Br. J. Pharmac. (1973), **49**, 214–225.

Influence of reserpine-induced depletion of noradrenaline on the negative feed-back mechanism for transmitter release during nerve stimulation

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Summary

1. The effects of depletion of endogenous noradrenaline by reserpine-pretreatment on [^{3}H]-noradrenaline overflow elicited by nerve stimulation were determined in the isolated nerve-muscle preparation of the cat's nictitating membrane.

2. Reserpine pretreatment (0·3 mg/kg, s.c., 4 days prior to the experiment) reduced the noradrenaline levels in the smooth muscle of the nictitating membrane to about 10% of the control values while granular retention of [^{3}H]-noradrenaline had recovered to nearly 40% of the controls.

3. In the reserpine-pretreated tissue the fraction release per shock induced by nerve stimulation was 2·2-fold higher than the value obtained in the untreated tissues. This effect was correlated with the degree of depletion of the noradrenaline stores rather than with the decrease in the response of the effector organ.

4. Phenoxybenzamine, 2·9 μM reduced the responses to nerve stimulation to the same extent in control and in reserpine-pretreated tissues. Yet, this concentration of phenoxybenzamine increased by 13-fold the overflow of the labelled transmitter in the controls and only by 3-fold in reserpine-pretreated tissues.

5. The decrease in effectiveness of phenoxybenzamine in enhancing transmitter overflow after reserpine-pretreatment appears to be due to the decrease in the total release of the transmitter.

6. The results obtained support the view that in reserpine-pretreated tissues decreased transmitter output reduces the activation of the presynaptic α-adrenoceptors which mediate the negative feed-back mechanism that regulates transmitter release by nerve stimulation.

Introduction

The presence of presynaptic α-receptors in adrenergic nerve endings has been postulated recently (Langer, Adler, Enero & Stefano, 1971 ; Farnebo & Hamberger, 1971b ; Kirpekar & Puig, 1971 ; Enero, Langer, Rothlin & Stefano, 1972 ; Starke, 1972b). Stimulation of these α-receptors inhibits the release of noradrenaline and it has been suggested that they form part of a negative feed-back control mechanism through which the transmitter noradrenaline may inhibit its own release. It might be expected that this mechanism would operate most effectively

when the quantity of transmitter released by each nerve impulse is high. Consequently, after depletion of the transmitter by the administration of reserpine, the release of noradrenaline may be reduced to near or below the threshold necessary for stimulation of the presynaptic receptors. This possibility was tested by studying the overflow of [^{3}H]-noradrenaline elicited by nerve stimulation in the cat's nictitating membrane after reserpine pretreatment (0·3 mg/kg, 4 days prior to the experiment). Under these experimental conditions there is a considerable depletion of endogenous noradrenaline while granular retention of [^{3}H]-noradrenaline has already partially recovered.

Methods

Cats of 2·0 to 4·0 kg body weight and of either sex were anaesthetized with sodium pentobarbitone 35 mg/kg (i.p.) and the trachea was cannulated. The eyeball was excised and the nictitating membrane with all the adjoining tissue was placed in slightly modified Krebs solution previously equilibrated with 95% O_2 and 5% CO_2. The composition of the Krebs solution was as follows (mM): NaCl, 118·0; KCl, 4·7; $CaCl_2$ 2·6; $MgCl_2$, 1·2; NaH_2PO_4 1·0; $NaHCO_3$, 25·0; glucose 11·1; 1·5 mg of the disodium salt of ethylendiamine tetraacetic acid (EDTA) and 20 mg of ascorbic acid were added to 1 litre of Krebs solution. The medial muscle was dissected free with the postganglionic sympathetic fibres arising from the infratrochlear nerve which innervates this smooth muscle of the nictitating membrane (Thompson, 1958). The cartilage on which the fibres of the medial muscle are inserted was fixed to the bottom of a 10 ml organ bath. The upper end of the muscle was connected to a Grass FT03 force-displacement transducer and the tension developed by the muscle was recorded on a Grass polygraph. The temperature was maintained at 37° C and the organ bath was bubbled with a 95% O_2: 5% CO_2 mixture. The infratrochlear nerve was pulled through shielded bipolar platinum electrodes for stimulation with monophasic rectangular pulses of 0·5 ms duration and supramaximal voltage delivered by a Grass S44 stimulator.

A period of 30 min was allowed to elapse before the start of the experiment. During this period the Krebs solution was replaced every 10 minutes. The resting tension of the muscle was repeatedly readjusted to 2·5 g and it reached steady conditions after 30 to 40 minutes.

Thirty minutes after the smooth muscle was set up in the organ bath it was incubated for 30 min with 60 μCi (6 μCi/ml) of (±)-[7-^{3}H]-noradrenaline (New England Nuclear Corporation, Boston, Mass. specific activity 5–10 Ci/mM). At the end of the incubation period, the tissue was washed eight times for one minute each with fresh Krebs solution. Subsequently, the Krebs solution was replaced every 10 min during the following 30 min and thereafter every five min for an additional 60 min period. The repeated washings ensured the elimination of extraneuronally bound [^{3}H]-noradrenaline. The spontaneous outflow of radioactive products from the tissue into the bathing fluid was monitored by counting 0·5 ml samples of the fluid which had been in contact with the tissue for five minutes. Ninety minutes after the end of the incubation with [^{3}H]-noradrenaline the spontaneous outflow of radioactive products had levelled off and the collection of samples for the assay of total radioactivity began. When the drugs were added

to the organ bath they were replaced every time that the bathing fluid was renewed. The nerves were stimulated at 10 Hz for 2 or 5 minutes.

The outflow of radioactive products was measured before, during and after the period of stimulation. Overflow induced by nerve stimulation was calculated by subtracting the spontaneous outflow assumed to have occurred in each sample during and after the period of nerve stimulation. The value of the spontaneous outflow subtracted from these samples was the basal resting release obtained in the period immediately before stimulation. The 'total overflow of the transmitter' was the sum of all increases above spontaneous levels induced by the period of nerve stimulation.

Samples of 1 ml of the bathing solution which had been in contact with the tissue for 5 min were collected. The radioactivity was measured with a mixture of Toluene 666 ml; Triton X-100, 333 ml; 2–5 diphenyloxazole, (PPO) 5·5 g and 1–4 bis (2 (5-phenyloxazolyl)) benzene, (POPOP) 0·1 g as scintillator. One ml of the samples was dissolved in 10 ml of the scintillator. Quenching was corrected for by the addition of tritiated water standards.

In all experiments, two stimulation periods were applied, the second 50 min after the first. As previously reported, in the normal tissue there was only a small decrease (less than 10%) in the [^{3}H]-noradrenaline content of the stimulated muscles at the time of the second stimulation (Enero *et al.*, 1972). In the control experiments the overflow of radioactivty in two consecutive periods of stimulation was the same when expressed as a fraction of the radioactivity remaining in the tissue at the time of stimulation. In experiments carried out with nictitating membranes from cats pretreated with reserpine (0·3 mg/kg, s.c. 4 days prior to the experiment) the [^{3}H]-noradrenaline content of the tissue decreased by 21·10 ± 0·97% (mean ± S.E. of the mean of 10 observations) between the first and the second stimulation period. Yet, there was no difference in the fraction of the tissue radioactivity released by nerve stimulation between the first and second periods of stimulation.

For determinations of [^{3}H]-noradrenaline and its metabolites in the smooth muscle of the nictitating membrane at the end of each experiment the tissues were blotted dry, weighed and homogenized with 5 ml of cold 0·4 N perchloric acid containing 1·0 mg/ml EDTA and 1·25 mg/ml Na_2SO_3. Portions of the tissue homogenates were brought to pH 8·2 by the addition of 2 volumes of Tris-HCl buffer (0·5 M, pH 9·0). The samples were then poured into a column packed with 200 mg of alumina (Merck, Darmstadt, Germany). The column was washed with 5 ml of distilled water, the effluent and washing containing the *O*-methylated metabolites of noradrenaline. The [^{3}H]-noradrenaline and the deaminated glycol, 3,4 dihydroxyphenylglycol (DOPEG) were eluted with 3 ml of 0·2 N acetic acid (Graefe, Stefano & Langer, 1973). The deaminated acid, 3,4 dihydroxymandelic acid was eluted with 3 ml of 0·2 N HCl. [^{3}H]-noradrenaline was separated from [^{3}H]-DOPEG by means of strong cation exchange resin column (Dowex 50W-x8, H^+ form, 200–400 mesh). The column was washed with 3 ml of distilled water. The effluent and washing contained the deaminated glycol, DOPEG. Noradrenaline was then eluted with 2 ml of 2 N HCl (Graefe *et al.*, 1973).

The fluorometric analysis of noradrenaline was carried out by the method of Laverty & Taylor (1968) on an aliquot of the 0·2 N acetic acid eluate from the

alumina column. Recoveries for noradrenaline were 95%, and the results were not corrected for recoveries.

Statistical calculations were performed according to conventional procedures (Snedecor & Cochran, 1969).

The following drugs were used: reserpine phosphate (stock solution 10 mg/ml dissolved in 20% ascorbic acid solution) and phenoxybenzamine hydrochloride.

Results

Endogenous noradrenaline levels and neuronal accumulation of [^{3}H]-noradrenaline in normal and in reserpine-pretreated nictitating membranes

Four days after the administration of 0·3 mg/kg of reserpine there was a pronounced decrease in the endogenous noradrenaline levels in the cat's nictitating membrane (Table 1). Yet, as shown previously by Andén & Henning (1966) granular retention of noradrenaline had already partially recovered to approximately 40% of the control values (Table 1). The radioactivity retained in the tissue was predominantly due to noradrenaline both in control and in reserpine pretreated tissues (Table 1), indicating that [^{3}H]-noradrenaline was retained in an intravesicular compartment under both experimental conditions. The specific activity of [^{3}H]-noradrenaline retained in the reserpine-treated tissue was 5 times higher than that observed in the control preparations (Table 1).

Spontaneous and nerve stimulation-induced transmitter overflow in normal and in reserpine-pretreated nictitating membranes

Spontaneous outflow of labelled products was determined in 5 min samples and was expressed as a fraction of total radioactivity in the tissue. In the samples taken before the first period of nerve stimulation this value was higher in reserpine pretreated tissues when compared with the corresponding controls (reserpine-pretreated: 0·021 ± 0·001 (n=12), controls 0·01 ± 0·0008 (n=8) $P<0·001$).

TABLE 1. *Effects of reserpine pretreatment (0·3 mg/kg, s.c., 4 days) on the endogenous noradrenaline (NA) content and on retention of labelled noradrenaline in the cat's nictitating membrane*

Experimental groups	n	Endogenous content NA μg/g (a)	Neuronal retention (90 min) [^{3}H]-NA ng/g (b)	Neuronal retention (180 min) [^{3}H]-NA ng/g (c)	[^{3}H]-NA as % of total tissue radioactivity (d)	Specific activity ng [^{3}H]-NA μg NA (e)
Control	8	7·28±0·96	1076±108	784±96	86·1±2·5	125·0 ± 9·6
Reserpine-pretreated (0·3 mg/kg, 4 days prior)	12	0·94±0·17**	435±38**	287±21**	88·3±2·7	681·7±134·8*

(a) Endogenous noradrenaline (NA): total NA determined fluorimetrically at the end of the experiment (i.e. 180 minutes after the end of the incubation with [^{3}H]-NA)
(b) [^{3}H]-NA retained in the smooth muscle of the nictitating membrane 90 min after the end of the incubation with [^{3}H]-NA.
(c) [^{3}H]-NA retained in the tissue at the end of the experiment (i.e. 180 min after the end of the incubation with [^{3}H]-NA).
(d) [^{3}H]-NA retained in the tissue at the end of the experiment expressed as a percentage of the total radioactivity.
(e) The specific activity in the tissue was calculated from the values of the [^{3}H]-NA retained in the tissue (column c) and of the unlabelled NA (total NA determined fluorimetrically minus [^{3}H]-NA). These values were determined at the end of each experiment.
n=number of experime nts; * $P<0·01$; ** $P<0·001$.
Values are mean±S.E.M.

Nerve stimulation elicited an increase in overflow of [^{3}H]-noradrenaline and its metabolites both in controls and in reserpine-pretreated tissues. The distribution of [^{3}H]-noradrenaline and its metabolites in the overflow elicited by nerve stimulation has been reported recently (Enero *et al.*, 1972; Langer, Stefano & Enero, 1972). Since the transmitter released by nerve stimulation is metabolized after leaving the nerve terminal (Langer, 1970; Langer *et al.*, 1972) the total overflow of tritium was considered a more relevant estimation of the transmitter released by stimulation. The total increase in outflow of tritium above spontaneous levels induced by stimulation was expressed as a fraction of the total radioactivity remaining in the tissue: total nCi released per shock divided by total nCi remaining in the tissue at the onset of stimulation.

If a threshold concentration of the adrenergic transmitter in the synaptic gap is required to trigger the presynaptic negative feed-back mechanism, a decrease in endogenous noradrenaline should be expected to lead to higher values of stimulation-induced fractional release of [^{3}H]-noradrenaline. Consequently, it was of interest to compare the stimulation-induced fractional release of [^{3}H]-noradrenaline for different tissue levels of endogenous noradrenaline.

Table 2 shows that the absolute values of total overflow of radioactivity induced by nerve stimulation did not differ when the control group was compared with the reserpine-pretreated tissues. However, when overflow was expressed as a

TABLE 2. *Transmitter release induced by nerve stimulation in the normal and in the reserpine-pretreated nictitating membrane*

Experimental groups	*n*	Nerve stimulation-induced overflow nCi/g (a)	Fraction released per shock ($\times 10^{-5}$) (b)
Control	8	360·2±74·2	1·16±0·24
Reserpine-pretreated (0·3 mg/kg, 4 days prior)	12	333·9±59·5	2·51±0·39*

(a) Total radioactivity released during the 2 min period of nerve stimulation (10 Hz, 0·5 ms duration, supramaximal voltage) expressed per g of tissue.
(b) Fraction of the total radioactivity in the tissue released per shock during the period of nerve stimulation.
Values are mean±S.E.M.; *n*=number of experiments; * $P<0.025$.

fraction of the radioactivity in the tissue the value obtained in reserpine-pretreated tissues was 2·2 times higher than the controls (Table 2). Since under identical experimental conditions in untreated tissues there was no difference between the specific activity of noradrenaline retained in the tissue and the specific activity of the transmitter released by nerve stimulation (Langer & Vogt, 1971) the assumption was made that the fractional release of tritiated noradrenaline was the same as the fractional release for the endogenous transmitter. Thus, the overflow of total noradrenaline was calculated from the fractional release of the labelled transmitter and the endogenous content of total noradrenaline in each experiment. The 'estimated overflow' of total noradrenaline was 79·0±17·3 (pg/shock)/g (n=8) in the controls, and 23·0±5·2 (pg/shock)/g (n=12) after reserpine pretreatment ($P<0.005$), in the groups stimulated for 2 minutes.

Figure 1 shows that in the reserpine-pretreated tissues the fractional release of [^{3}H]-noradrenaline induced by nerve stimulation was significantly higher in the group in which endogenous noradrenaline levels were below 1 μg/g ($P<0.001$).

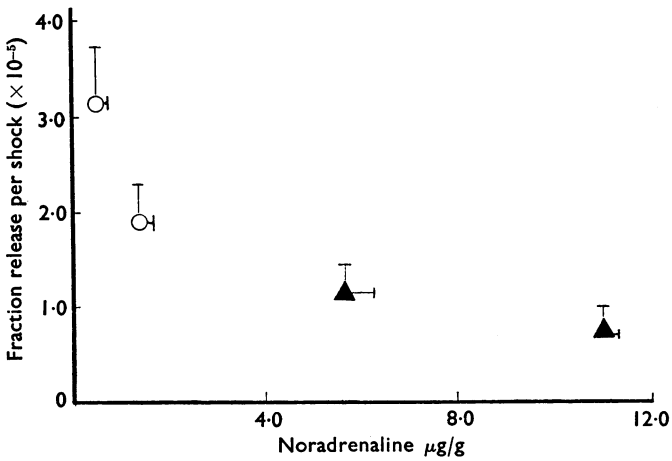

FIG. 1. Relationship between the endogenous noradrenaline content in the nictitating membrane and the fraction of the tissue radioactivity released per stimulus. Abscissae: endogenous noradrenaline content in the nictitating membrane. Ordinates: fraction release per shock ($\times 10^{-5}$) induced by a 2 min period of nerve stimulation (10 Hz, 0·5 ms, supramaximal voltage). ▲: Normal nictitating membranes: two groups were selected according to their endogenous noradrenaline content: (a) 4·0 to 7·0 μg/g (n=4); (b) 7·1 to 11·0 μg/g (n=4). O: Nictitating membranes from reserpine-pretreated cats (0·3 mg/kg, s.c., 4 days prior to the experiment): two groups were selected according to their endogenous noradrenaline content: (a) 0 to 0·9 μg/g (n=6); (b) 1·0 to 2·4 μg/g (n=6). Values are mean ± S.E.M. n=number of experiments.

For levels between 1 and 2 μg/g of noradrenaline the fractional release approached the values obtained in control tissues. There was no significant difference in fractional release between the two groups of normal tissues (4·0 to 7·0 μg/g and 7·0 to 11·0 μg/g of noradrenaline).

Responses to nerve stimulation in normal and in reserpine-pretreated tissues

The responses of the smooth muscle of the nictitating membrane to nerve stimulation in the experiments in which the overflow of [^{3}H]-noradrenaline was studied, are shown in Figure 2. In the controls the responses were well sustained during the 2 or 5 min of stimulation (Fig. 2a and b). As previously reported, responses were also sustained and of similar magnitude when the second period of stimulation was applied in the controls (Enero *et al.*, 1972).

Four days after reserpine-pretreatment, responses to nerve stimulation were only slightly reduced (Fig. 2c and d). These responses were sustained only for short periods of stimulation and declined rapidly after the second minute (Figure 2d).

Effects of phenoxybenzamine on transmitter overflow elicited by nerve stimulation in normal and in reserpine-pretreated nictitating membranes

Figure 3a shows that in the presence of 2·9 μM phenoxybenzamine responses to nerve stimulation were decreased to the same extent in control and in reserpine pretreated tissues

The ratio of the values for fractional release induced by stimulation in the presence of phenoxybenzamine over the corresponding controls are shown in Figure 3b. In the control group where the nerves were stimulated for 2 min

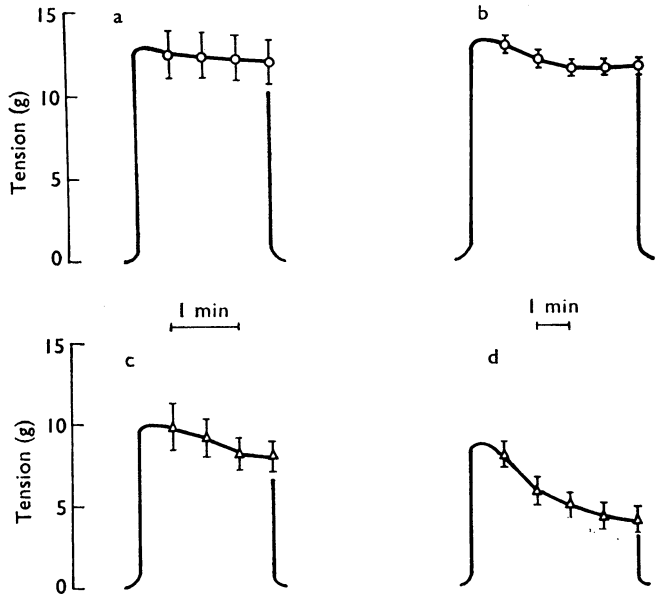

FIG. 2. Responses of the medial muscle of the nictitating membrane to nerve stimulation (10 Hz, 0·5 ms, supramaximal voltage). (a) Untreated muscles, 2 min stimulation ($n=8$). (b) Untreated muscles, 5 min stimulation ($n=24$). (c) Reserpine-pretreatment (0·3 mg/kg, s.c., 4 days before the experiment), 2 min stimulation ($n=6$). (d) reserpine pretreatment 0·3 mg/kg s.c. 4 days before the experiment) 5 min stimulation ($n=10$). Values are mean ± S.E.M. n=number of experiments.

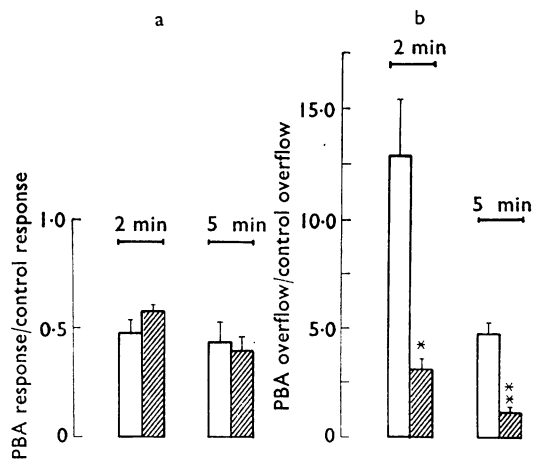

FIG. 3. Effects of phenoxybenzamine on responses and on transmitter overflow elicited by nerve stimulation in the normal and in the reserpine-pretreated nictitating membrane. a. Responses to nerve stimulation (10 Hz, 0·5 ms, supramaximal voltage). Ratio of the maximum responses to nerve stimulation in the presence of phenoxybenzamine (PBA, 2·9 μM) over the corresponding controls. b. Fraction release per shock ($\times 10^{-5}$) induced by nerve stimulation. Ratio of overflow of the labelled transmitter in the presence of phenoxybenzamine over the corresponding controls. Open columns=untreated. Cross-hatched columns=reserpine pretreated (0·3 mg/kg, s.c., 4 days before the experiment). 2 min and 5 min refer to the duration of the period of nerve stimulation. Values are mean ± S.E.M. $*P<0{\cdot}005$; $**P<0{\cdot}001$.

the ratio phenoxybenzamine over control was $12{\cdot}9 \pm 2{\cdot}5$ ($n=4$) while this value was only $3{\cdot}1 \pm 0{\cdot}4$ ($n=5$) in reserpine-pretreated tissues ($P<0{\cdot}005$). Similar differences between the controls and the reserpine-pretreated tissues were obtained in the groups in which the nerves were stimulated for 5 min (Figure 3b).

The considerable reduction in the effectiveness of phenoxybenzamine in increasing transmitter overflow after reserpine pretreatment is compatible with the view that activation of the presynaptic negative feed-back mechanism was decreased after depletion of the noradrenaline stores by reserpine-pretreatment.

Discussion

The hypothesis of the involvement of presynaptic α-adrenoceptors in the regulation of noradrenaline release by nerve stimulation is based on the increase in transmitter overflow obtained in the presence of α-adrenoceptor blocking agents. Both phenoxybenzamine and phentolamine increase transmitted overflow in concentrations in which neuronal uptake of noradrenaline is not interfered with (Langer *et al.*, 1971 ; Farnebo & Hamberger, 1971b ; Starke, Montel & Schümann, 1971 ; Enero *et al.*, 1972). In additino, these α-receptor blocking agents are effective in increasing noradrenaline overflow in tissues with either α- or β-adrenoceptors in the effector cell (Langer *et al.*, 1971 ; Starke, 1972a). Furthermore, α-receptor agonists can inhibit noradrenaline overflow elicited by nerve stimulation (Farnebo and Hamberger, 1971b ; Starke, 1972a ; Starke, 1972b). These results are compatible with the view that presynaptic α-receptors regulate the transmitter released by nerve stimulation through a negative feed-back mechanism in which the noradrenaline released inhibits its own release once a threshold concentration is achieved in the synaptic gap.

If the endogeneous levels of the neurotransmitter are depleted by the administration of reserpine the transmitter released during nerve stimulation might fall to levels close to or below the threshold required to activate the proposed negative feed-back mechanism. Under these experimental conditions two events can be predicted in connection with the overflow of labelled noradrenaline: (a) fractional release per shock should be increased because the presynaptic negative feed-back mechanism is not triggered by the neurotransmitter ; (b) the increase in transmitter overflow obtained in the presence of α-receptor blocking agents should be reduced or abolished because the presynaptic regulatory mechanism should be less effective at low transmitter output.

With the schedule of reserpine pretreatment employed in our experiments the endogenous levels of noradrenaline in the nictitating membrane were reduced to nearly 10% of the control values. Yet, as previously described by Andén & Henning (1966) on the fourth day after the administration of reserpine, granular retention of [^{3}H]-noradrenaline was approximately 40% of the controls and almost 90% of the radioactivity remaining in the tissue was accounted for by [^{3}H]-noradrenaline. The latter indicated the vesicular localization of the labelled transmitter which was retained in the tissue. The early recovery of the granular storage capacity after reserpine-pretreatment was also observed by Farnebo (1971) in the rat iris.

Four days after pretreatment with reserpine the overflow of tritium (expressed as the fractional release per shock) was significantly higher than in the controls

in the groups with the lowest levels of endogenous noradrenaline. These results indicated that in reserpine pretreated tissues total noradrenaline release might be reduced considerably and consequently would be less effective in activating the negative feed-back mechanism when compared with the controls. Therefore, it was of interest to estimate total overflow of noradrenaline in controls and in reserpine-pretreated tissues. These estimations, calculated from the fractional release of the labelled transmitter were based on the assumption that the specific activities of noradrenaline in the tissues and in the overflow elicited by stimulation were the same in the controls and in reserpine-pretreated tissues. While this information is available for untreated tissues (Langer & Vogt, 1971) the determination of the overflow of total noradrenaline after pretreatment with reserpine is below the sensitivity of our methods.

The 'estimated overflow' of transmitter in reserpine-pretreated tissues was decreased only to 30% of the controls, although the reduction in endogenous levels of noradrenaline was nearly 90% (Table 1). This discrepancy between the values of overflow and those of endogenous noradrenaline is probably the consequence of the increased fractional release of the transmitter that was observed in reserpine-pretreated tissues. The latter was probably due to the fact that the presynaptic negative feed-back mechanism was less operative in the tissues depleted of noradrenaline, since the values for fractional release were higher in those tissues in which depletion of endogenous noradrenaline was most pronounced.

An increase in fractional release of [^{3}H]-noradrenaline by field stimulation in tissues depleted of endogenous noradrenaline by α-methyl-*p*-tyrosine has been reported in brain slices (Farnebo, Hamberger & Jonsson, 1971). It is likely that in their experiments total noradrenaline released was near or below the threshold for the presynaptic negative feed-back mechanism, since this process has been postulated for noradrenaline release in the central nervous system as well (Farnebo & Hamberger, 1971a).

If the increase in fractional release of [^{3}H]-noradrenaline observed after reserpine pretreatment is due to a decrease in the activation of presynaptic α-receptors, blockade of these receptors by phenoxybenzamine should be less effective in enhancing transmitter overflow. The concentration of phenoxybenzamine employed in our experiments reduced to the same extent the mechanical responses to nerve stimulation in the controls and in the reserpine-pretreated group indicating comparable degrees of α-receptor blockade. Yet, in the groups which were stimulated for 2 min the increase in transmitter overflow was 13-fold in the controls while it only reached 3-fold after pretreatment with reserpine. The difference in effectiveness of phenoxybenzamine between the control and the reserpine-pretreated group was also obtained with 5 min stimulation periods.

In the control groups phenoxybenzamine increased transmitter overflow to a greater extent when 2 rather than 5 min periods of stimulation were applied. The greater effectiveness of phenoxybenzamine in increasing transmitter overflow with short periods of stimulation in untreated tissues was not related to fatigue. Responses were sustained during the 5 min period of stimulation and the overflow of tritium did not decline as a function of time when samples were analysed every minute (Enero & Langer, unpublished observations). However, in the presence of phenoxybenzamine the responses to nerve stimulation fade after the first minute of stimulation (Enero *et al.*, 1972) indicating a decline in the overflow

of transmitter as a function of time. Consequently, phenoxybenzamine should be more effective in increasing transmitter overflow when short periods of stimulation are applied.

The decrease in the effectiveness of phenoxybenzamine in enhancing transmitter overflow after reserpine-pretreament is compatible with the view that under these conditions total noradrenaline release fell near the threshold concentration required to activate the presynaptic α-receptors which are responsible for the negative feed-back mechanism.

Our results do not exclude the possibility of an interaction between reserpine and phenoxybenzamine at the level of the granule. Phenoxybenzamine in high concentrations has a granular site of action (Adler-Graschinsky, Langer & Rubio, 1972 ; Graefe *et al.*, 1973), and on the other hand a reserpine-like agent, Ro 4-1284 (2H-benzo-(a)quinolizine-2-hydroxy-2-ethyl1,3,4,6,7,11-b-hexahydro-3-isobutyl-9,10 - dimethoxy) increases the overflow of the neurotransmitter elicited by nerve stimulation in the cat's nictitating membrane (Enero & Langer, unpublished observations).

However, a direct interaction between reserpine and phenoxybenzamine under our experimental conditions appears unlikely because the effectiveness of phenoxybenzamine in increasing transmitter overflow can be restored when reserpine pretreated nictitating membranes are incubated with exogenous noradrenaline and a monoamine oxidase inhibitor to increase the tissue noradrenaline levels to the range of the controls (Enero & Langer, unpublished observations).

The concentration of phenoxybenzamine employed in this study has been shown to reduce neuronal uptake of noradrenaline to 40% of the controls (Enero *et al.*, 1972). Therefore, it could be argued that inhibition of neuronal uptake has been the main reason for the increase in transmitter overflow observed in the presence of the drug. However, inhibition of neuronal uptake by drugs is not very effective in increasing transmitter overflow during nerve stimulation (Blakeley, Brown & Ferry, 1963 ; Geffen, 1965 ; Ferry, 1967), and in the cat's nictitating membrane a similar inhibition of neuronal uptake in the presence of cocaine did not enhance transmitter overflow (Langer *et al.*, 1972). Furthermore, it has been shown in this tissue that the increase in transmitter overflow observed with several concentrations of phenoxybenzamine is well correlated with the degree of the α-adrenoceptor blocking effect of the drug (Enero *et al.*, 1972). Consequently, it appears unlikely that the decrease in effectiveness of phenoxybenzamine in enhancing transmitter overflow after reserpine pretreatment could be related to changes in the degree of inhibition of neuronal uptake of noradrenaline, because reserpine-pretreatment does not inhibit uptake of noradrenaline across the neuronal membrane (Lindmar & Muscholl, 1964). Nevertheless, the small increase in transmitter overflow obtained with phenoxybenzamine in reserpine-pretreated tissues might be related to inhibition of neuronal uptake of noradrenaline because phenoxybenzamine inhibits neuronal uptake of noradrenaline in a non-competitive manner while cocaine is a competitive inhibitor (Iversen & Langer, 1969).

The responses to nerve stimulation obtained after reserpine pretreatment may not be due entirely to the release of noradrenaline, because 1 and 2 days after reserpine pretreatment α-adrenoceptor blocking agents fail to antagonize the residual responses to nerve stimulation in the cat's nictitating membrane (Langer,

1972). Yet, 4 days after the administration of reserpine the responses to nerve stimulation were blocked by phenoxybenzamine to the same extent in the controls and after pretreatment with reserpine, indicating that at this time the residual responses were due predominantly to the release of noradrenaline.

Farnebo & Malmfors (1971) postulated that the response of the effector organ regulates the release of the transmitter and proposed a transynaptic regulation in the release of noradrenaline by nerve stimulation, enhancement in transmitter release being causally related to a decrease in the response of the effector organ. In our experiments the responses of the effector organ to the 2 min period of nerve stimulation were not significantly reduced by reserpine pretreatment when compared to the untreated tissues. Yet, the fractional release of [^{3}H]-noradrenaline was increased more than 2-fold. Since this increase in overflow obtained in reserpine-pretreated tissues was related to the depletion of endogenous noradrenaline and not to a decrease in the response of the effector organ, our results do not support a transynaptic mechanism in the regulation of transmitter release. Instead, our results indicate that a threshold concentration of the noradrenaline released by nerve stimulation is required for the activation of the presynaptic α-receptors which trigger the negative feed-back mechanism in the regulation of transmitter release by nerve stimulation.

The authors are indebted to Dr. R. A. McLean of Smith, Kline & French Laboratories (Philadelphia, U.S.A.) for the supply of phenoxybenzamine and to Dr. H. Brunner of Ciba-Geigy Laboratories (Basel, Switzerland) for reserpine.

The technical assistance of Miss María J. Rodriguez and Mrs. Noemí Elena Fernandez is gratefully acknowledged.

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(*Received December* 29, 1972)