histochemically that the smooth muscle cells of several tissues are capable of accumulating noradrenaline.

Cells in suspension lend themselves particularly well to a study of the kinetic properties of the accumulation process. Normetanephrine added to the incubation medium appears to be a simple competitive inhibitor whilst in the presence of 17-β-oestradiol the accumulation process shows all the characteristics of substrate inhibition. It is possible that at high concentrations noradrenaline combines with 17-β-oestradiol to form a complex that will further inhibit the accumulation process. It is noticeable that 17-β-oestradiol also inhibits intracellular normetanephrine formation, possibly by inhibiting the enzyme COMT. Cohn & Axelrod (1971) have reported, however, that 17-β-oestradiol does not inhibit COMT from the liver of rat. It is not possible to explain the discrepancy in these observations other than on the basis of a species or tissue difference.

Both 3,4-dihydroxy-mandelic acid and 3,4-dimethoxy-5-hydroxy-benzoic acid potentiate accumulation in a non-competitive manner and both also inhibit the formation of intracellular normetanephrine. 3,4-Dimethoxy-5-hydroxy-benzoic acid is a known inhibitor of COMT whilst 3,4-dihydroxy-mandelic acid is a substrate for COMT and could thus inhibit by competing with noradrenaline. The lower limit for the detection of extracellular metabolites is 50 pmol. It is unlikely, therefore, that the increased accumulation caused by 3,4-dimethoxy-5-hydroxy-benzoic acid could be accounted for solely by an inhibition of the metabolism of noradrenaline after uptake. This would require at least 400 pmol of normetanephrine to be released into the incubation medium in the absence of any drugs. Such levels are unlikely to go undetected. It may be that uptake is normally a self-limiting process with inhibition occurring as the levels of intracellular normetanephrine increase. It is even possible from Fig. 1 to calculate an approximate IC50 for intracellular normetanephrine of $2{\cdot}1 \times 10^{-4}$M, if it is assumed that normetanephrine is not held in a bound form but distributed throughout the intracellular fluid. This value is close to the IC50 for extracellular noradrenaline calculated from Fig. 2 or Figure 3.

Transport of noradrenaline across the cell membrane is thought to occur in both directions (Gillespie, Hamilton & Hosie, 1970) and intracellular normetanephrine could possibly sequester the carrier molecule on the inner surface of the membrane and thus inhibit the flow of noradrenaline from outside in a non-competitive manner. This contrasts with the possibility that normetanephrine external to the cell could modulate the entry of noradrenaline (Eisenfield, Landsberg & Axelrod, 1967). It is not inconceivable that both processes could occur simultaneously. It seems probable, however, that intracellular concentrations of normetanephrine will in most cases always exceed extracellular concentrations.

It is of interest that metabolites of MAO in the incubation medium can potentiate the accumulation process although admittedly only at relatively high concentrations around 5×10^{-4}M. It is not known whether such concentrations could be reached under physiological conditions but, since similar high concentrations of noradrenaline are themselves required for extraneuronal uptake and since extraneuronal uptake does appear to occur under certain physiological conditions (Avakian & Gillespie, 1968), it is possible that high localized concentrations of metabolites could occur.

It is probable that the increased accumulation caused by pargyline simply represents a decreased conversion of intracellular noradrenaline to metabolites by MAO although pargyline has been shown to increase uptake, probably extraneuronal, of dopamine and noradrenaline by the cat spleen (Street, Farmer & Roberts, 1971; Blakeley, Powis & Summers, 1971). The available evidence suggests that this is a direct action of pargyline and not dependent upon an inhibition of MAO (Blakeley, Powis & Summers, 1973). The possibility should not be excluded, however, that the immediate product of MAO activity 3,4-dihydroxy-mandelic aldehyde could be inhibiting the accumulation process, although it is usually considered that aldehyde dehydrogenase in the cell rapidly converts this compound to the acid form.

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