INFLUENCE OF IONS ON NOREPINEPHRINE STORAGE, TRANSPORT AND SYNTHESIS IN ISOLATED PERFUSED RAT HEART

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ABSTRACT

The effects of differing concentrations of the cations Ca^{++} (0 to 5 mM), Mg^{++} (0 to 16 mM), K^{+} (0 to 20 mM) and Na^{+} (0 to 145 mM) on the storage, uptake, subcellular distribution, release and synthesis of norepinephrine in the isolated perfused rat heart were investigated. Decreasing the concentration of Na^{+} in the perfusion medium reduced the level of endogenous norepinephrine in hearts, whereas changes in the concentrations of K^{+}, Ca^{++} and Mg^{++} were without effect. The uptake of ^{3}H-norepinephrine by the isolated perfused rat heart was not influenced by K^{+} or Mg^{++}, whereas increase in the concentration of both Na^{+} and Ca^{++} in the perfusion medium enhanced the uptake. However, Ca^{++} failed to influence ^{3}H-norepinephrine uptake in the absence of Na^{+}. The subcellular distribution of ^{3}H-norepinephrine in the heart was not altered by changing the concentrations of K^{+}, Ca^{++} and Mg^{++}, while increasing the concentration of Na^{+} in the perfusion medium caused an increase in the amount of ^{3}H-label in the granular fraction and a decrease in the soluble fraction. The spontaneous efflux of ^{3}H-norepinephrine was not influenced by changes in the concentrations of K^{+}, Ca^{++}, or Mg^{++} in the perfusion media, whereas increasing the concentrations of Na^{+} reduced the release of ^{3}H-label. The release of ^{3}H-norepinephrine due to Na^{+}-lack in the absence of Ca^{++} was more than that observed in the presence of Ca^{++}. The uptake of ^{14}C-catecholamine synthesis was decreased by increasing the concentration of Ca^{++} or Na^{+} but was not altered by K^{+} or Mg^{++}. The retention of newly synthesized ^{14}C-catecholamines in the heart was increased by increasing the concentration of Na^{+}. These results indicate the importance of Na^{+} and Ca^{++} in the storage, uptake, release and synthesis of norepinephrine in the rat heart.

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INTRODUCTION

Norepinephrine, the sympathetic neurotransmitter, is mainly localised in the adrenergic nerve terminals (Norberg and Hamberger, 1964) where it is stored in specific intracellular membrane bound granules (Euler and Hillarp, 1956; Potter and Axelrod, 1963b). Although extensive studies have been undertaken on the processes of storage, uptake, intracellular distribution, release and synthesis of norepinephrine, very little attention has been paid to the role of important cations in these processes in heart. The present investigation was undertaken to study the effects of certain cations on the transport and synthesis of norepinephrine in myocardium. For this purpose, the isolated, perfused, rat heart was employed as an experimental model. The influence of Ca^{++}, Mg^{++}, K^{+} and Na^{+} on endogenous stores of norepinephrine and on the uptake, subcellular distribution and release of exogenous ^{3}H-norepinephrine was investigated. In addition, the effects of these cations on the uptake of ^{14}C-tyrosine, the precursor of the neurotransmitter, and on the biosynthesis of ^{14}C-catecholamines from ^{14}C-tyrosine were also studied. The results of this study reveal the importance of Na^{+} and Ca^{++} ions in the transport, storage and synthesis of norepinephrine in the adrenergic nerve terminals in the heart.

REVIEW OF LITERATURE

Euler (1946) established that norepinephrine was the sympathetic neurotransmitter and various other investigators have confirmed this observation (Bacq and Fisher, 1947; Gaddum and Goodwin, 1947; Schmiterlow, 1948). Many excellent review articles concerning the studies on storage, uptake, release and biosynthesis of catecholamines have appeared in the literature (Stjarne, 1964; Acheson, 1966; Iversen, 1967; Axelrod and Kopin, 1969; Euler, 1969; Geffen and Livett, 1971). Although the primary objective of the present survey of literature was to obtain information about the influence of cations on synthesis, storage, uptake and release of norepinephrine, an attempt has also been made to outline various relevant features of these processes in adrenergic neurons.

Synthesis of Norepinephrine

The biosynthetic pathway of catecholamines from tyrosine involving hydroxylation, decarboxylation, β-oxidation was first proposed by Blaschko (1939). Since then it has been confirmed both *in vivo* and *in vitro* for adrenal medulla (Demis *et al.* 1955; Kirshner and Goodall, 1956; Udenfriend and Wyngaarden, 1956). The ability of rat and cat brain slices to synthesize dopamine and norepinephrine when incubated with ^{14}C-tyrosine, was reported by Masuoka *et al.* (1961). Similarly in the isolated dog and rabbit hearts the synthesis of norepinephrine was demonstrated by perfusion with labelled tyrosine or dopamine (Chidsey *et al.* 1963; Musacchio and Goldstein, 1963). It is now clear that the adrenergic nerve terminals contain all the necessary enzymes required for the synthesis of norepinephrine from its precursor, tyrosine. The rate of synthesis in the isolated perfused guinea pig heart was shown to be comparable with that found *in vivo* (Spector *et al.* 1963). Moreover, the level of activity of those enzymes involved in the biosynthesis of catecholamines was found to be greatly reduced in tissues after denervation of the sympathetic supply or in immunosympathectomised animals (Carlsson and Waldeck, 1963; Fischer *et al.* 1964; Andén *et al.* 1965; Klingman, 1965).

Tyrosine hydroxylase, the enzyme which catalyzes the conversion of l-tyrosine to l-dopa, has been studied in sympathetically innervated tissues, adrenal

medulla and brain (Nagatsu et al. 1964; Udenfriend, 1966b). A partially purified preparation of this enzyme from adrenal medulla was found to require tetrahydropteridine cofactors. The enzyme was stimulated by Fe^{++}, showed specificity for its substrate, l-α-methyl-p-tyrosine, and was inhibited both in vivo and in vitro by certain iodo-tyrosine derivatives (Goldstein and Weiss, 1965; Spector et al. 1965).

The decarboxylation of l-dopa into dopamine is catalyzed by dopa decarboxylase, which was discovered by Holtz et al. (1938) and purified by Fellman (1959). This enzyme was found to require pyridoxal phosphate as a cofactor. The decarboxylation of other aromatic l-amino acids to the corresponding aromatic amines is also catalyzed by this enzyme (Lovenberg et al. 1962).

The β-oxidation of dopamine to norepinephrine was catalyzed by dopamine-β-hydroxylase which is located in storage granules (Kirshner, 1959). This copper-containing enzyme required the presence of ascorbic acid and oxygen for its activity (Friedman and Kaufman, 1965). Tyramine and α-methyl dopamine also serve as substrates.

The conversion of norepinephrine to epinephrine has been shown to be catalyzed by the enzyme phenylethanolamine N-methyl transferase in the adrenal medulla (Axelrod, 1962). The activity of this enzyme has been demonstrated in the supernatant fraction of adrenal homogenates with S-adenosyl methionine serving as the methyl donor. However this enzyme is generally believed to be absent from the peripheral sympathetic nerves.

From studies of in vitro synthesis of norepinephrine from tyrosine, dopa and dopamine in various fractions of the bovine splenic nerve homogenate, Stjarne (1966b) has concluded that hydroxylation of tyrosine and decarboxylation of dopa take place in the extra-granular axoplasm while the formation of norepinephrine from dopamine occurs in the storage granule. However, Udenfriend (1966a) is of the opinion that all these enzymes are normally localized in a synthesizing organelle which may be the same as the norepinephrine storage vesicle. Levitt et al. (1965) have reported that the rate limiting step in the formation of

norepinephrine in the sympathetic nervous system is the conversion of l-tyrosine to dopa, catalyzed by tyrosine hydroxylase. In isolated perfused guinea pig hearts, the rate of norepinephrine synthesis was found to increase linearly on increasing the concentration of dopa or dopamine but not of tyrosine. The apparent K_m for the over-all reaction, tyrosine→norepinephrine, was shown to be comparable to that observed for the conversion of tyrosine to dopa by purified tyrosine hydroxylase. The activity of this enzyme was much lower than that of the other enzymes involved in the biosynthesis of norepinephrine. Stjarne (1966b) has observed that the synthesis of norepinephrine is regulated by the norepinephrine concentration in the tissues in two ways: by product inhibition of the enzymic process at the level of hydroxylation of tyrosine and by competitive inhibition of dopamine binding to the hydroxylation sites.

Acceleration of norepinephrine synthesis by nerve stimulation has been reported by several investigators (Alousi and Weiner, 1966; Gordon et al. 1966; Roth et al. 1966). The impulse induced acceleration of norepinephrine synthesis in the adrenergic neurone is found to occur only from tyrosine and not from dopa (Sedvall and Kopin, 1967). The synthesis is, therefore, stimulated at or before the tyrosine hydroxylation step. The rate of norepinephrine synthesis in this case is considered to be controlled by tyrosine transport, product inhibition, endogenous inhibitors or cofactors of tyrosine hydroxylase. It is currently believed that a reduction in the concentration of norepinephrine due to its release on nerve stimulation results in the activation of tyrosine hydroxylase and subsequently in the increase of norepinephrine biosynthesis (Neff and Costa, 1966). A reciprocal relationship between neuronal norepinephrine and its turn-over in rat brain and heart has been demonstrated (Neff and Costa, 1968). Thus, tyrosine hydroxylase appears to regulate the synthesis of norepinephrine in the sympathetic nervous system.

The effect of Ca^{++} ions on ^{14}C-dopamine biosynthesis from ^{14}C-tyrosine was investigated in slices from the striatum of rats by Goldstein et al. (1970). The addition of Ca^{++} to the incubation media was found to produce a dose dependent decrease of ^{14}C-dopamine biosynthesis. K^{+} was found to

accelerate the synthesis of norepinephrine in the guinea pig vas deferens (Boadle-Biber et al. 1970).

It may be noted that results concerning the effect of ions on the biosynthesis of norepinephrine are fragmentary in nature and that no detailed studies have as yet been published.

Storage of Norepinephrine

Norepinephrine stores are associated with adrenergic neurones and become depleted after degeneration of nerves (Euler and Purkhold, 1951). Histochemical fluorescence studies have revealed that norepinephrine is mainly concentrated in the terminal parts of the adrenergic neurones. It has been shown that nerve cell bodies have a low amine content and that practically all of the adrenergic transmitter is localised in the varicosities of the synaptic terminals (Norberg and Hamberger, 1964). Dahlstrom and Haggendal (1966) have estimated the norepinephrine content of the cervical sympathetic ganglia of cat and of the organs innervated by these ganglia and found that the ratio between cell bodies and terminals for norepinephrine content was about 1:300.

It has been shown by Blaschko and Welch (1953) and by Hillarp et al. (1953) that catecholamines are stored in specific intracellular membrane-bound granules in the adrenal medulla. Euler and Hillarp (1956) have demonstrated the existence of similar norepinephrine storing vesicles in bovine splenic nerves and rat spleen. The norepinephrine storage granules of the adrenergic nerves have also been demonstrated by electronmicroscopy (Wolfe et al. 1962). These storage granules are considered to be synthesized in nerve cell bodies (Dahlstrom et al. 1965) and transported via the axons down to the varicosities of the terminals, where they are present in abundance (Malmfors, 1965). Dalhstrom and Haggendal (1966) have calculated that the rate of transport of amine storage granules in sciatic nerve is 5 - 6 mm/hr and their life span is about 35 days. The number of amine granules in each varicosity is calculated to be about 1500 in rat iris and vas deferens (Dahlstrom et al. 1966). The physical properties, such as stability in various media and spontaneous amine release rate of splenic nerve vesicles (Euler and Lishajko, 1961a, b, 1963a, b; Stjarne, 1964) were found to

be similar to those from rat heart (Potter and Axelrod, 1963b; Snyder et al. 1964; Potter, 1966). Although a number of similarities between the granules from heart and adrenal medulla have been observed, these granules were also found to differ in some respects. For example, the half time for norepinephrine release from cardiac granules is about 10 min, whereas in the case of medullary granules it is about 108 min at 37°C (Euler, 1966). Furthermore, Ca^{++} accelerated the spontaneous rate of amine release from the medullary granules but was without any effect on the heart granules (Schumann et al. 1964). Thus, it appears that the norepinephrine storage granules in adrenergic nerves innervating different organs share some identical properties, but also possess some specific characteristics.

In addition to norepinephrine, ATP is also found to be present in storage granules (Schumann, 1958; Euler et al. 1963). Weak catecholamine-ATP complexes have also been demonstrated in vitro by nuclear magnetic resonance spectroscopy (Weiner and Jardetzky, 1964). Norepinephrine storage in vivo in the granules may be an amine-ATP complex in association with a characteristic protein (Kirshner et al. 1966; Schumann et al. 1966).

Although studies have demonstrated that the binding mechanisms for norepinephrine in storage granules are not specific for norepinephrine alone, a certain correlation has been established between chemical structure and binding. The quarternary N^{+} group and the β-hydroxyl group are important for binding (Weiner and Jardetzky, 1964). Stereospecificity of the β-hydroxyl group has been observed in that l-norepinephrine is preferred (Kopin and Bridgers, 1963).

Experiments concerning differential drug responses (Trendelenburg, 1961; Crout et al. 1962; Obianwu, 1968), and release rates of labelled norepinephrine in the rat heart (Axelrod et al. 1961; Kopin et al. 1962) suggest that norepinephrine is present in more than one "pool" in sympathetic nerves. However, Costa et al. (1966) have demonstrated that tracer amounts of ^{3}H-norepinephrine disappear from rat heart by a single exponential phase and that tyramine and reserpine cause a rapid and exponential release of norepinephrine stores, revealing that the norepinephrine is transfered so rapidly between compartments

that they behave essentially as a single pool. Studies on the subcellular distribution of norepinephrine in sympathetic nerves have shown that a considerable proportion of the total norepinephrine content is recovered in the soluble supernatant fraction obtained after centrifugation at 105,000 x g for 1 hr. However, it has been argued that supernatant norepinephrine might have arisen from the destruction of storage granules during the procedures of homogenization and centrifugation (Potter and Axelrod, 1963a; Carlsson, 1966).

Stjarne (1966a) has suggested that the soluble form of norepinephrine is an important source of the transmitter released by nerve stimulation. But studies of the miniature end plate potentials in sympathetically innervated guinea pig vas deferens have revealed norepinephrine release as quantal in nature (Burnstock and Holman, 1966). It was suggested that norepinephrine discharged from the axon by depolarization of the axonal membrane is immediately derived from nerve granules, which represent the anatomical substrate for quantal release. Studies on the time course of depletion of norepinephrine in rat heart and brain showed that norepinephrine present in the soluble cytoplasmic fraction was depleted more rapidly than the corresponding particle bound fraction (Westfall, 1970). It was considered that on nerve stimulation norepinephrine from the adrenergic neuron is either derived first from a supernatant pool or from granular fraction followed by rapid redistribution of the neurotransmitter to the particulate fraction at the expense of cytoplasmic amine.

Thus, the adrenergic neurotransmitter is stored in granules as an amine-ATP-protein complex but opinion is divided whether it is present in more than one pool. The importance of Na^+ ions for the process of norepinephrine storage in sympathetic nerve endings of rat heart slices was revealed by the observation, that retention of ^3H-norepinephrine in rat heart slices was reduced in the absence of Na^+ in the incubation media (Bogdanski and Brodie, 1966). Similar results were obtained in the absence of K^+ or Ca^{++} but the retention of norepinephrine was found to be normal in the presence of half the amount of the usual K^+ concentration in the incubation media (Gillis and Paton, 1967). It was suggested that the reduced retention of the amine observed under

these conditions is due to a combination of impaired active transport into the cell and interference with intracellular binding. Thus, it is apparent that very little information concerning the influence of cations on the ability of heart to retain and store norepinephrine is available in the literature.

Uptake of Norepinephrine

After injecting labelled norepinephrine into animals, the accumulation of the amine has been found to be well confined to the sympathetically innervated organs (Whitby et al. 1961). The normal ability of an organ to accumulate exogenously administered catecholamine is severely impaired or lost after chronic sympathetic denervation (Hertting et al. 1961; Stromblad and Nickerson, 1961) or in immunosympathectomized animals (Sjoqvist et al. 1965; Zaimis et al. 1965). Further proof of localization of catecholamine uptake has come from techniques such as autoradiography, electronmicroscopy and fluorescent histochemistry (Marks et al. 1962; Wolfe et al. 1962; Hamberger et al. 1964). The results from such experiments have led to the conclusion that the uptake of norepinephrine takes place in the sympathetic nerve terminals.

The physiological significance of the norepinephrine uptake process is considered to be in terminating the action of circulating amine (Celander, 1954). Similarly the reuptake of the released neurotransmitter on nerve stimulation results in the termination of its action (Brown and Gillespie, 1957). The reuptake process also serves to maintain endogenous stores of norepinephrine in tissues and thus helps in transmitter economy. Studies by Axelrod et al. (1959), Muscholl (1961) and Whitby et al. (1961) have clearly revealed that the uptake of catecholamines takes place against a concentration gradient. Dengler et al. (1961) have suggested that the uptake of norepinephrine in cat heart and brain slices might be mediated by a saturable membrane transport process. This was confirmed by Iversen (1963) who studied the kinetics of ^{3}H-norepinephrine uptake in the isolated perfused rat heart and found that the data fitted the Michaelis-Menten equation. The transport of norepinephrine is considered to be an energy dependent process (Berti and Shore, 1967; Hamberger, 1967).

Iversen (1965) has reported a second type of norepinephrine uptake, designated as uptake$_2$, in the isolated perfused rat heart when high external concentrations of norepinephrine (1 to 40µg/ml) were used. Lightman and Iversen (1969) have claimed that this extraneuronal uptake$_2$ may also have some physiological importance in terminating the action of catecholamines. Jacobowitz(1967) has reported intense catecholamine-fluorescent cells in the confines of the atrial ganglia of many species: these chromaffin cells are regarded as an extraneuronal catecholamine pool and may be involved in the uptake$_2$.

Exogenous norepinephrine is rapidly incorporated into the intracellular storage sites in rat and rabbit hearts (Michaelson et al. 1964; Stjarne, 1964). However, Iversen (1963) has shown that a large part (75%) of the endogenous pool exchanged very slowly, if at all, with exogenous norepinephrine in isolated perfused rat heart. Initial uptake of norepinephrine through the neuronal membrane can be distinguished from its incorporation into the intraneuronal storage vesicles. From an analysis of the uptake of ^{3}H-norepinephrine in the isolated rat heart, Iversen (1963) has concluded that there is a biphasic entry of the catecholamine into the sympathetic nerves: a rapid uptake into the axoplasm and a slow accumulation of the amine in the intraneuronal storage granules.

The uptake process has been shown by many workers to exhibit structural and stereochemical specificity. The uptake of norepinephrine by adrenergically innervated tissue was found to be more than that of epinephrine (Axelrod et al. 1959; Whitby et al. 1961). A trend of decreasing affinity for the uptake of amines by isolated rat heart was observed when the side chain at the N-position was increased (Burgen and Iversen, 1965). Beaven and Maickel (1964) have reported that the rate of uptake of l-norepinephrine by rat heart is several times more rapid than that of d-norepinephrine. But, Draskoczy and Trendelenburg (1968) failed to observe any stereospecificity in the uptake of norepinephrine in the neuronal membrane of isolated perfused rabbit heart. Sachs (1970) also showed a small preference for the uptake of l-isomers in the intact nerves of both mouse atria and rat iris; however, this

phenomenon was not apparent after blockade by reserpine of the Mg^{++}-ATP dependent uptake mechanism in the storage granules. This would indicate that the stereospecificity of norepinephrine uptake in the adrenergic nerve terminals is associated with the amine storage granules.

Kirshner (1962) and Carlsson et al. (1963) have shown that the storage particles isolated from the adrenal medulla accumulate exogenous epinephrine and norepinephrine and that this uptake is greatly increased by the addition of ATP and Mg^{++}. Likewise, the experiments by Euler and Lishajko (1961b, 1963a,b) demonstrated the ability of a suspension of bovine splenic nerve particles to take up exogenous norepinephrine at a higher external concentration in the medium and to a larger extent in the presence of ATP and Mg^{++}. However, Potter and Axelrod (1963b) have reported that the rat heart particles accumulated exogenous norepinephrine at low concentrations in the medium and in the absence of ATP and Mg^{++}. Some differences have also been noted in norepinephrine uptake by nerve particles and intact tissue. For example, norepinephrine uptake is quantitatively more in the intact tissue. Furthermore, norepinephrine uptake by isolated granules was apparent only when exposed to a relatively higher concentration of catecholamines in the medium (Iversen, 1967).

Thus, uptake of norepinephrine takes place in the adrenergic nerve terminals. This uptake is an energy dependent, carrier-mediated transport process that exhibits structural stereochemical specificity and is believed to be located in the axonal membrane of the adrenergic neuron. The current concept of the uptake of norepinephrine includes the presence of a Mg^{++}-ATP dependent uptake mechanism in the intraneuronal storage granules (Sachs, 1970).

Studies on the influence of cations on the uptake of norepinephrine have revealed a dependency of the transport mechanisms on Na^{+} in rat heart (Iversen and Kravitz, 1966; Gillis and Paton, 1966, 1967; Horst et al. 1968; Bogdanski and Brodie, 1969; Keen and Bogdanski, 1970), in rabbit heart (Surgue and Shore, 1969) in cat spleen (Kirpekar and Wakade, 1968) and in brain synaptosomes obtained from rat and rabbit (Bogdanski et al. 1968; Tissari et al. 1969).

Gillis and Paton (1966) have observed a significant reduction in the accumulation of ^{3}H-norepinephrine in rat heart slices in K^{+}-free medium. However, these workers have shown that concentrations of K^{+} higher than 6 mM in the medium also produced a decrease in the accumulation of norepinephrine. On the other hand, Bogdanski and Brodie (1969) observed this decrease in norepinephrine uptake only when the concentration of K^{+} in the medium was increased over 26 mM and suggested that high K^{+} level antagonise the effect of Na^{+} on uptake and storage of norepinephrine. But, for maximum transport of norepinephrine, the presence of K^{+} in the medium was essential (Bogdanski et al. 1970a,b). However, Horst et al. (1968) did not observe an effect of K^{+} on the uptake of norepinephrine by the isolated perfused rat heart. Thus, it appears that results concerning the effects of K^{+} on norepinephrine uptake are conflicting. Mg^{++} does not seem to have any effect on the uptake of norepinephrine in rat heart (Gillis and Paton, 1966; Horst et al. 1968).

A reduction in the uptake of ^{3}H-norepinephrine into rat heart slices due to Ca^{++} removal by the addition of EDTA (5 mM) to the medium, has been reported by Gillis and Paton (1966). On the other hand, norepinephrine uptake was shown to be enhanced in the absence of Ca^{++} and decreased in the presence of high concentrations of Ca^{++} in the isolated perfused rat heart (Horst et al. 1968). These workers have suggested that Ca^{++} and Na^{+} have a common but antagonistic site of action. Keen and Bogdanski (1970), on the other hand, have shown that the uptake of norepinephrine in rat heart slices is solely dependent upon the Na^{+} concentration. The presence or absence of Ca^{++} had little effect. Thus, it can be seen that contradictory reports regarding the effects of Ca^{++} on the uptake of norepinephrine have been published.

A model for amine transport has been suggested by Bogdanski and Brodie (1969). According to their hypothesis, the amine is transported together with Na^{+} across the neuronal membrane by a carrier-mediated process. This transport depends upon the asymmetric distribution of Na^{+} and K^{+} across the membrane. This concept is supported by the findings of Tissari et al. (1969),

who have shown that ouabain blocks the uptake of amine after a lapse of time as a result of the inhibition of Na^+ and K^+ dependent ATPase [(Na^+-K^+)-ATPase]. However, Leitz and Stefano (1970) could not correlate the onset of inhibition of norepinephrine uptake due to ouabain with changes in the intracellular Na^+ and K^+ concentration in guinea pig myocardium. Similarly, White and Keen (1970) have demonstrated that differences in internal Na^+/K^+ ionic composition had no effect on the uptake of ^{3}H-norepinephrine by rat brain synaptosomes; although raising the external concentration of Na^+ increased the uptake of ^{3}H-norepinephrine, this did not occur after pretreatment with metabolic inhibitors. Moreover, concentrations of ouabain (0.1 and 0.01 mM) and strophanthidin (1 mM) which inhibit (Na^+-K^+)-ATPase activity, do not depress the uptake of norepinephrine. At a concentration of 1 mM, ouabain inhibited both (Na^+-K^+)-ATPase activity and norepinephrine uptake. Thus, ouabain can inhibit norepinephrine uptake by a mechanism which does not involve (Na^+-K^+)-ATPase (White and Keen, 1971). These findings cast some doubt on the involvement of (Na^+-K^+)-ATPase in the uptake of norepinephrine.

Release of Norepinephrine

Release of norepinephrine from the adrenergic nerve terminals has been shown to occur on stimulation of the sympathetic nervous system. Various isolated perfused organs, which are innervated by the sympathetic nervous system have been employed as models for studying the mechanisms of norepinephrine release (Brown, 1965). The bovine splenic nerve granule preparation has been extensively employed to elucidate the characteristics of norepinephrine release (Euler and Lishajko, 1961a, 1963a,b, 1965). The amount of norepinephrine released can be measured biologically in terms of its effect on sensitive effector organs or chemically by spectrophotofluorometric methods. However, radioactive methods are most commonly employed for studying the release of norepinephrine under various experimental conditions (Hertting and Axelrod, 1961).

The release of norepinephrine from the adrenergic nerves in response to stimulation is a Ca^{++} dependent process. This phenomenon has been extensively documented by various workers using different preparations. In the isolated perfused rabbit heart, the response to sympathetic nerve stimulation was reduced when Ca^{++} concentration of the perfusion medium was lowered (Hukovic and Muscholl, 1962). Likewise, Boeles et al. (1963) have reported that the response to nerve stimulation in the isolated rat hypogastric nerve-vas deferens preparation was abolished in a Ca^{++} deficient medium. Similar results were reported for guinea pig vas deferens by Kuriyama (1964). Calcium dependence of norepinephrine release due to sympathetic stimulation has also been reported for rat brain and heart slices (Baldessarini and Kopin, 1966), isolated cat colon (Boullin, 1966, 1967), and cat spleen (Kirpekar and Misu, 1967).

Hukovic and Muscholl (1962) observed a basal rate of norepinephrine discharge of 2 ng/min, which could be increased more than 100 fold on stimulation of the adrenergic nerve supply in the perfused rabbit heart. Burnstock and Holman (1962) have provided supporting evidence by demonstrating small spontaneous depolarizing potentials in sympathetically innervated smooth muscles. Thus,it is considered that there is a small but significant amount of norepinephrine which is released spontaneously from the adrenergic nerve terminals in the absence of nerve impulses.

Boullin (1967) has shown that nerve stimulation releases ^{3}H-norepinephrine from the cat colon when the perfusion fluid contains Ca^{++}; but this phenomenon is not dependent upon the amount of Ca^{++} in the medium. Moreover, the spontaneous release of norepinephrine was not altered by the addition of Ca^{++} and was rather augmented by removal of Ca^{++}. It was suggested that Ca^{++} is essential for norepinephrine release by nerve stimulation but not for the spontaneous output that occurs in the absence of nervous activity.

The enhancement, induced by Ca^{++}, of norepinephrine release from adrenergic nerves in response to electrical stimulation is antagonized by Mg^{++} (Burn and Gibbons, 1964). Calcium was found capable of reversing the depressing

effect of Mg^{++} on the release of neurotransmitter by nerve stimulation (Farmer and Campbell, 1967). Keen and Bogdanski (1970) reported that the rate constant of norepinephrine efflux in rat heart slices is dependent upon the concentration of Ca^{++} present in the medium. The release of ^{3}H-norepinephrine from the heart slices was correlated with an increased uptake of ^{45}Ca by tissues incubated in Na^{+}-deficient media.

It is considered that the mechanism of norepinephrine release from adrenergic nerve terminals by nerve impulses is similar to that postulated for the adrenal medullary secretion of catecholamine induced by acetylcholine (Douglas, 1968). In both instances the release of transmitter is dependent upon Ca^{++} and is promoted by depolarizing concentrations of extracellular K^{+}. The "stimulus-secretion coupling" proposed for the release of catecholamines from adrenal medullary chromaffin cells by Douglas and Rubin (1961) may serve as a model system for understanding the mechanism of release of norepinephrine from sympathetic nerve terminals. The norepinephrine release rate is greatly influenced by temperature, the pH of the medium, the addition of ATP or ADP to the medium and even by the concentration of norepinephrine in the incubation medium (Euler, 1966). However, it is important to recognise that there are differences between nerve granules and the adrenal medullary granules. For example, nerve granules have a much higher rate of spontaneous release of norepinephrine than the medullary granules at 37°C.

It has been shown that acetylcholine increases ^{45}Ca uptake in the adrenal medulla as well as catecholamine release (Douglas and Poisner, 1962). In perfusion experiments employing cat adrenal gland, it was found by Douglas and Rubin (1961, 1963) that acetylcholine still retained its ability to release catecholamines even when Na^{+}, K^{+}, Mg^{++} and Cl^{-} were absent; only Ca^{++} was found to be required. The finding that K^{+} stimulated catecholamine secretion from the medulla (Vogt, 1952) was explained by the fact that K^{+} enhances Ca^{++} uptake (Douglas and Poisner, 1961). Although the secretory mechanism in adrenal medulla is normally considered to be Ca^{++} dependent, other divalent ions such as Ba^{++} or Sr^{++} can be substituted for Ca^{++} (Douglas

and Rubin, 1964).

Catecholamine release from the adrenal medulla in response to various stimuli was associated with the appearance of ATP and its metabolites in the perfusate. The molar ratios of released catecholamines to ATP and its metabolites in the perfusate were similar to those found in the chromaffin granules i.e. 4 mol of catecholamines for 1 mol of ATP. Thus it was evident that chromaffin granules might be the source of catecholamines released from the adrenal medulla (Douglas et al. 1965). As well as ATP, a characteristic soluble protein constituent of chromaffin granules was also detected in the perfusate (Banks and Helle, 1965; Blaschko et al. 1967; Kirshner et al. 1967). No changes in the phospholipid and cholesterol content of granules or in the perfusate was observed on stimulation (Poisner et al. 1967; Schneider et al. 1967). It has been suggested that catecholamine secretion from medullary chromaffin cells involves the granules directly and occurs by exocytosis or some closely related process (Douglas, 1966, 1968).

Banks (1966) has proposed that the actual role of Ca^{++} in "stimulation-secretion coupling" is by neutralization of the net negative surface charges on the granules which bring about the attachment and incorporation of granular membrane to the plasmalemma. A considerable amount of ATPase activity has been demonstrated in the granular membrane (Hosie, 1965). It has been found that ATP stimulates the release of catecholamines from chromaffin granules in media rich in Na^{+}, or K^{+} (Oka et al. 1965). The releasing effect of ATP could be blocked by inhibiting the ATPase activity of the granule (Poisner and Trifaro, 1967; Trifaro and Poisner, 1967). By splitting the ATP present on plasmalemma, granular membrane-bound ATPase might result in an increase in cross linkage and fusion of the granular membrane with plasmalemma. Douglas (1968) speculated that such an interaction between ATP and ATPase is promoted in situ by Ca^{++} with the membrane fusion and to the release of the contents of the granule. Rubin (1969) has observed that energy is required for the secretory action of Ca^{++} on medullary chromaffin cells. Lishajko (1970a, b) has reported that the enhancing effect of Ca^{++} on the release of catecholamines occurs only

in conjunction with precipitation of calcium phosphate while RNA strongly potentiates the releasing effect of Ca^{++}. The entry of Ca^{++} *in vivo* into the chromaffin cell influences catecholamine secretion in the intact gland by increasing the intracellular level of free Ca^{++} and making more Ca^{++} available for the process of catecholamine release. Thus, Ca^{++} is essential for the release of neurotransmitter on nerve stimulation, but the precise step involving Ca^{++} in the mechanism of release is still speculative.

MATERIALS AND METHODS

Perfusion of Heart

Each male hooded rat, weighing about 300 g, was sacrificed by decapitation, the heart rapidly removed, placed in ice-cold oxygenated Krebs-Henseleit bicarbonate buffer and freed from adipose and connective tissue. The aorta was tied to a cannula of the perfusion apparatus for coronary perfusion by the conventional Langendorff technique (Dhalla and McLain, 1967; Dhalla et al. 1970). After equilibrating the hearts for 10 minutes with control perfusion medium, the hearts were perfused with a medium of desired ionic composition for a period of 40 minutes unless otherwise indicated in the text. The coronary flow was maintained at a rate of 10 ml/min with a peristaltic pump.

Krebs-Henseleit bicarbonate buffer of the following composition (mM) was used as a control medium in all experiments: Na^{+}, 145; K^{+}, 6; Mg^{++}, 1.2; Ca^{++}, 1.25; Cl^{-}, 126; HCO_3^{-}, 25; $SO_4^{=}$, 1.2; $PO_4^{\equiv}$, 1.2 and dextrose, 11.1. When the concentration of a cation was varied in the medium, the osmolarity was maintained by adding an appropriate amount of sucrose. The perfusion media were equilibrated with 95% O_2 and 5% CO_2 gas mixture and the pH of the media was 7.4. The temperature of the perfusion media was maintained at 37°C.

Experiments on Endogenous Stores of Norepinephrine

The hearts were perfused in a closed circulation system with 50 ml of the perfusion medium for 40 minutes, removed from the cannula, blotted, weighed and norepinephrine content estimated.

The total norepinephrine in the heart was assayed chemically by the method of Anton and Sayre (1962). The hearts were homogenized in a motor-driven glass homogenizer at 4°C with 20 ml of ice cold 0.4 N perchloric acid. The tissue homogenate was centrifuged and the norepinephrine present in the clear supernatant was adsorbed on specially treated alumina at pH 8.6, washed thrice with cold water and eluted by vigorously shaking with 0.05 N

perchloric acid. An aliquot of the clear eluate obtained after refrigerated centrifugation was converted to its trihydroxyindole derivative through oxidation by potassium ferricyanide and the fluorescence measured on an Aminco-Bowman Spectrophotofluorometer with excitation and emission wave lengths (mμ) set at 400 and 520 respectively. Internal standards were run together with experimental batches. The recovery was 75 - 79%, and the results reported in this study were not corrected for this.

Experiments on ^{3}H-norepinephrine Uptake and Subcellular Distribution

The hearts were perfused in a closed circulation apparatus with 50 ml of perfusion medium containing 10 μCi of ^{3}H-norepinephrine (178 ng) and 500 ng of non-labelled norepinephrine. The radioactive ^{3}H-7-d, l-norepinephrine (specific activity 9.5 Ci/mmole) was obtained from the New England Nuclear Corporation, Boston, Mass. Ascorbic acid (1 mg) was also added to prevent auto-oxidation of norepinephrine. One milliliter samples of the effluent perfusate were withdrawn at different intervals of perfusion. Each sample was added to 15 ml of scintillation liquid (Bray, 1960) and the radioactivity counted in a Packard Tri Carb Liquid Scintillation Spectrometer (Model 3375).

At the end of 40 minutes the hearts were perfused for 2 minutes with norepinephrine-free perfusion medium to wash out the extra cellular norepinephrine. Studies with ^{14}C-sorbitol in the isolated perfused rat heart revealed that the extracellular space is almost cleared by a wash out perfusion period of this duration (Morgan et al. 1961). The hearts were blotted, weighed and the sub-cellular distribution of ^{3}H-norepinephrine determined by the method of Iversen et al. (1965) as described below.

The hearts were homogenized in 10 ml of ice-cold 0.25 M sucrose solution in an all glass homogenizer with a loose fitting motor driven pestle for 75 seconds. The homogenate was centrifuged in a Sorvall RC 2-B refrigerated centrifuge at 1000 x g for 10 minutes to remove the coarse fraction containing cell debris. One milliliter of the supernatant was pipetted for counting total radioactivity and the remainder was centrifuged in a Beckman L2-65B ultra-

centrifuge at 105,000 x g for 1 hour to obtain granular (sediment) and soluble (supernatant) fractions. The granular fraction was resuspended in 5 ml of ice-cold 0.25 M sucrose solution. An aliquot of one milliliter from each fraction was added to 15 ml of Bray's solution and the radioactivity counted.

Experiments on ^{3}H-norepinephrine Release

The hearts were perfused for 20 minutes with control medium containing 10 μCi of ^{3}H-norepinephrine (178 ng), 500 ng of non-labelled norepinephrine and 1 mg of ascorbic acid in a closed circulation perfusion apparatus. After an initial wash out period of 2 minutes with norepinephrine-free medium to remove the extracellular ^{3}H-norepinephrine, these hearts were then switched to a medium of desired ionic composition and perfused at the rate of 10 ml/min. One milliliter aliquot of the effluent was taken after 1,3,5,10, and 20 minutes of perfusion with the test solution and the radioactivity counted as before. At the end of the perfusion, the heart was removed, blotted and weighed. The exponential release of ^{3}H-norepinephrine was plotted according to the method of least squares. The rate constant of efflux (k) and half-time (T 1/2) of release of ^{3}H-label were calculated from the slope of decline between 3 and 20 minutes.

Experiments on ^{14}C-tyrosine Uptake and ^{14}C-catecholamine Synthesis

The experiments on the uptake of ^{14}C-tyrosine were performed in a similar manner to that previously described for ^{3}H-norepinephrine except that the perfusion medium of desired ionic composition contained 1 μCi of ^{14}C-tyrosine (498 ng) and 500 ng of non-labelled tyrosine. The radioactive ^{14}C (U)-l-tyrosine (specific activity 364 mCi/mmole) was obtained from New England Nuclear Corporation, Boston, Mass. One milliliter of perfusate was drawn at different intervals of perfusion and the radioactivity measured.

At the end of the perfusion the hearts were removed, blotted, weighed and the synthesized ^{14}C-catecholamine content estimated as follows. The hearts were homogenized in ice-cold 0.4 N perchloric acid. The ^{14}C-catecholamine was separated by alumina adsorption as described earlier. An

aliquot of the eluate containing the ^{14}C-catecholamine was added to 15 ml of Bray's solution and counted. The perfusate at the end of the 40 minutes perfusion period with ^{14}C-tyrosine was also analyzed for its content of ^{14}C-catecholamine, by acidifying with perchloric acid (0.4 N final concentration) and adsorbing on alumina as described above. The rate of newly synthesized norepinephrine was calculated by the method of Spector et al. (1963).

All the results reported in this study were subjected to statistical inference according to Dixon and Massey (1969).

RESULTS

Influence of Ions on the Endogenous Levels of Norepinephrine

The levels of norepinephrine in isolated rat hearts perfused for 40 minutes with media containing varying amounts of cations were determined and the results are shown in Tables 1 and 2. Changing the concentrations of Ca^{++} (0 to 5 mM), Mg^{++} (0 to 16 mM) and K^{+} (0 to 20 mM) had no significant effect ($P > 0.05$) on norepinephrine content of the myocardium whereas the levels of norepinephrine in hearts perfused with media containing Na^{+} (25 to 145 mM) were higher in comparison to those in hearts perfused with medium without Na^{+} ($P < 0.05$).

Influence of Ions on the Uptake of ^{3}H-norepinephrine

Isolated rat hearts were perfused for 40 minutes with media containing varying amounts of cations in the presence of ^{3}H-norepinephrine and the uptake of ^{3}H-label determined at different intervals of perfusion. Increasing the concentration of Ca^{++} from 0 to 5 mM in the perfusion medium was found to increase ($P < 0.05$) the uptake of ^{3}H-norepinephrine whereas Mg^{++} (0 to 16 mM) had no effect (Table 3). The ^{3}H-norepinephrine uptake by the isolated rat heart was not influenced ($P > 0.05$) by changing the concentration of K^{+} (0 mM to 20 mM) in the perfusion medium (Table 4). On the other hand, the ^{3}H-norepinephrine uptake by hearts perfused with media containing 85 or 145 mM Na^{+} was significantly ($P < 0.01$) higher than those perfused with 0 or 25 mM Na^{+} (Table 4).

The effect of Ca^{++} on ^{3}H-norepinephrine uptake by the heart was also studied in the absence or presence of Na^{+} in the perfusion medium. Figure 1 shows that in the presence of 145 mM Na^{+}, 1.25 mM Ca^{++} markedly increased ^{3}H-norepinephrine uptake but no such effect ($P > 0.05$) was observed in the absence of Na^{+}.

Influence of Ions on the Subcellular Distribution of ^{3}H-norepinephrine

The hearts were perfused for 40 minutes with media containing different concentrations of cations in the presence of ^{3}H-norepinephrine and the distribution

TABLE 1

Effect of Ca^{++} and Mg^{++} on the Endogenous Norepinephrine Levels in the Isolated Perfused Rat Heart.

Ions in perfusion media (mM)	Concentration of Norepinephrine (μg/g heart)
A. Calcium	
0	0.665 ± 0.034
1.25	0.699 ± 0.042
5	0.639 ± 0.032
B. Magnesium	
0	0.650 ± 0.036
4	0.698 ± 0.046
16	0.634 ± 0.031

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with media containing different concentrations of $CaCl_2$ or $MgCl_2$ after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

TABLE 2

Effect of K^+ and Na^+ on the Endogenous Norepinephrine Levels in the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	Concentration of norepinephrine (μg/g heart)
A. Potassium	
0	0.705 ± 0.025
5	0.703 ± 0.024
20	0.688 ± 0.019
B. Sodium	
0	0.570 ± 0.042
25	0.681 ± 0.019*
85	0.704 ± 0.040*
145	0.716 ± 0.026*

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with media containing different concentrations of KCl or NaCl after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

*Significantly more than the value for 0 mM Na^+ ($P < 0.05$).

TABLE 3

Effect of Ca^{++} and Mg^{++} on the Uptake of ^3H-norepinephrine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^3H-norepinephrine uptake (cpm x 10^5 per g heart)			
	5 min	10 min	20 min	40 min
A. Calcium				
0	15.08 ± 2.53	15.70 ± 1.48	16.61 ± 2.51	16.47 ± 1.15
1.25	17.54 ± 1.89	20.90 ± 1.44*	25.44 ± 2.97*	26.45 ± 3.85*
5	17.61 ± 1.30	21.09 ± 1.61*	26.78 ± 1.83*	30.37 ± 1.95*
B. Magnesium				
0	18.09 ± 0.87	20.18 ± 0.77	25.86 ± 0.91	33.02 ± 1.04
4	18.48 ± 2.62	21.20 ± 1.31	28.46 ± 1.88	35.70 ± 2.23
16	16.20 ± 1.73	18.94 ± 1.14	24.53 ± 1.16	31.07 ± 1.41

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^3H-norepinephrine (10 μCi) in different concentrations of $CaCl_2$ or $MgCl_2$ after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

*Significantly more than the respective values for 0 mM Ca^{++} ($P < 0.05$).

TABLE 4

Effect of K^+ and Na^+ on the Uptake of ^3H-norepinephrine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^3H-norepinephrine uptake (cpm x 10^5 per g heart)			
	5 min	10 min	20 min	40 min
A. Potassium				
0	20.66 ± 0.72	23.65 ± 1.17	29.01 ± 1.86	33.45 ± 2.80
5	21.18 ± 0.33	25.96 ± 0.74	32.18 ± 1.48	33.25 ± 2.28
20	18.63 ± 0.72	22.48 ± 0.82	27.18 ± 1.27	28.60 ± 1.47
B. Sodium				
0	12.17 ± 1.07	12.71 ± 1.95	14.65 ± 1.42	14.36 ± 2.50
25	14.63 ± 1.58	14.95 ± 1.44	17.10 ± 1.64	16.11 ± 1.15
85	21.92 ± 0.94*	23.46 ± 1.77*	29.00 ± 1.19*	30.38 ± 2.14*
145	20.12 ± 1.87*	22.61 ± 2.84*	26.95 ± 3.10*	28.54 ± 3.18*

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^3H-norepinephrine (10 μCi) in different concentrations of KCl or NaCl after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

*Significantly more than the respective values for 0 or 25 mM Na^+ ($P < 0.01$).

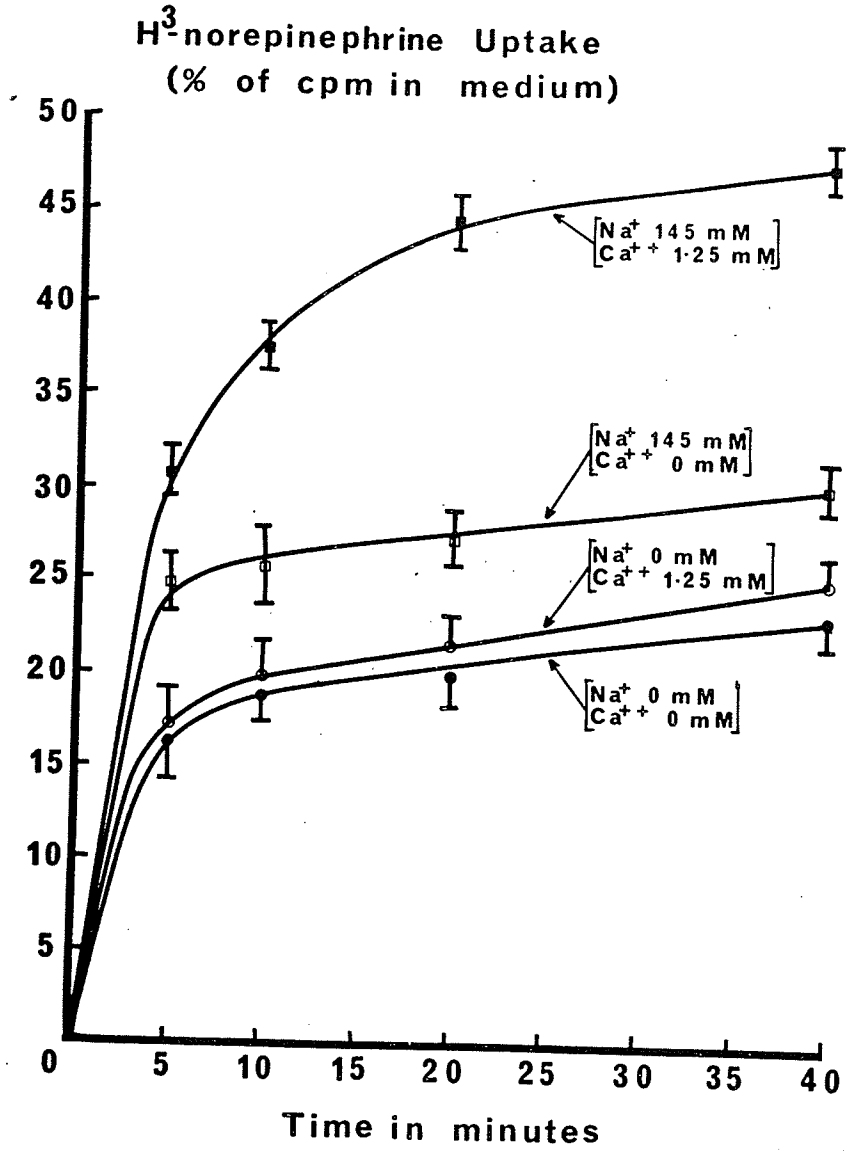

FIGURE 1: Effect of Na^{+} and Ca^{++} on the uptake of ^{3}H-norepinephrine by the isolated perfused rat heart. Each point is a mean ± SE of 4 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^{3}H-norepinephrine (10 μCi) in desired concentrations of NaCl and $CaCl_2$ after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

of ^{3}H-label in the granular and soluble fractions was studied. The results in Tables 5 and 6 indicate that the distribution of ^{3}H-norepinephrine was not altered ($P > 0.05$) on perfusing the hearts with varying amounts of Ca^{++} (0 to 5 mM), Mg^{++} (0 to 16 mM) and K^{+} (0 to 20 mM) while, on the other hand, increasing the concentration of Na^{+} from 0 to 145 mM in the perfusion medium increased ($P < 0.001$) the ^{3}H-label in the granular fraction and decreased ($P < 0.001$) it in the soluble fraction (Table 6).

Influence of Ions on the Release of ^{3}H-norepinephrine

Isolated rat hearts were labelled by perfusing with Krebs-Henseleit solution containing ^{3}H-norepinephrine for 20 minutes. The release of ^{3}H-norepinephrine at different time intervals was then studied by switching on to perfusion media containing varying concentrations of cations. The spontaneous release of ^{3}H-label was not influenced ($P > 0.05$) by the presence of different amounts of Ca^{++} (0 to 5 mM), Mg^{++} (0 to 16 mM) or K^{+} (0 to 20 mM) in the perfusion medium (Tables 7 and 8) whereas increasing the concentration of Na^{+} from 0 to 145 mM was found to decrease it significantly ($P < 0.05$).

The effect of Na^{+}-lack on the release of ^{3}H-norepinephrine was also studied in the absence or presence of Ca^{++} and the results are shown in Fig.2. In the presence of 1.25 mM Ca^{++} the spontaneous release of ^{3}H-norepinephrine due to the absence of Na^{+} was significantly increased ($P < 0.01$). In the absence of Ca^{++} the effect of Na^{+}-free medium on the spontaneous release of ^{3}H-norepinephrine became more pronounced ($P < 0.001$). Table 9 shows the effect of Na^{+} and Ca^{++} on the rate constant (k) and half-time (T 1/2) g ^{3}H-norepinephrine release from the isolated perfused rat heart. The absence of Na^{+} in the perfusion medium resulted in significant increase in the rate constant of efflux (k). When Ca^{++} was also omitted, the k value further increased ($P < 0.001$). The increased spontaneous release in the absence of Na^{+} and Ca^{++} in the medium was also revealed by the decrease in half-time (T 1/2) of decline in ^{3}H-label in the heart.

TABLE 5

Effect of Ca^{++} and Mg^{++} on the Subcellular Distribution of ^{3}H-norepinephrine in the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	Distribution of ^{3}H-label (% of ^{3}H-label in 1000 x g supernatant)	
	Granular Fraction	Soluble Fraction
A. Calcium		
0	34.47 ± 1.42	65.53 ± 1.42
1.25	36.46 ± 1.15	63.54 ± 1.15
5	35.99 ± 1.13	64.01 ± 1.03
B. Magnesium		
0	38.65 ± 2.37	61.35 ± 2.37
4	39.06 ± 2.70	60.94 ± 2.70
16	39.26 ± 1.90	60.74 ± 1.90

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with media containing ^{3}H-norepinephrine (10 μCi) in different concentrations of $CaCl_2$ or $MgCl_2$. The 1000 x g supernatant contained 56 to 60% of ^{3}H-label in the heart. The granular fraction refers to 1000 to 105,000 x g sediment whereas soluble fraction is the post 105,000 x g supernatant.

TABLE 6

Effect of K^+ and Na^+ on the Subcellular Distribution of ^3H-norepinephrine in the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	Distribution of ^3H-label (% of ^3H-label in 1000 x g supernatant)	
	Granular Fraction	Soluble Fraction
A. Potassium		
0	39.29 ± 2.96	60.71 ± 2.96
5	37.59 ± 3.88	62.41 ± 3.88
20	38.00 ± 2.29	62.00 ± 2.29
B. Sodium		
0	5.40 ± 0.47	94.60 ± 0.47
25	28.56 ± 1.62*	71.44 ± 1.62*
85	35.38 ± 3.25*	64.62 ± 3.25*
145	37.68 ± 3.15*	62.32 ± 3.15*

Each value is a mean ± SE of 6 experiments. The hearts were perfused for 40 min in a closed circulation with media containing ^3H-norepinephrine (10 μCi) in different concentrations of KCl or NaCl. The 1000 x g supernatant contained 57 to 63% of ^3H-label in the heart. The granular fraction refers to 1000 to 105,000 x g sediment whereas soluble fraction is the post 105,000 x g supernatant.

*Significantly different from the respective values for 0 mM Na^+ ($P < 0.001$).

TABLE 7

Effect of Ca^{++} and Mg^{++} on the Release of ^3H-norepinephrine from the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^3H-norepinephrine release (cpm x 10^2 per ml) at				
	1st min	3rd min	5th min	10th min	20th min
A. Calcium					
0	18.93 ± 2.67	11.85 ± 1.29	10.09 ± 1.55	8.91 ± 2.48	3.83 ± 0.38
1.25	22.32 ± 5.15	13.51 ± 3.08	12.00 ± 2.54	8.76 ± 2.10	4.48 ± 0.36
5	16.89 ± 1.36	11.73 ± 1.54	9.25 ± 0.96	6.95 ± 0.57	3.98 ± 0.25
B. Magnesium					
0	21.00 ± 1.10	13.75 ± 1.13	11.01 ± 1.07	7.56 ± 0.69	4.06 ± 0.39
4	26.56 ± 4.94	15.01 ± 3.33	12.83 ± 2.99	7.48 ± 0.57	4.06 ± 0.39
16	22.02 ± 3.74	16.72 ± 2.31	13.37 ± 1.68	9.19 ± 0.92	5.22 ± 0.34

Each value is a mean ± SE of 4 to 6 experiments. The hearts were labelled by perfusing with ^3H-norepinephrine (10 μCi) for 20 min in a closed circulation apparatus and the ^3H-label release was studied by switching over to the constant-flow open system with media containing different concentrations of $CaCl_2$ or $MgCl_2$.

TABLE 8

Effect of K^+ and Na^+ on the Release of ^{3}H-norepinephrine from the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^{3}H-norepinephrine release (cpm x 10^2 per ml) at				
	1st min	3rd min	5th min	10th min	20th min
A. Potassium					
0	23.84 ± 3.48	14.84 ± 2.45	10.09 ± 1.47	8.97 ± 1.02	4.25 ± 1.37
5	20.63 ± 2.73	12.60 ± 1.92	10.32 ± 1.48	7.61 ± 0.73	4.77 ± 0.13
20	23.89 ± 1.75	14.40 ± 1.14	11.47 ± 0.93	8.77 ± 0.43	4.84 ± 0.41
B. Sodium					
0	35.90 ± 3.72	26.97 ± 3.25	22.35 ± 1.75	20.48 ± 1.96	17.05 ± 1.04
25	27.51 ± 2.68*	19.84 ± 1.34*	15.88 ± 1.42*	11.09 ± 0.75*	7.80 ± 0.46*
85	25.68 ± 2.81*	16.86 ± 2.18*	12.50 ± 1.66*	8.67 ± 1.14*	5.00 ± 0.78*
145	20.33 ± 1.66*	13.02 ± 0.65*	10.41 ± 0.59*	7.35 ± 0.19*	4.67 ± 0.29*

Each value is a mean ± SE of 4 to 6 experiments. The hearts were labelled by perfusing with ^{3}H-norepinephrine (10 μCi) for 20 min in a closed circulation apparatus and the ^{3}H-label release was studied by switching over to the constant-flow open system with media containing different concentrations of KCl or NaCl.

*Significantly less than the respective values for 0 mM Na^+ ($P < 0.05$).

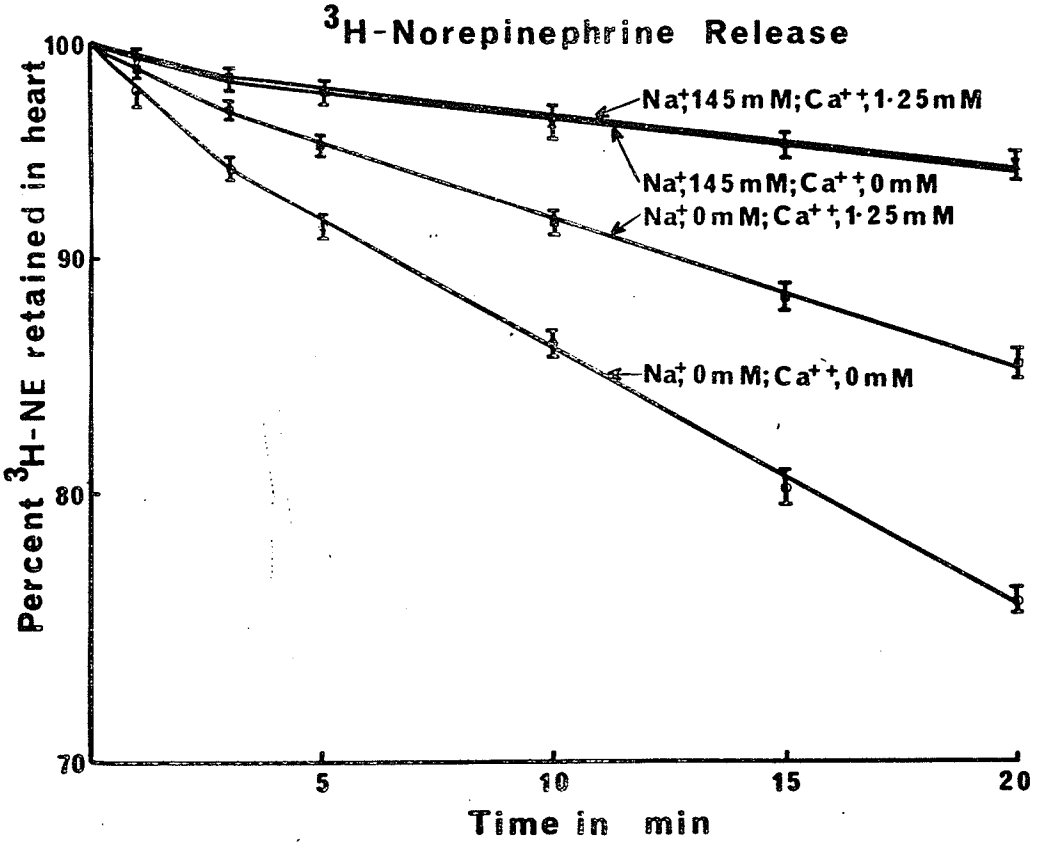

FIGURE 2: Effect of Na^+ and Ca^{++} on the rate of ^{3}H-norepinephrine release from the isolated perfused rat heart. Each point is a mean ± SE of 4 experiments. The hearts were labelled by perfusing with ^{3}H-norepinephrine (10 µCi) for 20 min in a closed circulation apparatus and the ^{3}H-label release was studied by switching over to the constant-flow open system with media containing desired concentrations of NaCl and $CaCl_2$. The lines were drawn according to the method of least squares.

TABLE 9

Effect of Na^{+} and Ca^{++} on the Rate Constant (k) and Half-time (T 1/2) ^{3}H-norepinephrine Release from the Isolated Perfused Rat Heart

Ions in perfusion media (mM)		k (min^{-1})	T 1/2 (min)
Na^{+}	Ca^{++}		
145	1.25	1.14×10^{-3}	264
145	0	1.20×10^{-3}	251
0	1.25	3.22×10^{-3}*	93*
0	0	5.48×10^{-3}*	55*

Each value is a mean of 4 experiments. The hearts were labelled by perfusing with ^{3}H-norepinephrine (10 μCi) for 20 min in a closed circulation apparatus and the ^{3}H-label release was studied by switching over to the constant flow open system with media containing the desired concentration of NaCl and $CaCl_2$. The rate constant of efflux (k) and half-time (T 1/2) were calculated from the slope of exponential decline between 3 and 20 min.

*Significantly different from the values for 145 mM Na^{+} and 1.25 mM Ca^{++} ($P < 0.001$).

Influence of Ions on the Uptake of ^{14}C-tyrosine and the Synthesis of ^{14}C-catecholamines

The synthesis of ^{14}C-catecholamines in the isolated rat heart was studied by perfusion with media containing varying concentrations of cations in the presence of the precursor ^{14}C-tyrosine. The uptake of ^{14}C-tyrosine by these hearts are shown in Tables 10 and 11. The uptake of ^{14}C-tyrosine at different intervals of perfusion did not change ($P > 0.05$) with variations in the concentrations of both Ca^{++} (0 to 5 mM) and Mg^{++} (0 to 16 mM). On the other hand, the uptake of ^{14}C-tyrosine was greater ($P < 0.05$) in hearts perfused with 5 and 20 mM K^{+}, in comparison to that in hearts perfused with a K^{+}-free medium (Table 11). Likewise, the ^{14}C-tyrosine uptake by hearts perfused with 85 and 145 mM Na^{+} but not with 25 mM Na^{+} was more ($P < 0.05$) than that by the hearts perfused in the absence of Na^{+}.

The amount of newly synthesized ^{14}C-catecholamine, present in both hearts and perfusate, as well as the rates of synthesis of ^{14}C-catecholamine in hearts perfused with media containing different concentrations of cations in the presence of ^{14}C-tyrosine are given in Tables 12 and 13. Increasing the concentration of Ca^{++} from 0 to 5 mM was found to decrease the rate of synthesis as well as the concentrations of ^{14}C-catecholamine in the heart and perfusate ($P < 0.01$). On the other hand, changes in the concentrations of Mg^{++} from 0 to 16 mM and K^{+} from 0 to 20 mM had no effect ($P > 0.05$). The rate of ^{14}C-catecholamine synthesis as well as its concentrations in the perfusate were significantly lower ($P < 0.01$) in hearts perfused with 85 and 145 mM Na^{+} but not with 25 mM Na^{+}, in comparison to those in hearts perfused in the absence of Na^{+}. The amount of ^{14}C-catecholamines present in hearts perfused with 85 and 145 mM Na^{+} was higher than that in hearts perfused in the absence of Na^{+} ($P < 0.01$).

Table 14 summarizes the effects due to the omission of cations from the perfusion media on endogenous concentration, uptake, subcellular distribution, release and the rate of synthesis of norepinephrine in adrenergic nerve endings of the isolated perfused rat heart.

TABLE 10

Effect of Ca^{++} and Mg^{++} on the uptake of ^{14}C-tyrosine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^{14}C-tyrosine uptake (cpm x 10^5 per g heart)			
	5 min	10 min	20 min	40 min
A. Calcium				
0	9.37 ± 0.50	11.15 ± 0.54	13.60 ± 0.27	15.11 ± 0.34
1.25	9.57 ± 0.38	11.96 ± 0.61	13.40 ± 0.43	15.27 ± 0.30
5	9.02 ± 0.39	10.70 ± 0.31	13.03 ± 0.31	15.10 ± 0.26
B. Magnesium				
0	9.49 ± 0.23	11.00 ± 0.24	13.35 ± 0.21	15.46 ± 0.35
4	9.31 ± 0.47	11.13 ± 0.46	13.78 ± 0.67	15.22 ± 0.19
16	9.58 ± 0.41	12.03 ± 0.27	13.17 ± 0.48	15.20 ± 0.23

Each value is a mean ± SE of 4 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^{14}C-tyrosine (1 μCi) in different concentrations of $CaCl_2$ or $MgCl_2$ after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

TABLE 11

Effect of K^+ and Na^+ on the Uptake of ^{14}C-tyrosine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^{14}C-tyrosine uptake (cpm x 10^5 per g heart)			
	5 min	10 min	20 min	40 min
A. Potassium				
0	8.41 ± 0.32	9.50 ± 0.28	10.68 ± 0.34	13.90 ± 0.29
5	9.79 ± 0.26*	10.87 ± 0.23*	12.62 ± 0.30*	15.19 ± 0.43*
20	10.89 ± 0.28*	12.37 ± 0.30*	13.62 ± 0.32*	16.87 ± 0.44*
B. Sodium				
0	7.96 ± 0.14	9.28 ± 0.25	11.34 ± 0.58	13.62 ± 0.33
25	7.71 ± 0.23	9.08 ± 0.31	11.34 ± 0.48	13.83 ± 0.30
85	9.32 ± 0.22**	10.87 ± 0.40**	13.04 ± 0.49**	15.32 ± 0.26**
145	9.52 ± 0.27**	11.19 ± 0.36**	13.39 ± 0.42**	15.74 ± 0.33**

Each value is a mean ± SE of 4 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^{14}C-tyrosine (1 μCi) in different concentration of KCl or NaCl after an initial equilibration period of 10 min with control Krebs-Henseleit medium.

*Significantly more than the respective values for 0 mM K^+ ($P < 0.05$).

**Significantly more than the respective values for 0 mM Na^+ ($P < 0.05$).

TABLE 12

Effect of Ca^{++} and Mg^{++} on the Synthesis of ^{14}C-catecholamine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^{14}C-catecholamine (cpm x 10^3 per g heart)		Rate of Synthesis (ng/g/hr)
	In heart	In perfusate	
A. Calcium			
0	7.23 ± 0.29	19.86 ± 0.65	41.82 ± 1.65
1.25	4.47 ± 0.24*	15.71 ± 0.75*	29.73 ± 2.06*
5	4.21 ± 0.15*	10.10 ± 0.32*	23.07 ± 1.04*
B. Magnesium			
0	4.97 ± 0.48	15.40 ± 0.19	29.72 ± 0.80
4	4.99 ± 0.28	15.69 ± 0.78	30.78 ± 2.19
16	5.64 ± 0.16	16.62 ± 0.81	31.32 ± 2.06

Each value is a mean ± SE of 4 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^{14}C-tyrosine (1 μCi) in different concentrations of $CaCl_2$ or $MgCl_2$ and ^{14}C-catecholamine was recovered by the alumina adsorption technique.

*Significantly less than the respective values for 0 mM Ca^{++} ($P < 0.01$).

TABLE 13

Effect of K^+ and Na^+ on the Synthesis of ^{14}C-catecholamine by the Isolated Perfused Rat Heart

Ions in perfusion media (mM)	^{14}C-catecholamine (cpm x 10^3 per g heart)		Rate of Synthesis (ng/g/hr)
	In heart	In perfusate	
A. Potassium			
0	5.11 ± 0.51	14.76 ± 1.25	29.81 ± 1.86
5	5.61 ± 0.54	15.75 ± 1.40	29.66 ± 2.88
20	6.34 ± 0.95	17.07 ± 2.22	29.13 ± 2.25
B. Sodium			
0	2.11 ± 0.30	28.17 ± 2.19	46.38 ± 2.21
25	3.29 ± 0.50	23.85 ± 1.68	41.12 ± 1.85
85	4.97 ± 0.69*	19.01 ± 0.89**	32.93 ± 1.55**
145	5.45 ± 0.76*	16.88 ± 0.90**	29.70 ± 0.96**

Each value is a mean ± SE of 4 experiments. The hearts were perfused for 40 min in a closed circulation apparatus with 50 ml of media containing ^{14}C-tyrosine (1 μCi) in different concentrations of KCl or NaCl and ^{14}C-catecholamine was recovered by the alumina adsorption technique.

*Significantly more than the value for 0 mM Na^+ ($P < 0.01$).

**Significantly less than the respective values for 0 mM Na^+ ($P < 0.01$).

TABLE 14

Summary of the effects of Ca^{++}-free, Mg^{++}-free, K^{+}-free or Na^{+}-free perfusion media on storage, transport and rate of synthesis of norepinephrine in isolated perfused rat heart

Perfusion medium	Endogenous norepinephrine concentration	^{3}H-norepinephrine			Rate of Synthesis
		Uptake	In granular fraction	Spontaneous release	
Ca^{++}-free	No effect	Decrease	No effect	No effect	Increase
Mg^{++}-free	No effect	No effect	No effect	No effect	No effect
K^{+}-free	No effect	No effect	No effect	No effect	No effect
Na^{+}-free	Decrease	Decrease	Decrease	Increase	Increase

DISCUSSION

Norepinephrine in tissues with sympathetic innervation, such as heart, is considered to be localized mainly in nerve endings (Euler and Purkhold, 1951). It is now generally accepted that exogenous norepinephrine is taken up from the external medium by an active transport across the nerve membrane and then stored in the granules of the peripheral adrenergic nerve endings (Iversen, 1965). For the purpose of synaptic transmission, norepinephrine is released from the nerve endings and then inactivated by a recapture mechanism located in the axonal membrane (Brown, 1965). Furthermore, adrenergic nerve endings have been demonstrated to be capable of synthesizing norepinephrine (Goodall and Kirshner, 1958; Spector et al. 1963). In the present study, we have not made any attempt to isolate adrenergic nerve endings from the heart nor have we studied the characteristics of such endings for norepinephrine uptake, synthesis, storage and release. However, on the basis of the above mentioned literature we assume that the results presented here apply essentially to the adrenergic nerve endings present in heart. The values for norepinephrine content of the isolated perfused rat heart obtained in this study are in general agreement with those reported in the literature concerning cardiac catecholamines.

Experiments on the subcellular distribution of ^{3}H-norepinephrine in the heart reveal that this amine exists in two forms: a bound form which is associated with the microsomal fraction and a free form which is present in the soluble fraction. Iversen (1963) has observed that the accumulated ^{3}H-norepinephrine did not mix freely with endogenous norepinephrine stores and its slow exchange with these stores did not exceed 30% of endogenous norepinephrine in the isolated rat heart. On the other hand, Potter and Axelrod (1963a) have shown that the exogenously given ^{3}H-norepinephrine is distributed quite rapidly between granular and soluble fraction of the myocardium and there is a remarkable constancy in the proportion of ^{3}H-norepinephrine recovered from these fractions (Iversen and Whitby, 1963; Stjarne, 1964). In this study about 35% of the total ^{3}H-norepinephrine, which accumulated under our experimental conditions, was

observed to be present in the bound form. It is, however, assumed that ^{3}H-norepinephrine is accumulated and bound in the storage vesicles since most of these particles are considered to be present in the microsomal fraction.

In this study it was demonstrated that the absence of Na^{+} in the medium decreased endogenous stores of norepinephrine in the isolated perfused rat heart. This could be due either to an increase in the release of norepinephrine or diminished ability of the nerve endings to reuptake the spontaneously released norepinephrine. The results reported here have revealed that, in Na^{+}-free medium, there was a marked increase in the leakage as well as impaired uptake of ^{3}H-norepinephrine in the perfused heart. These observations are in agreement with previously published reports that Na^{+} is required for the uptake of norepinephrine by the perfused heart (Iversen and Kravitz, 1966; Horst et al. 1968) and heart slices (Bogdanski and Brodie, 1966, 1969; Gillis and Paton, 1967; Keen and Bogdanski, 1970). The release of ^{3}H-norepinephrine from adrenergically innervated tissues has also been reported due to lack of Na^{+} (Bogdanski and Brodie, 1966, 1969; Gillis and Paton, 1967). Keen (1967) has observed that ^{3}H-norepinephrine accumulation is prevented under conditions of Na^{+} deficiency in which norepinephrine depletion did not occur. Furthermore, Horst et al. (1968) failed to find a significant change in the proportion of deaminated ^{3}H-norepinephrine due to the lack of Na^{+} in rat heart. On the basis of the fact that an increase in deaminated metabolites has been observed in hearts of the animals treated with reserpine which is known to impair the intraneuronal binding sites (Iversen et al. 1965), Horst et al. (1968) have interpreted their data as suggesting that Na^{+} does not play any role in the storage of norepinephrine in the intraneuronal vesicles. We believe that such an interpretation be considered with certain reservations because the results of the present study show that the amount of ^{3}H-norepinephrine in the granular fraction of the hearts perfused with Na^{+}-free medium was considerably less than that in the control heart. It may also be noted that in comparison with the hearts perfused with 85 mM Na^{+}, no significant change in norepinephrine content or subcellular distribution of ^{3}H-norepinephrine was observed in hearts perfused with 25 mM Na^{+} while the up-

take of ^{3}H-norepinephrine was significantly reduced. Furthermore, in comparison to the hearts perfused with 25 mM Na^+, there was a decrease in the content of exogenous labelled norepinephrine content and ^{3}H-norepinephrine in the granular fraction and an increase in ^{3}H-norepinephrine content of the soluble fraction of hearts perfused with Na^+-free medium whereas no significant change in ^{3}H-norepinephrine uptake was noted. These results support the view that the loss of endogenous norepinephrine due to Na^+-lack is due to the impairment of both the mechanism for the retention of norepinephrine in the nerve granules and for the reuptake of the norepinephrine at the nerve membrane. Other investigators (Bogdanski and Brodie, 1966; Gillis and Paton, 1967) have also suggested that Na^+ is not only required for the uptake of norepinephrine but is also necessary for the storage of norepinephrine.

On the basis of kinetic studies concerned with the influence of Na^+ on norepinephrine uptake by heart slices, Bogdanski and Brodie (1969) have calculated the K_m value for the uptake process to be about 30 mM Na^+. In the present study we have observed that ^{3}H-norepinephrine uptake by hearts perfused with 145 mM Na^+ was not significantly higher whereas that by hearts perfused with 25 mM Na^+ was lower than the values obtained for hearts perfused with 85 mM Na^+. From this, it can be interpreted that 145 mM Na^+ is above and 25 mM Na^+ is below the required Na^+ concentration for the optimal uptake of norepinephrine. Even in the absence of Na^+, a considerable amount of norepinephrine was still taken up by the perfused heart. This may be due to the presence of small amounts of Na^+ in the vicinity of nerve membrane under our experimental condition or to some mechanism other thanthat requiring Na^+ for norepinephrine uptake in the nerve terminal.

Although the reduction of norepinephrine uptake by the adrenergic nerve terminals in the absence of Na^+ is considered to be due to an impairment in the carrier mechanism involved in this process (Bogdanski and Brodie, 1969), other ions such as Ca^{++} has been shown to influence the action of Na^+ in this regard. For example, Horst et al. (1968) have found that norepinephrine uptake, diminished by perfusion with low-Na^+ medium, is dramatically increased by the

omission of Ca^{++} from the perfusion medium; however, omission of Ca^{++} had no effect in the total absence of Na^{+}. These workers have suggested that Ca^{++} and Na^{+} have a common but antagonistic site of action. On the other hand, Keen and Bogdanski (1970) failed to show any increase in the uptake of the norepinephrine due to lack of Ca^{++} in the presence of low concentrations of Na^{+}. In the present study we have demonstrated that 145 mM Na^{+}, in the absence of Ca^{++}, significantly increased the uptake of ^{3}H-norepinephrine when compared with Na^{+}-free medium. Furthermore, the increase in the norepinephrine uptake by Na^{+} is much more in the presence of Ca^{++} than in its absence. The reasons for this discrepancy in results from various laboratories concerning the interaction of Na^{+} and Ca^{++} on norepinephrine uptake are not clear. At any rate, the present results clearly suggest that Ca^{++} ion is required for the optimal effect of Na^{+} on the norepinephrine uptake process. Whether or not the effect of Ca^{++} is mediated through its influence on the suggested carrier mechanism for norepinephrine uptake is not clear at present.

A modification of norepinephrine release due to Na^{+}-lack by ions such as Ca^{++} and K^{+} has also been shown by some investigators. The net efflux of ^{3}H-norepinephrine from Na^{+} deficient heart slices by Na^{+} deficiency was greater in the presence of K^{+} (Bogdanski and Brodie, 1966). Horst et al. (1968) showed that the absence of Ca^{++} did not affect the rate of spontaneous release of norepinephrine from the isolated hearts perfused with a medium containing 25 mM Na^{+}. On the other hand, Keen and Bogdanski (1970) have reported that the release of ^{3}H-norepinephrine by Na^{+}-lack was markedly reduced in the absence of Ca^{++} in heart slices. Ca^{++} in concentrations less than 5 mM was shown to increase the efflux of norepinephrine due to Na^{+}-lack, whereas it decreased the efflux of norepinephrine in concentrations of 5 mM or higher. In the present study, absence of Ca^{++} in the perfusion medium was demonstrated to increase the release of ^{3}H-norepinephrine due to Na^{+}-lack from the isolated heart. It appears that the conflicting results concerning the influence of Ca^{++} on the efflux of norepinephrine induced by Na^{+}-lack are due to differences in experimental design. It may particularly be noted that Ca^{++} is known to be a membrane stabilizer

(Shanes, 1958) and its absence would alter the nerve membrane so that more amine could leak out. This is exactly what was observed in the absence of Na^{+} and Ca^{++} in the present study.

It was observed that ^{3}H-norepinephrine uptake by isolated heart perfused with 1.25 mM Ca^{++} was more than that in the absence of Ca^{++}. These results are in agreement with those of Gillis and Paton (1967) who showed that the uptake of ^{3}H-norepinephrine was reduced by Ca^{++}-free medium and this effect was further enhanced by the addition of EDTA. In contrast to these observations, Ca^{++} was found to have no effect on ^{3}H-norepinephrine uptake by heart (Iversen and Kravitz, 1966; Keen and Bogdanski, 1970) and rat uterus (Green and Miller, 1966). The uptake of ^{3}H-norepinephrine was also shown to increase in the absence of Ca^{++} and decrease in the presence of high concentrations of Ca^{++} in the perfused heart (Horst et al. 1968). It may be noted that these workers have studied norepinrphrine uptake only during a 2 min period whereas we have carried out experiments for 40 min. This may explain the differences in our results. In view of our observations that 1.25 mM Ca^{++} failed to increase ^{3}H-norepinephrine uptake from the perfusion medium in the absence of Na^{+} and that ^{3}H-norepinephrine uptake by hearts perfused with 5 mM Ca^{++} was not different from that with 1.25 mM Ca^{++}, it is likely that Ca^{++} is required for norepinephrine uptake by the adrenergic nerve endings in the myocardium. That Ca^{++} is essential for optimal retention of norepinephrine by rabbit heart slices was also shown by Dengler (1965).

Although it is well established that Ca^{++} is required for the release of norepinephrine due to nerve stimulation (Burns and Gibbons, 1965) the role of Ca^{++} in spontaneous release of norepinephrine is not clear. Boullin (1966) has demonstrated that Ca^{++} is essential for release of the sympathetic transmitter by nerve stimulation in isolated cat colon but not for the spontaneous output that occurs in the absence of the nervous activity. Bogdanski and Brodie (1969) have shown that the efflux of ^{3}H-norepinephrine from heart slices is increased in the absence of Ca^{++} and is further enhanced by the presence of EDTA. Perfusion of the isolated rat heart with Ca^{++}-free medium has also been demonstrated to

cause norepinephrine liberation (Taylor and Nash, 1966). The possibility that the release of norepinephrine in Ca^{++} deficient media in the presence of 145 mM Na^{+} is masked, can not be ruled out since it was apparent when Na^{+} was not present in the medium. Further experiments will be necessary to resolve the differences between results from different laboratories.

We have shown here that neither K^{+} nor Mg^{++} had any effect on the content of norepinephrine as well as the uptake, subcellular distribution and release of ^{3}H-norepinephrine from the isolated perfused heart. Although Gillis and Paton (1967) could not demonstrate any effect of an Mg^{++}-free medium on retention of norepinephrine by heart slices, these investigators have shown that complete elimination of K^{+} greatly reduced ^{3}H-norepinephrine retention. In medium containing one-half the usual amount of K^{+} (3 mM) the retention of the amine was within normal limits. On the other hand, low extracellular K^{+} has been shown to facilitate the uptake and storage whereas high extracellular K^{+} antagonizes the effect of Na^{+} on the uptake and storage of ^{3}H-norepinephrine in heart slices (Bogdanski and Brodie, 1969). In brain slices, high concentrations of K^{+} (16 - 66 mM), which depolarize cell membranes have been shown to liberate ^{3}H-norepinephrine (Baldessarini and Kopin, 1966). In view of the uncertainty of metabolic status of the tissue slices, it is difficult to interpret the data obtained from such experiments. From the data obtained with isolated perfused hearts where coronary perfusion is maintained we are of the view that K^{+} or Mg^{++} does not play any role in the transport or storage of ^{3}H-norepinephrine in the nerve terminals of myocardium. However, it is possible that sufficient intracellular K^{+} or Mg^{++} was still present in our experimental conditions when hearts were perfused with media deficient of these ions.

Increasing the concentrations of Na^{+} and K^{+} in the perfusion media has been observed to increase the uptake of ^{14}C-tyrosine, a well known precursor of norepinephrine, whereas Ca^{++} or Mg^{++} had no effect. These results are in general agreement with the concept of amino acid transport for which Na^{+} is considered to be essential for the uptake of amino acids and K^{+} has been shown to facilitate the process (Crane, 1965; Kipnis and Parrish, 1965). Since amino

acid transport is inhibited by ouabain (Dengler et al. 1961; Berti and Shore, 1967) it is likely that the Na^+-K^+-ATPase system is involved in this process. However, such a suggestion may only be considered as a matter of speculation until direct evidence in this regard is available. It should, however, be mentioned that Goldstein et al. (1970) have also recently reported that ^{14}C-tyrosine uptake by slices of rat striatum was not affected by alteration in the Ca^{++} concenfration of the medium.

In this study K^+ was found to have no effect on the rate of ^{14}C-norepinephrine synthesis by the adrenergic nerves in the heart. On the other hand, Boadle-Biber et al. (1970) have shown that K^+ stimulates norepinephrine biosynthesis in guinea pig vas deferens. These workers neither found any effect of K^+ on tyrosine hydroxylase nor observed the stimulating effect of K^+ if ^{14}C-DOPA was used as the starting substrate instead of ^{14}C-tyrosine. It is likely that the effect of K^+ on net synthesis of norepinephrine is due to its facilitating effect on the transport of ^{14}C-tyrosine into the cell without any alteration in the rate of norepinephrine synthesis. It should also be pointed out that tyrosine is not only taken up by nerve endings but also by cardiac cells while the synthesis of norepinephrine is considered to occur mainly in the adrenergic nerve terminals. We also failed to observe any effect of K^+ on the retention of newly synthesized norepinephrine in myocardium. Likewise, the data reported here reflect that Mg^{++} has no role in the synthesis or retention of norepinephrine in the heart.

The rate of synthesis of ^{14}C-catecholamine from tyrosine was significantly more in the hearts perfused with Na^+-free media. Neff and Costa (1966) have shown that increase in norepinephrine levels were associated with a decrease in norepinephrine synthesis and suggested that a negative feedback system may control catecholamine synthesis in the adrenergic nerve terminals. Norepinephrine has also been found to inhibit its own synthesis in sympathetic nerves (Alousi and Weiner, 1966). As Na^+-deficiency has been demonstrated not only to release norepinephrine and decrease its endogenous stores but also to release the newly synthesized ^{14}C-catecholamines, it is possible that increased

synthesis of norepinephrine in hearts perfused with Na^+-free medium may be explained on the basis of a negative feedback mechanism. At present we do not have any information concerning the direct action of Na^+-deficiency on the activities of the enzymes involved in the synthesis of norepinephrine.

Norepinephrine synthesis in hearts perfused with Ca^{++}-free medium was also increased. This finding is in agreement with the results reported by Goldstein et al. (1970) in the slices of rat striatum. However, it is difficult to explain these results on the basis of a negative feedback mechanism as we did not find a significant decrease in the endogenous catecholamine content or release of norepinephrine in the absence of Ca^{++}. Neither can it be explained on the basis of increased availability of the substrate on the nerve cell since the transport of ^{14}C-tyrosine was not affected by the presence or absence of Ca^{++} in the medium. The possibility that a small fraction of norepinephrine which is responsible for negative feedback mechanism was released from the cell in Ca^{++}-deficiency but was not detected under our experimental conditions can not be ruled out. This is in agreement with our observation that norepinephrine was released from hearts due to Ca^{++} deficiency in the absence of Na^+. Alternatively the increase in norepinephrine synthesis due to Ca^{++} deficiency can be considered due to change in the activities of the enzymes involved in norepinephrine synthesis. However Ikeda et al. (1966) have reported that Na^+, K^+, Ca^{++} ions have no effect on the activity of tyrosine hydroxylase in vitro. But, the present studies reveal the possibility of intraneuronal "free" Na^+ and Ca^{++} levels being definitely amongst the factors, which regulate the in vivo synthesis of the neurotransmitter, norepinephrine, in the adrenergic nerve endings.

SUMMARY AND CONCLUSIONS

The effects of Ca^{++} (0 - 5 mM), Mg^{++} (0 - 16 mM), K^{+} (0 - 20 mM) and Na^{+} (0 - 145 mM) on the endogenous level of norepinephrine and on the uptake, subcellular distribution and release of exogenously given ^{3}H-norepinephrine were investigated in the isolated perfused rat heart. The uptake of ^{14}C-tyrosine as well as the synthesis of ^{14}C-catecholamines as influenced by these cations in rat heart was also studied. The following observations were made:

Endogenous Level of Norepinephrine: Changes in the concentrations of Ca^{++}, Mg^{+} and K^{+} in the perfusion media had no influence on the endogenous level of norepinephrine, but the absence of Na^{+} resulted in a decrease.

Uptake of ^{3}H-norepinephrine: Na^{+} is found to be required for the process of uptake. Addition of Ca^{++} to the perfusion media containing Na^{+} increased the uptake of exogenous ^{3}H-norepinephrine, whereas Mg^{++} or K^{+} had no effect.

Subcellular Distribution of ^{3}H-norepinephrine: Increasing the concentration of Na^{+} from 0 to 145 mM in the perfusion medium increased the ^{3}H-label in the granular fraction and decreased it in the soluble fraction; Ca^{++}, Mg^{++} or K^{+} did not show any effect.

Spontaneous Efflux of ^{3}H-norepinephrine: The release was not influenced by the presence or absence of Ca^{++} or Mg^{++} or K^{+} in the medium. Absence of Na^{+} was found to accelerate the spontaneous efflux of ^{3}H-norepinephrine; this effect was more apparent when Ca^{++} was also omitted from the perfusion medium.

Uptake of ^{14}C-tyrosine: The uptake of ^{14}C-tyrosine by rat hearts was found to be greater in the presence than in the absence of K^{+} or Na^{+} in the medium whereas Ca^{++} and Mg^{++} did not have any effect.

Synthesis of ^{14}C-catecholamines: Increasing concentrations of Ca^{++} or Na^{+} in the

perfusion medium was found to decrease the rate of synthesis in rat heart whereas Mg^{++} and K^{+} showed no effect.

It is concluded that Na^{+} is important for the storage as well as the transport of norepinephrine across the neuronal membrane. Ca^{++} may have a facilitating role for the norepinephrine uptake process. Intraneuronal concentrations of Ca^{++} as well as Na^{+} appear to regulate the synthesis of the neurotransmitter in the adrenergic nerve terminals in rat heart.

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