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THE OUTPUT OF SYMPATHETIC TRANSMITTER FROM THE SPLEEN OF THE CAT

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The recorded action potential of mammalian C fibres can be increased by appropriate repetitive stimulation of the nerve (Brown & Holmes, 1956). This potentiation was attributed by Brown & Holmes to an increase in the action potentials of individual nerve fibres. Recent work by Ritchie & Straub (1956) has, however, suggested that summation of negative after-potentials of the individual fibre responses may be responsible for a large part of the phenomenon. There are many examples of 'post-tetanic' facilitation in peripheral tissues (cf. Hutter, 1952), and the responses of many tissues to nerve stimulation are dependent upon the frequency of the nerve impulses incident upon them (cf. Garry & Gillespie, 1955). Many of these phenomena could be explained if it were assumed that the amount of transmitter liberated peripherally were related to the size of the impulse reaching the terminals of the nerve. If this assumption were correct then conditions which cause an increase in action potential should cause a corresponding increase in the output of transmitter.

We accordingly turned our attention to the possibility of measuring the output of sympathin from an organ supplied by post-ganglionic sympathetic nerve fibres of the type showing the phenomenon of post-tetanic enhancement. Our preliminary experiments showed that some tissues previously reported as giving measurable outputs of sympathin, the rabbit's ear, the colon of the cat and of the rabbit, did not yield enough for quantitative analysis under the rigorous conditions necessary for our experiments. In the end, the spleen of the cat was found consistently to yield in its venous outflow amounts of sympathin large enough for accurate assay. Although our original intention was to investigate the effects of post-tetanic enhancement of action potential on the output of sympathetic transmitter, the experiments yielded information of such interest on the metabolism of the transmitter as to justify detailed investigation.

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In this paper we show the relationship between the output of sympathetic transmitter and the frequency of stimulation of the splenic nerves. The amount of transmitter in the venous blood is not affected by treating the tissue with substances inhibiting monoamine oxidase. The output of transmitter is, however, greatly increased by inactivation of the tissue receptors with substances like dibenzyl-β-chloroethylamine (dibenamine). It would appear that the most important factor in the disappearance of the sympathetic transmitter after liberation is its utilization at the receptors upon which it acts.

A preliminary account of this work has already appeared (Brown & Gillespie, 1956).

METHODS

The method, in outline, consisted of stimulating the splenic nerves using a predetermined number of stimuli, collecting the venous blood from the organ and assaying its content of sympathin as noradrenaline on the blood pressure of the pithed rat. The final result was expressed as the output of sympathin in nanogram (10^{-9} g) per stimulus.

Cats were anaesthetized with chloralose; the abdomen was opened in the mid line, and the intestines were removed from the mid duodenum to the terminal colon. In removing the intestines the superior mesenteric vein was cleaned as far as its junction with the splenic vein and cut between ligatures, leaving a stump of sufficient length for subsequent cannulation. The splenic nerves were dissected from the splenic artery and tied but not cut. The vascular connexions between the spleen and the stomach and omentum were tied and cut; the omentum was removed. Both splanchnic major nerves were cut to prevent escaping current stimulating them and possibly causing a reflex liberation of sympathin from some region other than the spleen. Both adrenal glands were removed. A ligature was placed round the portal vein just beyond the junction of the splenic and superior mesenteric veins but proximal to the entry of the left gastric vein; the cat was heparinized. The stump of the superior mesenteric vein was cannulated with a polythene cannula, the tip of the cannula lying just proximal to the entrance of the splenic vein. The freed bundle of splenic nerves was slipped on to shielded platinum electrodes. The nerves were stimulated with rectangular electrical pulses of 20 V/0·5 msec so arranged that the cathode was peripheral. Previous experiments had shown that such a voltage was several times that required to produce a maximal compound action potential in these fibres. Since we were interested in the influence of the frequency of stimulation on the output of sympathin, the total number of stimuli was kept constant. In all experiments 200 stimuli were used irrespective of their frequency. Periods of stimulation were repeated at regular 10 min intervals, the first of these was used to empty the spleen without collecting a sample. The abdomen was filled with warm paraffin agitated by bubbling through it a mixture of 95% O_2 + 5% CO_2.

The vascular supply of the spleen, the position of the collecting cannula and the stimulating electrodes are shown in Fig. 1. By pulling on the loose ligature round the portal vein the venous outflow from the spleen could be diverted to the polythene cannula. This blood was collected in chilled, silicone-coated, calibrated centrifuge tubes containing solid heparin (5 mg to a 10 ml. tube). These precautions (suggested by Gaddum, Peart & Vogt, 1949) prevented the development of vasopressor activity unrelated to sympathin in the blood. Blood was collected for the period of nerve stimulation plus the first 20 sec of the post-stimulatory period. This standard collection period was based on experiments, to be detailed later, which showed that over 80% of the sympathin coming from the organ was collected within such a period. More prolonged collection had the double disadvantage of bleeding the animal unnecessarily and of diluting the activity of the plasma. Immediately samples were collected their volumes were noted, the plasma and cells were separated by centrifugation and the volume of plasma was measured. A sample of plasma was removed, avoiding the region of the buffy coat; this separated plasma was kept on ice until

assayed. Under these conditions sympathin was relatively stable. The activity of a sample of plasma, assayed immediately and again some 2 hr later, was unchanged. The loss of sympathin from the plasma to the red blood cells was also negligible. This was checked by taking plasma to which noradrenaline had been added and mixing it with red blood cells. The decline in activity of the plasma was followed for 1 hr. The loss of activity within the first 20 min was barely detectable.

In some experiments noradrenaline was injected into the arterial blood going to the spleen. For this purpose the central stump of the superior mesenteric artery was cannulated, with the cannula directed towards the aorta. An occlusion ligature was placed round the aorta immediately below the superior mesenteric artery. All major branches of the coeliac axis were tied with the exception of the splenic artery. With the aorta occluded below the superior mesenteric artery, noradrenaline was injected and was carried in the blood stream to the spleen. To prevent the liberation by saline of vasoactive polypeptides from the blood plasma (Schachter, 1956) the noradrenaline (1–10μg) was made up in 0·5 ml. of fresh plasma from the same cat.

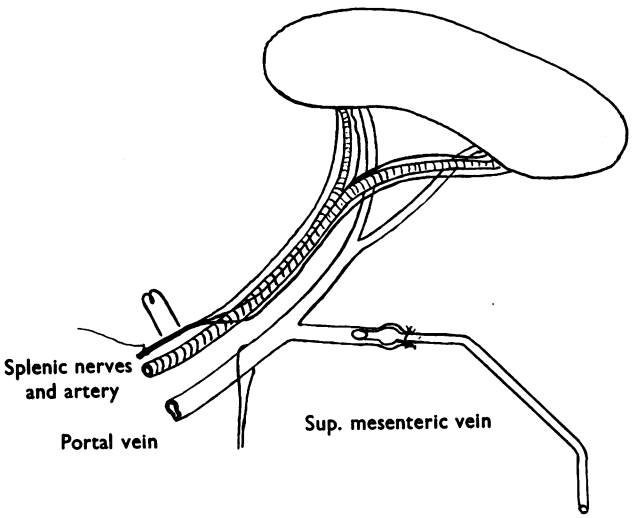

Fig. 1. A simple diagram showing the blood supply and venous drainage of the spleen when finally isolated. The position of the stimulating electrodes on the splenic nerves, and the arrangement whereby the venous blood can be diverted from the portal vein to the polythene collecting cannula, are also shown.

Two adrenergic blocking agents were used. *NN*-Dibenzyl-β-chloroethylamine (dibenamine) and the related *N*-phenoxyisopropyl-*N*-benzyl-β-chloroethylamine (dibenzyline, dibenyline). The effect of dibenamine is slow in onset and development so that it was given intravenously in doses of 20–30 mg/kg 20 hr before the experiment proper. Using this drug it was not possible to compare the output of sympathin before and after blocking the receptors, but only to compare the output in a cat which had been given dibenamine, with the average output in untreated animals. Dibenzyline acts in a way similar to dibenamine, but its effect comes on more rapidly. With this drug it was therefore possible, in the same animal, to determine the output both before and after inactivating the receptors. The dose of dibenzyline used was 5–20 mg/kg, usually 10 mg/kg. With neither drug was any difficulty experienced from the blocking agent being carried over in the cat plasma to the rat and there interfering with the assay, either by reducing the sensitivity to noradrenaline or itself having a vasopressor effect.

The rats used in the assay were in the weight range 180–240 g. They were given atropine 0·5 mg subcutaneously and then under ether anaesthesia the trachea was cannulated and the animal's

brain and spinal cord destroyed by running a skewer through one orbit and down the vertebral canal. The animal was immediately artificially respired with a small pump. The temperature was maintained at 30° C by means of a heating lamp operated by a thermistor in the rectum. The left femoral vein was cannulated, the animal heparinized and the arterial blood pressure was recorded from the right carotid artery. The cannula in the left femoral vein was attached with soft rubber tubing to a 1·0 ml. burette filled with saline. This was used to wash in drugs or plasma samples injected into the rubber tubing. The total volume injected was adjusted to 0·3 ml. In assaying plasma it was found important not to dilute the plasma if its activity was high, otherwise the vasopressor activity increased and was prolonged. The blood pressure of the pithed rat was 50–55 mm Hg, and it responded to as little as 0·5 ng of noradrenaline.

In some experiments the adrenaline and noradrenaline were extracted from the plasma, separated by paper chromatography and assayed. The method used was similar to that described by Vogt (1952). The noradrenaline eluted from the chromatogram was assayed on the pithed rat's blood pressure, the adrenaline on the rat's uterus stimulated with carbachol (Gaddum & Lembeck, 1949). In each experiment a sample of plasma, to which adrenaline 10 ng/ml. and noradrenaline 20 ng/ml. had been added, was subjected to the same process of extraction and chromatographic separation of the amines as the stimulation samples. This allowed some estimate of the losses in these processes.

RESULTS

The appearance of sympathin on stimulating the splenic nerves

Venous blood from the spleen collected before stimulation of the splenic nerves had little or no vasopressor activity. The organ, in the absence of any constrictor nerve discharge, was engorged with blood; stimulation of the splenic nerves caused a contraction of the capsular smooth muscle with a sudden increase in the venous outflow. This increase was due to the expulsion of blood, mainly red cells, stored in the spleen and was accompanied by a rise in the cell/plasma ratio. The blood flow then diminished but with a train of stimulating pulses as short as 200 the flow did not cease. After stimulation the flow remained slow for some time and then gradually returned to the pre-stimulatory rate. With stimulation a vasopressor substance appeared in the venous blood from the spleen. Previous qualitative work has shown that this is mainly, if not entirely, noradrenaline (Peart, 1949; Mann & West, 1950). This we have confirmed by chromatographic separation and separate assay of the two amines; only noradrenaline could be identified.

Effect of the frequency of stimulation on output

The results of all our experiments on the effect of frequency of stimulation on the output of noradrenaline in the venous blood are summarized in Fig. 2.

It is clear that there is a considerable variation in the output according to the frequency of stimulation. At frequencies below 10/sec no sympathin could be detected in the venous blood. At 10/sec the output was usually just sufficient to make an assay possible. This was not, of course, the lowest frequency capable of causing splenic contraction; much lower frequencies were still effective (cf. Celander, 1954). As the frequency of stimulation increased above 10/sec so the output of noradrenaline increased to reach a maximum at

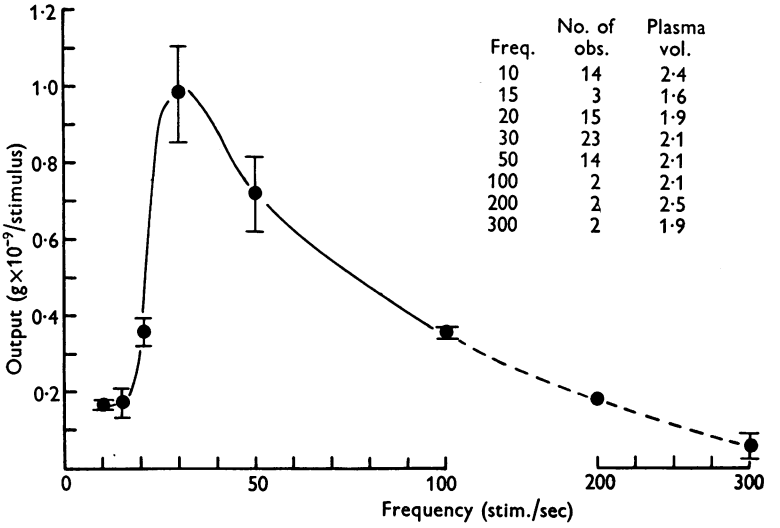

Fig. 2. The average output/stimulus of noradrenaline in the splenic venous blood of the cat after stimulation of the splenic nerves plotted against the frequency of stimulation. The vertical lines are the standard errors of the mean. The average plasma volume of the samples at each frequency is shown in the table.

about 30/sec, thereafter declining so that at 300/sec there was little detectable. The number of observations above 50/sec was limited, and attention was concentrated on the rising part of the curve between 10 and 30/sec. The difference in output between 30 and 50/sec may be a little exaggerated because more of the observations at 50/sec were in earlier experiments with a possibly less refined technique. Direct comparison in the same experiment between 30 and 50/sec showed little difference between the two, the output at 30/sec being slightly greater. The important point in Fig. 2, the great difference in the output between 10 and 30/sec, was repeatedly confirmed in direct comparison between the two frequencies in the same animal. An illustrative assay from one such experiment is shown in Fig. 3.

In comparing the output of noradrenaline at different frequencies of stimulation certain practical difficulties were encountered. The first of these was that repeated sampling of splenic venous blood in the same animal was not possible because of exsanguination. Attempts to prevent this by returning the spun red cells resuspended in isotonic saline solution led to a rise in the vasopressor activity of the control plasma. Restoring the blood volume with dextran was found to interfere with the rat assay, a finding consistent with the known toxicity of dextran for the albino rat (Briot & Halpern, 1952). After the first two or three experiments it was clear that the most effective frequency of stimulation was in the region 30/sec. In subsequent experiments, therefore,

the output at 30/sec was used as a standard against which to gauge the effectiveness of other frequencies of stimulation. In practice, the frequency under investigation was bracketed by a period of stimulation at 30/sec. By this rather cumbersome method it was possible to compare the effectiveness of various frequencies of stimulation.

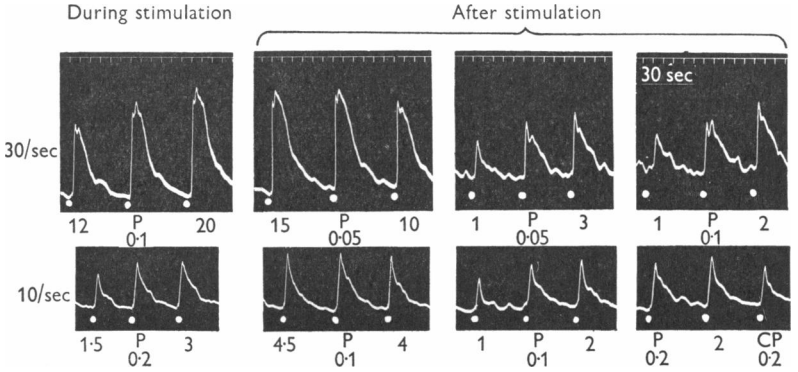

Fig. 3. Assay on the pithed rat's B.P. of plasma from the splenic venous blood (P) of a cat after stimulation of the splenic nerves at 30 and 10/sec. The volume of each injection of plasma is given in ml. Samples were collected during stimulation and in successive periods after stimulation. At 30/sec the response to plasma of each period is much greater than the corresponding response at 10/sec even though only half the volume of plasma is injected. In the final specimen the activity at both frequencies has almost returned to that of the control plasma (CP). The responses bracketing the plasma responses are to noradrenaline: the amount is shown in ng. Time marker, 30 sec.

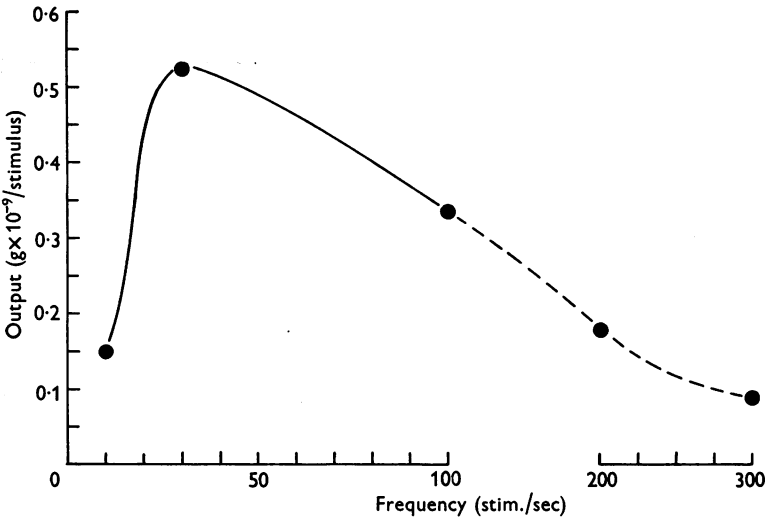

Fig. 4. The output/stimulus of noradrenaline in the splenic venous blood of one cat after stimulation of the splenic nerves at different frequencies.

On occasions, however, the blood pressure did remain high enough for us to take sufficient samples to construct in one animal an output : frequency curve similar to that of Fig. 2. Such a curve is shown in Fig. 4; the same variation in output/stimulus is present, with a peak at 30/sec.

Effect on output of the plasma volume of the venous sample

A second, and potentially more serious, difficulty arose out of experiments in which the output was measured in successive similar periods of stimulation, the object being to determine whether identical amounts of noradrenaline appeared. The splenic nerves were stimulated every 10 min with 200 stimuli at a set frequency. No blood was collected during the first period of stimulation, which was used only to empty the spleen. Samples were collected in each of the subsequent periods of stimulation, and the total output of noradrenaline

TABLE 1. Relation between the output of noradrenaline from the splenic nerves of the cat and the plasma volume of the sample. Results from two experiments are shown. In the first the plasma volume of successive samples is constant and the output/stimulus is constant. In the second the plasma volume varies and the output/stimulus varies similarly. In neither is the output related to the whole blood volume of the samples

Freq./sec of 200 stimuli	Blood vol. (ml.)	Plasma vol. (ml.)	Output/stimulus (pg)
Expt. 1			
20	6·2	1·5	210
20	4·5	1·6	220
20	3·9	1·5	245
Expt. 2			
30	12·5	4·1	1790
30	8·9	3·8	1470
30	5·3	1·4	360
30	4·2	1·8	820

was determined in each sample. At first it seemed that reproducible outputs could not be obtained. As more experiments were done it became clear that the variations in output accompanied similar variations in the plasma volume of the samples. Because of the progressive and unavoidable exsanguination of the cat these volumes usually fell. When this happened the output also invariably declined; in those experiments, however, in which successive samples of equal volume were obtained the outputs of noradrenaline were always equal within the limits of assay. On the rare occasions in which the volume of the sample rose the output correspondingly increased. Table 1 shows this dependence of output on the volume of the sample in two typical experiments out of twelve. A point of interest also brought out in this table is the lack of correlation between the *whole blood* volume of the sample and the output of noradrenaline. In each sample the animal lost proportionately more cells than plasma so that successive samples usually showed a fall in blood volume associated with a fall in the haematocrit. These changes were not necessarily reflected in any change in plasma volume.

This variation of output with the volume of the sample might have introduced a serious error into our curve of variation in output with the frequency of stimulation (Fig. 2) if the volumes of the samples at the higher frequency were consistently greater than at the low. That this could not completely explain the variation with frequency was clear from experiments in which frequencies of 10 and 30/sec produced samples of equal plasma volume but still differing greatly in their noradrenaline content. To reduce the error to the minimum in constructing the curve of Fig. 2, we excluded samples with a plasma volume less than 1 ml. since experience had shown us that below this volume the output was greatly reduced. The average volumes of plasma in Fig. 2 are similar and it therefore appears most unlikely that the shape of Fig. 2 was influenced by variations in plasma volume.

The duration of collection of samples

Once it had been shown that a variation in the output with the frequency of the nerve impulses did exist, various possible explanations had to be investigated. The first of these was the time factor of liberation. At low frequencies liberation of the transmitter took place over a longer period. It was possible therefore that the fraction of the total output collected was less at these low frequencies. The rate and duration of the output of noradrenaline at the critical frequencies of 10 and 30/sec was therefore studied. Four separate consecutive blood samples were collected during and after the stimulation period, the duration of each collection depending on the rate of flow. The concentration of noradrenaline in each sample was then assayed and the total output in each period was estimated. The results of one experiment are shown as a histogram in Fig. 5. The assay from this same experiment is illustrated in Fig. 3. At 30/sec the output is of course faster during stimulation but at both frequencies it is practically complete within 20 sec of the end of stimulation. Two other interesting points emerged from these experiments: first, that the haemoconcentration was maximal not during the stimulation period but immediately after it—very high haematocrit values, up to double the control, were found. The second was that the concentration of noradrenaline was also maximal in the first post-stimulatory period although the total output was less. This point is brought out in Fig. 3. Whether this high concentration (up to 500 ng/ml. plasma) was responsible for the reduced blood flow and high haematocrit, or was the consequence of it, cannot be stated. On the basis of these experiments, the duration of collection in all experiments was fixed as the period of stimulation, together with the subsequent 20 sec.

These experiments showed that the variations of output with frequency could not be explained on the grounds of incomplete collection of the transmitter liberated at low frequencies.

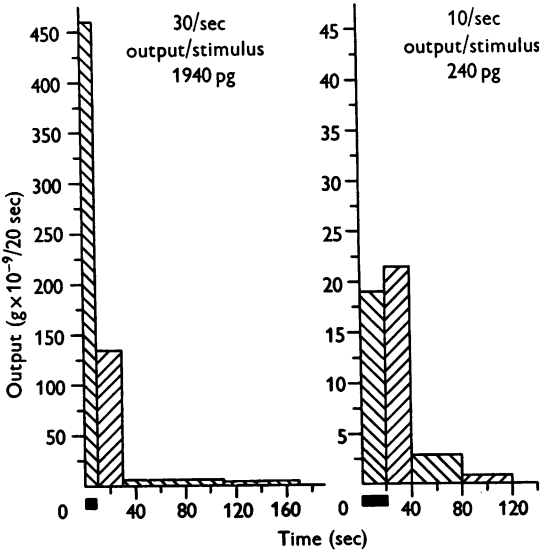

Fig. 5. Histogram of the output with time of noradrenaline in the splenic venous blood of a cat after stimulation of the splenic nerves at 30 and 10/sec. The period of nerve stimulation is shown as a solid rectangle on the time scale. There is a ten times difference in the ordinate scale at the two frequencies.

Effect of amine oxidase

Another possible explanation of the output: frequency relationship was that the destruction of the amine by monoamine oxidase varied with its rate of liberation. At high frequencies the rate of noradrenaline release might exceed the maximum rate at which amine oxidase could destroy it. The accumulating transmitter would then spill over into the blood stream where it would be protected from destruction (Bain & Batty, 1956). This possibility was investigated by studying the effect of an inhibitor of monoamine oxidase on the output of sympathin at 10 and 30/sec. *iso*Nicotinylisopropyl hydrazine (iproniazid, Marsilid, Roche Products) was given intravenously in amounts known to inhibit monoamine oxidase (Corne & Graham, 1957). No difference was found in the output of noradrenaline before and after Marsilid at either frequency. The results of one experiment are shown in Fig. 6 and Table 2. Table 2 shows that allowing for the differences in plasma volume the calculated output/stimulus was unchanged. Table 2 also shows the results of another experiment using a frequency of 10/sec to stimulate the nerves and following the effects of Marsilid for a longer period. Again the output was unaffected.

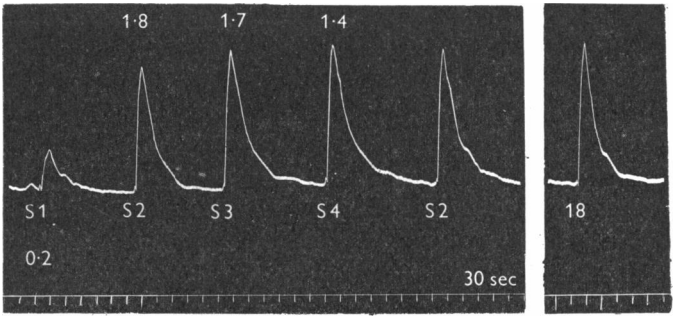

Fig. 6. Assay on the rat B.P. of plasma from the splenic venous blood of a cat. S1 is 0·2 ml. of plasma before stimulation of the splenic nerves; S2, S3 and S4, 0·1 ml. of plasma after stimulating the splenic nerves at 30/sec. Between S2 and S3 Marsilid 10 mg/kg was given to the cat to inhibit monoamine oxidase. The slight increase in the activity of the plasma after Marsilid is entirely due to the decrease in volume of the samples (shown in ml. above each response). The similarity between the response to plasma containing sympathin and to noradrenaline (18 ng) is shown. Time marker, 30 sec.

TABLE 2. The effect of an anti-amine oxidase (Marsilid) on the output of noradrenaline. The output is unaltered after this drug both at 10/sec when the output is low and at 30/sec when it is high

Expt.	Freq./sec	Output/stimulus before Marsilid (pg)	Output/stimulus after Marsilid (pg)			
			10 min	20 min	30 min	100 min
1	30	1370	1375	1130	—	—
2	10	175	—	155	135	190

The ratio of adrenaline to noradrenaline

In the experiments so far described we have assumed that the different vasopressor activity, measured on the rat blood pressure, did reflect a corresponding difference in the total amount of sympathin in the plasma. The same apparent variations might however have been due to a difference in the proportions of noradrenaline and adrenaline, since noradrenaline is a more effective pressor agent. To fit the observed variations the noradrenaline would have had to be present in higher proportion at the higher frequencies of stimulation. If noradrenaline is a precursor of adrenaline then it is feasible that with a higher frequency of stimulation the rate of liberation might exceed the maximum rate of methylation; Rapela (1956), for instance, measured the liberation of adrenaline and noradrenaline from the adrenals stimulated via the splanchnic nerve and found the percentage of noradrenaline increased with increasing frequencies of stimulation to reach a maximum at 40/sec.

This point we have investigated directly by measuring the output of the two amines separately. The amines were chemically extracted from the plasma samples, separated by paper chromatography, eluted and each assayed in

saline solution. The method is complicated and involves several transfers of dilute solutions of the amines from one vessel to another and evaporating these solutions to dryness. In our hands it was not possible in recovery experiments to get back much more than 40% of noradrenaline and adrenaline added to samples of plasma. For this reason a sample of control plasma to which adrenaline and noradrenaline had been added was subjected to the same extraction, separation, elution and assay. This gave in each experiment an estimate of the losses in the processing of the samples. The results of one such experiment were as follows. Four samples were used, control plasma before stimulating the nerves; control plasma to which adrenaline 10 ng/ml. and noradrenaline 20 ng/ml. had been added; plasma after stimulating at 10/sec and plasma after stimulating at 30/sec. In this experiment 40% of the amines added to the control plasma was recovered. The activity of the control plasma itself was negligible ($<0{\cdot}05$ ng/ml. adrenaline, $<0{\cdot}5$ ng/ml. noradrenaline). At 30/sec noradrenaline equal to 600 pg/stimulus was found which, allowing for the losses, probably represented an actual output of about 1500 pg; no adrenaline was detected. At 10/sec the noradrenaline in the eluate was just detectable; no adrenaline was found. Thus, although the output/stimulus of 15 pg was unusually low, presumably because of losses in processing, there was no evidence that at this frequency adrenaline was being liberated in place of noradrenaline.

Effects of blocking the receptors for noradrenaline

The appearance in the venous blood of amounts of noradrenaline which varied with the frequency of nerve stimulation could be explained therefore neither by variations in the fraction of the total output which was collected, nor by variations in destruction by monoamine oxidase, nor by variations in the proportion of noradrenaline and adrenaline at these different frequencies. We were left then with the possibility that the amount appearing in the venous blood either reflected exactly the amount liberated at the nerve terminals or that it was influenced by the utilization of the transmitter by the receptors on which it acts. To test this latter possibility an attempt was made to block the receptors and to determine the effect of block on the output of noradrenaline in the venous blood. Two related drugs, dibenamine and dibenzyline, were used. Dibenamine was given the day before the experiment, dibenzyline in the course of the experiment.

Dibenamine. After dibenamine the relation between noradrenaline output and the frequency of stimulation was quite altered: at 10/sec the output was increased about sixfold and equal to the average peak output at 30/sec in animals untreated with dibenamine. The output at 30/sec was little altered. The results of these experiments are shown in Fig. 7. The first part of the curve in Fig. 2 has been redrawn with an extended scale for the frequency of stimula-

tion; superimposed are the individual results for those experiments in which dibenamine had previously been given to the animal. The output/stimulus now appears equal at all frequencies up to about 50/sec. In our experiments dibenamine never abolished completely all responses of the spleen to nerve stimulation, but contraction was reduced. Whether for this reason or because vasoconstriction of the splenic arterioles was prevented, the average blood flow in these experiments was greater than in those in which the animals were not given dibenamine. This increase in the flow rate was not the explanation of the increase in the output at low frequencies. In many experiments flow rates comparable with those in untreated animals were found, yet the output was greatly increased. If repeated samples at a frequency of stimulation of 10/sec were taken until, with exsanguination, the blood flow was less than the average

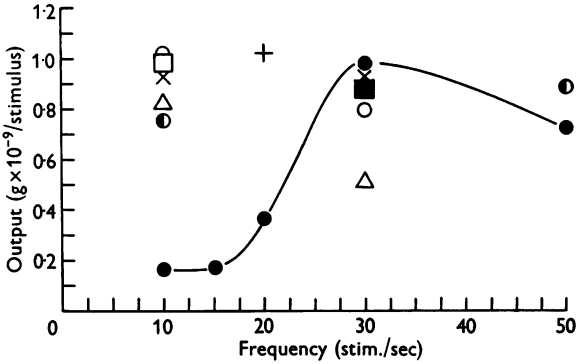

Fig. 7. Individual results for the output/stimulus of noradrenaline after dibenamine, superimposed on the graph showing the average output in untreated animals (solid line).

in untreated animals, for example, in one experiment until a plasma volume of only 0·6 ml. was reached, the output of noradrenaline still remained high. In this experiment the output/stimulus was just under 600 pg, compared with an average output/stimulus at 10/sec in animals untreated with dibenamine of 160 pg, this latter figure being based on samples restricted to those over 1·0 ml. Because of the difference in blood flow after dibenamine the rate and duration of the output of noradrenaline was investigated to see if there was any difference from the untreated animal and to ensure that our standard collection period was still applicable. The results are shown in Fig. 8. The pattern of output with time was similar with or without dibenamine and the use of the standard period of collection was justified.

Dibenzyline. The first experiment with dibenzyline was similar in design to that with dibenamine, i.e. it was given 20 hr before the experiment. The results were similar to those using dibenamine, the output at 10 and at 30/sec was equal. These results have been included with those of dibenamine in the graph of Fig. 7. Dibenzyline was next used in acute experiments to demonstrate

that the output at low frequencies of stimulation was augmented after injecting the drug. In the first experiment the output at 10/sec before dibenzyline was 195 pg/stimulus, a figure close to the average output at this frequency (160 pg/stimulus). After dibenzyline this was increased to 3100 pg/stimulus. A second period of stimulation later in the experiment gave a slightly lower figure of 2300 pg/stimulus. Such large outputs after dibenzyline prompted us to examine the output at much lower frequencies of stimulation than we had so far considered, and in this same experiment the output of noradrenaline at 1/sec was determined. This was 3000 pg/stimulus, i.e. equal to the output/stimulus at a frequency of 10/sec. Using a frequency of 1/sec the total number of pulses was reduced to 100 to avoid the loss of blood resulting from long

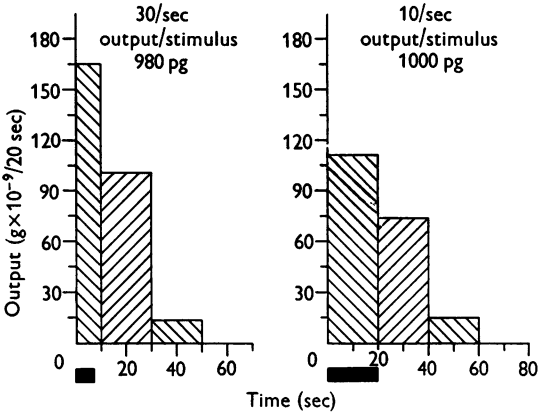

Fig. 8. Histogram of the output with time of sympathin in the splenic venous blood of a cat previously treated with dibenamine. The splenic nerves were stimulated at a frequency of 30 and 10/sec during the period shown by the solid rectangles on the time scale.

periods of collection. In the next experiment the output at 1/sec was again examined, but using smaller trains of pulses. Before dibenzyline, using a total of 20 stimuli, no sympathin was detected. Dibenzyline was given and stimulation was repeated. Noradrenaline was now present in measurable quantities, the output/stimulus equalling 500 pg. In this experiment the output/stimulus at 10/sec after dibenzyline was 700 pg; the difference between these two figures is within the error of our methods. It seemed probable that at a frequency as low as 1/sec the output/stimulus would be constant irrespective of the duration of stimulation so that even though trains of impulses shorter than the standard 200 were used the results could be compared with the output/stimulus at higher frequencies. This constancy was checked by using a train of 50 stimuli. The output/volley was 540 pg in comparison with 500 pg using 20 stimuli.

The output/stimulus at frequencies of stimulation above 50/sec in animals untreated with either dibenamine or dibenzyline falls. At frequencies of

200/sec and upwards the output/stimulus was similar to that at 10/sec. We decided to investigate the effect of blocking receptors with dibenzyline on the apparently similar output at these two frequencies. The assay from the experiment is illustrated in Fig. 9. The outputs at 200 and 10/sec before dibenzyline were respectively 225 and 105 pg/stimulus. After dibenzyline the output at 10/sec had jumped to 1595 pg/stimulus, whereas that at 200/sec had fallen slightly to 150 pg/stimulus. It is clear that the similarity in outputs between very low and very high frequencies conceals a great difference in the amount of noradrenaline liberated.

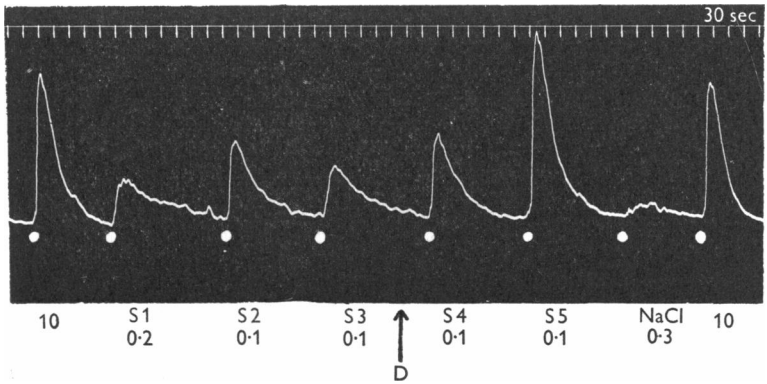

Fig. 9. Assay on the rat B.P. of the sympathin content of cat plasma after stimulation of the splenic nerves. S1 is the response to 0·2 ml. of plasma before nerve stimulation; S2 and S4 to 0·1 ml. of plasma after stimulation at 200/sec and S3 and S5 after stimulation at 10/sec. Between S3 and S4 dibenzyline 10 mg/kg was given to the cat. The activity of the plasma when the nerves were stimulated at 200/sec is unaltered; at 10/sec there is a great increase. The response to 10 ng of noradrenaline and the saline artifact are also shown. Time marker, 30 sec.

After dibenamine, although the output of noradrenaline is increased at frequencies of stimulation below 30/sec there is no apparent increase at this frequency. Several further experiments were done in which the splenic nerves were stimulated at 30/sec before and after dibenzyline to try to determine whether or not the output at this frequency was increased by blocking the receptors. The results were equivocal, sometimes there was a small increase, sometimes not. None of the increases was greater than the possible error introduced by the method of bioassay.

Arterial injection of noradrenaline

Since large quantities of noradrenaline liberated at nerve endings seemed to be removed by the tissues, and since this was prevented by inactivating the receptors, it was of interest to see whether a similar destruction of noradrenaline injected into the splenic arterial blood occurred, and whether this destruction in its turn could be prevented by receptor inactivation. The injection of

10μg of noradrenaline was needed to produce a response of the spleen similar to that produced by nerve stimulation. As we had expected, little, about 2%, of the injected noradrenaline appeared in the venous blood; over 90% of this large amount of noradrenaline had been destroyed by the tissues. Dibenzyline 10 mg/kg was then injected and, unlike its effect on the response to nerve stimulation, completely abolished the effect of a succeeding injection of 10μg of noradrenaline. Surprisingly, in the first of these experiments even less noradrenaline was recovered after giving dibenzyline; 45 ng compared with 165 ng. This was a most puzzling observation. The period of blood collection we used was based on the nerve stimulation experiments, i.e. the period of injection plus 20 sec. This might have been insufficient if the injected noradrenaline was slower in traversing the spleen than we imagined. Consequently, in the next experiment, we followed the time course of the output before and after dibenzyline. Before giving dibenzyline the noradrenaline appeared in the venous blood with a timing roughly similar to the output of sympathin when the splenic nerves were stimulated, i.e. almost all of it was collected within 20 sec of the end of the injection. After dibenzyline the whole picture was changed: noradrenaline continued to appear in the venous blood in each sample, which in this experiment meant for 2 min after stimulation, and there was no fall in the concentration of the drug between the first and last collection periods.

These experiments are difficult to interpret, but they do at least serve to show that it is not practicable by arterial injection to imitate the liberation of noradrenaline at nerve endings. The fact that the noradrenaline concentration in the venous blood after dibenzyline remains constant for such a long period may indicate a failure of the mechanism responsible for its destruction. Because of the difficulties in interpretation and the obvious lack of correspondence between injected noradrenaline and liberated noradrenaline these experiments were discontinued.

Anomalous results

In two of the sixty cats used in these experiments the results were so unusual as to require separate consideration. The preparation and condition of the animals was unexceptional, and the response of the spleen on stimulating the splenic nerves was excellent. The haematocrits of the blood leaving the spleen after nerve stimulation were the highest ever recorded, 75 and 78 respectively. Nerve stimulation, however, was not followed by the appearance of noradrenaline in the usual amounts in the venous blood. The outputs at 30/sec were 225 and 190 pg/stimulus. In the first experiment, administration of dibenzyline increased the output to 880 pg/stimulus although at this frequency there is normally no significant change in output after this drug. The low output of noradrenaline was therefore apparently due to a more complete utilization of the transmitter with its consequent destruction. We can offer no explanation of these results.

DISCUSSION

Our experiments have shown that appropriate stimulation of the post-ganglionic sympathetic fibres to the spleen causes the appearance in the venous blood of a vasopressor substance. This appears to be noradrenaline, and our results are therefore in conformity with those of other workers who have specifically pursued the identification of the splenic nerve transmitter (Peart, 1949; Mann & West, 1950). Under no circumstances has splenic nerve stimulalation been followed by the appearance of adrenaline in detectable quantities. The noradrenaline which we have found in the venous effluent from the spleen has appeared as the direct result of nerve stimulation; it is not due to the contraction of the splenic capsule expelling preformed material, because injection into the splenic artery of histamine causes a contraction of the spleen, but this is not accompanied by the appearance in the venous blood of noradrenaline. Conversely, when the nerves are stimulated after dibenzyline, the contraction of the spleen is reduced, but the output of noradrenaline is increased.

We were, initially at least, mainly interested in the effect of the frequency of nerve stimulation on the output of noradrenaline. The relationship of the output/stimulus to the frequency of stimulation was determined over a wide range of frequencies from 1 to 300/sec. The most striking variations appeared within the range 10–50/sec. In this range we found a sixfold increase in the average output/stimulus on increasing the frequency of stimulation from 10 to 30/sec. This increase could not be explained by variations in the fraction of noradrenaline collected, since experiment had shown that at both frequencies over 80% of the total output of noradrenaline was accounted for. Nor were variations in the destruction of the noradrenaline by amine oxidase the cause of the increase, because inhibition of monoamine oxidase had no effect on the output at either frequency. There was only one way in which the output: frequency relationship could be altered and that was by administering the drugs dibenamine and dibenzyline, whose action is to inactivate the receptors for noradrenaline. After either of these drugs the output at all frequencies up to about 30/sec became equal, and equal to the maximum output in untreated animals. This increase in output at low frequencies enabled us not only to identify but also to assay the output of noradrenaline at frequencies as low as 1/sec. We are now faced with the problem of accounting for the sixfold increase in output on increasing the frequency of stimulation from 10 to 30/sec and the constancy of the output in this range after dibenamine. The amount of transmitter appearing in the venous blood, which we assay, does not necessarily reflect directly the amount liberated at the nerve endings, but is the difference between the amount liberated and the amount destroyed after liberation. A simple explanation, consistent with the observed facts, is that the amount of noradrenaline liberated is constant between frequencies of

1 and 30/sec, and that the smallness of the amounts appearing in the venous blood at the lower frequencies is due to destruction of the transmitter. On this basis the interval between successive nerve volleys at frequencies below 10/sec would be sufficient for the destruction of all the transmitter liberated so that none would escape into the blood stream. Above 10/sec the interval between volleys would be insufficient for the complete destruction of the noradrenaline liberated which would therefore accumulate at the nerve endings, diffuse into the interstitial fluid and thence into the blood stream. Such an accumulation of transmitter at frequencies above about 10/sec has already been suggested by Folkow (1952) as an explanation of the delay in the onset and rate of relaxation of the smooth muscle of blood vessels after sympathetic nerve stimulation.

The constancy of the output at frequencies between 1 and 30/sec after dibenamine would be explicable on the ground that the dibenamine had prevented the destruction of the transmitter. The known effect of dibenamine is to block the tissue receptors for noradrenaline, and we must conclude therefore that combination with the receptors is a necessary prelude to the destruction and removal of liberated noradrenaline. It is, indeed, possible that the receptors themselves may destroy noradrenaline in the way suggested by Župančič (1953) for acetylcholine. Although this hypothesis can explain both the characteristic frequency:output relationship in animals untreated by dibenamine or dibenzyline and the equality of output at all frequencies up to about 30/sec after these drugs, it is surprising that the output at frequencies of 30/sec is not increased. The explanation may be in the fraction of liberated noradrenaline utilized at this frequency. If, for example, only 20% of the noradrenaline was utilized by the tissue receptors and the remaining 80% escaped into the blood stream, then prevention of the utilization could lead to an increase of only 20% in the venous blood, an increase which would not necessarily be detected by bioassay.

The idea that the tissue receptors for noradrenaline are responsible for its destruction, and that receptor inactivation results in an accumulation and overflow of transmitter, is supported by many observations. Of these, perhaps the most relevant is the finding of Cannon & Bacq (1931) that more sympathin appeared in the blood stream, as judged by its effect on the heart, when the sympathetic nerves to the hind quarters or to the colon were stimulated after a small, blocking dose of ergotamine. Further indirect evidence is provided by the observation that many drugs, which block the tissue response to adrenaline or to adrenergic nerve stimulation by acting at receptor sites, also potentiate these responses if used in low concentration. Such potentiation of the responses of several tissues has been demonstrated by Jang (1940*b*) for three typical adrenergic blocking agents, 933F, yohimbine and ergotoxine, and a similar potentiation by dibenzyline of the responses of the rat's uterus to

adrenaline has been described by Holzbauer & Vogt (1954). These anomalous effects of drugs whose action otherwise is to block receptors could be explained if the destruction of the transmitter was a function of the receptor site. The potentiating effect with small doses would imply that the activity of the receptor site in destroying noradrenaline was more susceptible to depression than was its activity as a receptor. Such discrimination between the receptor itself and the closely linked mechanism for transmitter inactivation might also explain why, in our experiments, the output of noradrenaline from the spleen increased after dibenamine or dibenzyline in spite of the fact that the response of the spleen to nerve stimulation was reduced and not abolished. A similar action might account for the well-known ability of ephedrine to potentiate the response of many tissues to adrenaline or to stimulation of adrenergic nerves (Gaddum & Kwiatkowski, 1938). These authors attributed this effect to the prevention of destruction of adrenaline, since the output of transmitter from the rabbit's ear, after stimulation of the sympathetic nerves, was increased if ephedrine was present in the perfusate. Ephedrine is known to inhibit monoamine oxidase, so that this enzyme was presumed to be the one responsible for the destruction. Ephedrine, however, is active at receptor sites, and in high concentration blocks the response to either nerve stimulation or adrenaline (Finkleman, 1930; Gaddum & Kwiatkowski, 1938; Ambache, 1951). Ephedrine has, therefore, much in common with the adrenergic blocking drugs, differing from them only in the greater discrimination between the concentration required to potentiate and that required to block the response. It is possible therefore that ephedrine also protects noradrenaline from destruction, not by inactivating monoamine oxidase, but by acting directly on the receptors for noradrenaline. Further evidence against the idea that ephedrine acts by virtue of its inhibitory effect on amine oxidase is provided by Jang's (1940*a*) finding that corbasil (dihydroxynorephedrine) a substance structurally like, and pharmacologically almost indistinguishable from, adrenaline has its action equally potentiated by ephedrine and yet is resistant to amine oxidase. Finally, several other sympathomimetic amines resembling ephedrine and adrenaline share this ability to potentiate the effects of adrenaline and nerve stimulation, but differ widely in their ability to inhibit monoamine oxidase (Jang, 1940*a*).

Although our experiments have shown that monoamine oxidase plays no apparent part in the destruction of the transmitter in the spleen, there is considerable indirect evidence associating this enzyme with adrenergic nerves (Blaschko, 1954; Burn & Robinson, 1952). The inhibitor Marsilid, which we used, has been claimed by Schayer & Smiley (1953) to halve the oxidation of intravenously injected noradrenaline. Von Euler & Zetterstrom (1955), however, have been unable to show that amine oxidase plays any part in inactivating circulating adrenaline or noradrenaline in man. Recently, Kamijo,

Koelle & Wagner (1956) have shown that the potentiating action of Marsilid on the effects of nerve stimulation and of injected noradrenaline is unrelated to its ability to inhibit monoamine oxidase.

The frequencies of stimulation needed to discharge demonstrable quantities of noradrenaline into the splenic venous blood in the absence of dibenamine or dibenzyline are probably higher than any occurring in life. According to Folkow (1952) the maximum response of blood vessels that can be produced reflexly can be matched by artificial stimulation of the efferent sympathetic fibres at 6–8/sec. At these frequencies no noradrenaline appeared in the venous blood in our experiments, which therefore confirm the view of Celander (1954) that circulating sympathin is a curiosity seen only when nerves are stimulated at frequencies much higher than those occurring in life.

We have not, so far, considered the output of noradrenaline at frequencies of nerve stimulation above 50/sec. At these frequencies the output in the untreated animal falls progressively to reach its lowest level at 300/sec, the highest frequency which we used. Treatment of the animal with dibenzyline does not increase the output. It is evident, therefore, that at these frequencies the amount of transmitter liberated at the nerve ending is diminished. The smallness of the nerve fibres constituting this post-ganglionic trunk makes it remotely unlikely that the nerve conducts a complete train of full-sized impulses at frequencies much above 50/sec, and the impulses reaching the peripheral nerve endings may be deficient either in size or number.

The increase in the output/stimulus of noradrenaline after dibenamine or dibenzyline we have attributed to the action of this drug in blocking receptor sites for noradrenaline. Although this seems the most likely explanation it is not the only one. The blood flow through the spleen after these drugs was often greater, especially with dibenamine, than that usually observed, and the response to nerve stimulation was of course diminished. That increased flow rate itself was not responsible for the increased output was clearly shown in the experiments in which the animal was repeatedly bled until the blood flow was greatly reduced. In spite of the very low flow rates the output of noradrenaline remained high. Another possibility, more difficult to deal with, is that after dibenzyline the pattern of blood flow through the spleen is altered. Such a redistribution might conceivably ensure a more effective removal of the liberated noradrenaline and thereby increase the output. This possibility we cannot exclude; it is one of the drawbacks inherent in using an organ with a circulation so complex as the spleen. It is not, however, a likely explanation; if the circulation after dibenzyline was more effective in washing out the noradrenaline liberated at the nerve endings, we would have expected some increase in the output at high frequencies.

In the course of these investigations we have had the opportunity of observing the output of vasopressor substances from the spleen both after

stimulation of the splenic nerves and after the arterial injection of noradrenaline. Gross & Schneider (1952) reported that injection of adrenaline or noradrenaline into the splenic artery of a cat released a vasopressor substance into the venous blood. This substance appeared to be noradrenaline. Stimulation of the splenic nerves at 50/sec also led to a release of this same substance. We have been unable to confirm these results. Noradrenaline injected into the splenic artery not only does not lead to a greater amount of noradrenaline appearing in the venous blood, but only a small fraction of the noradrenaline injected appears on the venous side. In our experiments the amounts of noradrenaline injected varied from ten times that injected in the experiments of Gross & Schneider down to the same amount. It is true that stimulating the splenic nerves at 50/sec as they did would release some noradrenaline into the circulation, but both this and intra-arterial injection of the amines would also cause splenic contraction with expulsion of cell-rich blood from the spleen. The increased circulatory volume and blood viscosity plus the increased peripheral resistance of a constricted spleen might well be responsible for the observed effects, an increase in blood pressure and fall in heart rate.

SUMMARY

1. The post-ganglionic sympathetic nerves to the spleen of the chloralosed cat were stimulated electrically and the output of sympathin in the venous blood from the organ was measured.

2. Chemical extraction and chromatographic separation of this sympathin from the plasma showed it to be noradrenaline. No adrenaline was detected.

3. The effect of the frequency of 200 maximal stimuli on the output of noradrenaline per stimulus was studied. At frequencies of stimulation below 10/sec no noradrenaline could be detected. At 10/sec sufficient was present to make an assay possible (average 160 pg/stimulus). With increasing frequency of stimulation the output per stimulus increased, reaching a maximum at about 30/sec when the average output per stimulus was about six times that at 10/sec (980 pg/stimulus). Above 30/sec the output fell progressively until, at 300/sec, noradrenaline was again just detectable.

4. This characteristic output:frequency relationship was not due to variations in the destruction of the transmitter by monoamine oxidase since administration of the anti-amine oxidase *iso*propylisonicotinyl hydrazine (Marsilid) did not alter the output at either frequency.

5. The output:frequency relationship could be altered by administering the adrenergic blocking agents dibenamine (*NN*-dibenzyl-β-chloroethylamine) or dibenzyline (*N*-phenoxyisopropyl-*N*-benzyl-β-chloroethylamine). After either of these drugs the output of noradrenaline at frequencies of stimulation below 30/sec was increased so that the output per stimulus at all frequencies in the

range 1–30/sec became equal. Dibenamine or dibenzyline did not increase the output of noradrenaline at frequencies over 50/sec.

6. It is suggested that the amount of noradrenaline liberated at adrenergic nerve endings by each nerve impulse is the same, at least up to a frequency of 30/sec. The amount of transmitter reaching the blood stream varies with the frequency of stimulation. At frequencies below 10/sec the interval between successive impulses is sufficient for the complete destruction of the transmitter. At higher frequencies this interval is insufficient, noradrenaline accumulates in the tissues and spills into the blood stream. At frequencies above 50/sec the decline in output per stimulus is due, not to destruction of transmitter, but to a failure of liberation.

7. The mechanism for the destruction of noradrenaline at the nerve endings is linked to the receptors for this substance and can be inactivated with them.

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