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Release of noradrenaline and dopamine by nerve stimulation in the cat spleen perfused with ^{3}H-dopamine

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Summary

1. Recovery of ^{3}H-dopamine and ^{3}H-L-dopa infused intra-arterially at a constant rate of 20 ng/min into the cat spleen perfused with Krebs bicarbonate solution was 52·6±3·8% and 90·5±3·1% respectively. Recovery of ^{3}H-dopamine in spleens treated with α-methyl-*p*-tyrosine was 67·7±6·7% and was not significantly different from untreated spleens.

2. Release of ^{3}H-dopamine by nerve stimulation after an infusion of ^{3}H-dopamine resembled release of endogenous noradrenaline. Release of ^{3}H-L-dopa and ^{3}H-L-tyrosine after their infusion did not occur in any consistent manner by nerve stimulation.

3. Preferential release of ^{3}H-noradrenaline formed from ^{3}H-dopamine was not observed during continuous nerve stimulation. Specific activity of released ^{3}H-noradrenaline remained constant during any single stimulation period with or without ^{3}H-dopamine infusion. Treating the cats with α-methyl-*p*-tyrosine did not change the time course of ^{3}H-noradrenaline release.

4. Output of noradrenaline expressed as a percentage of the first minute output in both normal and α-methyl-*p*-tyrosine treated spleens was not significantly different at various times during continuous nerve stimulation.

5. Specific activity of released noradrenaline formed from ^{3}H-dopamine was always greater than the specific activity of the spleen in normal spleens and spleens treated with α-methyl-*p*-tyrosine.

6. It is concluded that newly synthesized or infused noradrenaline initially mixes with a more rapidly turning pool and only slowly with the entire tissue store. During continuous nerve stimulation there is no further preferential release of newly synthesized noradrenaline. Released noradrenaline truly represents the state of the releasable pool and will vary with the latter.

Introduction

Brown & Gillespie (1957) showed that stimulation of the splenic nerves results in liberation of noradrenaline (NA) into the venous blood. A number of investigators have also shown the remarkable ability of sympathetic nerves to take up and conserve the released transmitter (Iversen, 1967). It is also well known that sympathetically innervated organs maintain their NA content under a variety of experimental conditions, and only intensive electrical stimulation will produce some depletion of endogenous stores of NA (Dearnaley & Geffen, 1966). Tissue stores

are probably maintained either by reuptake of released NA or by enhanced synthesis.

This investigation was undertaken to study the uptake and release of tyrosine, dopa and dopamine from the perfused cat spleen. Attempts were also made to study the role of synthesis in maintaining the release of NA during continuous nerve stimulation.

Methods

Cats were anaesthetized with ether, followed by chloralose (60 mg/kg, i.v.). The abdomen was opened by a midline incision, and the stomach, adrenals, intestines and colon were removed. The spleen was isolated and perfused *in situ* with Krebs bicarbonate solution at a constant rate of about 7 ml/min, at 35° C (Kirpekar & Misu, 1967). ^{3}H-dopamine (280 mCi/mmol) was infused into the splenic artery for 90 min at a constant rate of 20 ng/min in a volume of 0·05 ml by placing a cannula in the hepatic artery so that the tip of the cannula was at the junction of the hepatic artery with the main coeliac artery. Samples of venous effluent were taken before dopamine infusion began and at the 10th, 20th, 30th, 40th and 50th minute of infusion period. In the course of the infusion, the splenic nerves were continuously stimulated at 10 Hz for 3–9 minutes. Stimulation was also repeated 15 min after the termination of infusion. Venous samples were successively collected for 1 or 2 min during each stimulation period and for 1 min before each stimulation for determining the background activity. Similar experiments were also done using ^{3}H-L-dopa (1 Ci/mmol) or ^{3}H-L-tyrosine (36 Ci/mmol). In experiments using ^{3}H-L-tyrosine the perfusing Krebs solution contained 5×10^{-5}M tyrosine.

Venous samples were centrifuged for 10 min at 2,000 rev/min in a clinical centrifuge. Supernatant was measured, and a convenient volume was added to a similar volume of 0·8N perchloric acid. Samples made in this way were stored at −20° C until the next day. Catecholamines in the extract were adsorbed on alumina according to the method of Anton & Sayre (1962), but elution was carried out with 3 ml of acidified methanol (0·1N HCl).

A portion (0·5 ml) of the eluate was added directly to the scintillation fluid and counted in a liquid scintillation spectrophotometer, using external automatic standard. Another 1·0 ml portion of the eluate was evaporated to dryness at −20° C in a freeze dryer, and the residue was redissolved in 1 ml of 0·1N perchloric acid. This was then used for the fluorimetric determination of NA. Another 1·0 ml of acid extract was applied to paper for separating ^{3}H-NA/^{3}H-dopamine by ascending paper chromatography, using phenol/HCl as the solvent system in an atmosphere of N_2. Standard solutions were applied to a separate part of the paper. After about 24 h, the entire chromatogram was cut into strips of 1 cm, and these were eluted separately with 1 ml of 0·1N perchloric acid.

Recovery of standard ^{3}H-NA and ^{3}H-dopamine by this procedure was greater than 80%. Separation of NA and dopamine was relatively easy in this solvent system because of their different R_F values (NA, 0·27; dopamine, 0·43). Separation of L-dopa could not be achieved by this procedure, however, because this compound hardly moved from the origin. Separation of dopa from NA and dopamine was achieved first by paper electrophoresis (2,000 V for 1 h), using formic acid as a solvent (0·25M). The same paper was then run in another dimension in phenol-HCl

for the separation of dopamine and NA. After locating the positions of the three compounds, strips were cut and eluted as described before. Recovery of L-dopa by this combined procedure was about 60%. Tyrosine is not adsorbed on alumina, so separation of tyrosine from dopa, dopamine and NA was achieved by collecting the effluent from alumina.

At the end of each experiment, spleens were homogenized in 30 ml of perchloric acid (0·4N). After centrifugation, sediment was resuspended in 10 ml perchloric acid and recentrifuged. This was repeated once more and all the supernatants were added together. In some experiments, small portions of the spleen were taken before and after stimulation, in order to compare the specific activity of NA released by nerve stimulation with that of spleen. Small portions of spleen were homogenized in 3 ml of perchloric acid, and sediment after centrifugation was washed twice with 1·5 ml perchloric acid. Extracts were then processed as described previously, and catecholamines were determined by the method of Anton & Sayre (1962).

α-Methyl-*p*-tyrosine (100 mg/kg) was given intravenously 2 h before the start of the perfusion. Specific activity of NA is expressed as pmol ^{3}H-NA × 100/pmol NA.

Drugs used

The drugs used were: ^{3}H-dopamine, ^{3}H-L-dopa and ^{3}H-L-tyrosine (Nuclear Chicago); α-methyl-*p*-tyrosine (Merck, Sharp and Dohme).

Results

*Venous recovery of infused ^{3}H-dopamine in normal and α-methyl-*p*-tyrosine-treated spleens*

Table 1 shows the recovery of ^{3}H-dopamine in normal and α-methyl-*p*-tyrosine-treated spleens. In normal spleens, 52·6±3·8% of the infused amount of dopamine was recovered in the venous perfusate during 10–50 min, whereas in spleens treated with α-methyl-*p*-tyrosine the average recovery was 67·7±6·7% ($P>0·05$). However, the recovery of ^{3}H-L-dopa when infused in a similar manner was 90·5±3·1% (three experiments), indicating thereby that only a small portion of infused dopa is taken up into the sympathetic nerves.

Effect of continuous nerve stimulation on the release of ^{3}H-dopamine and ^{3}H-NA during or in the absence of infusion of ^{3}H-dopamine

Figure 1 shows a typical experiment in which splenic nerves were stimulated twice at 10 Hz for 3 min during ^{3}H-dopamine infusion (20 ng/min) and once 15 min after the termination of infusion. Venous samples collected over 1 min periods were analysed for net increases in NA, ^{3}H-NA and ^{3}H-dopamine. During continuous nerve stimulation there was initially (in the first minute) a very marked increase in NA output, which progressively declined during the subsequent 2 min stimulation period. In two experiments, nerve stimulation was continued for as long as 9 min, but the NA output from the third minute onward remained fairly constant. Release of ^{3}H-NA followed the same time course as endogenous NA, so that the specific activities of NA released during the entire stimulation period were comparable. However, the release of ^{3}H-dopamine did not follow a similar time course, and varied considerably in different experiments. During second stimulation, which was

TABLE 1. *Recovery of infused ^{3}H-dopamine at various time intervals in the perfused cat spleen*

Treatment	^{3}H-dopamine infused in 60 s (ng)	^{3}H-dopamine recovered in 60 s (ng)±S.E. 10 min	20 min	30 min	40 min	50 min	Mean recovery %
Normal (n=5)	20	10·00±1·91	10·09±0·62	10·09±0·62	10·89±0·69	11·72±0·35	52·6±3·8
α-methyl-*p*-tyrosine (n=4)	20	11·43±0·51	11·99±1·09	13·94±1·85	14·50±1·48	14·40±1·70	67·7±6·7

^{3}H-dopamine was infused at a rate of 20 ng/min and the samples of venous effluents were taken for 1 min at the 10, 20, 30, 40 and 50th minute of infusion.

repeated after 37 min, release of NA and ^{3}H-NA was comparable, while release of ^{3}H-dopamine was not related to NA release. Initial output of NA during the second stimulation was reduced, compared with the corresponding period of the first stimulation. During the second stimulation, specific activity of released NA was greater than that released during the first stimulation period. Stimulation of splenic nerves 15 min after the termination of ^{3}H-dopamine infusion also resulted in initial

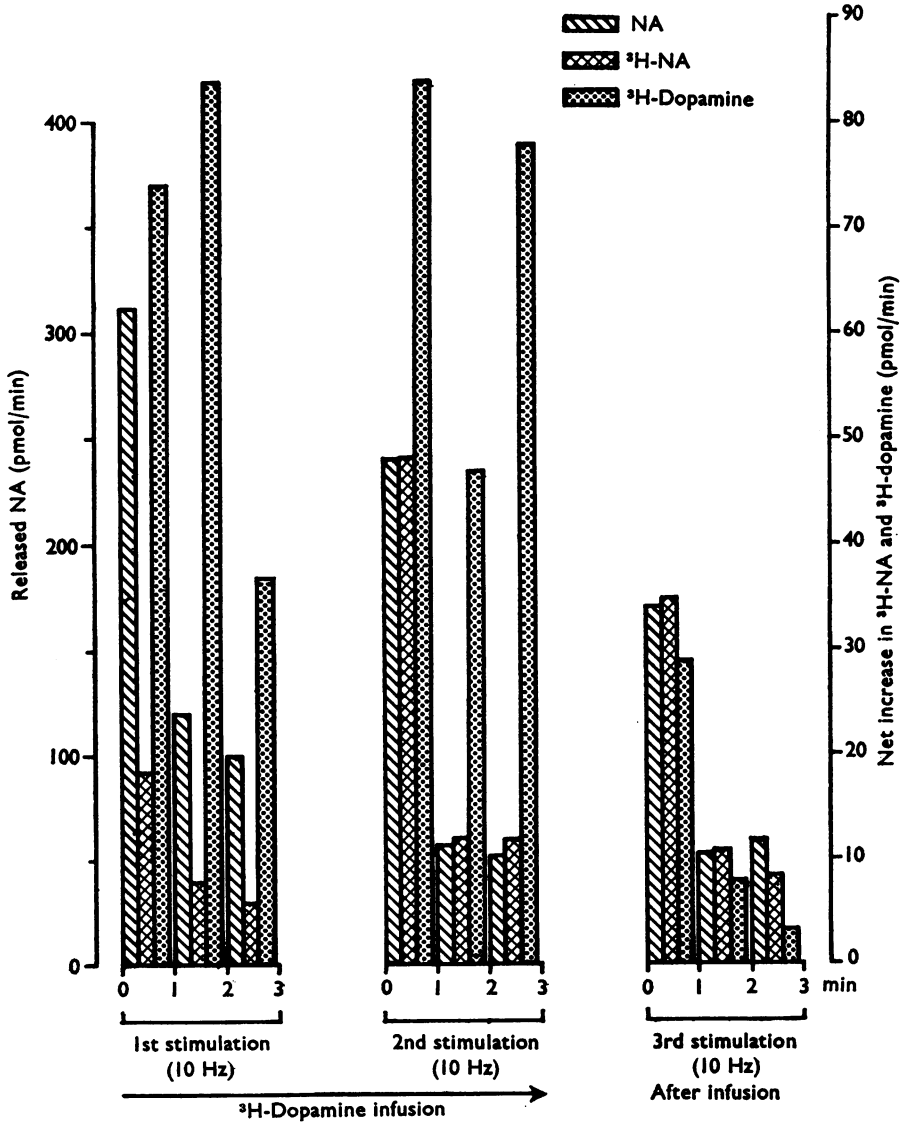

FIG. 1. Effect of continuous nerve stimulation on the release of NA, ^{3}H-NA, and ^{3}H-dopamine from a normal spleen. ^{3}H-dopamine was infused at a rate of 20 ng/min for 90 minutes. Splenic nerves were stimulated at 10 Hz for 3 min periods during the course of ^{3}H-dopamine infusion, first at 45 min and then at 85 minutes. Nerves were also stimulated 15 min after the end of ^{3}H-dopamine infusion. Each panel shows the net increases in NA, ^{3}H-NA and ^{3}H-dopamine at 1 min intervals during a 3 min stimulation period. Left hand ordinate shows output of NA (pmol/min) and the right hand ordinate shows output of ^{3}H-NA and ^{3}H-dopamine (pmol/min). Note the difference in scales.

release of endogenous NA, which was only 50% of that of the first stimulation period. Once again, the rate of release of ^{3}H-NA was comparable with the release of endogenous NA. Release of ^{3}H-dopamine also followed a time course similar to ^{3}H-NA and NA. In all four experiments the specific activities of released NA during any 3 min stimulation period, with or without infusion of dopamine, were comparable. In two experiments extending the stimulation period to 9 min also gave similar results. Table 2 shows specific activities of released NA during 3 min continuous nerve stimulation at 10 Hz during infusion of ^{3}H-dopamine.

Experiments with dopa and tyrosine carried out in this way were difficult to interpret because of poor incorporation and failure to separate small quantities of ^{3}H-NA from radioactive dopa and tyrosine. Nor was it possible to release either dopa or tyrosine by nerve stimulation, following their infusion.

*Effect of α-methyl-*p*-tyrosine on the release of NA, ^{3}H-NA and ^{3}H-dopamine during or in the absence of infusion of ^{3}H-dopamine*

Further experiments were carried out with α-methyl-*p*-tyrosine—a well known inhibitor of NA synthesis—in order to determine the contribution of newly synthesized NA in maintaining transmitter output during continuous nerve stimulation. If newly synthesized NA did contribute largely towards release then after treatment with α-methyl-*p*-tyrosine there should be a very marked increase in the specific activity of NA released during later periods of stimulation as compared with the initial period of stimulation. Figure 2 shows the effect of α-methyl-*p*-tyrosine on the release of NA, ^{3}H-NA ^{3}H-dopamine. In the first part of the figure, splenic nerves were stimulated at 10 Hz for 9 min during the infusion of ^{3}H-dopamine. Treatment with α-methyl-*p*-tyrosine did not significantly alter the pattern of release of either NA or ^{3}H-NA. Initially, there was a very marked increase in NA and ^{3}H-NA output, which subsequently declined and remained at a steady-state level from the third minute onward. Specific activities of NA released at different times throughout the stimulation period were not significantly different from those of the initial period. Table 2 shows a comparison of the specific activities of NA released in normal and α-methyl-*p*-tyrosine treated spleens during the first 3 min stimulation period. None of the results were significantly different. In this experiment, release of ^{3}H-dopamine during infusion of ^{3}H-dopamine also showed an initial increase, followed by a decline. When stimulation was repeated 35 min after terminating ^{3}H-dopamine infusion, release of endogenous NA and ^{3}H-NA followed a similar time course, and the specific activities of the released NA from the first to the last minute of the stimulation period remained fairly constant. In four experiments using α-methyl-*p*-tyrosine, similar results were obtained, although the release of ^{3}H-dopamine during ^{3}H-dopamine infusion varied considerably in different experiments.

TABLE 2. *Specific activity of ^{3}H-NA released (pmol ^{3}H-NA × 100/pmol NA) ±S.E. by continuous nerve stimulation at 10 Hz during perfusion with ^{3}H-dopamine (20 ng/min)*

Treatment	Specific activity of ^{3}H-NA 0–1 min	1–2 min	2–3 min
Normal (n=4) (%)	4·3±0·87 (100)	4·76±1·31 (104·7±12·5)	4·62±1·20 (103·8±11·7)
α-Methyl-*p*-tyrosine (n=4) (%)	4·92±1·73 (100)	6·53±2·46 (138·6±15·7)	7·38±2·10 (184·4±44·6)

*NA release in normal and α-methyl-*p*-tyrosine treated spleens*

Noradrenaline contents of normal and α-methyl-*p*-tyrosine treated spleens were not significantly different. The mean NA content of thirteen normal spleens was

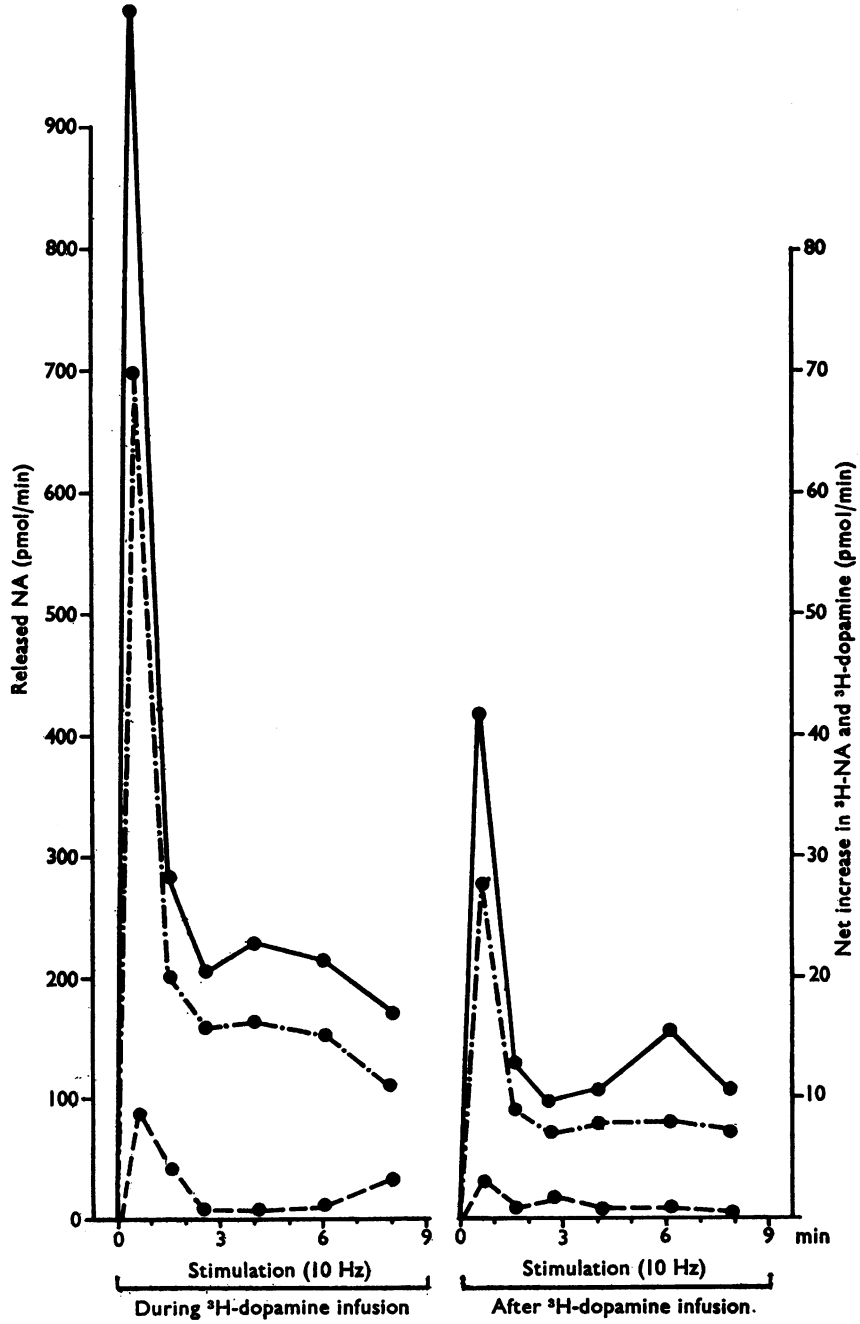

FIG. 2. Effect of continuous nerve stimulation on the release of NA (●——●), ³H-NA (●—·—·—●) and ³H-dopamine (●– – –●) from α-methyl-*p*-tyrosine treated spleens. ³H-dopamine was infused at a rate of 20 ng/min for 90 minutes. Splenic nerves were stimulated for 9 min at 10 Hz commencing 55 min after the start of ³H-dopamine infusion. Nerves were also stimulated for a similar period 35 min after the end of ³H-dopamine infusion.

14,506 ± 1,853 pmol/g, and of four α-methyl-*p*-tyrosine treated spleens was 10,939 ± 3,192 pmol/g. Since NA contents of spleens varied considerably, the outputs of NA following nerve stimulation were also scattered over a very wide range. For this reason, we have made comparisons of endogenous NA outputs in normal and α-methyl-*p*-tyrosine treated spleens by assigning the first minute output as 100%, and then the following outputs are expressed as percentages of the initial output. Figure 3 shows that the output of NA, expressed as a percentage of the first minute output, in both normal and α-methyl-*p*-tyrosine treated spleens, was not significantly different at various times during continuous stimulation.

Comparison of the specific activity of the released NA with that of the spleen

Table 3 summarizes the results from normal and α-methyl-*p*-tyrosine treated spleens. In these experiments a portion of the spleen was removed immediately after stimulation (10 Hz) and the specific activities of released and splenic NA were expressed. Specific activities of either released NA or splenic NA were not significantly different in the two groups. In every experiment, however, the specific activity of the released NA was always greater than that of the spleen from which NA was released. The ratio of specific activities of released NA to splenic NA in normal and α-methyl-*p*-tyrosine treated spleens was approximately 2.

Discussion

In these experiments we have studied the removal of dopamine and dopa infused intra-arterially at a steady rate into the perfused spleen of the cat. The recovery rate for infused dopamine was about 50%, and comparable with the rate of recovery of radioactive NA infused at much higher rates (Gillespie & Kirpekar, 1966). However, uptake of L-dopa by the spleen was very low, more than 90% of infused dopa was recovered in the venous effluent. Recovery studies with infused tyrosines were difficult to interpret, for uptake of tyrosine could occur in the smooth muscle cells of the spleen as well as the sympathetic nerves. As infused NA is principally inactivated by uptake into the postganglionic sympathetic nerve endings, it is probably fair to assume that the same mechanism is responsible for the removal of dopamine, but not dopa.

Release of ^{3}H-dopamine by nerve stimulation, following an infusion of ^{3}H-dopamine, probably occurs in a manner similar to release of endogenous NA. Thus, the rate of release of ^{3}H-dopamine in the postinfusion period follows a time course which was very similar to the release of NA and ^{3}H-NA at various times during nerve stimulation. This would mean that infused dopamine probably was stored at the same site as the endogenous transmitter and hence released in an analogous manner. Release of ^{3}H-dopamine during the course of ^{3}H-dopamine infusion was rather variable, and might suggest some interference in the uptake of dopamine during continuous nerve stimulation. Experiments with dopa and tyrosine showed that they were not released during sympathetic nerve stimulation.

TABLE 3. *Comparison of specific activity (pmol ^{3}H-NA × 100/pmol NA) ±S.E. of splenic and nerve stimulation (10 Hz) induced ^{3}H-NA*

Treatment	Specific activity of ^{3}H-NA		Released NA/splenic NA (%)
	Released NA	Splenic NA	
Normal (n=9)	4·77±1·74	1·92±0·78	242·7±50·1
α-Methyl-*p*-tyrosine (n=13)	6·07±1·64	3·88±0·83	164·1±19·3

Transmitter output during continuous nerve stimulation is probably maintained either by synthesis of NA or by the reuptake and reuse of released transmitter. Kopin, Bresse, Krauss & Weise (1968) have provided evidence that release is largely maintained by newly synthesized NA. Collier (1969) has also suggested that newly synthesized acetylcholine is preferentially released from the perfused superior cervical ganglion during continuous stimulation of its preganglionic nerves. However, Hedquist & Stjärne (1969) have concluded that contribution of newly synthesized NA in maintaining the transmitter output during nervous activity is only very small. Kuperman, Gillis & Roth (1970) have also shown that the release of newly synthesized NA from pulmonary veins is actually inhibited if the sympathetic nerves were stimulated at a high frequency.

The present investigation would also be against the idea that newly synthesized NA plays any significant part in the release of NA during continuous nerve activity. During infusion of dopamine, the specific activity of released NA did not appreciably change over the entire period of nerve stimulation, and there was no evidence that ^{3}H-NA formed from dopamine was preferentially released. Second, after discontinuation of ^{3}H-dopamine infusion, specific activity of released NA was constant

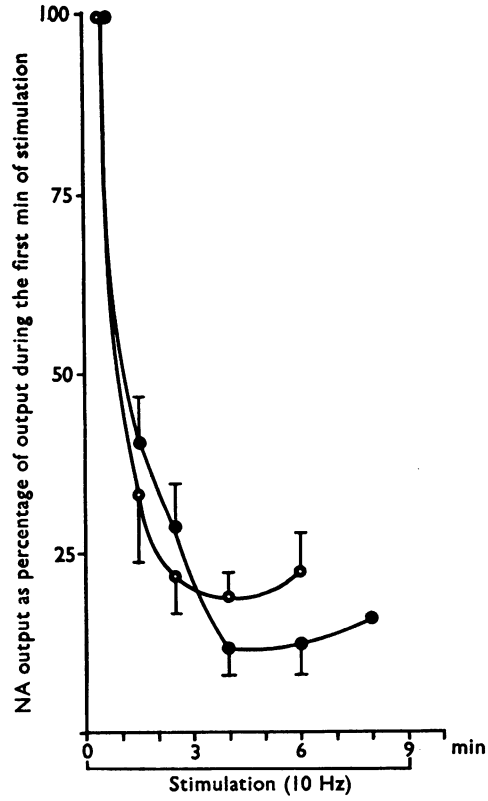

FIG. 3. Effect of α-methyl-*p*-tyrosine on release of NA during continuous nerve stimulation. Outputs are expressed as a percentage of the output during the first min of stimulation. Each point on α-methyl-*p*-tyrosine curve (○——○) is the mean of four experiments. Each point on the normal curve (●——●) is the mean of two to ten experiments. Last point on the normal curve is a single observation. Vertical bars represent standard error of the mean.

from the first to the last minute of stimulation. Results obtained with the inhibitor of NA synthesis—α-methyl-*p*-tyrosine—also showed that the specific activity of released NA during or after the infusion of ^{3}H-dopamine did not significantly vary during continuous stimulation.

Synthesis of NA from tyrosine is relatively slow, since the activity of tyrosine hydroxylase, which is many times lower than that of dopa-decarboxylase or dopamine β-hydroxylase, is adjusted to tissue requirements of NA (Levitt, Spector, Sjoerdsma & Udenfriend, 1965). A question therefore arises whether newly synthesized NA formed from tyrosine and dopamine would be handled in an identical manner by the sympathetic nerves. If NA formed from dopamine is treated differently, then the specific gravity of released NA should continuously fall during nerve stimulation without dopamine infusion. Second, following treatment with α-methyl-*p*-tyrosine the specific activity should rise during stimulation, since conversion of dopamine to ^{3}H-NA should not be affected by this treatment, whereas synthesis of endogenous NA would be depressed. This was not found to be the case. The present investigation, therefore, shows that newly acquired NA probably mixes homogeneously with the releasable pool and is not released in any preferential manner.

During stimulation, the rate of release of endogenous NA, which is initially very high, subsequently falls and remains at a steady level of about 10–25% of the initial output throughout the stimulation period. This rate of decline in NA output is not significantly different in normal and α-methyl-*p*-tyrosine treated spleens. If output of NA were maintained by newly synthesized NA during continuous stimulation, then we should have seen a very marked reduction in NA release in α-methyl-*p*-tyrosine treated spleens.

Even though the specific activity of released NA did not change during continuous stimulation of the sympathetic nerves, the specific activity of the released NA was always greater than that of the spleen in normal as well as α-methyl-*p*-tyrosine treated animals. These observations suggest that newly formed NA is preferentially taken up into the releasable pool, which is probably a reflection of the rapid turnover rate of the releasable pool. Since this pool has a higher specific activity than the spleen, the released NA would also have a higher specific activity, and perhaps in that sense newly synthesized NA is preferentially released.

Releasable pool is also preferentially labelled after infusion of ^{3}H-NA. Chidsey & Harrison (1963) showed that a period of about 5 h is required for exogenous NA to mix homogenously with the entire endogenous pool, and during this period the specific activity of NA released by nerve stimulation was greater than that of the heart. These observations suggest that newly acquired NA—whether infused or synthesized—initially mixes with a more rapidly turning pool and equilibrates only slowly with the entire tissue store.

It is concluded that postganglionic sympathetic nerves are chiefly responsible for the removal of infused dopamine, and that the release of dopamine after nerve stimulation occurs in a manner similar to the release of NA. It is also suggested that during continuous nerve stimulation mobilization of stored NA plays a much more important role than synthesis in maintaining release.

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