

## THE NATURE OF SYMPATHIN RELEASED IN THE RABBIT'S EAR \*

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It is now generally accepted that most mammalian adrenergic nerves release a mediator consisting largely of noradrenaline, which may be admixed with a varying but small proportion of adrenaline. The adrenergic nerves in the rabbit's ear were investigated by Gaddum and Kwiatkowski (1938, 1939) and Gaddum, Jang, and Kwiatkowski (1939), and found to release adrenaline. In view of the fact that no evidence was found for the release of noradrenaline these nerves have been quoted as exceptions to the general rule (Euler, 1950). Since there are now more sensitive and specific tests for the identification of the adrenergic mediators it was decided to reinvestigate the nature of those released in the rabbit's ear.

### METHOD

A large rabbit (over 2.5 kg. where possible) was anaesthetized with pentobarbitone sodium ("Nembutal" Abbott, containing 1 grain per ml.) in a dosage of 1.0 ml./kg. intraperitoneally, repeated every 15 minutes till anaesthesia was complete (0.5–1.5 hour).

The animal was fixed in a supine position on a warmed table. The trachea was quickly cannulated and artificial respiration instituted. The common carotid artery was identified and followed headwards to its terminal branches. The aim of the dissection throughout was to identify the arterial branches rather than to clean the structures surrounding the vessels. All the branches of the common carotid artery were tied off, including the terminal ones, but excluding the artery to the ear which arises laterally about the level of the superior cervical ganglion. The tied vessels were not divided.

The cervical sympathetic trunk was traced up to the superior cervical ganglion and the fine post-ganglionic fibres, running laterally, carefully dissected for 0.2–0.5 cm. Where these post-ganglionic fibres were in close proximity to the auricular artery no attempt was made to separate them from the vessel, but where the anatomy permitted, the nerves were cleared separately. The great auricular vein was freed. For identification, a loose ligature was placed around its union with the external jugular vein. The common carotid artery was then cannulated and the perfusion with salt solution commenced. The arterial cannula was usually of a simple straight type, but where the preparation was to be used later for assays (Gaddum, Peart, and Vogt, 1949) the special cannula described by Gaddum and Kwiatkowski (1938) was used. As soon as the salt solution was flowing into the ear the external jugular vein was cut with scissors to allow the perfusate to flow out freely. The tissues of the neck were completely severed with a scalpel as low down as possible, and the vertebral column was cut with bone forceps, thus decapitating the animal. In order to prevent leakage, the tissues of the neck were tied with a stout cord at a level below the arterial cannula and the cut in the jugular

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vein. Any possible leaking vessels were thus compressed against the cervical vertebrae. The vertebral canal was blocked tightly with plasticine and a cork.

A straight glass cannula was then inserted into the great auricular vein through the orifice in the jugular vein and tied therein. The reservoir containing the perfusing fluid was fixed about 2–3 m. above the preparation. The fluid passed through a 3–4 cm. length of capillary tubing fitted with a by-pass of rubber tubing with a clip. When the clip was closed the only passage available was through the capillary. The by-pass enabled the preparation to be washed out. The clip was generally opened slightly when the perfusion commenced, but tightened when the effluent from the ear no longer contained evident traces of blood. A water manometer of small-bore glass tubing was interposed between the capillary tube and the arterial cannula. The apparatus was similar to that devised by Gaddum and Kwiatkowski (1938) and was intended to make the rate of outflow independent of the degree of contraction of the vessels of the ear. The "resting" perfusion pressure was usually 10–15 cm. water, but on stimulation of the nerves the pressure rose owing to vasoconstriction.

The perfusing fluid was equilibrated with 5 per cent carbon dioxide in oxygen before perfusion started, and as finally evolved had the composition NaCl 9, KCl 0.21, CaCl<sub>2</sub> 0.24, NaHCO<sub>3</sub> 0.12, glucose 1 g./litre with ascorbic acid (10<sup>-3</sup>) and ephedrine hydrochloride (2.5 × 10<sup>-6</sup> of base). In a few experiments, the ephedrine was omitted at first and introduced later. The purpose of using half the usual concentration of potassium was to reduce interference in the biological assays; one-quarter the usual bicarbonate was used in order to render the solution somewhat less alkaline (Bülbring, 1944) and so preserve any amines liberated into the vessels. The perfusion was carried out at room temperature.

For stimulation of the nerves, a unipolar electrode was used, the "earth" terminal being a piece of brass gauze wrapped in saline-wetted cotton wool and placed behind the head. Stimulation was by alternating current (50 cycles, 5–10 volts) for 10–20 seconds in every minute for 10–15 minutes. The voltage in the first stimulation period was that which elicited a good contraction when tested on a voluntary muscle, but this was increased in succeeding periods in order to ensure that adequate stimulation was obtained. Continuous stimulation for the whole period of collection of the sample and intermittent stimulation by "square" pulse waves of different durations and frequencies were also tried, but had no advantages.

*Procedure.*—When the perfusing fluid was clear of blood the collection of samples was commenced. The outflow from the venous cannula was collected in ice-cooled centrifuge tubes, containing solid ascorbic acid (1 mg./10 ml.). Each sample was collected over 10–15 minutes and, in any particular experiment, the samples obtained were of approximately the same volume, since the rate of flow was kept constant by the perfusion device used. As soon as possible after collection the samples were centrifuged (2,000 r.p.m. for 5 min.) to bring down any cells, and the supernatant fluid was transferred to test-tubes and immediately acidified with 2–3 drops of 0.15 N-hydrochloric acid.

After one or more control samples had been taken the electrode was placed under the cleared nerves, or under the nerves and artery if the two had not been separated by dissection. Stimulation was applied intermittently during the sampling. Usually, several "stimulation" samples were collected, interrupted by periods of rest during which control samples were again taken.

*Estimation of adrenaline and noradrenaline.*—In the first series of experiments these substances were estimated by using several different tests on each sample and calculating the concentrations of the two amines by means of appropriate formulae. The biological methods were those described by Gaddum, Peart, and Vogt (1949) and Gaddum and Lembeck (1949). Shaw's colorimetric test was also used (Shaw, 1938).

In some assays on the rat's uterus and colon the control samples had a stimulant action owing to interfering substances. When these were present the effects of active samples were compared with those of the control samples with added adrenaline (cf. Peart, 1949).

In order to test for the presence of interfering substances which, without being actually stimulating, might have a "masking" effect on small amounts of adrenaline, that amine was added to control samples and this mixture was compared with standard solutions of adrenaline. Down to an adrenaline concentration of 25  $\mu\text{g./ml.}$  not more than a 20 per cent discrepancy on the rat uterus was found, and usually there was no appreciable difference.

In the second series of experiments a preliminary direct test was carried out on the rat's uterus, and then the two amines were estimated independently after separation by paper chromatography.

Each sample (4-6 ml.) was placed in a  $2.5 \times 9$  cm. centrifuge tube with a further 2-3 drops of 0.15 N-HCl and 1 mg. ascorbic acid. To each sample 10 ml. ethanol was added. The tubes were left in the refrigerator for 0.5 hour and then centrifuged (2,000 r.p.m. for 15 minutes). The supernatant fluid was transferred to a 50 ml. round-bottomed Quickfit and Quartz flask, the tube being washed with about 2 ml. alcohol which was also transferred. A little whitish residue was sometimes left in the tube. The mixture in the flask was evaporated to dryness *in vacuo* at a temperature not exceeding  $60^\circ \text{C.}$  (external temperature) and the flask allowed to cool with the suction still on. The flask was then opened and the inside leached three times with 1, 0.5, and 0.5 ml. alcohol saturated with sodium chloride. Potassium salts are very insoluble in this mixture so that interference in the assays by potassium was minimized (Barsoum and Gaddum, 1935). The leachings from two or more control or stimulation samples were usually pooled by transferring them to another 50 ml. flask. The contents were evaporated *in vacuo* and taken up in 0.75 ml. acid alcohol (0.1 per cent (v/v) conc. HCl in ethanol). This was applied to a cylinder of filter paper for chromatography. The flask was washed with a further 0.2 ml. acid alcohol which was also applied. Attempts were made to keep the total original volumes of the pooled control samples and the pooled stimulation samples approximately equal.

The application of the acid alcohol extracts to the paper, development of the chromatogram with phenol as solvent, elution of the appropriate strips of paper containing adrenaline and noradrenaline, and the assays of these amines on the rat's blood pressure preparation have been described by Crawford and Outschoorn (1951).

The effects of ascorbic acid on the biological tests were investigated. Ascorbic acid ( $10^{-3}$ ) was used in the tests described by Gaddum, Peart, and Vogt (1949), and did not cause interference. The tests were repeated with five and ten times this amount, the solutions having been acidified with HCl similarly to the samples of the rabbit's ear effluent and made almost neutral with solid sodium bicarbonate before assay on the rat's uterus and colon. The sodium ascorbate did not affect the responses of these tissues. In the chromatographic method, however, much higher concentrations might have occurred in the solutions used in the assay. Standard mixtures of adrenaline and noradrenaline (2  $\mu\text{g./ml.}$  of each) containing ascorbic acid in concentrations of 1.5, 10, and 50  $\text{mg./100 ml.}$  were therefore chromatographed and assayed on the rat's blood pressure preparation. The percentage recoveries all agreed closely, irrespective of the amounts of ascorbic acid present originally, and were of the order found by Crawford and Outschoorn (1951). These results indicate that for good recoveries of adrenaline and noradrenaline concentrations of ascorbic acid above  $10^{-5}$  are unnecessary but not disturbing.

The same amounts of adrenaline and noradrenaline were chromatographed with ephedrine hydrochloride ( $10^{-5}$  as base) alongside "blank" ephedrine solutions of the

same concentration. Assayed on the rat's blood pressure preparation, the "blanks" showed no difference from equal volumes of saline, while the adrenaline and noradrenaline were recovered to the same extent as without ephedrine. In addition, noradrenaline added to "blank" ephedrine eluates after chromatography showed no "masking" when compared with standard solutions of noradrenaline.

Attempts were made to find out the fate of ephedrine subjected to this method of chromatography and to compare it with that of adrenaline and noradrenaline. About 2 mg. ephedrine in  $2 \times 10^{-3}$  solution was applied on a 12 cm. line alongside a control spot of an adrenaline-noradrenaline mixture. After 22 hours' development the paper was washed in benzene and dried. The ephedrine portion was sprayed with 10 per cent ninhydrin in water, allowed to dry again, and sprayed with 10 per cent sodium carbonate solution. The adrenaline-noradrenaline strip was developed with ferricyanide in the usual way. The ephedrine showed up at the edge of the solvent, having travelled much faster than the adrenaline. For an average solvent flow of 27.5 cm. the mean RF values (taken from the centres of the coloured areas) were: ephedrine 0.85, adrenaline 0.57, and noradrenaline 0.29. The ephedrine was thus well away from the portion of paper which would have contained the adrenaline had this been present in the solution analysed.

The effect of ephedrine on the rat's blood pressure preparation was tested by injecting volumes of up to 0.4 ml. of solutions from  $10^{-3}$  to  $10^{-6}$ . None of the effects was distinguishable from those of saline injections. Dilutions made in the same way, and with the same pipettes, from the same stock solutions of adrenaline and noradrenaline in saline, and in saline containing ephedrine ( $10^{-5}$ ) were also compared. No differences were observed in the pressor responses. It is generally agreed that low concentrations ( $10^{-6}$ – $10^{-7}$ ) of ephedrine synergize where high concentrations ( $10^{-4}$ ) antagonize the effects of adrenaline (Gaddum and Kwiatkowski, 1938; Jang, 1940; West, 1947). Neither effect was noted on the rat's blood pressure over a period of about two hours. It is possible that intermittent injections of ephedrine over a longer period might have made these effects evident, but this was not tested. In any event, as estimates of the amines were made by comparison with standard solutions, it is unlikely that any sensitization by ephedrine would have introduced an error into these estimates.

In the other biological tests, concentrations of ephedrine above  $10^{-5}$  were found to produce definite effects and to potentiate those of standard adrenaline solutions. These effects were most marked on the rat's colon and were less on the rabbit's ear and rat's uterus. Smaller concentrations of ephedrine did not have these effects, and one-fourth the average threshold concentration was selected as suitable for the perfusing fluid. The existence of unusual sensitivity to ephedrine in any of the biological tests would have been brought to light when control samples, containing ephedrine only, were tested. No effects of any significance were found. Ephedrine is known to be without effect on the results of Shaw's chemical test (Gaddum and Kwiatkowski, 1938).

In assays of the final eluates on the rat's blood pressure preparation, injections of samples from some experiments had depressor effects, whereas those from others did not. The results of assays were checked by diluting standard noradrenaline (1 in 10) with eluates of the noradrenaline or adrenaline strips of the control samples collected when the nerves had not been stimulated. The concentrations of these dilutions were made to contain 100–250  $\mu\text{g}$ . noradrenaline per ml.; if the eluates had depressor properties, these concentrations of noradrenaline were often completely "masked"; if the eluates were pressor, their "masking" effect was either small (20 per cent) or absent.

The results described below were those obtained by actual comparison with standard noradrenaline with no correction for masking. They are therefore often underestimates. Since the occurrence of depressor effects did not appear to be related to the volume of original sample concentrated and chromatographed, and since all the

samples from a particular experiment either did, or did not, prove depressor, the ability to obtain a result of an assay apparently depended on the relative insensitivity of the rat used for the assay to the interfering substances present. It was impracticable to do a preliminary test on one (control) sample and in the event of there being depressor effects prepare another rat in the hope that it would prove more satisfactory.

### RESULTS

In some experiments the activity of the effluent was too low to be detected by direct tests, and in others stimulation of the nerve had no apparent effect, but in eight experiments there was clear evidence by at least two methods that the samples after stimulation were more active than control samples. This result was obtained not only by individual assays of the samples against standard solutions but also by direct comparison of the control and stimulation samples in the same biological tests. The results obtained by each of the tests used are given in Table I. By this direct comparison and assay, increased activity due to stimulation was shown by the rat's uterus 9/10 times, by the rat's colon 3/5 times, by the rabbit's ear 2/2 times, and by Shaw's test 5/5 times. Table II shows the results of tests on the active samples collected during and after stimulation when the increase due to stimulation was shown by at least two tests.

TABLE I  
RESULTS OF ASSAYS OF SAMPLES BY DIFFERENT TESTS

Test	Exp. No.	Dose ratio Nor./Adr. for equal responses	Adrenaline equivalents (m $\mu$ g./ml.)		
			Control	During stimulation	After stimulation
Rat's uterus	4	100	<2	2.5	1.8
	5	15	1	1	7.9
	6	150	1.87	5.8	3.3
	9	150	1.8	3.7	5
	11	100	<2.5	<1.25	<1.25
	12	100	1.8	3.7	<1.25
	13	75	<1.25	60	—
	16	100	3.7	15	—
	17	150	<5	5	5
	19	75	<2.5	3.7	—
Rat's colon	4	1	<125	750	375
	5	1	187	187	375
	6	0.75	<125	<125	<125
	13	1.1	—	750	—
	16	1.5	—	33	—
	17	2	<250	<50	82
	19	0.5	<300	<300	—
Rabbit's ear	5	3	—	—	10
	6	1.5	4	8	7.5
	13	0.75	—	3,000	—
	16	3.7	7.5	15	—
Shaw's test	9	} 13.5	9	106	17
	11		26	25	70
	12		15	30	—
	13		20	112	—
	16		38	110	—

TABLE II  
RESULTS OF TESTS ON SAMPLES COLLECTED DURING AND AFTER STIMULATION

Exp. No.	Adrenaline equivalents (mµg./ml.)				Concentrations calculated from uterus and colon results (mµg./ml.)		Percentage content of adrenaline
	Shaw's test	Uterus	Colon	Ear	Adrenaline	Nor-adrenaline	
4 {	—	2.5	750	—	(-5)	747	} 0
5 {	—	1.8	375	—	(-1.9)	373	
6 {	—	7.9	375	10	(-17.1)	367	} 0
9 {	—	5.8	<125	8	—	—	
12 {	—	3.3	<125	7.5	—	—	} 6.2
13 {	106	3.7	—	—	—	—	
16 {	17	5	—	—	—	—	} 34
17 {	30	3.7	—	—	—	—	
17	112	60	750	3,000	50	759	6
	110	15	33	15	14.5	28	34
	—	5	82	—	4.5	72	6

The estimates of the adrenaline equivalent with the uterus were nearly always much lower than those given by Shaw's test or by the colon or the ear. This fact itself shows that adrenaline was not the only substance present, and could be explained by the presence of noradrenaline. The equivalent concentrations of these two substances were calculated from the results obtained with the uterus and colon by the formulae used by Gaddum and Lembeck (1949) and others.

In Table II the concentrations of adrenaline and noradrenaline calculated in this way show in some instances "negative" values for adrenaline (experiments 4 and 5). These minus values are a measure of the error of the assay methods when a small amount of adrenaline is present in a mixture with a relatively larger amount of noradrenaline, i.e., when the percentage content of adrenaline is below ten. Gaddum and Lembeck (1949) have shown that in such assays only the calculated noradrenaline figure can be accepted as reasonably accurate. The replacement of the minus values by zero (i.e., no detectable adrenaline) is considered unjustifiable. It is doubtful whether all the results can be explained by the presence of these two amines, but the results do not justify speculation about the possible presence of other substances.

The results in Table III provide better evidence for the conclusion that adrenaline and noradrenaline are both released when the nerve is stimulated. In these experiments, noradrenaline was estimated on the rat's blood pressure after chromatographic separation from adrenaline and impurities. It was not possible to estimate adrenaline in the same way, since the rat's blood pressure is not sensitive enough to detect these very small amounts of adrenaline, and the rat's uterus is too sensitive to interfering substances coming from the paper itself. The estimates of adrenaline given in Table III were therefore obtained by testing samples taken from the unconcentrated extracts before chromatography. The amounts of noradrenaline found would not affect the results of those tests, since the rat's uterus was never less than 100 times as sensitive to adrenaline as it was to noradrenaline. The evidence in

TABLE III

Minute output of adrenaline and noradrenaline, calculated from results of estimates in pooled samples of effluent. Adrenaline estimated directly with the rat's uterus; noradrenaline estimated with the rat's blood pressure after chromatographic separation.

Exp. No.	Control samples		Samples collected during stimulation		
	Adrenaline (m $\mu$ g.)	Noradrenaline (m $\mu$ g.)	Adrenaline (m $\mu$ g.)	Noradrenaline (m $\mu$ g.)	% Adrenaline content
25	<0.33	Depressor	0.60	Depressor	—
27	0.12	<4.17	0.34	<6.25	<2.5
29	0.68	<8.33	1.00	12.50	7.4
30	<1.41	Depressor	1.45	Depressor	—
31	<0.81	<3.33	0.82	1.43	36.5
32	<0.45	<3.38	0.71	3.75	15.9
34	0.84	<2.07	0.60	<2.07	>22.5
35	0.42	<1.67	0.48	3.12	13.3
36	<0.52	<8.33	0.30	<3.12	>8.8
39	<0.43	<0.83	0.59	1.10	34.9

Tables II and III that the substance measured by these tests on the rat's uterus was adrenaline was thus not very good; it was certainly not noradrenaline or any other substance which is known to occur in the body. If it is true that these results give an estimate of adrenaline, then this substance was present in all the samples collected during stimulation. The amounts present in control samples were generally too small to be detected, and in 8 of the 10 experiments (Table III) there was evidence that adrenaline was present after stimulation in higher concentration than before.

Noradrenaline was detected in 5 of the 10 samples (Table III) collected during stimulation, and in each of these samples the concentration was greater than in the corresponding control samples. The identification of this substance as noradrenaline depends upon the fact that it moved up the paper at the same rate as noradrenaline.

These results show that both amines were present in increased amounts after stimulation. The percentage content of adrenaline in the samples collected during stimulation varied between 7.4 and 36.5 per cent.

#### DISCUSSION

The results recorded here show that both adrenaline and noradrenaline are released during stimulation of the nerves to the rabbit's ear. The percentage of adrenaline was often much higher than that found for other nerves, so that it is perhaps not surprising that Gaddum and his co-workers failed to detect the simultaneous liberation of noradrenaline. The concentrations of both amines were very low and it was difficult to get satisfactory results even with the improved methods now available. The results of the direct tests shown in Tables I and II prove that adrenaline was not the only substance present, since the test on the uterus gave lower estimates than the other tests. This test has a higher dose-ratio (noradrenaline/adrenaline) than any other known test and is therefore particularly useful in experiments of this type. Gaddum and Kwiatkowski (1939) used the hen's rectal caecum, which also has a fairly high dose-ratio, and did find that the adrenaline equivalent in this test was sometimes lower than that on the frog's heart. This might have

suggested the presence of noradrenaline, but the evidence was not convincing and was neglected. The specific test described by Shaw (1938) proved that adrenaline was present and the facts did not justify the theory that the two amines were both present.

In the present experiments, only Shaw's ordinary test was applied, and although the results are similar to Gaddum and Kwiatkowski's the discrepancy found between the adrenaline equivalent obtained by it and with the rat's uterus indicates that in the chemical method some other reactor with the colour reagent was also being estimated, since the effluent did not contain any substances which "masked" the effect of adrenaline added to control samples and assayed on the rat's uterus.

As previously shown by Gaddum and Kwiatkowski (1938), the addition of ephedrine to the perfusing fluid was essential in order to obtain a high yield of sympathin. The reason they assumed for this phenomenon was the inhibition of amine oxidase by ephedrine. Recent evidence in favour of their hypothesis was obtained when Thompson and Tickner (1951) were able to demonstrate the presence of the enzyme in rabbit ear vessels. It was further shown by Burn and Robinson (1951) that the potentiation of the effects of injected amines was lost when the enzyme had apparently disappeared after prolonged perfusion. The results in Table IV show the effect of adding ephedrine to the perfusing fluid.

TABLE IV  
RESULTS OF ASSAYS ON THE RAT'S UTERUS

Plain Locke 1.15 p.m.			Ephedrine-Locke 3.30 p.m.		
Sample	Time p.m.	Adrenaline equivalent (m $\mu$ g./ml.)	Sample	Time p.m.	Adrenaline equivalent (m $\mu$ g./ml.)
Control	2.15	<1.25	Control	4.00	<1.25
Stim.	2.30	<1.25	Stim.	4.15	1.25
Stim.	3.00	<1.25	Stim.	4.50	1.87
			Stim.	5.05	1.25

The low concentrations obtained show that it would not have been possible, even now, to prove that the two amines were both present without the use of concentrated extracts and chromatography. The evidence still does not exclude the possibility that other active substances besides adrenaline and noradrenaline are released.

#### SUMMARY

1. The sympathetic nerves were stimulated in rabbit ears perfused with salt solutions and the effluent tested for sympathomimetic amines by colorimetric and biological methods, aided by chromatography.

2. The release of adrenaline has been confirmed, but evidence is presented that it is accompanied by larger amounts of noradrenaline.

3. The percentage adrenaline content was generally higher than that usually found after stimulation of other adrenergic nerves, but there was no evidence of any qualitative differences between sympathetic nerves to the rabbit's ear and those to other tissues.



## REFERENCES

- Barsoum, G. S., and Gaddum, J. H. (1935). *J. Physiol.*, **85**, 1.  
Bülbring, E. (1944). *J. Physiol.*, **103**, 55.  
Burn, J. H., and Robinson, J. (1951). *Brit. J. Pharmacol.*, **6**, 101.  
Crawford, T. B. B., and Outschoorn, A. S. (1951). *Brit. J. Pharmacol.*, **6**, 8.  
Euler, U. S. v (1950). *Ergeb. Physiol.*, **46**, 261.  
Gaddum, J. H., Jang, C. S., and Kwiatkowski, H. (1939). *J. Physiol.*, **96**, 104.  
Gaddum, J. H., and Kwiatkowski, H. (1938). *J. Physiol.*, **94**, 87.  
Gaddum, J. H., and Kwiatkowski, H. (1939). *J. Physiol.*, **96**, 385.  
Gaddum, J. H., and Lembeck, F. (1949). *Brit. J. Pharmacol.*, **4**, 401.  
Gaddum, J. H., Peart, W. S., and Vogt, M. (1949). *J. Physiol.*, **108**, 467.  
Jang, C. S. (1940). *J. Pharmacol.*, **70**, 347; **71**, 87.  
Peart, W. S. (1949). *J. Physiol.*, **108**, 491.  
Shaw, F. H. (1938). *Biochem. J.*, **32**, 19.  
Thompson, R. H. S., and Tickner, A. (1951). *J. Physiol.*, **115**, 34.  
West, G. B. (1947). *J. Physiol.*, **106**, 418.