Br. J. Pharmac. (1969), 37, 627–637.

Effects of phenoxybenzamine on the uptake and metabolism of noradrenaline in the rat heart and vas deferens

L. L. IVERSEN AND S. Z. LANGER*

Department of Pharmacology, University of Cambridge, and Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

1. In the isolated rat heart perfused with various concentrations of (±)-^{3}H-noradrenaline (^{3}H-NA) the addition of phenoxybenzamine, cocaine or desipramine to the perfusion medium resulted in an inhibition of ^{3}H-NA uptake which appeared on kinetic analysis to be of a competitive nature.

2. Phenoxybenzamine also blocked the formation of labelled metabolites of ^{3}H-NA in the heart at all perfusion concentrations of ^{3}H-NA; this effect appeared to be unrelated to the inhibition of the neuronal uptake of NA produced by phenoxybenzamine, since no blockade of ^{3}H-NA metabolism was produced by cocaine in similar experiments.

3. In slices of rat vas deferens incubated with various concentrations of ^{3}H-NA, cocaine and desipramine and phenoxybenzamine were also shown to act as competitive inhibitors of NA uptake. Cocaine and phenoxybenzamine were less potent inhibitors of uptake in the vas deferens than they were in the heart; desipramine was equally potent in both tissues.

4. When vas deferens slices were incubated in a medium containing phenoxybenzamine for 30 min before the addition of ^{3}H-NA, the resulting inhibition of ^{3}H-NA uptake was increased and changed to a non-competitive type of interaction.

5. In hearts or vasa deferentia taken from animals pretreated *in vivo* with phenoxybenzamine (20 mg/kg), a significant inhibition of ^{3}H-NA uptake was found when the tissues were exposed to low concentrations of ^{3}H-NA but not when higher concentrations of ^{3}H-NA were used.

Following the original observations by Brown & Gillespie (1957), several reports have confirmed that treatment with phenoxybenzamine leads to a marked increase in the outflow of noradrenaline (NA) from isolated perfused organs during sympathetic nerve stimulation (Kirpekar & Cervoni, 1963; Thoenen, Hürlimann & Haefely, 1964; Brown, 1965; Langer, 1968). It was at first thought that phenoxybenzamine produced this effect by interfering with the binding of released NA at

* Present address: Instituto de Investigaciones Farmacologicas, Paraguay 2155, Piso 7°, Buenos Aires, Argentina.

adrenergic receptor sites (Brown & Gillespie, 1957). However, later findings suggested that the recapture of released NA by a transport mechanism in adrenergic nerve terminals was important in terminating the actions of the released amine, and it was shown that this uptake system was inhibited by phenoxybenzamine (Hertting, 1965; Iversen, 1965). In addition to inhibiting the neuronal uptake of NA, phenoxybenzamine has also been found to inhibit the extraneuronal metabolism of NA in the perfused rat heart (Eisenfeld, Axelrod & Krakoff, 1967). Recent experiments on the fate of ^{3}H-NA after its release from the sympathetic innervation of the cat nictitating membrane or rat vas deferens suggested that phenoxybenzamine promoted the overflow of NA by both inhibiting the recapture of ^{3}H-NA and blocking the extensive metabolism of the released amine which normally occurred.

The present studies were undertaken to examine in more detail the effects of phenoxybenzamine on NA uptake, since previous studies have not established the type of inhibition produced by this drug or its potency in comparison with other drugs which inhibit NA uptake.

Methods

Rat heart perfusions

Rat heart perfusions were performed as described previously (Iversen, 1963). The hearts were perfused for 10 min with a medium containing ($\pm$)-^{3}H-NA (0·05 μc/ml.) diluted with various amounts of non-radioactive ($\pm$)-NA to give perfusion concentrations of ($\pm$)-NA in the range 10–300 ng/ml. At the end of the perfusion the hearts were analysed for total radioactivity and for ($\pm$)-^{3}H-NA by ion-exchange chromatography and liquid scintillation counting (Iversen, 1963). Radioactive metabolites were estimated as the difference between total radioactivity and ($\pm$)-^{3}H-NA radioactivity, after correction of ($\pm$)-^{3}H-NA results for an average recovery of 80% for the ion-exchange procedure. Results were corrected for the presence of extracellular ($\pm$)-^{3}H-NA, assuming an average value of 33% of wet weight as the extracellular space (Iversen, 1963).

Uptake of ($\pm$)-^{3}H-NA in vas deferens

Vasa deferentia were cut into segments approximately 0·5 cm long, and each segment was cut longitudinally into three slices, using a razor and frosted glass slide. The resulting slices were incubated with shaking at 37° C in groups of eight to ten in 2 ml. of incubation medium. After a 30 min incubation, ($\pm$)-^{3}H-NA (0·5 μc/ml.) diluted with various amounts of non-radioactive ($\pm$)-NA was added to give NA concentrations in the range 25–200 ng/ml. and incubation was continued for 10 min; the slices were then transferred to vials containing 5 ml. of NA-free medium for a further 10 min incubation to wash out extracellular ($\pm$)-^{3}H-NA. The slices were blotted, weighed and ($\pm$)-^{3}H-NA was extracted by soaking the small tissue slices for 60 min in 0·2 ml. distilled water in a counting vial; radioactivity was then measured after the addition of 4 ml. of ethoxy-ethanol and 10 ml. of toluene scintillator (0·4% butylPBD (CIBA) in toluene). This extraction procedure yielded results similar to those found after slices were extracted by soaking overnight in 1 ml. of hyamine (0·1 M in methanol) or by homogenization in 0·4 N perchloric acid, indicating that essentially all the accumulated ($\pm$)-^{3}H-NA was extracted by the osmotic shock treatment. Preliminary experiments showed that the presence of

pargyline and pyrogallol in the incubation medium effectively prevented the formation of any radioactive metabolites of (±)-^{3}H-NA without influencing the rate of accumulation of the unchanged amine.

Animals for all experiments were adult male albino Wistar rats. Phenoxybenzamine solutions were prepared shortly before each experiment by dissolving the drug in a small volume of acid ethanol and diluting this solution with normal saline. (±)-^{3}H-noradrenaline (specific activity=3·6 c/m-mole) was obtained from the Radiochemical Centre, Amersham. Perfusion medium for heart experiments was Krebs-Henseleit bicarbonate modified as previously described (Iversen, 1963). Incubation medium for vas deferens slices was oxygenated Krebs phosphate solution with the following additions: glucose (1 g/l.); ascorbic acid 20 mg/l.; EDTA-disodium salt (10 mg/l.); pargyline (0·1 mM) and pyrogallol (1·0 mM).

Results

Effects of phenoxybenzamine, cocaine and desipramine on the uptake of (±)-^{3}H-NA in the perfused rat heart

Rats were pretreated with phenoxybenzamine 1 mg/kg or 3 mg/kg given intraperitoneally, and 30 min later the hearts were removed and perfused with a medium containing various concentrations of (±)-^{3}H-NA together with phenoxybenzamine (1·0 or 3·3 μM respectively). The results thus obtained were compared with those from control hearts, using a Lineweaver–Burke plot (Fig. 1). The inhibition of (±)-^{3}H-NA uptake produced by phenoxybenzamine was of a simple competitive type, and was similar to that produced in other experiments of this type by cocaine or desipramine (Fig. 2). *Ki* values calculated from these results are summarized in Table 1; the values for cocaine and desipramine are similar to previous estimates of the potency of these drugs (Iversen, 1965; Berti & Shore, 1967). The apparent *Km* for (±)-NA (Table 1) is lower than that previously reported for this preparation (Iversen, 1963), probably because the present method of measuring NA uptake after a fixed time interval yielded only an approximation of the initial rate of uptake of the amine, particularly at the higher concentrations of NA. The results, nevertheless, show that phenoxybenzamine has a potency comparable with that of cocaine as an inhibitor of NA uptake in the rat heart in these conditions. In animals treated 1 hr previously with larger *in vivo* doses of phenoxybenzamine (20 mg/kg), the accumulation of (±)-^{3}H-NA in hearts perfused with (±)-^{3}H-NA (10 ng/ml.) was significantly reduced (to 53% of control) even when the perfusion medium contained

TABLE 1. *Kinetic constants for (±)-NA uptake and drug inhibition of uptake in rat heart and vas deferens*

Compound	Apparent *Km* or *Ki* values (μM) Heart	Vas deferens
(±)-NA	0·26	1·03
PBA (competitive)	0·86	11·30
PBA (non-competitive)	—	7·40
Cocaine	0·58	2·94
Desipramine	0·06	0·04

no added phenoxybenzamine. However, no inhibition of NA uptake was found in such hearts when higher concentrations of ($\pm$)-^{3}H-NA were used, indicating that the inhibition of uptake produced by phenoxybenzamine after *in vivo* treatment was predominantly of a competitive type.

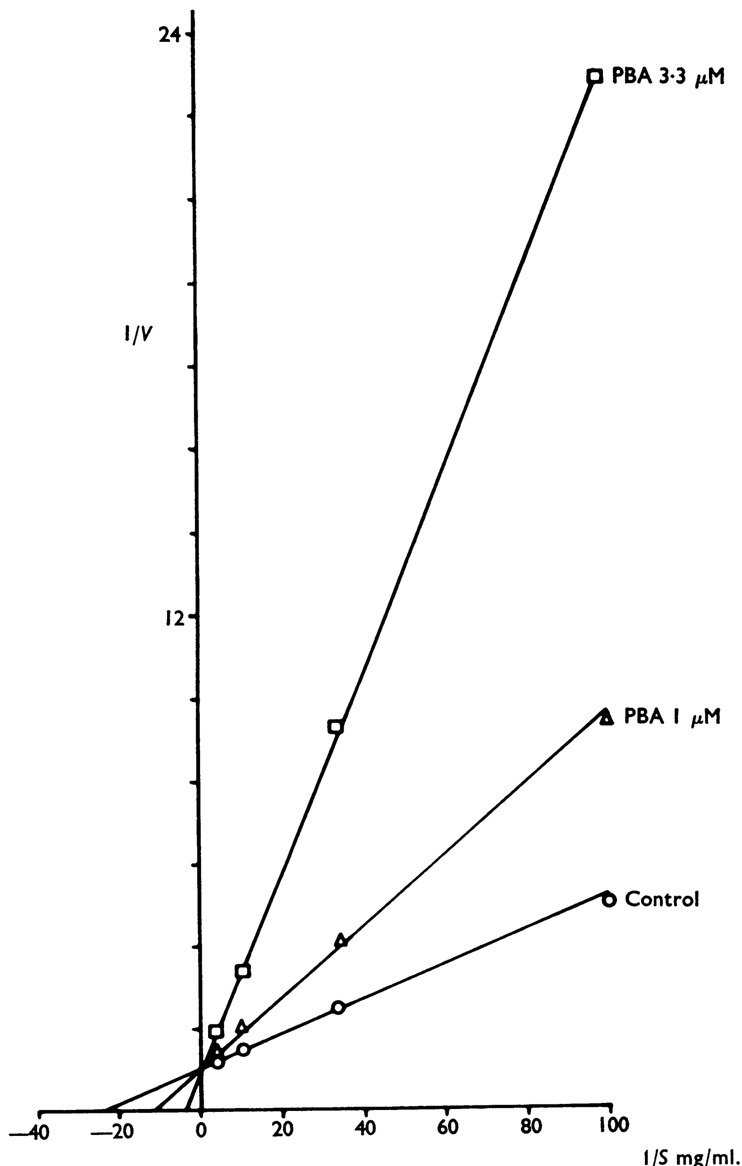

FIG. 1. Inhibition of ($\pm$)-^{3}H-NA uptake by phenoxybenzamine (PBA) in the perfused rat heart. Hearts were perfused for 10 min with various concentrations of ($\pm$)-^{3}H-NA in a medium containing phenoxybenzamine (1 μM or 3·3 μM), the tissue was analysed for ($\pm$)-^{3}H-NA at the end of the perfusion. *V*, Uptake of ($\pm$)-^{3}H-NA (μg/g); *S*, perfusion concentration of ($\pm$)-^{3}H-NA (mg/ml.). Each point is the mean of six to twelve values.

Effects of phenoxybenzamine and cocaine on the metabolism of $(\pm)$-^{3}H-NA in the rat heart

In control hearts perfused for 10 min with a medium containing a low concentration of $(\pm)$-^{3}H-NA (10 ng/ml.) only negligible amounts of radioactive metabolites were recovered, amounting to less than 5% of the total radioactivity in the tissue extracts. As the perfusion concentration of NA was increased, however, considerable amounts of labelled metabolites accumulated in the heart, amounting to about 20% of the total radioactivity in the tissue after perfusion with the highest concentration tested (300 ng/ml.) (Fig. 3). The presence of cocaine in the perfusion medium had no significant effect on the formation of labelled metabolites, even though the

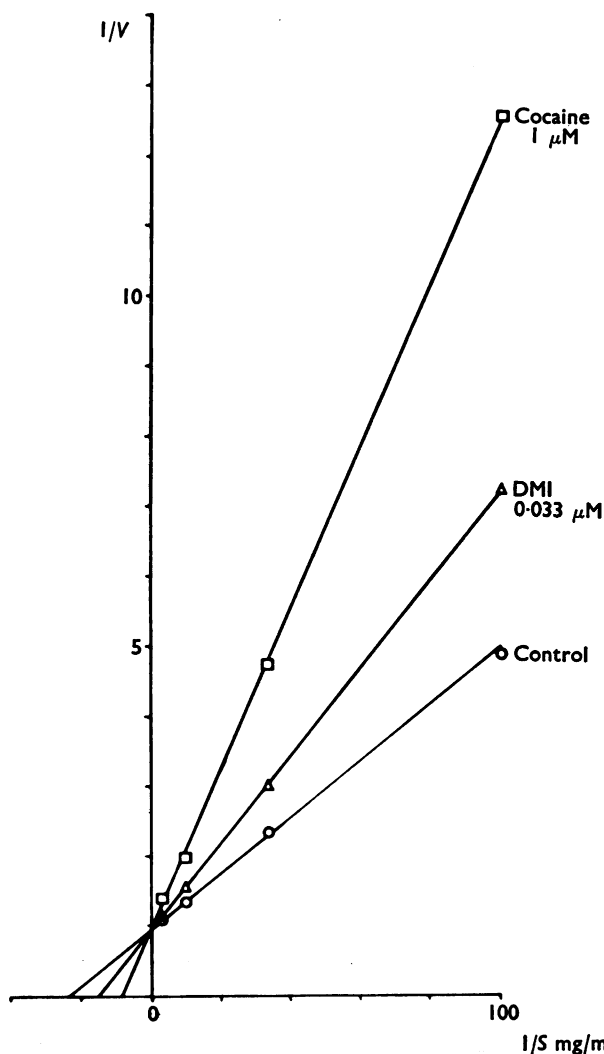

FIG. 2. Inhibition of $(\pm)$-^{3}H-NA uptake by cocaine (1 μM) or desipramine (DMI) (0·033 μM) in the perfused rat heart. (As Fig. 1.)

drug produced a marked inhibition of (±)-^{3}H-NA uptake (Fig. 3). In contrast, the presence of phenoxybenzamine (3·3 μM) led to a complete inhibition of the formation of labelled metabolites at all perfusion concentrations of NA, although at the higher NA concentrations the inhibition of NA uptake produced by the drug was only partial. This would suggest that phenoxybenzamine has some independent action leading to an inhibition of the metabolism of extracellular NA in the heart, and this action appears to be considerably more potent than its effects on the NA uptake system.

Effects of phenoxybenzamine and other drugs on NA uptake by slices of rat vas deferens

When small slices of rat vas deferens were incubated with (±)-^{3}H-NA in the presence of inhibitors of catechol-O-methyl transferase and monoamine oxidase they rapidly accumulated the unchanged amine. At the end of a 10 min incubation period with a low concentration of (±)-^{3}H-NA (25 ng/ml.) the concentration of (±)-^{3}H-NA in the tissue slices was 8·8 times that in the incubation medium (mean

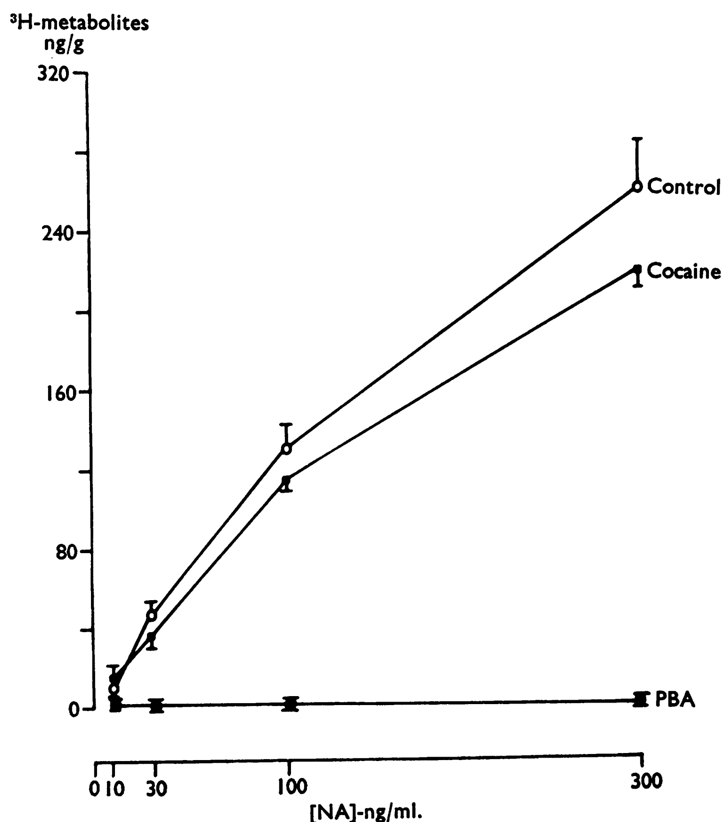

FIG. 3. Effects of cocaine (1 μM) or phenoxybenzamine (3·3 μM) on the formation of labelled metabolites of (±)-^{3}H-NA in the rat heart during a 10 min perfusion with various concentrations of (±)-^{3}H-NA. ^{3}H-Metabolites are expressed as ng/g heart, each point is the mean of six to eight values, ±S.E. of mean.

of sixteen experiments). The accumulation of (±)-^{3}H-NA progressed linearly with time for at least 15 min. There was no significant loss of the accumulated amine during a 10 min washout period after the exposure to labelled NA. This washout procedure was therefore routinely used in order to remove extracellular (±)-^{3}H-NA from the tissue samples before analysis.

When slices of vas deferens were exposed to (±)-^{3}H-NA and phenoxybenzamine (6·6 μM) simultaneously the inhibition of NA uptake produced by the drug was again of a simple competitive nature, and resembled that produced under similar conditions by cocaine (Fig. 4). The calculated *Ki* values for both drugs were considerably higher than those found in the perfused heart (Table 1). Desipramine, on the other hand, was a very potent inhibitor of NA uptake in the vas deferens; the inhibition being also of a simple competitive type. When vas deferens was exposed to phenoxybenzamine (6·6 μM) for 30 min before the addition of (±)-^{3}H-NA, the

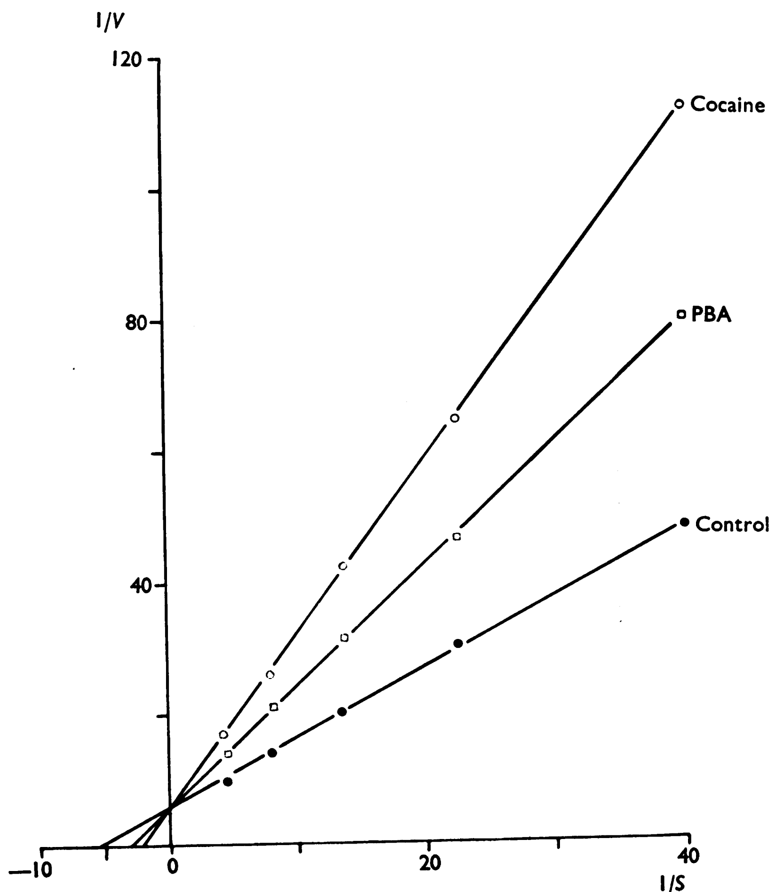

FIG. 4. Inhibition of (±)-^{3}-NA uptake by cocaine (6·6 μM) or phenoxybenzamine (6·6 μM) in slices of rat vas deferens incubated for 10 min with various concentrations of (±)-^{3}H-NA. *V*, Uptake of (±)-^{3}H-NA (μg/100 mg), *S*, concentration of (±)-^{3}H-NA in medium (mg/ml.). Each point is the mean of four to eight values.

inhibition of NA uptake produced by the drug was increased, and now appeared to be of a non-competitive nature (Fig. 5, Table 1). Pretreatment of vas deferens slices with cocaine (6·6 μM), however, in similar conditions, produced no change in the competitive inhibition seen previously with this drug.

Studies of NA uptake in slices prepared from animals at various times after *in vivo* treatments with phenoxybenzamine showed that the drug can produce long-lasting effects on NA uptake (Fig. 6). Kinetic studies of the inhibition produced by such *in vivo* treatments, however, indicated that the effects of the drug were largely of a competitive nature, as in the heart under similar conditions.

Discussion

The present results confirm that phenoxybenzamine is an inhibitor of the noradrenaline uptake process in sympathetic nerves. The studies were designed to examine the type of inhibition of uptake produced by phenoxybenzamine and to obtain an estimate of the potency of this drug as an uptake inhibitor. Since NA uptake was measured after exposure of the tissues for a fixed time interval the results give only approximate estimates of the initial rates of uptake, particularly at the higher concentrations of external NA. However, the results clearly show that

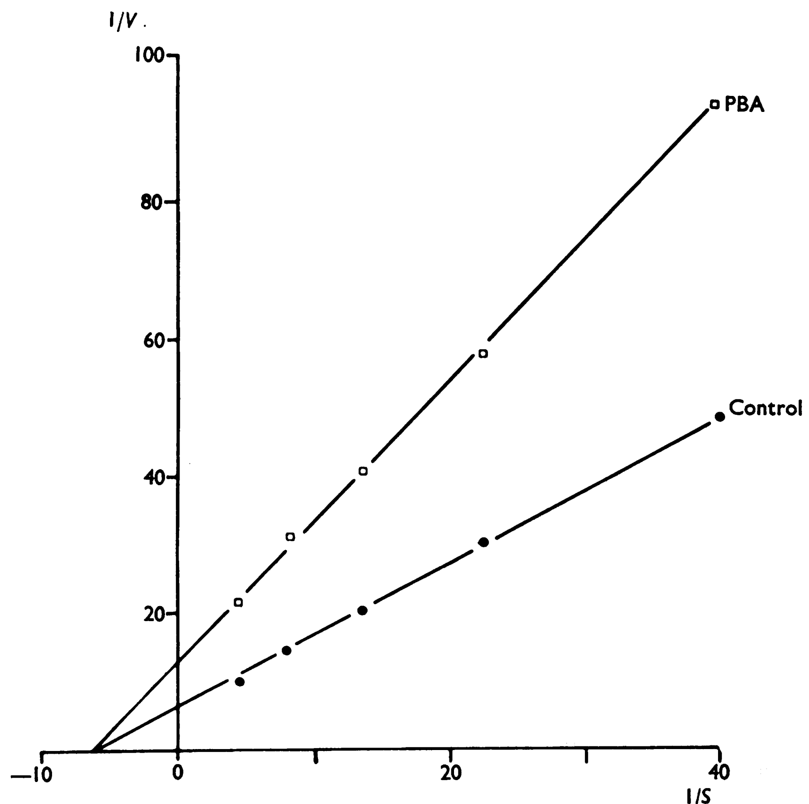

FIG. 5. Inhibition of $(\pm)$-^{3}H-NA uptake by phenoxybenzamine (6·6 μM) in slices of rat vas deferens ; as Fig. 4 except that the tissue was exposed to the drug for 30 min before the addition of $(\pm)$-^{3}H-NA.

the inhibition of uptake produced by phenoxybenzamine was of a simple competitive type when heart or vas deferens tissue was exposed to the drug and to NA simultaneously. An interesting change occurred, however, when vas deferens tissue was exposed to phenoxybenzamine for 30 min before the addition of NA ; in this case the inhibition of uptake appeared to be of a non-competitive nature. No such difference was found in the actions of cocaine, which remained a competitive inhibitor even after prolonged exposure of the vas deferens tissue to the drug. The

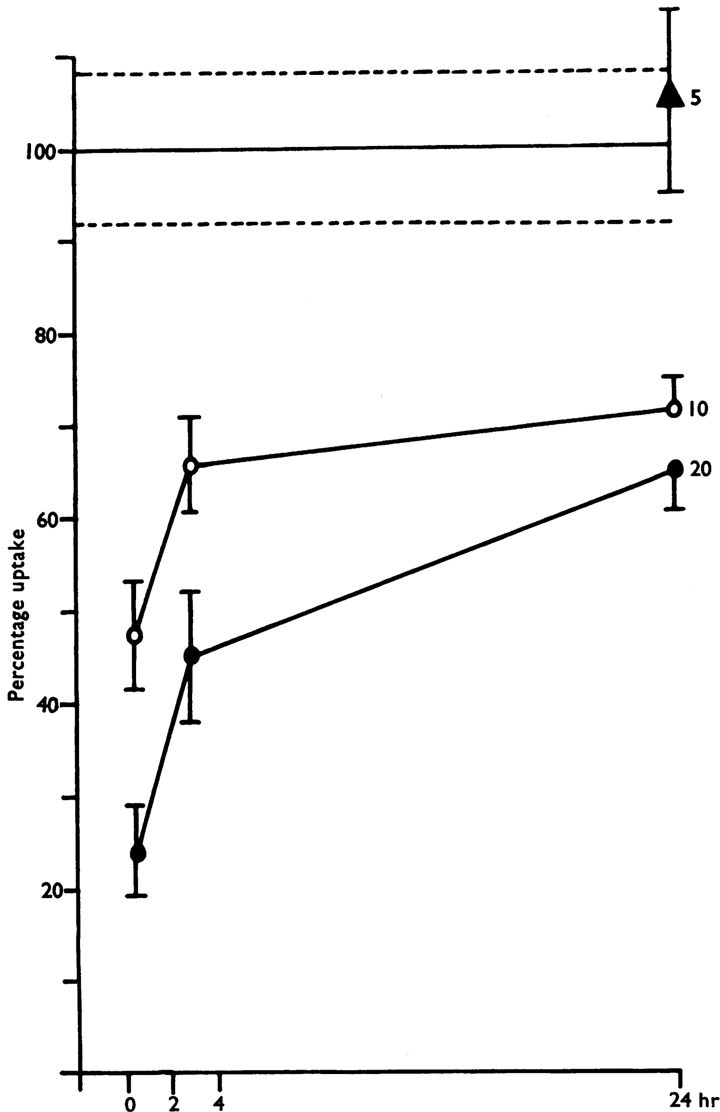

FIG. 6. Effect of *in vivo* treatment with various doses of phenoxybenzamine (5–20 mg/kg, intraperitoneally) on the *in vitro* uptake of (±)-^{3}H-NA by slices of rat vas deferens incubated for 10 min with (±)-^{3}H-NA (10 ng/ml.). Each point is the mean ±S.E. of mean for four to eight values. Control values for tissues from untreated animals are expressed as 100% ; dotted lines indicate S.E. of mean for control. Zero time is the time of phenoxybenzamine administration.

gradual appearance of a non-competitive interaction between phenoxybenzamine and NA uptake sites, following an initial competitive effect, is reminiscent of the sequence of events which leads to an irreversible blockade of adrenergic receptors by this type of drug, following an initial period of competitive antagonism (Nickerson, 1959). Phenoxybenzamine is thus one of the few drugs which inhibit NA uptake by a non-competitive mechanism. This phenomenon, however, could only be demonstrated in *in vitro* experiments. Although the *in vivo* administration of the drug led to a long-lasting inhibition of NA uptake in both heart and vas deferens, this effect appeared only at low concentrations of NA, and thus seemed to be of a competitive nature.

The present results also confirm that phenoxybenzamine produces a complete inhibition of the metabolism of extracellular NA in the rat heart. This effect of phenoxybenzamine appears to be unrelated to its actions on NA uptake, for no such effects were seen in hearts perfused with cocaine, which produces a similar competitive inhibition of NA uptake. The inhibition of NA metabolism produced by phenoxybenzamine is thought to be related to an effect on an extraneuronal uptake mechanism (Uptake$_2$) which allows noradrenaline to gain access to the cells which contain the degradative enzymes (Lightman & Iversen, 1969).

The effects of phenoxybenzamine on the neuronal and extraneuronal uptake of NA do not appear to be related to the well known activity of the drug as an α-adrenergic receptor antagonist, since a variety of drugs which have little or no antagonist activity may act as uptake inhibitors (Iversen, 1967a; Eisenfeld, Landsberg & Axelrod, 1967).

Estimates of the potency of phenoxybenzamine and cocaine as inhibitors of neuronal NA uptake in the rat vas deferens and heart indicated that both drugs are more potent inhibitors of the uptake system in the heart; desipramine, however, was extremely potent in both tissues. These findings are in agreement with other results which indicate that the uptake processes in the rat heart and vas deferens show considerable differences in their sensitivity to inhibition by various drugs (Iversen, 1967b).

In conclusion, the present results confirm that phenoxybenzamine is a potent inhibitor of NA uptake after both *in vitro* and *in vivo* administration. In most cases the inhibition appears to be of a simple competitive nature, but prolonged exposure of tissues to the drug *in vitro* may lead to a non-competitive interaction. The inhibition of NA uptake produced by phenoxybenzamine is probably insufficient by itself to account for the effects of this drug on adrenergic transmitter outflow in release experiments, and prevention of the metabolism of the released transmitter might be another contributory factor. In a subsequent study the effects of phenoxybenzamine on the extraneuronal uptake and metabolism of NA in the rat heart have been examined in more detail (Lightman & Iversen, 1969).

S.Z.L. was supported by a Fellowship from the Wellcome Foundation. The study was supported by a grant to L.L.I. from the Mental Health Research Fund.

REFERENCES

BERTI, F. & SHORE, P. A. (1967). A kinetic analysis of drugs that inhibit the adrenergic neuronal membrane amine pump. *Biochem. Pharmac.*, **16**, 2091–2094.

BROWN, G. L. (1965). The release and fate of the transmitter liberated by adrenergic nerves. *Proc. R. Soc. B.*, **162**, 1–19.

BROWN, G. L. & GILLESPIE, J. S. (1957). The output of sympathetic transmitter from the spleen of the cat. *J. Physiol., Lond.*, **138**, 81–102.

EISENFELD, A. J., AXELROD, J. & KRAKOFF, L. (1967). Inhibition of the extraneuronal accumulation and metabolism of norepinephrine by adrenergic blocking agents. *J. Pharmac. exp. Ther.*, **156**, 107–113.

EISENFELD, A. J., LANDSBERG, L. & AXELROD, J. (1967). Effect of drugs on the accumulation and metabolism of extraneuronal norepinephrine in the rat heart. *J. Pharmac. exp. Ther.*, **158**, 378–385.

HERTTING, G. (1965). Effects of drugs and sympathetic denervation on noradrenaline uptake and binding in animal tissues. In *Pharmacology of Cholinergic and Adrenergic Transmission*, ed. Douglas and Carlsson, pp. 277–288. Oxford: Pergamon Press.

IVERSEN, L. L. (1963). The uptake of noradrenaline by the isolated perfused rat heart. *Br. J. Pharmac. Chemother.*, **21**, 523–537.

IVERSEN, L. L. (1965). The inhibition of noradrenaline uptake by drugs. *Adv. Drug Res.*, **2**, 5–23.

IVERSEN, L. L. (1967a). *The Uptake of Noradrenaline by Sympathetic Nerves.* London: Cambridge Univ. Press.

IVERSEN, L. L. (1967b). Characteristics of noradrenaline uptake in the iris/ciliary body and other peripheral tissues of the rat. *Arch. exp. Path. Pharmak.*, **259**, 179.

KIRPEKAR, S. M. & CERVONI, P. (1963). Effects of cocaine, phenoxybenzamine and phentolamine on the catecholamine output from spleen and adrenal medulla. *J. Pharmac. exp. Ther.*, **142**, 59–70.

LANGER, S. Z. (1968). The effects of phenoxybenzamine on metabolism of ^{3}H-noradrenaline released from the isolated nictitating membrane. *Br. J. Pharmac.*, **34**, 222P.

LIGHTMAN, S. & IVERSEN, L. L. (1969). The role of Uptake$_2$ in the extraneuronal metabolism of noradrenaline in the isolated rat heart. *Br. J. Pharmac.*, **37**, 638–649.

NICKERSON, M. (1959). Blockade of the actions of adrenaline and noradrenaline. *Pharmac. Rev.*, **11**, 443–461.

THOENEN, H., HÜRLIMANN, W. & HAEFELY, W. (1964). Wirkung von Phenoxybenzamin Phentolamin und Azapetin auf adrenergische Synapses der Katzenmilz. *Helv. physiol. pharmac. Acta*, **22**, 148–161.

(*Received July* 29, 1969)