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VARIATION IN NORADRENALINE OUTPUT WITH CHANGES IN STIMULUS FREQUENCY AND TRAIN LENGTH: ROLE OF DIFFERENT NORADRENALINE POOLS

J. HUGHES[1]
Department of Pharmacology, University of Aberdeen, Scotland

R.H. ROTH
Department of Pharmacology, Yale University, New Haven, Connecticut, U.S.A.

1 During adrenergic nerve stimulation the output/pulse of noradrenaline from the rabbit vas deferens and portal vein is not constant but increases as the stimulus frequency or train length is increased. Depending upon the stimulus frequency and train length the fractional release of noradrenaline may vary from less than 10^{-7} to greater than 10^{-4}.

2 Endogenous tissue stores of noradrenaline were labelled by incubation with (−)-[^{3}H]-noradrenaline and [^{14}C]-tyrosine. The output/pulse of newly synthesized [^{14}C]-noradrenaline remained constant as the train length was increased whilst the output/pulse of [^{3}H]-noradrenaline increased under the same conditions. This phenomenon was independent of the stimulus frequency. Newly synthesized noradrenaline also appeared in the superfusate following nerve stimulation more rapidly than exogenously loaded noradrenaline.

3 Both [^{3}H]-noradrenaline and [^{14}C]-tyrosine were found to label an easily releasable store of noradrenaline. Mobilization from this store was the same at low and high frequencies of nerve stimulation.

4 It is concluded that at least two functional stores of noradrenaline exist within the adrenergic nerve ending. Newly synthesized noradrenaline is probably only a minor constituent of transmitter output under normal conditions of adrenergic nerve activity.

5 At least two mechanisms control the amount of noradrenaline released per pulse during nerve stimulation. Facilitation of release with increasing train lengths appears to be due to the mobilization of transmitter from a secondary store. Facilitation of release with increasing stimulus frequency is not dependent on mobilization from any particular store and at present there is no satisfactory explanation for this phenomenon.

Introduction

Estimates of the fractional release of noradrenaline provide a useful comparison of noradrenergic release mechanisms at different junctions; some authors have also used these estimates in an attempt to assess the role of adrenergic nerve vesicles in the release process (Smith & Winkler, 1972). Surprisingly, most authors have appeared to ignore the possibility that the fractional release may not be constant under varying conditions of nerve stimulation. Hughes (1972) showed that the noradrenaline output per pulse increases with the frequency of nerve stimulation in the rabbit vas deferens and portal vein, and that the fractional release was certainly not constant in these tissues at frequencies between 1 and 16 Hz. It also appeared probable from previous reports that the fractional release changes with the length of the stimulus train (Burnstock, Holman & Kuriyama, 1964; Haefely, Hurlimann & Thoenen, 1965).

We have now investigated the relationship between train length and noradrenaline output in the rabbit vas deferens and portal vein.

A preliminary report of this work has been given to the British Pharmacological Society (Hughes & Roth, 1972).

[1] Present address: Unit for Research on Addictive Drugs, Marischal College, University of Aberdeen, Aberdeen AB9 1AS.

Methods

Male, albino rabbits of 2-3 kg were killed by cervical dislocation and the portal vein and vasa deferentia were dissected out and prepared as described previously (Hughes & Vane, 1967; Hughes, 1972). The vas deferens was carefully stripped of adhering connective tissue and of its outer serous coat in order to remove any peripheral sympathetic ganglia. The isolated tissues were either suspended in 3 ml organ baths as described by Hughes (1972), or mounted in a superfusion apparatus as described by Hughes & Roth (1971). The tissues were bathed in, or superfused with, modified Krebs solution equilibrated with 95% O_2 and 5% CO_2 and maintained at 37 ± 0.5° C. The intramural nerves of the vas deferens and portal vein were stimulated with supramaximal, rectilinear electrical pulses of 1 ms duration (current strength 80-100 mA). Two parallel platinum electrodes were used to stimulate the superfused preparations; the tissues were positioned between the two electrodes which lightly brushed the preparation along its whole length; a similar arrangement was also used for the bathed preparations in the organ bath. Changes in tissue tension were monitored with isometric transducers coupled to a Grass Polygraph recorder.

Estimation of noradrenaline output

Endogenous noradrenaline output. Both chemical and biological assay methods were used. The bioassay has been previously described in detail (Hughes, 1972); briefly the bioassay consists of transferring the Krebs solution bathing the donor tissue to a superfusion system where the noradrenaline is determined by bracket assay on spiral preparations of the rabbit aorta and iliac artery. This technique was used to estimate the noradrenaline output at different train lengths and stimulus frequencies. Only one train length at a set frequency was studied in any one experiment. The noradrenaline output was measured for two stimulus trains given at 20 min intervals and values averaged in each experiment. The tissue was removed from the bath at the end of the experiment and the endogenous noradrenaline content determined as described below. The resting output of noradrenaline was also determined in each experiment and this was deducted from the total output after electrical stimulation.

For fluorimetric assay the Krebs solution bathing the donor tissue was removed after electrical stimulation and the noradrenaline isolated by column chromatography (see below). The noradrenaline was then estimated by the trihydroxyindole method (O'Hanlon, Campuzano & Horvath, 1970). The fluorimetric assay procedure was only used when we wished to determine the specific activity of labelled noradrenaline released from phenoxybenzamine-treated tissues after electrical stimulation for 300 pulses at 2 or 16 Hz.

Labelled noradrenaline output. The tissue stores were labelled by incubating the tissues with either (−)-[^{3}H]-noradrenaline or L-[^{14}C]-tyrosine. Both dual and single labelling procedures were used with the following protocols:

(a) Preincubation for 20 min in Krebs solution at 37° C; 30 min incubation with [^{3}H]-noradrenaline (10 or 100 ng/ml) specific activity 40 μCi/μg in each case; 40 min wash with fresh Krebs solution every 5 minutes.

(b) Preincubation for 20 min at 37° C; 60 min incubation with [^{14}C]-tyrosine (2.5 μCi/ml, 2.58 μg/ml); 40 min wash as above.

(c) Preincubation for 20 min at 37° C; 30 min incubation with [^{3}H]-noradrenaline (10 or 100 ng/ml); 10 min wash; 60 min incubation with [^{14}C]-tyrosine; 30 min wash with fresh Krebs solution every 5 minutes.

The incubations and washings were carried out in flasks maintained at 37° C and the tissues were transferred to the organ bath or superfusion system after the final wash period; this procedure avoided undue contamination of the apparatus. Samples of the bathing fluid from the organ baths and superfusion system were counted in a Packard liquid scintillation counter (Model 2425 or 3375) after column chromatography as described below. Standard methods were used for dual and single label counting with corrections for channel spillover and chemical quenching.

Estimation of tissue noradrenaline

The endogenous content of noradrenaline was determined fluorimetrically. The tissue was blotted to remove excess bathing solution, frozen on solid carbon dioxide and weighed. The samples were homogenized at 4° C with 5 ml of 0.4 M perchloric acid containing disodium diaminoethane tetra-acetate and sodium bisulphite (1 mg/ml of each). The homogenates were allowed to stand for 15 min at 4° C, then they were shaken to remix the contents and the tubes were centrifuged at 10,000 *g* for 15 minutes. The supernatants were carried through both Amberlite CG 120 and alumina column procedures in label experiments, and through alumina columns only in all other cases.

Column chromatography

Alumina (B.D.H.) column chromatography was carried out as described by Boadle-Biber, Hughes & Roth (1970). Amberlite CG 120 columns (1 x 0.5 cm) were prepared in the sodium form and samples applied at pH 4.5; for this purpose tissue supernatants were adjusted with KOH to precipitate perchlorate. The Amberlite column was then washed with 15 ml of 0.2 M phosphate buffer at pH 6 and then with 5 ml of distilled water; the column was eluted with 8 ml of 1 M HCl. A combination of alumina and CG 120 procedures was used to isolate noradrenaline in those experiments in which tissue and effluent specific activity were determined. The samples were first passed through the CG 120 columns and the eluates from these were then adjusted to pH 8.4 and passed through the alumina columns. Estimations of the chemical and radioactive concentration of noradrenaline were made on the final alumina column eluate (2 ml).

Dowex-50-X8 columns (4 x 0.5 cm) in the sodium form were used to isolate noradrenaline and normetanephrine in those experiments in which only the output of label was measured. Samples of the perfusate or the bathing medium were adjusted to pH 4.5 with 0.1 M HCl and applied to the column. Tyrosine, deaminated and neutral metabolites of noradrenaline were washed through with 20 ml sodium acetate buffer (0.2 M, pH 6) and 10 ml of distilled water. Noradrenaline and normetanephrine were then eluted with 4 ml 2 M ammonium hydroxide; only radioactive determinations were made on this eluate.

Recovery of noradrenaline in the alumina procedure averaged 75% and this was corrected for when determining the endogenous tissue levels for calculating the fractional release of noradrenaline.

Solutions

A modified Krebs solution of the following composition (mM) was used; NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 0.93, $MgSO_4$ 1.19, $NaHCO_3$ 25, glucose 11.1, disodium edetate 0.027, sodium ascorbate 0.1, L-tyrosine 0.025. L-tyrosine was omitted in those experiments involving labelled tyrosine.

Noradrenaline standards for the bioassay were diluted freshly in Krebs solution; these solutions were kept in an ice bath and protected from strong light. Stock solutions of noradrenaline (1 mg/ml) were prepared in 0.01 M HCl with ascorbic acid (1 mg/ml) and kept at -20° C for periods not exceeding one month.

Phenoxybenzamine was dissolved in eight parts of propylene glycol and two parts of distilled water to give a stock solution of 10 mg/ml. This solution was protected from strong light.

Scintillation fluid for radioactivity measurements consisted of one litre each of dioxane, toluene and ethanol containing 0.3 g 1,4-bis-2(4,methyl-5-phenyloxyazolyl)-benzene, 15 g of 2,5-diphenyloxazole and 240 g of naphthalene.

Chemicals and drugs

All drug concentrations are quoted as base. Cocaine hydrochloride (MacFarlan Smith Ltd); (−)-noradrenaline bitartrate (British Drug Houses); (−)-[7-^{3}H]-noradrenaline (Amersham Radiochemicals); phenoxybenzamine hydrochloride (Smith, Kline & French); L-[^{14}C]-tyrosine, uniformly labelled (Amersham Radiochemicals).

Labelled noradrenaline and tyrosine were purified on alumina columns and the specific activity checked before use.

Definitions

Output. Refers to the amount of noradrenaline which diffuses from the donor tissue into the surrounding medium and which is then subsequently measured. This output cannot be directly equated with total amount of noradrenaline released during adrenergic stimulation, although it is assumed to be proportional to the total release.

Fractional output. This was calculated as that fraction of the total tissue store of noradrenaline released per pulse. In calculating this fraction the total noradrenaline released during electrical stimulation was added to that found in the tissue at the end of the experiment.

Results

Facilitation of output during repetitive stimulation

The fractional output was determined in tissues that had been treated with phenoxybenzamine (10 μg/ml) for 1 hour. In both the vas deferens and portal vein the fractional output increased as the number of pulses per stimulus train was increased. Figure 1 shows the results of a series of experiments in which the vas deferens was stimulated at 2 or 16 Hz for train lengths varying from 5 to 900 pulses. The fractional output increased from 3×10^{-6} to 2.4×10^{-4} when the train length was increased from 5 to 300 pulses at 16 Hz. Similarly at 2 Hz the fractional output increased from 2×10^{-6} to 8×10^{-5} when the

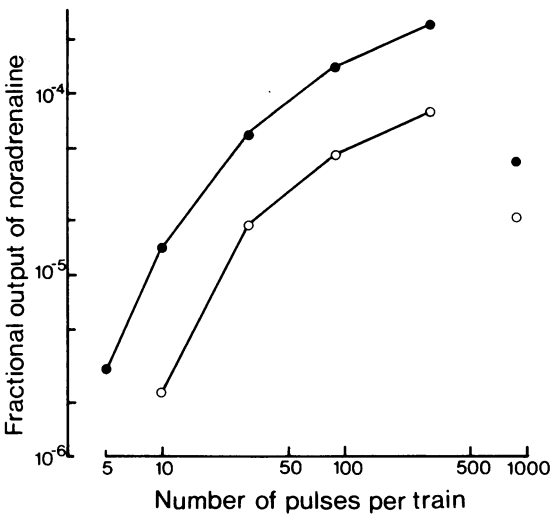

Fig. 1 Facilitation of noradrenaline output with increasing train length and stimulus frequency. Rabbit vas deferens treated with phenoxybenzamine (10 μg/ml for 1 hour). Each point is the mean of four separate experiments; the standard errors have been omitted to improve clarity, these did not exceed 14% at any point. The fractional output of noradrenaline increased from 3×10^{-6} at 16 Hz (●) for trains of 5 pulses to 2.4×10^{-4} at 300 pulses. Similarly at 2 Hz (○) the fractional release increased from 2×10^{-6} at 10 pulses to 8×10^{-5} at 300 pulses.

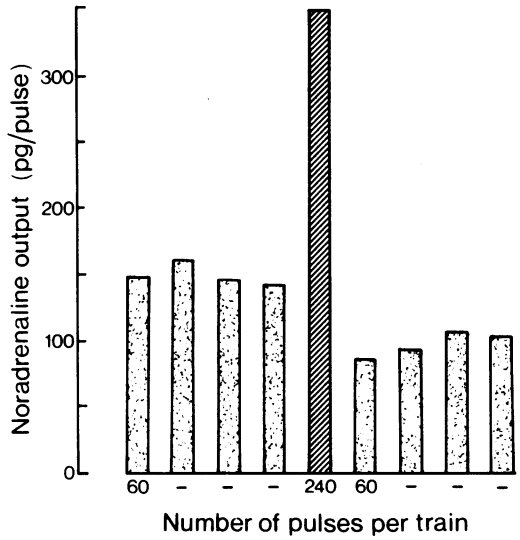

Fig. 2 Interaction between trains of different lengths. Rabbit vas deferens treated with phenoxybenzamine (10 μg/ml for 1 hour). Stippled columns, trains of 60 pulses at 16 Hz; hatched column, train of 240 pulses at 16 Hz. The tissue was stimulated every 15 min and the noradrenaline output determined by bioassay.

number of pulses was increased from 10 to 300. The facilitation of output with increasing train length was approximately the same at 2 and 16 Hz, but the fractional release was consistently greater at 16 Hz than at 2 Hz for any given train length. It was not possible to determine accurately the outputs for train lengths of less than five pulses at 16 Hz, or less than ten pulses at 2 Hz. It would appear that contractions elicited by single pulses or very short trains of pulses are associated with very small outputs of transmitter which represent a fractional release of 10^{-7} or less.

When long trains of 900 pulses were studied it was found that there was a rapid decline in transmitter output, so that a second train given 20 min after the first elicited only 25-30% of the output of the initial train. This explains the apparent decline in fractional output at 900 pulses in Fig. 1, since duplicate estimations were made in each tissue and a mean of the two values taken as the result for that tissue. The rapid decline in output with successive trains was not apparent at the shorter train lengths where the maximum decline was of the order of 8-10% for 400 pulses. Further studies showed that trains of different lengths showed marked interactions. The output elicited by a given stimulus train was invariably depressed if a longer stimulus train, at the same frequency, was applied before the shorter train. For example, it can be seen in Fig. 2 that the output elicited by 60 pulses at 16 Hz every 15 min declined quite slowly. However, the interposition of a 240 pulse train in this sequence caused a marked diminution in the output elicited by successive 60 pulse trains. Subsequent outputs at 60 pulses tended to return to the control levels but this recovery was never complete (Figure 2). The depression of output observed in this type of experiment became more pronounced as the length of the interposed stimulus train was increased. Thus the introduction of a 900 pulse train reduced the output evoked by 60 pulses to between 20-25% of the control value.

The output of noradrenaline from the portal vein is very much less than that from the vas deferens and so it was not possible to study such a wide range of train lengths as in the vas deferens. However, it was possible to show that the fractional output increased with train length in the portal vein as in the vas deferens. Thus the fractional release at 16 Hz for 30 pulses was 2.8×10^{-5} (s.e. mean = $\pm 0.4 \times 10^{-5}$; $n = 4$), whilst

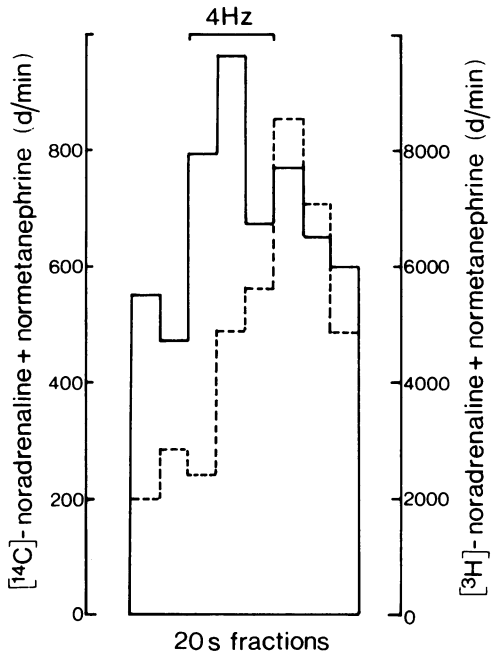

Fig. 3 Efflux of ^{3}H- and ^{14}C-labelled transmitter from rabbit portal vein during continuous electrical stimulation. Untreated tissue labelled as in protocol C of methods section ([^{3}H]-noradrenaline 100 ng/ml), and superfused with Krebs solution at 3 ml/minute. Twenty-second fractions of the superfusate were collected before, during and after electrical stimulation at 4 Hz for 1 minute. Labelled noradrenaline and normetanephrine were isolated on Dowex-50 columns as described in the methods section. Ordinates: total label output per 20 s of ^{3}H-labelled (broken line) and ^{14}C-labelled (continuous line) noradrenaline + normetanephrine.

it increased to 2.8×10^{-4} (s.e. mean = $\pm 0.3 \times 10^{-4}$; $n = 4$) when the train length was increased to 400 pulses.

Studies on the output of labelled noradrenaline

Time course of transmitter release. These experiments were done on superfused portal veins that had been labelled with [^{3}H]-noradrenaline and [^{14}C]-tyrosine as described in protocol (C) in the methods section. Consecutive samples were collected for 10 or 20 s before, during and after electrical stimulation at 2, 4 or 20 Hz for 1 minute. The samples were chromatographed on Dowex-50 columns as described in the methods section. A comparison of the efflux patterns revealed that there was a consistent difference in the time courses of the labelled compounds. It was found that the ^{14}C efflux (labelled noradrenaline and normetanephrine derived from tyrosine) reached a peak before that of the ^{3}H-efflux. A typical experiment is shown in Fig. 3, the ^{14}C-efflux reached a peak within 40-60 s during stimulation at 4 Hz, whereas the peak ^{3}H-efflux occurred some 20-40 s later. This difference in efflux patterns was observed in 7 of our 9 experiments. It was calculated that in these experiments 69 ± 9% of the total ^{14}C-efflux was released within 60 s whereas only 34 ± 12% of the total ^{3}H-efflux was released within this period. This effect was independent of the stimulus frequency but it was more difficult to see at 20 Hz because of the necessity for short collection periods consequent upon the much faster increase in transmitter output with time. The difference in efflux patterns became less evident as the length of the experiment was extended and there was no detectable difference in efflux patterns 5-7 h after labelling the tissue.

Comparison of ^{3}H- and ^{14}C-efflux at different train lengths. A possible explanation for the preceding results was that incubation with [^{3}H]-noradrenaline and [^{14}C]-tyrosine results in [^{3}H]- and [^{14}C]-noradrenaline entering different neuronal pools. Each of these pools is then mobilized during electrical stimulation but the contribution of each to the total output is dependent on the train length. To test this hypothesis the total outputs of both labels were determined in both the portal vein and vas deferens after a fixed number of pulses; in each experiment the output for 40 pulses was compared for that for 240 pulses.

In both tissues it was found that there was a proportionately greater increase in the ^{3}H-label output compared to the ^{14}C-label output when the train length was increased from 40 to 240 pulses. A typical experiment is shown in Fig. 4, a six-fold increase in the train length led to a six-fold increase in ^{14}C-label output from the portal vein. In contrast there was a 15-20 fold increase in ^{3}H-label efflux. Thus increasing the train length led to a three- to four-fold increase in the ^{3}H-output/pulse whilst the ^{14}C-output/pulse remained almost constant. The same result was obtained in six experiments with the portal vein and in five experiments with the vas deferens. In one experiment with the portal vein and two experiments with the vas deferens there was only a three- to four-fold increase in ^{14}C-catecholamine efflux and an eight- to ten-fold increase in ^{3}H-catecholamine efflux when the train length was increased. A consistent feature in all these experiments was the depression of the [^{3}H]-catecholamine efflux due to 40 pulses after the 240

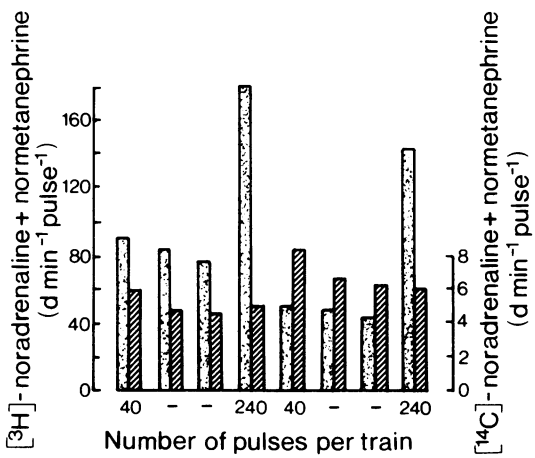

Fig. 4 Output of ^{3}H- and ^{14}C-labelled transmitter during stimulus trains of discrete lengths. Untreated rabbit portal vein labelled with [^{3}H]-noradrenaline (100 ng/ml) and [^{14}C]-tyrosine as described in the methods section. The vein was stimulated electrically every 15 min at 10 Hz for either 40 or 240 pulses. Labelled noradrenaline and normetanephrine were isolated on Dowex-50 columns; the basal output for an equivalent time period has been deducted from the stimulated output. Note that the output per pulse of the ^{3}H-label increased two- to three-fold when the train length was increased from 40 to 240 pulses, whereas the ^{14}C-label output remained constant. Ordinates: output per pulse of [^{3}H]- and [^{14}C]-noradrenaline + normetanephrine, stippled and hatched columns, respectively

pulse train and a 25-50% increase in the [^{14}C]-catecholamine efflux (Figure 4).

Selective labelling of neuronal stores. Hughes (1973) was able to demonstrate the existence of an 'easily releasable' noradrenaline store in vasa deferentia that had been labelled with low (10 ng/ml) concentrations of [^{3}H]-noradrenaline. We decided to investigate the possibility that release of transmitter from this 'easily releasable pool' might vary with the frequency of nerve stimulation. Pairs of vasa deferentia were labelled with [^{3}H]-noradrenaline (10 ng/ml) (protocol A) and then treated with phenoxybenzamine for 1 hour. Paired experiments were carried out in which one vas was stimulated for 300 pulses at 2 Hz and the other for the same number of pulses at 16 Hz. In each experiment (Table 1) the specific activity of the released noradrenaline greatly exceeded that of the tissue stores. However, there was no significant difference between the specific activity of the noradrenaline output at 2 Hz compared to that at 16 Hz. Thus although the total noradrenaline output per pulse was two to four times greater at 16 Hz compared to 2 Hz, there was also a correspondingly greater output of [^{3}H]-noradrenaline at the higher frequency.

A further series of experiments was carried out on vasa deferentia labelled with [^{3}H]-noradrenaline (10 ng/ml) and with [^{14}C]-tyrosine (protocol C). The tissues were stimulated 1, 3, 5 or 9 h after the initial labelling procedure and the specific activities of the released and stored noradrenaline compared at each time point (separate experiments). For up to 5 h after the beginning of the experiment the specific activities of both [^{3}H]- and [^{14}C]-noradrenaline released during stimulation were significantly greater than the specific activity of the corresponding labelled noradrenaline contained in the vas deferens (Table 2). However, the difference between the stored and released [^{3}H]-noradrenaline declined gradually and at 9 h the specific activity of the released noradrenaline was only marginally greater than that of the stored material (Table 2). In contrast there was little or no decline in the difference between the specific activities of the stored and released ^{14}C-labelled noradrenaline, even after 9 hours. In these experiments the tissue

Table 1 Specific activity of [^{3}H]-noradrenaline released during nerve stimulation, at different stimulus frequencies, compared to tissue stores

	Specific activity (d min^{-1} ng^{-1})			
	Vas A		*Vas B*	
	Tissue	*Medium (2 Hz)*	*Tissue*	*Medium (16 Hz)*
Means (n = 5) ± s.e.	138 ± 10	543 ± 50	139 ± 9	511 ± 41

Paired experiments in which each pair of vas deferentia was incubated with [^{3}H]-noradrenaline (10 ng/ml) as in protocol A in the methods section. The tissues were treated with phenoxybenzamine (10 μg/ml) for 1 h after labelling and then stimulated at 2 Hz (Vas A) and 16 Hz (Vas B) for 300 pulses. In each experiment the specific activity is calculated from the mean of two stimulated outputs obtained at 20 min intervals. The specific activity of the tissue stores was corrected for loss of labelled and endogenous transmitter during stimulation.

Table 2 Specific activity of noradrenaline released at different times from the rabbit vas deferens labelled with (−)-[^{3}H]-noradrenaline and [^{14}C]-tyrosine

	Specific activity (d min^{-1} ng^{-1})			
	[^{3}H]-noradrenaline		*[^{14}C]-noradrenaline*	
Time after labelling (h)	*Tissue*	*2 Hz*	*Tissue*	*2 Hz*
1	259 ± 25	691 ± 35	14 ± 2	49 ± 5
3	220 ± 15	426 ± 36	20 ± 3	40 ± 3
5	150 ± 14	246 ± 19	16 ± 4	40 ± 6
9	132 ± 12	170 ± 20	17 ± 2	38 ± 7

Vas deferens labelled with (−)-[^{3}H]-noradrenaline (10 ng/ml) and [^{14}C]-tyrosine (2.58 μg/ml) as in protocol C of the methods section. Four separate tissues were used at each time point, the tissues were stimulated at 2 Hz for 300 pulses at the times indicated after the end of the incubation procedure. The tissues were not stimulated before the indicated time point. The specific activity of the [^{14}C]-noradrenaline output was significantly greater ($P < 0.005$) than that of the tissue stores at each time point. The same was true for the ^{3}H-label up to 5 h ($P < 0.01$), but not at 9 h ($0.05 < P < 0.1$).

content of [^{14}C]-tyrosine (perchloric acid soluble ^{14}C-activity not retained on alumina) declined by 18 ± 3% between the 1 and 9 h periods. The results obtained with tissues loaded with low concentrations of labelled noradrenaline (10 ng/ml) suggest that under these conditions the labelled noradrenaline enters a store that has a higher turnover rate than the total noradrenaline store in the phenoxybenzamine treated tissue.

Discussion

Noradrenergic nerve terminals appear to possess a remarkable ability to vary the amount of transmitter that is released under different conditions of nerve stimulation. In the phenoxybenzamine-treated rabbit vas deferens the fractional release per pulse increases from 10^{-6}, or less, for trains of less than 5 pulses, to greater than 10^{-4} for trains of 100-400 pulses. The degree of flexibility is even greater when we consider that the output/pulse is facilitated when the stimulus frequency is increased over the range 0.5 to 16 Hz. The same mechanisms appear to operate in the portal vein, although we were unable to investigate such a wide range of train lengths in this tissue as in the vas deferens.

To our knowledge, there has been no other study in which noradrenaline output has been systemically estimated during discrete trains of different lengths. Direct measurements have been made for fixed intervals during continuous splenic nerve stimulation (Brown, 1965; Haefely, *et al.*, 1965). However, these studies with perfused spleens gave only an approximate estimate of the output/pulse during any one part of a stimulus train. Electrophysiological studies in the guinea-pig and mouse vas deferens (Burnstock *et al.*, 1964; Bennett, 1973a,b), have shown that the amplitude of excitatory junction potentials, elicited by nerve stimulation, increase for only the first 6-12 pulses in a stimulus train. These workers also showed a facilitation of excitatory junction potentials with increasing stimulus frequency, but only between 0.1 and 2 Hz. There is, therefore, a general agreement between our results and the electrophysiological measurements in that noradrenaline release is facilitated with successive pulses or increasing stimulus frequencies. However, there is a considerable discrepancy between the results concerning the time course and absolute magnitude of the facilitation with successive pulses. Moreover, direct estimates of noradrenaline output indicate that the output/pulse increases through the range 0.5 to 16 Hz in a number of different tissues (Hughes, 1972; Henderson, Hughes & Thompson, 1972; this paper). These relationships appear to be true reflections of the release process since they can be observed in the presence of cocaine, cocaine plus corticosterone, phenoxybenzamine and indomethacin, as well as in untreated preparations (Hughes, 1972 and unpublished results). We may have to wait for more electrophysiological experiments of the type performed by Bennett (1973a, b), preferably combined with direct release studies, before we can resolve the contradictory results obtained by the two techniques.

Both newly synthesized [^{14}C]-noradrenaline and low concentrations of exogenous [^{3}H]-noradrenaline appear to be incorporated in a store which has different properties compared with the total neuronal store of noradrenaline and with the store which is entered by high concentrations of exogenous [^{3}H]-noradrenaline. Thus in our

experiments the specific activity of the noradrenaline released by nerve stimulation was two to four times greater than that of the total tissue store. The labelled transmitter may be incorporated into vesicles close to the neuronal membrane, these vesicles, because of their proximity to the site of release, would then constitute a 'readily releasable' transmitter pool. Morphological evidence for this hypothesis has been obtained by Budd & Salpeter (1969). Another possibility is that 'young vesicles' (recently transported from the cell body) are particularly active in the storage and release of noradrenaline (Haggendal & Dahlström, 1972). It could be that these 'young vesicles' constitute an 'easily releasable pool' in comparison to the 'older storage vesicles', which may only accumulate noradrenaline when it is present in relatively high concentrations.

During nerve stimulation, noradrenaline appears to be mobilized from two distinct stores. Mobilization from the 'easily releasable pool' appears to be a continuous process from the onset of stimulation. However, this pool does not appear to contribute to the facilitation of output that is seen with increasing numbers of pulses (see Figures 3 and 4). The output/pulse of newly synthesized noradrenaline remains constant between 40 and 300 pulses, whereas that of the total noradrenaline output significantly increases. The two-pool hypothesis offers a partial explanation for our results concerning the facilitation of output with increasing train length. The 'readily releasable pool' is immediately mobilized upon stimulation and release is maintained from this pool for at least 300 pulses. However, with successive pulses, the probability of release from the second pool increases and the total output/pulse is boosted. This could be due to the migration of deep lying vesicles towards the neuronal membrane.

The above hypothesis does not explain the facilitation of output with increasing stimulus frequency. There was no significant difference between the specific activities of the noradrenaline released at high and low frequencies of stimulation in tissues where the 'easily releasable pool' has been selectively labelled. Moreover, the mobilization of noradrenaline from different stores during a train of pulses was seen at both low and high frequencies of stimulation. Therefore it does not appear that additional transmitter stores are recruited as the stimulus frequency is increased; the facilitation of output must involve a mechanism which involves all the stores of noradrenaline. The most intriguing aspect of these studies is that although we would expect all autonomic C-fibres to possess similar biophysical properties, it is a fact that the relation of transmitter output to train length and frequency is not at all similar at cholinergic and adrenergic neuroeffector junctions (Paton, 1963; Henderson *et al.*, 1972).

The proposal that newly synthesized noradrenaline constitutes a major fraction of the transmitter output (Kopin, Breese, Krauss & Weise, 1968: Gewirtz & Kopin, 1970) from adrenergic nerves should perhaps be questioned. The evidence for a major role for newly synthesized transmitter is largely based on results from experiments in which extreme conditions of nerve stimulation were used (high frequencies for long time periods in the presence of uptake blocking agents) and in which the tissue stores were rapidly depleted leaving *de novo* synthesis as the only source of releasable transmitter. It is also noteworthy that a significant acceleration of noradrenaline synthesis is seen only under conditions of intensive nerve stimulation (Alousi & Weiner, 1966; Roth, Stjärne & von Euler, 1966; Austin, Livett & Chubb, 1967). Even under conditions of near maximal rates of synthesis, a rate of 7-8 pg g^{-1} s^{-1} is exceptional (Boadle-Biber *et al.*, 1970) and does not compare with the rate of release which may vary from 100-1000 pg g^{-1} s^{-1} in the rabbit vas deferens in the presence of cocaine or phenoxybenzamine (Hughes, 1972). However, since 80-90% of the noradrenaline released by nerve stimulation is recaptured by the nerves (Hughes, 1972), the amount lost by overflow (5-20 pg g^{-1} s^{-1}) could be made up by *de novo* synthesis. We thus agree with Hedqvist & Stjärne (1969) that under normal conditions the noradrenaline recaptured by the neurone is of far greater importance than synthesis in maintaining the functional transmitter store.

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