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Release of noradrenaline from the cat spleen by sodium deprivation

A. G. GARCIA AND S. M. KIRPEKAR

Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Summary

1. The endogenous noradrenaline content of cat spleen slices was markedly reduced when the slices were incubated at 37° C in a medium in which sodium was replaced by sucrose, lithium, choline or potassium. Depletion of tissue noradrenaline was accounted for by its release into the incubating medium. At an external sodium concentration of 20 mM, about 50% depletion was obtained in 2 hours.

2. The enhanced release induced by sodium deprivation occurred in the absence of calcium, with or without ethyleneglycol-bis (β-aminoethyl ether) *N,N'* tetraacetic acid. Manganese potentiated release, while magnesium was without effect.

3. Ouabain caused a dose-dependent release of noradrenaline which was partially calcium-dependent. Removal of potassium from the incubation medium caused some release, which was potentiated in 25 mM sodium Krebs solution or by ouabain.

4. At 4° C, the release did not occur in sodium-free medium.

5. Dinitrophenol did not affect the loss of noradrenaline caused by sodium withdrawal. Iodoacetic acid and *N*-ethylmaleimide caused a time-dependent depletion of noradrenaline. Tetracaine caused release and partly opposed the release caused by sodium deprivation. Tetrodotoxin had no effect. Guanethidine, but not phenoxybenzamine, released noradrenaline and potentiated the release induced by sodium withdrawal.

6. The rate of release of ^{3}H-noradrenaline from reserpine-treated spleen slices was not altered by sodium withdrawal.

7. Uptake-retention of ^{3}H-noradrenaline in slices depleted of their endogenous noradrenaline content by sodium deprivation was about 60% of the control slices. This was effectively blocked by cocaine. Release of ^{3}H-noradrenaline evoked by high potassium from both control and treated slices was calcium-dependent.

8. It is suggested that sodium-potassium-activated ATPase maintains the integrity of the axonal membrane, and any procedure which depresses the activity of the enzyme or the sodium-potassium pump would cause transmitter release by causing temporary disturbance in the membrane. Evidence is presented to suggest that vesicles depleted of their endogenous noradrenaline content by sodium deprivation are re-used for the storage and release of transmitter.

Introduction

Several investigators have previously reported that sympathetically innervated tissues lose their noradrenaline content when exposed to a low sodium environment. The sodium requirement for the retention of catecholamines has been observed in the perfused adrenal gland (Douglas & Rubin, 1961), spleen (Kirpekar & Wakade, 1968a, b), and isolated heart slices (Bogdanski & Brodie, 1969 ; Keen & Bogdanski, 1970). Similarly, sodium requirement for the retention of acetylcholine has been demonstrated in the myenteric plexus of the guinea-pig ileum (Paton, Vizi & Zar, 1971) and in the motor nerves of the frog sartorius (Birks & Cohen, 1968).

The spontaneous release of endogenous noradrenaline in a sodium-deficient medium may be attributed to the failure of the uptake mechanism, since the transport mechanism for the uptake of exogenous catecholamine, and presumably the uptake of endogenous noradrenaline, is mediated by a specific sodium-dependent carrier mechanism (Iversen & Kravitz, 1966 ; Gillis & Paton, 1967 ; Kirpekar & Wakade, 1968a) located in the axonal membrane. In addition, the observation that the evoked release of noradrenaline is potentiated in a sodium-free medium (Kirpekar & Wakade, 1968a) suggests that a competition between sodium and calcium ions may exist at the sympathetic nerve endings to modulate the release of noradrenaline. Competition between these ions has been observed in the contraction of the heart (Lüttgau & Niedergerke, 1958) and the release of acetylcholine at cholinergic nerve endings (Birks, Burstyn & Firth, 1968).

The present investigation was undertaken to study the mechanism of noradrenaline release from spleen slices by sodium deprivation and included studies of uptake and release of ^{3}H-noradrenaline in spleens depleted of their noradrenaline content by prior exposure to sodium-free medium.

Methods

Cats were anaesthetized with sodium pentobarbitone (30 mg/kg) or with ether, followed by chloralose (40–60 mg/kg, i.v.). The abdomen was opened by a midline incision, and the spleen was quickly removed and placed in a Petri dish containing ice-cold Krebs–bicarbonate solution.

Preparation of slices

Spleens were cut into sections of about 0·5–0·7 mm thickness by a tissue slicer. Slices (about 200 mg) were selected randomly, washed twice in Krebs–bicarbonate solution, blotted on filter paper and weighed as quickly as possible. They were then thoroughly rinsed in the modified solution to be studied, and transferred to beakers containing 10 ml of that solution for incubation in air for different periods up to 2 h at 37° C, with continuous shaking in a water bath. Control experiments carried out in normal Krebs solution showed that incubation of slices under these conditions for up to 6·5 h only slightly depleted their endogenous noradrenaline content.

Incubation media

The normal incubation medium was Krebs–bicarbonate solution with the following composition (mM): NaCl, 119 ; KCl, 4·7 ; $CaCl_2$, 2·5 ; $MgSO_4 \cdot 7\ H_2O$, 1·2 ; $NaHCO_3$, 25 ; KH_2PO_4, 1·2 ; glucose, 11·0: and the disodium salt of ethylenediaminetetraacetic acid (EDTA), 0·03. This solution was equilibrated with 95% O_2: 5% CO_2, and the final pH was 7·4 to 7·5. Sodium-substituted Krebs solutions

contained equivalent osmolar quantities of sucrose, lithium chloride, choline chloride or potassium sulphate ; these solutions, which contained Tris buffer (1 mM) and no bicarbonate or phosphate, were equilibrated with 100% O_2. Calcium-rich solutions were prepared by adding calcium chloride up to 50 mM. In some experiments calcium was removed from the Krebs solution and ethyleneglycol-bis (β-aminoethyl ether) *N,N*[1] tetraacetic acid (EGTA, 0·1 mM) was added. Other modifications of the incubation solutions will be described under the appropriate sections.

Assay of noradrenaline and ^{3}H-noradrenaline

At the end of each incubation period slices were blotted on a filter paper and homogenized in 5 ml (final volume) of ice-cold 0·4 N perchloric acid containing 0·1% sodium meta-bisulphite and 0·05% EDTA. Incubation media were immediately cooled in ice, and acidified with perchloric acid to a final concentration of 0·2 N ; EDTA and sodium meta-bisulphite were added in a proportion similar to that used for tissues. Adsorption, elution and assay of noradrenaline were carried out following the method of Anton & Sayre (1962), as modified by Shellenberger & Gordon (1971). Recovery of standard amounts of noradrenaline in concomitant assays was $78{\cdot}6 \pm 1{\cdot}0\%$ ($n=41$). All values are corrected for recovery, and they are expressed as μg/g wet weight of tissue, or as a percentage of the noradrenaline content of slices at the start of the incubation period (0 minutes).

In experiments with ^{3}H-noradrenaline, both total radioactivity and ^{3}H-noradrenaline were measured. In analyses of total radioactivity in the tissues and the media, 0·5 ml of the perchloric acid extract was added to 10 ml of scintillation mixture, and radioactivity was counted in a 3-channel Packard Tri-Carb liquid scintillation spectrometer. In analysis of ^{3}H-noradrenaline, 0·5 ml of eluate from alumina, derived from the Shellenberger & Gordon (1971) procedure, was added to 10 ml of scintillation mixture and the radioactivity counted. All samples were corrected for quenching with an automatic external reference standard.

^{3}H-noradrenaline efflux from spleen slices prepared from reserpine-treated animals

Reserpine (Serpasil, Ciba) was administered i.p. to cats in a dose of 5 mg/kg. Spleens were removed 18–24 h later, and slices prepared. Such slices were first treated with pheniprazine (30 μg/ml) for 15 min, followed by 3 washes with normal Krebs solution over a 45-min period. They were then incubated with 60 ng/ml of (−)-^{3}H-noradrenaline (sp. act. 6·6 Ci/mmole) for 30 min, and then thoroughly washed. Three additional washes were given with normal Krebs solution over the subsequent 60-min period. After incubation with ^{3}H-noradrenaline in this way, the slices were incubated in normal or in sodium-free (sucrose) Krebs solution, and the rate of efflux of ^{3}H-noradrenaline was determined by measuring ^{3}H-noradrenaline remaining in the slices at intervals over a 2-h period.

^{3}H-Noradrenaline efflux from spleen slices previously incubated in sodium-free solution

Slices were first exposed to sodium-free (sucrose) Krebs solution for 2 h, and then washed (4 ×) with normal Krebs solution at 37° C over a 60-min period. They were then incubated with ^{3}H-noradrenaline, as previously described. After incuba-

tion with ^{3}H-noradrenaline, the slices were incubated in normal or in sodium-free (sucrose) Krebs solution, and the rate of efflux of ^{3}H-noradrenaline was studied as in slices obtained from reserpine-treated animals. The total incubation period from the beginning to the end of an experiment was 6·5 hours.

Release of ^{3}H-noradrenaline by potassium

Experiments were done on normal slices and on slices whose endogenous noradrenaline content was severely depleted by prior exposure to sodium-free medium. Slices were incubated with ^{3}H-noradrenaline according to the procedure described above. To determine background release, slices were incubated for 10 min in 10 ml of hypertonic Krebs solution made either with sucrose or sodium sulphate (140 mM) or in normal Krebs solution. Background release was comparable in both normal and hypertonic Krebs solution. To evoke release of ^{3}H-noradrenaline, slices were then exposed to 10 ml of high potassium sulphate (140 mM) Krebs solution for 10 minutes. These solutions were hypertonic since excess potassium was added to the normal Krebs solution.

In order to study the effect of calcium on the evoked release of ^{3}H-noradrenaline, slices preincubated with ^{3}H-noradrenaline (as described above) were incubated for 10 min in 10 ml of calcium-free Krebs solution. They were then transferred for the second time to 10 ml of calcium-free Krebs solution for 10 more min to deter-

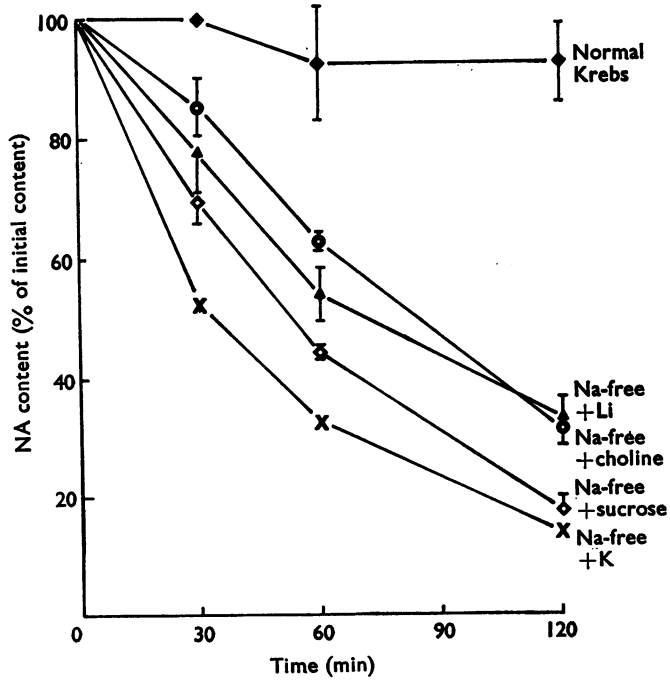

FIG. 1a. Effect of total sodium-deprivation on the noradrenaline (NA) content of spleen slices. Sodium was replaced by sucrose (✧——✧), lithium (▲——▲), choline (○——○) or potassium (×——×). The initial noradrenaline content of spleen slices in normal Krebs solution (◆——◆) has been taken as 100%, and the subsequent values at 30, 60 and 120 min, on treatment with sodium-free Krebs solution, are expressed as % of the initial content. Vertical bars show the S.E.M. of 3–6 experiments. Lowest curve with potassium was obtained from a single experiment.

mine the background release. This precaution was taken to remove any calcium contamination from the previous incubation. Finally, they were incubated in 10 ml of high potassium (140 mM) calcium-free Krebs solution for 10 minutes. The total duration of these experiments was 5 hours.

Results

The effect of sodium ions on the efflux of noradrenaline from spleen slices

The incubation of spleen slices in normal Krebs solution for 2 h did not appreciably deplete their endogenous noradrenaline content. However, replacement of the sodium in the incubation medium with sucrose, lithium, choline or potassium induced a very marked depletion of noradrenaline over the same period of time (Fig. 1a). During incubation in sodium-free (sucrose) medium, the spleen, which originally contained $1{\cdot}86 \pm 0{\cdot}28$ μg/g of noradrenaline, lost $30{\cdot}1 \pm 4{\cdot}0\%$ of its noradrenaline content during the first 0·5 h, $55{\cdot}4 \pm 1{\cdot}0\%$ in 1 h, and $82{\cdot}7 \pm 2{\cdot}8\%$ in 2 hours. Replacement of sodium with either lithium or choline had a depleting effect similar to sucrose over the first hour; however, depletion after 2 h was significantly less ($P<0{\cdot}05$) than that in the sucrose medium (about 70%, as compared to 82·7% in sucrose Krebs solution). In one experiment, first hour depletion of noradrenaline was seen to be greatest when sodium was replaced by potassium (72·5%, as compared to 55·4% in sucrose Krebs solution); by the second hour a

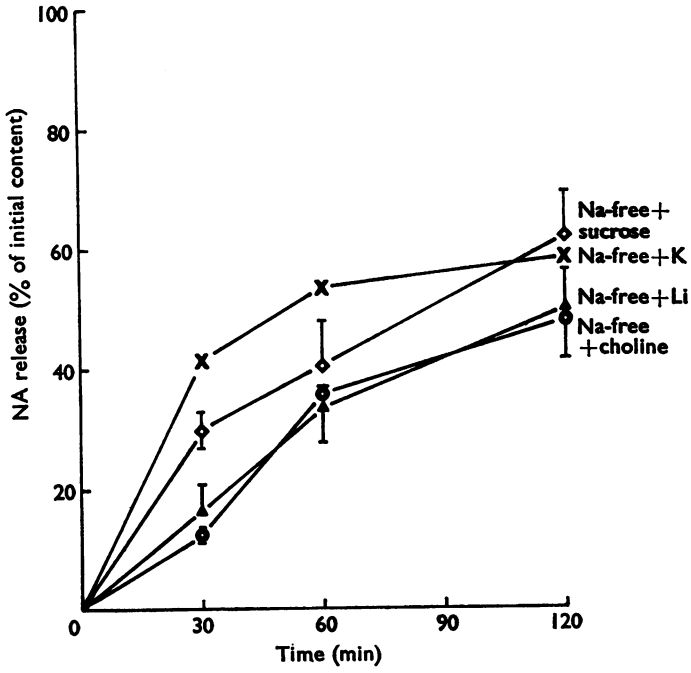

FIG. 1b. Noradrenaline (NA) release by total sodium-deprivation. Results are obtained from the same experiments shown in Fig. 1a. Sodium was replaced by sucrose (◇——◇), lithium (▲——▲), choline (○——○) or potassium (×——×). Rate of noradrenaline release in normal Krebs solution is very low and therefore has been taken as zero. The subsequent rates of release at 30, 60 and 120 min, on treatment with sodium-free Krebs solution, have been expressed as % of the initial noradrenaline content of the slices before treatment. Vertical bars show S.E.M. of 3–6 experiments. Potassium curve was obtained from a single experiment.

difference was not evident. The amount of noradrenaline lost from the tissue appears to be delivered quantitatively into the sodium-free medium. Thus, if one adds the amount of noradrenaline present in the tissue and the amount of noradrenaline present in the bathing medium (sucrose) after a 30-min incubation period, almost 100% of the endogenous noradrenaline is recovered (Fig. 1b). However, as the incubation is prolonged to 1–2 h, the recovery of noradrenaline lost from the tissue to the medium is not quantitatively accounted for. Thus, after 2 h of incubation, about 20% of the released noradrenaline appears to be metabolized, probably due to oxidation. Similar recoveries were also obtained in sodium-free Krebs solution in which choline, lithium or potassium was substituted for sodium. Oxidation of pure noradrenaline in normal Krebs solution at 37° C for 2 h is much more extensive, and it appears that removal of sodium from the bathing medium and the presence of tissue slices offer considerable protection against oxidation of noradrenaline. In consideration of these observations, we have concluded that the loss of noradrenaline from the tissue during exposure to sodium-free solution is due to release into the bathing medium.

The effect of graded sodium concentrations on the efflux of noradrenaline from spleen slices

Figure 2 shows the effect of different sodium concentrations on the loss of noradrenaline from spleen slices. As the concentration of sodium in the incubation

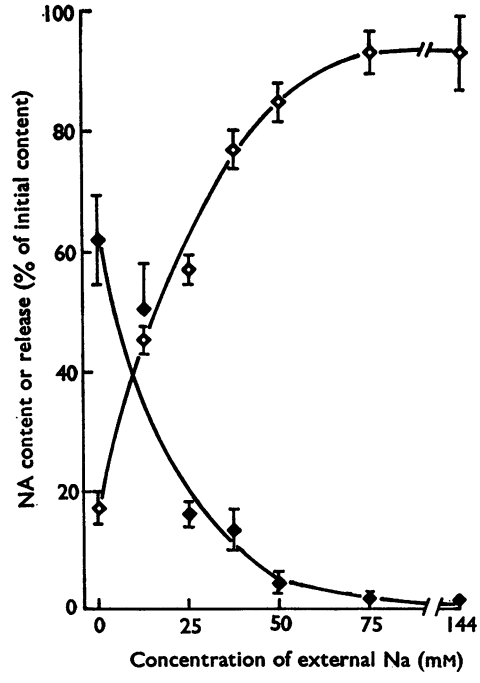

FIG. 2. Effect of graded concentrations of sodium on the content and release of noradrenaline (NA) from spleen slices. Slices were incubated in solutions containing different concentrations of sodium for 2 h, and the noradrenaline concentrations both in the tissue slices and the medium were determined. Results on noradrenaline content and release, shown by open (◇——◇) and filled (◆——◆) symbols, respectively, are expressed as % of the initial value before treatment. Vertical bars show S.E.M. of 4–6 experiments.

medium was increased from 0 mM to 144 mM, the depletion of noradrenaline was progressively diminished. It can be seen that 50% depletion was obtained at an external sodium concentration of about 20 mM, while at 75 mM sodium, practically no noradrenaline loss occurred. In most experiments the noradrenaline lost from the tissue was also quantitatively accounted for by its presence in the incubation medium (Fig. 2).

The effect of calcium on the efflux of noradrenaline induced by sodium deprivation

Since calcium is required for the evoked release of noradrenaline (Huković & Muscholl, 1962; Kirpekar & Misu, 1967) from sympathetically innervated tissues, experiments were done to determine the effect of calcium removal on noradrenaline loss induced by sodium deprivation. Figure 3a shows that removal of calcium alone, or removal of calcium plus addition of EGTA (0·1 mM), did not have any appreciable effect on the efflux of noradrenaline. Thus, slices incubated for 2 h in calcium- and sodium-free Krebs solution containing EGTA lost 81·5 ± 1·2% of their endogenous noradrenaline, which was similar to the loss that occurs in sodium-free Krebs solution containing the usual amount of calcium (2·5 mM). Even though noradrenaline loss in calcium- and sodium-free solution appeared to be slightly less than the loss in sodium-free solution alone, the results obtained from experiments with calcium-free plus EGTA solution rule out any role of calcium in this type of noradrenaline release. An increase in the calcium concentration of the bathing medium to 25 mM or 50 mM did not appreciably affect noradrenaline loss induced

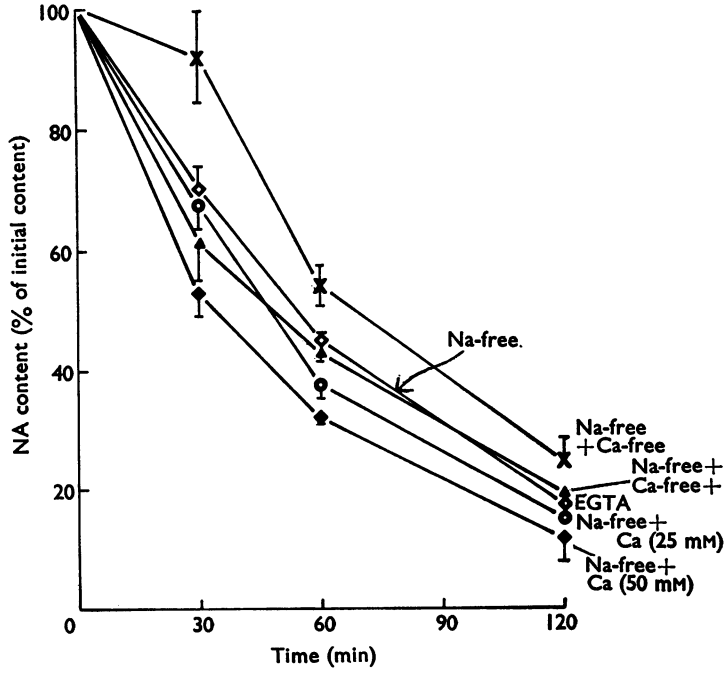

FIG. 3a. Effect of calcium on the depletion of endogenous noradrenaline (NA) by total sodium-deprivation. Note that neither lack of calcium nor excess calcium affected the rate of release of noradrenaline in sodium (sucrose)-free medium. Vertical bars show the S.E.M. of 3–6 experiments.

by sodium deprivation (Fig. 3a). However, this enhanced concentration of calcium in the bathing medium appears to give considerable protection against destruction of released noradrenaline in the incubation medium. Thus, in high-calcium solutions, noradrenaline lost from the tissue was almost quantitatively recovered, so that the sum of the noradrenaline remaining in the tissue, and that lost to the medium from the tissue, equalled 100% at each of the three incubation periods (Fig. 3b).

We also made a very curious observation concerning the protective effect of calcium against noradrenaline loss in hypotonic solutions. Thus, incubation of the slices in hypotonic solution (245 m-osmole) containing 25 mM calcium appeared to give a considerable amount of protection against the loss induced by sodium deprivation. At 30 min there is virtually no loss of noradrenaline ($98{\cdot}7 \pm 6{\cdot}7\%$ remaining in tissue), and at 2 h the loss is only $54{\cdot}7 \pm 3{\cdot}9\%$, as compared to $82{\cdot}4 \pm 2{\cdot}1\%$ in isotonic sodium-free Krebs solution containing 25 mM of calcium ($P<0{\cdot}02$, $n=6$).

Since magnesium and manganese ions compete with calcium ions for transmitter release (del Castillo & Katz, 1954; Douglas & Rubin, 1961; Kirpekar & Misu, 1967; Kirpekar, Dixon & Prat, 1970a), the effects of these ions were also investigated on noradrenaline loss due to sodium deprivation. Magnesium (25 mM) did not appreciably alter the rate of loss, whereas manganese (1 mM) potentiated it. In the presence of manganese the endogenous noradrenaline stores decreased by $60{\cdot}6 \pm 3{\cdot}8\%$ in the first 0·5 h, $82{\cdot}4 \pm 1{\cdot}2\%$ in 1 h, and $95{\cdot}4 \pm 0{\cdot}7\%$ in 2 hours.

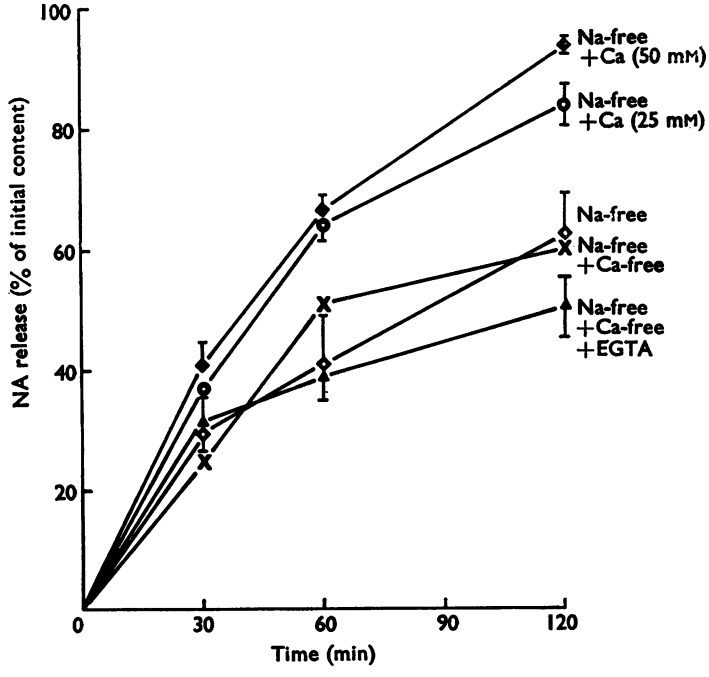

FIG. 3b. Effect of calcium on the release of noradrenaline (NA) into the incubation medium by sodium-deprivation. Data are obtained from the experiments of Fig. 3a. Note that the recovery of endogenous noradrenaline was almost 100% when the incubation medium contained excess calcium without sodium. Vertical bars show S.E.M. of at least 3–6 experiments.

Recovery of noradrenaline released in the medium was very low because of a chemical interaction of noradrenaline with manganese (Löffelholz & Scholz, 1970). In 3 experiments, manganese (1 mM) by itself caused a loss of $26{\cdot}2 \pm 2{\cdot}8\%$ in the first 0·5 h, and did not cause much further loss during the remaining 1·5 h of the incubation period ($38{\cdot}7 \pm 3{\cdot}2\%$).

The effect of ouabain on the efflux of noradrenaline from spleen slices

In normal Krebs solution ouabain causes a loss of noradrenaline in a dose-dependent manner, with a maximal release occurring at a concentration of about 10^{-5}M. When the ouabain concentration was increased from 10^{-5} to 10^{-4}M the release was not further augmented. The maximum rate of loss occurred over the first 30 min, and the tissues continued to lose noradrenaline during the remaining 1·5 h of incubation. Ouabain caused $45{\cdot}7 \pm 4{\cdot}3\%$ depletion in the first 0·5 h, and $53{\cdot}6 \pm 4{\cdot}0\%$ and $66{\cdot}3 \pm 7{\cdot}1\%$ depletion in 1 and 2 h, respectively. Depletion caused by ouabain was partially antagonized by removal of calcium, or was potentiated by addition of excess calcium (25 mM) to the incubation medium (Fig. 4). Depletion caused by ouabain was also markedly potentiated by removal of potassium from the Krebs solution (Fig. 5). Thus, the release was further enhanced to $70{\cdot}2 \pm 1{\cdot}3\%$ and $87{\cdot}5 \pm 0{\cdot}7\%$, at 1 and 2 h, respectively, in potassium-free Krebs solution.

Ouabain also enhanced noradrenaline release induced either by 25 mM sodium or total sodium deprivation. However, the results were difficult to interpret because of the severe depletion of endogenous noradrenaline caused by the combined treatment.

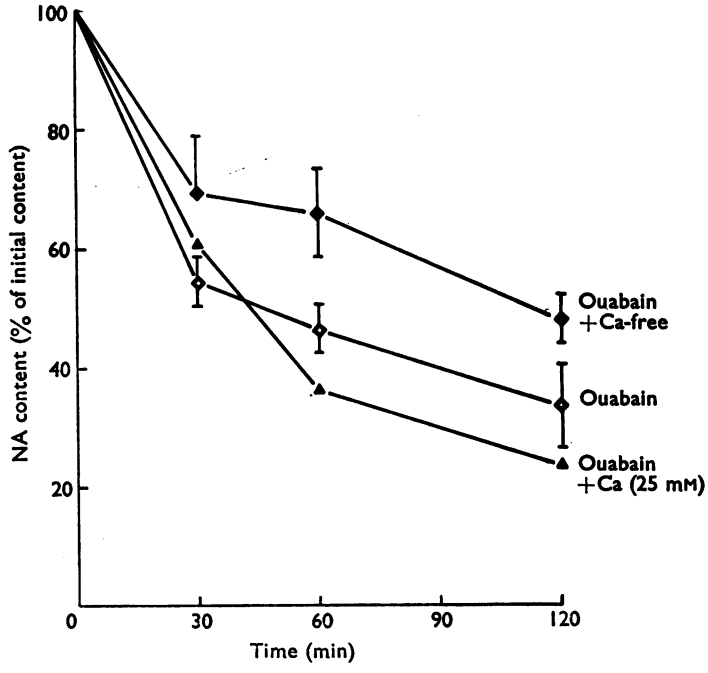

FIG. 4. Effect of ouabain on the endogenous noradrenaline (NA) content of spleen slices. Note that the depletion caused by ouabain was partially calcium-dependent. Vertical bars show S.E.M. of at least 3–5 experiments. Only 2 experiments were done to obtain the lowest curve.

The effect of potassium removal on the efflux of noradrenaline from spleen slices

Even though removal of potassium from the normal Krebs solution did not appreciably deplete the noradrenaline content in 1 h, this treatment produced about 30% depletion in 2 hours. The depleting effect of 25 mM sodium was potentiated in potassium-free solution (Fig. 5). Thus, in 2 h the tissues lost $65{\cdot}5 \pm 5{\cdot}6\%$, as against $49{\cdot}5 \pm 5{\cdot}0\%$ loss in 25 mM sodium Krebs solution. Removal of potassium also potentiated the depletion induced by ouabain (see above).

The effect of metabolic inhibitors on the efflux of noradrenaline from spleen slices

We have examined the effects of inhibitors of glycolysis and oxidative phosphorylation on release of noradrenaline. Table 1 shows that treatment with either *N*-ethylmaleimide (NEM) (5×10^{-4}M), or iodoacetic acid (IAA) (5×10^{-4}M), which inhibit glycolysis, produced a gradual decline in the noradrenaline content of spleen slices incubated in normal Krebs solution. Thus, after 2 h of incubation with NEM and IAA, $74{\cdot}5 \pm 5{\cdot}8\%$ and $70{\cdot}1 \pm 4{\cdot}6\%$ of the endogenous noradrenaline was depleted, respectively. Because of the marked depletion of the noradrenaline produced by treatment with IAA or NEM, we did not study the effects of these agents on the depletion caused by sodium deprivation. Dinitrophenol (5×10^{-4}M) did not modify the release induced by sodium deprivation.

Incubation of the slices in sodium-free Krebs solution at 4° C prevented release completely (Table 1).

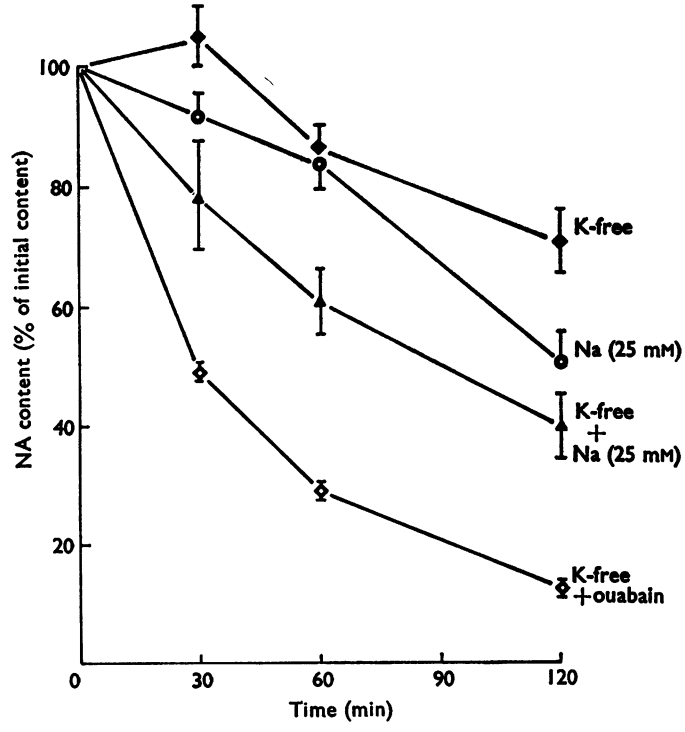

FIG. 5. Effect of potassium on noradrenaline (NA) depletion induced by low sodium (25 mM) or ouabain. In potassium-free Krebs solution the effects of low sodium and ouabain are augmented. Vertical bars are S.E.M. of at least 3–6 experiments.

TABLE 1. *Effects of various agents and temperature on the noradrenaline (NA) content of cat spleen slices incubated in normal and sodium-free (sucrose) Krebs solution*

Agent	No. of observations	Control NA content before treatment μg/g	NA content as percentage of control: 30 min after start of treatment	60 min after start of treatment	120 min after start of treatment
Control	6	1·69	100·0±13·6	92·3± 9·5	92·3± 6·5
N-ethylmaleimide (5×10^{-4} M)	3	2·11	82·4± 4·3	50·4± 2·6	25·5± 5·8
Iodoacetic acid (5×10^{-4} M)	3	1·01	78·9± 5·5	53·1± 5·4	29·9± 4·6
Dinitrophenol (5×10^{-4} M)+sodium-free Krebs	3	1·24	77·2± 2·4	47·6± 0·6	15·1± 0·9
4° C+sodium-free Krebs	5	2·06	94·9± 4·5	98·7±12·4	104·6±13·5
Tetracaine (2×10^{-4} M)	4	2·88	94·1± 5·3	78·3± 6·2	54·1± 2·5
Tetracaine+sodium-free Krebs	4	2·06	70·3± 3·1	65·7± 1·9	33·0± 1·9
Tetrodotoxin ($3\cdot13\times10^{-6}$ M)	3	2·37	100·0± 1·5	85·1± 6·1	84·9± 6·3
Tetrodotoxin+sodium-free Krebs	3	1·89	50·9± 4·0	33·9± 3·1	10·4± 0·8
Guanethidine ($1\cdot51\times10^{-6}$ M)	3	1·72	91·8± 5·7	81·5± 1·8	68·7± 5·5
Guanethidine+sodium-free Krebs	3	2·24	49·5± 2·0	30·4± 1·6	12·9± 1·5
Phenoxybenzamine ($3\cdot28\times10^{-5}$ M)	3	1·72	89·5± 0·6	93·2± 4·1	88·3± 1·9
Phenoxybenzamine+sodium-free Krebs	1	1·97	72·0	55·6	32·3

The effects of local anaesthetics on the efflux of noradrenaline induced by sodium deprivation

Table 1 shows the effects of tetracaine (2×10^{-4}M) and tetrodotoxin ($3\cdot13\times10^{-6}$M) on the efflux of noradrenaline, either in normal or in sodium-free Krebs solution. Tetracaine produced 45·9 ± 2·5% depletion of the endogenous noradrenaline content in 2 h, whereas tetrodotoxin had no effect. Tetracaine depressed the rate of noradrenaline release in sodium-free Krebs solution after 1 and 2 h of incubation by 21 and 15%, respectively ($P<0\cdot004$). Tetrodotoxin had no effect on the noradrenaline loss induced by sodium deprivation.

The effect of guanethidine and phenoxybenzamine on the efflux of noradrenaline induced by sodium deprivation

Since sodium deprivation inhibits the uptake of noradrenaline, experiments were done to study the effects of agents such as phenoxybenzamine and guanethidine, which also prevent uptake of noradrenaline (Furchgott & Kirpekar, 1963 ; Gillespie & Kirpekar, 1965 ; Iversen, 1965). Guanethidine, in addition to preventing uptake, markedly blocks the release of noradrenaline by nerve stimulation (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960). Guanethidine produced 31·3 ± 5·5% depletion of the endogenous noradrenaline content in 2 h, whereas phenoxybenzamine had no effect. Guanethidine enhanced the rate of release of noradrenaline in sodium-free Krebs solution during the first hour (69·6 ± 1·6%, as compared to 55·4 ± 1·0% loss in sodium-free Krebs solution alone). Phenoxybenzamine did not have any appreciable effect on release induced by sodium deprivation (Table 1).

The efflux of ^{3}H-noradrenaline from spleen slices of reserpine-treated animals in normal and in sodium-free Krebs solution

In order to determine whether sodium deprivation would cause loss of noradrenaline from extragranular sites ('surplus' noradrenaline, see **Discussion**), experiments were done with tissues from reserpine-treated cats. Even though

tissues of reserpine-treated animals markedly depleted of their noradrenaline content fail to retain exogenous noradrenaline, retention in such tissues can be greatly enhanced by pretreatment with a monoamine oxidase (MAO) inhibitor (Furchgott & Sánchez-Garcia, 1968). In order to increase the retention of ^{3}H-noradrenaline in spleen slices from reserpine-treated cats the slices were first exposed to pheniprazine (MAO inhibitor) before incubation with ^{3}H-noradrenaline. Efflux of ^{3}H-noradrenaline from reserpine- and pheniprazine-treated slices in normal and sodium-free Krebs solution is shown in Figure 6. During the first 0·5 h, the efflux of ^{3}H-noradrenaline in sodium-free solution was more rapid than the efflux in normal Krebs solution. From this point onwards the two rates were comparable for the rest of the incubation period, and it appears that the loss of extragranular or surplus noradrenaline is not appreciably accelerated by sodium deprivation. However, we have no explanation for the rapid loss of radioactivity during the first 30 min, when slices were first exposed to either Krebs solution or to sodium-free Krebs solution.

The uptake and release of ^{3}H-noradrenaline from spleen slices previously exposed to sodium-free Krebs solution

Since incubation of the spleen slices in sodium-free Krebs solution for 2 h markedly depletes the endogenous noradrenaline content, it was of interest to study uptake and release of noradrenaline from such slices, and to compare some of the features of uptake and release with those of reserpine-treated and normal tissues. In addition, if uptake and release of ^{3}H-noradrenaline could be shown to occur from treated slices in response to a physiological stimulus, we might get some idea

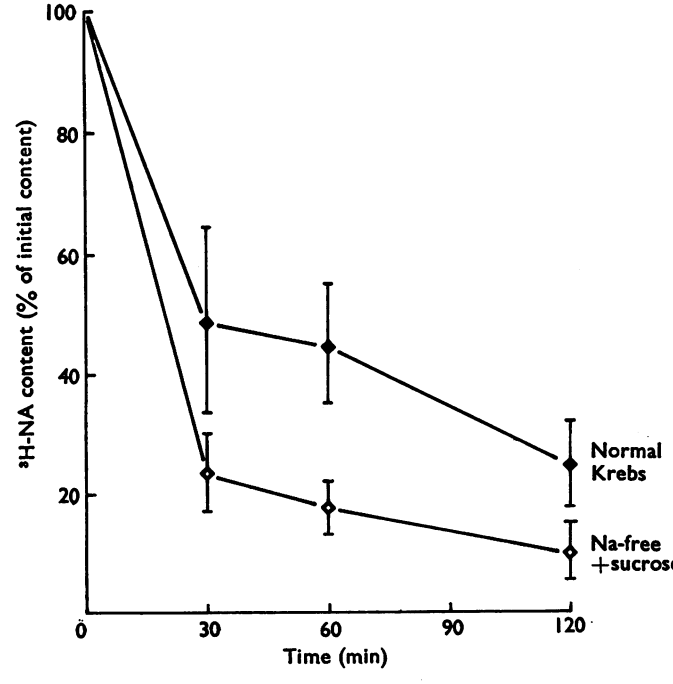

FIG. 6. Rate of loss of ^{3}H-noradrenaline from (^{3}H-NA) spleen slices of reserpine-treated cat in normal and sodium-free Krebs solution. For experimental details see **Methods.** Vertical bars are S.E.M. of 4–5 experiments.

about the re-use of the vesicle for storage and release of noradrenaline once it discharges its transmitter.

The retention of ^{3}H-noradrenaline in slices previously depleted of their noradrenaline content by exposure to sodium-free Krebs solution was 56·9 ± 4·5% of that in control slices, despite the fact that the endogenous noradrenaline content of the treated slices was less than 15% of that in the control slices (Fig. 7a, b). This retention is in marked contrast to the lack of retention of noradrenaline in tissues depleted of their endogenous noradrenaline by reserpine (Axelrod, Hertting & Potter, 1962). It should be pointed out that slices depleted of their catecholamines by sodium deprivation for 2 h continue to lose noradrenaline at a slow rate, even if they are incubated in normal Krebs solution. This might account for the reduction in the noradrenaline content from about 18% (see Fig. 1a) to 13% in the present experiments over a period of about 2·5 h, during which the slices were exposed to normal Krebs solution after their initial exposure to sodium-free solution for 2 h (see **Methods**).

In order to rule out the possibility that exposure of the spleen for 2 h to sodium-free solution did not cause irreversible tissue damage, the rate of loss of ^{3}H-noradrenaline from tissues previously depleted of their endogenous noradrenaline content by sodium deprivation was examined in normal and in sodium-free Krebs solution. Figure 7c shows that the rate of loss of ^{3}H-noradrenaline was much

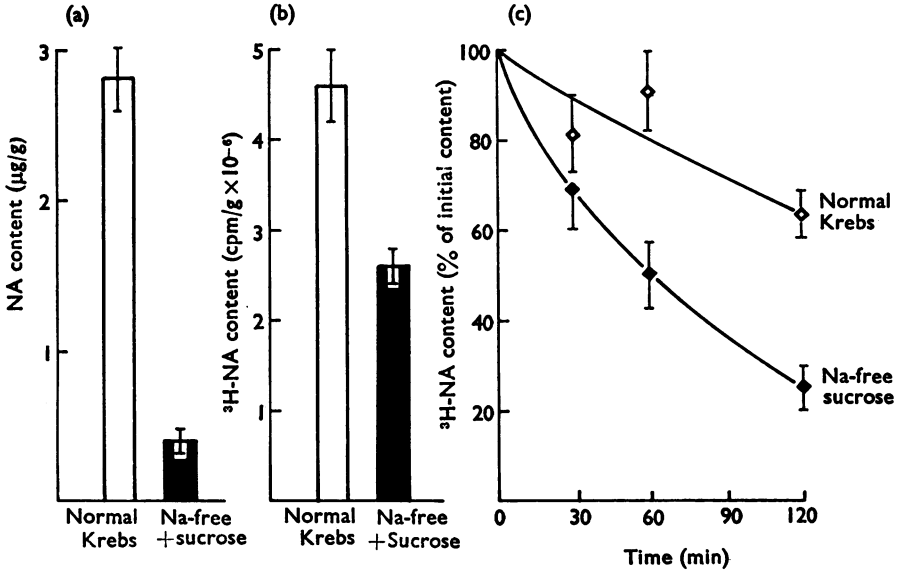

FIG. 7. Uptake and release of ^{3}H-noradrenaline from spleen slices previously exposed to sodium-free Krebs solution. Slices were first incubated for 2 h in normal or sodium-free sucrose medium and washed for 1 h with normal Krebs solution. They were then incubated with ^{3}H-noradrenaline for 0·5 h followed by 4 washes with normal Krebs solution over 1 h; (a) shows the effect of sodium deprivation on the endogenous noradrenaline (NA) content; (b) shows that retention of ^{3}H-noradrenaline (^{3}H-NA) in treated slices was considerably higher than its noradrenaline content; (c) shows that the rate of loss of ^{3}H-noradrenaline from slices whose endogenous noradrenaline content was severely depleted by sodium deprivation was much greater in sodium-free than in normal Krebs solution. Vertical bars in (a), (b) and (c) are S.E.M. of 6, 6 and 8 experiments respectively. Open columns show noradrenaline content and ^{3}H-noradrenaline uptake in control slices. Filled columns show noradrenaline content and ^{3}H-noradrenaline uptake in treated slices.

greater in sodium-free Krebs solution than in normal Krebs solution, and is comparable to the loss of noradrenaline from normal slices during exposure to sodium-free Krebs solution. The fact that the treated slices gradually lose ^{3}H-noradrenaline, even in normal Krebs solution (Fig. 7c), suggests that prolonged exposure to sodium-free solution might cause some damage to the storage of noradrenaline in sympathetic nerves.

A question arises whether the uptake of ^{3}H-noradrenaline in slices depleted of their noradrenaline content by sodium deprivation occurred in the sympathetic nerves. This could be easily tested by studying uptake in the presence of cocaine. Cocaine (1 μg/ml) blocked uptake of ^{3}H-noradrenaline by about 70% in both control and treated slices. Increase in the concentration of cocaine to 10 μg/ml almost completely blocked uptake in normal and in treated slices. Since cocaine specifically blocks uptake of noradrenaline into the sympathetic nerves of the spleen (Gillespie & Kirpekar, 1965), and since it blocked uptake in the treated slices in a similar manner, we would like to conclude that ^{3}H-noradrenaline uptake also occurred inside the sympathetic nerves after their noradrenaline content was severely reduced by sodium deprivation.

The release of ^{3}H-noradrenaline by potassium from spleen slices previously exposed to sodium-free Krebs solution

Figure 8 shows the release of radioactivity by potassium (140 mM) from normal as well as from treated slices whose noradrenaline content was severely depleted by previous exposure to sodium-free Krebs solution. Results are expressed in terms of total radioactivity released. In two experiments it was ascertained that excess

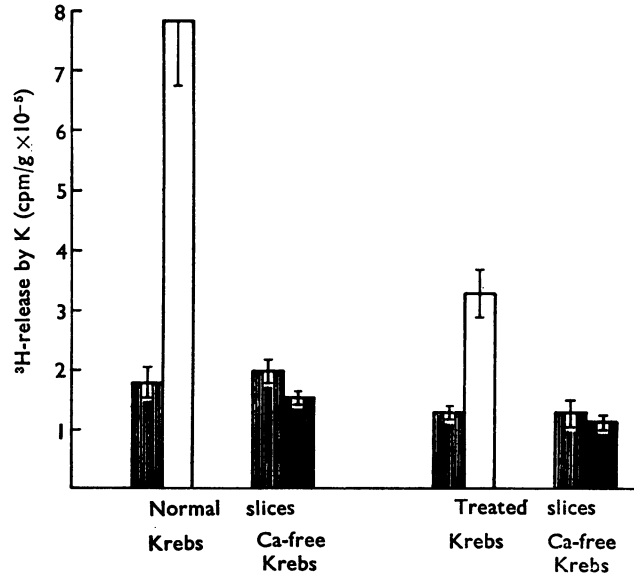

FIG. 8. The release of radioactivity induced by potassium from normal slices, and slices which were previously exposed to sodium-free Krebs solution for 2 h. Open columns show release induced by potassium. Filled columns show release in absence of calcium. Lined columns show the background release. Vertical bars are S.E.M. of 6 experiments. For experimental details, see **Methods.**

radioactivity released by potassium was due to release of ^{3}H-noradrenaline. Background activity of control samples taken before exposure to potassium was only slightly higher than the treated samples. This is probably due to the fact that the treated slices retained less ^{3}H-noradrenaline than the normal slices (Fig. 7b). Release from control and treated slices, obtained by exposing them to high potassium, amounted to $12{\cdot}8 \pm 1{\cdot}7$ and $7{\cdot}03 \pm 0{\cdot}9\%$ of their respective ^{3}H-noradrenaline contents. The amount of excess radioactivity released by potassium from normal slices was almost three times greater than that released from treated slices. This could be attributed to two factors: first, treated slices retained less ^{3}H-noradrenaline; second, prolonged exposure to sodium-free solution may cause some irreversible damage to the release mechanism. In spite of these adverse effects of sodium deprivation on uptake and release, the release from these slices was entirely calcium-dependent. Thus, the evoked release from normal and treated slices was completely blocked in the absence of calcium (Fig. 8). We conclude, on the basis of these experiments, that vesicles depleted of their endogenous noradrenaline content by sodium deprivation can take up exogenous noradrenaline and subsequently release it in a physiological manner in response to potassium.

Discussion

Experiments described in this paper show quite clearly that a sympathetically innervated organ such as the spleen rapidly loses its adrenergic transmitter when exposed to sodium-free solution. Replacement of sodium by sucrose, lithium, choline or potassium had qualitatively similar depleting effects. The rate of depletion of noradrenaline in sodium-deprived Krebs solution (sucrose being substituted for sodium) was related to the sodium concentration in the medium, and about 50% depletion was obtained after a 2 h incubation in an external sodium concentration of 20 mM. Replacement of the sodium with potassium, if anything, augmented release.

Depletion of endogenous noradrenaline caused by sodium deprivation is almost completely due to release, since the amount of transmitter lost from the tissue appears quantitatively in the incubating medium. It is unlikely that there is any significant synthesis of endogenous noradrenaline in the slices during the incubation period, since Blakeley & Brown (1963) have calculated the rate of noradrenaline synthesis in spleen to be only 0·3 (μg/h)/spleen.

Sodium deprivation appears to have rather a specific effect on endogenous noradrenaline, which is normally stored in small granules. This conclusion is based on the observation that the rate of release of ^{3}H-noradrenaline from reserpine-treated spleens was not appreciably altered by sodium deprivation. Reserpine-treated tissues are capable of storing exogenous noradrenaline after MAO inhibition, yet electrical stimulation of the sympathetic nerves of such tissues does not release noradrenaline (Furchgott, Kirpekar, Rieker & Schwab, 1963), presumably because exogenous noradrenaline present in the cytoplasm outside the granules is not available for the evoked release. Reserpine-treated spleen therefore serves as a useful tool to study the effect of sodium deprivation on 'surplus' noradrenaline, which is probably analogous to surplus acetylcholine, normally destroyed by acetylcholine esterase, and which is also not available for release (Birks & MacIntosh, 1961).

A number of observations made in this study suggest that the release of noradrenaline induced by sodium deprivation is different from the release of noradrenaline evoked either by nerve stimulation or by potassium. Evoked release of noradrenaline is calcium-dependent (Huković & Muscholl, 1962 ; Burn & Gibbons, 1965; Kirpekar & Misu, 1967 ; Boullin, 1967), whereas noradrenaline release induced by sodium deprivation appears to be calcium-independent. Incubation of splenic slices in sodium- and calcium-free solution containing EGTA caused loss of noradrenaline at the same rate as that from slices incubated in sodium-free Krebs solution alone. Addition of manganese, which presumably opposes calcium entry into the neurone to inhibit transmitter release (Katz & Miledi, 1969 ; Kirpekar *et al.*, 1970a), also did not modify release caused by sodium deprivation, but appeared to potentiate it. Guanethidine, which blocks the evoked release of noradrenaline (Thoenen, Huerlimann & Haefely, 1966 ; Kirpekar, Wakade, Dixon & Prat, 1969), also did not prevent loss, but slightly potentiated it. However, Blaszkowski & Bogdanski (1971) have observed that the release of ^{3}H-noradrenaline from heart slices incubated in sodium-free medium containing choline was inhibited if calcium in the bathing medium was eliminated. On the basis of this observation, the authors have suggested a possible interaction between calcium and sodium ions in the release of noradrenaline in adrenergic nerve endings. We did not observe such a protective effect of calcium removal on loss of noradrenaline in sodium-free Krebs solution containing sucrose. By the same token, we did not observe any further loss of noradrenaline in sodium-free Krebs solution when the calcium concentration was elevated. Paton *et al.* (1971) have also reported that removal of calcium from the incubation medium in which sodium was replaced by sucrose, lithium or trometamol did not protect against loss of acetylcholine from guinea-pig intestine. From evidence such as this, it appears to us that calcium does not play a significant role in the release of noradrenaline induced by sodium deprivation.

Prevention of re-uptake of endogenous noradrenaline also does not seem to be a contributing factor in the loss of noradrenaline mediated by sodium deprivation. Inhibition of uptake by phenoxybenzamine neither caused appreciable loss of noradrenaline from normal tissues nor modified the rate of loss induced by sodium deprivation.

Release of noradrenaline by sodium deprivation appears to be independent of metabolic energy derived by oxidative phosphorylation, since dinitrophenol does not have any significant effect of its own on depletion (Kirpekar, Prat & Yamamoto, 1970b), nor does it modify the rate of loss of noradrenaline induced by sodium deprivation. Inhibitors of glycolysis, such as IAA or NEM, produced significant depletion of endogenous noradrenaline stores, yet prolonged glucose deprivation has been previously found not to cause any loss of endogenous noradrenaline in guinea-pig isolated atria (Wakade & Furchgott, 1968). Hence, IAA and NEM may be causing noradrenaline loss by a mechanism other than inhibition of glycolysis. It is interesting to note that the release of noradrenaline evoked by nerve stimulation, or by potassium which was reduced by treatment with *p*-chloromercuribenzoate, IAA or NEM, was not appreciably affected when only oxidative metabolism was blocked by nitrogen or dinitrophenol (Kirpekar *et al.*, 1970b).

Depletion of noradrenaline by incubation in sodium-free solution was completely inhibited when the incubation was carried out at 4° C. This inhibition is probably due to a marked slowing at this low temperature of those cellular processes that

are initiated by exposure to a sodium-free solution and which result in a rapid loss of noradrenaline at 37° C.

Loss of noradrenaline in sodium-free Krebs solution appears to be related in some way to the inhibition of sodium-potassium-activated ATPase in sympathetic nerve endings. Thus, removal of sodium or its replacement by lithium or choline in the external medium would indirectly depress the activity of the sodium-potassium pump (Skou, 1957) as the sodium concentration inside the cell is reduced. Since the demonstration by Schatzmann (1953) that cardiac glycosides specifically inhibit cation transport, several investigators have shown their effectiveness against ATPase in a number of organ systems (Skou, 1957; Dunham & Glynn, 1961; Bonting, Caravaggio & Hawkins, 1962). NEM and *p*-hydroxymercuribenzoate also inhibit ATPase (Skou, 1963; Rendi, 1965; Banerjee, Wong, Khanna & Sen, 1972). It is also known that ATPase activity depends on potassium (Skou, 1957), and that the efflux of sodium is reduced in the absence of external potassium ions (Hodgkin & Keynes, 1955). Tetracaine, a local anaesthetic, may slow down the pump activity indirectly by blocking the movement of cations across the cell membrane. Since all these procedures that are known to inhibit the sodium-potassium-activated ATPase activity in a variety of tissues induced release of noradrenaline, it is reasonable to assume that inhibition of ATPase activity in the sympathetic nerves would also lead to release. Paton *et al.* (1971) have also found that ATPase inhibitors cause a marked release of acetylcholine from the plexus of guinea-pig ileum longitudinal strips and have proposed that physiological release of acetylcholine is also mediated by inhibition of ATPase activity. However, it appears to us that the integrity of the axonal membrane probably depends upon the ATPase activity, and once it is inhibited the intragranular noradrenaline diffuses out of the nerve endings.

It is well established that treatment with reserpine causes a marked depletion of catecholamines, and a reduction in the retention of infused noradrenaline in sympathetically innervated organs. Even though reserpine-treated tissues pretreated with a monoamine oxidase inhibitor retain noradrenaline following exposure to exogenous noradrenaline, they fail to release it in response to electrical stimulation of sympathetic nerves (Furchgott *et al.*, 1963). Wakade & Kirpekar (1971) showed release of ^{3}H-noradrenaline from reserpine-treated guinea-pig vas deferens by high potassium but this release was calcium independent, in contrast to the release of noradrenaline from normal vas deferens and spleen which is calcium dependent. Splenic slices depleted of their adrenergic transmitter by sodium deprivation were able to accumulate ^{3}H-noradrenaline from the normal solution. Retention of exogenous ^{3}H-noradrenaline in such tissues amounted to nearly 60% of the retention that occurred in control tissue slices. It is interesting to note that even though the treated slices contained only one-tenth the amount of endogenous noradrenaline of the control slices, uptake was still very high. This observation suggests that vesicles which were depleted of their transmitter are reutilized for the storage of exogenous noradrenaline. This suggestion is strengthened by the observation that an increase in the potassium concentration of the Krebs solution induced the release of ^{3}H-noradrenaline from severely depleted slices and that this release was entirely calcium-dependent. It therefore appears that vesicles depleted of their endogenous transmitter by sodium deprivation are capable not only of taking up exogenous noradrenaline, but also of releasing it in response to a stimulus. We cannot be

sure, however, whether vesicles which empty their contents in response to nerve stimulation are also similarly reutilized for the storage and release of its transmitter.

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