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Evaluation of mechanisms controlling the release and inactivation of the adrenergic transmitter in the rabbit portal vein and vas deferens

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Summary

1. A method is described for the detection and assay of picogramme quantities of noradrenaline. This involves transferring Krebs solution containing noradrenaline to a cascade system where the catecholamine may be bioassayed on superfused preparations of the rabbit aorta and iliac artery.

2. Electrical field stimulation of the rabbit vas deferens and portal vein caused the release, into the bathing medium, of a material which was identified by pharmacological and chemical tests as noradrenaline.

3. Cocaine (0·3–5 μg/ml) caused a marked increase in noradrenaline output after electrical stimulation of the portal vein and vas deferens. This effect appeared to be maximal at a concentration of 2·4 μg/ml ; when the cocaine concentration was increased above 10 μg/ml the noradrenaline output was greatly reduced.

4. Phenoxybenzamine (5 μg/ml) caused a 4–8 times greater increase in noradrenaline output than cocaine ; however, the increase in output due to phenoxybenzamine was much smaller in tissues pretreated with cocaine.

5. Corticosterone (20 μg/ml) increased noradrenaline output by 30–40% in untreated vas deferentia, but caused a 300% increase in output in tissues pretreated with cocaine. Cocaine also caused a much greater increase in output in tissues pretreated with corticosterone than in untreated tissues.

6. Treatment with pargyline plus tropolone caused a 100–200% increase in noradrenaline output ; this effect was not modified by cocaine, but was abolished when the tissues were pretreated with either phenoxybenzamine or corticosterone.

7. When tissues were stimulated for 240 pulses at 1–16 Hz, the output per pulse of noradrenaline increased linearly with the logarithm of the frequency of stimulation. This relationship between frequency and output was seen in both untreated tissues, and in tissues treated with cocaine, phenoxybenzamine, corticosterone or pargyline plus tropolone.

8. It is concluded that cocaine enhances output by blocking the neuronal reuptake of noradrenaline, and corticosterone by blocking the extraneuronal uptake and subsequent metabolism of noradrenaline. Phenoxybenzamine acts by blocking both neuronal and extraneuronal uptake mechanisms. There appears to be a dynamic balance in the distribution of noradrenaline between the two uptake mechanisms after the release of the transmitter from the nerve endings.

9. It is calculated that more than 90% of the noradrenaline released by nerve stimulation (240 pulses at 2–16 Hz) is inactivated by neuronal and extra-neuronal uptake mechanisms.

10. It is calculated that the fraction of the total noradrenaline store that is released by one pulse at 2 Hz is $6{\cdot}6 \times 10^{-5}$ in the portal vein and $5{\cdot}6 \times 10^{-5}$ in the vas deferens; the corresponding values at 16 Hz were $15{\cdot}9 \times 10^{-5}$ and $16{\cdot}2 \times 10^{-5}$.

Introduction

It would be an advantage to be able to measure the release of endogenous noradrenaline from isolated tissues since the *in vitro* situation is much simpler than the more commonly used, but more complex, perfused systems of the heart and spleen. This paper describes a method with which it is possible to detect and assay endogenous noradrenaline release from isolated smooth muscle preparations. Two different types of adrenergically innervated tissues have been examined; first, the rabbit portal vein which has a relatively sparse, two dimensional adrenergic plexus (Holman, Kasby, Suthers & Wilson, 1968), and, second, the vas deferens which has a dense, three dimensional innervation (Norberg & Hamberger, 1964). A study has been made of the relationship between the frequency of nerve stimulation and noradrenaline output and of the various inactivation mechanisms which appear to function at adrenergic neuroeffector junctions. A possible re-evaluation of adrenergic transmitter mechanisms is discussed.

Methods

Preparation of tissues

Donor tissues

Male, albino rabbits, weighing 1·5–3 kg, were killed by breaking the neck and exsanguination. The portal vein was dissected out and split into a flat strip as described by Hughes & Vane (1967). The two vas deferens were dissected out from the epididymis to the seminal vesicle; they were then split open longitudinally and cotton threads attached to both ends. The vas deferens was often too long to be accommodated by the donor bath and in this case the tissue was folded double and the two loose ends secured together by cotton thread. The donor tissues were secured to the bottom of the donor bath by cotton thread and connected to an isometric transducer by the upper thread. The tissues were bathed in Krebs solution maintained at 37·5° C and gassed with 95% oxygen and 5% carbon dioxide. The portal vein was initially stretched until it maintained a resting tension of 3 g, the vas deferens was similarly adjusted to a resting tension of 3 g.

Assay tissues

Spiral preparations of the rabbit aorta and iliac arteries were used. These were dissected out with the donor tissues from the same animal. The aorta was removed from below the left renal artery to its bifurcation, mounted on a fixed needle and cut in a right, continuous spiral from the caudal to the cephalic end. The resulting strip was divided crosswise to give two preparations approximately 35×3 mm. The two iliac arteries were also cut in a right spiral; these strips were approximately 25×15 mm and were not further divided. The assay tissues were mounted for

superfusion inside special cascade baths (Fig. 1), the outer jacket of these baths contained circulating water maintained at 38° C. The upper end of each artery was attached by cotton thread to the arm of an isometric transducer and the lower end fixed to the outlet of the cascade bath. The tissues were superfused with modified Krebs solution at the rate of 2·5–4 ml/minute. The aortic strips were initially stretched to 2 g tension and the iliac arteries to 1·5 g tension. They were then allowed to equilibrate for 2 h, after which time a further 0·5 to 1·0 g tension was applied.

Measurement of endogenous transmitter release

Bioassay

The technique depends on transferring the Krebs solution bathing the donor tissue to a cascade system where the active material is assayed (Fig. 1). The donor bath has a 2·5 ml capacity and internal dimensions of 60 × 7 mm, a needle fixed into the base supplies 95% O_2+5% CO_2 at a sufficiently vigorous rate to ensure rapid mixing within the bath. Both the donor and assay baths (110 × 25 mm) are surrounded by constant temperature water jackets (38° C) supplied by a Churchill heater-pump. Krebs solution is supplied from a reservoir through the main heating coil and the donor bath is washed out by overflow by opening the 3-way tap T_1. T_2 is normally adjusted so that the roller pump (Watson-Marlow) draws Krebs solution through the main and secondary heating jackets to supply the cascade system. When T_2 is closed and T_1 opened towards the roller pump the fluid in the donor bath is drawn through the cascade system. The roller pump was normally set to

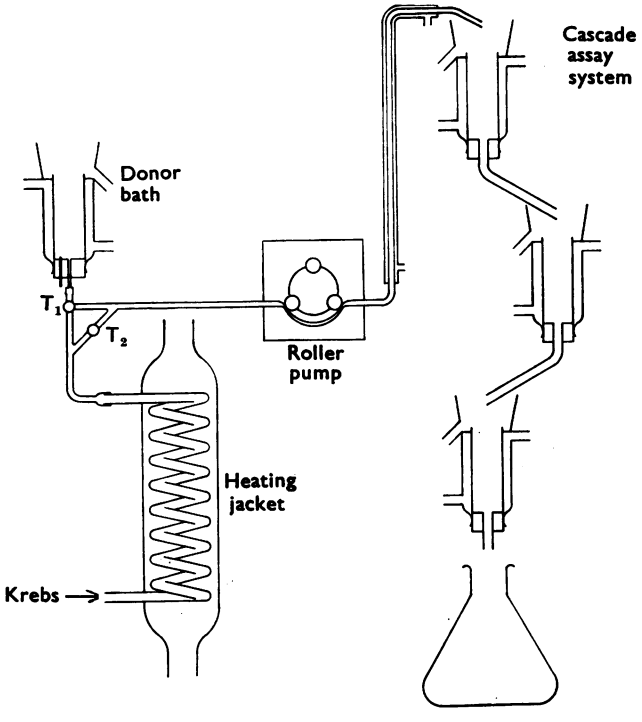

FIG. 1. Experimental arrangement for detecting transmitter release. T_1 and T_2 are three-way taps. The platinum electrodes for transmural stimulation have been omitted from the diagram.

deliver 2·5 ml/min, this meant that the donor fluid was in contact with the assay tissues for one minute.

In practice, two identical donor-cascade systems were used, thus permitting two experiments to be carried out simultaneously. In most experiments two assay tissues were used in each cascade system. The outputs from all the transducers were displayed on a six channel Grass polygraph. Contractions of the assay tissues to standard amounts of noradrenaline were obtained by adding the drug to the donor bath via a microsyringe (volumes $<0{\cdot}05$ ml); 30 s was allowed for mixing and then the bath contents were drawn over the assay tissues. The output of transmitter from the donor tissue was determined by bracketing contractions due to the released material with contractions elicited by standard doses of noradrenaline.

To avoid errors arising from contamination of the donor baths and tubing it was necessary to ensure complete cleanliness of the apparatus. This entailed soaking the tubing in ' Decon-75 ' for 2 h followed by a thorough wash with hot tap water; a further soaking for 30 min in 0·1 N HCl was followed by a final rinse with at least 10 litres of distilled water.

Fluorimetric assay

Donor fluid samples were collected directly from the cascade system into cooled flasks containing 0·2 ml of 1 N HCl and 0·2 ml of 10% disodium edetate. The samples were immediately concentrated over alumina columns as described by Boadle-Biber, Hughes & Roth (1970) with the modification that the eluant (0·15 N perchloric acid) was left in contact with the alumina for 10 min before opening the column tap and collecting the eluate. Column recovery of adrenaline or noradrenaline was between 70 and 75% with 2 ml of eluant. Standard amounts of noradrenaline were also processed through the cascade system and the column procedure in order to determine the total recovery of the amine, this averaged 61% in ten determinations.

All determinations were carried out on 0·5 ml samples of the alumina eluate. The method of O'Hanlon, Compuzano & Horvath (1970) was used with the modification that the sample pH was adjusted with 0·5 M K_2HPO_4 to 6·75 for noradrenaline oxidation. The method depends on the differential oxidation of adrenaline and noradrenaline at pH 4·0 and 6·75 respectively to yield the appropriate trihydroxyindole derivatives. The assay gave a fluorescence reading 1·5 times that of the blank with a sample containing 2 ng of noradrenaline.

Nerve stimulation

Electrical field stimulation was used to excite intramural nerves. Platinum electrodes were fixed vertically on opposite sides of the donor tissue and as close to the tissue as possible. Supramaximal stimuli (150 mA, 1·0 ms rectilinear pulses) were used throughout. In each experiment a standard train of 240 pulses was used at each stimulus frequency.

The possibility of destruction of noradrenaline by the electrical current was considered. Standard amounts of noradrenaline (0·5–5 ng) were added to the donor bath, without any donor tissue, and current was passed through the electrodes at frequencies of 2 and 16 Hz for 240 pulses. In 4 such experiments less than 20% of the noradrenaline was destroyed. Further experiments were carried out in the pre-

sence of the portal vein (three experiments) and the vas deferens (three experiments) treated with bretylium (5 μg/ml): no destruction of noradrenaline could be detected in these experiments or in another experiment where the vein was treated with tetrodotoxin (0·5 μg/ml).

Solutions

A modified Krebs-Henseleit solution of the following composition was used throughout: (mM) NaCl, 118 ; KCl, 4·75 ; $CaCl_2$, 2·54 ; KH_2PO_4, 0·93 ; $MgSO_4$, 1·19 ; $NaHCO_3$, 25·0 ; glucose, 11·1 ; disodium edetate, 0·027 ; Na ascorbate, 0·1 ; L-tyrosine, 0·025.

Noradrenaline standard solutions for bioassay were diluted freshly in the modified Krebs solution. Stock noradrenaline solutions (1 mg/ml) were made by dissolving noradrenaline bitartrate in 0·01 N HCl and adding 1 mg/ml of sodium ascorbate and the stock solution kept at −20° C. The standard solutions (1·0, 0·1 and 0·01 μg/ml) were maintained at the temperature of melting ice and protected from light throughout the experiment. When the standards were checked against fresh dilutions no deterioration of the original dilution could be detected over a period of 8–10 hours.

Phenoxybenzamine and corticosterone were freshly dissolved in propylene glycol and water (8 : 2 by volume) and protected from strong light. These drugs were never added in volumes greater than 0·005 ml/ml of Krebs solution.

Drugs

All drug concentrations are quoted in terms of the base (μg/ml). (−)-Adrenaline bitartrate (B.D.H.), bretylium tosylate (Burroughs Wellcome), cocaine hydrochloride (MacFarlan Smith Ltd.), 1,3,5 (10)-estratrien-3, 17β-diol (corticosterone, Koch-Light), (−)-noradrenaline bitartrate (B.D.H.), pargyline hydrochloride (Abbott Laboratories), phenoxybenzamine hydrochloride (Smith, Kline & French), phentolamine hydrochloride (Ciba), tropolone (Aldrich Chemicals).

Definition of terms

Output of transmitter is used in this paper to describe the amount of noradrenaline which diffuses from the donor tissue into the bathing medium and which is then subsequently measured. This output cannot be directly equated with the total amount *released* from the sympathetic nerve endings, although it is assumed that output is proportional to the actual amount released, and that the distinction between the two terms becomes less when transmitter inactivation is blocked.

Results

Bioassay

In preliminary experiments it was found that spiral preparations of the abdominal aorta, iliac artery, mesenteric artery and renal artery were equally capable of detecting as little as 0·02–0·05 ng of noradrenaline. However, the first two preparations had the advantage of being easier to prepare and were more consistent in their responses over a long period of time.

An example of the assay procedure is shown in Fig. 2. Preliminary experiments showed that untreated preparations of the portal vein released between 0·05 and 0·5 ng of noradrenaline-like material when stimulated with 240 pulses at 2 Hz, and the vas deferens between 0·1 and 1·0 ng. Accordingly, responses of the two assay tissues to noradrenaline in these dose ranges was first obtained at the beginning of each experiment. The donor tissue was then stimulated electrically and an approximate estimate of the output made.

The assay tissues were normally able to discriminate between noradrenaline doses varying by a factor of 1·3. The accuracy of the assay was assessed by double-blind estimations of a range of prepared noradrenaline samples. In a series of thirty estimations there was a mean standard error of 12%. It was found that a considerable increase in sensitivity could be obtained by using arteries stored for 1–3 days in Krebs solution at 4° C. This type of preparation was rarely used since freshly prepared tissues were normally quite adequate for the purposes of this investigation. In any one bracket the results from the two assay tissues were averaged ; however, in some cases (≏20%) one of the assay tissues proved insensitive and the assay was based on the remaining sensitive tissue. In most experiments it was possible to use a dose interval of 6–10 minutes. This meant that the donor tissue could be electrically stimulated once every 12–20 minutes.

The problem of allowing adequate time for the diffusion of the transmitter from the donor tissue into the bathing medium was considered. It was necessary to compromise between obtaining the maximum amount of ' recoverable transmitter ' and keeping the collection time down to a reasonable length to enable the maximum

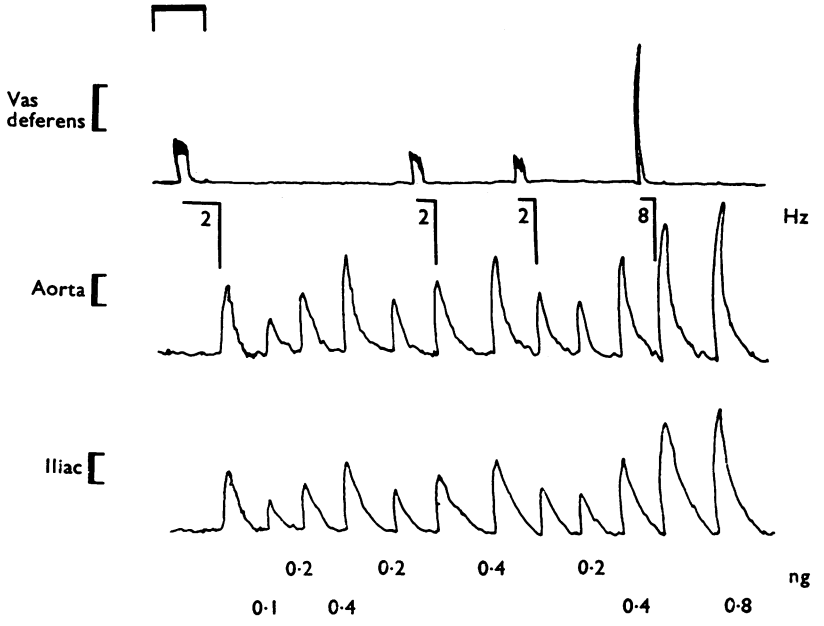

FIG. 2. Bioassay of transmitter released from the rabbit vas deferens. Upper trace: donor vas deferens stimulated by electrical field stimulation at 2 or 8 Hz for 240 pulses; vertical calibration=2 g. Lower two traces: assay tissues, aorta and iliac artery; vertical calibrations=0·2 g. Assay tissue responses to 0·1, 0·2 and 0·4 ng of noradrenaline were first obtained; the output from the vas after stimulation at 2 Hz was then bracketed by 0·2 and 0·4 ng amounts of noradrenaline. Similarly the output after stimulation at 8 Hz was bracketed between 0·4 and 0·8 ng of noradrenaline. Horizontal time mark=10 minutes.

number of observations to be made in an experiment. It was also necessary to allow for the fact that when the transmitter output was increased by various drugs, a longer collection period was needed to obtain a similar proportion of the output as in the untreated tissue.

In the portal vein, 3 min were allowed for diffusion and mixing in the donor bath. This time, measured from the end of the stimulation period, gave a recovery of 85–95% of the total 'recoverable transmitter' (2 and 16 Hz for 240 pulses). In the presence of cocaine, corticosterone or pargyline+tropolone, a collection time of 4 min gave recovery values of 80–90%. In the presence of phenoxybenzamine the time was extended to 4·5 min to give recoveries of 75–80%. The diffusion times were somewhat longer in the vas deferens, in untreated tissues 80–85% recovery was obtained with a 3·5 min collection period. This time was extended to 5 min in the presence of drugs which increased transmitter output. This gave recoveries of 80–90% in the presence of cocaine, corticosterone and pargyline plus tropolone, and 70–80% in the presence of either phenoxybenzamine or of cocaine plus corticosterone. The results in the following sections are uncorrected for the above recovery values.

Identification of material released by electrical stimulation

Bretylium (two experiments, 5 μg/ml) abolished contractions of the portal vein by electrical stimulation and reduced the output of the active material to levels undetectable by the bioassay ($<$0·01 ng). In the vas deferens even prolonged exposure to bretylium (up to 3 h, 5 μg/ml) never abolished the contractions to electrical stimulation but again the outputs fell to undetectable levels. These reductions represented at least a 90–95% decrease in output.

Phentolamine was infused directly over the assay tissues to give a final concentration of from 0·1 to 0·5 μg/ml; this procedure abolished contractions to noradrenaline and to the material released by electrical stimulation of the donor tissue but did not reduce contractions elicited by equiactive doses of histamine and 5-hydroxytryptamine.

For fluorimetric assay, the outputs were increased by pretreating the donor tissues with phenoxybenzamine (see following sections). After bioassay of the active material, the superfusate was collected in cooled flasks containing 0·2 ml of 1 N HCl and 0·2 ml of 10% EDTA. These samples were concentrated by alumina chromatography and aliquots of the eluate taken for the differential fluorimetric assay of adrenaline and noradrenaline. Table 1 shows the correlation obtained between the bioassay and the fluorimetric assay for noradrenaline. A comparison of the two methods using the paired t test showed no significant difference between the two sets of results ($P>0·2$). When the adrenaline assay was corrected for noradrenaline fluorescence no significant amounts of adrenaline could be detected. In a small number of experiments (Table 1) the alumina eluate was also taken for bioassay on the cascade system. These results also agreed with those obtained with the chemical estimation and with the original bioassay.

In view of these results the material released by electrical stimulation will now be referred to as noradrenaline. It is accepted, however, that these results do not constitute an absolute identification of the released material.

Variability of noradrenaline output

In any one experiment a decline in the stimulated output was seen with consecutive stimulus trains, this effect was observed at all frequencies of stimulation. The decline in output depended on the interval between the stimulus trains, the outputs declining by 8–15% per stimulus train with intervals of 10–14 min, and by 4–8% per stimulus train with intervals of 20 minutes. In all the following experiments the stimulus interval was kept between 14 and 18 min, this ensured that any one output was at least within 10% of the previous output. Experiments in which the output declined by more than 10% per stimulus train were either rejected or further controls were obtained until the outputs were within 10% of each other. When the effects of drugs on noradrenaline output were examined at least two control outputs were obtained at any one frequency and these values were averaged.

TABLE 1. *Estimation of noradrenaline output by chemical and biological assay*

Sample nos.	Amount of noradrenaline in sample (ng): Bioassay during superfusion	Fluorimetric assay of alumina eluate	Bioassay of alumina eluate
1	108	90	—
2	105	110	—
3	80	60	—
4	51	50	—
5	60	30	—
6	51	38	—
7*	45	45	—
8*	28	25	—
9*	17	16	—
10	40	55	—
11	45	48	—
12	33	30	—
13	70	75	67
14*	37	43	35
15*	30	41	33
16	102	105	119
17	110	66	79
18	100	82	106
19	85	87	80
	Paired *t* test mean difference ± S.E. = 5·3 ± 3·4 ($P > 0·2$)		

Rabbit vas deferens treated with phenoxybenzamine (5 μg/ml) for 45 minutes. Transmural stimulation for 240 pulses at 16 Hz or 2 Hz (samples marked by *). The output after each stimulus train was first estimated by cascade bioassay, the samples were then collected and pooled (two or three samples) for alumina column separation. The results from the cascade bioassay were totalled to allow direct comparison with the assays on the pooled samples. The results from the assays on the alumina eluate are corrected for loss of noradrenaline in both the cascade system and in the alumina column procedure (mean recovery of standards with identical procedure = 61%).

TABLE 2. *Basal output of noradrenaline from resting donor tissues*

		Basal output ((pg/g)/min) (*n*)	Range	Mean
Portal vein	Untreated	29	45–200	110
	Cocaine	6	80–300	175
	Phenoxybenzamine	10	460–920	690
Vas deferens	Untreated	13	65–240	155
	Cocaine	5	55–340	200
	Phenoxybenzamine	12	1,100–3,500	2,000

Values are calculated from 8, 10 or 15 min outputs; a large number of resting outputs could not be estimated with any degree of certainty and these have been omitted.

Basal release of noradrenaline

In several experiments the presence of a noradrenaline-like substance could be detected in the medium bathing resting donor tissues. It was often difficult to detect the presence of this substance, which presumably reflects the spontaneous release of noradrenaline. Table 2 shows the range of values for the basal output of noradrenaline in the portal vein and vas deferens under a variety of experimental conditions. In general, the basal output per unit of time was considerably less than the stimulated output, although at low frequencies of stimulation (1–2 Hz) the minute basal output might represent as much as 10–20% of the observed stimulated output. In view of the difficulties involved in accurately measuring the basal output no attempt has been made to correct for this spontaneous release of noradrenaline in the following results. In most experiments at least one attempt was made to measure the basal release in order to check that the stimulated outputs were not being distorted by an anomalously high basal output.

Relationship between frequency of electrical stimulation and noradrenaline output

A very noticeable feature of the outputs from the vein and vas deferens was an increase in the output per pulse with increasing frequencies of stimulation. In order to reduce errors arising from declining outputs and possible interactions between different frequencies, the output evoked by a train of 240 pulses was determined at 1, 2, 4, 8 and 16 Hz first in an ascending order, and then in a descending order and the values averaged. Figure 3 shows the results obtained in a series of portal veins ; when the output per pulse was plotted against the frequency of stimulation on a logarithmic scale, the output was seen to increase linearly with the stimulus frequency. Similar results were obtained in the vas deferens and Fig. 5 shows the linear relationship between output and frequency that was also observed in this tissue.

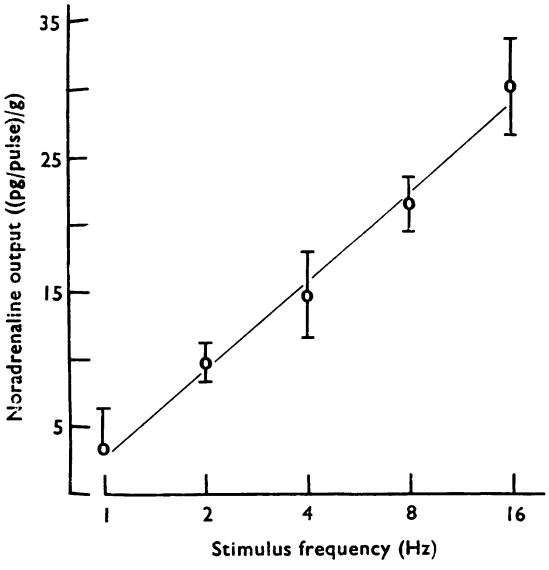

FIG. 3. Relation between stimulus frequency and noradrenaline output in the rabbit portal vein. Results from six untreated portal veins (means ± S.E.). Ordinate: noradrenaline output ((pg/pulse)/g). Abscissa: stimulus frequency, 1, 2, 4, 8, and 16 Hz for 240 pulses.

The frequency-response curve for the portal vein reached a maximum at 30–40 Hz, and a 50% maximal response was usually obtained at 4–6 Hz. At any one frequency, the contraction reached a maximum height within 150–200 pulses, and was usually maintained at this level for at least a further 100 pulses. The frequency-response curve of the vas was similar to that of the vein. However the vas responded to single pulses (contraction height⌓10 to 15% maximal response) and the response to repetitive stimulation above 1 Hz was biphasic. There was an initial quick contraction which tended to fade after fifteen to twenty pulses, the tension then once more increased with continued stimulation to reach a plateau after 100–150 pulses. The secondary increase in tension was usually maintained for at least a further 100 pulses.

Effect of drugs on noradrenaline output

Cocaine

Preliminary experiments showed that cocaine markedly increased the output of noradrenaline in both the vas deferens and portal vein. The variation in output with varying concentrations of cocaine was examined in both tissues (three experiments

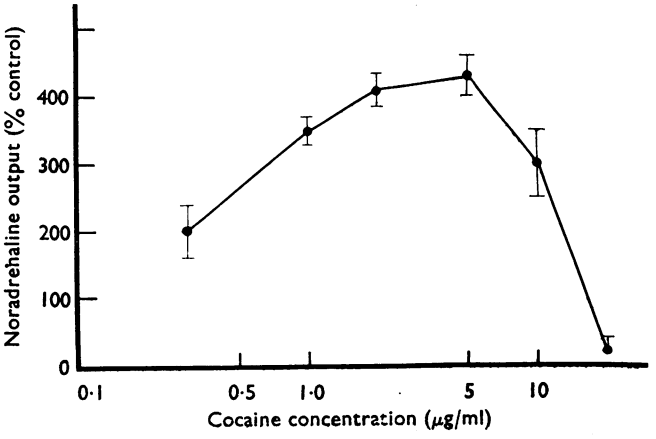

FIG. 4. Effect of cocaine on noradrenaline output from the rabbit portal vein. Means ± S.E. of three experiments. Ordinate: noradrenaline output as a percentage of the output in the absence of cocaine. Abscissa: cocaine concentration.

TABLE 3. *Effect of cocaine on noradrenaline output*

Tissue	Stimulus* frequency (Hz)	(*n*)	Noradrenaline output ((pg/pulse)/g) Control	Cocaine†	Alteration in output‡
Portal	2	(7)	9·3±3	25± 7	3·5±0·3
Vein	16	(7)	24±8	82±20	3·8±0·6
Vas	2	(9)	16±3	58±12	4·6±0·4
Deferens	16	(9)	41±7	190±29	5·0±0·6

* In these and all the following experiments the stimulus train consisted of 240 pulses. † Cocaine concentration = 5 μg/ml. ‡ Alteration in output:

$$\frac{\text{Output from cocaine treated tissue}}{\text{Output from untreated tissue}}$$

The values for the alteration in output were calculated from the individual experiments and not from the mean outputs of all the experiments. All values in this and the following tables refer to the mean ± the standard error.

each). A significant increase was seen with 0·1 μg/ml of cocaine; this effect was increased with higher concentrations of cocaine until a maximal potentiation occurred at 2–5 μg/ml (Fig. 4). A marked decrease in output occurred when the concentration of cocaine was increased to above 10 μg/ml (Fig. 4). In these and all following experiments, cocaine (5 μg/ml) was always present in the Krebs solution for the duration of the experiment; the effects of cocaine were normally fully developed within 10–15 minutes.

In both the portal vein and vas deferens, the increase in noradrenaline output was independent of frequency in the range of 1 to 16 Hz. Thus treatment with cocaine caused a parallel shift of the frequency-output curve in both tissues. The effect of cocaine on the noradrenaline output at 2 and 16 Hz is summarized in Table 3. In both tissues there was no significant difference in the effect of cocaine at the two frequencies. A 3·5–3·7 fold mean increase in output occurred in the portal vein, whilst a slightly greater mean increase of 4·5 to 5·0 was seen in the vas deferens. The basal efflux of noradrenaline was increased by cocaine in both tissues; however this efflux still only represented a small proportion of the stimulated output and was ignored when calculating the stimulated output.

The height and duration of contractions elicited by electrical stimulation were markedly increased by cocaine in the portal vein at low frequencies of stimulation. Above 6 Hz there was little increase in the height of the contractions but the duration of the response was greatly increased. In the vas deferens, cocaine had little or no effect on the height of the response, irrespective of the stimulus frequency. The most predominant effect was the appearance of a series of secondary contractions following the decline of the contraction at the end of the stimulus period.

Phenoxybenzamine

Phenoxybenzamine was added to the Krebs solution bathing the donor tissue, and was left in contact with the tissue for 45 minutes. At the end of this period the donor tissue was thoroughly washed to remove any free phenoxybenzamine. Preliminary experiments showed that a maximal increase in noradrenaline output could be obtained with a phenoxybenzamine concentration of 5 μg/ml. The increase in output after treatment with phenoxybenzamine was very large; the mean increase was 36–38 fold in the vas deferens, and 11–16 fold in the portal vein (Table 4). As with cocaine, this increase was seen at all frequencies of stimulation in the range 1–16 Hz. Figure 5 shows that phenoxybenzamine caused a parallel shift in the frequency-output curves.

TABLE 4. *Effect of phenoxybenzamine on noradrenaline output*

Tissue	Stimulus frequency (Hz)	(*n*)	Noradrenaline output ((pg/pulse)/g) Control	Phenoxybenzamine	Alteration in output*
Portal Vein	2	(5)	4±0·8	53±15	16±4
	16	(7)	14±2·5	146±15	11±3
Vas Deferens	2	(8)	19±5	560±111	36±8
	16	(8)	48±6	1,350±150	38±5

Phenoxybenzamine concentration = 5 μg/ml. * Alteration in output = $\frac{\text{Output after phenoxybenzamine}}{\text{Output of untreated tissue}}$

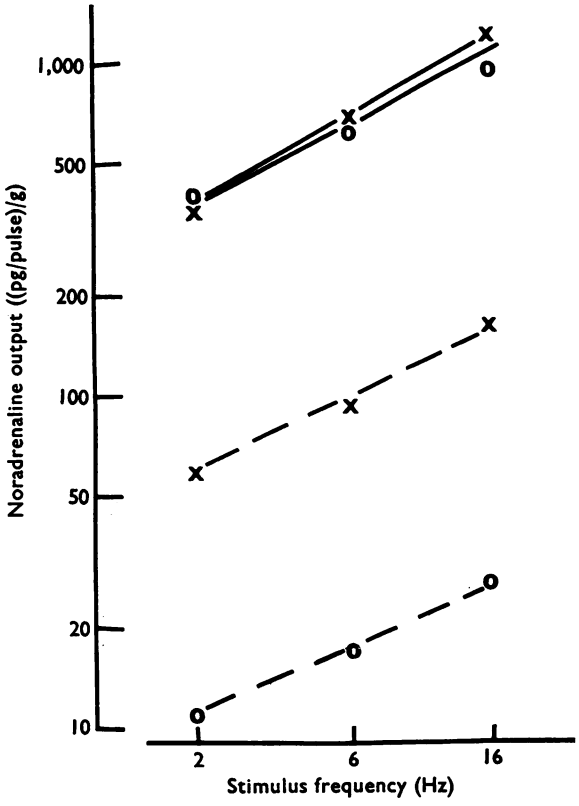

FIG. 5. Effect of phenoxybenzamine on noradrenaline output in untreated and cocaine treated vas deferens. Paired vas deferentia, one untreated (○---○), one treated with cocaine, 5 μg/ml (×---×). After phenoxybenzamine, 5 μg/ml (solid lines) there was a parallel shift of the frequency-output curves. Note the much greater increase of output caused by phenoxybenzamine in the untreated vas, leading to almost equivalent outputs from the two tissues after treatment. Ordinate: noradrenaline output ((pg/pulse)/g). Abscissa: Stimulus frequency.

TABLE 5. *Interaction between cocaine and phenoxybenzamine in the rabbit vas deferens*

	Experimental conditions	(*n*)	Noradrenaline output ((pg/pulse)/g) 2 Hz	16 Hz	Alteration in output* 2 Hz	16 Hz
a	Untreated	(5)	22± 6	53± 10	—	—
	Phenoxybenzamine	(5)	440±100	1,230±180	36±9	40±8
b	Cocaine	(5)	73± 15	200± 20	—	—
	Cocaine+ Phenoxybenzamine	(5)	500± 80	1,100±200	9±4	8±1

Results from paired vas deferentia a and b. The increase in output after phenoxybenzamine (5 μg/ml) was determined in the one untreated vas (a), and the increase in output in the presence of cocaine (5 μg/ml) was determined in the contralateral vas (b).

* Alteration in output = (a) $\frac{\text{Output after phenoxybenzamine}}{\text{Output from untreated tissue}}$

(b) $\frac{\text{Output in presence of cocaine+PBZ}}{\text{Output in presence of cocaine}}$

The effect of phenoxybenzamine appeared to be irreversible since the increased outputs were maintained over a period of 6–8 h with only a 10–15% decline per stimulus train. Also, further treatment of the donor tissue with phenoxybenzamine did not cause a second increase in noradrenaline output. The increase in basal efflux of noradrenaline was approximately 3–15 fold after treatment with phenoxybenzamine ; however, the amount was still small enough to be ignored for purposes of calculating the stimulated output.

In view of the large increase in output caused by phenoxybenzamine, a series of experiments were designed to test the possibility of an interaction between cocaine and phenoxybenzamine. Paired vas deferentia were examined simultaneously, one was pretreated with cocaine (5 μg/ml) and the other left untreated. The stimulated outputs from these tissues were then measured before and after treatment with phenoxybenzamine. From Table 5 it can be seen that phenoxybenzamine increased the output of the tissues to the same level irrespective of the presence or absence of cocaine. However, there was only an 8 to 9 fold increase in output in the cocaine treated tissues, compared with a 35–37 fold increase in the untreated tissues. A comparison of the results given in Tables 3 and 5 shows that, if the increase caused by cocaine alone is multiplied by that caused by phenoxybenzamine in the presence of cocaine, then the product closely approaches the figure for the increase caused by phenoxybenzamine in untreated tissues. Three further experiments on both the vas deferens and portal vein showed that cocaine did not increase the noradrenaline output in tissues pretreated with phenoxybenzamine.

After treatment with phenoxybenzamine, the contractions of the portal vein to electrical stimulation were abolished. The secondary contraction of the vas deferens was also abolished, however the initial quick contraction appeared to be little affected by phenoxybenzamine.

Corticosterone

Preliminary experiments indicated that treatment with corticosterone caused a significant increase (30–40%) in noradrenaline output from the portal vein and vas deferens. This effect took up to 60–75 min to develop and appeared to be maximal at a corticosterone concentration of 20 μg/ml.

In a further series of experiments it appeared that the effect of corticosterone was increased when the tissues had been treated with cocaine. The effect of corticosterone (20 μg/ml) was compared in experiments on pairs of vas deferentia of which only one was pretreated with cocaine (5 μg/ml). In the untreated tissues, corticosterone increased the output 1·36 fold whilst in the cocaine-treated tissues it caused a 3·9 fold increase (Table 6). The effect of corticosterone was independent of the frequency of stimulation and almost identical increases in output were seen at 2 and 16 Hz (Table 6).

Figure 6 shows the effect of corticosterone in an experiment in which one of a pair of vas deferentia was treated with cocaine and the other untreated. In both tissues treatment with corticosterone caused an increase in output, the effect reaching a maximum after approximately 60 minutes. It is quite clear, however, that the effect of corticosterone was much greater in the cocaine-treated tissue. When the corticosterone was washed out, the noradrenaline output rapidly fell below the initial control outputs.

In three further experiments with paired vas deferentia, the order of treatment with cocaine and corticosterone was reversed. In untreated tissues, cocaine caused a 4·6±0·3 fold increase in output; however, in tissues treated with corticosterone, cocaine caused a 13·3±0·4 fold increase in noradrenaline output. The interaction between these two drugs was therefore independent of the order of drug treatment. It was of interest to compare the relative effectiveness of corticosterone and phenoxybenzamine in paired vas deferens pretreated with cocaine. In three such experiments there was a 4 fold increase in output with corticosterone, and an 8 fold increase with phenoxybenzamine.

Corticosterone tended to reduce the height of the contractions to electrical stimulation at 2 and 16 Hz in both the portal vein and vas deferens, however the responses

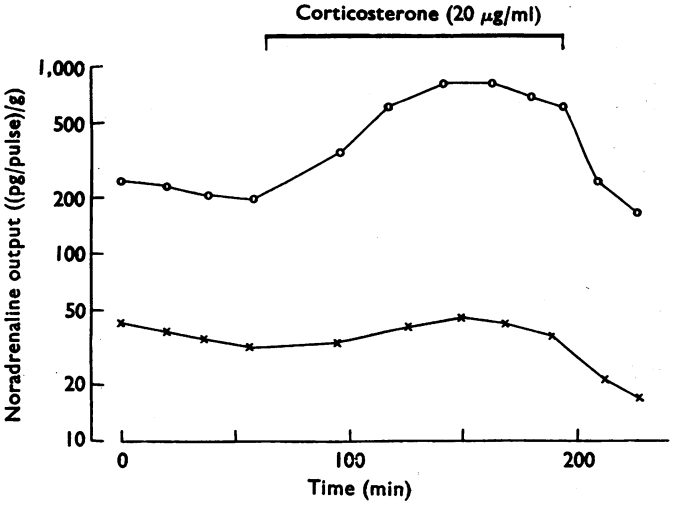

FIG. 6. Effect of corticosterone on noradrenaline output in untreated and cocaine treated vas deferens. Paired vas deferentia, one initially untreated (×——×), the other treated with 5 μg/ml of cocaine (○——○). Corticosterone (20 μg/ml) caused a much greater increase in noradrenaline output in the cocaine treated vas deferens. When the corticosterone was washed out, the noradrenaline output of both tissues rapidly declined. Ordinate: noradrenaline output ((pg/pulse)/g). Abscissa: time (min). Stimulus frequency=16 Hz.

TABLE 6. *Interaction between cocaine and corticosterone on noradrenaline output*

	Treatment	(*n*)	Noradrenaline output ((pg/pulse)/g) 2 Hz	16 Hz	Alteration in output (a) 2 Hz	16 Hz
A	None	(4)	12± 4	66± 24	—	—
	Corticosterone	(4)	19± 5 (b)	99± 37 (c)	1·37±0·1	1·36±0·04
B	Cocaine	(4)	48± 8	188± 32	—	—
	Cocaine+ Corticosterone	(4)	139±12	726±110	3·8 ±0·4	3·9 ±0·2

Paired vas deferens, a and b, as in Table 4. In each experiment the effect of corticosterone (20 μg/ml) was determined (a) in one untreated vas, and (b) in one treated with cocaine (5 μg/ml).

(a) Alteration in output = (A) $\frac{\text{Output in presence of corticosterone}}{\text{Output of untreated tissue}}$ (B) $\frac{\text{Output in presence of cocaine+corticosterone}}{\text{Output in presence of cocaine}}$

(b) Significantly greater than controls ($P<0·05$). Paired t test mean difference±S.E.=4·4±0·9.

(c) Significantly greater than controls ($P<0·025$). Paired t test mean difference ± S.E.=28±8.

were never reduced by more than 20%. A further interesting feature was that the rate and amplitude of the spontaneous activity in the portal vein was considerably reduced after treatment with corticosterone.

Pargyline + tropolone

Deaminated and *O*-methylated metabolites are released from the portal vein and vas deferens during electrical stimulation (Hughes & Roth, 1971 ; Hughes, unpublished results). The appearance of these metabolites can be almost completely prevented by incubating the tissues with pargyline (20 μg/ml) and tropolone (10 μg/ml) for 60 min (Hughes, unpublished results). In the present work, a maximal increase in noradrenaline output was seen after incubation of the tissues for 60–80 min with these enzyme inhibitors. There was a 2·5–2·8 fold increase in output after simultaneous treatment with pargyline and tropolone (Table 7) ; identical effects were observed in untreated and cocaine treated tissues. In a further series of experiments pargyline and tropolone did not increase the noradrenaline output in tissues that had been pretreated with cocaine and corticosterone, with corticosterone alone, or with phenoxybenzamine.

Treatment with pargyline and tropolone tended to reduce the height of contractions elicited by electrical stimulation, although the duration of the response was somewhat prolonged. The reduction in the contraction height was more predominant at 2 Hz, at this frequency a 20–25% decrease in response was seen in both tissues. The reduction in the contraction height at 16 Hz never exceeded 10%.

Propylene glycol

Since phenoxybenzamine and corticosterone were dissolved in a mixture of propylene glycol and water, the possible effects of this vehicle on noradrenaline output were investigated. Noradrenaline output was determined in the cocaine treated vas deferens, before and after treatment with propylene glycol. In three such experiments the output of noradrenaline was increased 1·3, 1·8 and 2·8 fold when the tissues were incubated with 2, 5 and 10% (v/v) propylene glycol respectively. The effect became maximal within 20 min and was reversed within 20 min after

TABLE 7. *Effect of pargyline+tropolone on noradrenaline output*

Tissue		Treatment	(*n*)	Noradrenaline output ((pg/pulse)/g) 2 Hz	16 Hz	Alteration in output 2 Hz	16 Hz
Vas deferens	a	None	(4)	17± 4	29± 6	—	—
		Pargyline+ Tropolone	(4)	38± 5	75± 9	2·6±0·3 (1)	2·8±0·4 (1)
	b	Cocaine	(4)	58±12	96±17	—	—
		Pargyline+ Tropolone	(4)	139±15	260±13	2·4±0·4 (2)	2·5±0·3 (2)
Portal vein		None	(3)	—	18± 3	—	—
		Pargyline+ Tropolone	(3)	—	56±11	—	3·1±0·4 (1)

The effects of pargyline and tropolone (20 μg/ml each) in the vas deferens were determined in paired tissues, one untreated (a) and the other treated with cocaine (b).

Alteration in output (1) = $\frac{\text{Output in presence of pargyline+tropolone}}{\text{Output of untreated tissue}}$

(2) = $\frac{\text{Output in presence of cocaine+pargyline+tropolone}}{\text{Output in presence of cocaine}}$

washing the tissue; reintroduction of propylene glycol after washing out again caused an increase in noradrenaline output. Ethanol was considered as an alternative vehicle; concentrations less than 1% (v/v) did not affect the output of noradrenaline, but above 1% there was a definite decrease in output.

It should be noted that in experiments involving corticosterone and phenoxybenzamine, the final concentration of propylene glycol in the donor bath never exceeded 0·5%.

Endogenous noradrenaline content of the portal vein and vas deferens

Freshly dissected samples of the portal vein and vas deferens contained $1{\cdot}5 \pm 0{\cdot}3$ (mean $\pm$ S.E.) and 10 ± 1 μg/g of noradrenaline respectively. However, when the tissues were incubated in Krebs solution at 38° C for 3 h, without electrical stimulation, there was some loss of endogenous noradrenaline. The content of the vein fell to $0{\cdot}9 \pm 0{\cdot}2$ μg/g ($n=6$) and that of the vas to $8{\cdot}5 \pm 0{\cdot}8$ μg/g ($n=6$). These latter figures were used to calculate the fraction of the endogenous store that was released, per pulse, at 2 and 16 Hz (Table 8). The mean output per pulse was taken from the outputs measured in phenoxybenzamine-treated tissue (Table 4). If phenoxybenzamine can actually facilitate the release of noradrenaline (see **Discussion**) then the values for the fractional release may be too high by a factor of two or three. In fact the values arrived at in Table 8 are remarkably similar in both the portal vein and vas deferens and are in agreement with the values obtained by other workers in other tissues. However, it should be emphasized that these values represent the mean fractional output of 240 pulses and give no information about any individual pulse.

Discussion

One of the problems measuring small amounts of biologically active material is that it is often necessary to extract and reduce the volume of the original sample for assay. This is time-consuming and involves a variable loss of material. Direct sampling of small donor bath volumes by cascade bioassay surmounts this problem. The assay described in this paper has a high degree of sensitivity for noradrenaline and has an accuracy similar to that of other bioassays. Substances other than noradrenaline may be released from the portal vein and vas deferens, and these may interfere with the bioassay. However, the correlation between bioassays (before and after alumina separation) and chemical assay suggests that such interference does not occur. Both the pharmacological and chemical evidence indicate that the

TABLE 8. *Fraction of tissue noradrenaline store released per pulse*

Tissue	Frequency (Hz)	Fractional release	Reference
Rabbit vas deferens	2	$6{\cdot}6 \times 10^{-5}$	This paper
	16	$15{\cdot}9 \times 10^{-5}$	—
Rabbit portal vein	2	$5{\cdot}6 \times 10^{-5}$	—
	16	$16{\cdot}2 \times 10^{-5}$	—
Cat hind limb	6	2×10^{-5}	Folkow, Häggendal & Lisander (1967)
Cat hind limb	6	6×10^{-5}	Stjärne, Hedquist & Bygdeman (1969)
Rat portal vein	4	1×10^{-5}	Häggendal, Johansson, Jonason & Ljung (1970)
Cat nictitating membrane	4–6	4×10^{-4}	Langer & Vogt (1971)

Values for the rabbit portal vein calculated from a mean endogenous content of 0·9 μg/g, and a mean of 8·5 μg/g for the vas deferens. The values for the output/pulse are taken from Table 2.

material released during transmural stimulation is noradrenaline; there was no evidence to suggest that adrenaline was present in significant amounts.

Cocaine is a potent inhibitor of the neuronal uptake ($Uptake_1$) process for noradrenaline (Iversen, 1967). In the portal vein and the vas deferens, a maximal increase in noradrenaline output was seen with a cocaine concentration of 2–4 μg/ml. Cocaine concentrations above 10 μg/ml reduced both the noradrenaline output and the response of the donor tissue, presumably due to the local anaesthetic effect of cocaine. The maximal increase in output caused by cocaine may be the result of a maximal inhibition of $Uptake_1$, or a combination between enhanced output (due to partial inhibition of $Uptake_1$) and decreased neuronal activity (due to an increasing local anaesthetic effect). This dilemma is difficult to resolve; however, direct estimations indicate that more than 90% of the neuronal uptake of noradrenaline is inhibited by cocaine in concentrations of 2–4 μg/ml (Iversen, 1967; Hughes & Roth, 1971).

In the vas deferens, the increase in noradrenaline output caused by cocaine was somewhat greater than that seen in the portal vein; this finding may reflect the greater density of the adrenergic innervation in the vas deferens. The 3·5–3·8 fold increase in noradrenaline output in the vein is comparable to the 2–4 fold increase observed in the spleen (Cripps & Dearnaley, 1970, 1971). The enhancement of the output in both the vas deferens and portal vein was independent of frequency between 2 and 16 Hz, for a train of 240 pulses. This is contrary to the view that the reuptake of noradrenaline is only significant at low stimulus frequencies (Gillis & Schneider, 1967). The present results indicate that neuronal uptake is equally active at all frequencies in the physiological range. However, there appears to be a paradox, in that cocaine is known to cause a greater potentiation of tissue responses at low frequencies of sympathetic nerve stimulation (Cairnie, Kosterlitz & Taylor, 1961; Haefely, Hürlimann & Thoenen, 1964; Gillis & Schneider, 1967). This is also true in the portal vein where cocaine may cause a 3 fold increase in the contraction height between 1 and 5 Hz, but little increase at greater frequencies. The paradox can be explained by the fact that the output per pulse is much greater at high than at low stimulus frequencies, and that the change in tissue response per pulse is maximal at high frequencies. Thus, cocaine will increase the apparent output per pulse at all frequencies, but can only increase the response at low frequencies since this is submaximal. In the present experiments, the output per pulse between 6 and 16 Hz, was always 2–4 times greater than that at 2 Hz.

Since a similar proportion of the transmitter is removed by neuronal uptake between 2 and 16 Hz, it appears that the uptake mechanism is not saturated by noradrenaline in this frequency range. $Uptake_1$ is saturated by exogenous noradrenaline at a concentration approaching 1 μg/ml (Iversen, 1967); this suggests that this concentration is not exceeded at the nerve terminals during sympathetic stimulation in the vas deferens and portal vein. However, it is possible that nerve depolarization increases the uptake of noradrenaline (Gillis, Schneider, Van Orden & Giarman, 1966; Bhagat & Zeidman, 1970), and therefore the extraneuronal concentration of noradrenaline may exceed 1 μg/ml and still not saturate the uptake process during nerve stimulation.

Phenoxybenzamine inhibits both the neuronal and extraneuronal uptake of noradrenaline in several tissues (Iversen, 1967; Avakian & Gillespie, 1968; Lightman & Iversen, 1969). Both these effects appear to contribute to the apparently irrever-

sible action of phenoxybenzamine in the vas deferens and portal vein. An inhibition of neuronal uptake is indicated by the fact that the effects of phenoxybenzamine and cocaine were additive. Moreover, the effect of cocaine was no longer seen when the tissues had been pretreated with phenoxybenzamine.

The increase in output caused by phenoxybenzamine in the presence of cocaine may be due to inhibition of extraneuronal uptake, which appears to be more important in the vas deferens than in the portal vein for the removal of noradrenaline. In the presence of cocaine, phenoxybenzamine caused a 3·5 fold increase in output in the portal vein, whilst there was an 8–9 fold increase in the vas deferens. In the spleen, phenoxybenzamine causes a 10–15 fold increase in noradrenaline output at a stimulus frequency of 10 Hz (Brown & Gillespie, 1957 ; Kirpekar & Misu, 1967 ; Cripps & Dearnaley, 1971) ; cocaine is less effective, causing a 2–4 fold increase (Cripps & Dearnaley, 1970). The pattern of inactivation in the spleen is thus comparable to that in the portal vein. In the cat nictitating membrane, phenoxybenzamine causes only a 2–4 fold increase in ^{3}H-noradrenaline output (Langer & Vogt, 1971). It is likely, therefore, that the various inactivation processes may vary in importance at different tissue sites and in different species.

Iversen & Salt (1970) have shown that corticosterone is a highly selective inhibitor of $Uptake_2$ (extraneuronal uptake) in the rat heart. The enhancement of noradrenaline output by corticosterone provides further evidence that extraneuronal uptake plays a role in the inactivation of the sympathetic transmitter. The effect of corticosterone was much greater in tissues pretreated with cocaine, when the outputs were already increased due to inhibition of neuronal uptake. The effect of cocaine was also enhanced in tissues pretreated with corticosterone. This interaction is unlikely to be due to additive effects on the same process since, at the concentrations used, the drugs had a maximum effect in promoting output when they were given alone. One explanation of these results is that there is a dynamic balance in the distribution of noradrenaline between the neuronal and extraneuronal uptake mechanisms. When one of these mechanisms is blocked, more noradrenaline becomes available to the remaining process and its relative importance increases. Thus, treatment with corticosterone will divert a proportion of the transmitter, normally removed by $Uptake_2$, to $Uptake_1$. In support of this view, normetanephrine, which blocks $Uptake_2$, enhances the neuronal uptake of ^{3}H-noradrenaline in the rat vas deferens (Iversen, Fischer & Axelrod, 1966).

It follows from these considerations that it is impossible to estimate the contribution which each of the two uptake processes makes to the inactivation of noradrenaline under normal physiological conditions. It can be calculated, however, that, when $Uptake_1$ is blocked, $Uptake_2$ is capable of removing up to 75% of the transmitter ; on the other hand, $Uptake_1$ can remove at least 90% when $Uptake_2$ is blocked. These two processes, together, probably inactivate slightly more than 90% of the transmitter under physiological conditions. These conclusions assume that the drug effects are solely due to the inhibition of the respective uptake processes, and that the output of noradrenaline, in the presence of both drugs, represents the total transmitter release. The latter point is a likely source of error, since other inactivation processes may still operate in the presence of these drugs. This may explain why phenoxybenzamine is twice as effective as corticosterone in cocaine-treated tissues. The additional effect of phenoxybenzamine may be due to its α-adrenoceptor blocking activity (Cripps & Dearnaley, 1971), or to an effect on the noradrenaline release mechanism (Langer, 1970). A third possibility is that it

is more effective in blocking the uptake processes due to the non-competitive nature of its blocking action.

Inhibition of catechol-*O*-methyltransferase and of monoamine oxidase caused an increased output of noradrenaline in the portal vein and vas deferens; this effect was not observed when the tissues were pretreated either with corticosterone or with phenoxybenzamine. These results support the view that $Uptake_2$ is intimately concerned with the metabolism of noradrenaline at low concentrations and its sequestration at high concentrations (Lightman & Iversen, 1969; Iversen, 1971).

Most of the data relating to the correlation of noradrenaline output with stimulus frequency have been obtained in the perfused spleen. This is a complex tissue and the possibility of errors arising from variations in the recovery of the transmitter may explain the conflicting results. Some authors conclude that the output per pulse is constant or declines with increasing frequencies of stimulation (Brown & Gillespie, 1957; Stjärne, 1970), whilst others have shown an increase in output with frequency, but with the maximum output occurring between 2 and 10 Hz (Haefely, Hürlimann & Thoenen, 1965; Kirpekar & Misu, 1967; Davies & Withrington, 1968). In the vas deferens and portal vein, the output per pulse increased linearly with the logarithm of the stimulus frequency between 1 and 16 Hz. It is unlikely that the increase in output with frequency was due to differences in the rate or degree of inactivation at different frequencies, since the effects of cocaine, corticosterone, phenoxybenzamine and of pargyline plus tropolone were independent of frequency, between 2 and 16 Hz. The increase in output per pulse with increasing frequency of stimulation might be due to an enhancement of the amplitude of the action potentials with diminishing pulse intervals (Brown & Ferry, 1963), leading to a recruitment of additional nerve terminals or varicosities. Another possibility is that different neuronal stores of noradrenaline may be mobilized in response to different frequencies of stimulation. It remains to be seen whether the relation between frequency and output is the same at all adrenergic synapses.

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REFERENCES

AVAKIAN, O. V. & GILLESPIE, J. S. (1968). Uptake of noradrenaline by adrenergic nerves, smooth muscle and connective tissue in isolated perfused arteries and its correlation with the vasoconstrictor response. *Br. J. Pharmac. Chemother.*, **32**, 168–184.

BHAGAT, B. & ZEIDMAN, H. (1970). Increased retention of norepinephrine-^{3}H in vas deferens during nerve stimulation. *Am. J. Physiol.*, **219**, 692–696.

BOADLE-BIBER, M. C., HUGHES, J. & ROTH, R. H. (1970). Acceleration of noradrenaline biosynthesis in the guinea-pig vas deferens by potassium. *Br. J. Pharmac.*, **40**, 702–720.

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

BROWN, G. L. & FERRY, C. B. (1963). Facilitation of output of sympathetic transmitter. *J. Physiol., Lond.*, **167**, 48*P*–49*P*.

CAIRNIE, A. B., KOSTERLITZ, H. W. & TAYLOR, D. W. (1961). Effect of morphine on some sympathetically innervated effectors. *J. Pharmac. exp. Ther.*, **17**, 539–551.

CRIPPS, H. & DEARNALEY, D. P. (1970). The effect of cocaine on the noradrenaline overflow from the isolated blood-perfused cat spleen. *J. Physiol., Lond.*, **209**, 37*P*–38*P*.

CRIPPS, H. & DEARNALEY, D. P. (1971). Evidence suggesting uptake of noradrenaline at adrenergic receptors in the isolated blood-perfused cat spleen. *Proceedings Physiological Society, April* 1971, 6*P*–7*P*.

DAVIES, B. N. & WITHRINGTON, P. G. (1968). The release of noradrenaline by the sympathetic post-ganglionic nerves to the spleen of the cat in response to low frequency stimulation. *Archs int. Pharmacodyn. Thér.*, **171**, 185–197.

FOLKOW, B., HÄGGENDAL, J. & LISANDER, B. (1967). Extent of release and elimination of noradrenaline at peripheral adrenergic nerve terminals. *Acta. physiol. scand.*, **72**, Suppl. **307**, 1–38.

GILLIS, C. N., SCHNEIDER, F. H., VAN ORDEN, L. S. & GIARMAN, N. H. (1966). Biochemical and microfluorometric studies of norepinephrine redistribution accompanying sympathetic nerve stimulation. *J. Pharmac. exp. Ther.*, **151**, 46–54.

GILLIS, C. N. & SCHNEIDER, F. H. (1967). Frequency dependence potentiation by various drugs of the chronotropic response of isolated cat atria to sympathetic nerve stimulation. *Br. J. Pharmac. Chemother.*, **30**, 541–553.

HAEFELY, W., HÜRLIMANN, A. & THOENEN, H. (1964). A quantitative study of the effect of cocaine on the response of the cat nictitating membrane to nerve stimulation and to injected noradrenaline. *Br. J. Pharmac. Chemother.*, **22**, 5–21.

HAEFELY, W., HÜRLIMANN, A. & THOENEN, H. (1965). Relation between the rate of stimulation and the quantity of noradrenaline liberated from sympathetic nerve endings in the isolated perfused spleen of the cat. *J. Physiol., Lond.*, **818**, 48–58.

HÄGGENDAL, J., JOHANSSON, B., JONASON, J. & LJUNG, B. (1970). Correlation between noradrenaline release and effector response to nerve stimulation in the rat portal vein *in vitro*. *Acta physiol. scand.*, Suppl. 349, 17–32.

HOLMAN, M. E., KASBY, C. B., SUTHERS, M. B. & WILSON, A. F. (1968). Some properties of the smooth muscle of rabbit portal vein. *J. Physiol., Lond.*, **196**, 111–132.

HUGHES, J. & VANE, J. R. (1967). An analysis of the responses of the isolated portal vein of the rabbit to electrical stimulation and to drugs. *Br. J. Pharmac. Chemother.*, **30**, 46–66.

HUGHES, J. & ROTH, R. H. (1971). Evidence that angiotensin enhances transmitter release during sympathetic nerve stimulation. *Br. J. Pharmac.*, **41**, 239–255.

IVERSEN, L. L., FISCHER, J. E. & AXELROD, J. (1966). Enhancement of ^{3}H-norepinephrine uptake in rat tissues by O-methylated metabolites of catecholamines. *J. Pharmac. exp. Ther.*, **154**, 56–63.

IVERSEN, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*. London: Cambridge University Press.

IVERSEN, L. L. & SALT, P. J. (1970). Inhibition of catecholamine Uptake$_2$ by steroids in the isolated rat heart. *Br. J. Pharmac.*, **40**, 528–530.

IVERSEN, L. L. (1971). Role of transmitter uptake mechanisms in synaptic transmission: Third Gaddum Memorial Lecture. *Br. J. Pharmac.*, **41**, 571–591.

KIRPEKAR, S. M. & MISU, Y. (1967). Release of noradrenaline by splenic nerve stimulation and its dependence on calcium. *J. Physiol., Lond.*, **188**, 219–234.

LANGER, S. Z. (1970). The metabolism of ^{3}H-noradrenaline released by electrical stimulation from the isolated nictitating membrane of the cat and the vas deferens of the rat. *J. Physiol., Lond.*, **208**, 515–546.

LANGER, S. Z. & VOGT, M. (1971). Noradrenaline release from isolated muscles of the nictitating membrane of the cat. *J. Physiol., Lond.*, **214**, 159–171.

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

NORBERG, K. A. & HAMBERGER, B. (1964). The sympathetic adrenergic neuron. *Acta physiol. scand.*, **63**, Suppl. 238.

O'HANLON, J. F., CAMPUZANO, H. C. & HORVATH, S. M. (1970). A fluorimetric assay for subnanogram concentrations of adrenaline and noradrenaline in plasma. *Analyt. Biochem.*, **34**, 568–581.

STJÄRNE, L., HEDQUIST, P. & BYGDEMAN, S. (1969). Neurotransmitter quantum released from sympathetic nerves in cat's skeletal muscle. *Life Sci.*, **8**, 189–196.

STJÄRNE, L. (1970). Quantal or graded secretion of adrenal medullary hormone and sympathetic neurotransmitter. In: *New Aspects of Storage and Release Mechanisms of Catecholamines*, ed. Schumann, H. J. & Kroneberg, G., pp. 112–126. Berlin: Springer-Verlag.

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