

## THE RELEASE OF ADRENALINE AND NORADRENALINE FROM THE ADRENAL MEDULLA OF THE CAT DURING SPLANCHNIC STIMULATION

BY

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Cats were anaesthetized with chloralose, eviscerated and the right adrenal gland was removed. The venous outflow from the left adrenal gland was collected during 10 five-minute periods of stimulation of the left splanchnic nerve. The amounts of adrenaline and noradrenaline in the venous outflow and in the stimulated and unstimulated glands were determined by a fluorimetric method. In eight experiments a mean of 50.5  $\mu$ g. of total catechol amine was recovered from the effluent blood. The mean difference in amine content between the stimulated and unstimulated glands was 50  $\mu$ g., representing a loss of 29% from the stimulated gland. The proportions of the two amines in the effluent blood were very similar to those found in the gland. The results provide no evidence for an increase in the rate of synthesis of catechol amines during splanchnic stimulation.

In 1912 Elliott reported a "very slight" or "almost inappreciable" loss of adrenaline from the stimulated adrenal glands of cats in which he stimulated the splanchnic nerve for periods of 2 to 7 hr. Elliott (1912) had expected to find a greater degree of depletion since the blood pressure responses indicated that pressor amine had been discharged from the gland during each period of stimulation. Tscheboksaroff (1911) had made similar experiments in dogs and reported that the stimulated gland contained more adrenaline at the end of the experiment than the unstimulated gland. West (1950) obtained similar results in rabbits. A possible explanation of these findings is that the rate of synthesis had kept pace with the rate of release of amine so that depletion did not occur. Recently Holland and Schümann (1956) provided further evidence in support of this theory. In a series of experiments in eviscerated cats they showed that the adrenal gland lost 25% of its amine content when stimulated through the splanchnic nerve, but the amount of amine estimated to have passed into the circulation was more than 63% of that originally present in the gland. They concluded that the difference (38%) must have been synthesized during splanchnic stimulation. This evidence implies a rapid rate of amine synthesis during stimulation.

Such an interpretation is not in agreement with other information available concerning the rate of synthesis of medullary amines. Insulin hypoglycaemia causes depletion of adrenal medullary amines, and recovery of the normal amine content of the gland is not complete for several hours or days in cats (Burn, Hutcheon, and Parker, 1950) or in rats (Höckfelt, 1951; Outschoorn, 1952). Outschoorn (1952) also found that, after depletion with  $\beta$ -tetrahydronaphthylamine, recovery of the initial amine concentration in the gland took about 16 hr. A similar slow replacement of discharged amines has been demonstrated by Van Arman (1950) using eserine in rats. Further, Butterworth and Mann (1957a) found that restoration of the normal total amine content of the cat adrenal glands required 7 days following depletion by repeated doses of acetylcholine. The delayed recovery of normal amine content after insulin hypoglycaemia,  $\beta$ -tetrahydronaphthylamine or eserine, which act by way of the splanchnic nerves, or after acetylcholine, indicates a slow rate of synthesis of medullary amine for which more evidence has recently been obtained by Butterworth and Mann (1957b). They found that the adrenaline and noradrenaline lost from the cat adrenal gland, stimulated by repeated doses of acetylcholine, could be recovered quantitatively in the adrenal blood.

It is difficult to reconcile these findings with the suggestion that rapid synthesis occurs during electrical stimulation of the splanchnic nerves. For this reason we decided to reinvestigate the problem. We estimated directly the amount of catechol amines appearing in the adrenal venous effluent during electrical stimulation of the splanchnic nerve and compared this with the difference in amine content of the stimulated and unstimulated glands.

#### METHODS

Cats of either sex, weighing 2 to 4 kg., were anaesthetized with ether followed by chloralose, 60 mg./kg. intravenously. The stomach, intestines, spleen and pancreas were removed; the blood supply to the liver was interrupted by a ligature round the coeliac artery. In three experiments the renal vessels were also tied off. The blood pressure was recorded from a carotid artery. The right adrenal gland was removed and immediately weighed, ground in 0.5 ml. of 0.15 N-HCl and frozen. Heparin (116 i.u./kg.) was injected intravenously. Artificial respiration was begun and the left splanchnic nerve was freed by dissection under the diaphragm and cut as high up as possible. The lesser splanchnic nerves and the sympathetic chain on the same side were also cut.

For collection of the adrenal venous outflow, the usual procedure was to insert a polyethylene cannula into the adreno-lumbar vein just lateral to the adrenal gland. When the adrenal vein was tied close to the inferior vena cava, blood from the gland flowed into the cannula. This blood could then be collected, or returned to the animal by a second polyethylene tube in the right renal or other convenient vein.

The splanchnic nerve was stimulated with rectangular pulses at a rate of 20/sec. (duration 2.5 msec., intensity 5 V.). The venous effluent from the gland was collected during 10 periods of stimulation. Each collection was continued for 15 sec. longer than the period of stimulation (4 min. 45 sec.) to complete the recovery of released amine. There was a rest period of 7 min. between each collection. The volume of blood collected in 5 min. varied from 3.4 to 11.0 ml. in different cats. To maintain the blood volume, arterial blood from another cat (anaesthetized with ether and chloralose and given heparin) was injected after every second collection of adrenal venous blood; the volume injected was equal to that lost by the experimental animal. Control samples of adrenal venous blood were collected without stimulation of the splanchnic nerve.

At the end of the experiment the left gland was removed, weighed, ground in 0.5 ml. of 0.15 N-HCl and frozen.

*Standard Solutions.*—Double-distilled water was used for the preparation of solutions and of samples for assay. Stock solutions of adrenaline hydrogentartrate and noradrenaline hydrogentartrate monohydrate containing 1 mg./ml. free base were prepared

in 0.1 N-HCl and diluted as required to provide standard solutions. Values for adrenaline and noradrenaline were calculated as the free base.

*The Assay of Adrenaline and Noradrenaline in Plasma.*—The venous effluent was collected in cold, graduated centrifuge tubes containing 12 i.u. of heparin and immediately centrifuged for 5 min. at 600 g. The "stimulated" plasma was collected and pooled as required, and either frozen or kept at 2° for 12 to 14 hr. before purification.

1.5 to 3 ml. of plasma was diluted to 10 times the original volume with Versene buffer (0.7% Versene in 1 M-sodium acetate solution, adjusted to pH 8.5 with NaOH). The diluted plasma was passed down columns (0.6 cm. internal diameter) containing 2 g. of acid-washed aluminium oxide (Merck—reagent grade) which had been washed in the Versene buffer solution; the rate of flow was 36 drops/min. Following adsorption, the column was washed with 5 ml. of Versene buffer and 5 ml. of water. The amines were eluted from the column at a flow rate of 9 drops/min. using 5 ml. of 0.2 N-acetic acid followed by 5 ml. of water.

Duplicate determinations of the adrenaline and noradrenaline content of the purified plasma were made using a differential fluorescence method similar to that described by Millar and Benfey (1958). The adrenaline and noradrenaline were oxidized by potassium ferricyanide at pH 6.0 and the fluorescence intensity, developed after treatment with sodium hydroxide and ascorbic acid, was measured at 365 m $\mu$  and 436 m $\mu$  using a Beckman D.U. fluorescence attachment. Calculation of the amount of each amine present in the eluate was made using a simultaneous equation derived from the fluorescence shown by the "stimulated" plasma minus that shown by the control plasma and the fluorescence values of standard solutions containing 0.1  $\mu$ g. of adrenaline or noradrenaline.

*The Assay of Adrenaline and Noradrenaline in Adrenal Glands.*—The amines were separated by paper chromatography and estimated separately by fluorimetry after elution from the paper. The homogenates of the stimulated and non-stimulated glands were thawed and transferred with washing (0.01 N-HCl) to centrifuge tubes and centrifuged at 11,000 g for 10 min. at 0°. The supernatant fluid was carefully removed to avoid, as far as possible, contamination with the floating lipid layer. The sediment was twice washed with 0.3 ml. of 0.01 N-HCl and the washings were added to the supernatant. The final volume of the gland extract varied between 2.2 ml. (stimulated) and 3.0 ml. (unstimulated) so that the final concentration of amine was similar in each extract. For chromatography, 0.07 ml. of extract was applied, along a 4 cm. origin, to Whatman No. 1 filter paper which had been previously washed by three successive immersions for 12 hr. in 0.01 N-HCl. Ascending chromatography in the dark for 15 hr. at 26° in phenol-HCl (Vogt, 1952) was used for separation of the amines. The chromatograms were

washed in reagent grade benzene to remove excess phenol. For each extract, a portion of the same extract to which 3  $\mu\text{g.}$  of adrenaline and 3  $\mu\text{g.}$  of noradrenaline had been added was run as a control. The control strip was sprayed with alkaline potassium ferricyanide to develop the adrenaline and noradrenaline spots, which were used as a guide for cutting out 5  $\times$  7 cm. areas of filter paper for elution. These sections were cut into small pieces and extracted for 8 hr., with occasional shaking, in 0.02 N-acetic acid. The extracts were assayed in duplicate for adrenaline and noradrenaline by measuring the fluorescence at 365 m $\mu$ . Standard curves for each amine (0.1 to 0.3  $\mu\text{g.}$ ) were prepared for each determination.

*The Recovery of Adrenaline and Noradrenaline from Whole Blood.*—In a separate series of experiments, blood from the inferior vena cava of cats anaesthetized with chloralose and injected with heparin was separated into 5 ml. portions and chilled in an ice bath. A total volume of 0.65 ml. of 0.001 N-HCl in 0.9% NaCl solution, containing both adrenaline and noradrenaline (0.3 to 2.0  $\mu\text{g.}$  of either amine), was added to each sample except the control, to which acid saline only was added. The samples were mixed and centrifuged for 5 min. at 600 g and the plasma was collected. The adrenaline and noradrenaline present in the plasma were determined as previously described. In 10 tests, the mean recovery of adrenaline was 77.8%  $\pm$  3.4 (S.E.) and of noradrenaline 70.35%  $\pm$  2.8 (S.E.). As there was no significant difference between the two means, they were averaged to give a mean recovery value of 74.1%. The experimental values for plasma were suitably corrected to give values for whole blood.

## RESULTS

A summary of the results obtained in eight experiments is shown in Table I. An average of 50  $\mu\text{g.}$  of catechol amine was released from the adrenal gland during 10 periods of stimulation (lasting a total of 50 min.) as determined by

TABLE I

THE RELEASE OF CATECHOL AMINES FROM THE ADRENAL MEDULLA DURING SPLANCHNIC NERVE STIMULATION

Left gland stimulated, right not stimulated. Amine content of blood adjusted for loss in extraction: see text.

Cat No.	Total Amine Content ( $\mu\text{g.}$ /Gland)		Amine Released ( $\mu\text{g.}$ )	Amine in Blood ( $\mu\text{g.}$ )	% Amine Released
	Right	Left			
1	134	94	40	47	30
2	232	197	35	24	15
3	168	111	57	59	34
4	208	157	51	54	24.5
5	87	67	20	13	23
6	135	101	34	35	25
7	218	144	74	73	34
8	185	95	90	107	49
Mean	171	121	50	51.5	29

the difference in amine content between the right, non-stimulated, gland and the left, stimulated, gland (171–121=50  $\mu\text{g.}$ ). This value represents a mean release of catechol amines during splanchnic stimulation of 29%. The catechol amine present in the venous blood collected from the adrenal gland during splanchnic stimulation ranged from 13 to 107  $\mu\text{g.}$ , with a mean of 51.5  $\mu\text{g.}$  The stimulated gland always contained less catechol amine at the end of the experiment than did the non-stimulated gland which was removed at the beginning. In each experiment, the amount of amine present in the venous outflow was similar to that estimated to have been lost from the stimulated gland. Similar results have been obtained in three other experiments in which the frequency of stimulation was varied and the plasma samples were assayed individually without pooling.

In Table II, further results from the same eight experiments are presented. The values shown are

TABLE II

ADRENALINE AS % OF TOTAL CATECHOL AMINE CONTENT OF ADRENAL GLANDS AND OF POOLED PLASMA SAMPLES

Left gland stimulated, right not stimulated. No. of plasma samples pooled for analysis shown in brackets. Cat No. 1 excluded from means.

Cat No.	% Adrenaline in Gland		% Adrenaline in Plasma (Pooled)		
	Right	Left	1 (5)	2 (5)	3
1	57	62	46	43	
2	72	75	(3) 63	(3) 67	(4) 68.5
3	67	67	57	58.5	67.5
4	67	69.5	44	51	56
5	61	62	57.5	59	53
6	37	36	43	44	48
7	55	60	47	56.5	58
8	73	77	54	60	74
Mean (2–8)	62	64	52	58	61

for adrenaline as % total catechol amine (adrenaline + noradrenaline) in the adrenal glands and in the pooled plasma samples. There does not appear to be any important alteration in the proportion of adrenaline and noradrenaline in the stimulated gland or in the effluent blood, irrespective of the degree of depletion observed or the initial amount of noradrenaline present in the gland. The mean value for adrenaline in the non-stimulated gland was 62% and that in the stimulated gland 64%. The greatest difference found was a 5% increase in the adrenaline of the stimulated gland of cat No. 7. There appears to be a

smaller proportion of adrenaline in the first pooled plasma samples than in the final pooled samples. The mean value for adrenaline in the first pooled sample of plasma was 52%, compared with 61% in the last sample.

#### DISCUSSION

The results provide no evidence for an increase in the rate of formation of catechol amines during electrical stimulation of the splanchnic nerve. This finding differs from that of Holland and Schümann (1956). They reported a mean depletion of the stimulated gland of 25%, which is very similar to the 29% depletion we observed under similar experimental conditions. However, we were unable to confirm their finding that synthesis occurred to the extent of 38% of the amine content of the unstimulated gland (mean of twelve experiments). The reason for this difference is not known, but two possibilities suggest themselves. First, the assay method used by these workers may not be suitable for estimating the output of medullary amines in the same animal. It is open to question whether the infusion of a 50:50 mixture of adrenaline and noradrenaline at a constant rate into the external jugular vein is capable of duplicating quantitatively the effect of amine released into the circulation by stimulation of the splanchnic nerve. Second, the possibility cannot be overlooked that part of the pressor response which these authors observe after splanchnic stimulation is due to the release of pressor substances from areas other than the adrenal gland. In five of our eight experiments no pressor effect was observed during the periods of splanchnic stimulation with collection of the blood from the adrenal gland. In one cat in which the branches of the coeliac artery were ligated as close as possible to the organs which they supplied, a pressor response was observed. Following the application of a second ligature to the coeliac artery close to its origin from the aorta, there was no pressor response to any of the subsequent periods of splanchnic stimulation. In the other two cats each period of splanchnic stimulation was accompanied by some rise in blood pressure. After removal of the stimulated gland in these cats, splanchnic stimulation still caused a rise in blood pressure in the now bilaterally adrenalectomized animal. These observations make it unlikely that the pressor response was due to the escape of amine from the stimulated gland but do support the possibility that pressor substances may arise from elsewhere than the adrenal gland.

It is clear from previous work (Elliott, 1912; Crowden, 1929; Van Arman, 1950) that synthesis of amine by the adrenal medulla is not necessarily dependent upon the integrity of the sympathetic nerve supply. These workers found that the amine content of a depleted adrenal gland could return to normal despite cutting of the ipsilateral splanchnic nerve. As pointed out earlier, it is difficult to reconcile the rapid synthesis of amine postulated by Holland and Schümann (1956) with the fact that, after insulin and other agents which eventually act through splanchnic nerves to deplete the gland, recovery of the normal content of amine requires several hours or days. Tscheboksaroff (1911) found that in the dog anaesthetized with morphine and curarized, stimulation of the cut left splanchnic nerve led to release of pressor substance; at the end of the experiment the pressor activity of an extract of the stimulated gland was generally greater than that of a similar extract from the right unstimulated gland. He concluded that adrenaline "wird auch in grösserer Menge gebildet und im Drüsenparenchym selbst angesammelt." However, the splanchnic nerve supply to the unstimulated gland apparently remained intact throughout the experiment and the gland was therefore subject to depletion caused by the central action of morphine. The apparent increase in activity in the left gland might equally well represent a greater depletion of the right gland. It is therefore difficult to accept these experiments as evidence for an increase in the rate of synthesis in the stimulated gland. Elliott (1912) never succeeded in demonstrating more than "a very slight loss" of medullary amines when using the method of measuring the residual adrenaline in the gland after stimulation. However, in the one cat experiment (out of 10) which he quotes in detail, there was a 34% depletion of the gland which had been stimulated intermittently for 7 hr. In the three dog experiments the stimulated gland showed a 3% loss in one, a gain of 7% in another, and a loss of 13% in the third. These experiments, like those of Tscheboksaroff (1911), do not necessarily support the idea that splanchnic stimulation causes a rapid rate of synthesis of catechol amine. The results reported by West (1950) show that the stimulated adrenal gland of the rabbit contained an average of 36% more adrenaline than the unstimulated gland. Like Tscheboksaroff (1911), he apparently did not cut the right splanchnic nerve and used an anaesthetic (urethane) which Elliott (1912) had shown to cause a depletion of the adrenal medulla. From these observations it is difficult to conclude

that an increase in the rate of synthesis of catechol amine with stimulation of the splanchnic nerve has been adequately demonstrated.

In six of our eight experiments we observed a small increase (1 to 5%) in the % of adrenaline in the stimulated compared with the non-stimulated gland. These small changes may be insignificant. If they do have any meaning, two explanations should be considered. The first is that they represent, as Holland and Schümann (1956) concluded, an increase in the rate of methylation of noradrenaline. The second explanation, that more noradrenaline than adrenaline was released from the store in the gland, is consistent with our finding that the earlier samples of plasma do contain a higher proportion of noradrenaline than the stimulated gland. The proportion of noradrenaline in later samples of plasma approximates more closely to that in the glands.

While the rate of new formation of amines by the gland may be fast enough to keep pace with the resting secretion it does not appear to be sufficient to meet the demand of any severe or

prolonged stimulus; under these conditions most of the amine liberated will be drawn from the storage depots, thus depleting them.

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