Br. J. Pharmac. (1971), 43, 359–369.

Effect of flow-stop on noradrenaline release from normal spleens and spleens treated with cocaine, phentolamine or phenoxybenzamine

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Summary

1. Cat spleens were perfused with Krebs-bicarbonate solution, using a constant-flow pump at a rate of about 7 ml/min at 33–35° C. Noradrenaline (NA) overflow by nerve stimulation at 10 Hz for 20 s was determined with or without flow-stop before and after treatment with cocaine, phentolamine or phenoxybenzamine. In order to determine the effect of flow-stop on overflow, the arterial and the venous flows were occluded by clamping the inflow and outflow tubes during the period of stimulation plus 30, 60 or 120 seconds.

2. Without flow-stop, NA output was 0·93±0·25 ng/stimulus, which was significantly increased after cocaine (123±6·6%), phentolamine (415±93%) and phenoxybenzamine (578±107%). Phentolamine and phenoxybenzamine were much more effective than cocaine in enhancing overflow.

3. Before treatment with drugs, flow-stops of 30, 60 and 120 s reduced NA outputs to 70±6·6, 27·5±2 and 7%, respectively, of the control outputs without flow-stop. None of the drugs significantly influenced the percentage reductions in NA outputs during a 30 s flow-stop. However, the percentage outputs after cocaine or phenoxybenzamine treatment during a 60 s flow-stop significantly increased to 45±2·5% and 57±6%, respectively, as compared to the percentage output of 27·5±2% from untreated spleens during a corresponding flow-stop period. During flow-stop, there was no appreciable metabolism of the released transmitter.

4. Diffusion of the released transmitter from the site of liberation plays only a minor role in the removal of the released NA.

5. It is suggested that the NA released by nerve stimulation acts on the presynaptic α sites to inhibit its own release by a negative feedback mechanism. Adrenoceptor blocking agents enhance the NA overflow from spleen because they remove this autoinhibition by blocking the presynaptic α sites.

Introduction

Marked potentiation of the effects of exogenous NA in sympathetically denervated organs has been principally attributed to the inability of the degenerated sympathetic nerves to remove infused NA, thereby allowing higher concentrations of NA to reach the effector site (Kirpekar, Cervoni & Furchgott, 1962). Also, potentiation

by cocaine of the actions of NA has been explained on the basis of block of neuronal uptake by this agent (Iversen, 1967). Besides potentiating the actions of exogenous NA, agents such as cocaine which prevent uptake should also potentiate the actions of NA released by sympathetic nerve stimulation, and greatly enhance the overflow of adrenergic transmitter in the venous blood on sympathetic nerve stimulation. The literature on this point is inconclusive. Trendelenburg (1959) reported no increase in the release of NA from splenic nerve stimulation after cocaine. Blakeley, Brown & Ferry (1963) and Kirpekar & Cervoni (1963) reported no increase, or only a small increase, after cocaine (5 mg/kg). Subsequent treatment with hydergine or phenoxybenzamine increased the output very markedly. However, Theonen, Huerlimann & Haefely (1964) reported that cocaine increased NA output from saline perfused spleen when nerves were stimulated at low frequencies. Muscholl (1963) also reported that cocaine increased NA output by nearly 2-fold from saline perfused rabbit heart. In contrast to the conflicting reports on cocaine, there is almost a unanimous agreement that α-adrenoceptor blocking agents such as phenoxybenzamine, hydergine and phentolamine substantially increase the transmitter output from the spleen after nerve stimulation (Brown & Gillespie, 1957; Blakeley *et al.*, 1963; Kirpekar & Cervoni, 1963; Thoenen, Huerlimann & Haefely, 1966).

The increased overflow of NA from the spleen with adrenoceptor blocking agents has been attributed to the effects of these drugs in inhibiting NA reuptake, and also to their ability to block vasoconstriction by released NA and thus prevent a decrease in blood flow. On the basis of this latter concept, α-adrenoceptor blocking agents, by maintaining blood flow during nerve stimulation, should increase overflow by allowing the transmitter readily to diffuse away from the nerve endings; whereas agents such as cocaine, which enhance the reduction in blood flow by vasoconstriction during nerve stimulation, should allow the transmitter to remain in contact with the nerves for a longer time and thus facilitate reuptake (Hertting, 1965).

The work described here was undertaken to reinvestigate the effects of cocaine, phentolamine and phenoxybenzamine in spleens perfused with Krebs-bicarbonate solution. The saline perfused spleen was selected for this study, since most of the results about the action of cocaine on transmitter release have been obtained from saline perfused organs (Muscholl, 1963; Theonen *et al.*, 1964, 1966). Spleens were perfused with a constant outflow pump to keep the perfusion rate constant when different drugs were used. In addition, the effect of stopping flow for different periods on the recovery of venous NA released by nerve stimulation was also determined in normal and drug treated spleens. A preliminary report of some of these findings has appeared (Puig & Kirpekar, 1970).

Methods

Cat spleens were perfused with Krebs-bicarbonate solution at 33–35° C according to the method described by Kirpekar & Misu (1967). Cats were anaesthetized by ether induction, followed by chloralose (60 mg/kg, i.v.). The abdomen was opened by a midline incision, and the stomach, intestines and colon were removed. Both adrenals were also removed. The arrangement for splenic nerve stimulation and sample collection were similar to those previously described. The perfusion rate was kept constant at about 7 ml/min by means of a pump (Sigmamotor AL4E).

Splenic nerves were stimulated at 10 Hz for 20 s (25V, 1 ms) in normal spleens and spleens treated with cocaine, phentolamine and phenoxybenzamine. In some experiments without any drugs, nerves were also stimulated at 30 Hz for 6·7 seconds. Venous samples were collected for 60 seconds. In some experiments the endogenous pool of NA was labelled by infusing into the spleen ^{3}H-NA (specific activity 3·7 Ci/mM) at 20 ng/min for 30 minutes. After the infusion of ^{3}H-NA, spleens were perfused with normal Krebs solution for 20–30 min before nerves were stimulated.

Generally, the spleen was first perfused with Krebs solution for about 15 min and nerves were stimulated at 10 Hz to obtain the initial control stimulation sample. The spleen was then perfused with a drug. Cocaine or phentolamine (2 μg/ml or 3 μg/ml, respectively) were perfused for 20–25 min, and nerves were then stimulated in their presence. Phenoxybenzamine (2 or 10 μg/ml) was perfused for 20–30 min, and nerves were stimulated after perfusion with regular Krebs solution (free of phenoxybenzamine) had been reinstituted. In most experiments the spleen was perfused with phenoxybenzamine solution after the perfusion with cocaine or phentolamine. In other experiments perfusion with phentolamine followed that with cocaine. In two experiments all three drugs were used in the same spleen.

To study the effect of flow on NA overflow by nerve stimulation, the flow in the spleen was stopped by simultaneously clamping the inflow and outflow tubes. The pump was turned off during flow-stop. Flow was stopped during the period of stimulation plus an additional 30, 60 or 120 s, and venous samples were taken for 60 s immediately after resumption of flow. Experiments of this type were performed in normal and drug treated spleens. In each experiment the stimulation with 30 s flow-stop preceded, and the stimulation with 60 s flow-stop succeeded, a stimulation period without flow-stop. In two experiments the occlusion period was extended to 120 seconds.

Assay of NA

The NA content of the venous samples was determined by the method of Anton & Sayre (1962), except that 3 ml perchloric acid (0·05 N) was used for elution. In experiments using ^{3}H-NA, total radioactivity or ^{3}H-NA content of the venous samples was measured in a Tricarb liquid scintillation counter by adding 0·5 ml of the sample to 10 ml of Bray's scintillation fluid. To determine the net increment in radioactivity or ^{3}H-NA output due to nerve stimulation, the background activity of the same volume of solution in the absence of nerve stimulation was subtracted from the total output of radioactivity or ^{3}H-NA by nerve stimulation.

Results

Release of NA from untreated spleens by nerve stimulation

NA overflow after stimulation of the splenic nerves at 10 Hz for 20 s was 0·93 ng$\pm$0·25 ng/stimulus (n=12). Output of NA at this frequency in saline perfused spleens is considerably greater than the corresponding outputs in the blood-perfused spleens (Brown & Gillespie, 1957).

Effect of cocaine on NA overflow

Since Thoenen *et al.* (1964) have demonstrated that cocaine (2 μg/ml) effectively prevents the uptake of exogenous NA and enhances the transmitter output by nerve stimulation, we have used the same concentration of cocaine in these experiments. Figure 1 shows that cocaine enhanced NA output over the control value, but only by a small margin. Thus, cocaine increased the output from a control value of

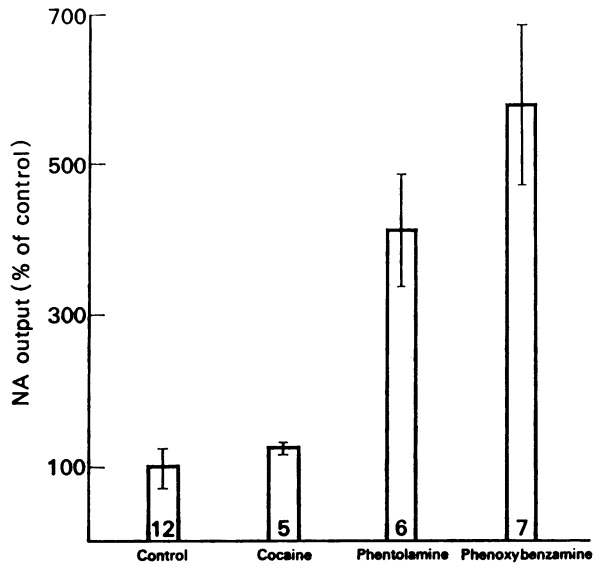

FIG. 1. Effect of cocaine, phentolamine and phenoxybenzamine on NA output. Nerves were stimulated at 10 Hz for 20 seconds. Each column represents mean NA output, expressed as a percentage of the initial control output before treatment. Numbers in each column represent the number of experiments, and vertical lines are the standard errors of the mean.

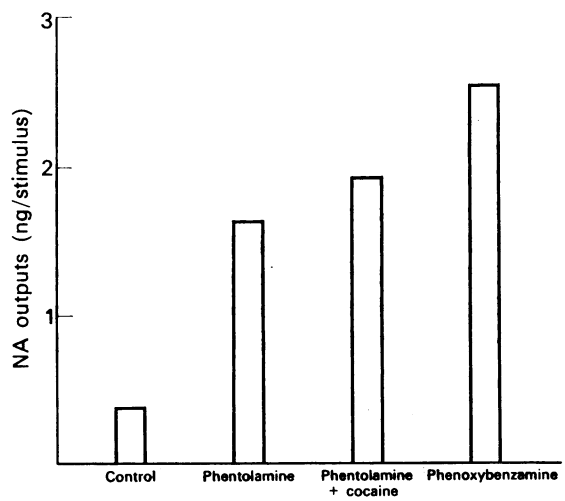

FIG. 2. A typical experiment showing the effect of cocaine, phentolamine and phenoxybenzamine. Nerves were stimulated at 10 Hz for 20 s, and each column represents NA output (ng/stimulus). Phentolamine (3 μg/ml) was perfused for 20 min before nerves were stimulated. The spleen was then perfused with a solution containing both phentolamine and cocaine (2 μg/ml). The last column represents NA output after the spleen was perfused with phenoxybenzamine (10 μg/ml) for 20 min, followed by normal Krebs solution.

1·3±0·66 to 1·55±0·65 ng/stimulus. Expressing the output after cocaine treatment as a percentage of the initial control output, cocaine significantly increased it to 123 ± 6·6% ($P<0·05$, paired data comparison).

Effect of phentolamine on NA overflow

Figures 1 and 2 show the effects of phentolamine on NA overflow. Figure 2 shows a single experiment in which phentolamine, cocaine and phenoxybenzamine were used in the same animal. Phentolamine enhanced the NA overflow by nearly 4-fold on nerve stimulation. Figure 1 shows that phentolamine significantly increased the transmitter output by nearly 4-fold, to 415±93% of the control output ($P<0·001$). In six paired experiments, the NA overflow after phentolamine treatment increased to 2·48±1·0 ng/stimulus from the corresponding control output of 0·54±0·22 ng/stimulus.

Effect of phenoxybenzamine on NA overflow

Phenoxybenzamine markedly increases NA output by nerve stimulation. In seven spleens treated with phenoxybenzamine, the NA output was 4·74±0·8 ng/stimulus, whereas the control output without treatment was 1·18±0·41 ng/stimulus. Figure 1 shows that phenoxybenzamine increased NA output to 578±107% of the control value ($P<0·001$).

Comparison of the effects of cocaine, phentolamine and phenoxybenzamine in the same spleen

The experiment illustrated in Fig. 2 shows that phentolamine alone increased the transmitter output by several fold. Cocaine added after phentolamine produced an additional small increment in transmitter output. Phenoxybenzamine was only slightly more effective than the combined treatment with phentolamine and cocaine. Similar results were obtained in one more experiment.

Effect of flow-stop on NA output by nerve stimulation in untreated spleens

In untreated spleens NA output was 208 ± 60 ng ($n=10$). After a 30 s flow-stop period, the output was reduced to 119±44 ng ($n=7$), which was further reduced to 50±11 ng after a 60 s flow-stop period. In order to calculate the significance of the differences in NA outputs, the control outputs without flow-stop were taken as 100%, and the outputs after 30, 60 or 120 s flow-stop periods were expressed as percentages of the initial control output. Results calculated in this manner showed that NA outputs were significantly reduced to 70 ± 6·0% and 27·5 ± 2% after 30 and 60 s flow-stop periods, respectively ($P<0·004$). In one experiment, extending the flow-stop period to 120 s reduced the NA output to 7% (Fig. 3).

Effect of phentolamine, cocaine and phenoxybenzamine on NA output by nerve stimulation after flow-stop

In spleens treated with phentolamine the control output of NA was 499±208 ng, which was reduced to 187±16 ng (65±5·6%) and 136±20 ng (36±6·2%) after 30 and 60 s flow-stop periods, respectively. NA outputs after 30 and 60 s flow-stop periods were significantly reduced ($P<0·004$) as compared to the phentolamine

control output without flow-stop. Differences in outputs between 30 and 60 s flow-stop periods were also highly significant ($P<0.001$). Even though the percentage reductions in outputs after flow-stop periods were not significantly different after phentolamine treatment as compared to those with no treatment, the NA outputs

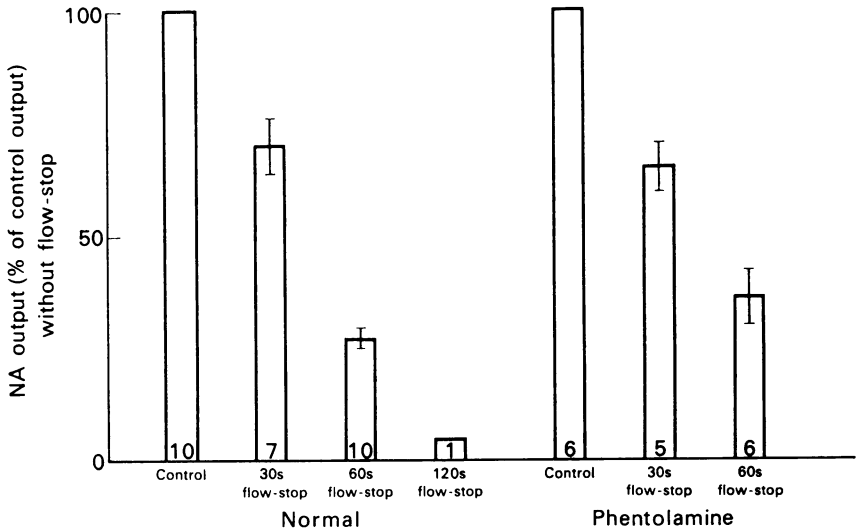

FIG. 3. Effect of flow-stop on NA overflow expressed as a percentage of the control output without flow-stop. Left and right hand parts of the figure show experiments in normal spleens and spleens treated with phentolamine. Nerves were stimulated at 10 Hz for 20 seconds. Flow was stopped for the duration of stimulation +30, 60 or 120 seconds. Control output was 208±60 ng in normal spleens. In spleens treated with phentolamine control output without flowstop was 499±208 ng.

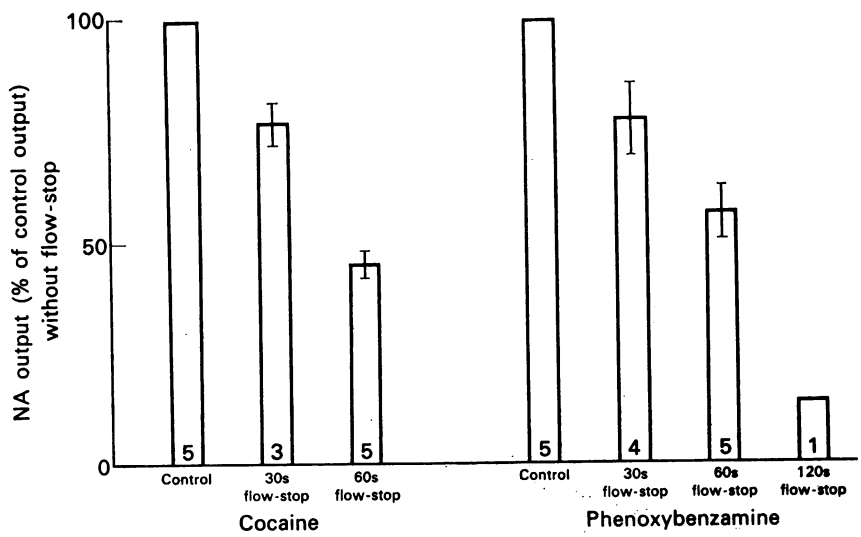

FIG. 4. Effect of flow-stop on NA overflow from spleens treated with cocaine or phenoxybenzamine. Nerves were stimulated at 10 Hz for 20 seconds. Control output without flow-stop in spleens treated with cocaine was 315±132 ng. Control output in spleens treated with phenoxybenzamine was 1,011±105 ng. Other details same as Fig. 3. Note that after treatment with cocaine and phenoxybenzamine the percentage reduction in NA overflow after 60 s flow-stop was significantly less than the reduction in normal spleen after a flow-stop of a similar duration.

remained at least 4–5 times greater than the corresponding control outputs with or without flow-stop periods (Fig. 3).

In the presence of cocaine, NA output without flow-stop was 315±132 ng, which was reduced to 231±159 ng (76±4·6%) and 154±74 (44·6±2·6%) after 30 and 60 s flow-stop periods, respectively. The percentage outputs obtained after 60 s flow-stop only, are significantly greater ($P<0·05$) than the corresponding NA outputs after 60 s flow-stop in untreated spleens (Fig. 4).

In spleens treated with phenoxybenzamine, NA output during a control stimulation period without flow-stop was 1,011 ± 105 ng. This was reduced to 788 ± 165 (77±8%) and 585±93 ng (57±6%) after stopping the flow for 30 and 60 s, respectively. In one experiment, stopping the flow for 120 s reduced NA output to 14%. The percentage outputs obtained after 60 s flow-stop were significantly greater ($P<0·01$) than the corresponding NA outputs after 60 s flow-stop in untreated spleens (Fig. 4).

Effect of flow-stop on the metabolism of NA released by nerve stimulation

Effect of flow-stop on the overflow of NA by nerve stimulation was observed in normal spleens at stimulation frequencies of 10 and 30 Hz. To ascertain the extent of metabolism of released NA during flow-stop, the endogenous pool of NA was first labelled by infusing ^{3}H-NA into the spleen before nerve stimulation. Since the net increase in radioactivity by nerve stimulation is primarily due to release of ^{3}H-NA (Hertting & Axelrod, 1961; Gillespie & Kirpekar, 1966), and if metabolism of released NA did not occur during flow-stop, the net increase in radioactivity should be largely accounted for as ^{3}H-NA without the formation of metabolites. Results of a typical experiment using a stimulation frequency of 30 Hz are shown in Fig. 5. The net increase in radioactivity by nerve stimulation with or without flow-stop can be mostly accounted for as ^{3}H-NA, since the two lower columns representing net ^{3}H-NA increase and NA output roughly correspond to the respective columns in the top panel. Assuming net increase in total radioactivity as 100% during the control period of stimulation without flow-stop, increments in total radioactivity during 30 and 60 s flow-stop periods were 67 and 26%, respectively, which corresponded to the increments in ^{3}H-NA columns during the corresponding flow-stop periods. Similar experiments were performed using a stimulation frequency of 10 Hz. In five experiments, net increases in total radioactivity during 30 and 60 s flow-stop periods were 69±10·2 and 39±10% of the control stimulation period without flow-stop. After extracting the samples for ^{3}H-NA and expressing the outputs as a percentage of the control output, net increments in ^{3}H-NA output during 30 and 60 s flow-stop periods were 67±5·0 and 31±6·5%, respectively, and were comparable to the net increases in radioactivity during the corresponding flow-stop periods. We conclude, on the basis of these observations, that appreciable metabolism of NA released by nerve stimulation does not occur during the flow-stop periods.

Discussion

These experiments on the effects of cocaine, phentolamine and phenoxybenzamine on the release of NA by nerve stimulation in saline perfused cat spleens confirm observations previously reported by Trendelenburg (1959), Kirpekar & Cervoni

(1963), and Blakeley, Brown & Ferry (1963) in the intact spleen. Cocaine increased NA output significantly, but only by a very small percentage, whereas phentolamine and phenoxybenzamine increased it by nearly 4–5 times. The increments in NA outputs after phentolamine and phenoxybenzamine were much greater than after cocaine alone ($P<0.01$). A statistical comparison between NA outputs after phentolamine and phenoxybenzamine treatment did not show a significant difference due to wide variations in transmitter outputs from different spleens; yet in two paired experiments, phenoxybenzamine increased NA output considerably as compared to phentolamine alone. Recently, Häggendal (1970) and Farnebo & Hamberger

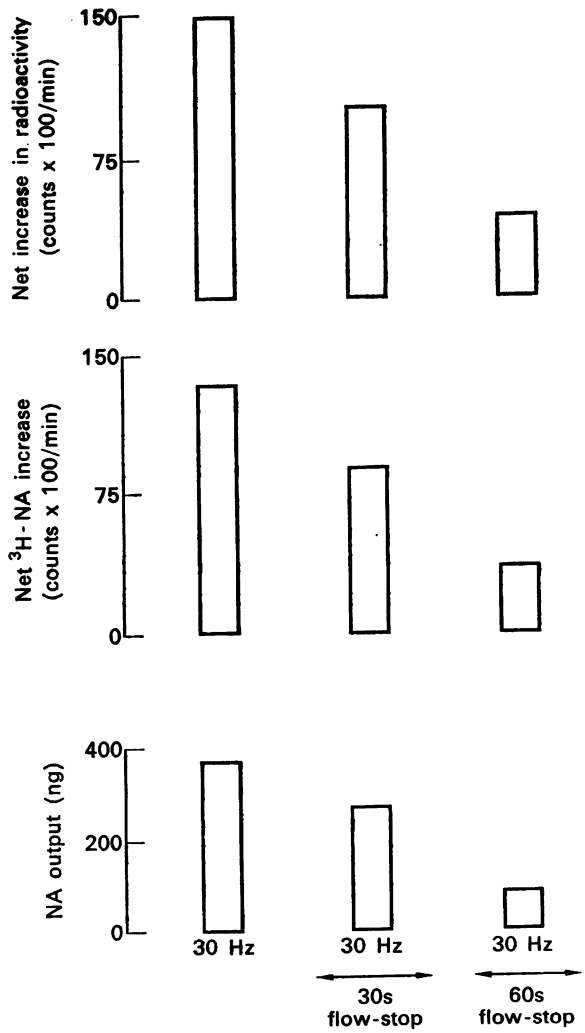

FIG. 5. Effect of flow-stop of NA overflow from spleen. ^{3}H-NA (20 ng/min) was infused intraarterially into the spleen for 30 minutes. After the end of the ^{3}H-NA infusion, the spleen was perfused with normal Krebs solution for 20 min before the nerves were stimulated. During 1 min before each stimulation period, control samples were taken to determine the background activity. Nerves were stimulated at 30 Hz for 6·7 seconds. Each column in the top panel shows net increase in radioactivity due to nerve stimulation during control and flow-stop periods. Flow was stopped for the duration of nerve stimulation +30 or 60 seconds. Middle and bottom panels show ^{3}H-NA and NA values of the same samples shown in the top panel after treatment of the samples with alumina.

(1970) showed that adrenoceptor blocking agents are more effective in increasing the transmitter output from skeletal muscle, iris and cerebral cortex than is desipramine, which effectively blocks the neuronal uptake of NA. They have concluded that the effector organ somehow influences the amount of transmitter released. Langer (1970), however, showed that phentolamine did not increase the transmitter output (as determined by the release of total radioactivity) by field stimulation of the isolated cat nictitating membranes.

The differences in NA overflows between spleens treated with cocaine and phenoxybenzamine or phentolamine are not due to differences in flow rates. In the first place, the spleens were perfused with a constant outflow pump; hence, the venous volumes collected after nerve stimulation in control and drug treated spleens were not appreciably different. Second, flow-stop experiments showed that restriction on flow cannot alone explain the disparity in transmitter output, for example, between cocaine and phentolamine treatment. If the increase in transmitter output after phentolamine treatment was mainly due to its action in blocking vasoconstriction and thus improving flow rates, then restriction on flow should have caused a much greater percentage reduction in phentolamine treated than in untreated or cocaine treated spleens. However, restricting the flow for 30 s resulted in the removal of roughly the same percentage of NA in normal spleens and spleens treated with cocaine or phentolamine.

Flow-stop experiments have clarified the role of diffusion on transmitter output. NA outputs from untreated spleens were depressed by 90 and 160 ng during flow-stop periods of 30 and 60 s, respectively. Since flow-stop does not result in an appreciable increase in metabolism of the released NA, the net effect of flow-stop was to decrease overflow at a rate of about 3 ng/s (2 ng/s if the stimulation period of 20 s during which flow was stopped is also included). Assuming that NA overflow after phenoxybenzamine treatment represents total release, then the difference in output of nearly 800 ng between normal spleens and spleens treated with phenoxybenzamine represents the amount of transmitter removed during the course of nerve stimulation in untreated spleens, which amounts to a removal rate of about 40 ng/second. We know from flow-stop experiments that the maximum amount of NA that the nerves could possibly remove because of the severe restriction on flow imposed by nerve stimulation is of the order of 3 ng/s or 60 ng/stimulation period, which is far too small to account for the differences in outputs from normal spleens and spleens treated with phenoxybenzamine. It must therefore be concluded that the removal of over 90% of the released transmitter is probably uninfluenced by such factors as diffusion and flow rate. Second, reuptake of the released NA must have occurred into the nerve terminals located in the immediate area of release. This conclusion is probably valid, since during flow-stop the diffusion of the released NA is restricted to a small area and the NA overflow was depressed without the formation of metabolites for the duration of the flow-stop period. The reduction in overflow of NA cannot be attributed to reduction in transmitter release by nerve stimulation during flow-stop, because the decrease in overflow was roughly proportional to the duration of flow-stop.

Blakeley & Brown (1964) and Yamamoto & Kirpekar (1968) have shown that the uptake of infused NA during nerve stimulation is either reduced or not affected at all. Yet, the results reported in this investigation show that a large fraction of

the released NA was removed during the course of nerve stimulation. These two conflicting observations could be reconciled if one imagines than an α site on the presynaptic nerve terminal has an inhibitory role in the release of NA. It is suggested that the NA released by nerve stimulation acts on these α sites of the presynaptic membrane to inhibit its own release. On such a theory, the outputs of NA from normal spleens and spleens treated with cocaine, phentolamine or phenoxybenzamine could possibly be explained. In normal spleens and spleens treated with cocaine the released transmitter would act on these hypothetical α sites to inhibit its own release. In the presence of adrenergic blocking agents the transmitter output would be enhanced, since the inhibitory α sites on the nerve terminals are blocked. These are probably not the same sites through which NA is taken up into the nerve endings, because cocaine, which effectively blocks the uptake of infused NA into the sympathetic nerves, does not substantially enhance release.

This work was supported by a U.S. Public Health Service Grant No. HE 05237. Dr. Margarita Puig was a holder of a Fundacion Juan March Fellowship. We are grateful to Professor Robert F. Furchgott for his interest in this work.

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(*Received June* 18, 1971)