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POSSIBLE ROLE OF SODIUM AND CALCIUM IONS IN RETENTION AND PHYSIOLOGICAL RELEASE OF NOREPINEPHRINE BY ADRENERGIC NERVE ENDINGS

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Abstract—Calcium and barium ions deplete tritium labeled stores of norepinephrine (NE) from rat heart slices incubated in Na^+-free media containing choline$^+$. The reciprocal of the rate constant of appearance of ^{3}H-NE in the medium, plotted as a function of the reciprocal of the calcium^{2+} or barium^{2+} concentration, forms a linear curve. Sodium$^+$ is a non-competitive antagonist of calcium^{2+}. The calcium^{2+} requirement for release is greater in the presence of choline$^+$ or potassium$^+$ than in the presence of sucrose. The possible roles of sodium$^+$ and calcium^{2+} in the storage of norepinephrine in adrenergic nerve endings, and in the release of norepinephrine by the nerve impulse, are discussed.

THE REQUIREMENT of sodium ion for retention of amines by adrenergic nerve endings has been shown with heart slices,[1–5] perfused organs[6–8] and isolated nerve endings (synaptosomes) prepared from brain.[9]

Sodium fulfills a dual role in the sequence of reactions taking part in the storage of exogenous and endogenous norepinephrine (NE). The presence of Na^+ in the extracellular fluid is required for the retention of stored NE by nerve endings.[1,2] Moreover, the transport mechanism for the uptake of exogenous NE, and presumably, re-uptake of endogenous amine, is mediated by a Na^+-dependent carrier mechanism located in the plasma membrane.[2–4,10–12]

Calcium ion is required for the release of neurotransmitter by the nerve impulse, shown first for cholinergic nerve endings[13–16] and for adrenergic nerve endings.[17–21] The calcium requirement for neurosecretion has also been demonstrated for the adrenal medulla[6,22] and the posterior pituitary.[23]

Evidence obtained by experiments with striated muscle[24] preceded the discovery of the mutual antagonism between the effects of Na^+ and Ca^{2+} at the adrenal medulla,[6] the cholinergic nerve ending,[25–28] at the adrenergic nerve endings[7,12,29,30] and the neurohypophysis.[23] The antagonistic relationship between Na^+ and Ca^{2+} may be an important factor in the process by which the nerve impulse releases transmitter from the nerve endings.

The present report explores the Ca^+–Na^+ antagonism in terms of the kinetic properties of norepinephrine release from nerve endings. Also considered are some effects of various Na^+ replacements and some effects of barium on the release of NE.

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METHODS

Heart slices were prepared as previously described.[30] Male Sprague–Dawley rats (180–220 g) were injected with 270 ng (20 μc) *dl*-norepinephrine-7-^{3}H (12·5 c/mM, New England Nuclear Corp.) and killed by cervical dislocation 18 hr later. The hearts were bisected longitudinally, rinsed and cooled in ice-cold Krebs-bicarbonate solution, pH 7·4. Heart ventricle slices, weighing 60–100 mg/slice, were then prepared with a Stadie–Riggs tissue slicer and stored in ice-cold Krebs-bicarbonate solution while others were being prepared. Fifteen slices were then transferred to each of several beakers containing 40 ml of Krebs-bicarbonate solution, and preliminarily incubated 1 hr at 37°. Six slices were then transferred to each of several beakers containing 20 ml of the various media to be tested, and incubated at 37° in an Eberbach Corp. water bath shaker, in an atmosphere of 95% O_2–5% CO_2. The usual incubation period was 150 min.

For the estimation of efflux of radioactivity from slices, 0·5-ml samples of incubation medium were transferred to scintillation counting vials at 10, 20, 35, 50, 65, 80, 95, 110, 130 and 150 min. Variations from this procedure are given with the figures. At the conclusion of the experiments the slices were blotted, weighed and homogenized in sufficient ice-cold 0·4 N perchloric acid to make 5 ml of homogenate. The homogenates were centrifuged and 0·5-ml aliquots of the supernatant fluid were counted. The radioactivity remaining in the slices at each time interval was expressed as a percentage of the amount of radioactivity (90% of which is ^{3}H-NE) originally present in the slices at the beginning of the test incubation. The percentages were plotted logarithmically against time on semilogarithmic graph paper and the half lives of tissue radioactivity ($T_{\frac{1}{2}}$) were estimated from the exponential part of the curve. The rate constants of efflux (k) were calculated as follows: $k = 0{\cdot}693/T_{\frac{1}{2}}$.

The ionic compositions of the various media used are given in Table 1.

TABLE 1. COMPOSITION OF INCUBATION MEDIA*

Incubation medium	Concentrations of electrolytes (mM)						
	NaCl	$NaHCO_3$	KCl	K^+ as KH_2PO_4	$KHCO_3$	$MgSO_4$	$CaCl_2$
Krebs-bicarbonate	118	25	4·8	1·2		1·18	2·54
0 Na, 0·25 M sucrose†			4·8	1·2		1·18	2·54
0 Na, KCl			123	1·2	25	1·18	2·54
0 Na, choline	Choline Cl 118	Choline HCO_3 25	4·8	1·2		1·18	2·54
Na deficient, K_2SO_4	Na^+ as Na_2SO_4 25, 50 or 100		K^+ as K_2SO_4 118	1·2	25	$MgCl_2$ 0·25	2·54

* The basic composition of each of the media used in these experiments is listed. Variations are given in the text or with appropriate figures. All media contained dextrose, 2 g/l.

† pH adjusted to 7·3 with KOH.

RESULTS AND DISCUSSION

The effect of calcium and barium ions on the efflux of ^{3}H-NE from heart slices incubated in various media. Sodium free media, made isotonic with choline$^+$ and containing Ca^{2+}, deplete rat heart slices of their labeled stores of NE (Fig. 1). The efflux of the radioactivity from the slices begins slowly, becoming exponential after about 45 min (Figs. 1 and 2). The rate of efflux depends upon the presence of Ca^{2+}, but [Ca^{2+}] above 5 mM inhibit efflux.[30]

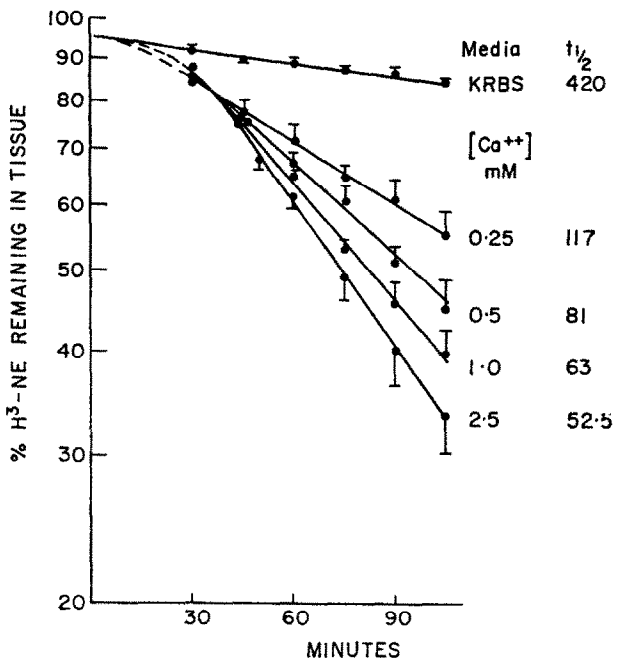

FIG. 1. Temporal plots of ^{3}H-NE remaining in rat heart slices incubated in Na^+-free media (choline$^+$) containing various [Ca^{2+}]. Control medium, Krebs-bicarbonate solution (KRBS). The Na-free media contained 143 mM choline$^+$ and all other constituents of Krebs-bicarbonate solution except $MgSO_4$. The concentrations of Ca^{2+} and half-lives of efflux of radioactivity ($T_{\frac{1}{2}}$) are listed on the figure. Each point represents an average of values determined in six to eight experiments, ± S.E.

The radioactivity accumulated in the incubation medium contains unmetabolized amine and deaminated metabolites. Equal absolute quantities of deaminated metabolite are recovered from Krebs solution and from Na^+-free media (sucrose, or choline$^+$). The metabolites enter the fluid at a rate corresponding to the half-life of NE *in vivo* suggesting that the overall turnover of NE in heart slices is mainly a function of intraneuronal metabolism, rather than release into the extracellular space. Unmetabolized NE accounts for the increment of radioactivity that accumulates in the Na^+-free media containing either choline$^+$ or sucrose.[3,30]

By contrast, the efflux of radioactivity from slices incubated in Na^+ and Ca^{2+}-free media (choline$^+$) containing Ba^{2+} also begins slowly, accelerates to an exponential

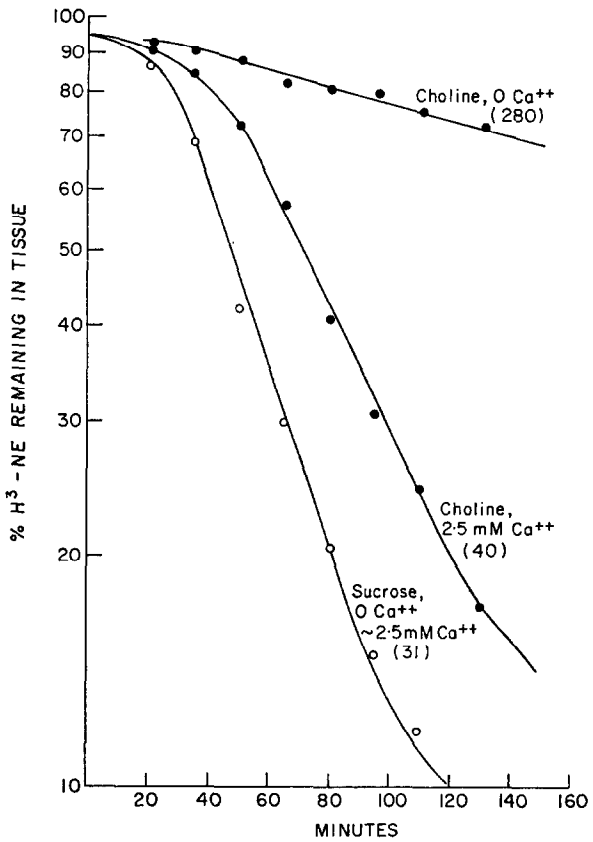

FIG. 2. Temporal plots of ^{3}H-NE remaining in rat heart slices incubated in Na-free media with and without 2·5 mM Ca^{2+}. The media contained either 0·25 M sucrose or 143 mM choline$^+$ as labeled on the figure. The media also contained all other compounds normally present in Krebs-bicarbonate solution except $MgSO_4$. Half-lives of efflux of radioactivity are given in parentheses on the figure. Each point represents an average of values determined in three experiments.

phase that lasts about 60 min, but then tapers off abruptly, giving the curves a distinct tri-phasic appearance (Fig. 3). Hence, the onset of the diminishing tertiary rate of efflux in response to Ba^{2+} is primarily dependent upon time regardless of the quantity of ^{3}H-NE remaining within the tissue, a fact of some interest though the reason for this phenomenon has not been established. The tertiary phase of efflux is also exponential and the calculated k for both exponential phases is dependent upon [Ba^{2+}]. Unlike Ca^{2+}, Ba^{2+} in concentrations up to 10 mM does not inhibit the efflux of ^{3}H-NE from slices.

Interpretation of rate constants of efflux in terms of principles of kinetics. The exponential curves illustrated in Fig. 1 and the secondary phases of the curves indicated in Fig. 3 were used to calculate the k for each of the concentrations of Ca^{2+} and Ba^{2+} contained in Na^+-free media (choline$^+$). The k calculated for the Na^+-free media was corrected for deaminated metabolites by subtraction of the k calculated for Krebs solution ($k - k_{Krebs}$). Double reciprocal plots of Lineweaver–Burk were then con-

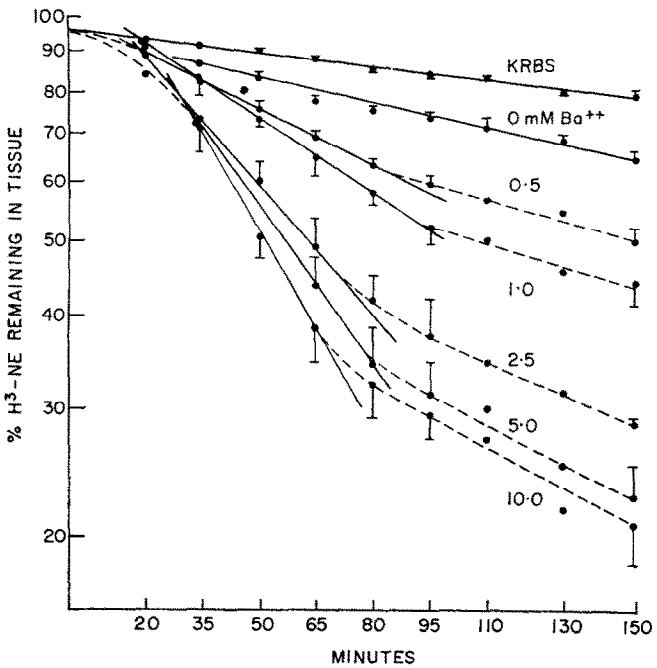

FIG. 3. Temporal plot of ^{3}H-NE remaining in rat heart slices incubated in Na^+-free media (choline$^+$) containing various [Ba^{2+}]. Control medium, Krebs-bicarbonate solution (KRBS). The Na^+-free media contained 143 mM choline$^+$ and all other constituents of Krebs-bicarbonate solution. The concentration of Ba^{2+} and half-lives of efflux of radioactivity in minutes ($T_{\frac{1}{2}}$) are listed on the figure. Dashed lines are the actual curves. The straight solid lines in these curves illustrate their linear component from which rate constants of efflux were calculated. Each point represents an average of values determined in five to six experiments, ± S.E.

structed, plotting $1/(k - k_{Krebs})$ vs. 1/[divalent ion], (Fig. 4). The linear plots so obtained are analogous to similar plots which illustrate Michaelis–Menten kinetics describing enzyme reactions. These plots suggest that Ca^{2+} and Ba^{2+} act upon a saturable intermediary in the process by which divalent ions apparently release previously stored ^{3}H-NE from storage vesicles in peripheral adrenergic nerve endings. It is premature to imply that the postulated intermediary functions in the classical enzymological sense. The fact that the efflux of ^{3}H-NE from slices incubated in Na^+-free media (sucrose or K^+) is temperature dependent, however, suggests that the efflux may be metabolically dependent (Table 2). This evidence is in accord with other evidence indicating that the release of NE from adrenal medullary granules is metabolically dependent.[31–34]

Comparison of the Ca^{2+} requirement for rapid-efflux using various replacements for Na^+. It was known that sucrose, K^+ and Li^+ cannot substitute for Na^+ in maintaining stores of ^{3}H-NE in heart slices.[2,3]

Table 3 lists the values of k as calculated for experiments in which the Ca^{2+} requirement was tested in the various media. In Na^+-free media containing sucrose for maintaining isotonicity, Ca^{2+} had little effect upon k as compared to Na^+-free media containing choline$^+$. A possible explanation for the apparent ineffectiveness of Ca^{2+} in this situation is that the Na^+-free (sucrose) medium alone increased the loss of

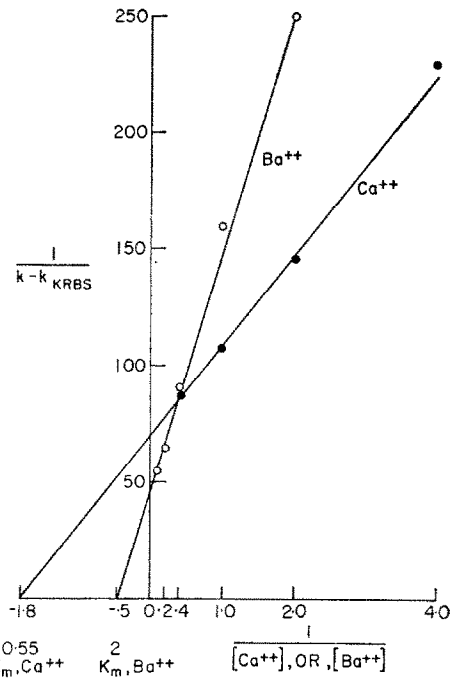

FIG. 4. Double reciprocal plots of the rate constants of efflux of ^{3}H-NE vs. $[Ca^{2+}]$ or $[Ba^{2+}]$. Rate constants were calculated from the half-lives of efflux of radioactivity determined from Figs. 1 and 3. The half-lives of efflux of radioactivity were converted to half-lives of efflux of ^{3}H-NE by subtracting deaminated products of ^{3}H-NE from the effluent recovered in the medium ($k - k_{Krebs}$, see Text). Media containing Ca^{2+} were Mg^{2+}-free. Media containing Ba^{2+} also contained Mg^{2+}.

TABLE 2. EFFECT OF TEMPERATURE ON THE EFFLUX OF ^{3}H-NE FROM RAT HEART SLICES INCUBATED IN Na-FREE MEDIA*

Medium	Initial rate, 37°	Rate after transfer, 0°	Initial rate 0°
Na^+-free, sucrose	0·018	0·00038	0·00030
Na^+-free, K^+	0·014	0·00030	0·00025

* Slices were placed in Na^+-free media (sucrose or K^+), at 0° from the start of incubation, or were first incubated at 37° to attain a rapid rate of efflux, then transferred to an identical medium at 0°. Rate constants were calculated from the results of 2 experiments.

amine from the tissues to an extent that Ca^{2+} could have little additional effect. The substitution of 25 or 100 mM Na^+ for an isosmotic quantity of sucrose slowed efflux which was then increased about 25 per cent in the presence of 2·5 mM Ca^{2+}.

By contrast, sodium free media containing KCl in concentrations equimolar to the Na^+ deficiency require Ca^{2+} in order to elicit maximal responses (Table 3, Fig. 5) resembling Na^+-free media (choline$^+$) in this regard.

TABLE 3. EFFECT OF Ca^{2+} ON THE RATE CONSTANT OF EFFLUX (k) OF RADIOACTIVITY FROM RAT HEART SLICES INCUBATED IN Na^+-DEFICIENT MEDIA*

Media	0 Na^+ (k, min^{-1})	25 Na^+ (k, min^{-1})	50 Na^+ (k, min^{-1})	100 Na^+ (k, min^{-1})
Choline$^+$, 0 Ca^{2+}	0·0025			
Choline$^+$, 2·5 mM, Ca^{2+}	0·0115			
Sucrose, 0 Ca^{2+}	0·0192			
Sucrose, 0·25, Ca^{2+}		0·0031		0·0017
Sucrose, 1·00, Ca^{2+}				0·0021
Sucrose, 2·50, Ca^{2+}	0·0200	0·0038		0·0022
K_2SO_4, 0 Ca^{2+}	0·0071	0·0060	0·0049	0·0044
K_2SO_4, 2·5, Ca^{2+}	0·0139	0·0108	0·0080	0·0069
KCl, 0 Ca^{2+}	0·0051	0·0046		
KCl, 2·5, Ca^{2+}	0·0100	0·0084		

* The basic composition of the various media are given in Table 1. Except for the media made with sulfate salts, Na^+ at 25 mM was added as the bicarbonate salt. Sodium in excess of 25 mM was added as the chloride. Sodium was added to the K_2SO_4 medium as the sulfate salt. Rate constants were calculated from the results of four to six experiments.

The curves shown on Fig. 5 illustrate the fact that the efflux of ^{3}H-NE from slices incubated in Na^+-free media containing K^+ begins rapidly, is well maintained throughout the experiment when Ca^{2+} is present, or slows after 30–40 min when Ca^{2+} is omitted from the medium (Fig. 5). The possibility arose that the initial rapid efflux of radioactivity from slices incubated in the various K^+ solutions, including the Ca^{2+}-free solution (contrast choline$^+$ and sucrose solutions, Figs. 1 and 2), was caused by the mobilization of tissue-bound Ca^{2+} by K^+. That possibility was tested in media containing various concentrations of ethylenediaminetraacetic acid (EDTA) in the media used for the preliminary and test incubations. In concentrations of 10^{-6} and 10^{-5} M, EDTA had no effect upon any component of efflux. In concentrations of 10^{-4} and 10^{-3} M, EDTA increased k to 0·0077 and 0·0160 min^{-1}, respectively, from 0·0039 min^{-1}, the data being analogous to data previously reported for standard Krebs-HCO_3 solution.[3] The initial rapid efflux of ^{3}H-NE in high [K^+] solutions is apparently not mediated by a component of tissue Ca^{2+} accessible to external EDTA.

A quantitative estimate of the relative increase in k calculated for ^{3}H-NE in Na^+-free media containing choline$^+$ or sucrose is of interest. In these media, the k with Ca^{2+} is about 2–3 times that in the Ca^{2+}-free media. Since the Ca^{2+}-deficient medium itself increases the k to about three times that for Krebs-bicarbonate solution, efflux in Na^+-deficient media containing Ca^{2+} is more properly compared to the efflux in standard Krebs-HCO_3 medium containing Ca^{2+}. Correcting total efflux for the radioactivity present as deaminated metabolites to obtain ^{3}H-NE ($k - k_{Krebs}$), the rate of efflux of unchanged ^{3}H-NE in Na^+-free medium containing 2·5 mM Ca^{2+} was increased by about 40 times for choline$^+$ and 65 times for sucrose.

The significance of choline$^+$ for demonstrating the Ca^{2+} requirement for rapid efflux was made clear by replacing sucrose with choline $^+$ according to the proportion 143 mM choline$^+$ to 0·25 M sucrose. Calcium ion was not clearly required until the concentration of choline$^+$ was somewhere between 25 and 50 mM. The response to

Ca^{2+} was roughly dependent upon the amount of $choline^{+}$ replacing the sucrose. For Na^{+}-free media, the relationship between Ca^{2+}-free and Ca^{2+} containing media made with either sucrose or $choline^{+}$, is illustrated in Fig. 2. Calcium ion had no significant effect on the efflux of NE into media containing 0·25 M sucrose but markedly stimulated the efflux of NE into media containing 143 mM $choline^{+}$ (see Table 3 also).

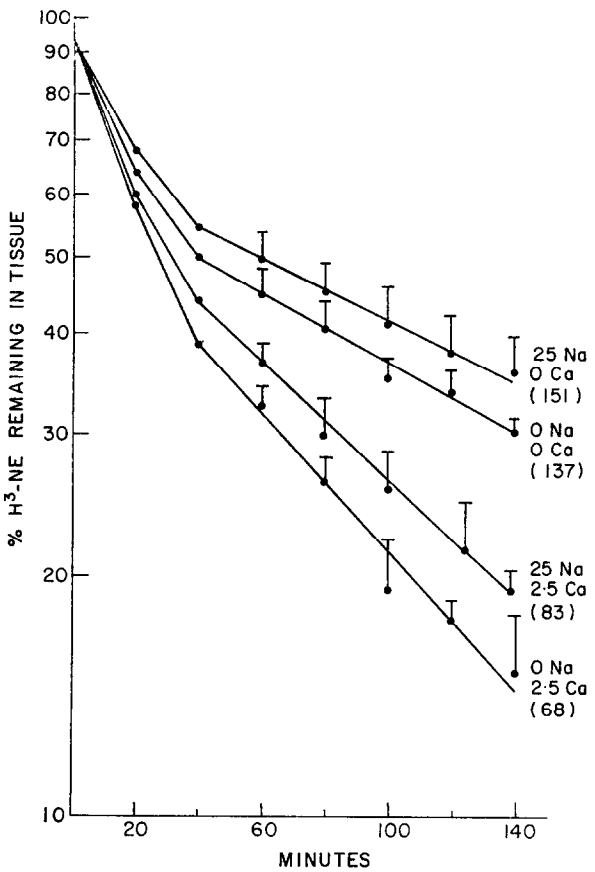

FIG. 5. Temporal plots of ^{3}H-NE remaining in rat heart slices incubated in Na-deficient media with and without 2·5 mM Ca^{2+}. The Na-free medium contained 118 mM KCl and 25 mM K HCO_3. The low-Na^{+} medium contained 118 mM KCl and 25 mM $NaHCO_3$. Both media were prepared with and without 2·5 mM Ca^{2+} and with all other compounds normally present in Krebs-bicarbonate solution. The curves are labeled as to Na^{+} and Ca^{2+} content. Half-lives of efflux of radioactivity are given in parentheses on the figure. Each point represents an average of values determined in five experiments ± S.E.

Antagonism of Ca^{2+} by Na^{+}. The results of the experiment described in the preceding sections are in accord with the hypothesis that Na^{+} and Ca^{2+} are mutual antagonists in the process by which various substances are released from the bound state (see introductory paragraphs). This hypothesis was tested by incubating heart slices in media containing various [Na^{+}] added to media containing 143 mM K^{+}. $Sodium^{+}$ was added to these media rather than substituted for K^{+} in order to prevent

effects which could be attributed to changes in $[K^+]$. Sulfate salts of Na^+ and K^+ were used in these experiments in order to minimize the tissue swelling that can occur with media containing high concentrations of KCl as a result of the accumulation of KCl by the cells. Cells will not accumulate the K^+ salt of $SO_4^=$, which is impermeable. Heart slices, having few intact cells, do not gain weight even in KCl solutions, but the precaution was taken against the possibility that the nerve endings would seal off and react to various media as though they were intact cells. The existence of a possible osmotic influence of hypertonicity on the efflux of ^{3}H-NE was eliminated by experiments showing no significant changes in k when slices were incubated in Krebs-bicarbonate solution or in the 143 mM K^+ solution (K_2SO_4) to which sucrose was added at 0·2 M. By contrast, hypertonic media increase the output of transmitter from cholinergic nerve endings.[35]

The question arises as to whether the increased depletion rate of ^{3}H-NE from slices incubated in media containing high concentrations of K^+ is dependent upon depolarization of the cell membrane or upon the Na^+ deficiency. Calcium ion is known to be required for the release of norepinephrine from adrenergic nerve endings by K^+[8] and by the nerve impulse. However, calcium-dependent release of cholinergic transmitter is not related solely to the lowering of membrane potential.[14,27] The increased rates of efflux in the present experimental media are related to Na^+ deficiency as indicated by the fact that Na^+ inhibited the Ca^{2+} stimulated efflux under conditions (143 mM K^+) in which the membrane was likely to be depolarized by K^+. However, Na^+-deficient media (K^+) containing 25 mM Na^+ produced higher values for k than similar media containing sucrose. The data suggest an influenced K^+ possibly as a Na^+ antagonist[2] in the overall process leading to efflux of ^{3}H NE.

Figure 6 shows double reciprocal plots of $k - k_{Krebs}$ vs. $[Ca^{2+}]$, which represent media containing Na^+ in concentrations of 25, 50 and 100 mM and 0·25, 0·5, 1·0 and 2·5 mM Ca^{2+}. The straight lines so formed intersect beyond the y axis, a result characteristic of noncompetitive inhibition. Gage and Quastel,[27] using frequency counts of miniature end-plate potentials in the rat diaphragm-phrenic nerve preparation, observed that Na^+ was a competitive inhibitor of Ca^{2+}.

The results of experiments in which choline$^+$ was substituted for Na^+ differ from those reported for K^+ (Fig. 7). The preliminary delay in the appearance of rapid efflux (Fig. 1) is lengthened when some Na^+ is present. Moreover, choline added to standard Krebs-bicarbonate solution at 100 mM concentration eventually increased efflux of ^{3}H-NE after 130 min incubation (Fig. 7). This observation modifies our previous report that choline$^+$ added to standard Krebs-bicarbonate solution did not increase k within 120 min.[30] In the case of the media containing 30 and 100 mM Na^+, efflux ultimately became exponential, the k calculated for the medium containing 100 mM Na, being about 1/2 that in the Na^+-free medium (Fig. 7). We conclude from these experiments, as well as from the experiments described in the preceding section, that the antagonism between Na^+ and Ca^{2+} can be observed in all media, but it is more pronounced in some media than in others.

Possible role for choline$^+$ and K^+ in causing rapid efflux. The mechanism by means of which amine is released from slices incubated in media containing choline^{2+} might involve the gradual loss of Na^+ from the cell or the entrance of choline$^+$ into the cell. The simultaneous presence of Na^+ and choline$^+$ in the external medium would retard the rate of net loss of cellular Na^+ accounting for the delayed onset of rapid efflux.

The delay in media containing sucrose (Fig. 2) or Li^+ (unpublished) is evidence against a mandatory role for choline$^+$ in causing efflux. Hence, loss of intracellular Na^+ appears to be the main factor leading to the rapid efflux of NE in Na^+-free media containing choline$^+$ and presumably K^+. However, both substitute ions retard efflux from slices incubated in Na^+ and Ca^{2+}-free media, contrasting with sucrose (Fig. 2), the efflux then being stimulated by addition of Ca^{2+} to the media.

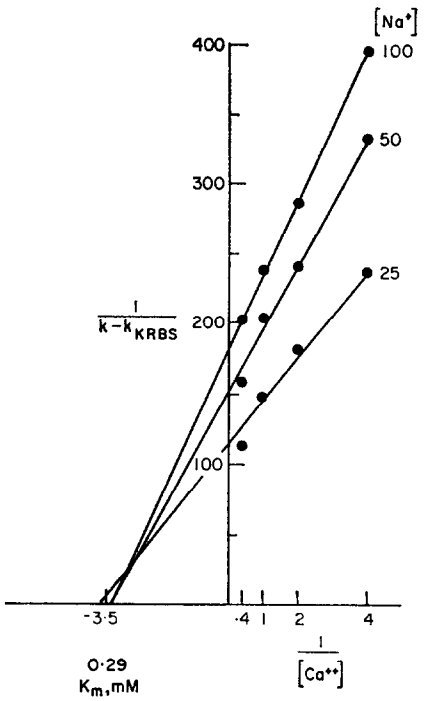

FIG. 6. Double reciprocal plots of the rate constants of efflux of ^{3}H-NE vs. $[Ca^{2+}]$. Rate constants ($k - k_{Krebs}$) were calculated as for Fig. 4. All media contained 144 mM K^+ (K_2SO_4) and 25, 50 or 100 mM Na^+ (Na_2SO_4), as labeled on the figure. The media contained all other compounds normally contained in Krebs-bicarbonate solution except $MgSO_4$ (Table 1). Each point represents an average of values determined in six experiments.

The data do not exclude from consideration the Burn and Rand[36] hypothesis that acetylcholine (or choline$^+$ in the present experiments) triggers the Ca^{2+} activated release mechanism. In testing this possibility we have been unable to inhibit the efflux of ^{3}H-NE into Na^+-free media (choline$^+$) with bretyllium in concentrations of 1, 5, 10, 20 and 50 μg/ml. In a concentration of 10^{-4} M, *d*-tubocurarine reduced the k efflux from 0·0133 min^{-1} to 0·0099 min^{-1}. Both inhibitors should have significantly reduced efflux if the hypothesis of Burn and Rand is applicable to choline$^+$.

Mechanism of action of Ca^{2+} and Ba^{2+} ions in releasing ^{3}H-NE. The evidence reported above suggests that Ca^{2+} and Ba^{2+} release stored ^{3}H-NE by means of a temperature sensitive reaction involving a saturable intermediary mechanism. Sodium and calcium ions are noncompetitive antagonists in the reaction. The relationship

between Na^+ and Ca^{2+} is most easily observed in Na^+-free media containing K^+ or choline$^+$ as substitutes for Na^+. These two Na^+ substitutes markedly decrease k when Ca^{2+} is absent (contrasting with sucrose or Li^+) and Ca^{2+} added to the media greatly increases k.

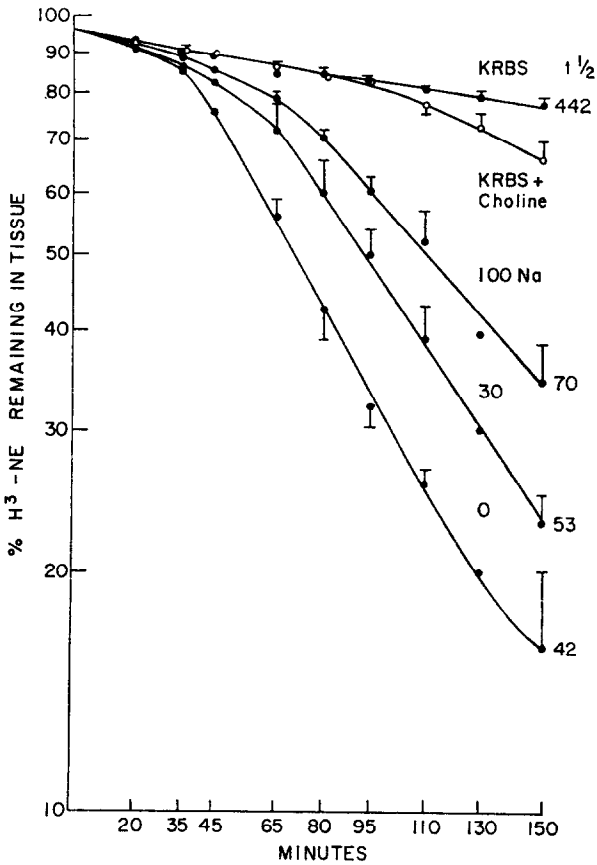

FIG. 7. Temporal plots of ^{3}H-NE remaining in rat heart slices incubated in Na^+-deficient media (choline$^+$). Control medium, Krebs-bicarbonate solution (KRBS). Choline$^+$ Cl (100 mM) was added to the solution marked KRBS and choline. The concentration of Na^+ and the half-lives of efflux ($T_{\frac{1}{2}}$) are labeled on the figure. The media contained all other compounds normally present in Krebs-bicarbonate solution. Each point represents an average of values determined in five experiments, ± S.E.

Although K^+ and choline$^+$ partially satisfy the Na^+ requirement for storage, these ions appear to intensify the releasing effect of Ca^{2+} in media containing low [Na^+], (Figs. 1, 2 and 5), as compared to the effect of Ca^{2+} in sucrose solutions containing low [Na^+] (Table 3). Weak agonists are often antagonists of a more powerful agonist, and K^+ is an antagonist of the NE retaining effect of Na^+ in heart slices.[2,3] Of interest is the fact that K^+ and choline$^+$ are both normal constituents of cells. Hauesler *et al.*[37] reported a special role for acetylcholine in the release of NE from perfused cat

heart in terms of an increased Ca^{2+} conductance of the membrane, but the effect of acetylcholine was also related to action potentials and to depolarization, which themselves increase Ca^{2+} uptake.

Although Ca^{2+} may serve as an intermediary in the process of stimulus (depolarization) secretion coupling,[6] membrane depolarization cannot be the sole cause of NE efflux in the present experiments as discussed in the section on Na^+-Ca^{2+} antagonism. Hence, the fact that the efflux of Na^+ from the squid axon is increased in Na^+ deficient media containing Li^+, choline$^+$ or dextrose,[38] may be of more relevance for efflux from slices than any concomitant decrease in membrane potential. Moreover, the Ca^+ requirement for efflux stimulation is greater for choline$^+$ than for Li^+ or sucrose. Li^+ may antagonize Ca^{2+}[39] obscuring the releasing effect of Ca^{2+} but that explanation will not hold for sucrose. Thus, the loss of intracellular Na^+, sometimes aided by the presence of Ca^{2+}, appears to be a common requirement for increasing the rate of efflux of ^{3}H-NE from rat heart slices.

Perhaps the simplest interpretations of the present results are that (1) Na^+ is required for normal storage regardless of the presence or absence of Ca^{2+}. (2) Choline$^+$ and K^+ partially satisfy the Na^+ requirement for storage. (3) Ca^{2+} releases unmetabolized amine by antagonizing the effects of low [Na^+], or Na substitutes, on storage. The effects of Ca^{2+} appear to be mediated by a metabolically dependent mechanism which is saturable. (4) The Na^+ requirement for storage can be defined in part as the prevention of the amine releasing effect of Ca^{2+}, and as a direct storage requirement.

What bearing do these experiments have upon the mechanism of release of transmitter by the nerve impulse? Birks and Cohen[40] postulated that the nerve impulse causes the accumulation of intracellular Ca^{2+} by mobilizing a membrane carrier mechanism from which Ca^{2+} is released by competition with intracellular Na^+. Calcium then releases cholinergic transmitter. Consistent with this view is the fact that Ca^{2+} influx is increased when the intraaxonal Na^+ concentration is raised.[38] In agreement with that idea, the stimulant effect of cardiac glycosides on efflux has been attributed to accumulation of intracellular Ca^{2+} in parallel with an increase of intracellular [Na^+].[40] We have interpreted similar results to suggest that the abolition of the membrane transport mechanism for the re-uptake of NE is the cause of increased efflux in this particular situation.[3]

The Na^+ requirement for storage of amine suggests the possibility that Ca^{2+} releases amine by competition with Na^+ at the storage level. The effect of Ca^{2+} is terminated when Ca^{2+} is pumped out of the cells by exchanging with extracellular Na^+.[38,41]

However, the influx of calcium into the squid axon increases when intracellular [Na^+] is either low or high.[38] An alternative idea to the above hypothesis, also consistent with our data, shifts the emphasis from inward movement of Ca^{2+} to outward movement of Na^+ as being of major importance for the release of transmitter. In principle, this speculation is in accord with the data of Baker *et al.*[38] showing that Ca^{2+} is transported into the cell in exchange for intracellular Na^+. The rate of exchange is increased by high concentrations of intracellular Na^+, such as occur, apparently,[42] in isolated nerve endings (synaptosomes). The nerve impulse might set in motion an inward flux of Ca^{2+} in exchange for intracellular Na^+. Since the idea is speculative, though supported by same data, it suffices to say that the optimum

conditions for demonstrating Na^+–Ca^{2+} exchange in the squid axon are not necessarily the same as the optimum conditions for demonstrating a Ca^{2+} requirement for stimulating the efflux of transmitter from rat heart slices incubated in Na^+-deficient media.

An apparent contradiction to the alternative suggestion described in the preceding paragraph arises from the fact that our results relate to Na^+ deficiency intracellularly, whereas Na^+ enters axons during the nerve impulse. However, ACh can be released from the cholinergic nerve ending by electrotonic current in the presence of tetrodotoxin and Ca^{2+}, indicating that the entry of Na^+ into the nerve ending (which may already be Na^+-rich,[42] is not essential for transmitter release.[16] Perhaps the apparent inconsistency can be resolved by an assumption that the nerve impulse mobilizes a component of cellular Na^+ or the Ca^{2+} carrier, either ion then exchanges with its antagonist resulting in a deficiency of the Na^+ required for storage of amine. The reaction would normally occur at a localized site, presumably at the junction of membrane and vesicles. Calcium might then be displaced by Na^+ entering the cell on the amine carrier during re-uptake[3] and then be transported away from the site of action.

Competition between Na^+ and Ca^{2+} in various reactions has frequently been mentioned (see introductory paragraphs), and it appears that the interaction between Na^+ and Ca^{2+} occurs at receptors mediating the movement of Ca^{2+} across the plasma membrane[38] as well as mediating the release of transmitter.[27,28] Sodium deficiency can increase the entrance of Ca^{2+} into cells.[30,38,43] Magnesium ion inhibits the Ca^{2+} stimulated efflux of ^{3}H-NE from various organs[19–21] and from rat heart slices incubated in Na^+-free media. Moreover, Mg^{2+} inhibits the increased uptake of Ca^{2+} by heart slices,[30] the squid axon[44] and the adrenal gland.[45] These data suggest that Ca^{2+} accumulation by cells is essential for increased outflow of neurotransmitter.

The above discussion is in accord with the point of view that stimulus-secretion coupling requires Ca^{2+} and involves a Na^+–Ca^{2+} interaction. For a precise significance and mechanism of the interaction a number of possibilities arise, some of them apparently contradictory, but all may contribute some part of the total picture.

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