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RELEASE OF NORADRENALINE FROM THE CAT SPLEEN BY POTASSIUM

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SUMMARY

1. Cat spleens were perfused with Krebs-bicarbonate solution by means of a constant flow pump. The amount of noradrenaline released by an injection of potassium chloride solution (3·7 M) was measured. The dependence of noradrenaline released by KCl on the ionic composition of perfusion medium was determined.

2. In normal cats, the average output was 166 ng following a low dose of KCl (0·2 ml.), and 507 ng following a high dose (0·8 ml.). After phenoxybenzamine treatment, the outputs with both doses were nearly doubled.

3. In both normal and phenoxybenzamine treated cats, removal of calcium from the perfusing solution nearly abolished the release of noradrenaline. Replacing the calcium in Krebs solution restored the output. Increasing the magnesium concentration to 20 mM also markedly reduced the noradrenaline output.

4. Lowering the sodium concentration to either 25 or 0 mM increased the noradrenaline output by nearly two- to threefold. In solutions with reduced concentrations of sodium, noradrenaline was released over longer periods following KCl injections. In phenoxybenzamine treated cats, reduction of sodium in the perfusing solution also increased the noradrenaline output by nearly twofold following either the low or the high dose of KCl.

5. It is concluded that calcium ions are necessary for the release of noradrenaline from post-ganglionic sympathetic nerves following depolarization by potassium ions. It is also suggested that sodium ions are specifically needed for the reincorporation of released transmitter into the sympathetic nerve endings.

INTRODUCTION

Kirpekar & Misu (1967) have shown that removal of calcium from Krebs solution markedly reduces the amount of noradrenaline released by sympathetic nerve stimulation. They tentatively suggested that calcium

is required as a link between excitation–secretion coupling at sympathetic nerve endings. However, they could not reach a definite conclusion because it is known that in a calcium-free medium nerves initially become hyperexcitable and then conduction fails (Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser, 1957; Huxley, 1959; unpublished observations of A. L. Hodgkin & R. D. Keynes reported by Huxley, 1959). It is conceivable that the orthodromic impulses failed to reach the nerve terminals because of a conduction block, and hence were unable to release the neurotransmitter. In order to test whether calcium is required for secretion without the complication of a conduction block, we have investigated the effect of calcium removal and other ionic alterations on the release of noradrenaline produced by potassium instead of by electrical stimuli. The results reported in this paper support the earlier conclusion that calcium is necessary for the release of noradrenaline from the sympathetic nerve endings.

METHODS

Cats were anaesthetized with ether followed by chloralose (40–60 mg/kg). The spleen was perfused *in situ* according to the method described by Kirpekar & Misu (1967). Potassium chloride was injected into a cannula placed in the hepatic artery. The spleen was perfused by means of a Sigmamotor pump (Model AL4E) at a rate of about 6 ml./min. Perfusion pressure was recorded as a measure of vascular resistance.

Experimental arrangement. The standard perfusion fluid was Krebs-bicarbonate solution. In some experiments calcium was omitted or the sodium concentration was reduced, the osmotic pressure being maintained with sucrose. In other experiments the magnesium concentration was increased to 20 mM and the sodium concentration was correspondingly reduced.

Generally the spleen was perfused for about 20–30 min with Krebs-bicarbonate solution before the first potassium injection. The spleen was then perfused with the test solution for 30 min and finally with the normal Krebs solution for another 30 min. During each perfusion period the first potassium injection was made about 20 min following the beginning of perfusion. In some experiments a second potassium injection was given 10 min after the first injection.

Potassium injections. The standard procedure consisted of administering 0·2 or 0·8 ml. of 3·7 M potassium chloride solution into the hepatic artery in a single rapid injection. Such injections will henceforth be referred to as low and high potassium injections. In some experiments, animals were pre-treated with phenoxybenzamine (10 mg/kg) at least half an hour before the start of the experiment. In other experiments, spleens were perfused for 30 min with Krebs solution containing phenoxybenzamine (20 μg/ml.). The splenic venous effluent was collected in chilled tubes for 150 sec, even though most of the released transmitter appeared in the effluent within 60–90 sec. Experiments reported under different sections were repeated on three to five animals.

Assay of catecholamines. Samples were assayed either biologically on the blood pressure of the pithed rat or fluorimetrically by the method of Anton & Sayre (1962). Samples injected into the rat did not exceed 0·1 ml. since higher volumes produced a sharp transient fall in blood pressure owing to the presence of excess potassium in these samples. All results are expressed in terms of noradrenaline base.

RESULTS

Release of noradrenaline from perfused spleen by potassium injections. The effect of potassium injection on the spleen perfused with Krebs-bicarbonate solution was similar to the effect of nerve stimulation. Following potassium injection the perfusion pressure sharply increased and this was accompanied by contraction of the spleen and an initial rapid expulsion of fluid. The flow returned to the original rate within 60 sec, whereas the increase in perfusion pressure was much more sustained.

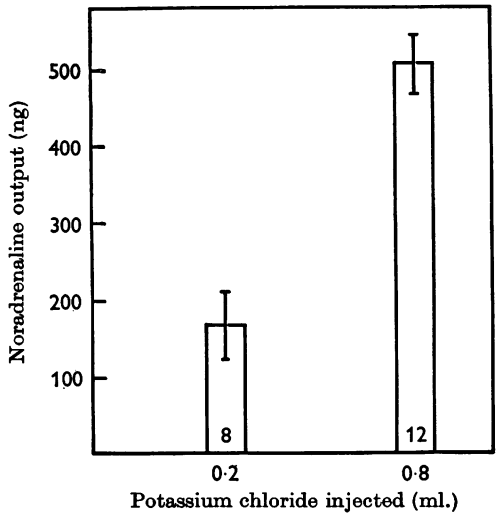


Fig. 1. Release of noradrenaline by potassium chloride injections. Noradrenaline values are expressed as ng/injection of potassium and the numbers under each column show the volume of potassium chloride (3·7 M) injected. The number of observations are shown within the columns, and the vertical lines indicate ± S.E.M.

The effect of potassium injection on the output of noradrenaline from the spleen is shown in Fig. 1. In eight experiments, the mean output of noradrenaline following the low potassium injection was 166 ± 42 ng. When the dose of potassium was increased fourfold the mean output in twelve experiments was 507 ± 39 ng. The left-hand portion of Fig. 4 shows the very fast time course of noradrenaline overflow into the perfusing fluid after injecting a high dose of potassium in a typical experiment. Most of the transmitter appeared in the perfusion fluid collected in the first 90 sec. The sample collected during the next 90 sec was almost free of noradrenaline. In a few experiments 60 sec samples were collected and the second contained variable amounts of noradrenaline. Therefore, the first sample was routinely collected for 90 sec.

Effect of phenoxybenzamine on noradrenaline release by potassium. Brown & Gillespie (1957) showed that phenoxybenzamine increased the transmitter output from spleen when splenic nerves were stimulated at low frequencies. Therefore, we did experiments to determine the effect of phenoxybenzamine on transmitter output by potassium injections. In these experiments the effects of low and high frequency stimulation are

TABLE 1. Effect of phenoxybenzamine (20 μg/ml.) on noradrenaline release from spleen by potassium

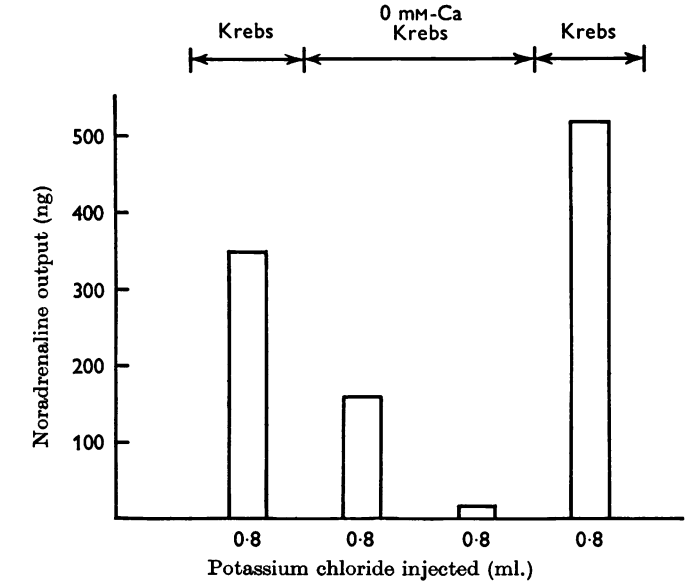
Expt. no.	Volume of KCl solution (3·7 M) injected (ml.)	Noradrenaline output (ng/injection)	
		Krebs solution	Phenoxybenzamine-Krebs solution
113	0·2	94	202
	0·8	384	813
114	0·2	232	423
	0·8	250	637
115	0·2	250	270
116	0·2	31	110
	0·8	586	455
126	0·2	660	665
	0·8	154	565
127	0·2	155	414
	0·8	435	260

mimicked by injections of low and high potassium solutions. Potassium injections were given before and after a 30-min period of perfusion with Krebs solution containing phenoxybenzamine (20 μg/ml.). The results are shown in Table 1. In four experiments the transmitter released by the low dose of potassium was nearly doubled following phenoxybenzamine. In two experiments no such effect of phenoxybenzamine was observed. Experiment 126 was rather unusual because the noradrenaline output before phenoxybenzamine in response to a low dose of potassium was very high, whereas it was low for a high dose of potassium. In three out of five experiments the transmitter output produced by the high dose of potassium was increased 2- to 3-fold by phenoxybenzamine.

Legend to Fig. 2

Fig. 2. *a*, The effect of removing calcium ions from the perfusing solution on noradrenaline output following potassium injection in phenoxybenzamine (10 mg/kg) treated cat. In calcium-free Krebs solution the noradrenaline output following potassium injection was markedly reduced. Note that following return to normal Krebs solution the noradrenaline output is greater than the initial control value.

b, The effect of excess magnesium on noradrenaline output following potassium injection in a cat pre-treated with phenoxybenzamine (10 mg/kg). In the presence of 20 mM magnesium Krebs solution the noradrenaline output following potassium injection was markedly depressed and was partially restored when the spleen was perfused with normal Krebs solution.



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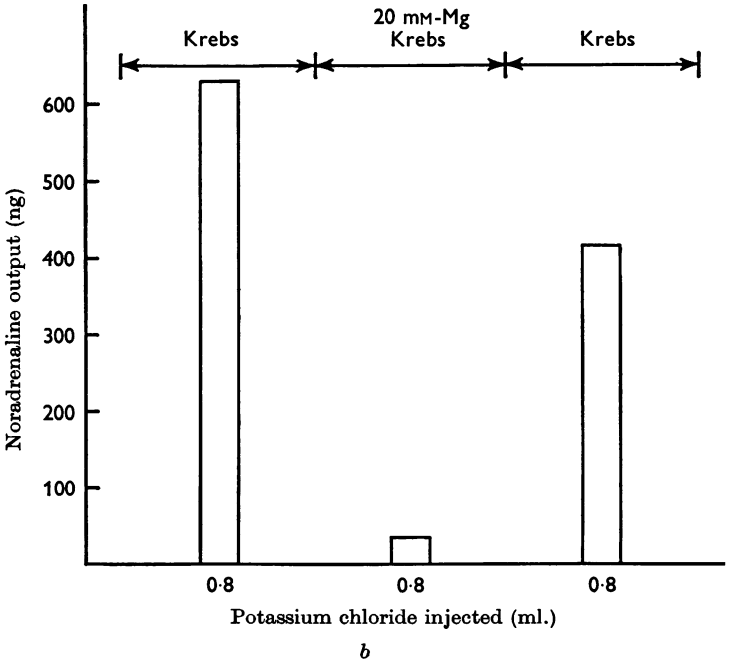


b

Fig. 2. For legend see opposite page.

In two cats pre-treated with phenoxybenzamine (10 mg/kg), the noradrenaline output produced by the low dose of potassium was considerably higher than the output in normal animals. The outputs were 401 and 669 ng.

The effect of calcium removal on the release of noradrenaline by potassium. Kirpekar & Misu (1967) showed that in calcium-free solution noradrenaline could not be released by nerve stimulation from either normal or phenoxybenzamine-treated spleens. The present experiments with potassium injections were intended to exclude the possibility that this was due to a failure in nerve conduction. In the spleen, following treatment with

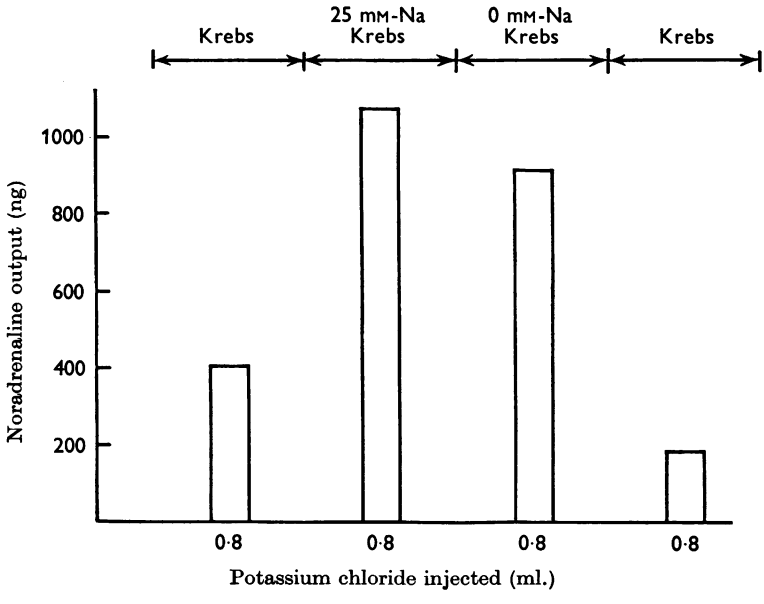
TABLE 2. Effect of perfusing Krebs solution without calcium on noradrenaline output from spleen following potassium injections

Expt. no.	Pre-treatment	Volume of KCl (3·7 M) solution injected (ml.)	Noradrenaline output (ng) in Krebs solution	Noradrenaline output (ng) in Ca-free Krebs solution	Noradrenaline output (ng) in Krebs solution after perfusion with Ca-free Krebs
49	—	0·2	32	Nil	68
50	Phenoxybenzamine (10 mg/kg)	0·8	349	158, 18	518
51		0·8	*	232, 58	789
52		0·8	1141	267, 158	811
53		0·8	622	Nil	160

* Sample spilt.

phenoxybenzamine, the rise in perfusion pressure by potassium injection was considerably reduced compared with the rise in the normal spleen. This reduction may have been due to the blockade of α adrenergic receptors by phenoxybenzamine. The background pressor activity of samples taken before stimulation either in normal or calcium-free solutions was negligible. During perfusion with normal Krebs solution, the noradrenaline output in response to a high dose of potassium was 349 ng. When the perfusion fluid was changed to calcium-free Krebs solution, this same dose of potassium released 158 ng of noradrenaline. After further perfusion for 15 min the transmitter output was decreased even more (18 ng), probably owing to more complete washout of calcium from the spleen. The effect of potassium injection on transmitter output was restored by returning to normal Krebs solution (Fig. 2*a*). Similar results were obtained in three other experiments. In one cat which had not been given phenoxybenzamine, removal of calcium from the perfusing solution eliminated noradrenaline release in response to a low dose of potassium (from 32 ng in normal Krebs to 0 ng in calcium-free Krebs) (Table 2).

Effect of excess magnesium on release of noradrenaline by potassium. Excess magnesium also decreased the release of noradrenaline by potassium in phenoxybenzamine pre-treated spleens. In the experiment illustrated



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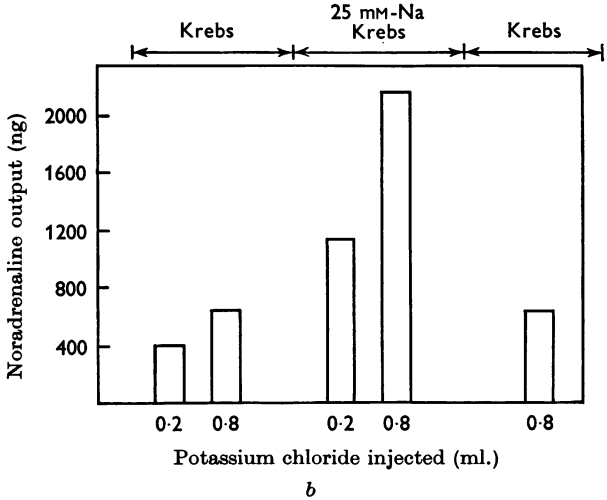

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Fig. 3. *a*, The effect of reducing sodium on noradrenaline output following potassium injection. During perfusion with Krebs solution containing 25 mM or 0 mM sodium, the noradrenaline output was increased by nearly twofold. Following return to normal Krebs, the output was very much less than the initial control value.

b, The effect of reducing sodium on noradrenaline output following potassium injection in phenoxybenzamine pre-treated cat. During perfusion with 25 mM sodium Krebs solution the noradrenaline output following low and high injections of potassium was markedly increased.

in Fig. 2*b*, the transmitter output in normal Krebs solution was 627 ng. In the presence of Krebs solution containing 20 mM magnesium the noradrenaline output was reduced to 32 ng. The output was increased to 419 ng on return to normal Krebs solution. In Krebs solution with excess magnesium, the increase in perfusion pressure due to potassium injection was less than the controls.

TABLE 3. Effect of perfusing Krebs solution containing 25 mM and zero sodium concentrations on noradrenaline output from spleen following potassium injections

Expt. no.	Pre-treatment	Volume of KCl (3·7 M) solution injected (ml.)	Noradrenaline output (ng) in sodium-deficient Krebs solution (mM-Na)			
			Krebs solution	25 mM	0 mM	Krebs solution after perfusion with Na-deficient Krebs
104	—	0·8	404	1071	913, 730	187
105	—	0·8	552	703	888, 479	180
106	—	0·8	432	—	361	Nil
123	—	0·2	330	573	—	—
		0·8	728	932	—	—
121	Phenoxybenzamine (10 mg/kg)	0·2	202	1041	318	—
		0·8	651	2008	738	255
124		0·2	401	1129	—	—
		0·8	642	2167	—	532
125		0·2	669	1029	—	—
		0·8	1286	2699	—	1165

Effect of reducing sodium concentration on release of noradrenaline. Since complete removal of sodium from the perfusing solution prevents the conduction in nerve fibres, Kirpekar & Misu (1967) could not decide whether sodium was necessary for the release of noradrenaline by nerve stimulation. When the sodium concentration was reduced to 25 mM, noradrenaline was not released by nerve stimulation and the failure of the transmitter to appear in the venous effluent was attributed to conduction block. Therefore, we conducted experiments to determine the effect of sodium deficiency on the release of noradrenaline by potassium. The results of a typical experiment are shown in Fig. 3*a*. The spleen was first perfused with Krebs solution, then with a solution containing 25 mM sodium, followed by 0 mM sodium and finally with normal Krebs solution again. The noradrenaline output in response to potassium injection during perfusion with sodium-deficient Krebs solution was about 2 times that released during perfusion with normal Krebs solution. In four experiments increments in noradrenaline output were obtained with both low and high potassium injections (Table 3).

Kirpekar & Misu (1967) also observed that the transmitter released by splenic nerve stimulation on returning to normal Krebs solution was invariably reduced by previous perfusion of the spleen with sodium-

deficient Krebs solution. We noted a similar effect of perfusion with sodium-free Krebs solution on the noradrenaline output produced by potassium injection. The transmitter output was reduced by half on returning to normal Krebs solution. However, if the spleens were perfused with Krebs solution containing 25 mM sodium instead of no sodium, the noradrenaline output in response to potassium on returning to normal Krebs solution in the final perfusion period was comparable to the output during the initial control period.

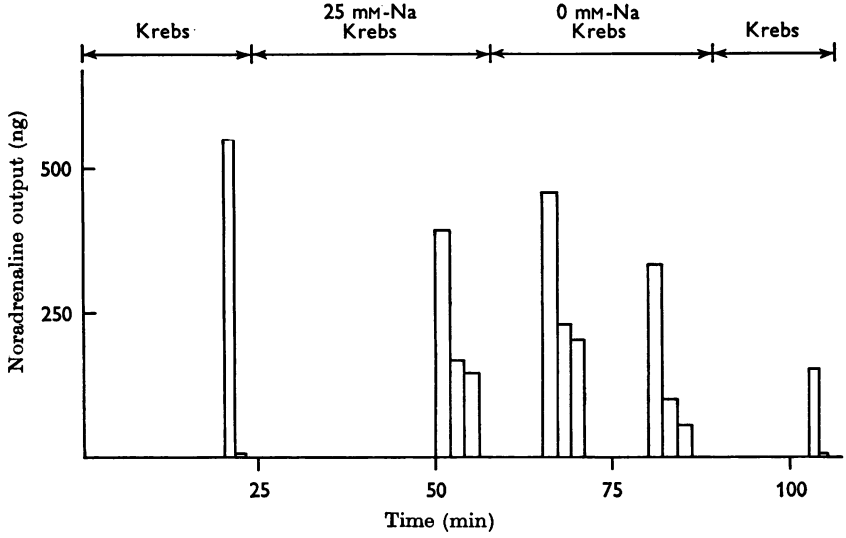

Fig. 4. Time course of the release of noradrenaline in normal and reduced sodium Krebs solution following potassium injections. Abscissa shows the time in minutes from the beginning of the experiment, and the ordinate shows the noradrenaline output following a high dose of potassium.

Not only does sodium deficiency increase the total output of noradrenaline in response to potassium injection, but it prolongs the duration of noradrenaline release. During perfusion with normal Krebs solution most of the transmitter released by potassium appeared in the venous effluent in 60–90 sec. However, during perfusion with sodium-deficient Krebs solution the transmitter output persisted for about 3–5 min. In the experiment illustrated in Fig. 4, a considerable amount of noradrenaline was present in the venous effluent during the third collection period after potassium injection in the presence of 25 mM sodium Krebs solution. In sodium-free solution potassium injection released greater amounts of noradrenaline and the output was similarly prolonged. When the spleen was once again perfused with normal Krebs solution, the same dose of potassium released considerably less noradrenaline and the recovery was

almost complete within 90 sec. The increase in noradrenaline output in sodium-free Krebs solution was not always as marked as in the experiment of Fig. 4, but it was consistently more than 2–3 times the amount released during the final period when perfusion with normal Krebs solution was restored.

During perfusion with sodium-deficient—especially with sodium-free—Krebs solution, noradrenaline was found in the venous effluent of control samples. The amount of noradrenaline present varied from zero to 25 ng/ml. In calculating the noradrenaline content of samples following potassium injections, the background pressor activity, if present, was always subtracted.

Perfusion with sodium-deficient solution produced a further increase in the perfusion pressure of about 50–100 mm Hg in about 10 min. The increase in perfusion pressure by potassium was also much more sustained in the presence of sodium-deficient Krebs solution.

Effect of sodium removal on the release of noradrenaline by potassium in phenoxybenzamine pre-treated cats. Animals were injected with phenoxybenzamine (10 mg/kg) at least half an hour before perfusing the spleen with Krebs solution. Potassium injections were given during perfusion with normal and sodium-deficient Krebs solution. The results of a typical experiment are shown in Fig. 3*b*. During the initial control period the noradrenaline outputs due to low and high doses of potassium were 401 and 642 ng, respectively. During perfusion with 25 mM sodium Krebs solution, the transmitter output was further increased by nearly threefold. Perfusion of the spleen with normal Krebs solution restored the transmitter output to the initial control level (Table 3).

DISCUSSION

We have shown that noradrenaline is released from the spleen in a dose-dependent fashion by injecting potassium into the splenic artery. Since chronic sympathectomy almost completely depletes the spleen of noradrenaline (Euler, 1946; Gillespie & Kirpekar, 1966*b*), endogenous noradrenaline is probably stored in sympathetic nerve endings. Therefore, the effect of potassium is probably due to depolarization of the nerve membrane and the subsequent release of noradrenaline stored in sympathetic nerve terminals. Thus we feel that this technique is useful for studying the release of the adrenergic transmitter in situations where the sympathetic nerves cannot be effectively activated by electrical stimulation. Since removal of calcium from the perfusion fluid markedly reduced the release of noradrenaline by potassium injection, it would appear that calcium is required in the excitation–secretion coupling at adrenergic

nerve endings. Previously, Kirpekar & Misu (1967) reported the inability of splenic sympathetic nerves to release noradrenaline following nerve stimulation when calcium was removed from the perfusing solution. The present results indicate that this failure of nerve stimulation to release noradrenaline may have been due to the inhibition of excitation–secretion coupling at the nerve ending rather than to a conduction block along the nerve axon. Recently, Katz & Miledi (1965) showed that impulses in motor nerves are propagated to the terminal arborizations even in the absence of calcium, yet acetylcholine is not released. It would therefore appear, from a number of observations (in sympathetic ganglia by Harvey & MacIntosh (1940); in neuromuscular junction by del Castillo & Katz (1954*a*, *b*) and Katz & Miledi (1965); in squid ganglia by Miledi & Slater (1966); in post-ganglionic sympathetic nerves by Kirpekar & Misu (1967)) that calcium ions are needed for the release of the transmitter at different synapses. Douglas & Rubin (1961) showed in adrenal glands a similar dependence of the catecholamine releasing process on calcium.

The decrease in the release of noradrenaline by potassium in the presence of excess magnesium probably results from a competitive antagonism between magnesium and calcium.

It was previously reported that in low sodium solutions noradrenaline was not released by stimulation of post-ganglionic sympathetic nerves. In contrast, we observed that the removal of sodium ions does not decrease but in fact increases the noradrenaline output produced by an injection of potassium. There are at least three possible explanations for this enhanced output of noradrenaline. First, it is possible that removal of sodium increases the amount of noradrenaline actually released from sympathetic nerves by potassium. The processes of excitation–contraction coupling in muscle and excitation–secretion coupling in nerves have many features in common, one of which is the requirement of calcium. Since the contractile response of cardiac muscle, for instance, increases as the ratio $[Ca^{2+}]/[Na^{+}]^{2}$ increases in the extracellular fluid (Lüttgau & Niedergerke, 1958; Niedergerke, 1963; Reiter, 1964) the secretory response may be dependent on a similar relationship of these ions. If this were so, then the same stimulus should be more effective in releasing noradrenaline in sodium-deficient solutions. However, Kirpekar & Misu (1967) showed that reducing the sodium concentration to 50 mM did not increase the transmitter output by nerve stimulation. Hence the enhanced noradrenaline release in sodium-deficient solutions is probably not due to facilitation of transmitter release. We should like to point out, however, that at low sodium and normal calcium concentrations in the perfusing solution noradrenaline leaks spontaneously in the venous effluent. It is therefore

conceivable that this release process could be further facilitated by potassium-induced depolarization.

Secondly, the increased noradrenaline output in sodium-deficient solutions may be due to the increased duration of potassium effect. One consequence of this prolonged effect would be to release more transmitter over a longer period of time without actually increasing the peak concentration. In many experiments the total amount as well as the peak concentration of noradrenaline in the first collecting period were higher. In the experiments illustrated in Fig. 3*a*, *b* the peak concentrations in sodium-deficient solutions were higher. However, in experiment of Fig. 4 the peak concentrations were not substantially different from the concentrations obtained when potassium was initially injected. But they were still greater in comparison with the last control collection period.

Finally, sodium ions may play an important role in the inactivation of the transmitter released from nerve endings. A great many investigations (Burn & Rand, 1958; Whitby, Axelrod & Weil-Malherbe, 1961; Furchgott, Kirpekar, Rieker & Schwab, 1963; Gillespie & Kirpekar, 1965; Gillespie & Kirpekar, 1966*a*, *b*) suggest that the noradrenaline released from sympathetic nerves is rapidly inactivated by reincorporation into the nerve endings. In addition, Iversen & Kravitz (1966), Kirpekar & Misu (1967) and Kirpekar & Wakade (1968) showed that sodium ions are necessary for the uptake of infused noradrenaline by the rat heart and cat spleen. Therefore, noradrenaline may be released normally in reduced sodium solutions by potassium but it may no longer be reincorporated into the nerve endings for lack of sodium. One consequence of this will be enhanced appearance of the transmitter in the venous effluent. We feel that this may be a reasonable explanation for the greater amounts of noradrenaline appearing in the venous effluent after potassium injection in sodium-deficient solutions. We would like to point out, however, that the present experiments do not completely rule out any of the three possibilities.

We showed that not only does a greater amount of noradrenaline appear in the perfusate in reduced sodium solutions but that it continues to appear in the venous effluent over a longer period after potassium injection. This finding can also be explained by the observation that sodium ions are needed for the re-uptake of noradrenaline by the nerve terminals. In normal Krebs solution, the released transmitter is efficiently removed from the receptor site by re-uptake, and diffusion is probably minimal. However, in reduced sodium solution, the released transmitter has to diffuse away from the receptor because uptake of noradrenaline is largely suppressed. Since diffusion is a relatively slower process than active uptake, this might explain the more prolonged appearance of noradrenaline in the venous effluent in reduced sodium solutions.

The transmitter output following perfusion with phenoxybenzamine was not consistently increased by low and high doses of potassium. However, the general trend was an increase in noradrenaline output. Pre-treatment of the cat with phenoxybenzamine was even more effective. These results, however, are consistent with the original observation of Brown & Gillespie (1957). The enhanced noradrenaline output has been attributed to receptor inactivation and subsequent failure of the nerve terminals to reabsorb the released transmitter (Hertting, Axelrod & Whitby, 1961; Gillespie & Kirpekar, 1965). Even in phenoxybenzamine-treated animals, reduction of sodium concentration in the perfusing medium led to an almost twofold increase in noradrenaline output by potassium. This finding was rather surprising in view of the current belief that phenoxybenzamine is an excellent blocker of noradrenaline uptake process, and it would appear from the present results that the re-uptake process is more efficiently blocked by reducing the sodium concentration in extracellular fluid. These observations would also suggest that phenoxybenzamine and low sodium probably act in different ways in preventing noradrenaline uptake into the sympathetic nerve endings.

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