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THE EFFECT OF SODIUM AND CALCIUM IONS ON THE RELEASE OF CATECHOLAMINES FROM THE ADRENAL MEDULLA: SODIUM DEPRIVATION INDUCES RELEASE BY EXOCYTOSIS IN THE ABSENCE OF EXTRACELLULAR CALCIUM

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SUMMARY

1. Perfusing bovine adrenal glands with Na^+-free Locke solution for 15–40 min did not modify the increase in the release of catecholamines from glands stimulated by acetylcholine. However, after 80–100 min of perfusion with Na^+-free solution, the response to acetylcholine stimulation was decreased or abolished.

2. Immediately after switching the perfusion medium to Na^+-free solution, there was a sharp increase (6–10 times over control values) in catecholamine output.

3. Graded substitution of Na^+ in the perfusion fluid enhanced the output of catecholamines. This increase in the output of amines was linearly related to the logarithm of the extracellular Na^+ concentration.

4. The release of catecholamines in the absence of Na^+ was not reduced by the presence of atropine and hexamethonium nor by the omission of Ca^{2+} in the presence of EDTA or EGTA.

5. Excess of Mg^{2+} in the perfusion fluid reduced (10 mM-Mg^{2+}) or blocked (20 mM-Mg^{2+}) the increase in the output of catecholamines induced by Na^+ deprivation in the presence or absence of extracellular Ca^{2+}.

6. Na^+ deprivation induced release of catecholamines during perfusion of the glands with depolarizing concentrations (56 mM) of K^+.

7. In the presence or the absence of extracellular Ca^{2+}, the increase in the output of catecholamines induced by Na^+ deprivation was accompanied by an increase in the output of dopamine β-hydroxylase, but not of lactate dehydrogenase. In addition, during perfusion with Ca^{2+} free solution, Na^+ deprivation induced a parallel increase in both catecholamine and adenosine triphosphate outputs.

8. The ratios of catecholamines to dopamine β-hydroxylase and catecholamines to adenosine triphosphate determined in the perfusates collected from glands during perfusion with Na^+-free medium were similar to those measured in the soluble contents of isolated chromaffin granules. These results provided biochemical evidence in favour of exocytosis as the mechanism of secretion during Na^+ deprivation.

INTRODUCTION

The mechanism of secretion of catecholamines from the adrenal medulla have been studied extensively (Smith & Winkler, 1972). Although the requirement of extracellular Ca^{2+} for the acetylcholine-induced release of catecholamines has been well established, the role of sodium ions in either the 'spontaneous' or the acetylcholine-evoked release is not clearly understood. It has been shown that when cat's adrenal glands were stimulated after a short period of perfusion with Na^+-free medium, there was a potentiation of the acetylcholine-evoked release of catecholamines (Douglas & Rubin, 1963). On the other hand, perfusion of bovine adrenal glands with Na^+-free solutions for longer periods of time decreased or completely abolished the response of the adrenal medulla to cholinergic stimulation (Banks, Biggins, Bishop, Christian & Currie, 1969). Furthermore, in both of these studies, an increase in catecholamine output was noted, after switching the perfusion from normal to Na^+-free medium (Douglas & Rubin, 1961; Banks *et al.* 1969).

The present study was undertaken to examine the role of sodium ions in the release of catecholamines, and in particular to study in more detail the release induced by exposure to Na^+-free medium. The results demonstrate that amines can be released by exocytosis in the absence of extracellular Ca^{2+}. A preliminary account of some of the findings has already been given (Trifaró & Lastowecka, 1973).

METHODS

Bovine adrenal glands of an average weight of 12 ± 2 g were perfused and stimulated *in vitro* as described by Trifaró *et al.* (1967). *Perfusion fluids*: (*a*) the principal perfusion medium was phosphate-buffered-Locke solution of the following composition (mM): NaCl, 154; $CaCl_2$, 2·2; KCl, 2·6; K_2HPO_4, 2·15; KH_2PO_4, 0·85; and, dextrose, 10; (*b*) Na-deficient and Na^+-free Locke solutions were of the same composition as the standard Locke solution except that NaCl was partially or totally replaced by equimolar concentrations of LiCl, choline chloride or osmotically equivalent concentrations of sucrose; (*c*) high potassium Locke solution contained 56 mM-K^+ of which 53 mM was as KCl and 3 mM as K_2HPO_4 and KH_2PO_4. In this solution NaCl was reduced by an equivalent amount (50·4 mM); (*d*) Ca^{2+}-free Locke solution was similar to the Locke solution mentioned in (*a*), except that $CaCl_2$ was

omitted and 2·0 mM-EDTA or 0·1 mM-EGTA were added to the medium. All solutions were equilibrated with 5% CO_2 in O_2, and the final pH of the solutions was 7·2. The glands were perfused at room temperature (25° C), at a rate of flow that was between 12 and 18 ml./min; for each gland the flow rate was kept constant during the entire perfusion period. Samples from the perfusate were collected at 1 min intervals in ice-chilled tubes containing 10 μl. of 1·9 N-HCl per ml. perfusate. When enzymes were determined in the perfusate the HCl was omitted.

Chemical determinations. The catecholamine content of the perfusates was measured by the tri-hydroxyindole fluorometric method (Anton & Sayre, 1962). Dopamine β-hydroxylase (DBH) was measured by the method of Friedman & Kaufman (1965) as modified by Viveros, Arqueros, Connett & Kirshner (1969). In this method the enzyme activity is assayed by determining the amount of [^{3}H]-octopamine formed from [^{3}H]-tyramine (10 μM). Lactate dehydrogenase (LDH) was measured as described by Wróblewski & La Due (1955). This method is based on the decrease in the optical density of reduced nicotinamide adenine dinucleotide phosphate ($NADH_2$), 1 unit of LDH activity being the amount of the enzyme that produces a fall of 0·01 O.D. unit per minute. Adenosine triphosphate (ATP) was measured by the firefly luminescence technique previously described (Douglas & Poisner, 1966*a*). When necessary, Ca^{2+} levels in the solutions were measured by atomic absorption spectrophotometry (Gimblet, Marney & Bonsnes, 1967). Results are expressed as mean ± S.E.

Isolation of chromaffin granules. Highly purified granules were obtained from homogenates of the adrenal medulla by the method of Smith & Winkler (1967).

Chemicals. The chemicals were obtained from the following sources: Reduced nicotinamide adenine dinucleotide phosphate ($NADH_2$), adenosine triphosphate (ATP), eserine sulphate, and firefly tail powder, Sigma Chemical Company; acetylcholine chloride, Welcker Laboratories; atropine sulphate and hexamethonium chloride, Nutritional Biochemical Corporation; ethyleneglycol-bis-(β-amino-ethyl-ether)-N,N′-tetra-acetic acid (EGTA), J. T. Baker Chemical Company; disodium ethylenediamine tetra-acetate (EDTA) and LiCl, Fisher Scientific Company; choline chloride, The British Drug Houses Limited; sucrose (density gradient grade), Schwarz-Mann; and [H^3]-tyramine (10 Ci/m-mole), New England Nuclear.

RESULTS

Effect of Na^+ omission on acetylcholine-evoked release of catecholamines

When adrenal glands were perfused with Na^+-free sucrose medium for 15–40 min and stimulated with acetylcholine (10^{-4} M) the output of catecholamines during stimulation was similar to that obtained during perfusion with normal Locke solution (Fig. 1*a*). On the contrary, perfusion of adrenal glands with Na^+-free Locke solution for longer periods of time decreased or abolished the response to acetylcholine stimulation (Fig. 1*b*). Similar results were obtained in six other experiments at each of the exposure times to Na^+-free medium. These results are in agreement with those previously reported by Banks *et al.* (1969, 1970), although in their experiments, Na^+ was substituted by either choline or Li^+ and stimulation was by carbamylcholine (10^{-2} M). Banks *et al.* interpreted the diminished response to carbamylcholine stimulation as due to a decrease in the entry

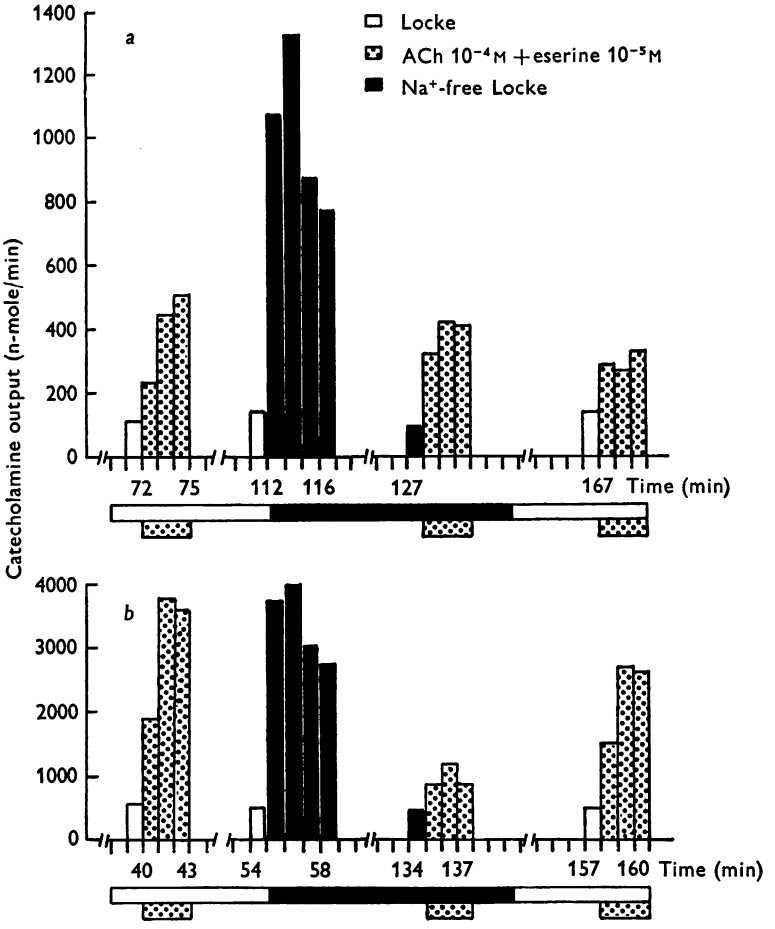

Fig. 1. Effect of Na^+ omission on acetylcholine-evoked release of catecholamines. In this, and in all subsequent Figures, the graphs show the rate of catecholamine output (n-mole/min) from perfused bovine adrenal glands. (*a*) and (*b*) show the stimulant effect of acetylcholine (10^{-4} M) in the presence of eserine (10^{-5} M) (▦), during perfusion with Locke solution (□), or Na^+-free Locke solution (■). The second acetylcholine stimulation was applied in (*a*) after 15 min and in (*b*) after 80 min of perfusion with Na^+-free solution. Glands were perfused at room temperature (25° C) with a flow rate of approximately 15 ml./min. The perfusing solutions were gassed with a mixture of 5% CO_2 in O_2. Samples were collected from the perfusates at 1 min intervals; these were assayed for catecholamine content as indicated in the Methods.

of Ca^{2+} into the chromaffin cell, this being a result of a progressive fall in the intracellular concentrations of Na^+.

Effect of Na^+ omission on the output of catecholamines

In all of these experiments there was a sharp and significant rise in catecholamine output (6–10 times over the control value) immediately after the perfusion medium was switched to a Na^+-free solution (Fig. 1). Therefore, it was decided to perform experiments to characterize this increase of amine output evoked by the omission of Na^+. Adrenal glands were perfused for three successive periods of 15 min each with Na^+-free Locke solution, and each of these 15 min periods was separated from the next by 20 min perfusion with normal Locke solution. Fig. 2 summarizes

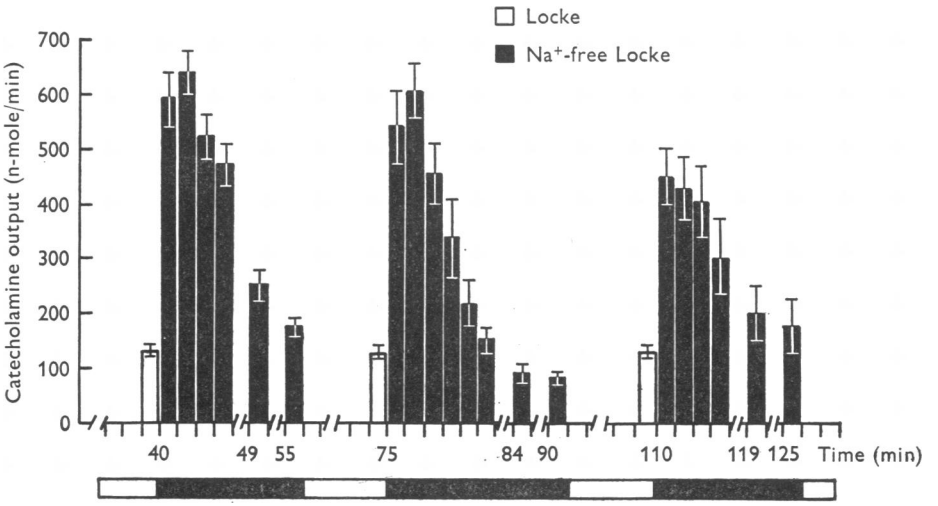

Fig. 2. Effect of Na^+ omission on the output of catecholamines. Bovine adrenal glands (n = 13) were perfused alternately with Locke solution (□) and Na^+-free Locke solution (■) for periods of 20 and 15 min respectively. The vertical bars represent the mean ± S.E. of mean of catecholamine outputs expressed in n-mole/minute. Other conditions were as described in Fig. 1.

the results of thirteen experiments. During the first 2 min of perfusion with Na^+-free Locke solution the catecholamine output increased 864 ± 65%, 805 ± 93%, and 611 ± 62% in the first, second and third stimulation periods respectively. Catecholamine output returned to control levels after 12–15 min of perfusion with a Na^+-free solution (Fig. 2).

It is known that during repeated stimulation of the adrenal glands with acetylcholine, the increase in catecholamine output in response to acetylcholine decreases with time (Douglas & Rubin, 1961). To see if a similar

pattern in catecholamine output could be observed during successive exposure to Na^+-free solutions, nine other adrenal glands were perfused with normal Locke solution for a period of 20 min followed by ten successive 15 min periods of perfusion with Na^+-free Locke solution. As with acetylcholine stimulation, the response to the omission of Na^+ from the extracellular environment decreased with time. The increase in the output of catecholamines produced during the ninth and tenth periods of perfusion with Na^+-free Locke solution were of $200 \pm 30\%$ and $180 \pm 25\%$ respectively.

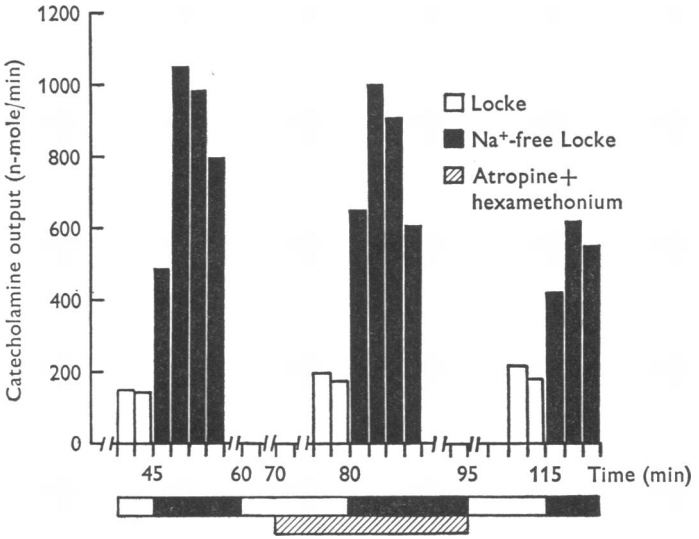

Fig. 3. The lack of effect of anticholinergic drugs on the release of catecholamines produced by the omission of Na^+ from the perfusion fluid. A bovine adrenal gland was perfused alternately with Locke solution (□) and Na^+-free Locke solution (■) for periods of 20–15 min respectively. During the second perfusion period, the Na^+-free Locke solution also contained atropine (10^{-5} g/ml.) and hexamethonium (5×10^{-4} g/ml.). Similar results were obtained in four other experiments. Other conditions were as described in Fig. 1.

Effect of anticholinergic drugs on the release of catecholamines produced by Na^+ omission

During the *in vitro* perfusion of bovine adrenals, the splanchnic nerve terminals which remained within the gland are closely associated with the chromaffin cells. Therefore, the possibility exists that the increased catecholamine output observed during the omission of Na^+ from the extracellular fluid was secondary to the effect of acetylcholine released from the cholinergic terminals. If this were the case, the response should then be blocked by atropine plus hexamethonium, but this was not so. The

results obtained in four experiments showed that when atropine (10^{-5} g/ml.) and hexamethonium (5×10^{-4} g/ml.) were present in the perfusion fluid the increase in catecholamine output in response to Na^+ omission was not diminished (Fig. 3).

Effect of graded substitution of Na^+ in the perfusion fluid on the output of catecholamines

Experiments were carried out to determine if partial substitution of Na^+ in the extracellular medium would produce an increase in catecholamine output, and if this were the case, to see if this increase in amine output was proportional or related to the extracellular concentration of Na^+. The catecholamine output rose between 40–168%, 200–250%, and 360–676%, in glands perfused with solutions containing 100, 50 and

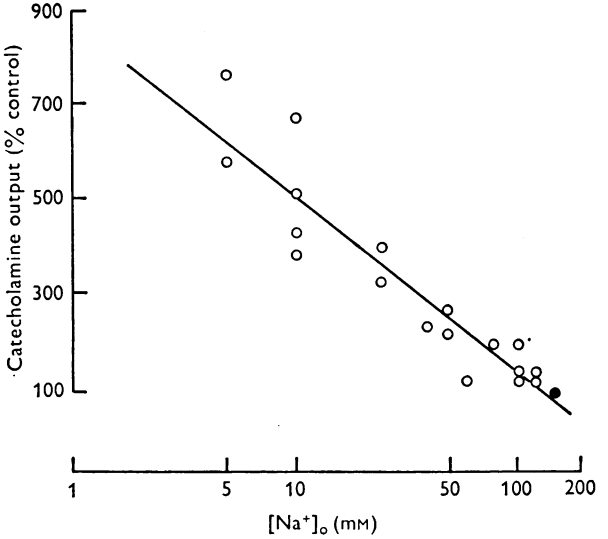

Fig. 4. Effect of graded substitution of Na^+ in the perfusion fluid on the output of catecholamines. Bovine adrenal glands (n = 18) were first perfused with Locke solution for 40 min. At the end of this perfusion period samples were collected for 2 min and assayed for catecholamine content. The values thus obtained were considered equal to 100% (●). After perfusion with Locke solution the glands were then perfused with modified Locke solutions (○) and samples were collected during the first 2 min of perfusion and assayed for catecholamines. The modified Locke solutions contained between 5 and 125 mM-NaCl. In these solutions NaCl was replaced by osmotically equivalent amounts of sucrose. The ordinate represents catecholamine output, as the percentage of the value obtained during perfusion with Locke solution. The abscissa represents the logarithm of the extracellular concentration of Na^+ ions. The line was computer fitted (correlation coefficient = −0·90). Other conditions were as described in Fig. 1.

10 mM-Na^+ respectively. When the logarithm of extracellular concentration of Na^+ was plotted against the percentage increase in catecholamine output during the first 2 min of perfusion with Na^+deficient solutions, a linear correlation was obtained (Fig. 4).

Differences obtained in the outputs of catecholamines during the substitution of Na^+ in the perfusion fluid by osmotically equivalent amounts of sucrose, choline or Li^+

In the experiments described above, Na^+ was substituted by osmotically equivalent amounts of sucrose. Therefore, experiments were also performed in which Na^+ was replaced by either choline or Li^+ ions. When a Na^+-free (choline) Locke solution reached the gland, there was a significant increase in catecholamine release (Fig. 5*a*). In three experiments the increase in amine output was between 300 and 450%. On the other hand, substitution of Na^+ by Li^+ did not produce a rise in catecholamine output (Fig. 5*b*), although switching the perfusion fluid from Li^+-Locke solution to a Li^+-free (sucrose) Locke solution produced a significant increase in catecholamine output.

Effect of successive stimulations with Na^+-free Locke solution on the responses of the adrenal gland to acetylcholine

As during acetylcholine stimulation, successive exposure of the adrenal glands to Na^+-free solutions decreases the responsiveness of the gland. It was, therefore, decided to perfuse glands with Locke solution and then expose the glands on five successive occasions to Na^+-free solution, followed by successive stimulations with acetylcholine (10^{-4} M). It was observed that the first acetylcholine stimulation, that is, the sixth stimulation, since the five previous stimulations were done by Na^+ omissions, produced a greater increase in catecholamine output than the three or four preceding stimulations done by Na^+-free solutions (Fig. 6). Similar results were obtained when the first five stimulations were done by acetylcholine. The sixth stimulation, that is, the first by Na^+ omission, produced an increase in amine output greater than the two preceding acetylcholine stimulations

Effect of increasing the concentration of K^+ in the perfusion fluid on the increased output of catecholamines produced by Na^+ omission

It is well known that acetylcholine is capable of inducing release of amines during perfusion of adrenal glands with solutions containing 56 mM-K^+ (Douglas & Rubin, 1963). To see the effect of depolarizing concentrations of K^+ on the Na^+-free effect, glands were perfused with Locke solution, stimulated for a first time by Na^+-free Locke solution,

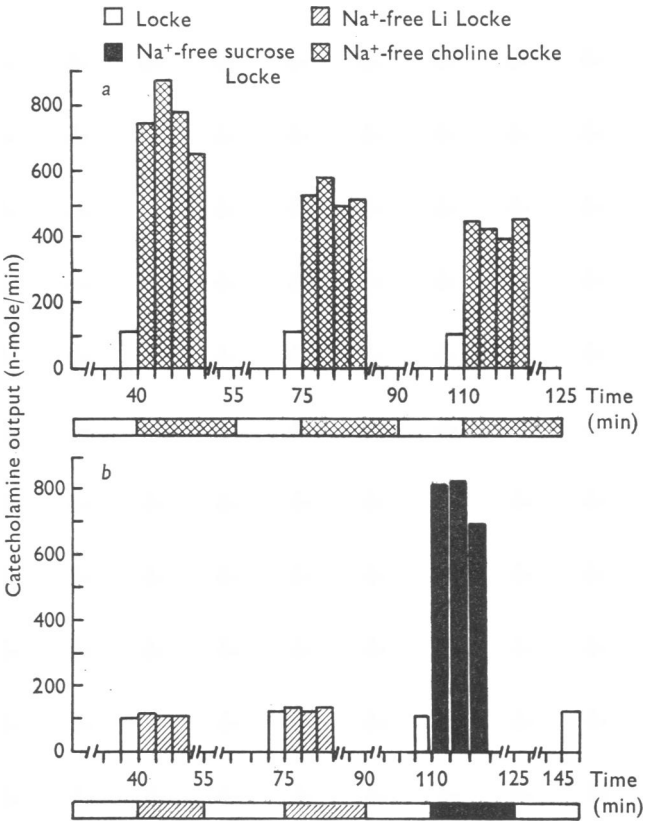

Fig. 5. Differences in catecholamine outputs obtained during the substitution of Na^+ in the perfusion fluid by osmotically equivalent amounts of sucrose, choline or Li^+. Bovine adrenal glands *a* and *b* were first perfused with Locke solution (□). Then gland *a* was perfused alternately with Locke solution and Na^+-free (choline) Locke solution (▨) for periods of 20 and 15 min respectively. Gland *b* was perfused alternately with Locke solution and two periods of Na^+-free (Li) Locke solution (▨). The perfusion was then continued with Locke solution for 20 min followed by a 15 min period of perfusion with Na^+-free Locke solution (■). Similar results were obtained in three other experiments. Other conditions were as described in Fig. 1.

and after 20 min of perfusion with normal Locke solution they were stimulated with 56 mM-K^+ medium; perfusion was switched to a 56 mM-K^+ Locke solution in which the 104 mM-Na^+ was substituted by an osmotically equivalent concentration of sucrose. The omission of Na^+ from a high K^+ solution during perfusion of the adrenal glands produced the characteristic increase in catecholamine output (Fig. 7).

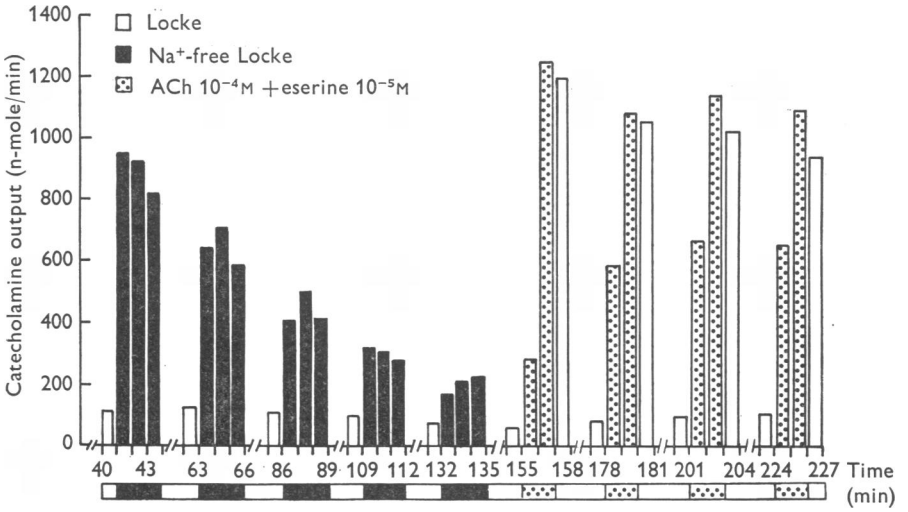

Fig. 6. Effect of successive stimulations by Na^+-free Locke solution on the response of the adrenal gland to acetylcholine. A bovine adrenal gland was perfused 5 consecutive times alternately with Locke and Na^+-free Locke solutions. This was followed by four periods of perfusion using Locke and acetylcholine-Locke solutions alternately. Similar results were obtained in four other experiments. Other conditions were as described in Fig. 1.

Effect of Mg^{2+} on the output of catecholamines evoked by Na^+ omission

If Na^+ is omitted from the extracellular environment there is an increase in the ratio between the intra and extracellular concentrations of Na^+ ($[Na^+]_i/[Na^+]_o$). This seems to be an ideal condition to increase the entry of Ca^{2+} into cells (Baker, 1970). It was, therefore, possible that the increased amine output observed during Na^+ deprivation was a result of an enhanced Ca^{2+} influx into chromaffin cells. If this were so, increasing the extracellular concentration of Mg^{2+} should partially or totally block amine released by Na^+ deprivation because Mg^{2+} has been shown to decrease the acetylcholine-evoked release of catecholamines from the adrenal medulla by competing with the Ca^{2+} entry into the cells (Douglas & Rubin, 1963). When glands were perfused with Na^+-free Locke solution in the presence of 10 mM-Mg^{2+} there was a decrease in the catecholamine output in response to Na^+ deprivation. Under these conditions, that is, in the presence of 10 mM-Mg^{2+}, the catecholamine output during the first 3 min of perfusion was $43{\cdot}6 \pm 6{\cdot}1\%$ ($n = 4$) of that obtained during the preceding stimulation in the absence of Mg^{2+}. This figure is significantly different from the value $91{\cdot}1 \pm 11{\cdot}1\%$ ($n = 13$, $P < 0{\cdot}005$) obtained when the second stimulation was carried out in the absence of Mg^{2+}. Increasing the

concentration of Mg^{2+} in the perfusion fluid to 20 mM produced a greater blocking effect ($P < 0.001$). The results obtained in five experiments are shown in Table 1. It should also be noticed that the third stimulation, which was carried out in the absence of Mg^{2+}, produced a response of $84.7 \pm 8.2\%$. This figure is not significantly different from the value of $73.7 \pm 11\%$, obtained for the third response when all responses to Na^+-free Locke solution were obtained in the absence of Mg^{2+}.

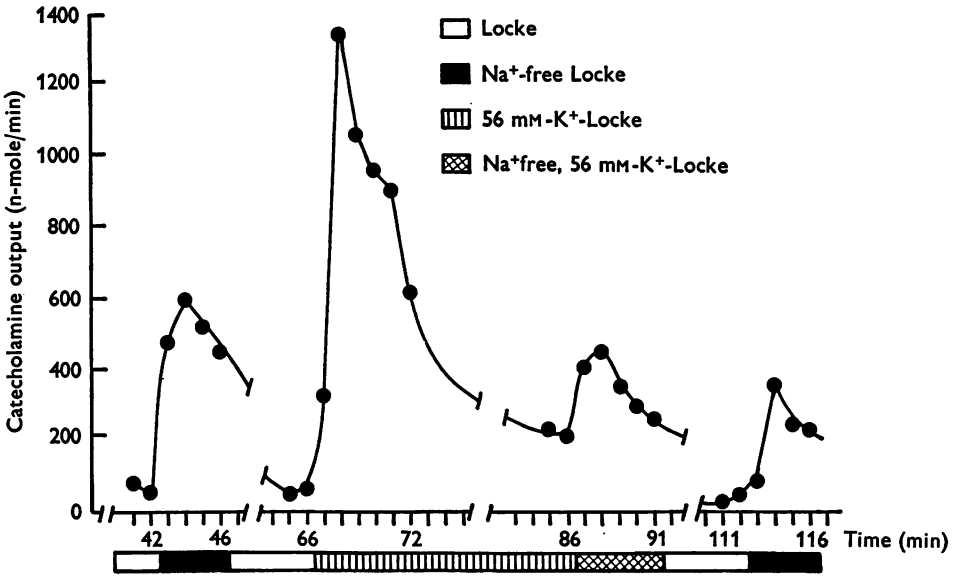

Fig. 7. Effects of Na^+-deprivation on the output of catecholamines during depolarization with KCl. A bovine adrenal gland was perfused with Locke solution for 40 min. Then perfusion was for 15 min with Na^+-free Locke solution. Perfusion was continued for a further 20 min with Locke solution followed by a 25 min period of perfusion with 56 mM-K^+-Locke solution. During the last 5 min of this perfusion period, 104 mM-NaCl was replaced by an osmotically equivalent amount of sucrose. After this period, perfusion was switched to Locke solution. This was followed by another period of perfusion with Na^+-free Locke solution. Similar results were obtained in four other experiments. Other conditions were as described in Fig. 1.

Effect of Ca^{2+} omission from the extracellular environment on the release of catecholamines produced during perfusion with Na^+-free Locke solution

If the blockage in Na^+-free stimulated amine release produced by raising the extracellular concentration of Mg^{2+} is due to the competition between this ion and Ca^{2+}, it should be possible to obtain similar results when Ca^{2+} is omitted from the Na^+-free solutions. In the Ca^{2+}-free, Na^+-free solutions, in which Na^+ was replaced by osmotically equivalent amounts of

TABLE 1. Effect of Mg^{2+} on the output of catecholamines evoked by Na^+ omission. Adrenal glands were perfused during the first and third stimulation periods with Na^+-free Locke solution. During the 2nd stimulation period, the Na-free Locke solution contained 20 mM-$MgCl_2$

Expt. no.	Increased catecholamine release (n-mole/min) 1st stimulation	2nd stimulation	3rd stimulation	Ratio of 2nd to 1st stimulation (%)	Ratio of 3rd to 1st stimulation (%)
1	+284	+11	+240	3·9	84·5
2	+331	+33	+323	9·9	97·6
3	+504	+26	+312	5·2	62·0
4	+734	+103	+788	14·0	107·4
5	+1227	+10	+883	0·8	72·0
			Mean ± S.E. of mean	6·8 ± 2·3	84·7 ± 8·2

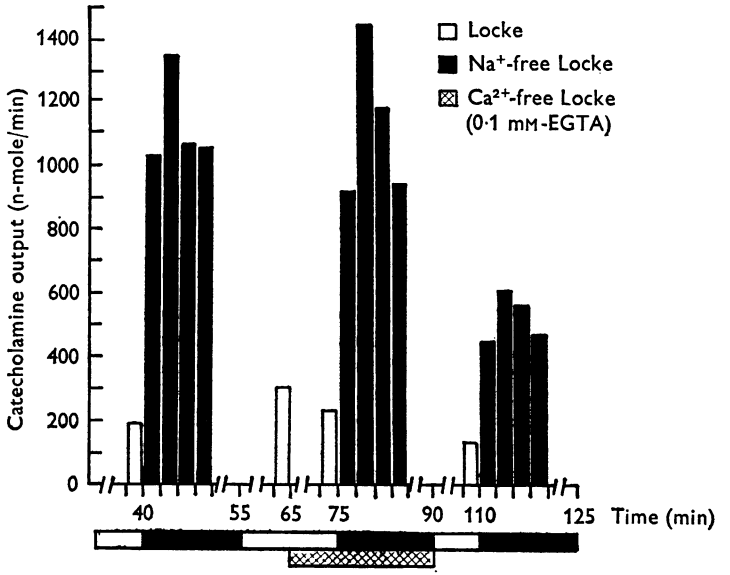

Fig. 8. Effect of Ca^{2+} omission from the extracellular environment on the release of catecholamines produced during perfusion with Na^+-free Locke solution. A bovine adrenal gland was perfused alternately with Locke solution (□) and Na^+-free Locke solution (■) for periods of 20 and 15 min respectively. During the second perfusion period, Ca^{2+} was omitted and 0·1 mM-EGTA was present in the Na^+-free Locke solution. Other conditions were as described in Fig. 1.

sucrose, there was $9{\cdot}96 \pm 0{\cdot}9\ \mu$M ($n = 5$) of Ca^{2+}. This amount can easily be chelated by adding EGTA or EDTA to the solutions.

Adrenal glands were perfused with Locke solution and three successive stimulations were performed by switching to a Na^+-free Locke solution. The second stimulation was done in the absence of Ca^{2+} and the presence of 0·1 mM EGTA. In the absence of Ca^{2+} the amine output produced by the Na^+-free medium was not diminished (Fig. 8). On the contrary, there was a small increase in amine output during Ca^{2+} omission. The results of

TABLE 2. Effect of Ca^{2+} omission on the release of catecholamines produced during perfusion with Na^+-free Locke solution. Adrenal glands were perfused during the 1st stimulation period with Na^+-free Locke solution containing 2·2 mM-$CaCl_2$. During the 2nd stimulation period, $CaCl_2$ was omitted and 0·1 mM EGTA was added to the Na^+-free medium

	Increased catecholamine release (n-mole/min)		Ratio of 2nd to 1st stimulations
Expt. no.	1st stimulation	2nd stimulation	(%)
1	+388	+696	206
2	+372	+383	103
3	+932	+930	99
4	+1004	+688	69
5	+1352	+1716	127
		Mean ± S.E. of mean	120 ± 23

five experiments are shown in Table 2. The value of $120 \pm 23\%$ ($n = 5$) is not significantly different from the figure $91{\cdot}1 \pm 11{\cdot}1\%$ ($n = 13$), obtained for the second stimulation in the control experiments. Six other experiments were done in which the Ca^{2+}-free, Na^+-free solution contained 2·0 mM-EDTA. Under these conditions, during the second stimulation, the Na^+-free solution evoked release of amines was of $101 \pm 24\%$ ($n = 6$). Two glands were stimulated to release catecholamines by acetylcholine; perfusion was then switched to Ca^{2+}-free medium and catecholamine release by Na^+-free medium and by acetylcholine was tested. The results obtained with two glands were similar, and the results obtained with one of them are shown in Fig. 9. These results should be compared with those of Fig. 1(*a*), and unlike those experiments, the response to acetylcholine stimulation was completely abolished. However Na^+ deprivation still induced catecholamine release. Furthermore, in order to see if the blockage of the acetylcholine response was due to the long exposure of the glands to Ca^{2+}-free medium, experiments were also performed in four other glands. Here, during the period between the two acetylcholine stimulations, the glands were perfused with Ca^{2+}-free Locke solution, and they were first exposed to acetylcholine for 3 min. Under these conditions the response to

acetylcholine stimulation was abolished. However, switching the perfusion fluid to Ca^{2+}-free, Na^{+}-free Locke solution produced a characteristic increase in catecholamine output.

Due to these unexpected results, it was decided to repeat the experiments described in the preceding section. However, on this occasion, the effect of 20 mM-Mg^{2+} on the stimulation produced by Na^{+} omission was

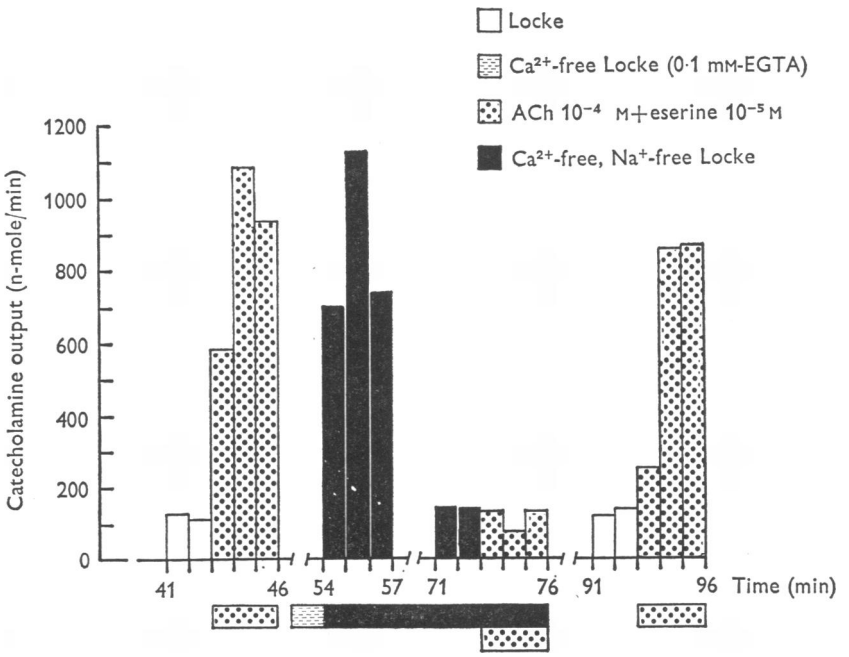

Fig. 9. Effect of Ca^{2+} and Na^{+} omission on acetylcholine-evoked release of catecholamines. A bovine adrenal gland was perfused with Locke solution and was subjected to two periods of stimulation by acetylcholine (▦). Between these two stimulation periods, the gland was first perfused with Ca^{2+}-free Locke solution (▤), and then there followed a 22 min period of perfusion with Ca^{2+}-free, Na^{+}-free Locke solution (■). During the last 3 min of this period, the gland was stimulated by acetylcholine (▦). Similar results were obtained with another gland. Other conditions were as described in Fig. 1.

tested in the absence of Ca^{2+} from the perfusion medium. Here again, the Na^{+} deprivation effect on amine release was blocked by Mg^{2+}. The response in the presence of Mg^{2+} was $6{\cdot}7 \pm 1{\cdot}8\%$ ($n = 3$) of that obtained in the absence of Mg^{2+}, and this value is similar to that obtained in the presence of extracellular Ca^{2+} (see Table 1, column 4).

Is exocytosis the mechanism of release of catecholamines during Na^+ omission?

The results of the experiments shown in Fig. 6 seem to suggest that the amines released during the omission of Na^+ from the extracellular environment came from different cellular pools or different cells than those amines secreted during acetylcholine stimulation. Furthermore, unlike during acetylcholine stimulation, the release produced by Na^+ omission occurs in the absence of extracellular Ca^{2+}, although our experiments do not rule out the possibility of mobilization of intracellular Ca^{2+} during sodium omission. Therefore, due to this discrepancy, it was decided to study the mode of secretion during Na^+ deprivation. This was done by perfusing adrenal glands with Na^+-free Locke solution in the presence or absence of extracellular Ca^{2+} and the perfusates were examined for other soluble constituents of the chromaffin granules, i.e. dopamine β-hydroxylase (DBH) and adenosine triphosphate (ATP).

(*a*) *DBH efflux.* Fig. 10 shows the results obtained with two glands: gland *a* was stimulated by acetylcholine whereas gland *b* was stimulated by Na^+ deprivation. The increase in catecholamine output produced by both forms of stimulation was associated with concomitant increases in DBH outputs. The ratios between catecholamines and DBH determined in the perfusates during either acetylcholine or Na^+ omission induced release were similar to those ratios determined in the soluble content of isolated chromaffin granules (Table 3). Na^+ deprivation also induces release of dopamine β-hydroxylase in the absence of extracellular Ca^{2+} (presence of EDTA), as indicated in Table 3.

(*b*) *ATP efflux.* Under the conditions used in these experiments, that is, absence of extracellular Ca^{2+} and Mg^{2+}, extracellular ATPases should be inhibited, and if catecholamines are released by exocytosis, ATP should be recovered in the perfusates. Eighteen tests were performed on six glands. The results obtained with three of these glands are shown in Fig. 11, which shows that, during stimulation, there was a parallel increase in both catecholamine and ATP outputs. The molar ratios in the perfusates between catecholamines and ATP are shown in Fig. 12. The catecholamine ATP molar ratio in the perfusates was of $3{\cdot}97 \pm 0{\cdot}18$ ($n = 18$), a figure which is not significantly different from the value of $4{\cdot}21 \pm 0{\cdot}44$ ($n = 7$), previously reported for the soluble content of isolated chromaffin granules (Trifaró & Dworkind, 1970). In addition, perfusates were also assayed for lactate dehydrogenase (LDH), an enzyme used as a cytoplasmic marker. If stimulation by Na^+ deprivation damages or produces a non-physiological increase in the permeability of the cell membrane which would account for the release of catecholamines together with DBH and ATP, one might expect a concomitant rise in the perfusates of the levels of this

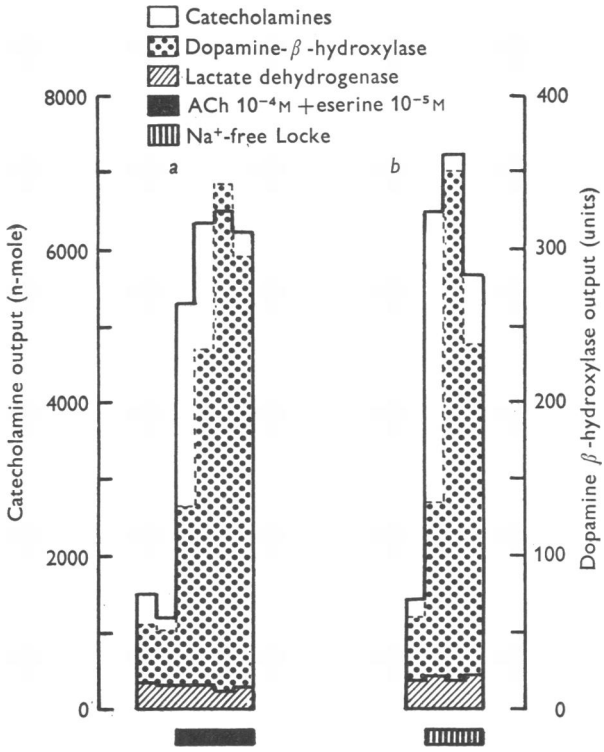

Fig. 10. Effect of Na^+ deprivation and acetylcholine stimulation on the outputs of catecholamines, dopamine β-hydroxylase, and lactate dehydrogenase. Two bovine adrenal glands were perfused with Locke solution and stimulated five consecutive times for 6–8 min by either acetylcholine (*a*) or Na^+ deprivation (*b*). Samples of the perfusates were collected before and during the stimulations, at 2 min intervals. The Figure represents the catecholamine (□) and dopamine β-hydroxylase (▦) outputs after combining the samples of the five stimulation periods in the following manner: samples taken during the first 2 min of the five stimulation periods were combined; similarly, the samples taken during the second 2 min of stimulation were also combined, etc. Aliquots were taken from these pooled samples and assayed for catecholamines and lactate dehydrogenase as described in the Methods section. The rest of the samples were dialysed for 48 hr against 0·5 mM phosphate buffer (pH 6·5) and later lyophilized. The resultant dried precipitates were suspended and assayed for dopamine β-hydroxylase as described in the Methods section. One unit of the DBH activity represents the formation of 10 p-mole of octopamine per hour. The Figure also shows the outputs of lactate dehydrogenase (Hatched bars, ▨), and for clarity, the units of LDH are not indicated in the graph. However, LDH outputs were similar to those indicated in Table 4.

cytoplasmic enzyme. Fig. 10 and Table 4 show that as during acetylcholine stimulation, Na^+ omission did not produce an elevation in the LDH levels of the perfusates. The LDH content of the medulla and cortex were of 4398 ± 85 ($n = 5$) and 5380 ± 24 ($n = 5$) units per gram of wet tissue respectively. The ratio of the cortex to the medulla (w:w) in the

TABLE 3. Ratios of catecholamines to dopamine β-hydroxylase in the soluble content of chromaffin granules and in the perfusates from glands stimulated by either acetylcholine or Na^+ deprivation

Condition	Ratio: $\frac{\text{Catecholamines}}{\text{Dopamine } \beta\text{-hydroxylase (n-mole/units*)}}$	
ACh evoked release	$20{\cdot}6 \pm 4{\cdot}6$	($n = 5$)†
Na^+-free evoked release	$22{\cdot}2 \pm 4{\cdot}6$	($n = 7$)
Ca^{2+}-free, Na^+-free evoked release	$26{\cdot}0 \pm 3{\cdot}0$	($n = 11$)
Soluble granule contents	$23{\cdot}6 \pm 4{\cdot}5$	($n = 14$)

* 1 unit = 10 p-moles of octopamine formed per hour.
† n = number of tests.

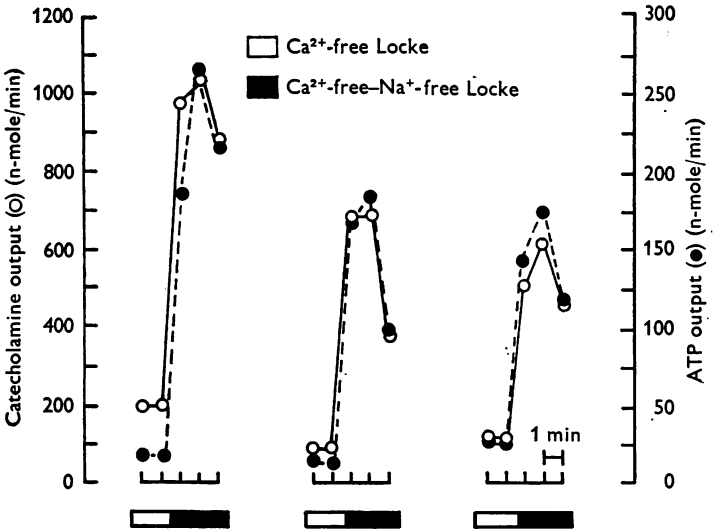

Fig. 11. Effect of Na^+ deprivation on the outputs of catecholamines and adenosine triphosphate in the absence of extracellular Ca^{2+}. Three bovine adrenal glands were perfused with Ca^{2+}-free Locke solution (□) and these followed a 3 min perfusion period with Ca^{2+}-free, Na^+-free Locke solution (■). Both solutions contained 1·0 mM-EDTA and 0·1 mM-EGTA. Samples of the perfusates were collected at 1 min intervals and they were assayed for catecholamines (○) and ATP (●) as described in the Methods. Similar results were obtained with three other glands. Other conditions were as described in Fig. 1.

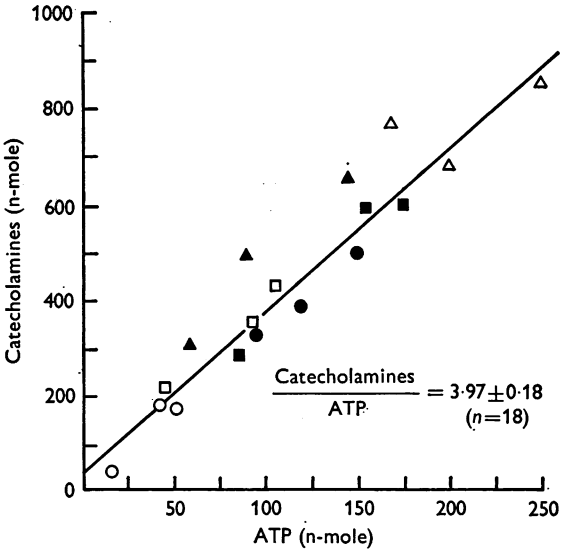

Fig. 12. Correlation of catecholamine output with ATP efflux during stimulation by Na^+ deprivation. A total of eighteen tests were performed on six glands where after perfusion with Ca^{2+}-free Locke solution, the perfusion was switched to Ca^{2+}-free, Na^+-free Locke solution. Both solutions contained 1·0 mM-EDTA and 0·1 mM-EGTA. The results of tests performed on each gland are represented by the same symbol. The line was computer fitted (correlation coefficient = 0·95).

TABLE 4. Efflux of catecholamines and lactate dehydrogenase during stimulation of adrenal glands by either acetylcholine or Na^+ deprivation

	Units*/min							
	Gland 1		Gland 2		Gland 3		Gland 4	
Condition	LDH	CA	LDH	CA	CA	CA	LDH	CA
Locke	2·9	140	2·0	106	4·3	142	5·1	246
Na^+-free Locke	3·2	614	1·9	230	4·7	812	5·2	634
Na^+-free Locke	3·2	690	1·9	458	4·4	822	5·3	988
Na^+-free Locke	3·2	576	2·0	230	4·4	602	5·1	804
Locke	3·0	131	—	—	—	—	5·4	337
Acetylcholine Locke	2·8	484	—	—	—	—	5·4	464
Acetylcholine Locke	2·8	1102	—	—	—	—	5·4	1753
Acetylcholine Locke	2·6	1271	—	—	—	—	5·4	1771

* 1 unit of lactate dehydrogenase (LDH) is the fall of 0·01 of an optical density unit per minute. 1 unit of catecholamine (CA) is 1 n-mole.

adrenal gland was of $3{\cdot}51 \pm 0{\cdot}15$ ($n = 9$). Because the average weight of the perfused gland was 12 ± 2 g, a gland of an average weight would contain about 61925 units of LDH. The LDH levels detected in the perfusates were of 1·9 to 5·4 units/min (Table 4). These values represent 0·003–0·009% of the total LDH content of the gland. Therefore, in 2 hr of perfusion, the total LDH released would be about 1% of the total content of the gland.

DISCUSSION

The need of extracellular Ca^{2+} for the acetylcholine-induced release of catecholamines from the adrenal medulla has been well established (Douglas & Rubin, 1963). However, it is the role of Na^+ on either the 'spontaneous' or the acetylcholine-evoked release of catecholamines which has not been clearly established. The present experiments have provided new evidence on the effect of sodium ions on the release of catecholamines from the adrenal medulla.

Na^+ deprivation and the output of catecholamines. During perfusion of the bovine adrenal glands, a sharp increase in the output of catecholamines was observed immediately upon switching the perfusion fluid to one in which Na^+ was substituted by either choline or sucrose. This effect of Na^+ withdrawal from the extracellular environment was briefly mentioned in an early publication by Douglas & Rubin (1961). They described this phenomenon as an increase in the 'spontaneous' release of catecholamines, but they did not perform further experiments in order to study this effect of the omission of Na^+. We thought that if this effect of Na^+ omission was not secondary to the effect of acetylcholine on chromaffin cells, it would provide the opportunity of studying the role of Na^+ on catecholamine release in the absence of secretagogues. Birks & Cohen (1968*a*, *b*) have shown that an increase in the $[Na^+]_i/[Na^+]_o$ ratio enhances the frequency of miniature e.p.p.s at the frog neuromuscular junction. Furthermore, evidence has been published recently of an increased release of acetylcholine from parasympathetic nerves and from brain cortex during Na^+ deprivation (Paton, Vizi & Zar, 1971; Vizi, 1972). Proof that the effect observed on the adrenal medulla was not a secondary effect of the acetylcholine released from the splanchnic nerve terminals during Na^+ deprivation was obtained in the experiments in which the increased catecholamine release was still observed when glands were perfused with a Na^+-free medium in the presence of atropine and hexamethonium.

Unlike the increase in catecholamine release in response to ouabain (Banks, 1967), Na^+ omission produces an immediate and sharp rise in the output of catecholamines, and as with the acetylcholine-evoked release, this high output of catecholamines is not maintained and declines to

resting levels in 12–15 min. We have not investigated the reason why the response of the gland died out with time. However, it is possible that if Na^+ deprivation somehow mobilizes and increases the intracellular ionized Ca^{2+}; and if the amount of Ca^{2+} available for the release reaction is limited, and once it is used, the release response would decrease. Another possibility is that a high intracellular Ca^{2+} level is related to a higher $[Na^+]_i/[Na^+]_o$ ratio, and that the release response declines when the intracellular Na^+ is being extruded from the cell. In either case, perfusion with regular Locke solution should then restore the cellular ionic equilibrium and make the cell available again for release. The results indicate that the response to Na^+ deprivation can be obtained 7–10 consecutive times provided that short periods of perfusion with normal Locke solution precede the omission of Na^+ from the perfusion medium.

Our results also indicate that the release of catecholamines was proportional to the concentration of the extracellular Na^+; the lower the concentration of this ion, the greater the output of catecholamines. Decreasing the concentration of extracellular Na^+ produces an increase in the $[Na^+]_i/[Na^+]_o$ ratio, and perhaps a concomitant increase in the intracellular Ca^{2+} levels, since it is known that extracellular Na^+ is necessary for a normal Ca^{2+} efflux (Blaustein & Hodgkin, 1969).

Na^+ deprivation and the acetylcholine-evoked release of catecholamines. Douglas & Rubin (1963) have shown that when cat's adrenal glands were perfused *in situ* for 14 min with a Na^+-free Locke solution, the response to acetylcholine stimulation was potentiated. On the contrary, Banks *et al.* (1969) were able to show that if bovine adrenal glands were perfused with Na^+-free solutions for longer periods of time, the response of the glands to carbamylcholine stimulation was decreased or completely abolished. The results of our experiments show clearly that if the acetylcholine stimulus is applied during the 15–40 min of perfusion with Na^+-free Locke solution, the secretory response is neither potentiated nor decreased. However, perfusion with Na^+-free medium for longer periods of time (80–100 min) decreases or completely abolishes the acetylcholine-evoked release of catecholamines. These latter results are in agreement with those published by Banks *et al.* (1969, 1970), who interpreted their observations to mean that the diminished secretory response was due to a decrease in the influx of Ca^{2+} into the chromaffin cell, which is a result of a fall in the intracellular concentration of Na^+ rather than the absence of extracellular Na^+.

Replacement of Na^+ by Li^+, choline or sucrose and the release of catecholamines. Replacement of Na^+ by Li^+ failed to release catecholamines from the adrenal medulla. The effect of Na^+ deprivation on release was observed only when Na^+ was replaced by either sucrose or choline. Our results with Li^+ can be explained if we assume that the external surface of the plasma

membrane of the chromaffin cell is not able to distinguish between Li^+ and Na^+ ions. This assumption is not unreasonable if we consider that Keynes & Swan (1959) have demonstrated that in the sartorius muscle the passive permeability mechanism responsible for generating the action potential does not discriminate between Na^+ or Li^+. Other membranes show similar behaviour to Li^+, for it seems that, in human red blood cells, the passive influx of Na^+ or Li^+ has similar rate constants (Maizles, 1954). In conclusion, it seems that Li^+ enters the cell as readily as Na^+, but that on the contrary, as demonstrated by Keynes & Swan (1959), the active transport mechanism extrudes Li^+ much more slowly than Na^+. Therefore, Li^+ tends to accumulate in excitable tissues at the expense of intracellular K^+, and after a prolonged exposure to Li^+ the membrane potential would fall (Keynes & Swan, 1959). This latter fact would also explain the observation to Banks *et al.* (1969) on the blockage of the cholinergic stimulation of the adrenal medulla after long perfusion with solutions containing Li^+.

Ca^{2+} and Mg^{2+} ions and the release of catecholamines induced by Na^+ deprivation. Douglas & Rubin (1963, 1964) have shown that increasing the concentration of Mg^{2+} in the extracellular environment decreases or blocks the effects of acetylcholine, high K^+ and Ba^{2+} on the release of catecholamines. They interpreted their findings as a result of a blockage of the entry of Ca^{2+} or Ba^{2+} into the cells. Since our results clearly indicate that Mg^{2+}, not only in the presence, but also in the absence of extracellular Ca^{2+}, blocks the release of catecholamines induced by Na^+ deprivation, here, another explanation for the effects of Mg^{2+} other than that of the competition with the entry of Ca^{2+} must be found. Possibilities include: (*a*) Mg^{2+} might block the combination of Ca^{2+} with intracellular receptors; (*b*) Mg^{2+} might also block the intracellular release or translocation of Ca^{2+}, since Mg^{2+} has been shown to be necessary for the uptake and binding of $^{45}Ca^{2+}$ into adrenal medullary microsomes (Poisner & Hava, 1970). On the contrary, Ba^{2+} interferes with Ca^{2+} binding by adrenal medullary microsomes (Poisner & Hava, 1970). These two explanations of the effect of Mg^{2+} assume that Na^+ deprivation would hyperpolarize the cells and that this would increase the intracellular levels of Ca^{2+}. Douglas, Kanno & Sampson (1967) have shown that withdrawal of Na^+ from the extracellular space produces hyperpolarization of the chromaffin cells. In addition, it has been suggested that hyperpolarization enhances transmitter release at other synapses (del Castillo & Katz, 1954); (*c*) Mg^{2+} might inhibit the Mg^{2+}-dependent ATPase of the chromaffin granules. From *in vitro* studies it has been suggested that the granule ATPase may play a role in the secretory process (Poisner & Trifaró, 1967; Trifaró & Poisner, 1967). The results show that concentrations of Mg^{2+} of 10 mM or more blocked cate-

cholamine release in response to Na^+ deprivation. The same concentration range has been shown to have an inhibitory effect on the chromaffin granule ATPase (Winkler, Hörtnagl, Hörtnagl & Smith, 1970).

Mechanism of release during Na^+ deprivation. Previous studies have demonstrated that the release of catecholamines by acetylcholine from the adrenal medulla is by exocytosis. The evidence for release by exocytosis included the demonstration of simultaneous release of catecholamines with ATP (Douglas & Poisner, 1966*a*, *b*), chromogranin A (Banks & Helle, 1965), and dopamine β-hydroxylase (Viveros, Arqueros & Kirshner, 1968). On the contrary, the membrane components of the chromaffin granules are not released into the perfusate (Trifaró *et al.* 1967; Schneider, Smith & Winkler, 1967), and are quantitatively retained within the cell (Poisner *et al.* 1967; Viveros, Arqueros, Connett & Kirshner, 1969). This process appears also to be the mechanism by which noradrenaline is released from sympathetic nerve endings (Smith & Winkler, 1972). In all these systems extracellular Ca^{2+} seems to be essential for the release process (Rubin, 1970). However, in the presence or in the absence of extracellular Ca^{2+}, the release induced by Na^+ deprivation was apparently by exocytosis because catecholamines released by Na^+-free medium were accompanied by ATP and dopamine β-hydroxylase. The ratio of catecholamines to dopamine β-hydroxylase or to ATP in the perfusates was similar to that in the granules. Furthermore, the increased release of dopamine β-hydroxylase was not accompanied by a parallel increase in the output of a cytoplasmic protein of smaller molecular weight, such as lactate dehydrogenase. The unchanged efflux of lactate dehydrogenase during the release of catecholamines induced by Na^+ omission also indicates that there was no cell damage during perfusion with the Na^+-free medium. While this paper was in preparation, a study was published on the effect of Na^+ deprivation on the content of catecholamines of cat's spleen slices (García & Kirpekar, 1973). The experimental conditions used in these experiments did not allow, as they did in our case, the study of the immediate effect of Na^+ deprivation on the release of catecholamines. Furthermore, contrary to our findings, 25 mM-Mg^{2+} did not block the loss of catecholamines from the spleen slices during incubation in Na^+-free medium. The authors' conclusion was that prolonged exposure to Na^+-free solution might cause damage to the storage of noradrenaline in sympathetic nerves. However, they did not measure either any cytoplasmic marker, as for example, lactate dehydrogenase, or any other soluble components of the storage vesicles in order to determine if the loss of amines from the incubated slices was due to release by exocytosis, which is the case for the adrenal medulla, as our results indicate.

General inferences. Release of catecholamines induced by Na^+ depriva-

tion has much in common with acetylcholine-induced release of catecholamines: the release is by exocytosis; the release is blocked by Mg^{2+}; the release effect decreases with the time of exposure to the stimulus; the stimulation of release in the presence of depolarizing concentrations of K^+. However, they differ in that, during acetylcholine stimulation, the chromaffin cell is depolarized whereas Na^+ substitution by osmotically equivalent amounts of sucrose hyperpolarizes the chromaffin cells (Douglas *et al.* 1967), and, furthermore, acetylcholine stimulation requires the presence of extracellular Ca^{2+} for release whereas Na^+ deprivation does not. Moreover, if we assume that, in response to different stimuli, release by exocytosis would involve similar cellular and molecular mechanisms, several questions arise from our results. First, is Ca^{2+} a necessary requirement for exocytosis? If it is, we must assume that during Na^+ deprivation there is an increase in intracellular Ca^{2+}. Experiments dealing with this question are now in progress in our laboratory. Secondly, why is extracellular Ca^{2+} necessary for catecholamine release during acetylcholine stimulation? Acetylcholine acts on the cell surface by combination with specific receptors. It may be that it has no ability to affect the intracellular levels of Ca^{2+} directly, and only increases intracellular Ca^{2+} by promoting the entry of Ca^{2+} into the cell. Pre-treatment with ouabain, which might increase Ca^{2+} entry, has been found to potentiate the release of catecholamines induced by cholinergic agents (Banks, 1967, 1970). It seems, therefore, that release by Na^+ deprivation would depend on the availability of intracellular Ca^{2+} whereas release induced by acetylcholine would depend on an increased entry of Ca^{2+}. If this is true, experiments like those shown in Fig. 6 may be interpreted as catecholamines released from the same cells, but whose release is triggered by two different mechanisms of increasing Ca^{2+} levels, rather than catecholamines released from different cells or different catecholamine pools.

With the exception of those experiments where the uptake of $^{45}Ca^{2+}$ by the adrenal medulla was measured (Douglas & Poisner, 1962; Rubin *et al.* 1967), no studies have been done on the passive movement or active transport of ions in this tissue and on the relationship between ion movements and the secretory process. Therefore, it would be of importance to perform on this tissue studies similar to those carried out by Baker (1970) on the squid axon. Such studies, if correlated to the events of secretion, should clarify the role of ions in the secretory process.

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