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RELEASE OF NORADRENALINE FROM SLICES OF CAT SPLEEN BY PRE-TREATMENT WITH CALCIUM, STRONTIUM AND BARIUM

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SUMMARY

1. Spleen slices pre-incubated for different periods at 4° C in Krebs solution containing varying concentrations of calcium, up to 96 mM, lost their endogenous noradrenaline stores when reincubated in normal Krebs solution at 37° C for 2 hr. Rate of loss of noradrenaline was roughly related to the calcium concentration of the pre-incubation medium and the pre-exposure time.

2. Pre-treatment with isotonic barium or strontium (96 mM) Krebs solution also induced release of noradrenaline from spleen slices when re-exposed to normal Krebs solution. Barium was more effective than either calcium or strontium.

3. The enhanced release induced by calcium pre-treatment occurred in the absence of calcium, with or without EGTA.

4. Tissue calcium concentration of spleen slices was 0·68 m-mole/kg. Pre-treatment of slices with normal or 96 mM calcium-Krebs solution for 4 hr at 4° C increased the calcium concentration to 2·57 and 9·9 m-mole/kg, respectively.

5. Ouabain, which caused a dose-dependent release of noradrenaline, did not modify the release induced by calcium pre-treatment.

6. Spleen slices prepared from cats anaesthetized with sodium pentobarbitone instead of ether were resistant to noradrenaline depletion by calcium pre-treatment.

7. Evoked release of [^{3}H]noradrenaline by high potassium from calcium-pre-treated slices did not occur in the absence of external calcium, even though the calcium pre-treatment enhanced the tissue concentration of this ion by nearly tenfold.

8. Net uptake of noradrenaline in normal and in treated slices whose noradrenaline content was severely reduced by barium pre-treatment or sodium withdrawal was comparable.

9. Specific activity of released and endogenous [^{3}H]noradrenaline increased as the tissue stores of noradrenaline were reduced.

10. It is suggested that the spontaneous loss of tissue noradrenaline after pre-treatment with high-calcium solution was due to inhibition of sodium-potassium-activated ATPase by intracellular accumulation of calcium ions. Evidence is presented to suggest that vesicles depleted of their endogenous transmitter by pre-treatment with calcium, strontium or barium, or by sodium withdrawal, are re-used for the storage and release of exogenous noradrenaline.

INTRODUCTION

A reduction in the calcium ion concentration of the external medium leads to a decrease in the amount of noradrenaline released by nerve impulses or by high potassium. Kirpekar, Prat, Puig & Wakade (1972) were unable to show a direct stimulating effect of calcium ions on the release of noradrenaline from sympathetic terminals in cat spleen without electrical stimulation. This observation suggested to us that the entry of calcium specifically required for transmitter release could only occur from outside during depolarization. Attempts were then made to determine whether transmitter release could be sustained in the absence of calcium ions in the external medium, in tissues previously loaded by exposure to high concentrations of calcium.

The unexpected finding of the experiments reported in this paper was that increased tissue calcium concentration induced spontaneous release of noradrenaline, yet release induced by high potassium remained dependent on extracellular calcium. Since calcium pre-treatment depleted the endogenous tissue stores of noradrenaline, additional studies on the uptake-retention and release of exogenous noradrenaline were carried out on such tissues to investigate the possibility of re-use of synaptic vesicles for storage and release of transmitter.

METHODS

Cats (about 2 kg) were anaesthetized with ether. The abdomen was opened by a mid line incision, and the spleen was quickly removed and placed in a beaker containing ice-cold Krebs-bicarbonate solution. In one group of experiments spleens were isolated from cats anaesthetized with sodium-pentobarbitone (30–40 mg/kg).

Preparation of slices. Spleens were cut into thin slices of about 0·5–0·7 mm thickness by a McIlwain tissue slicer. Slices (total wet weight about 200 mg) were washed 2–3 times in ice-cold Krebs-bicarbonate solution, blotted on a filter paper, and weighed as quickly as possible. They were then thoroughly rinsed in the respective modified solution, and finally transferred to beakers containing 10 ml. of that solution for pre-incubation at 4° C for different periods up to 24 hr.

At the end of the pre-incubation period, slices were transferred to beakers containing cold normal Krebs solution and washed repeatedly to remove extracellular

calcium, strontium or barium. Then slices were incubated in normal Krebs solution for different periods up to 2 hr at 37° C in an atmosphere of 95 % O_2–5 % CO_2 under continuous shaking in a water bath. At the end of each incubation period slices were blotted on filter paper and homogenized in cold 0·4 N perchloric acid (PCA) containing 0·1 % sodium metabisulphite and 0·05 % ethylenediamine tetra-acetate (EDTA).

Incubation media. Normal incubation medium was Krebs–bicarbonate solution having the following composition (expressed in mM): NaCl, 119; KCl, 4·17; $CaCl_2$, 2·5; $MgSO_4.7H_2O$, 1·2; $NaHCO_3$, 25; KH_2PO_4, 1·2; glucose, 11; and EDTA, 0·03. This solution was equilibrated with 95 % O_2–5 % CO_2, and the final pH was 7·4–7·5. Pre-incubation media contained calcium, strontium or barium in different concentrations up to 96 mM; sodium was omitted, and when necessary, tonicity was maintained with lithium chloride. Sodium-free solution was prepared by replacing all sodium by equiosmotic amounts of sucrose. Pre-incubating and sodium-free solutions contained Tris buffer (1 mM) in place of bicarbonate and phosphate, and were equilibrated with 100 % O_2; the pH was adjusted to 7·4 with HCl (1 N). In some experiments calcium was removed from the Krebs solution and ethyleneglycol bis β-aminoethyl ether N,N′ tetra-acetic acid (EGTA, 0·1–1 mM) was added. Other modifications of the incubating solutions will be described under appropriate sections.

Assay of noradrenaline and [^3H]noradrenaline. Adsorption, elution and assay of noradrenaline in tissue extracts were carried out according to the procedure of Shellenberger & Gordon (1971). 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 min). [^{3}H]noradrenaline measurements were made as previously described (Garcia & Kirpekar, 1973).

Uptake-retention of noradrenaline and [^3H]noradrenaline. In the first set of experiments, slices (total wet weight about 1 g) were first incubated in 20 ml. normal, high-calcium or high-barium (96 mM) Krebs solution at 4° C for 4 hr, followed by a 2-hr incubation in 20 ml. normal Krebs solution at 37° C. Slices were then exposed to [l-^{3}H]noradrenaline, 60 ng/ml. (specific activity 6·6 c/m-mole, New England Nuclear) for 30 min and thoroughly washed with fresh Krebs solution. Three additional washes were given over the subsequent 1-hr period, and about four to five slices (100–150 mg total) from each group were removed to determine their noradrenaline and [^{3}H]noradrenaline contents. The remaining slices were used for studies of cold (endogenous) as well as labelled (exogenous) noradrenaline release as described below.

In a second set of experiments, slices (total wet weight about 1 g each group) were treated in the following manner: one group was incubated for 3 hr in normal Krebs solution at 37° C; the second group was incubated in sodium-free (sucrose) solution for 2 hr at 37° C, followed by 1 hr in normal Krebs (four washes); the last group of slices was preincubated in high barium (96 mM) solution for 24 hr at 4° C, followed by 2 hr in normal Krebs at 37° C (two washes). After these pre-treatments the tissues were incubated with noradrenaline (10^{-5} g/ml.) for different periods up to 6 hr, fresh solution (containing the amine) being replaced every hr. At various times, four to five slices (total wet weight about 100–150 mg) were removed from each of the three groups and transferred to a beaker containing 10 ml. normal Krebs solution, thoroughly rinsed, washed 4 times over a period of 1 hr, and finally blotted, homogenized and assayed for their noradrenaline content (procedure used to obtain data of Fig. 8).

Release of noradrenaline and [^3H]noradrenaline. For the study of the release of [^{3}H]noradrenaline, tissues were preloaded with [^{3}H]noradrenaline as follows: about 2 g of slices were pooled in one beaker containing 20 ml. normal Krebs-bicarbonate

solution. After 15 min of equilibration at 37° C, slices were loaded with [^{3}H]noradrenaline as described under the uptake-retention section. After labelling the endogenous noradrenaline stores in this manner, groups of four to five slices (about 100 mg wet weight) were pre-incubated in 10 ml. of zero, 2·5, or 96 mM calcium-Krebs solution at 4° C for 4 hr. For each concentration of calcium in the pre-incubation medium, slices were divided into four groups for release studies as follows: (1) background release of [^{3}H]noradrenaline determined in normal Krebs solution for 5 min. Preliminary experiments showed that exposure of the slices to hypertonic solution (210 m-osmole over the tonicity of normal Krebs solution), made by adding either sucrose or sodium chloride, caused no further increase in the background release; (2) release of [^{3}H]noradrenaline evoked by incubating the slices for 5 min in Krebs solution containing 140 mM potassium (as potassium sulphate); (3) release by potassium in the absence of calcium; and finally; (4) release by potassium in the absence of calcium with EGTA (1 mM) (procedure used to obtain data of Fig. 7).

In order to measure specific activity of released noradrenaline (Table 1, Results), about 1 g (instead of 100–200 mg) of slices was used. After their pre-treatment with high-calcium or barium solution, or exposure to sodium-free solution, they were exposed to [^{3}H]noradrenaline as described under uptake-retention section for the first set of experiments. Background and potassium-evoked release were studied as described above. In spite of the large number of slices used in these experiments, we had considerable difficulty in determining the release of endogenous noradrenaline from slices when endogenous noradrenaline content was severely reduced by sodium withdrawal. Specific activity is expressed as ct/min of [^{3}H]noradrenaline per p-mole noradrenaline.

Calcium determination. Slices were prepared in an isotonic calcium-free medium and then subjected to two different pre-treatments. One group of splenic slices (about 100 mg) was preincubated in 10 ml. normal Krebs solution, and the other group in 10 ml. isotonic calcium-Krebs solution (96 mM) for 4 hr at 4° C. After this treatment the slices were incubated for different periods of time at 37° C in oxygenated normal Krebs solution. Following each incubation period, slices were thoroughly rinsed in cold isotonic sodium chloride, gently blotted on ashless filter paper, placed into a tared, fused, silica crucible and dried overnight at 95° C. The dried tissue was then ashed at 600° C and redissolved in 0·5 ml. 1 N-HNO_3. Aliquots of the ashed tissue extract were used for the estimation of total tissue calcium content according to the method described by Yanasigawa (1955) and modified by Kingsley & Robnett (1958). The values obtained for total tissue calcium content were corrected, assuming the extracellular space in this organ to be 21 ml./100 g wet wt., as estimated by Grossman & Furchgott (1964) for the guinea-pig atrium.

RESULTS

Effect of substituting sodium of the pre-incubation medium with different concentrations of calcium on the noradrenaline content of spleen slices. Fig. 1 shows the effect of prior incubation with different calcium concentrations on the noradrenaline content of spleen slices when they were subsequently exposed to normal Krebs solution for 2 hr. Pre-incubation of the slices in isotonic calcium (96 mM) Krebs solution for 4 hr at 4° C, and then in normal Krebs solution at 37° C, caused a time-dependent release of noradrenaline which was more pronounced during the first half hr of incubation. The tissue noradrenaline content after half an hr was 55·1 ± 1·9 %,

whereas after 2 hr it was only $34{\cdot}8 \pm 3\%$ of the initial content. The effect of prior incubation with calcium on the endogenous noradrenaline content was dependent upon the calcium concentration of the pre-incubation medium. Thus, pre-exposure to 0 mM calcium solution for 4 hr at 4° C did not cause any appreciable reduction in the endogenous levels of noradrenaline; however, increasing the calcium concentration of the pre-incubation medium from 0 to 96 mM induced proportionately greater losses. It was interesting to observe that pre-incubation of the slices in normal Krebs solution at 4° C also caused noradrenaline loss on subsequent exposure to normal Krebs solution. The magnitude of release from slices incubated in normal Krebs solution was comparable with those incubated in sodium-free (sodium replaced by lithium), 2·5 mM calcium solution (Fig. 1). The releasing effect of pre-incubation in high-calcium solution could not be prevented if the slices, loaded with calcium (see below) were repeatedly washed in cold normal or calcium-free Krebs solution for up to 2 hr.

Loss of endogenous noradrenaline by pre-exposure to high-calcium solution is probably due to its release. This point, however, could not be tested, because noradrenaline is easily oxidized when incubated in normal Krebs solution at 37° C (Garcia & Kirpekar, 1973), and thus we could not account for its loss by its presence in the incubating medium.

At the end of the pre-incubation period of 4 hr in any calcium-Krebs solution at 4° C, and just before the exposure to normal Krebs solution, the mean noradrenaline content was $1{\cdot}65 \pm 0{\cdot}22$ μg/g ($n = 7$), which was not significantly different from the initial noradrenaline content of the spleen slices ($1{\cdot}87 \pm 0{\cdot}29$ μg/g, $n = 6$). Furthermore, exposure of the slices to cold, *per se*, for 4 hr does not appear to damage the sympathetic nerves to cause release of noradrenaline, since incubation of the slices in calcium-free lithium-Krebs solution (sodium replaced by lithium) for 4 hr at 4° C and then exposing them to normal Krebs solution for 2 hr at 37° C resulted in only a slight loss.

The relationship between the calcium content of the spleen slices and the calcium concentration of the incubating medium. The total calcium content was determined for isolated spleen slices which were equilibrated in Krebs solutions containing 0, 2·5, or 96 mM calcium. After isolating the slices in cold isotonic saline, they were transferred to a medium containing either 2·5 or 96 mM calcium for 4 hr at 4° C. In three experiments, slices isolated in normal saline contained an average of $0{\cdot}68 \pm 0{\cdot}09$ m-mole/Ca^{2+}/kg wet tissue. After incubating the slices in normal Krebs solution or isotonic calcium-Krebs solution, the tissue calcium concentrations were $2{\cdot}57 \pm 0{\cdot}41$ and $9{\cdot}9 \pm 1{\cdot}32$ n-mole/kg, respectively (average of three experiments).

In another series, splenic slices were equilibrated in isotonic calcium-

Krebs solution for 4 hr at 4° C and then exposed to normal Krebs solution at 37° C for periods up to 2 hr. Fig. 2 shows that the slices lost nearly 50 % of their calcium content over the first 40 min. During the remaining 80 min, calcium loss occurred at a much reduced rate.

Effect of substituting sodium of the pre-incubation medium with calcium, strontium or barium on the noradrenaline content of spleen slices. Fig. 3 shows that barium and strontium (96 mM) were as effective as calcium in releasing noradrenaline. Barium pre-treatment was even more effective than calcium

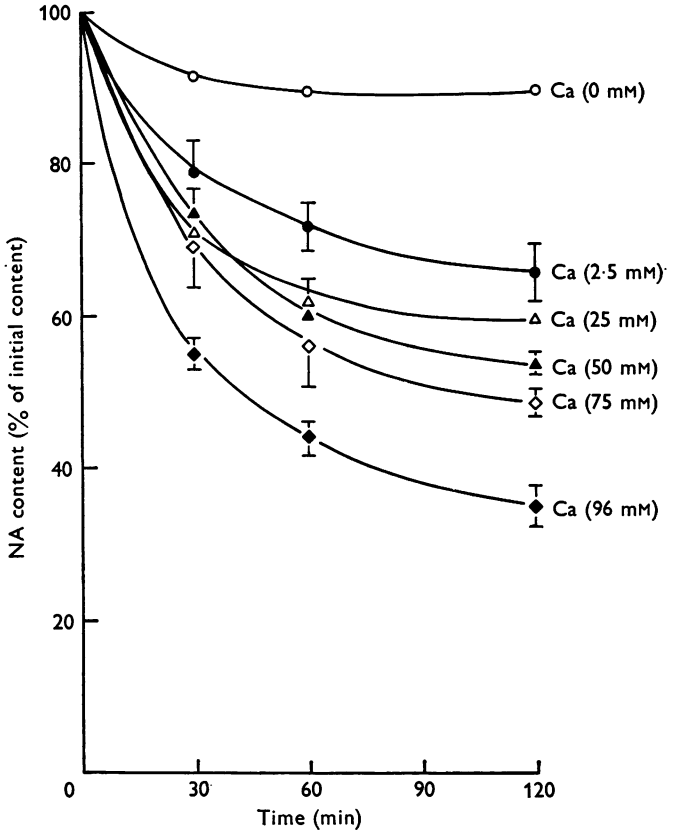

Fig. 1. Effect of pre-treatment with calcium on the noradrenaline (NA) content of spleen slices. Slices were pre-incubated in Krebs solution containing 0–96 mM calcium for 4 hr at 4° C. Isotonicity was maintained by lithium. Rate of loss of endogenous NA was followed for 2 hr in normal Krebs solution at 37° C. The initial NA content of spleen slices, determined immediately before the pre-incubation period, has been taken as 100 %, and the subsequent values at 30, 60 and 120 min are expressed as a % of the initial content. During the pre-incubation time no apparent loss of NA was observed. Vertical bars show S.E. of mean of three to seven experiments. Top curve with zero calcium was obtained from a single experiment.

pre-treatment, since it caused $89 \pm 1{\cdot}6\,\%$ depletion of endogenous levels of noradrenaline in 2 hr. The greater effectiveness of barium treatment was apparent over the first half-hour, since it caused $64 \pm 1\,\%$ depletion as compared to about $45 \pm 2\,\%$ depletion by calcium or strontium pre-treatment, respectively. From this point onward, the rate of release with any of the three pre-treatments was comparable. Incubation of slices in either barium- or strontium-Krebs solution at 4° C for 4 hr did not of itself cause a loss of endogenous noradrenaline.

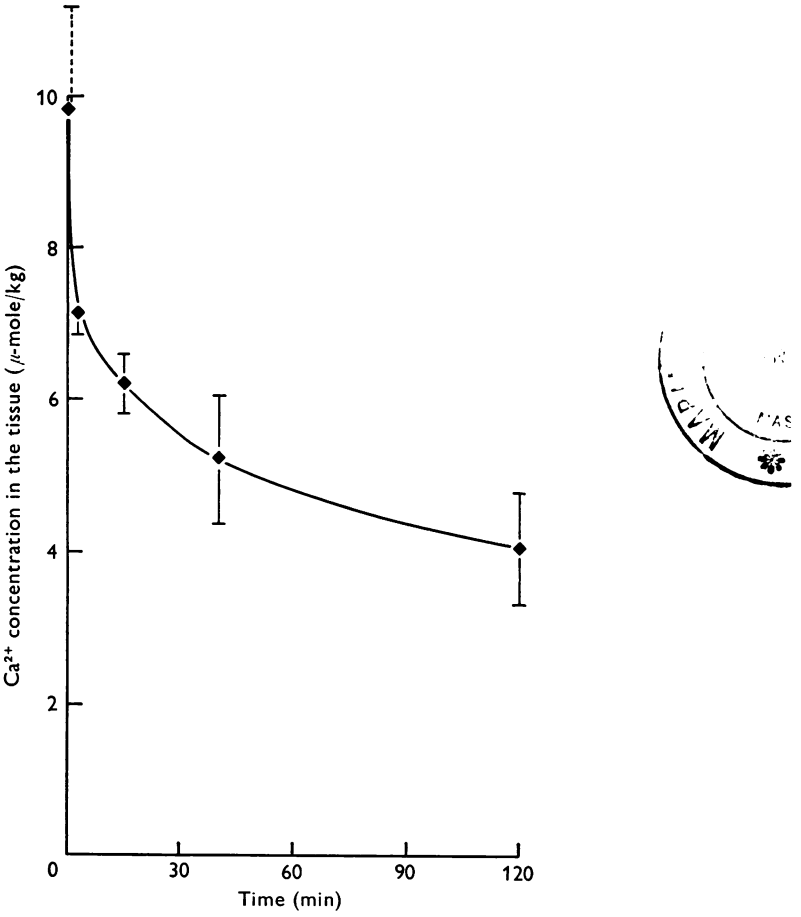

Fig. 2. Rate of loss of calcium from the calcium-preloaded spleen slices after incubation in normal Krebs solution. The zero-min value is obtained from slices which were exposed to 96 mM calcium-Krebs solution for 4 hr at 4° C and then repeatedly washed with cold saline. Subsequent calcium determinations were done during incubation of the slices in normal Krebs solution at 37° C. Vertical bars show S.E. of mean of three experiments.

Release of noradrenaline as a function of pre-exposure time to calcium or barium. Fig. 4 shows that pre-incubation of spleen slices in either isotonic calcium- or barium-Krebs solution (sodium replaced by calcium or barium) at 4° C for as little as 15 min caused 50–60 % loss of their endogenous noradrenaline content following re-exposure to normal Krebs solution for

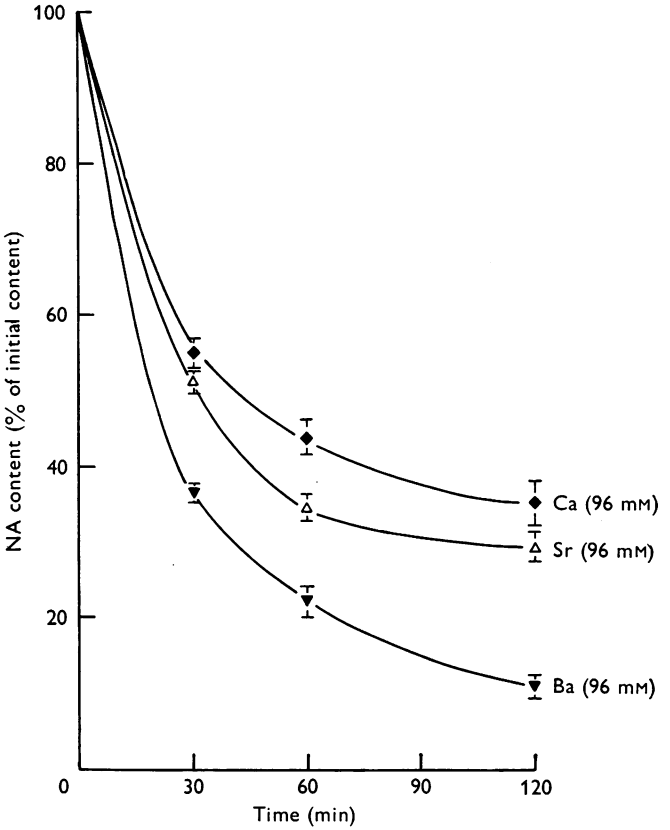

Fig. 3. Effect of pre-treatment with calcium, strontium or barium on the NA content of spleen slices. Slices were pre-incubated in isotonic calcium-, strontium- and barium-Krebs solution for 4 hr at 4° C. Experimental details same as Fig. 1. Note that barium pretreatment was much more effective than calcium pre-treatment in causing NA loss. Vertical bars show S.E. of mean of three to seven experiments.

2 hr at 37° C. As the pre-incubation time was prolonged from 15 min to 24 hr there was a greater loss of endogenous noradrenaline, so that the tissues were depleted of more than 90 % of their noradrenaline content by a 24-hr pre-incubation with either ion. Barium pre-treatment was more effective than calcium in reducing the endogenous noradrenaline levels

resulting from the first 6 hr of pre-incubation. On the basis of these experiments, we conclude that the cellular effects of pre-treatment with high-calcium or barium ions, which eventually lead to release of noradrenaline, must have occurred a short time after the beginning of the pre-treatment.

The effect of calcium on the efflux of noradrenaline induced by calcium or barium pre-treatment. Since calcium is indispensable for the release of neurotransmitters and hormones (Rubin, 1970), it was of interest to determine the effect of calcium removal on noradrenaline loss induced by calcium or barium pre-treatment. In three experiments, removal of calcium

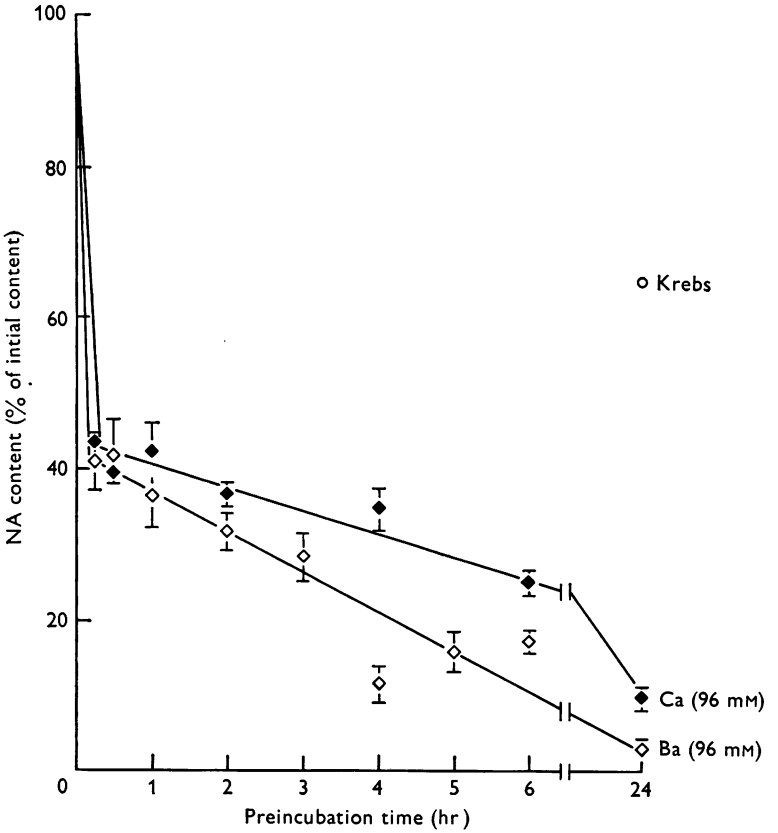

Fig. 4. Relationship between noradrenaline content and exposure time to pre-incubation solutions. Slices were pre-incubated from 15 min to 24 hr in isotonic calcium- or barium-Krebs solution at 4° C. After the specified pre-incubation time, they were exposed to normal Krebs solution for 2 hr at 37° C and their NA content measured. Note that the depleting effect of the pre-treatment was obtained in about 15 min after exposure, and that incubation in normal Krebs solution (2·5 mM calcium) for 24 hr at 4° C also caused about 35 % depletion. Vertical bars show S.E. of mean of three to five experiments.

alone, or removal of calcium plus addition of EGTA (0·1 mM) to the incubating solution did not appreciably affect the rate of loss of endogenous noradrenaline induced by calcium or barium pre-treatment. Increasing the calcium or magnesium concentrations of the incubating medium to 25 mM also did not affect the rate of noradrenaline loss by calcium or barium pre-treatment.

Effect of pentobarbitone or ether anaesthesia on noradrenaline release induced by pre-incubation in high-calcium Krebs solution. In the course of studying various procedures to antagonize the calcium effect on nor-

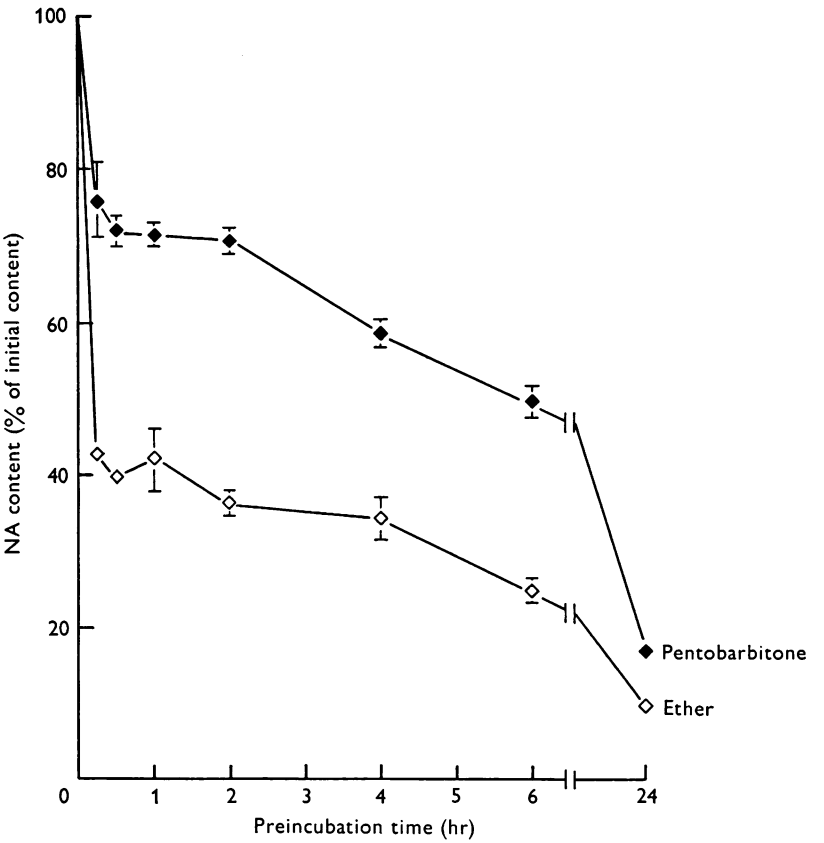

Fig. 5. Effect of pre-treatment with calcium on the NA content of spleen slices obtained from cats anaesthetized with sodium pentobarbitone (35–40 mg/kg) or ether. Slices were preincubated in isotonic calcium-Krebs solution from 15 min to 24 hr, and NA content was determined after exposing them for 2 hr in normal Krebs solution at 37° C. Note that the loss of NA was considerably less in slices obtained from cats anaesthetized with pentobarbitone than with ether. Vertical bars show S.E. of mean of four to five experiments.

adrenaline release, we found that spleen slices prepared from cats anaesthetized with pentobarbitone were resistant to depletion when pre-exposed to high-calcium solution. Thus, slices prepared from spleens of cats anaesthetized with sodium pentobarbitone and pre-incubated in high-calcium (96 mM) Krebs solution for 15 min at 4° C lost only $24 \pm 5\%$ of their endogenous noradrenaline as compared to a $57{\cdot}3 \pm 5\%$ loss obtained in slices prepared from spleens of cats anaesthetized with ether ($P < 0{\cdot}001$, Fig. 5). As the pre-incubation time was prolonged from 15 min to 6 hr, the depletion increased to 50 and 75% in pentobarbitone- and ether-treated slices, respectively. After 24 hr of pre-incubation the depletion was about 80–90% in both groups of slices.

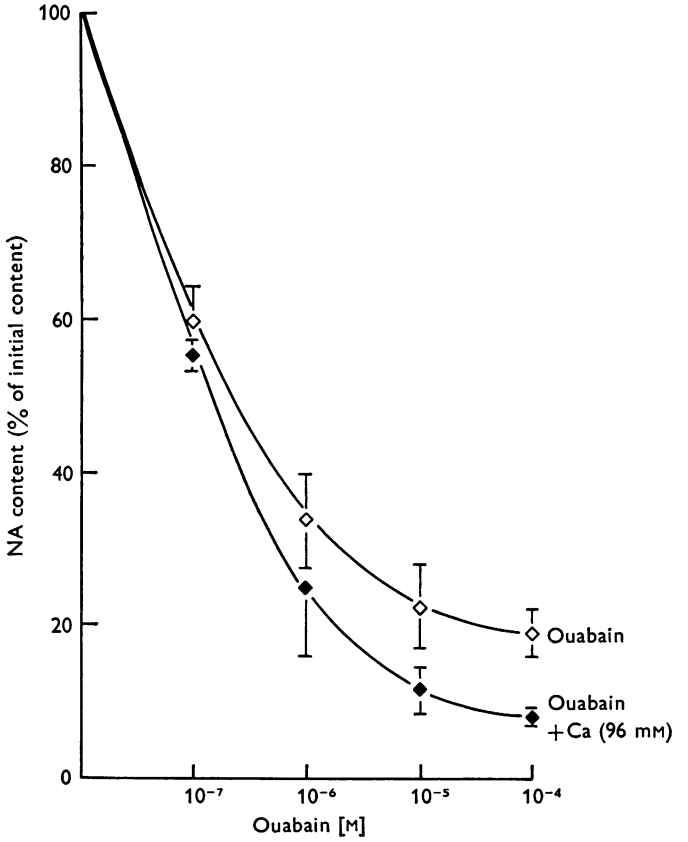

Fig. 6. Effect of ouabain and calcium pre-treatment on NA content of spleen slices. Normal slices, or slices pre-incubated in high-calcium (96 mM)-Krebs solution for 1 hr at 4° C were exposed for 2 hr to different concentrations of ouabain in normal Krebs solution. Note that higher concentrations of ouabain did not appreciably potentiate NA loss from calcium pre-treated slices. Vertical bars show S.E. of mean of two to four experiments.

Effect of ouabain on noradrenaline release induced by pre-incubation in high-calcium Krebs solution. Garcia & Kirpekar (1973) previously showed that ouabain (10^{-5}–10^{-4} M) caused a marked reduction in the noradrenaline content of spleen slices in 2 hr. Fig. 6 shows that ouabain (10^{-7}–10^{-5} M) does not appreciably enhance the release induced by 1 hr pre-treatment with high-calcium solution at 4° C, and at 10^{-4} M ouabain only slightly enhanced the release induced by high-calcium pre-treatment. Because of the marked effect of either treatment in causing noradrenaline release, the nature of the interaction between ouabain and high-calcium pre-treatment is not clear. The fact that the lower doses of ouabain were not potentiated by high-calcium pre-treatment may be due to the marked effect of the calcium pre-treatment alone, which will mask any further potentiation by the additional ouabain treatment.

Release of [^3H]noradrenaline from slices pre-treated with high-calcium solution. Since incubation of splenic slices in high-calcium Krebs solution at 4° C for 4 hr markedly increases tissue concentration of calcium, it is conceivable that the calcium concentration of the sympathetic nerves, which form only a small portion of the total tissue mass, should also be proportionately increased. If the intracellular calcium concentration of the sympathetic nerves is thus elevated and if it is available for transmitter release then it should be possible to evoke transmitter release in the complete absence of extracellular calcium.

To test this possibility, experiments were made on slices whose endogenous stores of noradrenaline were first labelled with [^{3}H]noradrenaline as described in Methods, and which were then transferred to high-calcium (96 mM) Krebs solution for 4 hr at 4° C. Release was induced by potassium after 3 min and 40 min washout of high-calcium solution with normal Krebs solution at 37° C, and the results are shown in Fig. 7. Background [^{3}H]noradrenaline released in 5 min before exposure to high-potassium solution was considerably higher at 3 min than at 40 min after washout. After exposure to high potassium (140 mM) the slices which were washed for only 3 min showed a marked increase in the release of [^{3}H]noradrenaline, which was not suppressed in the absence of external calcium, with or without EGTA (1 mM). However, if the slices were repeatedly washed over 40 min, then the release induced by high potassium was almost completely blocked in the absence of external calcium, with or without EGTA. In one experiment, when the washout period was limited to 15 min the release induced by potassium was also found to be calcium-independent. More than 90 % of potassium-induced release of radioactivity was accounted for as [^{3}H]noradrenaline. It should be pointed out that the net release of [^{3}H]-noradrenaline induced by potassium was much greater at 3 min than at 40 min of washout. This probably is due to a loss of about 50 % of the

endogenous noradrenaline content during the washout period of 40 min. The fact that the release was blocked by removing calcium after 40 min but not after 3 min washout suggests that, initially, the leakage of intracellular calcium was sufficiently high to provide enough concentration of this ion in the vicinity of sympathetic nerve endings to sustain release.

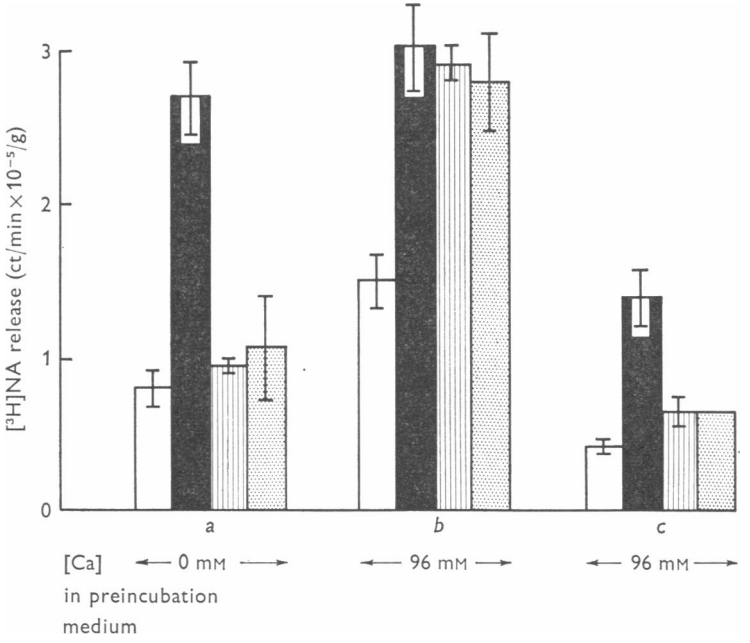

Fig. 7. Release of [³H]NA by potassium after 3- and 40-min washout of pre-incubation solution. Slices were pre-incubated in Krebs solution containing 0 or 96 mM calcium for 4 hr at 4° C. After this pre-treatment they were incubated in normal or calcium-free Krebs solution for 3 or 40 min, and release of [³H]NA was evoked by potassium (140 mM, 5 min). □ Background release; ■ release by potassium in the presence of calcium; ▥ release by potassium in the absence of calcium; ▦ release by potassium in the absence of calcium containing EGTA (1 mM). In *a* and *b*, release was obtained after 3 min, and in *c*, after 40-min washout of pre-incubation solution. Vertical bars represent S.E. of means of two to four experiments (see Methods).

In one experiment, in order to maximally remove or dilute the extracellular calcium still remaining, slices were washed 3 times for 1 hr at 4° C (to prevent noradrenaline depletion) with calcium-free Krebs solution after high-calcium pre-treatment. Potassium-evoked release of [³H]noradrenaline after a 3 min washout at 37° C was still partially calcium-independent. However, in the presence of 1 mM-EGTA the transmitter release was completely blocked.

Uptake-retention of noradrenaline in slices depleted of their endogenous noradrenaline content by sodium deprivation or pre-treatment with barium. In order to study uptake of noradrenaline, spleen slices incubated in sodium-free solution at 37° C for 2 hr (Garcia & Kirpekar, 1973), or pre-treated with high-barium solution for 24 hr at 4° C, were used, since these

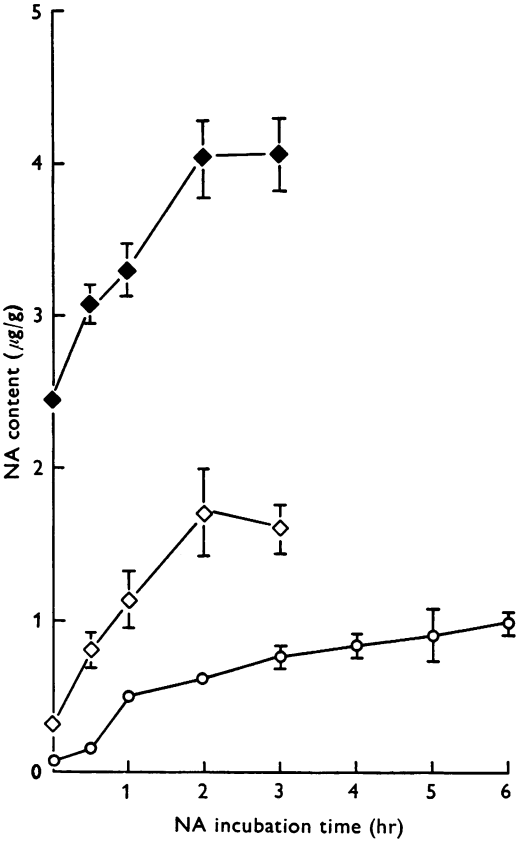

Fig. 8. Uptake of NA in normal and treated slices. Endogenous NA was markedly depleted by pre-treatment of the slices with barium (96 mM) for 24 hr at 4° C, or by exposing them to sodium-free solution for 2 hr. They were then washed for 1 hr with normal Krebs solution, and then incubated with NA (10^{-5} g/ml.) for various times up to 6 hr. Initial NA content of slices for all three groups was $2{\cdot}45 \pm 0{\cdot}16$ μg/ml. Vertical lines are S.E. of mean of two to twelve experiments: ◆ normal; ◇ sodium-free; ○ barium-pre-treated.

pre-treatments depleted the endogenous noradrenaline levels by over 90–95 %. Fig. 8 shows that after a 24 hr incubation in barium (96 mM)-Krebs solution at 4° C and subsequent exposure for 2 hr in normal Krebs solution the endogenous levels were reduced from $2{\cdot}45 \pm 0{\cdot}16$ to $0{\cdot}07 \pm$

0·01 μg/g. Slices were then exposed to noradrenaline (10^{-5} g/ml.) for various times up to 6 hr. It can be readily seen that very little retention occurred up to 30 min, and maximum retention of $0·96 \pm 0·08$ μg/g occurred only after 6 hr. We are not very clear about the reasons why uptake failed to occur during the first 15–30 min of noradrenaline incubation, but this experiment at least shows that the noradrenaline content of the depleted slices can be restored to about 50 % of the endogenous level existing before treatment by exposing them to exogenous noradrenaline. Retention of exogenous noradrenaline was even more impressive when depletion of the endogenous transmitter was caused by sodium withdrawal. Thus the noradrenaline content, which was markedly reduced from the normal value of $2·45 \pm 0·16$ μg/g to $0·34 \pm 0·06$ μg/g during sodium withdrawal, was partially restored to $1·7 \pm 0·31$ μg/g in 2 hr and $1·6 \pm 0·17$ μg/g in 3 hr. These uptake studies are very similar to those previously reported for the retention of [^{3}H]noradrenaline in such slices (Garcia & Kirpekar, 1973). Incubation of normal slices with noradrenaline also showed a gradual increase in the endogenous levels of noradrenaline. Thus, after a 3 hr incubation the noradrenaline content of the normal slices increased to $4·05 \pm 0·25$ μg/g.

If one calculates the net retention in the three groups, one finds that the retention of exogenous noradrenaline in normal and treated slices was comparable. Thus the net retention in control slices was 1·60 μg/g, whereas it was 1·26 μg/g in slices depleted of their endogenous noradrenaline content by sodium withdrawal. Retention of 0·89 μg/g in barium-treated slices was lower than the retention in control slices; however, this reduced retention may be attributed to the toxic action of barium ions on sympathetic nerves over long periods of exposure.

Uptake-retention and release of [^{3}H]noradrenaline from slices depleted of their endogenous noradrenaline content by various pre-treatments. In view of the demonstration that the noradrenaline content of the treated slices could be partially restored by their exposure to exogenous noradrenaline, it was of interest to compare the specific activity of the released transmitter to that of the spleen under experimental conditions in which the endogenous stores of noradrenaline have been variably depleted by different treatments. Depending upon whether the empty vesicles, resulting from sodium deprivation, calcium or barium pre-treatment, are re-used for storage and release, the specific activity of released transmitter should be inversely proportional to the endogenous stores of noradrenaline.

In order to measure the specific activity of noradrenaline released by high potassium, about 1 g (instead of 0·2 g) of slices was used in these experiments. Table 1 shows that after pre-incubation in high-calcium or barium (96 mM) solution at 4° C for 4 hr and subsequent exposure to normal

Krebs solution for 2 hr at 37° C, noradrenaline content was reduced by about 40 and 70%, respectively. Lesser effects of the two treatments in causing noradrenaline depletion in the present experiments, as compared to the experiments reported earlier, may be attributed to the high ratio of tissue slices/medium in these experiments. [^{3}H]noradrenaline uptake-retention studies were then carried out by exposing the tissues to a solution of [^{3}H]noradrenaline (see Methods).

TABLE 1. Relationship between endogenous noradrenaline and specific activity ± S.E. of splenic and potassium-induced [^{3}H]noradrenaline. Number of experiments is indicated in parentheses. Statistical comparisons were made versus the control values obtained in normal Krebs solution

Pre-treatment	n	Tissue noradrenaline content (μg/g)	Specific activity of [^{3}H]noradrenaline (ct/min × 100/p-mole noradrenaline)	
			Splenic	Released
Normal Krebs	(7)	3·94 ± 0·47	1·55 ± 0·17	6·39 ± 0·40
Calcium Krebs	(6)	2·32 ± 0·25*	2·11 ± 0·20*	7·14 ± 0·35
Barium Krebs	(6)	1·03 ± 0·14*	2·88 ± 0·37*	9·06 ± 0·83**
Sodium-free Krebs	(6)	0·59 ± 0·10***	8·87 ± 0·92***	

* $P < 0·01$
** $P < 0·05$
Paired comparison, Student's t test.

*** $P < 0·01$ Group comparison, Student's t test.

Table 1 shows that the tissue specific activity increases as the endogenous noradrenaline content is reduced by various treatments. Since calcium pre-treatment depleted the tissue stores by only 40%, and since the retention of [^{3}H]noradrenaline in such tissues was 70–80% of normal tissues, the tissue specific activity was also proportionately greater. Tissue specific activity after barium pre-treatment was also greater than normal. The greatest increase in tissue specific activity was obtained from slices whose noradrenaline was severely depleted by sodium withdrawal. Since retention of [^{3}H]noradrenaline was still 60% of the normal, the specific activity was nearly tenfold greater than normal. Results with barium and calcium pre-treatment were rather difficult to interpret, since prolonged treatment with these ions produces some damage, affecting both the uptake-retention and release of noradrenaline. In spite of this difficulty, the specific activity of pre-treated slices was almost 1·5- to 2-fold greater than that of normal slices.

Table 1 also shows that the specific activity of released noradrenaline from each group was about 2–3 times greater than that of the tissue from which it is released. Previously, Kirpekar & Yamamoto (1971) showed that the specific activity of noradrenaline released from normal and α-

methyl-*p*-tyrosine-treated spleens was about twofold greater than that of the spleen itself. Even though it is difficult to measure release of cold noradrenaline from calcium- and barium-pre-treated slices (it was impossible to measure release from slices severely depleted by sodium withdrawal), and hence calculate the specific activity of released noradrenaline, it was obvious that the specific activity of noradrenaline released from depleted slices was greater than that released from normal slices.

Comparison of the specific activity of released noradrenaline to that of tissue noradrenaline reveals that the former exceeds the tissue specific activity by a constant factor, irrespective of the endogenous levels of noradrenaline. If this relationship were to hold, then one could even predict the specific activity of released noradrenaline from slices which were severely depleted of their endogenous noradrenaline by sodium withdrawal and then refilled with [^{3}H]noradrenaline. Since tissue specific activity was very high after this latter treatment, the specific activity of released noradrenaline should also be several times greater than that released from normal slices.

Finally, it should also be pointed out that the uptake-retention and release of [^{3}H]noradrenaline from treated slices occurred in a manner comparable to normal slices. Thus, uptake was completely blocked by pre-treatment with cocaine (10^{-6}–10^{-5} g/ml.) or intraneuronal storage by reserpine (10^{-6} g/ml.), and potassium-evoked release was entirely calcium-dependent.

DISCUSSION

Experiments reported in this paper show that splenic slices previously exposed to high calcium lose their endogenous noradrenaline content when re-exposed to normal Krebs solution. Release was roughly proportional to the calcium concentration of the pre-incubation medium. When lithium instead of calcium was used in the pre-incubation medium, no subsequent release of noradrenaline occurred. Loss of noradrenaline after pre-treatment with high-calcium solution probably is due to accumulation of calcium inside the sympathetic nerves. This is a reasonable assumption, because total tissue-calcium content rose very markedly after incubation in high-calcium Krebs solution. Previously, Desmedt (1953) showed that the intracellular sodium concentration of frog sartorii can be raised by soaking these muscles in cold, potassium-free Boyle–Conway Ringer solution. Similarly, we have shown that the tissue calcium concentration can also be similarly raised by soaking the tissues in cold, isotonic, calcium-Krebs solution. Barium and strontium pre-treatment also resulted in the loss of endogenous noradrenaline. We have taken this as evidence to suggest that the release is specifically due to intraneuronal accumulation

of bivalent alkaline elements, and not due to some non-specific harmful effects of these ions on the sympathetic nerves of the spleen. It is also interesting to note that, even if the slices were pre-incubated in sodium-free (lithium) solution containing 2·5 mM calcium, substantial release occurred subsequently in the normal Krebs solution at 37° C. This is not really surprising, since the external calcium concentration of 2·5 mM is considerably greater than the tissue concentration of this ion (0·68 mM), and at this gradient with low temperature (4° C) the calcium ions will accumulate inside the smooth muscle cells and the sympathetic nerves and cause noradrenaline release.

Release of noradrenaline induced by pre-treatment with calcium or barium occurred in the absence of calcium in the external medium. This was not surprising, irrespective of the mechanism(s) involved in the release of noradrenaline by calcium. Calcium pre-treatment may induce release either by a direct action of calcium ions on the release mechanism, or by inhibition of ATPase due to intracellular accumulation of calcium ions. These experiments were originally undertaken with the view that accumulation of calcium would occur indiscriminately all along the sympathetic neurone, including the specific sites, which are usually associated with transmitter release evoked by depolarization. It was therefore expected that spontaneous release of noradrenaline would occur in the absence of external calcium. Garcia & Kirpekar (1973) have previously shown that inhibition of ATPase activity, either by removal of sodium from the bathing medium or by treatment with ouabain, also caused spontaneous release of noradrenaline. It is well known that calcium also inhibits sodium-potassium-activated ATPase (Skou, 1957; Dunham & Glynn, 1961), and effective inhibition of ATPase activity is obtained at very low concentrations of calcium. Since splenic slices were incubated with high-calcium concentrations, up to 96 mM at 4° C, it is reasonable to assume that the intracellular concentration of this ion was high enough to cause inhibition of ATPase activity. If the release was induced by ATPase inhibition, then it should continue to occur in a calcium-free medium as long as ATPase remains inhibited. Since ouabain did not further appreciably modify the effect of calcium pre-treatment on release, we tentatively suggest that the mechanism of release by calcium pre-treatment is due to ATPase inhibition. Conversely, partial protection afforded by sodium pentobarbitone against loss of noradrenaline induced by calcium pre-treatment can also be explained, since sodium pentobarbitone has been shown to interfere with the microsomal uptake of calcium and to stimulate the activity of the calcium-activated microsomal ATPase enzyme (Nayler & Szeto, 1972).

We have also shown that spleen slices depleted of their endogenous noradrenaline content by pre-treatment with calcium or barium can be

refilled with either cold noradrenaline or [^{3}H]noradrenaline, which subsequently could be released in high-potassium medium. The fact that the evoked release of [^{3}H]noradrenaline from the treated slices did not occur when calcium in the external medium was removed, suggests that the intraneuronally accumulated calcium is not used when release is induced by depolarization. Tissue calcium measurements clearly show that even after washing for 40 min, the slices still retained a substantial amount of calcium, which should have been more than adequate to sustain release provided intracellular calcium was used for release. This finding is similar to the one reported by Miledi & Slater (1966), who showed that mere intracellular injection of calcium ions was not enough to sustain evoked release of transmitter from squid preganglionic nerves in the absence of external calcium. One might assume from such a finding that the sites through which calcium enters during depolarization are available only from the outside.

Garcia & Kirpekar (1973) showed that slices depleted of their endogenous noradrenaline content by over 90 %, by incubating them in sodium-free solution, were able to accumulate [^{3}H]noradrenaline in a manner comparable to normal slices. We have suggested, on the basis of this finding, that the vesicles emptied of noradrenaline by sodium-deprivation were still present within the nerve endings and were available for restoring infused noradrenaline. Recent experiments in our laboratory support this assumption, because treatment of slices with a sodium-free medium for as long as 3 hr did not appreciably affect the activity of dopamine β-hydroxylase – a marker for the vesicles – even though tissue noradrenaline was largely depleted. Although pre-treatment with calcium or barium did not produce comparable depletion of noradrenaline, present results do suggest that the vesicles, depleted of their endogenous catecholamines, were probably capable of re-use for the storage and release of [^{3}H]noradrenaline. Comparison of specific activities of the endogenous and released noradrenaline shows that the specific activity increases as the endogenous level of noradrenaline progressively falls (Table 1). This would mean that once exogenous noradrenaline enters the neuronal membrane, it rapidly equilibrates with all the vesicles, empty or normal, and is probably uniformly distributed within all the vesicles. This conclusion seems justified, because comparative uptake studies in normal slices and in slices severely depleted of noradrenaline by barium pre-treatment or sodium withdrawal showed that, under identical conditions of incubation with cold noradrenaline, uptake and retention in the normal slices was only 50 % *over* the endogenous amine content, and in the treated slices it was about 50 % *of* the initial noradrenaline content of such slices; so the net uptake was comparable in normal and treated slices. This means that normal 'full'

vesicles are able to accumulate noradrenaline only moderately, and that the uptake in severely noradrenaline-depleted spleen slices (which is considerable, relative to their endogenous content) must have occurred in 'empty' vesicles of such tissues. Irrespective of whether each vesicle was partially depleted, or a large fraction of the total population of the vesicles was entirely depleted by sodium deprivation or calcium pre-treatment ('quantal release'), the specific activity of endogenous and released noradrenaline from such depleted tissues should be greater than that of normal slices. Present experiments at least suggest that the trend is in this direction, and it is quite probable that the specific activity of released noradrenaline from severely depleted slices would be several times greater than that released from normal slices, provided the empty vesicles were re-used for storage and release of noradrenaline.

A question may also be posed as to whether the entire retention of exogenous noradrenaline in treated tissues occurred in a few percent of the remaining normal vesicles. Since the [^{3}H]noradrenaline uptake in slices severely depleted by sodium withdrawal amounted to 60% of the control uptake (Garcia & Kirpekar, 1973), we would have to assume, in support of that argument, that the remaining normal vesicles in such tissues took up a 5–10 times greater amount of [^{3}H]noradrenaline than their counterparts in untreated control slices. Since there is no basis for making such an assumption, we shall have to conclude that the uptake was probably similar in normal vesicles derived either from normal or noradrenaline-depleted slices.

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