J. Physiol. (1966), **187**, *pp.* 51–68
With 8 text-figures
Printed in Great Britain

THE UPTAKE AND RELEASE OF RADIOACTIVE NORADRENALINE BY THE SPLENIC NERVES OF CATS

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(*Received* 7 *December* 1965)

SUMMARY

1. DL-[^{3}H]noradrenaline was infused close-arterially into the spleens of chloralosed cats at rates of 0·625 or 1·25 μg/min for 10 or 20 min and the recovery of noradrenaline and its metabolites in the venous blood measured during the infusion and after nerve stimulation at various times after the infusion.

2. During the infusion 41 % of the noradrenaline was recovered in the blood as such and 11 % as metabolites. The remaining 48 % was retained within the spleen.

3. The noradrenaline retained in the spleen was slowly released to appear as metabolites in the blood stream. In normal animals the rate of loss from the spleen was 0·22 % per minute. In animals given phenoxybenzamine after the end of the infusion this rate was several times greater.

4. Splenic nerve stimulation in normal animals or in animals treated with phenoxybenzamine resulted in an increase in the radioactivity of the blood leaving the spleen. Paper chromatography showed this to be radioactive noradrenaline.

5. In normal animals the specific activity of the transmitter liberated by nerve stimulation was less than that of the stores of noradrenaline within the spleen. In animals treated with phenoxybenzamine these two values were similar.

6. It is suggested that the infused noradrenaline retained in the spleen is largely taken up into nerve fibres and is available for subsequent release by nervous activity.

INTRODUCTION

We have already shown that when (−)-noradrenaline was infused into the arterial blood going to the cat spleen only some 30 % could be recovered in the venous blood leaving that organ. The ability of the spleen to remove noradrenaline from the blood was related to an intact post-ganglionic innervation since after cutting the splenic nerves and allowing

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time for degeneration the recovery of noradrenaline in the venous blood increased to just over 80 %. Phenoxybenzamine or cocaine increased the recovery of noradrenaline to a similar value. It was suggested that some 50–60 % of the noradrenaline was taken up by the splenic nerves and that this uptake was abolished by both phenoxybenzamine and cocaine. (Gillespie & Kirpekar, 1965*a*).

The present paper presents more direct evidence of uptake of noradrenaline by sympathetic nerves. We have infused tritium-labelled noradrenaline into the spleen that can later be released by electrical stimulation of the splenic nerves. We have also repeated our earlier experiments on the fate of the noradrenaline during the infusion. If some of the radioactive noradrenaline is metabolized, the radioactivity of any metabolites removed in the venous blood can be detected after separation from noradrenaline by paper chromatography. In earlier experiments with unlabelled noradrenaline there was no direct method of detecting such metabolites.

In the next paper (Gillespie & Kirpekar, 1966), results on the histological localization of noradrenaline are described that were obtained using the fluorescence technique of Falck (1962) for total catecholamines and autoradiography for radioactive infused noradrenaline.

A brief account of these results has already been published (Gillespie & Kirpekar, 1965*b*).

METHODS

The technique of infusing noradrenaline into the heparinized cat's splenic arterial blood has already been described (Gillespie & Kirpekar, 1965*a*). Briefly, in cats under chloralose the trachea was cannulated and the arterial pressure recorded from the cartoid artery. After the removal of the stomach, intestines and adrenal glands venous blood from the spleen was collected intermittently by means of a cannula in the superior mesenteric vein. Noradrenaline was infused into the splenic artery from the hepatic artery. The splenic nerves were stimulated electrically through bright platinum electrodes shielded in glass. Pulses of 1 msec duration and supra-maximal voltages were used; the frequencies are indicated in the text. DL-[^{3}H]noradrenaline, obtained from the Radiochemical Centre, Amersham (specific activity from 0·88 to 1·25 c/m-mole) was infused at rates of either 0·625 or 1·25 μg/min for 10 or 20 min. Plasma noradrenaline was assayed by its pressor activity in the pithed rat. Biological assay of a mixture of (−)-noradrenaline from nerve endings and DL-[^{3}H] noradrenaline from the infusion is complicated by the greater biological activity of the laevorotatory form. This problem was minimized by preparing the assay standards from whichever form was dominant, DL-[^{3}H]noradrenaline for the assay of plasma removed during an infusion and (−)-noradrenaline for that of the transmitter released by nerve stimulation. With nerve stimulation, the contribution of DL-[^{3}H]noradrenaline to the total was calculated from the increase in radioactivity and the biological assay then corrected for the small error introduced by assaying the total biological activity as (−)-noradrenaline. Plasma radioactivity was measured by adding 0·25 ml. drop by drop to 8 ml. of phosphor (Bray, 1960). The precipitated proteins were centrifuged down and the supernatant decanted into a measuring vial. The precipitate was washed with a further 2 ml. of phosphor, centrifuged and the supernatant added to the vial. Radioactivity was measured in a Packard Tri Carb

scintillation counter using internal standards to estimate quenching. The remainder of the plasma was extracted and noradrenaline separated by ascending paper chromatography using phenol/HCl as the solvent system in an atmosphere of N_2. The papers were treated in one of two ways. In the first, a control spot of noradrenaline was applied to a separate part of the paper, located the next day by spraying with potassium ferricyanide and a corresponding strip cut from the sample. The 1–2 cm of paper between the origin and the noradrenaline strip was discarded and the remainder of the paper arbitrarily divided into two parts; all three strips were then eluted with phosphate buffer at pH 4·0 and the radioactivity counted. In the second method, which gave a better resolution, the entire chromatogram was cut into strips of 1 cm length and these separately extracted with saline and counted. In calculating the recovery of noradrenaline and its metabolites from the radioactivity on these chromatograms two difficulties were encountered. The DL-[^{3}H]noradrenaline was not chromatographically pure and during the extraction procedure further decomposition occurred. These points are brought out in Fig. 1. In one experiment, impurities were eluted from the chromatogram and perfused through the spleen. All of the radioactivity was recovered in the venous blood. The removal of noradrenaline from such a mixture as it passed through the spleen would result in the impurities accounting for an increasing proportion of the total radioactivity. Decomposition of noradrenaline during extraction would increase this proportion still further. In all experiments allowance has been made for these sources of error. Impurities were measured by applying a solution of the noradrenaline directly to a paper chromatogram. The percentage of counts appearing as a single peak (94 % in Fig. 1) was taken as noradrenaline and the remaining 6 % as impurities. Some of the noradrenaline solution was extracted in the same way as plasma and applied to the paper. The percentage of counts appearing as noradrenaline diminished to 68% (Fig. 1). The remaining 32 %, minus the 6 % of impurities originally present, was taken to represent noradrenaline decomposed during extraction. Impurities were measured for each new batch of noradrenaline and decomposition products in each experiment. In some experiments the DL-[^{3}H]noradrenaline was purified by paper chromatography and eluted in saline; this material was infused through the cat spleen. In these experiments no allowance was made for impurities in the infused material.

At the end of the experiment small pieces of spleen were removed for fluorescence microscopy and for autoradiography. The remainder of the spleen was weighed, homogenized in 0·4N-perchloric acid and centrifuged; K_2CO_3 was added to bring the pH to 4·0. After recentrifugation the supernatant was applied to Dowex 50 columns. Noradrenaline was eluted with N-HCl and determined by the trihydroxyindole method of Bertler, Carlsson & Rosengren (1958), measuring the fluorescence with the Aminco-Bowman Spectrophotofluorimeter. The radioactivity of the eluate was also measured.

The following drugs were given intravenously in the amounts shown: Phenoxybenzamine, 10 mg/kg; Hydergine (Sandoz), 0·5 mg/kg; and phentolamine, 3 mg/kg.

RESULTS

Venous recovery of infused [^{3}H]noradrenaline. Since the radioactive noradrenaline infused was a racemic mixture it was possible that its removal by the spleen would differ in some respects from that of the unlabelled laevorotatory form used earlier. We therefore infused DL-[^{3}H]-noradrenaline and measured the recovery of noradrenaline and of radioactivity in the splenic venous blood sampled at the 10th, 15th and 20th min when steady-state conditions were reached. The results (Table 1) differed in two respects from those with unlabelled noradrenaline. First

the recovery of noradrenaline was greater, 41 % as against 30 %. Secondly, the recovery of radioactivity at 55 % exceeded the recovery of noradrenaline. This greater recovery of radioactivity could be due either to the presence of radioactive metabolites produced in the spleen or to radioactive impurities in the noradrenaline infused. The second possibility was excluded by first purifying the noradrenaline by paper chromatography. Infusion of noradrenaline eluted from the chromatogram still resulted in recoveries of radioactivity not accounted for by that of noradrenaline (last three experiments in Table 1).

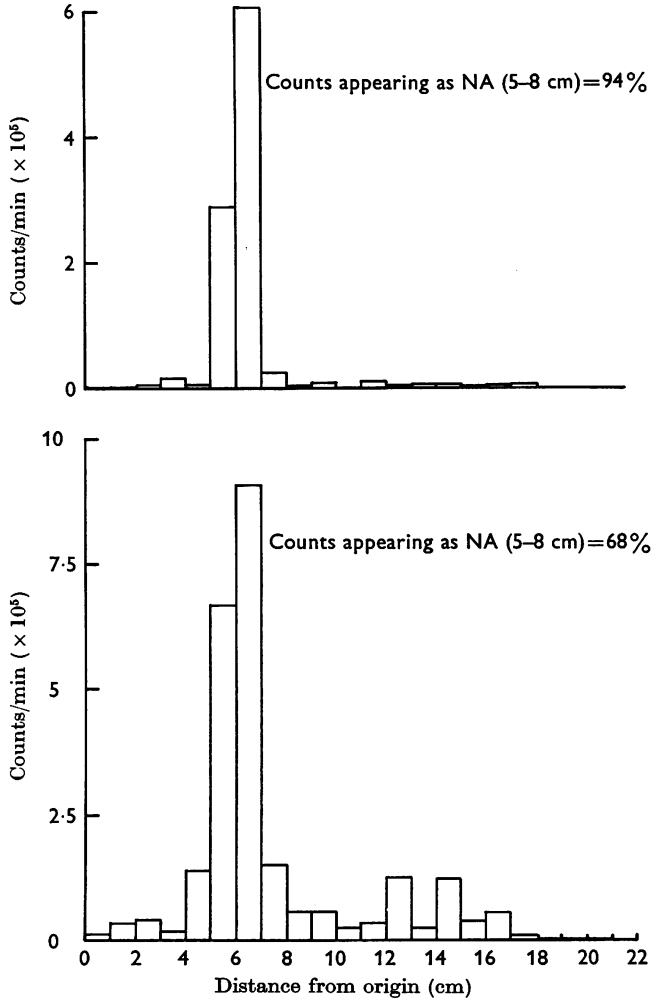

Fig. 1. Chromatographic distribution of radioactivity after applying DL-[^{3}H]-noradrenaline either directly to the paper (above) or after subjection to the procedure used for extracting noradrenaline from plasma (below).

The possible formation of metabolites of noradrenaline within the spleen was examined in the same experiments by extracting and separating the radioactivity in the venous blood. The results are shown in Table 1 and Fig. 2. The radioactivity shows two peaks, one corresponding to noradrenaline and the other travelling much faster on the chromatogram (Fig. 2). In blood taken after the end of the infusion the noradrenaline

TABLE 1. The recovery of noradrenaline and of radioactivity in the splenic venous blood during intra-arterial infusion of DL-[^{3}H]noradrenaline. In three experiments the radioactivity of the plasma was extracted, separated by paper chromatography and the recovery of noradrenaline and 'metabolites' separately measured

	Percentage recovery		Percentage of radioactivity recovered after chromatography		
Time (min)	Biological activity	Radioactivity	As NA	As metabolites	Percentage uptake in spleen
10	32	40	—	—	—
10	33	60	—	—	—
15	47	66	—	—	—
20	47	68	—	—	—
10	44	29	—	—	—
15	42	69	—	—	—
20	37	59	—	—	—
*15	—	40	31	9	60
20	—	41	30	11	59
*15	—	60	53	7	40
20	—	71	55	16	29
*15	42	54	44	10	46
20	46	57	41	16	43
Mean (±SE)	41±1·9	55±3·7	42±4·3	12±1·5	46±4·8

* In these experiments the tritiated noradrenaline was purified by chromatography.

peak disappears and only this second peak remains. Since this second peak is absent in the infused noradrenaline and small after extracting noradrenaline from plasma (Fig. 1), most of the radioactivity must be formed in the spleen and is presumably due to a metabolite. Table 1 shows that the radioactivity in this second peak accounts on average for 12 % of the infused noradrenaline. The other finding, the greater recovery of noradrenaline compared with our previous experiments using unlabelled (−)-noradrenaline, was not pursued. There is evidence that the dextrorotatory form of noradrenaline is less readily bound and this may be responsible for the increase (Iversen, 1963; Kopin & Bridgers, 1963; Maickel, Beaven & Brodie, 1963 and von Euler & Lishajko, 1964).

After the infusion the levels of both noradrenaline and radioactivity in the venous blood fell rapidly. The fall of radioactivity was followed most easily since only small blood samples were needed. Figure 3 shows an experiment of this kind.

The radioactivity fell rapidly at first and then much more slowly. The

figure also shows the radioactivity in the carotid blood taken at the same time. During the first few minutes the radioactivity in the blood leaving the spleen greatly exceeded that in the arterial blood, indicating the addition of a great deal of radioactivity by the spleen to the blood passing through it. From about the 15th min a large part of the radioactivity in the blood leaving the spleen was present in the blood entering it. Thus, while variations in blood flow through the spleen had a considerable effect on the concentration of radioactivity in the blood in the first few minutes after the end of the infusion, this effect was soon lost and the venous

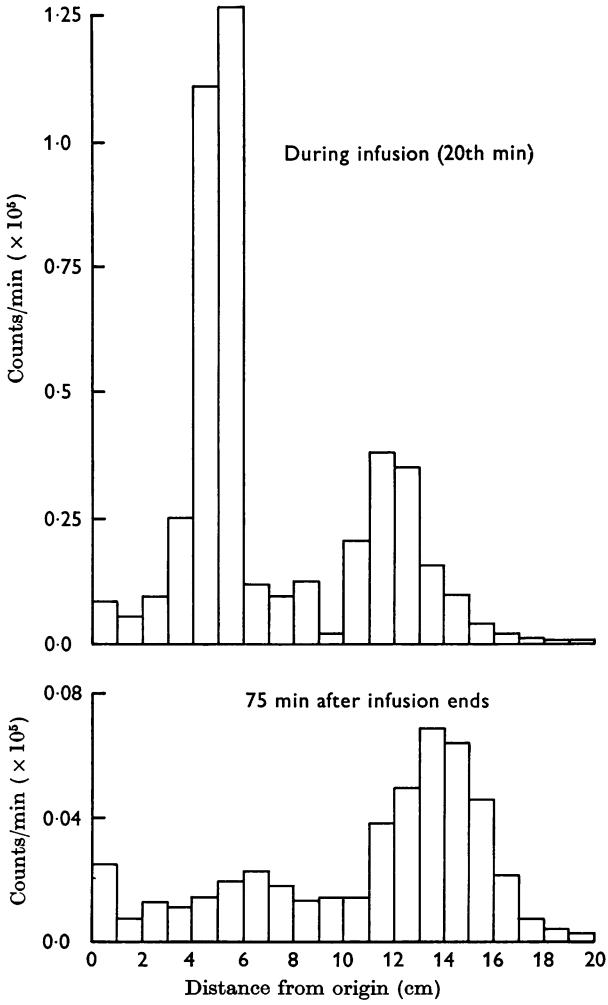

Fig. 2. Distribution of the radioactivity in chromatograms from samples of splenic venous blood during close arterial infusion of DL-[^{3}H]noradrenaline (above) and 75 min after the end of the infusion (below).

radioactivity then became substantially independent of blood flow (Figs. 4, 5).

Release of [^3H]noradrenaline by nerve stimulation. The splenic nerves were stimulated by trains of 240 stimuli at 30/sec, which are known to liberate easily measurable quantities of noradrenaline into the venous blood (Brown & Gillespie, 1957). Coincident with the appearance of noradrenaline, the radioactivity of the venous blood was increased; the

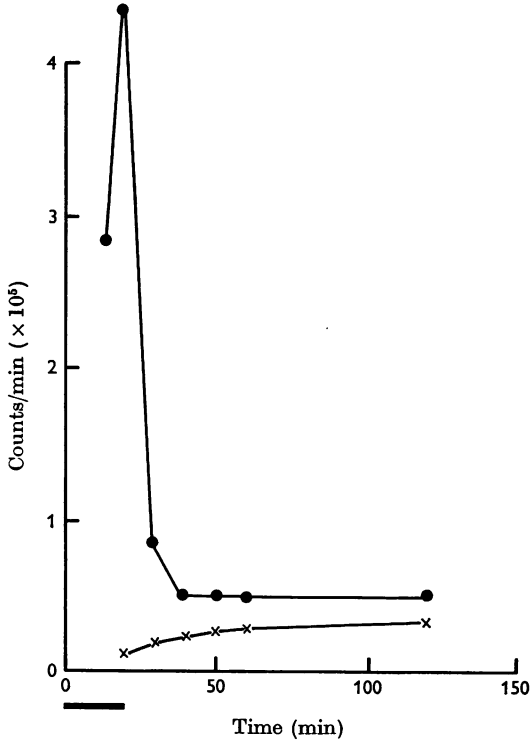

Fig. 3. Changes in plasma radioactivity in the last 5 min of an infusion of DL-[^{3}H]-noradrenaline and in the post-infusion period. Closed circles splenic venous blood, crosses arterial blood. The black bar represents the period of infusion.

results from four such periods of stimulation in one experiment are shown in Fig. 4. In Table 2 the increase in radioactivity has been expressed as an output of radioactive noradrenaline for each experiment. The possibility that the increase in radioactivity upon nerve stimulation was a secondary effect either of contraction of the spleen or of non-radioactive noradrenaline liberated by the nerves displacing radioactive noradrenaline from some other site was investigated in two animals by giving a close-arterial injection of 10 μg of noradrenaline base into the splenic artery. This caused an intense contraction of the spleen with expulsion of stored

blood. The plasma radioactivity showed either no change or only a small increase. In the same experiments stimulation of the splenic nerves caused a considerable increase in radioactivity. These results are included in Table 2.

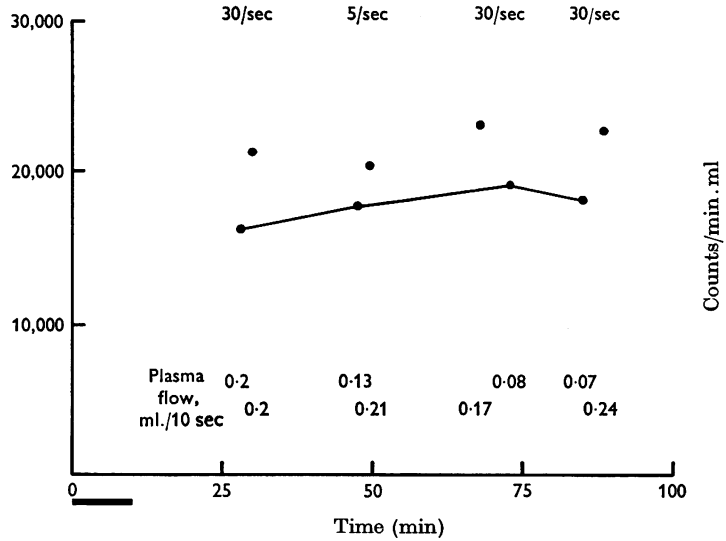

Fig. 4. The effect of splenic nerve stimulation on the radioactivity of the blood leaving the spleen. The frequency of stimulation is shown above each sample. Control samples were taken immediately before each period of stimulation. These control values have been joined by a continuous line. The black bar represents the period of infusion of DL-[^{3}H]noradrenaline. The plasma flow rate in ml./10 sec is given for all samples. Between the first and the third control sample there is an almost threefold reduction in plasma flow with little change in background radioactivity. In this experiment stimulation produces either no change in plasma flow (1st pair of observations) or an increase in flow.

The action of phenoxybenzamine. In normal cats the fraction of the transmitter liberated by nerve stimulation appearing in the venous blood falls as the frequency of stimulation is lowered from 30/sec; below 10/sec it becomes increasingly difficult to measure (Brown & Gillespie, 1957). The drug phenoxybenzamine increases the amount of released transmitter appearing in the venous blood at these low frequencies of stimulation. Phenoxybenzamine was given after the period of infusion so as to avoid interference with uptake during the infusion. The first period of nerve stimulation was at least 30 min after giving the drug. Figure 5 shows the results obtained in one animal. In each period of stimulation the radioactivity of the blood leaving the spleen almost doubled when compared with the control value immediately before nerve stimulation. This increase occurs in spite of the increase in blood flow during stimulation. In normal

animals during stimulation the blood flow often falls after the initial expulsion of stored blood, yet in both normal and phenoxybenzamine-treated animals the plasma radioactivity rises, further evidence against splenic contraction as the cause of the increased radioactivity. Table 3 summarizes our results for all cats given phenoxybenzamine.

TABLE 2. Normal animals. DL-[^{3}H]noradrenaline retained in the spleen after intra-arterial infusion and its subsequent release by nerve stimulation and injection of noradrenaline

		Noradrenaline output per stimulus			Noradrenaline in spleen			
Time after infusion (min)	Stimulation frequency pulses/sec	NA by bioassay (*pg*) (*a*)	^{3}H [NA] (*pg*) (*b*)	(*b*) as % (*a*)	Total μg/g (*c*)	^{3}H [NA] μg/g (*d*)	(*d*) as % of (*c*)	% infused [^{3}H] NA retained
7	30	1,240	33	2·7	—	—	—	—
15	10	220	13	5·8	—	—	—	—
35	30	900	104	11·0	1·18	0·194	16·4	—
7	10	82	—	—	—	—	—	—
17	30	520	29	5·6	—	—	—	—
26	10	—	1·5	—	—	—	—	—
37	30	280	46	16	1·86	0·277	15	13·0
7	30	580	70	12	—	—	—	—
21	30	313	33	10·7	—	—	—	—
43	30	196	13	6·2	0·21	0·051	24·3	6·0
7	30	1,350	50	3·7	—	—	—	—
21	30	820	33	4·0	—	—	—	—
49	30	870	38	4·4	—	—	—	—
62	30	210	33	16	—	—	—	—
73	30	1,300	30	3	1·76	0·42	24	12
15	30	2,700	654	24	—	—	—	—
34	30	2,300	246	10·7	3·4	1·47	43	47
51	30	1,600	25	1·5	—	—	—	—
62	30	2,180	38	1·7	3·9	0·21	5·6	8·7
20	30	500	75	15	—	—	—	—
40	*	—	5	—	—	—	—	—
72	30	600	42	6·5	1·54	0·51	33	17
41	*	—	0	—	—	—	—	—
63	30	520	8	1·5	—	—	—	—
81	*	—	0	—	—	—	—	—
94	30	830	13	1·5	0·69	0·16	24	6·9
20	30	860	21	2·5	—	—	—	—
40	5	0	18	—	—	—	—	—
58	30	1,400	29	2	—	—	—	—
78	30	1,500	38	2·5	2·8	0·19	7	7·9
20	30	500	4	1	—	—	—	—
40	5	0	4	—	—	—	—	—
66	30	780	17	2	—	—	—	—
85	5	0	4	—	0·92	0·009	1	8·6

* 10 μg (−)-noradrenaline infused.

The identity of the radioactive material appearing in the venous blood on stimulating the splenic nerves was investigated in five experiments by extracting the plasma and running the extract on a paper chromatogram. Figure 6 shows one such experiment. Four samples were applied to the

paper, first the radioactive noradrenaline infused into the spleen, then two samples collected after stimulating the nerves at either 5/sec or 30/sec and, finally, venous blood taken as a control before nerve stimulation. The samples obtained during nerve stimulation showed radioactivity in two peaks, the slower running peak corresponding to noradrenaline. In venous blood without nerve stimulation the radioactivity was restricted to a single broad peak corresponding to the second peak in the stimulated samples;

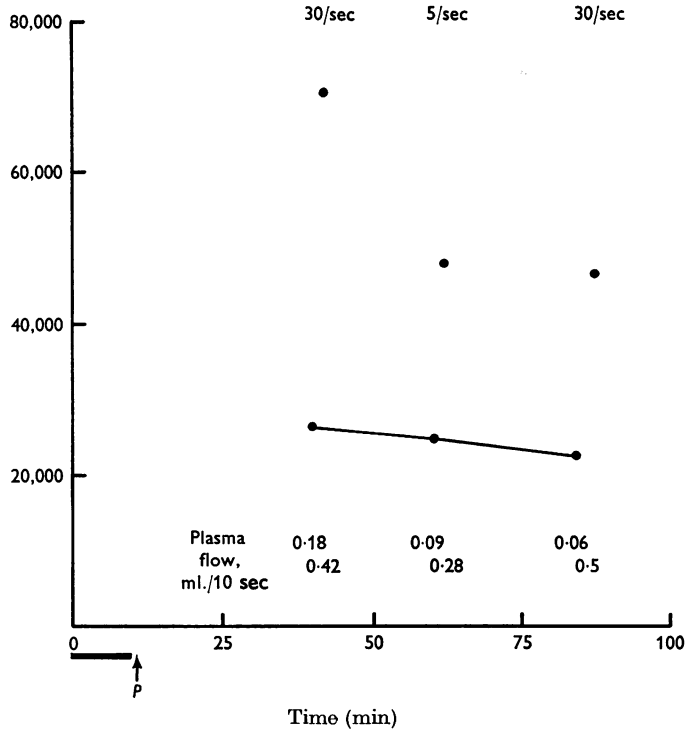

Fig. 5. The effect of splenic nerve stimulation on the radioactivity of the blood leaving the spleen in an animal treated with phenoxybenzamine (*P*). The frequency of stimulation is shown above each sample. Control samples were taken immediately before stimulation and these control values have been joined by a continuous line. The black bar represents the period of infusion of DL-[^{3}H]noradrenaline. The plasma flow rate in ml./10 sec is given for all samples. Changes in flow rate in the control periods have little effect on the background radioactivity. Stimulation causes a large increase in plasma flow.

this was presumably due to metabolites. The results in the other experiments were similar: after nerve stimulation the total radioactivity in the venous blood increased and the fraction of this radioactivity in the region of the chromatogram corresponding to noradrenaline increased. Whether or not nerve stimulation increased the amount of radioactive metabolites

was less clear. Calculations were possible in only two experiments. In one of these there was a slight increase, in the other, none.

Percentage of [^3H]noradrenaline in the spleen stores and in the noradrenaline liberated by nerve stimulation. From the biological assay of the total amount of noradrenaline liberated by the nerves together with the increase in radioactivity the percentage of radioactive noradrenaline in the transmitter liberated was calculated. These figures are included in Tables 2 and 3.

TABLE 3. The effect of phenoxybenzamine upon the release by nerve stimulation of DL-[^{3}H]-noradrenaline. The phenoxybenzamine was given at the end of the noradrenaline infusion and a period of at least 30 min allowed before stimulation

Time after infusion (min)	Stimulation frequency (pulse/sec)	Noradrenaline output per stimulus			Noradrenaline in spleen			% infused [^{3}H] NA retained
		NA by bioassay (*pg*) (*a*)	[^{3}H]NA *pg* (*b*)	(*b*) as % of (*a*)	Total μg/g (*c*)	[^{3}H]NA (μg/g) (*d*)	(*d*) as % of (*c*)	
30	30	1,260	130	10	—	—	—	—
44	5	550	29	5	—	—	—	—
60	5	530	66	12·5	—	—	—	—
71	30	710	79	11	1·42	0·11	7·5	6·8
32	30	1,820	238	13	—	—	—	—
52	5	1,140	163	14	—	—	—	—
77	30	1,000	113	11·3	1·49	0·17	11·7	10·2
50	5	1,170	129	11	—	—	—	—
70	30	960	58	6	—	—	—	—
90	5	850	71	8	—	—	—	—
110	30	730	18	2·3	0·95	0·013	1·3	1
40	5	1,890	8	0·4	—	—	—	—
55	30	1,370	87	6·5	0·72	0·032	4·4	2
30	5	10,400	100	1·0	—	—	—	—
41	30	5,800	50	1·0	—	—	—	—
51	5	5,000	4	0·08	1·0	0·003	0·2	0·2
27	5	12,600	533	4·2	—	—	—	—
37	30	4,500	292	6·1	2·39	0·22	9·2	7·4
30	5	3,000	100	3·3	—	—	—	—
39	30	2,000	38	1·8	2·55	0·19	7·3	9·4
32	5	12,100	493	3·7	—	—	—	—
38	30	4,900	242	4·8	2·74	0·155	5·7	12·3
37	5	3,700	200	5·4	—	—	—	—
47	30	1,700	92	5·6	1·1	0·078	7	8·6
30	5	1,490	50	3·4	—	—	—	—
42	30	1,600	38	2·4	1·96	0·075	3·8	3·6

In this calculation it was assumed that the increase in radioactivity was due to noradrenaline. This was justified by the evidence just given that increases in the metabolities were small or absent and by assuming that any increase in metabolites was secondary to the release of noradrenaline. At the end of the experiment the total noradrenaline content of the spleen was measured as well as the radioactive noradrenaline remaining in the organ. Comparison of the percentage of [^{3}H]noradrenaline in the liberated transmitter with that of the spleen stores showed an interesting difference between normal animals and those treated with phenoxybenzamine. Such

a comparison between the period of nerve stimulation immediately preceding removal of the spleen and the splenic contents is shown in Fig. 7.

The experiments have been arranged according to the interval between the end of the infusion and the removal of the spleen. In normal animals the percentage of [^{3}H]noradrenaline in the transmitter liberated by nerve stimulation is almost always less and often considerably less than the percentage of [^{3}H]noradrenaline in the stores in the spleen. This difference is shown in the mean figures which for the noradrenaline released was 6·1 % (S.E. ± 1·6) and for the noradrenaline in the spleen 19·3 % (S.E. ± 4·1).

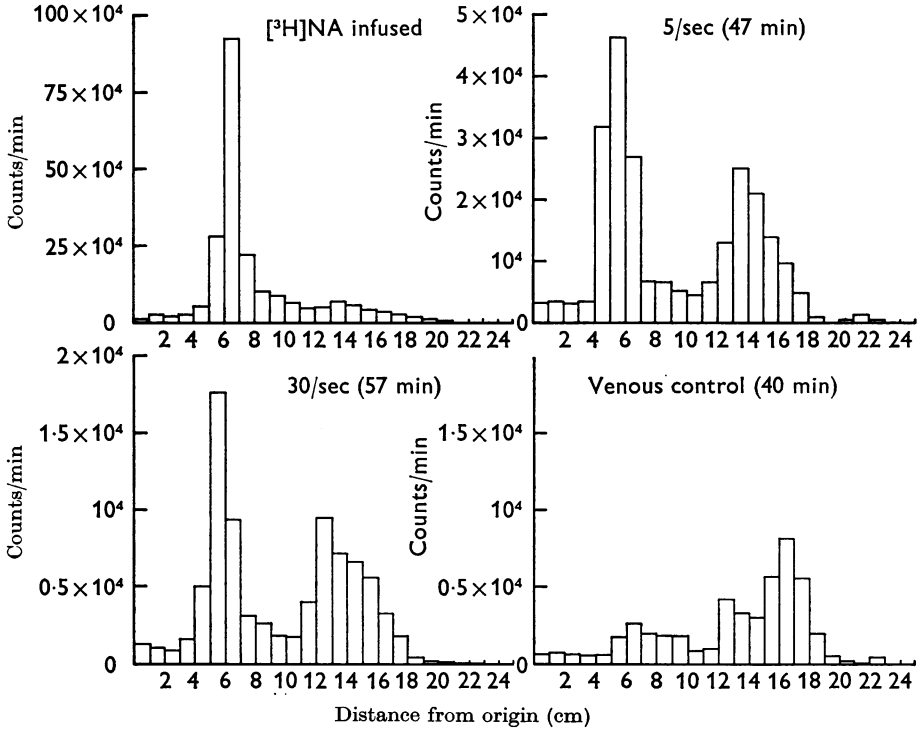

Fig. 6. Chromatographic distribution of the radioactivity of the solution of DL-[^{3}H]noradrenaline infused into a cat's spleen (upper left) and of the radioactivity extracted from the splenic venous blood after stimulating the splenic nerves at 5/sec (upper right) or 30/sec (lower left). The panel at lower right shows the distribution of radioactivity in the venous blood in the absence of nerve stimulation.

The liberated transmitter therefore was not representative of the total stores in the spleen. Figure 7 also shows that in normal animals the percentage of [^{3}H]noradrenaline in the transmitter liberated by nerve stimulation declines with time. In animals treated with phenoxybenzamine the average proportion of [^{3}H]noradrenaline in the transmitter liberated from the nerves is 5·5 % (S.E. ± 1·2), little different from the normal. There is no evidence of any change in this proportion with time. The average per-

centage of [^{3}H]noradrenaline in the spleen stores, 6·0% (S.E. ± 1·1), is much less than in normal animals and close to that of the transmitter liberated by nerve stimulation. After phenoxybenzamine therefore the transmitter liberated by the nerves reflects reasonably well the stores of noradrenaline within the spleen. The effect of the frequency of stimulation on the percentage labelling of the liberated transmitter was examined and Figure 8 summarizes the results for normal animals and those treated with either phenoxybenzamine, hydergine or phentolamine. In normal animals it was difficult to assay biologically the noradrenaline liberated at low frequencies of stimulation and so the bulk of the data refers to a frequency of 30/sec. In some instances at low frequencies an increase in radioactivity

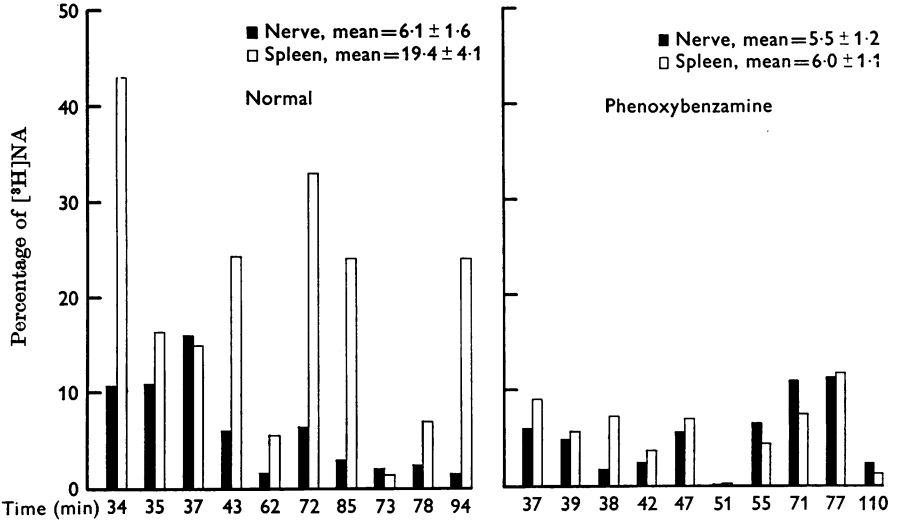

Fig. 7. Comparison of the percentage of radioactive noradrenaline in the spleen stores (open columns) with that in the transmitter liberated by splenic nerve stimulation (filled columns) in normal animals previously infused with DL-[^{3}H]-noradrenaline and animals treated with phenoxybenzamine following the infusion of radioactive noradrenaline. The period of stimulation chosen was that closest to the time of removal of the spleen. The time after the end of the infusion at which the spleen was removed is given below each experiment and these are grouped with the shortest interval to the left.

could be shown though the increase in noradrenaline was too small to measure. These results have been shown below the abscissa in Fig. 8. There are big differences in the total amount of noradrenaline appearing in the venous blood per stimulus, especially between the normal and phenoxybenzamine-treated animals. Within the group treated with phenoxybenzamine the amount of transmitter appearing per stimulus at 5/sec is almost twice that with a rate of 30/sec. In spite of this the scatter of

values for the percentage of [^{3}H]noradrenaline in the transmitter liberated in similar for both frequencies and for both groups.

Spontaneous release of [^{3}H]noradrenaline. In those experiments in which simultaneous samples of arterial and venous blood were taken it was possible to calculate the amount of radioactivity leaving the spleen per minute. An example is illustrated in Fig. 3. At the end of the experiment the spleen was removed and its content of [^{3}H]noradrenaline measured.

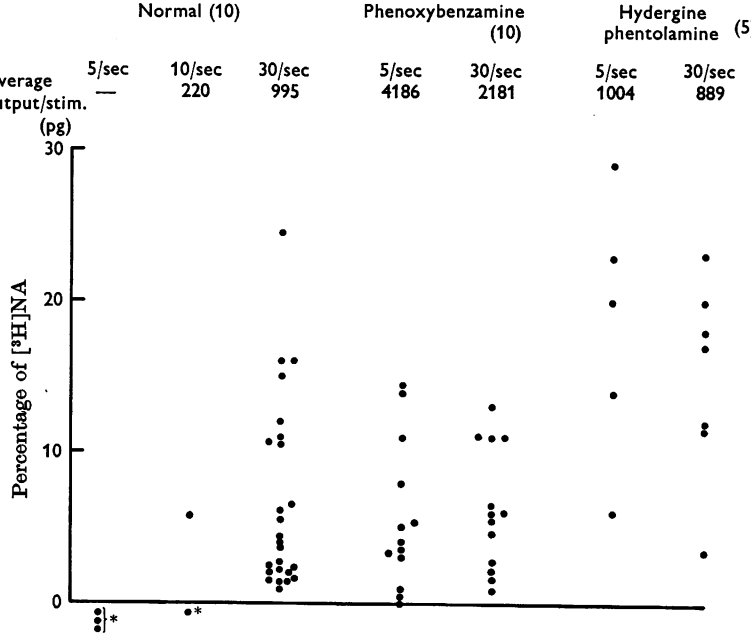

Fig. 8. The effect of frequency on the percentage of radioactive noradrenaline in the transmitter released by nerve stimulation in normal animals, in animals treated with phenoxybenzamine and in animals treated with either hydergine or phentolamine. The number of animals in each group is shown in brackets. In those results marked by an asterisk a percentage could not be calculated because the total recovery of noradrenaline was too low for biological assay. An increase in radioactivity was observed.

The rate of loss of [^{3}H]noradrenaline immediately before removing the spleen was calculated from the difference in radioactivity in arterial and splenic venous blood and the splenic blood flow. From these values the turnover rate of the radioactive noradrenaline was calculated assuming that the radioactivity released into the blood was derived from the stores of noradrenaline even though part of it may be metabolized before reaching the blood stream. At least at long intervals after the end of the infusion this is justifiable since at these times most of the radioactivity retained in the spleen is due to noradrenaline.

Turnover rates have been calculated both for normal animals and

animals treated with phenoxybenzamine after the noradrenaline infusion. The results are shown in Table 4. In normal animals the rate of loss is initially high and falls in the first 45 min after the end of the infusion. In the three experiments in which the spleen was removed more than 1 hr after the infusion the values were low and constant, averaging 0·22 % per minute.

TABLE 4. Spontaneous loss of [^{3}H]noradrenaline from the spleen in normal cats and cats given phenoxybenzamine at the end of the loading infusion of [^{3}H]noradrenaline. The loss was calculated from the difference in venous and arterial blood radioactivity and flow rate. The values used were those obtained immediately before removing the spleen for estimation of its noradrenaline content

Time after infusion (min)	[^{3}H]NA lost (ng/min)	Total [^{3}H]NA in spleen (ng)	% spleen stores lost/min
	Normal		
41	235	5,893	4·0
44	12·5	1,793	0·7
60	5·1	—	—
75	3·2	1,520	0·21
120	7·8	3,375	0·23
224	5·0	2,257	0·22
	Phenoxybenzamine 10 mg/kg		
75	15·3	—	—
100	24	1,180	2·0
120	16·6	1,530	1·3
100*	12·8	960	1·3

* Blood values at 100 min, spleen removed at 120 min.

Once again the results in the animals treated with phenoxybenzamine differed. In these the turnover rates were many times higher. As a result the total [^{3}H]noradrenaline content of these spleens was lower in the untreated animal.

DISCUSSION

Hertting & Axelrod (1961) have reported that in the isolated spleen of the cat perfused with blood, sympathetic nerve stimulation will release radioactive noradrenaline some time after the injection of labelled noradrenaline. Similar observations have been made in the dog's gracilis muscle preparation (Rosell, Kopin & Axelrod, 1963). Infused radioactive adrenaline can also be retained and later released by nerve stimulation (Rosell, Axelrod & Kopin, 1964). The present findings confirm these observations for the spleen in which the normal circulation is retained. Stimulation of the splenic nerves up to 90 min after the end of an infusion of [^{3}H]noradrenaline results in radioactive noradrenaline appearing in the venous blood. This noradrenaline must be held in some protected site. One possible site would be the receptors, another the adrenergic nerve endings. Several findings make receptor-bound noradrenaline unlikely. First, blocking agents, which should displace noradrenaline from these

receptors, do not prevent the appearance of labelled noradrenaline in the venous blood after nerve stimulation, Secondly, the release of receptor-bound radioactive noradrenaline by nerve stimulation would presumably be due to the endogenous noradrenaline liberated by the nerves displacing radioactive noradrenaline. Yet close-arterial injection of large quantities of unlabelled noradrenaline is ineffective in liberating [^{3}H]noradrenaline. The evidence suggests that the infused noradrenaline is taken up into the sympathetic nerve endings and is released from there by nerve stimulation.

In the experiments reported by Hertting & Axelrod (1961) and by Rosell *et al.* (1963) a continual leakage of radioactive noradrenaline, together with its metabolites, was found in the absence of nerve stimulation. A similar spontaneous loss of noradrenaline in the present experiments was responsible for the steady increase in radioactivity of the systemic blood long after the end of the infusion (Fig. 3). That the spleen was the only significant source of this radioactivity was shown in one experiment by removing the spleen immediately after the end of the infusion. In the next 2 hr the radioactivity in the arterial blood remained unaltered. The radioactive material leaving the spleen in the absence of nerve stimulation appeared in the venous blood as metabolites. Hertting & Axelrod (1961) and Rosell *et al.* (1963) found both noradrenaline and metabolites in the perfusing blood. Figures are available only for the gracilis muscle in which 23 % of the total radioactivity was noradrenaline. This difference in results may be a real one and related to the preservation of the normal circulation in our experiments. A small proportion of noradrenaline however may have been present in the blood leaving the spleen and masked by the accumulation of metabolites which recirculation of blood permits. In earlier experiments indirect evidence suggested that 15–20 % of (−)-noradrenaline infused through the spleen was destroyed by enzymes (Gillespie & Kirpekar, 1965*a*). In the present experiments the presence of metabolites has been confirmed by chromatography; they account for 11 % of the DL-noradrenaline infused. The difference between this figure and the 15–20 % unaccounted for in the previous experiments is small and could be due to a less rapid metabolism of the dextrorotatory form. It would seem reasonable to suggest on the basis of both groups of experiments that when (−)-noradrenaline is infused at rates comparable to the rate of liberation by nerve stimulation at 10/sec, 30 % passes unaltered through the spleen to appear in the venous blood, 15 % is metabolized in a single passage through the spleen and the remaining 55 % is incorporated into the nerve endings. The application of these figures to noradrenaline liberated physiologically at nerve endings is more difficult. At a stimulation frequency of 10/sec about 20 % of the transmitter liberated overflows into the venous blood (Brown & Gillespie, 1957; Brown, Davies & Ferry, 1961). The proportion of the re-

mainder taken up into nerve endings or metabolized is not known. As the frequency of stimulation falls there is an increase in the amount of transmitter liberated per stimulus but the amount overflowing into the blood stream diminishes and eventually disappears (Brown *et al.* 1961). The transmitter liberated at these low frequencies must therefore be dealt with by some combination of uptake into nerve endings and metabolism. If the removal were by metabolism then it would be expected that as the frequency of stimulation was lowered and less noradrenaline appeared in the venous blood its place would be taken by radioactive metabolites which would appear in large amounts. This was not found (Fig. 8). It seems likely that at a stimulation frequency of 10/sec the proportion of liberated transmitter re-incorporated into the nerve endings is somewhat greater than in the experiments where noradrenaline was infused at equivalent rates and that as the frequency is lowered the proportion re-incorporated increases. Evidence supporting such an increased re-incorporation at lower frequencies and emphasizing its importance in preventing a decline in transmitter output during continuous stimulation has been found (Blakeley & Brown, 1963; Haefely, Hürlimann & Thoenen, 1965).

Normal animals differ from those treated with phenoxybenzamine in two ways. First the specific activity of the noradrenaline in the spleen and that released by nerve stimulation are similar in animals treated with phenoxybenzamine, whereas in normal animals noradrenaline stored in the spleen has a much higher specific activity than that liberated from the nerves. Secondly, the rate of turnover of noradrenaline was several times greater after treatment with phenoxybenzamine. The value for normal animals of about 13 % per hour is similar to others given in the literature (Green & Erickson, 1960; Spector, Melmon & Sjoerdsma, 1962; Udenfriend & Zaltzman-Nirenberg, 1963; Montanari, Costa, Beaven & Brodie, 1963). These differences could be explained if there are at least two stores of noradrenaline in the spleen, only one of which is available to the nervous impulse. The rate of turnover of this available store would have to be greater than the other. As a result the specific activity of the transmitter liberated by nerve stimulation would fall below that of the noradrenaline in the spleen. On this theory these two stores become one in animals treated with phenoxybenzamine and this single store is available to the nerve impulse and has a fast rate of spontaneous loss. It is tempting but not necessary to locate the site of both stores at the nerve ending. The noradrenaline not available to the nerve impulse could be bound to tissue receptors. Alternatively, the intense vasoconstriction produced by noradrenaline may produce areas of the spleen which are effectively non-perfused. Against binding to tissue receptors is the observation to be reported in the following article that histologically, both by the fluore-

scence technique and by autoradiography, noradrenaline can be detected only in relation to nerve fibres.

We would like to thank the M.R.C. for a grant to one of us (J.S.G) for scientific assistance. Our thanks also go to Miss W. Quinn and Miss C. Muir for their careful technical assistance. The phenoxybenzamine was very kindly supplied by Messrs Smith, Kline and French to whom our thanks are due.

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