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Effects of McN-A-343 and DMPP on the uptake and release of ^{3}H-noradrenaline by guinea-pig atria

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Summary

1. McN-A-343 4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride and DMPP (*N,N*-dimethyl-*N'*-phenylpiperazinium iodide) inhibit the uptake of (±)-^{3}H-noradrenaline by guinea-pig atria, being approximately as potent as cocaine in this respect.

2. The inhibition of uptake produced by McN-A-343 or DMPP was not affected by atropine or hexamethonium in concentrations which antagonized actions on muscarinic and nicotinic receptors respectively.

3. McN-A-343 in the presence of atropine had a positive inotropic action on atria, but this was not accompanied by efflux of radioactivity from atria previously incubated with (−)-^{3}H-noradrenaline.

4. In the presence of McN-A-343, responses of atria to noradrenaline were increased and those to tyramine were decreased.

5. DMPP had positive inotropic and chronotropic actions on atria, and these effects were accompanied by an increase in efflux of radioactivity from atria previously incubated with (−)-^{3}H-noradrenaline.

Introduction

Cholinomimetic drugs have effects mimicking those of sympathetic nerve stimulation in various tissues including cardiac preparations. This is best known for drugs having a nicotinic action, such as nicotine itself, acetylcholine in the presence of atropine and DMPP (*N,N*-dimethyl-*N'*-phenylpiperazinium). These drugs have been shown to release noradrenaline from cardiac tissue (Lee & Shideman, 1959; Löffelholz, 1967, 1970; Lindmar, Löffelholz & Muscholl, 1968).

The cholinomimetic drug McN-A-343 (4-(*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium chloride), in the presence of atropine, also has an effect on guinea-pig isolated atria resembling that of sympathetic nerve stimulation (Bhagat, 1966). The positive inotropic action of McN-A-343 was antagonized by dichloroisoprenaline and was absent in atria from guinea-pigs pretreated with reserpine, suggesting that it was due to release of noradrenaline; however, it was not affected by concentrations of hexamethonium which abolished the actions of DMPP, indicating that it was not due to an action on nicotinic receptors that was unmasked by atropine. McN-A-343 stimulates sympathetic ganglion cells by acting on muscarinic receptors, this effect being antagonized by atropine (Roszkowski, 1961; Smith, 1966). It also acts on muscarinic receptors of the guinea-pig taenia caeci (Hobbiger, Mitchelson & Rand, 1969). In the isolated artery of the

rabbit ear, McN-A-343 causes a decrease in responses to sympathetic nerve stimulation at low frequencies, and this too is a muscarinic action being abolished by atropine (Rand & Varma, 1971). However, McN-A-343 causes increases in responses to high frequencies of stimulation and, in the presence of atropine, to stimulation at low frequencies. These findings suggested that McN-A-343 might facilitate noradrenaline release; alternatively, they could be due to inhibition of re-uptake of noradrenaline by McN-A-343.

The present experiments are concerned with a comparison of the effects of DMPP and McN-A-343 on the uptake and release of tritiated noradrenaline by isolated atria of the guinea-pig. A preliminary account was communicated to the Australian Society of Clinical and Experimental Pharmacologists, November, 1970 by Allen, Story & Varma (unpublished).

Methods

Guinea-pigs of 300–500 g were killed by cervical dislocation; they were exanguinated and the hearts rapidly removed. The atria were dissected free and suspended in a 10 ml organ bath containing Krebs-Henseleit solution of the following composition: NaCl, 118 mM; KCl, 4·7 mM; $NaHCO_3$, 25·0 mM; $MgSO_4$, 0·45 mM; KH_2PO_4, 1·03 mM; $CaCl_2$, 2·5 mM and D-(+)-glucose, 11·1 mM. To prevent oxidation of catecholamines, 25 μg/ml of disodium ethylenediamine tetra-acetic acid (0·067 mM) was added to the Krebs-Henseleit solution. The solution bathing the atria was gassed with a mixture of 95% oxygen and 5% carbon dioxide and maintained at a temperature of 37° C. The force of spontaneous contractions was recorded on a Brush MK 250 pen recorder using a high compliance transducer; the initial tension was adjusted to about 1 gf ($\simeq$9·8 mN). The atria were allowed to equilibrate under these conditions for 45 to 60 min before experimental procedures were started.

Uptake of ^{3}H-noradrenaline

Atria were incubated with (±)-^{3}H-noradrenaline (0·5 μCi/ml, 22 ng/ml) for 20 minutes. There was maximal incorporation of radioactivity by atria with this incubation time (Story & Story, 1969). The atria were then washed for 10 min with Krebs-Henseleit solution at 37° C, removed from the organ bath, blotted, weighed and homogenized in 3 ml of 0·4 M perchloric acid. The homogenate was centrifuged and 0·3 ml of the supernatant was added to a counting vial containing 10 ml of scintillation solution of the following composition: 5·5 g of 2,5-diphenyloxazole (PPO), 0·1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and 333 ml of Triton-X per litre of toluene. The vials were counted in a Packard Tricarb liquid scintillation counter and corrections for counting efficiency were made by the use of an external reference standard. Tissue radioactivity was calculated in disintegrations per minute (d/min)/mg of atrial wet weight.

In some experiments the tissues were assayed for radioactive noradrenaline and metabolites. For this, atria were homogenized in 6 ml of ice-cold 0·4 M perchloric acid, the homogenate was centrifuged and 5·0 ml of the supernatant was transferred to a 50 ml beaker containing 10 ml of disodium ethylenediamine tetra-acetic acid (10 mg/ml), 10 ml of sodium metabisulphite (1 mg/ml) and 400 mg of aluminium oxide (alumina). The radioactive catechol and non-catechol components were then separated by adsorption and elution from alumina as described by Anton &

Sayre (1962). The non-catechol and catechol components were estimated by counting 0·5 ml aliquots of the appropriate supernatants in 10 ml of scintillation solution.

Since acid catechol metabolites are not separated from catecholamines by the alumina adsorption procedure (Crout, 1964), the eluate from the alumina was tested for deaminated metabolites by the following procedure: a 2 ml portion of the eluate was added to a 40 ml stoppered centrifuge tube containing 0·4 ml of 6 M HCl. The tube was shaken for 3 min, centrifuged, and 2 ml of the ethyl acetate layer was added to a counting vial with 10 ml of scintillation solution. No acid catechol metabolites were detected by this procedure and therefore the radioactive catechol fraction eluted from the alumina was considered to consist almost entirely of ^{3}H-noradrenaline. Furthermore, under the incubation conditions used, the total tissue radioactivity was closely related to the actual levels of radioactive noradrenaline. Thus in most subsequent experiments only total radioactivity was measured.

The effects of drugs on the uptake of ^{3}H-noradrenaline were investigated by preincubating atria with the required concentrations of the drug for 15 min prior to the addition of the labelled noradrenaline.

Release of ^{3}H-noradrenaline

The effects of drugs in releasing noradrenaline were investigated in two ways ; by measuring the loss of radioactivity from tissues, and by measuring the efflux of noradrenaline into the surrounding fluid.

Atria were incubated with (±)-^{3}H-noradrenaline (0·5 μCi/ml ; 22 ng/ml) as described above and were then washed with noradrenaline-free solution for 10 min before being incubated with the cholinomimetic drugs. The amount of radioactivity remaining in the atria was then determined.

For measurement of efflux of radioactivity, atria were set up in a 2 ml organ bath. During the equilibration period, the solution bathing the atria was frequently replaced. After equilibration the atria were incubated with (−)-^{3}H-noradrenaline (5 μCi/ml ; 0·21 μg/ml) for 20 min and then washed with drug-free solution for 60 minutes. The efflux of radioactive noradrenaline from the atria into the surrounding solution was estimated after 30 s periods of contact with the atria in the absence and presence of drugs, a 0·5 ml aliquot being mixed with 10 ml of scintillation solution.

Radiochemicals and drugs

Tritiated racemic and laevo-noradrenaline, 7-(±)-^{3}H-noradrenaline hydrochloride (specific activity 3·8 Ci/mmol) and 7-(−)-^{3}H-noradrenaline acetate (specific activity, 4·1 Ci/mmol) were obtained from the Radiochemical Centre, Amersham. The concentrations of these radiochemicals referred to in the text are expressed in terms of noradrenaline base.

The following drugs were used: atropine sulphate (David G. Bull Laboratories, Melbourne), 4-(*m*-chlorophenylcarbamoyloxy-2-butynyltrimethylammonium chloride (McN-A-343 ; McNeil Laboratories), cocaine hydrochloride (Drug Houses of Australia), *N,N*-dimethyl-*N'*-phenylpiperazinium iodide (DMPP ; Fluka), hexamethonium bromide (May & Baker), (±)-isoprenaline hydrochloride (Winthrop),

(−)-noradrenaline bitartrate (Winthrop), tyramine hydrochloride (Sigma). All solutions of drugs were freshly prepared, and where not otherwise stated the concentrations are expressed in terms of the salts.

Results

Uptake of (±)-^{3}H-noradrenaline

Effect of McN-A-343

Inhibition of the uptake of tritium label was produced by McN-A-343 in a concentration range of 1×10^{-7} to 1×10^{-3}M (31·7 ng/ml to 317 μg/ml). Figure 1 shows that the degree of inhibition was dependent on concentration, the maximal effect being obtained with 5×10^{-4}M (158·5 μg/ml) with which the inhibition amounted to 93%.

To determine whether the inhibition of uptake was a reversible effect, experiments were carried out in which McN-A-343 was washed from the bath after the 15 min contact period and the atria were washed repeatedly with drug-free solution for a further 10 min period. The atria were then incubated with (±)-^{3}H-noradrenaline as before. The radioactivity accumulated by these tissues was not significantly different from that of control ($P > 0{\cdot}05$).

The chemical nature of the radioactivity taken up by atria was investigated in the absence of McN-A-343 and in the presence of a concentration of 1×10^{-4}M (31·7 μg/ml) which caused 80% inhibition of uptake. The results are summarized

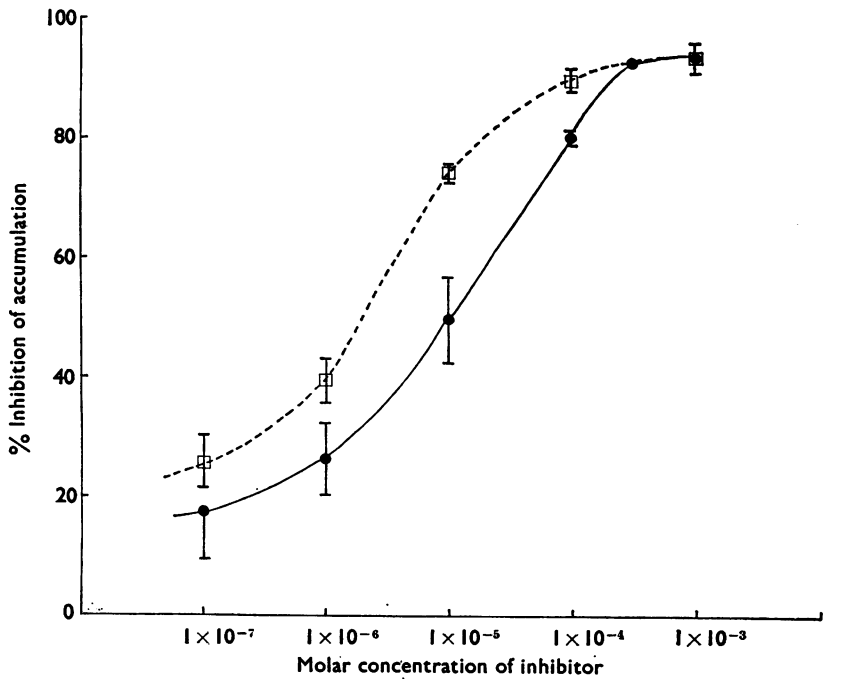

FIG. 1. Inhibition of uptake of (±)-^{3}H-noradrenaline (0·5 μC/ml; 22 ng/ml) by atria during 20 min incubation period by McN-A-343 (—●—) and cocaine (- -□- -). The symbols are means and the vertical bars are the standard errors of means. Ordinates: percentage inhibition of the uptake, control uptake was 2,360±100 (d/min)/mg wet weight of atrial tissue. Abscissae: molar concentrations of McN-A-343 and cocaine.

in Fig. 2. In control atria, 76% of the total radioactivity was recovered in the catechol fraction and none was in the acid catechol fraction; hence it may be attributed to (±)-^{3}H-noradrenaline. The non-catechol fraction was 13%.

In the presence of McN-A-343, the total radioactivity taken up by atria was reduced to 18%, and that recovered as catechol and non-catechol components was reduced to 17% and 32% respectively, of control values. The proportion of the total radioactivity taken up in the presence of McN-A-343 that was recovered in the catechol fraction was 22%. The effect of McN-A-343 was therefore substantially due to the inhibition of uptake of (±)-^{3}H-noradrenaline.

Comparison of McN-A-343 and cocaine

The inhibition of uptake produced by McN-A-343 was compared with that produced by cocaine in the same range of concentrations (Fig. 1). The maximal effect of both drugs occurred at the same concentration (5×10^{-4}M) and was of the same extent (93% inhibition); however, the concentration-effect curves had different shapes, and at lower concentrations than 5×10^{-4}M cocaine was more potent than McN-A-343 in inhibiting uptake.

Effect of DMPP

In a concentration of 1×10^{-4}M (31·7 μg/ml), DMPP was more potent than McN-A-343 in inhibiting uptake (Table 1). The mean inhibitions produced by these concentrations were 94·4 ± 2·3% for DMPP and 80·4 ± 0·8% for McN-A-343, the difference being statistically highly significant ($P<0.001$).

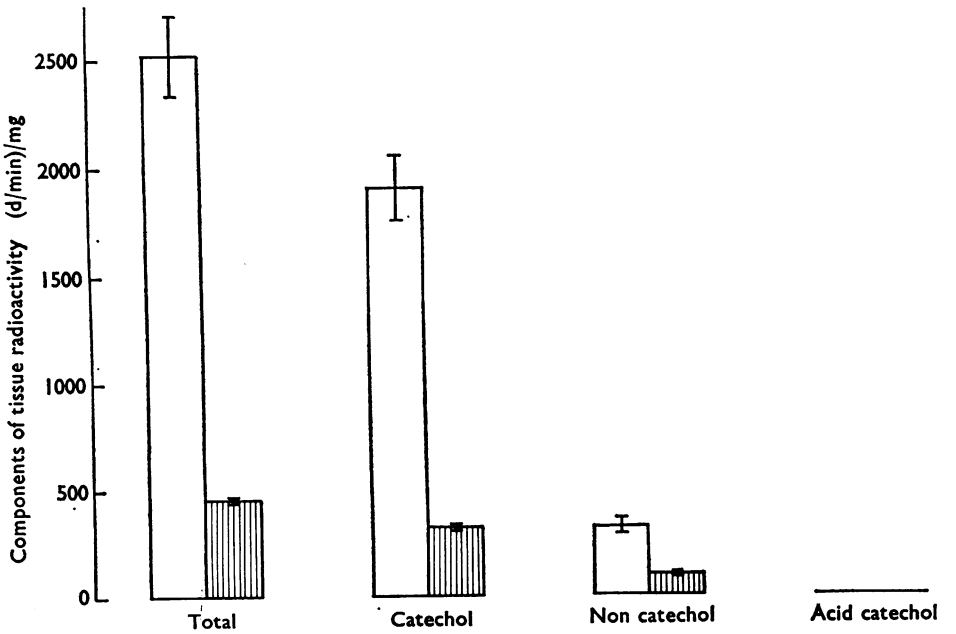

FIG. 2. Total radioactivity of atria and fractions recovered as catechol, non-catechol and acid catechol after incubation with (±)-^{3}H-noradrenaline. The catechol fraction corresponds to radio-labelled noradrenaline. The open columns are in the absence and the hatched columns are in the presence of McN-A-343 (1×10^{-4}M, 31·7 μg/ml). The vertical bars represent standard errors of means.

Effect of atropine and hexamethonium

To determine whether the inhibition of noradrenaline uptake by McN-A-343 and DMPP involved specific cholinoceptive sites of action, experiments were performed with muscarinic and nicotinic antagonists in sufficient concentrations to block the agonistic actions of McN-A-343 and DMPP involving these receptors. Atria were incubated initially with either atropine (0·12 μg/ml) or hexamethonium (200 μg/ml) for 15 min, then with the addition of DMPP or McN-A-343 in a concentration of 1×10^{-4}M for a further 15 min, and finally (±)-^{3}H-noradrenaline was added for 20 minutes. In control experiments atria were pre-incubated only with atropine or hexamethonium before the addition of the noradrenaline. In the concentrations used, neither atropine nor hexamethonium significantly affected the uptake of tritiated noradrenaline, and they did not diminish the uptake blocking effect of DMPP or McN-A-343 (Table 1).

Release of noradrenaline

Tissue levels

A possible explanation for the inhibition of uptake of radio-labelled noradrenaline produced by McN-A-343 and DMPP could be that there was release subsequent to uptake. This possibility was ruled out by the results of experiments in which these drugs were applied to atria which had been incubated with (±)-^{3}H-noradrenaline (Table 2). Neither McN-A-343 nor DMPP, nor cocaine which was also used, had significant effects on the content of radioactivity in atria ($P>0.05$).

Effect of McN-A-343 and DMPP on efflux of (−)-^{3}H-noradrenaline and responses of atria

In experiments to measure efflux of radioactivity the atria were first incubated with tritiated (−)-noradrenaline of high specific activity.

The force of contractions of atria was decreased by McN-A-343 in a concentration dependent manner in the range of about 2 to 50 μg/ml. The effect was short

TABLE 1. *Effects of atropine* (0·12 μg/ml) *and hexamethonium* (200 μg/ml) *on the inhibition of uptake of* (±)-^{3}*H-noradrenaline produced by McN-A-*343 *or DMPP* (1×10^{-4}M)

Treatment	Percentage inhibition of uptake Mean±S.E.	No. of experiments
McN-A-343	80·4±0·8	6
Atropine+McN-A-343	*83·6±0·3	3
Hexamethonium+McN-A-343	*84·4±1·1	4
DMPP	94·4±2·3	3
Atropine+DMPP	93·7±2·6	3
Hexamethonium+DMPP	91·8±0·4	3

* Significantly greater inhibition than with McN-A-343 alone ($P<0.05$).

TABLE 2. *Effects of McN-A-*343, *DMPP and cocaine, each in a concentration of* 1×10^{-4}M, *on the level of radioactivity in atria previously incubated with* (±)-^{3}*H-noradrenaline*

Drug	Radioactivity: mean (d/min)/mg ±S.E. (number of experiments)	
—	2,007±240	(3)
McN-A-343	2,012±140	(3)
DMPP	1,912±140	(3)
Cocaine	2,370±210	(3)

lasting and the amplitude returned to the control level, or even exceeded it, within 10 min even in the continued presence of the drug. In the presence of atropine (0·12 μg/ml), McN-A-343 caused a slowly developing increase in the force of contractions, as reported previously by Bhagat (1966). The effects of 31·7 μg/ml (1×10^{-4}M) of McN-A-343 in the absence and presence of atropine are illustrated in Fig. 3, centre and right hand panels.

McN-A-343 had no effect on the efflux of (−)-^{3}H-noradrenaline from atria in either the absence or the presence of atropine, despite the positive inotropic effect that occurred in the latter case (Fig. 3, right hand panel).

Cocaine, in concentrations having as great an effect as McN-A-343 in inhibiting noradrenaline uptake, did not produce any change in noradrenaline efflux from atria; however, it caused a small increase in amplitude of contractions of the atria, the effect being much less than that of McN-A-343. Similar results were obtained with another inhibitor of noradrenaline uptake, desipramine (D. Story, unpublished experiments).

On the other hand, DMPP caused a rapid increase in the force of contractions and this effect was accompanied by a marked increase in the efflux of radioactivity from the atria (Fig. 3, left hand panel).

Effects of McN-A-343 on responses of atria to noradrenaline, isoprenaline and tyramine

Control responses of about 50% maximal were obtained with noradrenaline (5 to 10 ng/ml), isoprenaline (0·5 to 5 ng/ml) and tyramine (2 to 20 μg/ml) in separate experiments (concentrations in terms of bases). McN-A-343 was added to the bath

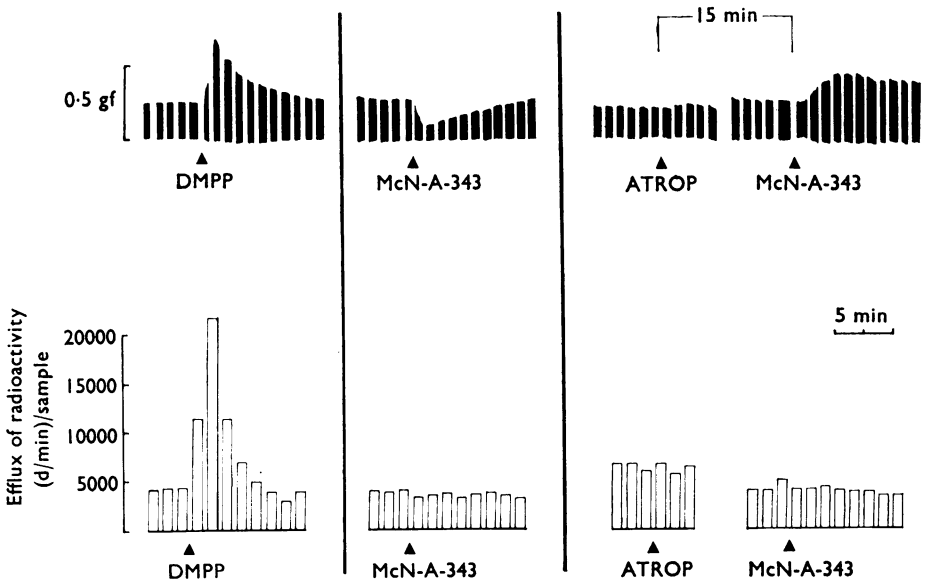

FIG. 3. Effects of DMPP (1×10^{-4}M, 31·7 μg/ml) and of McN-A-343 (1×10^{-4}M, 31·7 μg/ml) in the absence and presence of atropine (0·12 μg/ml) on the force of contractions of atria and the efflux of radioactivity, determined simultaneously. The atria had been previously incubated with (−)-^{3}H-noradrenaline (5 μCi/ml; 0·21 μg/ml). Upper records: force of contraction (1 gf≃9·8 mN); the tracing is interrupted at intervals when the bath fluid was removed and replaced with fresh drug-free or drug-containing fluid. Lower records: content of radioactivity in 0·5 ml aliquots of the bath fluid (bath volume=2 ml).

in a concentration of 31·7 μg/ml (1×10^{-4}M). After 15 min, the amplitude of contractions was constant but exceeded the control amplitude, the mean percentage increase in 10 experiments being $43{\cdot}7 \pm 4{\cdot}6\%$. In the continued presence of McN-A-343 the effects of noradrenaline, isoprenaline and tyramine were observed again. The responses to noradrenaline were significantly potentiated ($P<0{\cdot}01$) by McN-A-343 the mean percentage increase above the control responses being $22{\cdot}8 \pm 30\%$ (4 experiments). The responses to isoprenaline were also potentiated by McN-A-343, but to a lesser extent than those to noradrenaline ($P<0{\cdot}05$), the mean percentage increase in the isoprenaline responses being $8{\cdot}3 \pm 1{\cdot}2\%$ (3 experiments). On the other hand, the responses to tyramine were significantly reduced ($P<0{\cdot}05$) by McN-A-343, the mean percentage reduction being $21{\cdot}6 \pm 3{\cdot}6\%$ (3 experiments) of the control responses.

Discussion

The cholinomimetic drugs McN-A-343 and DMPP are potent inhibitors of the uptake of noradrenaline by guinea-pig atria. In concentrations of 1×10^{-4}M, DMPP was as effective as cocaine and McN-A-343 was only slightly less effective.

Neither DMPP nor McN-A-343 caused significant reductions in the tissue levels of radioactivity established in atria previously incubated in (±)-^{3}H-noradrenaline. It can be assumed therefore that the effect of these cholinomimetics in reducing the amount of radioactive noradrenaline incorporated by atria is in fact due to a block of uptake rather than release of the catecholamine subsequent to its uptake. Since cocaine under the same conditions was also effective in inhibiting the uptake of the labelled noradrenaline it can be concluded that this uptake was predominantly into sympathetic nerves (Iversen, 1967).

The inhibition of noradrenaline uptake by McN-A-343 and DMPP did not depend on the actions of these drugs on nicotinic or muscarinic receptors since neither atropine nor hexamethonium had any effect on the inhibition of uptake.

DMPP caused an efflux of radioactivity from atria which had been previously incubated with (−)-^{3}H-noradrenaline, and this coincided in extent and time with the positive inotropic and chronotropic responses of the atria. These findings are completely in accord with those reported by others (for references see **Introduction**), and the generally accepted explanation is that DMPP releases noradrenaline from sympathetic nerve terminals. Our findings suggest that the actions and efflux of the released noradrenaline may be enhanced by the additional effect of DMPP in inhibiting re-uptake of the released noradrenaline.

The release of noradrenaline from rabbit heart by nicotinic drugs, including DMPP, is terminated by a blockade of release which has been termed 'auto-inhibition' (Löffelholz & Muscholl, 1969, Löffelholz, 1970a, b). In guinea-pig atria, cocaine abolishes responses to DMPP and nicotine (Bhagat, 1966) and in the rabbit pulmonary artery the noradrenaline-releasing action of nicotine is reduced by phenoxybenzamine, desipramine and cocaine (Su & Bevan, 1970). These findings indicate that drugs which inhibit the neuronal uptake of noradrenaline (and other amines) abolish responses to DMPP and other nicotinic stimulants, and suggest that these drugs must be taken up by the neurones before they exert their noradrenaline releasing action. The phenomenon of 'auto-inhibition', at least with DMPP, may be due to self-inhibition of uptake. The difficulty in accepting the

suggestion that DMPP acts only after being taken up is that its actions are blocked by hexamethonium, but hexamethonium did not affect noradrenaline uptake in atria. This finding suggests that DMPP acts on nicotinic receptors. The possibility that uptake-blocking drugs may also block nicotinic receptors was discounted in the light of experiments by our colleague, Mr. W. A. Palmer, who showed that cocaine did not significantly affect responses of the toad *abdominus rectus* preparation to nicotinic stimulants. It is possible that hexamethonium may block the neuronal uptake of DMPP without affecting that of noradrenaline; alternatively, it may be that DMPP acts at two sites, nicotinic receptors and intraneuronally, to effect the release of noradrenaline. Further experiments will be required to distinguish between these possibilities.

McN-A-343, unlike DMPP, did not cause an increased efflux of radioactivity from atria in which the endogenous noradrenaline pools had been labelled with (−)-^{3}H-noradrenaline. This finding does not support the contention of Bhagat (1966) that the positive inotropic action of McN-A-343 in the presence of atropine is mediated through release of noradrenaline. A far more likely explanation is that the effect is due to accumulation in the vicinity of the receptors of spontaneously liberated noradrenaline as a consequence of inhibition of its re-uptake by McN-A-343. However, two other inhibitors of noradrenaline uptake, cocaine and desipramine, had much less positive inotropic action than McN-A-343. The difference may be attributable to the depressant action of cocaine and desipramine which partly counteracts the stimulant action of the accumulated, spontaneously released noradrenaline; alternatively, McN-A-343 may have a noradrenaline releasing action which is too slight to result in increased efflux but is sufficient to stimulate cardiac β-receptors in the vicinity of the sites of release, particularly when neuronal uptake is inhibited. The positive inotropic action of McN-A-343 in the presence of atropine is abolished by cocaine (Bhagat, 1966). It is possible that cocaine is preventing a noradrenaline-releasing action of McN-A-343 by blocking the entry of McN-A-343 into adrenergic neurones. However, a simpler explanation is that cocaine has pre-empted the effect of McN-A-343 in blocking reuptake of spontaneously released noradrenaline.

Presumably, there is no increased efflux of noradrenaline in the presence of McN-A-343 because other sources of loss of noradrenaline, such as non-neuronal uptake, can cope with the slight excess of extra-neuronal noradrenaline. In the presence of McN-A-343, the proportion of non-catechol radioactivity incorporated into atria was higher than in its absence (22% against 13%). The major non-catechol component formed from the added ^{3}H-noradrenaline is the 3-methoxy derivative following the action of catechol-*O*-methyltransferase after uptake into cardiac muscle cells, as determined by Iversen (1963) for rat heart and by D. Story (unpublished observations) for guinea-pig atria.

McN-A-343 closely resembles cocaine in terms of its effects on responses of atria to noradrenaline and tyramine, the former being enhanced, the latter reduced. These effects are indubitably a consequence of inhibition of neuronal uptake of these amines. There was a slight potentiation of responses to isoprenaline, as occurs with other inhibitors of neuronal uptake (Basset, Cairncross, Hacket & Story, 1969). Inhibition of uptake by McN-A-343 must be taken into account when evaluating its interaction with adrenergic mechanisms. However, it does not appear to account for the increases in responses to sympathetic nerve stimulation

in isolated arteries produced by McN-A-343 since in this tissue the responses to noradrenaline were not potentiated (Rand & Varma, 1971). Furthermore, other cholinomimetic drugs, including acetylcholine, methacholine and pilocarpine, also facilitated responses of the ear artery to sympathetic nerve stimulation under certain circumstances (Rand & Varma, 1970); however these cholinomimetics were considerably less active than McN-A-343 in inhibiting noradrenaline uptake (Allen, Rand & Story, unpublished observations).

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