THE EFFECT OF SODIUM AND CALCIUM IONS ON THE RELEASE OF CATECHOLAMINES FROM THE ADRENAL MEDULLA

by

Anna Lastowecka

A thesis submitted to the Faculty
of Graduate Studies and Research
in partial fulfillment of the
requirements for the Degree of
Master of Science

Department of Pharmacology and Therapeutics
McGill University,
Montreal, Quebec

August, 1975

Short Title:

SODIUM OMISSION AND CATECHOLAMINE RELEASE

Anna Lastowecka
Department of Pharmacology & Therapeutics M.Sc.

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Abstract

Bovine adrenal glands were perfused in vitro. Immediately after switching the perfusion medium to a Na^+-free solution, a sharp increase in catecholamine output was obtained (6-10 times over control values). This increase was linearly related to the logarithm of the extracellular Na^+ concentration, and was not reduced in the presence of atropine and hexamethonium thus indicating that Ach from preganglionic nerve terminals is not responsible for this release. Excess Mg^{2+} reduced (10 mM-Mg^{2+}) or blocked (20 mM Mg^{2+}) the Na^+-free induced amine output both in the presence and absence of extracellular Ca^{2+}. Depolarizing the chromaffin cell membrane with 56 mM K^+ did not affect the Na^+-free induced amine release. Perfusion with a Na^+-free medium in the presence or absence of calcium, released catecholamines, DBH and ATP in the same proportions as measured in the soluble contents of isolated chromaffin granules. LDH, a cytosol-localized enzyme was not released. These results provide physiological and biochemical evidence that exocytotic release of catecholamines from the adrenal medulla can be induced by Na^+/omission in the absence of extracellular Ca^{2+}.

Anna Lastowecka
Department of Pharmacology & Therapeutics M.Sc.

L'EFFET DES IONS SODIUM ET CALCIUM SUR LA LIBERATION DE CATECHOLAMINES PAR LA MEDULLOSURRENALE

Condensé

Des glandes surrénales de bovins ont été perfusées in vitro. Aussitôt après le changement du médium de perfusion pour une solution ne contenant pas de Na^+, on obtient une augmentation de la libération de catécholamines (6-10 fois les valeurs contrôles). Cette augmentation est reliée linéairement au logarithme de la concentration extracellulaire de Na^+, et n'est pas modifiée par la présence d'atropine et d'hexaméthonium, ce qui indique que cette libération n'est pas dûe à l' ACh venant des terminaisons nerveuses préganglioniques. Un excès de Mg^{2+} réduit (10 mM-Mg^{2+}) ou bloque (20 mM Mg^{2+}) la libération de catécholamines causée par l'abscence de Na^+, en l'abscence et en présence de Ca^{2+} extracellulaire. La dépolarization de la membrane de la cellule chromaffine à l'aide de 56 mM K^+ n'affecte pas la libération causée par l'abscence de Na^+. La perfusion avec un médium ne contenant pas de Na^+, en présence ou en l'abscence de Ca^{2+}, libère les catécholamines, le DBH et l'ATP dans les mêmes proportions que celles determinées dans le contenu soluble de granules chromaffines isolés. Le LDH, un enzyme localisé dans le cytosol, n'est pas libéré. Les résultats procurent l'évidence physiologique et biochimique que la libération, par exocytose de catécholamines, par la médullosurrénale, peut être induite par l' omission de Na^+ en l'abscence de Ca^{2+} extracellulaire.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Dr. J.M. Trifaro, for his guidance and encouragement during my research studies in his laboratory.

Also, I am grateful to Dr. J.E.B. Pinto for his valuable and sincere assistance in the preparation of this thesis.

A special acknowledgement is due to Dr. Brian Collier for his helpful advice on many occasions during the course of my studies.

A sincere thank-you to those whose technical skills contributed to the preparation of this thesis: Mr. Gordon Boyd for the figures and Mr. Royal Raymond who photographed them; Anita Colaviti and Bonnie McDonald, who provided technical assistance and a happy atmosphere in the laboratory; Linda Pfeiffer, Louise Robert and Sylvia Cohen for typing and preparing the manuscripts; M. Jean-Pierre Coty-Matricon and Pierre Lavoie for the French translations.

My graduate education was supported by a studentship from the Medical Research Council of Canada, and made possible by the Department of Pharmacology under the Chairmanship of Professor M. Nickerson.

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ABBREVIATIONS USED

A	adrenaline
Ach	acetylcholine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CA	catecholamines
COMT	catechol-O-methyltransferase
DA	dopamine
DBH	dopamine β-hydroxylase
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycol tetra-acetic acid
MAO	monoamine oxidase
MW	molecular weight
NA	noradrenaline
$NADH_2$	reduced nicotine adeninedinucleotide
PNMT	phenylethanolamine-N-methyl-transferase
VMA	3-methoxy-4-hydroxymandelic acid
UTP	uridine triphosphate

INTRODUCTION

The adrenal medulla or more precisely its anatomical unit - the chromaffin cell - can be considered from a physiological point of view as a peripheral bridge between two of the main regulatory mechanisms of the higher living organisms; the endocrine axis and the nervous system. In fact the adrenal medulla represents, at the peripheral level, the best example of a neuro-endocrine transducer as the hypothalamus does at the central nervous system. A feature of this endocrine tissue, embryologically derived from neuroblast cells is that it synthetizes, stores and secretes to the internal medium substances with an identical chemical structure to the neurotransmitter released by sympathetic neurons (Coupland, 1956).

The following introduction is a brief review of the structure and function of this tissue and an attempt to integrate and describe in a concise way the main known steps involved in the catecholamine release-reaction. The possible role of ions in the stimulus-secretion coupling mechanism within the chromaffin cell is also described.

Embryology & Anatomy

In higher vertebrates, including man, the adrenal glands are small, paired structures lying on either side of the midline of the abdominal cavity above the kidneys. Each gland is a double organ composed of two distinct types of tissue of different origin and function, an outer cortex of mesodermal origin and an inner medulla of neuroectodermal origin. The medullary chromaffin cells develop

from primitive cells of the sympathetic ganglion originating from the neural crest and remain intimately connected with the splanchnic sympathetic system. Thus, the medulla is considered to be part of the post-ganglionic sympathetic system and shares some properties with the adrenergic neurons (Kohn, 1902; Soulié, 1903a, b; Yntema & Hammond, 1947; Coupland, 1956; Wurtman, 1965).

Chromaffin tissue similar to that of the adrenal medulla is also found in the organ of Zuckerkandl, in the thorax, neck and carotid body, and in small bodies adjoining the paravertebral sympathetic ganglion chain ("paraganglia") which in some lower vertebrates represents the adrenal medullary tissue. In mammals, most of these cells atrophy after birth, whereas those of the adrenal medulla mature into medullary cells and become the major source of circulating adrenaline (Zuckerkandl, 1912; Wurtman, 1965).

The close anatomic association of cortex and medulla in higher mammals was suggested to be functional in that the adrenal glucocorticoids may influence adrenaline biosynthesis in the medulla (Shepherd & West, 1951; Coupland, 1953). For example, foetal medullary tissue cannot synthetize adrenaline until it establishes anatomical and circulatory contact with mesodermal cortical tissue (Shepherd & West, 1951; Brundin, 1965), and, in the dogfish, the steroid secreting cells are anatomically distant from the chromaffin cells which contain only noradrenaline (Coupland, 1953). Recent evidence has shown that the concentration of glucocorticoids draining from the cortex to the medulla maintain the activity of the phenylethanolamine-N-methyl transferase (PNMT), an enzyme which catalyses the conversion of nora-

drenaline to adrenaline (Kirshner & Goodall, 1957; Axelrod, 1962; Wurtman & Axelrod, 1966). Furthermore, in the snake, "Xenodon merremii", the regions containing adrenaline and noradrenaline can be separated and only the part which forms adrenaline contains PNMT (Wurtman et al., 1967). Thus, a recognition of the adrenal cortico-medullary interaction and its physiological significance was established. More recently, immunofluorescence microscopy studies have also shown that in mammalian adrenal medullae the adrenaline storing cells were the richest in PNMT, (Goldstein et al., 1972).

Histology & Innervation

The medulla is composed of interlacing cords of densely innervated granule-containing cells called 'chromaffin' cells due to their characteristic brown colour when exposed to potassium bichromate (Kohn, 1902). Early microscopic studies reported the presence of two different and distinct types of cell which differ histologically and histochemically; for example the noradrenaline-storing cells can be selectively stained by the iodate reaction which leaves the adrenaline-storing cells uncoloured (Hillarp & Hökfelt, 1953); also, the noradrenaline-storing cells show a formalin-induced fluorescence when exposed to ultra violet light (Eränkö, 1955b). Each type of cell was given several names: adrenaline-containing and noradrenaline-containing cells (Eränkö, 1955a, b); Type I and Type II cells (Kanno, 1959); adrenaline-storing and noradrenaline-storing cells (Coupland et al., 1964); light and dark cells (Benedeczky et al., 1966). Using differential staining techniques the medullary distributions of the two types of cells have been obtained (Coupland et al., 1964). Electro-

microscopic observations have shown that both types of cell contain specific amine-storing vesicles ('chromaffin' granules) in addition to the normal cell organelles (Blaschko & Welch, 1953; Lever, 1955; Banks, 1965; Coupland, 1965). Further support for two types of chromaffin cell is in the studies on snake adrenals showing that amines are stored in two distinct ribbons of cells, each ribbon storing one amine type (Houssay et al., 1962; Wassermann & Tremazzani, 1963).

The noradrenaline-storing cells run in cords along the blood vessels derived from the adrenal medullae arteries; their granules are electron dense with eccentrically placed cores. The adrenaline-storing cells, arranged in palisade form along the cortico-medullary venous sinuses, have well-defined arterial and venous poles; their granules are less dense and centered in the cell (Coupland, 1965). The adrenaline-containing cells have been found to have a larger number of secretory granules per cell than the noradrenaline-containing cells (Pohorecky & Rust, 1968).

Unlike the adrenal cortex which is controlled by hormonal input (eg. ACTH from the pituitary gland), the adrenal medulla secretes its products in response to neural input reaching the gland via the greater thoracic splanchnic nerves (Dreyer, 1899). These nerves are derived from the spinal cord segments T3 to L3. The nerves pass through the adrenal cortex, run adjacent to large blood vessels, and finally form a plexus from which fine nerve fibres emerge and pass between adjacent chromaffin cells. Terminal boutons from these fibres form true synapses with the chromaffin cells, with their plasma membranes corres-

ponding to the pre- and to the post-synaptic thickenings (Maycock & Heslop, 1939; Young, 1939; Lever, 1955; Coupland, 1962, 63, 65). Although the splanchnic nerves regulate the secretory activity of the medulla, they have no trophic influence on the cells; denervated or isolated-perfused glands secrete just as well in response to acetylcholine or other secretagogues that directly influence the chromaffin cell (Houssay & Molinelli, 1925, 1926; Cannon & Rosenblueth, 1937; Douglas & Rubin, 1961).

Blood Supply

The adrenal medulla is irrigated by an intra-adrenal portal circulation although there is species and individual variations, the mammalian gland is richly supplied by a number of arteries that enter the gland from different points. The major ones arise from the aorta or one of its major somatic or splanchnic branches, or from the adrenolumbar artery. From these, multiple small adrenal arteries converge on the gland; these arteries form a plexus in the gland capsule and then form an anastomosing sinusoidal network of cortical arteries surrounding the cortical cells. The sinusoids became progressively wider and coalesce as they approach the medulla center. A few capsular arteries penetrate the medulla directly where they, too, branch repeatedly to form a capillary network around groups of chromaffin cells. The medullary capillaries empty into the same venous system as that draining the cortex and which eventually forms the single adrenolumbar vein (Harrison & Huey, 1960; Coupland, 1965).

The significance of the specialized vascularization of the

adrenal medulla is that: a) the blood passing from the cortex to the medulla is undiluted and rich in cortical steroids and b) the chromaffin cells have become specialized to secrete their products into the general circulation to interact with distant receptors - hence the term "hormones" for the medullary catecholamines.

Physiology

Oliver & Schäfer (1894) first observed that extracts of adrenal glands of sheep and dog had powerful vasopressor actions. Moore (1895) showed that the active substance was confined to the medulla of the gland. The active principle was isolated and identified chemically by Aldrich (1901) and Takamine (1901) who named the substance "epinephrine" and "adrenaline" respectively. A pressor substance was also extracted from the 'organ of Zuckerkandl' and assumed to be adrenaline (Biedl & Wiesel, 1902). Adrenaline thus became the first hormone to be identified.

Work on the nature of the pressor substance secreted by the adrenal medulla proceeded in parallel with investigations of chemical mediators at sympathetic nerve endings, and adrenaline and noradrenaline were found to be separate chemical mediators released at adrenergic nerve endings (Elliott, 1904, 1905; Greer et al., 1938; Bergström et al., 1949). A noradrenaline-like substance was later demonstrated in adrenal gland extracts (Holtz et al., 1947). Finally, both adrenaline and noradrenaline were demonstrated in the cat's adrenal venous blood during splanchnic nerve stimulation (Bülbring & Burn, 1949), and both amines were also extracted from fresh cattle adrenal glands (Euler & Hamberg, 1949).

In addition to adrenaline and noradrenaline, small quantities of dopamine (Goodall, 1951) and N-methyladrenaline (Axelrod, 1960) have been extracted from mammalian adrenal glands. No major physiological role was assumed for these substances.

The function of adrenomedullary activity has been thought by Cannon and others to be one of adaptation - an emergency mechanism helpful in preparing an animal for "flight or fight" or other form of stress (Cannon, 1931). Stimulation by pain, cold, anoxia, emotional excitement, exercise, hemorrhage, hypotension or by hypoglycemia, showed a greatly increased secretion of the adrenomedullary hormones (Houssay & Molinelli, 1925; Cannon, 1931; Euler, 1956). All of these stimuli to adrenomedullary secretion are transmitted via hypothalamic nervous centers and the sympathetic innervation of the gland which then differentially control the individual secretion of adrenaline and noradrenaline (Houssay & Molinelli, 1925; Nagoun et al., 1937). According to Folkow & Euler (1954), stimulation of certain areas in the hypothalamus enhances the secretion of adrenaline more than that of noradrenaline, whereas stimulation of other areas nearby has the converse effect. Furthermore, different external stimuli may elicit adrenal secretion in which the proportions of the two hormones differ characteristically (Malmejac, 1964; Euler, 1967). Thus, two distinct systems may exist for the control of adrenomedullary activity (Euler & Luft, 1952; Holtzbauer & Vogt, 1954).

The Secretory Process

The endocrine and the nervous systems function as a complex process of handling different kinds of biological information. These biological messages are handled by a dynamic interplay of excitation and inhibition within and between highly developed cells. Generally speaking, most biological communication is structured on a series of transduction processes involving mainly chemical and electrical energy. In focusing these events to a specific endocrine tissue such as the adrenal medulla one should point out that the main consequence of the physiological stimulation of the chromaffin cell is the extrusion of catecholamines and other soluble constituents of the granules to the cell exterior as the result of the secretory process. Although many attempts, using multidisciplinary methodology, have been done to clarify the release reaction in exocrine and endocrine glands as well as nervous tissue, many of the events associated with this process of secretion still remain to be elucidated.

Therefore for the sake of simplicity one should divide the secretory process, regardless of importance or priorities, as schematically composed of four main events: a) synthesis of the hormone or neurotransmitter; b) storage of the hormone in granules; c) mechanism of hormone release and d) hormone inactivation mechanisms. A brief description of these events is given below. Furthermore, as an integrative attempt, the role of ions, and the series of events triggered by the interaction of acetylcholine with the cholinergic receptors of the chromaffin cell is briefly discussed under the generally accepted denomination of "stimulus-secretion coupling".

a) Biosynthetic Pathway:

The in vivo formation of adrenaline and noradrenaline is from the dietary source of phenylalanine and ℓ-tyrosine (Gurin & Deluva, 1947; Udenfriend et al., 1953) which are taken up by the chromaffin cells. ℓ-Tyrosine is then hydroxylated to ℓ-dopa by the enzyme tyrosine hydroxylase (Blaschko, 1939; Nagatsu et al., 1964). This first enzymatic step of the biosynthetic pathway is the slowest and, physiologically, represents the main regulatory reaction of all the sequential enzymatic processes in the synthesis of catecholamines (Spector et al., 1963a; Levitt et al., 1965). It is at this level where cytoplasmic noradrenaline or adrenaline, and perhaps some metabolites, exert the so-called feed-back end product inhibition on this rate-limiting step (Nagatsu et al., 1964; Stjärne, 1966; Udenfriend, 1966). This effect could be mediated directly on tyrosine hydroxylase or by inhibiting the pteridine cofactor of this enzyme (Thoenen et al., 1969). Actually, several different kinds of stimuli or conditions have been shown to be capable of altering the tyrosine hydroxylase activity within the chromaffin cell, not only modifying the enzymatic activity but also the levels of the enzyme as well (Bygdeman et al., 1960; Gordon et al., 1966; Thoenen et al., 1969).

A second step in this synthetic pathway is the decarboxylation of ℓ-dopa to dopamine by the enzyme ℓ-dopadecarboxylase or also known as aromatic aminoacid decarboxylase (Holtz, 1939; Goodall, 1951; Udenfriend & Wyngaarden, 1956; Goodall & Kirshner, 1957). Like tyrosine hydroxylase, this enzyme seems to be localized in the cytosol of the chromaffin cell (Lovenberg et al., 1960).

The third step, the biocatalytic conversion of dopamine to noradrenaline requires the presence of dopamine β-hydroxylase, an enzyme present within the storage granules (Blaschko, 1939: Kirshner, 1957; Goodall & Kirshner, 1957; Levin et al., 1960; Goldstein et al., 1965).

The chromaffin cells of the adrenal medulla, especially in mammals, has developed a synthetic step capable of synthesizing adrenaline from noradrenaline as a final step of the biosynthetic pathway. The enzyme phenylethanolamine N-methyl transferase (PNMT) converts noradrenaline to adrenaline by transfering a methyl group from S-adenosylmethionine to the amine nitrogen of noaradrenaline (Bülbring, 1949; Kirshner & Goodall, 1957; Axelrod, 1962). PNMT seems to be a soluble component within the chromaffin cell (Kirshner & Goodall, 1957; Axelrod, 1962). The physiologic importance of this enzymatic step results in the ability of the medulla to have two distinct functional populations of cells, one containing adrenaline and the other storing noradrenaline. This characteristic seems to be mainly the result of the anatomical and endocrine interrelationship between the adrenal cortex and the medulla (Kirshner & Goodall, 1957; Axelrod, 1962; Wurtman & Axelrod, 1966). As has been mentioned above, glucocorticoids released to the adrenal portal system in high concentrations are responsible for the induction of PNMT activity (Coupland, 1953; Wurtman & Axelrod, 1965, 1966).

b) Storage of catecholamines

It is now well established that the chromaffin cell stores

catecholamines in special intracytoplasmic membrane-limited organelles (chromaffin granules) which are involved in the uptake, biosynthesis, storage and secretion of hormones. This feature is shared with other secretory tissues such as some endocrine glands and nerve endings (Coupland, 1956). These granules are, from the physiological point of view, a cellular mechanism able to store and prepare hormones and transmitters for release and at the same time protect those substances from degradation by cytoplasmic enzymes. Biochemically, these granules contain mainly water (70%), proteins (11.5%), lipids (7%), catecholamines (6.7%) and ATP (4.5%) (Kirshner, 1974). Isolated chromaffin granules can be separated into water soluble and water insoluble components after lysis by hypoosmotic shock followed by centrifugation. Among the soluble constituents the main feature is the striking and unusually high concentrations of adenine nucleotides, ATP being the major component. Smaller amounts of other nucleotides such as ADP, AMP, UTP, etc. have also been detected (Hillarp & Thieme, 1959; Da Prada & Pletscher, 1970). Different experimental evidence has suggested that nucleotides are involved in the storage process forming a non-diffussible complex with catecholamines within the chromaffin granules (Hillarp, 1958). This complex consists basically of catecholamines and ATP in a molar ratio of 4:1. This catecholamine-ATP complex is stabilized by soluble proteins (chromogranins) present in the granules (Blaschko et al., 1967a; Smith & Kirshner, 1967). The main component of this group of proteins, which represents 70% of the total granular proteins, is chromogranin A, an acidic protein having a MW of 80,000 (Smith &

Kirshner, 1967; Smith & Winkler, 1967). Another soluble protein is dopamine β-hydroxylase (DBH). In the bovine adrenal medulla half of the total granular enzyme is present as a soluble component whereas the other 50% is membrane bound (Winkler et al., 1970; Duch et al., 1968). As has been mentioned above this enzyme is involved in the conversion of dopamine to noradrenaline (Blaschko, 1939; Goldstein et al., 1965). The catalytic site of the enzyme faces the interior of the granule, so the substrate must enter the vesicle before it can be oxidized (Kirshner, 1962; Kirshner et al., 1967; Stjärne & Lishajko, 1966). In connection to this event it is important to point out that a transport mechanism (uptake) for catecholamines has been already described at the level of the membrane (Taugner, 1971, 1972). This is an ATP-Mg^{2+} dependent active transport which seems to be the result of the activity of a membrane-bound ATPase (Kirshner et al., 1966). The kinetics of this transport system would suggest that a carrier molecule might be involved in this uptake process (Slotkin & Kirshner, 1973). However, evidence for the possible role of the granule membrane ATPase in the release process has also been published (Poisner & Trifaró, 1967; Trifaró & Poisner, 1967; Trifaró & Dworkind, 1971). Another enzyme, present in the granule membrane is phosphatidylinositol kinase (Trifaró, 1973; Trifaró & Dworkind, 1975; Muller & Kirshner, 1975). This enzyme catalyzes the transphosphorylation from ATP to monophosphatidylinositol with the formation of diphosphatidylinositol (Trifaró, 1973; Trifaró & Dworkind, 1975; Muller & Kirshner, 1975). Among the granule membrane-bound

proteins an electron transport system of unknown function has also been identified. This consists of cytochrome b561 and a NADH: cytochrome b561 reductase (Spiro & Ball, 1961; Flatmark & Terland, 1971).

The electrophoretic studies of the membrane proteins of chromaffin granules done by Winkler et al. (1971, 1972) indicated the existence of at least two main proteins. They have proposed the name chromomembrins A and B for these proteins (Winkler et al., 1970; Hörtnagl et al., 1972). Chromomembrin A has been identified as dopamine β-hydroxylase (Hörtnagl et al., 1972; Winkler & Hörtnagl, 1972). The remaining protein, chromomembrin B, has not yet been characterized. Phospholipids and cholesterol are the main lipid components of vesicle membranes. These membranes are very rich in cholesterol and have an unusually high content of lysolecithin (Blaschko et al., 1967b; Trifaró, 1973).

Finally, the origin of these secretory granules is not clearly understood. Electron microscopic studies have suggested the Golgi apparatus as the place of origin of the chromaffin granules (De Robertis & Vaz Ferreira, 1957). However there is no biochemical evidence to substantiate this hypothesis.

c) Mechanism of hormone release

The release to the extracellular medium of the soluble components of the chromaffin granules represents the culmination of the secretory process. From the physiological point of view, catecholamine release from the adrenal medulla is the last of a sequence of events of an homeostatic autonomic neuroendocrine reflex. Normally, the adrenal medulla seems to maintain a low rate of release of cate-

cholamines to the blood; the gland being the main source of circulating adrenaline. Catecholamine levels in plasma increase several fold as a reflex response to any stressing stimuli (Smith & Winkler, 1972). From a subcellular and molecular point of view the chromaffin cell has rendered useful information about the release process (Trifaró, 1970; Smith & Winkler, 1972). At present, strong experimental evidence supports the idea that the adrenal medulla secretes its products by a cellular mechanism known as exocytosis (Smith & Winkler, 1972). This process, originally called reversed pinocytosis or emiocytosis, involves the attachment and fusion of granular and plasma membranes coupled with a molecular rearrangment resulting in a transient membrane discontinuity through which the soluble intragranular content can reach the extracellular medium. This mechanism of release, which does not seem to be restricted to the adrenal medulla, has received solid and relevant support mainly from morphological and biochemical studies (Trifaró, 1970; Smith & Winkler, 1972). Biochemical evidence in favour of the exocytotic mechanism has been provided by analysing catecholamines and other granular and extragranular constituents from the whole gland, or from the different particulate fractions following stimulation of the tissue (Trifaró, 1970; Smith & Winkler, 1972). Another useful experimental approach was the study of chromaffin cell components present in the effluent of blood leaving the gland before, during and after acetylcholine or splanchnic nerve stimulation (Douglas & Poisner, 1966; Viveros et al., 1968). Summing up the numerous and different kinds of evidence, one may conclude that during stimulation, the soluble components of the nucleotide-rich chromaffin granules dis-

appear from the gland and are quantitatively recovered in the effluents escaping from the gland (Douglas & Poisner, 1966; Viveros et al., 1968). Equally important, is the evidence suggesting that the insoluble membrane-bound components of the chromaffin granules are not released to the extracellular medium but are quantitatively retained within the chromaffin cell (Trifaró et al., 1967; Poisner et al., 1967; Schneider et al., 1967). This conclusion on the fate of granular components correlates with electron-microscopic figures of exocytosis obtained in the hamster adrenal gland (Diner, 1967) and with the fact that there is no detectable increase in release of cytosol marker-enzymes (Schneider et al., 1967) associated with catecholamine secretion, thus strengthening the idea of an all-or-none exocytotic mechanism of release in the chromaffin cell.

The exocytotic mechanism was useful in explaining a crucial step in the release mechanism, but at the same time it has raised many unanswered questions. Practically nothing is known of the intimate process of attachment or how, and for how long, the chromaffin granule remains fused to the cell membrane. Furthermore, there is disagreement concerning the fate of chromaffin granules after they release their contents. There is some evidence suggesting a retrieval process from the plasma membrane with subsequent reincorporation into the cytoplam, but conclusive evidence on the final fate and the possibility of reusage of the granules is still lacking.

d) Inactivation of catecholamines

There are two enzymes within the organism that are responsible

for the initial metabolic transformation of most of the catecholamines that enter the circulation. These are monoamino-oxidase (MAO) and catechol-O-methyltransferase (COMT). Both enzymes are widely distributed in the organism, specially MAO, which in the liver and the kidney is intracellulary localized in the mitochondria, whereas COMT is present in the cytosol (Blaschko et al., 1937; Blaschko, 1952; Axelrod, 1957; Axelrod & Tomchick, 1958). It has been shown that most of the catecholamines coming from the adrenal medulla or from exogenous administration are first methylated by COMT into metanephrine and normetanephrine (Axelrod, 1959). However, MAO contributes also to the metabolic disposal of catecholamines. This is reflected in the urine where the major metabolite of catecholamines excreted is 3-methoxy-4 hydroxymandelic acid (VMA) (Axelrod, 1957; Axelrod et al., 1958). The quantitive relationship between enzymatic pathways involved in the metabolic disposition of catecholamines reveals that 40% is excreted as VMA, 40% as metanephrine (free and conjugated), 7% as 3-methoxy-4 hydroxyphenyl glycol sulfate and small amounts as unchanged epinephrine and 3-4 dihydroxymandelic acid (Kopin & Gordon, 1963). It is also important to point out that the chromaffin cell lacks an active amine uptake mechanism such as that present in adrenergic nerve terminals (Iversen, 1965). This mechanism is also involved in the inactivation process of catecholamines that have reached the internal medium (Kalsner & Nickerson, 1969b).

Stimulus-secretion coupling

One of the common and striking features of the secretory process in different cells which store their secretory products in granules is the physiological dependency of the release process on extracellular calcium (Douglas & Rubin, 1961). This well demonstrated and established fact has allowed some researchers to speculate about the different steps taking place in the chromaffin cell - from the interaction between acetylcholine and membrane receptors to the final exocytotic release of intragranular soluble contents. All these events have been condensed by Douglas and Rubin (1961) in the widely accepted expression "stimulus-secretion coupling" patterned from the previous phrase "excitation-contraction coupling" introduced by Sandow (1952) to describe the chain of events occuring during muscle activation. As quoted by Douglas (1968): "the true stimulus-secretion coupling is thus intended to embrace all the events occurring in the cell exposed to its immediate stimulus that lead finally to the appearance of the characteristic secretory product in the extracellular environment." One of the most important consequences of the release mechanism by exocytosis is that all the steps involved in the stimulus-secretion coupling are directly or indirectly related to the plasma membrane. The aim of this section is to describe the known events taking place at the plasma membrane during stimulus-secretion coupling, (especially those associated with the role of ions). At the same time, it is important to notice that the sequence of steps, illustrated in the following scheme (modified from Smith & Winkler, 1972), is far from being complete, definitive

or totally correct. Many different experimental subcellular and molecular approaches are required in order to obtain new evidence to elucidate this fascinating chain of events.

Interaction between
acetylcholine and the receptor

↓

Increased Ionic permeability of
plasma membrane

↓

Depolarization

↓

Increased Calcium Influx

↓

Attachment of the chromaffin
granule to the plasma membrane

↓

Fusion of granular and
plasma membranes

↓

Transient Membrane discontinuity
allowing a direct communication
between intragranular and extra-
cellular compartments

↓

Release of granule soluble
contents to the extracellular medium

↓

Fission of granular membrane
from the cell membrane

↓

Granules Recycling ?

The earliest event in stimulus-secretion coupling involves the interaction between the acetylcholine released from the pre-ganglionic autonomic fibres and the cholinergic receptors of the

plasma membrane. By extrapolating from electrophysiological evidence obtained by Del Castillo & Katz (1955) in the neuromuscular junction, it is now accepted that acetylcholine exerts its action on the outer surface of the chromaffin cell membrane (Douglas, 1968). It is important to point out that, similar to the sympathetic ganglion, the cholinergic transmission in the adrenal medulla is a consequence of the activation of nicotinic and muscarinic receptors (Douglas & Poisner, 1965; Lee & Trendelenburg, 1967). Furthermore, in the cat adrenal gland, it has been postulated that release of adrenaline occurs in response to muscarinic stimulation whereas nicotinic receptors are involved in the secretion of both adrenaline and noradrenaline (Douglas & Poisner, 1965).

Regarding synaptic transmission, it is generally accepted that changes in ionic permeability accompanied by a concomitant modification in cell polarization follows the activation of postsynaptic receptors (Eccles, 1964). Electrophysiological studies done in the adrenal medulla have shown that acetylcholine acts at the medullary synapse similar to its actions at other synapses (Douglas, 1968). It has been demonstrated in isolated gerbil chromaffin cells (which have a resting potential of 30 mV), that those cells undergo a transient, non-spike membrane depolarization in the presence of acetylcholine. By modifying the extracellular ionic composition, it was found that the depolarizing effect induced by acetylcholine was due to inward currents of sodium and calcium (Douglas, Kanno & Sampson, 1967a, b). Although in other excitable tissues, such as muscle, depolarization represents a necessary requirement in the genesis of the final response this does not seem to be the case

in the chromaffin cell. This statement is fundamentally important because, although an inward sodium current is mainly responsible for acetylcholine depolarization, the omission of this cation from the extracellular fluid fails to modify the magnitude of the release response to acetylcholine (Douglas, 1968). Moreover, tetrodotoxin, a specific blocking agent of the inward sodium current, does not inhibit the release of catecholamines from perfused ox adrenals stimulated by carbamylcholine (Smith & Winkler, 1972). Furthermore, Douglas and Rubin (1961, 1963) found that omission of either K^+, Mg^{2+} or Cl^- from the perfusion medium does not prevent the acetylcholine-induced release of catecholamines from the cat adrenal gland. It is at this point where the crucial physiological role of extracellular calcium should be emphasized.

The role of calcium in secretion

Douglas and Rubin (1961) showed that the inward calcium current associated with acetylcholine depolarization was an essential link in the chain of events leading to catecholamine secretion in the adrenal medulla. They demonstrated that omission of Ca^{2+} from the extracellular medium abolished the secretory response to acetylcholine in the in situ perfused adrenal gland of the cat. Convincing evidence linking Ca^{2+} entry to the action of acetylcholine came from experiments showing increased $^{45}Ca^{2+}$ uptake into adrenal glands during stimulation with acetylcholine (Douglas & Poisner, 1963). This role of Ca^{2+} has been described as 'ion-specific', i.e. it is a sufficient and essential requirement for

the secretory process in the adrenal medulla (Douglas, 1968). This same requirement for Ca^{2+} has been shown for other secretagogues such as histamine, serotonin, high extracellular concentrations of K^+, angiotensin, and others (Poisner & Douglas, 1966).

Reintroducing Ca^{2+} into the medium after perfusing adrenal glands with a Ca^{2+}-free solution results in a marked release of catecholamines (Douglas & Poisner, 1961). Electrophysiological investigation of this interesting observation revealed a small depolarizing current of about 5 mV when the chromaffin cell is exposed to acetylcholine in the absence of extracellular Na^+. This current has been interpreted to be due to inward movement of Ca^{2+} (Douglas et al., 1967). Furthermore, both the Ca^{2+} inward current and the associated release process can be blocked by tetracaine without altering the magnitude of the inward depolarizing Na^+ current (Douglas & Kanno, 1967).

These and other observations have led to the conclusion that the inward movement of calcium, rather than depolarization per se, is required for secretion (Douglas, 1968).

The mechanism by which Ca^{2+} promotes the release of catecholamines from adrenal chromaffin cells is still unknown. Many hypotheses have been put forward, e.g., a charge neutralization effect (Banks, 1966; Matthews et al., 1972); inducing changes in viscosity (Poste and Allison, 1973); an interaction with microtubules (Schmitt, 1968; Lacy et al., 1968), or with a contractile mechanism of release associated with a membrane ATPase (Poisner and Trifaro, 1967). However, such evidence is indirect, and there is no clear agreement regarding the molecular mechanism linking influx of extracellular

Ca^{2+} to final exocytotic release of granular components, nor do they indicate how Ca^{2+} is involved in the attachment and fusion of granular and plasma membranes. These questions require more experimental evidence.

Experiments in other cells can offer some clues on Ca^{2+} action. An increase in intracellular Ca^{2+} by iontophoresis, in a Ca^{2+}-free medium was shown to induce release in mast cells (Kanno et al., 1973; Cochrane and Douglas, 1974), and in the giant synapse of the squid (Miledi, 1973). From these and other studies (Baker, 1974), the idea that the intracellular Ca^{2+} concentration may be the main regulatory factor modulating the release process is progressively gaining acceptance. In connection with this hypothesis, it is now recognized that the inward movement of extracellular Ca^{2+} is only one of many factors affecting the intracellular Ca^{2+} concentration (Baker, 1972; 1974). A Na^{+}-dependent Ca^{2+} transport through the plasma membrane has been shown in different excitable tissues (Baker, 1972; Reuter, 1974). Furthermore, intracellular Ca^{2+} buffer systems such as those localized in mitochondria and endoplasmic reticulum have been postulated to play an important role in maintaining a low intracellular free Ca^{2+} concentration (Baker and Reuter, 1975).

Role of other ions in secretion

The activities of other ions have provided information on the role of Ca^{2+} in secretion. Magnesium is known to be a competitive antagonist of Ca^{2+} in biological systems. In the adrenal medulla, Mg^{2+} interferes competitively with Ca^{2+} entry and inhibits the secretory response to acetylcholine (Douglas and Rubin, 1963). This effect is reversed by increasing the Ca^{2+} concentration in the extra-

cellular medium. Recent evidence indicates that Mg^{2+} has an intracellular site of action (Rubin, 1975).

It has also been shown that Ba^{2+} can replace Ca^{2+} and will release acetylcholine from cholinergic nerves. By adding Ba^{2+} to the extracellular medium, it can evoke catecholamine release in the adrenal medulla; this effect is blocked by Mg^{2+}, this indicating that Ba^{2+} in some way mimics the Ca^{2+} action (Douglas & Rubin, 1963).

The removal of Na^{+} or K^{+} from the extracellular medium potentiates the response of the adrenal medulla to acetylcholine stimulation (Douglas and Rubin, 1973). Possible explanations of this effect could be that low Na^{+} or K^{+} solutions facilitate Ca^{2+} movement into cells, or that removal of Na^{+} hyperpolarizes the cell membrane. In support of the latter, Na^{+} omission was shown to raise the membrane potential of chromaffin cell (Douglas et al., 1967b). This hyperpolarizing effect of low or Na^{+}-free solutions suggested that the chromaffin cell membrane may be more permeable to Na^{+} than other cell membranes and that it has properties similar to those of mammalian C fibers (Douglas et al., 1967b).

Another interesting observation is that acetylcholine can induce secretion from the chromaffin cell which has been already depolarized by exposure to high K^{+} concentrations (Douglas and Rubin, 1963). Related to this observation, acetylcholine has been shown to increase membrane permeability in depolarized membrane preparations (Del Castillo and Katz, 1955).

Several ions are intricately involved in the spontaneous release of catecholamines. In Ca^{2+}-free solutions, the spontaneous release of catecholamine is low (Douglas and Rubin, 1961; Banks, 1967),

but in Na^+-deficient solutions, an increase in spontaneous release is found which was not abolished by Ca^{2+} omission (Douglas and Rubin, 1961; 1963). These stimulant effects of Na^+ and K^+ were thought to represent a competition between these monovalent ions and Ca^{2+}. The observation that ouabain (Bank, 1967) can increase the spontaneous release of medullary catecholamines should be borne in mind as this indicates the presence of an active Na^+-K^+ pump in the chromaffin cell membrane (Rubin, 1970).

Finally, it has been shown that Ca^{2+} has little stimulant effect on isolated chromaffin granules (Banks, 1966; Poisner and Trifaro, 1967), however, ATP causes a dose-dependent release of catecholamines from the chromaffin granules in vitro (Poisner and Trifaro, 1967). These findings suggest that the secretory action of Ca^{2+} is energy-dependent and also implicating a Ca^{2+} interaction with high-energy phosphates as a critical event in the secretory process.

STATEMENT OF THE PROBLEM

In the last decade, a great deal of information has accumulated to show that chromaffin cells secrete catecholamines by exocytosis. However, the ionic mechanisms involved have not been fully resolved.

It has been well established that extracellular calcium is required for the acetylcholine-induced release of catecholamines, but the role of sodium ions in resting or stimulated release is not clearly understood. During perfusion of cat adrenal glands with Na^+-free medium, a potentiation of acetylcholine-induced catecholamine release was found (Douglas & Rubin, 1963). On the other hand, perfusion of bovine adrenal glands with Na^+-free solutions for longer periods of time resulted in a decrease, or almost abolished, the response of the adrenal medulla to cholinergic stimulation (Banks et al., 1969). In both of the above studies, an increase in catecholamine output was noted immediately after switching the perfusion from normal to Na^+-free medium. This substantial, but transitory, rise in catecholamine secretion in the absence of secretagogues was only briefly alluded to and suggested to be due to the removal of the sodium inhibition of calcium influx into the chromaffin cell (Douglas & Rubin, 1961, 1963; Banks et al., 1969).

The present investigation was undertaken to examine the role of sodium ions in catecholamine release, and especially to study in more detail the release induced by Na^+-free medium.

Some of the results presented in this thesis have already been published by the author (Lastowecka & Trifaro, 1974).

METHODS AND MATERIALS

A. Preparation of adrenal glands for perfusion

Fresh bovine adrenal glands were obtained from the local slaughter-house, they were placed into plastic bags on ice for transport and used within two hours post-mortem. Glands of an average weight of 12 ± 2 g were used. They were cleaned of excess adipose and connective tissue, and the capsule membrane was removed as efficiently as possible, taking care not to damage the cortex. The adrenolumbar vein was trimmed and freed from the base of the gland to facilitate tying around a cannula. Only glands with a single venous orifice were used.

B. Preparation of perfusion fluids

1) The regular perfusion fluid was a phosphate buffered Locke solution of the following composition (mM): NaCl 154; KCl 2.6; $CaCl_2$ 2.2; KH_2PO_4 0.85; K_2HPO_4 2.15; dextrose 10. The final concentration of K^+ was 5.6 mM. In solutions containing excess K^+, the NaCl was reduced by an equivalent amount. 2) Na^+-deficient and Na^+-free solutions had the same composition as regular Locke except that NaCl was partially or totally replaced by equimolar concentrations of lithium chloride or choline chloride, or by an osmotically equivalent concentration of sucrose. The cryoscopic point of the solutions was determined to show that 0.9 g NaCl/100 mls H_2O was equal to 9.25 g sucrose/100 mls H_2O. This same conversion factor had been calculated from osmotic coefficients listed by Lifson and Visscher (1944). 3) Ca^{2+}-free Locke solution was regular Locke without $CaCl_2$, and to which 2.0 mM EDTA or 0.1 mM EGTA was added. 4) High potassium Locke solution contained 56 mM of which 53 mM was as KCl and 3 mM as K_2HPO_4 and KH_2PO_4.

In this solution NaCl was reduced by an equivalent amount (50.4 mM). All solutions were equilibrated with 5% CO_2 in O_2 and their pH adjusted to 7.0 - 7.2 by the addition of NaOH (1N) to regular Locke and KOH (1N) to Na^+-deficient solutions. Overall change in molarity due to addition of alkali did not exceed 2 mM. Acetylcholine chloride (10^{-4} M) and other drugs were added to the solutions prior to pH adjustment.

Throughout all the experiments and preparations of solutions, the water source used was deionized and purified by means of special columns supplied by Hydro Service and Supplies, Durham, N. Carolina.

C. Perfusion protocol

Adrenal glands were perfused *in vitro* at room temperature (25° C), retrogradely via the adrenal vein according to the method described by Trifaro *et al.* (1967). Perfusates were introduced through a cannula of narrow polyethylene tubing (gauge No. PE 205) one end of which was inserted into the vein and fixed by means of a strong nylon thread passed around and tied at the base of the vein. The other end of the cannula carried a 16 gauge needle, 2 inches long. The needle was inserted into the rubber tubing which was pulled over the outflow tube of Marriot flasks containing the solutions. This last procedure was carried out prior to insertion of the cannula into the vein thus eliminating the dead space by the issuing fluid under pressure; this also ensured absence of air bubbles entering the gland which may block capillary passages. Perfusion was speedily switched from one solution to another by manually inserting the needle into the rubber tubing of another solution flask. Perfusion pressure was generated by connecting the perfusion bottles to a cylinder containing 5% CO_2 in O_2, and the flow rates were between 10 - 20 ml/min - which were maintained constant in each

experiment by adjusting the perfusion pressure over the range of 90 - 130 mm Hg.

In all experiments perfusion for the first 40 minutes was with regular Locke solution. This allowed washout of blood and ensured a steady resting output of catecholamines. During this time, final adjustments of flow rate and perfusion pressure were carried out. The glands were placed into polyethylene funnels fixed onto clampstands . Samples were collected at one or two minute intervals in test-tubes standing on ice. To each tube, 1.9 N HCl (10 µl/ml perfusion fluid) was added to prevent oxidation of the catecholamines. Samples of the perfusates were stored at -15^{o} C for future catecholamine determination. After the initial 40 minutes perfusion, the glands were subjected to a period of stimulation lasting from three to fifteen minutes. Usually three stimulations were performed. Drugs to be tested, or any changes in the composition of the perfusion fluid, were introduced during the second period. Each stimulation period was followed by a washout with regular Locke solution for a period of 30 minutes, thus comparison of drug effects could be made.

Other modifications of the protocol will be described in detail in the Results section.

D. Preparation of adrenal homogenates

A sample of medullary tissue (2 g) was dissected from the glands and chopped finely with scissors. All preparations were carried out over ice and ice-cold solutions were used. The medullary tissue was then homogenized in a 0.3 M sucrose solution (pH 7.0) (4 volumes to 1 g tissue) using a smooth glass tube with Teflon pestle (clearance 0.075 mm). A motor driven homogenizer (Cole-Parmer Model 4420) at a speed of 3000 rpm was used, and the

pestle was passed up and down three times to ensure uniformity and consistency in homogenization procedures. The homogenate was then centrifuged at 800 g for 10 minutes in a refrigerated centrifuge. Rotor head No. 870 of an International Model B-20 was used. The obtained pellet containing intact cells and nuclei was discarded, and the supernatant ('low speed supernatant') was further utilized in the preparation of the 'crude granule fraction'.

E. Isolation of chromaffin granules

Differential centrifugation of the 'low speed supernatant' at 20,000 g for 20 minutes in the same rotor head yielded a supernatant, which was discarded, and a pellet ('crude granule fraction', CGF). This sediment was further suspended in 2.0 mls of ice-cold 0.3 M sucrose and then layered onto 7.5 ml of a 1.6 M sucrose solution (pH 7.0). The tubes were centrifuged at 100,000 g for 60 minutes in a No. 40 rotor head of a Beckman Model L ultracentrifuge. This procedure of discontinuous density gradient centrifugation separates intact granules from broken or emptied granules, mitochondria and lysosomes (Smith & Winkler, 1967). The supernatant was decanted off and the pellet containing purified chromaffin granules washed with 1.6 M sucrose twice and the loose white upper sediment on the pellet discarded. The rest of the sediment was lysed - a procedure which breaks the membranes of the chromaffin granules. Lysing was carried out by suspending the granule pellet in 2.5 mls of weak phosphate buffer (0.005 M, pH 6.0). The contents were transferred into Sorval tubes which were then placed into acetone on dry ice (-80° C) for one minute. Thawing was attained by placing the tubes into water at 37° C, after which they were centrifuged at 100,000 g for 60 minutes to remove the granule membranes. The supernatant containing soluble proteins, catecholamines and ATP was decanted and retained and the remaining

pellet subjected to the lysing procedure a further three times to ensure total lysis of the granules. The supernatants after each lysing stage were collected, pooled, and aliquots removed for catecholamine assay. The remaining supernatant was dialyzed against a weak phosphate buffer for 24 hours, and finally concentrated by lyophilizing to a volume of 1 ml. Aliquots from this concentrated solution were taken for dopamine β-hydroxylase assay.

F. Determination of catecholamines in perfusates

The total catecholamine (adrenaline and noradrenaline) content of the perfusates was determined using the quantitative method as described by Anton & Sayre (1962). This is a very sensitive analytical method, is direct and speedy, and is adaptable for the estimation of catecholamines in a variety of biological material. The procedure also allows the simultaneous estimation of adrenaline and noradrenaline in the same sample. As the perfusates contained large quantities of catecholamines, there was no need to concentrate the amines by absorption onto aluminium oxide. The specific reaction forming the basis of this assay is usually termed the trihydroxyindole (THI) procedure resulting in the formation of highly fluorescent hydroxyindole derivatives of adrenaline (adrenolutine) and noradrenaline (noradrenolutine). Potassium ferricyanide is used as the oxidizing agent to form the aminochrome derivatives which have the fluorescent characteristics. Oxidation at two different pH's differentially analyses adrenaline from noradrenaline. Addition of strong alkaline ascorbic acid solutions stops the reaction and yields the trihydroxyindoles stabilized by the ascorbic acid.

Frozen perfusate samples were thawed carefully at room temperature by rigorous shaking in a mechanical vibrator. Sample dilutions of 1 ml in 20 mls of ice-cold water were made and the tubes placed on ice throughout the analysis.

Estimation of the diluted sample fraction took place in the following sequence: 0.2 ml aliquots were pipetted into small test-tubes containing 0.1 ml buffer solution (phosphate buffer 0.5 M, pH 7.0 or acetic acid 1.6 M, pH 2.0). Then 0.02 ml of $K_3Fe(CN)_6$ (a 0.25% solution) was added and the contents allowed to stand for exactly one minute. 0.2 ml of alkaline ascorbic acid (10 mg of sodium ascorbate in 10 mls of 10 N NaOH) was added and the volume made up to 1 ml by addition of 0.5 ml of water. The sample was transferred to a quartz cuvette (10 mm light path) and the fluorescence read in not less than one minute or more than 5 minutes after addition of the alkaline ascorbate.

Readings were taken in an Aminco-Bowman Spectrophotofluorometer (SPF-125) using slit arrangement #2. Optimal activation and fluorescent wavelengths at pH 7.0 were 400 and 500, and at pH 2.0 they were 415 and 520. Series of ten samples were assayed at one time. Internal blanks for both pH's were made using the same water source as in the preparation of the dilutions, and the blanks were assayed in a similar manner except that the ferricyanide was omitted.

Standard catecholamine solutions were prepared by dilution of 6 mM stock solutions of commercial adrenaline and noradrenaline; they were assayed at the beginning and end of the experimental series and the mean of the readings were taken.

Values were corrected for blanks and results calculated according to the following formula:

$$T = \frac{(X_7 - a)A - (X_2 - d)_B}{C}$$

where T = Total catecholamines in nanomoles/ml

A = Difference in specific fluorescence between standard Adrenaline and Noradrenaline at pH 2 (E_2sp.- N_2sp.)

B = Difference in specific fluorescence between standard

C = N_7sp.E_2sp - N_2sp.E_7sp.

a = Blank at pH 7

d = Blank at pH 2

X_7 = Test fluorescence at pH 7

X_2 = Test sample fluorescence at pH 2

This formula is a modification of the one used by Anton & Sayre, 1962.

This formula was programmed onto a magnetic card. An Olivetti Underwood Programma 101 electronic desk computer was used for all calculations. Total catecholamine output was expressed as nanomoles/minute.

G. Determination of catecholamines in soluble contents of chromaffin granules

Adrenal chromaffin granules were prepared as described in E. After lysing the granules, 0.5 mls aliquots of the supernatant were each treated with 0.5 mls of perchloric acid (0.8 N) to precipitate the proteins. The preparations were centrifuged at 15,000 g for 10 minutes and the supernatants thus obtained were kept for catecholamine assay. The supernatants were diluted with ice-cold water (1 ml in 10 mls of water) and the assay followed the same procedure as described in the previous section for perfusates.

H. Determination of dopamine β-hydroxylase in perfusates

Dopamine β-hydroxylase (DBH) was assayed by the method of Friedman &

Kaufman (1965) as modified by Viveros et al., (1969). The technique measures the amount of H^3-octopamine formed from H^3-tyramine in the presence of DBH. Tyramine has been shown to replace dopamine and can be hydroxylated by DBH to form octopamine in the chromaffin cell (Schumann & Phillippu, 1962ab; Musacchio et al., 1966).

Since resting levels of DBH in adrenal medulla perfusates are very low, the assay procedure required the pooling of corresponding one-minute samples of perfusates from several glands in order to obtain sufficient enzyme quantities for measurement. The pooled perfusates were then dialyzed against a weak phosphate buffer (0.5 mM, pH 6.0) for 48 hours to remove catecholamines and ATP, and then concentrated by lyophilization. The concentrated preparation was dissolved in 1 ml of weak phosphate buffer (0.005 M, pH 6.0) and an aliquot was taken for assay. H^3-Tyramine was used as substrate. The final concentration of tyramine in the incubation medium was 10 µM and the specific activity of this isotope was of 10 Ci/m-mole. Other conditions were as previously described (Viveros et al., 1969). Radioactivity was measured in a Nuclear Chicago Liquid Scintillation Spectrometer using "Aquasol" as scintillation fluid. The counting efficiency was between 20-35%, and correction for quenching was made to 100% efficiency by the external channel-ratio method.

DBH activity was expressed in units whereby 1 unit represents 1 picomole of octopamine formed per hour.

I. Determination of dopamine β-hydroxylase in the soluble contents of chromaffin granules

Adrenal chromaffin granules were prepared as described in Section E. The supernatants obtained after lysing the granules were dialysed and then lyophilized and assays were performed according to the same procedure as

described in the preceding section.

J. Determination of lactate dehydrogenase in perfusates

The enzyme, lactate dehydrogenase (LDH) is found in the cytosol of all mammalian cells, and its function is to convert pyruvate to lactate in the presence of reduced nicotine adenine dinucleotide ($NADH_2$).

The method used for the determination of the enzyme was the U.V. technique of Wróblewski & La Due (1955) which is based on the measurement of the decrease in optical density of $NADH_2$ during conversion of pyruvate to lactate. Readings were taken at a wavelength of 340 nm in a Zeiss PMQ II Spectrophotometer. Quartz cells of 10 mm light path were used. The unit of measurement of LDH activity used was the amount of enzyme which will produce a fall of 0.01 OD units/minute.

K. Determination of lactate dehydrogenase in adrenal homogenates

Adrenal glands were perfused for 20 minutes with Locke solution and then the medullae were separated from the cortices. Both tissues were separately homogenized in Locke solution by means of a glass homogenator. The preparations were centrifuged at 20,000 g for 20 minutes and aliquots of the supernatants were taken for assay. Determinations were carried out as indicated in the preceding section, and results were expressed in units per gram of wet tissue.

L. Determination of ATP

Adenosine triphosphate was determined by Douglas and Poisner's modification (1966) of the firefly technique of Strehler (1963). The reaction was carried out in a 5 ml quartz cuvette (10 mm light path) and the light produced during the enzymatic reaction was measured in a modified cuvette holder (Douglas & Poisner, 1966) by means of a photomultiplier tube

(Aminco-Bowman) and recorded with a linear pen recorder. The standard curve was prepared with concentrations of ATP ranging from 0.2 to 2 nmoles ATP/ml.

M. Determination of calcium

When necessary, Ca^{2+} levels in the solution were measured by atomic absorption spectrophotometry as described by Gimblet et al. (1967).

N. Chemicals

The chemicals were obtained from the following sources:

Reduced nicotinamide adenine nucleotide phosphate ($NADH_2$), adenosine triphosphate (ATP), eserine sulphate, and firefly tail powder, Sigma Chemical Company;

Acetylcholine chloride, Welcker Laboratories;

Ascorbic acid, atropine sulphate and hexamethonium chloride, Nutritional Biochemical Corporation;

Ethyleneglycol-bis-(β amino-ethyl-ether)-N,N'-tetra-acetic acid (EGTA) and dextrose, J.T. Baker Chemical Company;

Disodium ethylenediamine-tetra-acetate (EDTA), LiCl, Phosphate buffer SO-β-109, Phenol SO-P-24, Fisher Scientific Company;

Choline Chloride, The British Drug Houses Limited;

Sucrose (density gradient grade), Schwarz-Mann;

H^3-tyramine (10 Ci/m-mole), New England Nuclear;

(-)-Arterenol bitartrate hygrade β grade, L-epinephrine bitartrate β grade, Calbiochem;

Total protein standard, Harlenko;

Acetic acid, Anachemia.

All other chemicals were Baker or Fisher analysed products.

O. Calculation of results

Results were expressed as Mean ± S.E. of the mean. Statistical significance between the difference of two means was obtained by the Student's t-test.

RESULTS

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

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

B. Effect of Na^+ omission on the output of catecholamines

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

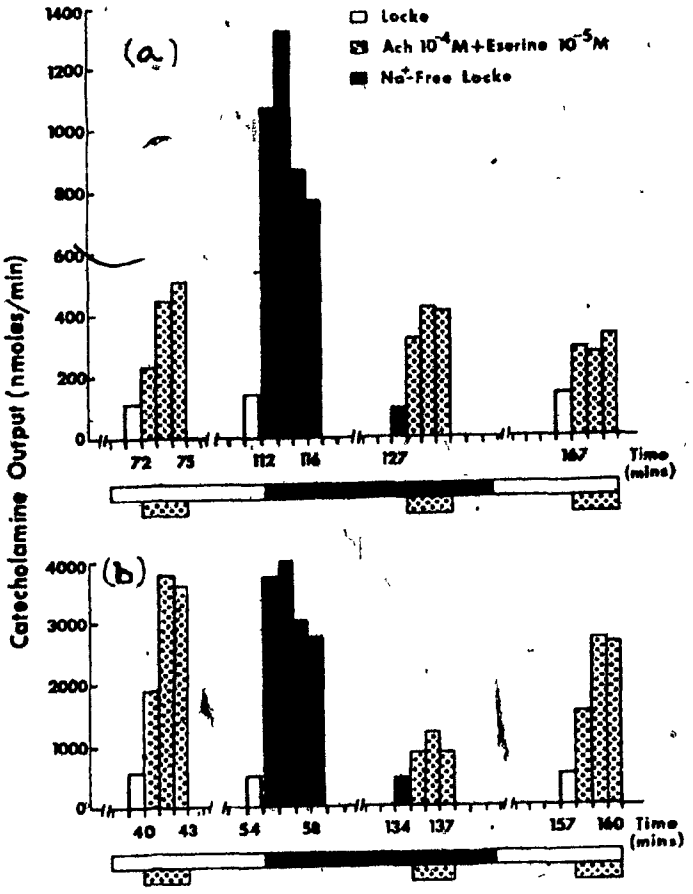


Fig. 1. Effect of Na^+ omission on acetylcholine-evoked release of catecholamines. In this, and in all subsequent Figures, the graphs show the rate of catecholamine output (n-mole/min) from perfused bovine adrenal glands. (a) and (b) show the stimulant effect of acetylcholine (10^{-4} M) in the presence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

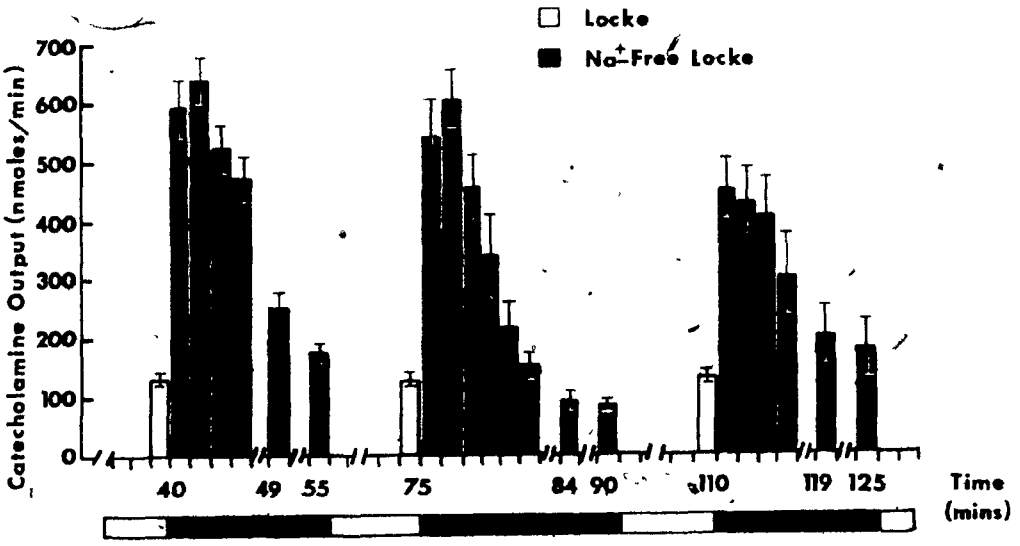


Fig. 2. Effect of Na^+ omission on the output of catecholamines. Bovine adrenal glands (n = 13) were perfused alternately with Locke solution (□) and Na^+-free Locke solution (■) for periods of 20 and 15 min respectively. The vertical bars represent the mean±S.E. of mean of catecholamine outputs expressed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

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

It is known that during repeated stimulation of the adrenal glands with acetylcholine, the increase in catecholamine output in response to acetylcholine decreases with time (Douglas & Rubin, 1961). To see if a similar pattern in catecholamine output could be observed during successive exposure to Na^+-free solutions, nine other adrenal glands were perfused with normal Locke solution for a period of 20 min followed by ten successive 15 min periods of perfusion with Na^+-free Locke solution. As with acetylcholine stimulation, the response to the omission of Na^+ from the extracellular environment decreased with time (Fig. 3). The increase in the output of catecholamines produced during the ninth and tenth periods of perfusion with Na^+-free Locke solution were of 200 ± 30% and 180 ± 25% respectively.

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

During the in vitro perfusion of bovine adrenals, the splanchnic nerve terminals which remained within the gland are closely associated with the chromaffin cells. Therefore, the possibility exists that the increased catecholamine output observed during the omission of Na^+ from the extracellular fluid was secondary to the effect of acetylcholine released from the cholinergic terminals. If this were the case, the response should then be blocked by atropine plus hexamethonium, but this was not so. The results obtained in four experiments showed that when atropine (10^{-5} g/ml) and hexamethonium (5×10^{-4} g/ml) were present in the perfusion fluid the increase in catecholamine output in response to Na^+ omission was not

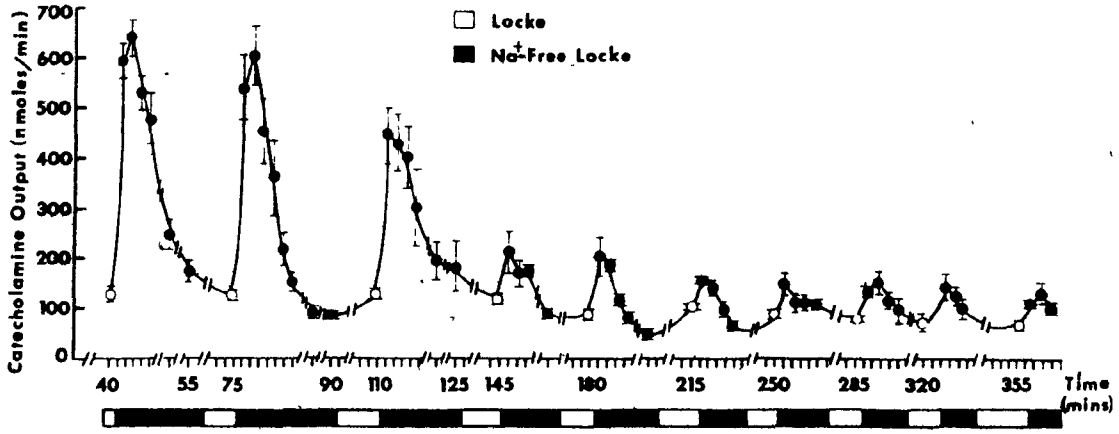


Fig. 3. The effects of successive introductions of Na^+-free Locke solution on catecholamine output. Bovine adrenal glands were perfused ten alternate periods with Locke solution and Na^+-free Locke solution, 20 and 15 minutes respectively. Each point represents the Mean ± S.E. of nine different experiments. Other conditions were as described in Figure 1.

diminished (Fig. 4).

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

Experiments were carried out to determine if partial substitution of Na^+ in the extracellular medium would produce an increase in catecholamine output, and if this were the case, to see if this increase in amine output was proportional or related to the extracellular concentration of Na^+. The catecholamine output rose between 40 - 168%, 200 - 250%, and 360 - 676%, in glands perfused with solutions containing 100, 50 and 10 mM-Na^+ respectively (Fig. 5). When the logarithm of extracellular concentration of Na^+ was plotted against the percentage increase in catecholamine output during the first 2 min of perfusion with Na^+ deficient solutions, a linear correlation was obtained (Fig. 6).

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

In the experiments described above, Na^+ was substituted by osmotically equivalent amounts of sucrose. Therefore, experiments were also performed in which Na^+ was replaced by either choline or Li^+ ions. When a Na^+-free (choline) Locke solution reached the gland, there was a significant increase in catecholamine release (Fig. 7a). In three experiments the increase in amine output was between 300 and 450%. On the other hand, substitution of Na^+ by Li^+ did not produce a rise in catecholamine output (Fig. 7b), although switching the perfusion fluid from Li^+-Locke solution to a Li^+-free (sucrose) Locke solution produced a significant increase in catecholamine output.

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

As during acetylcholine stimulation, successive exposure of the adrenal

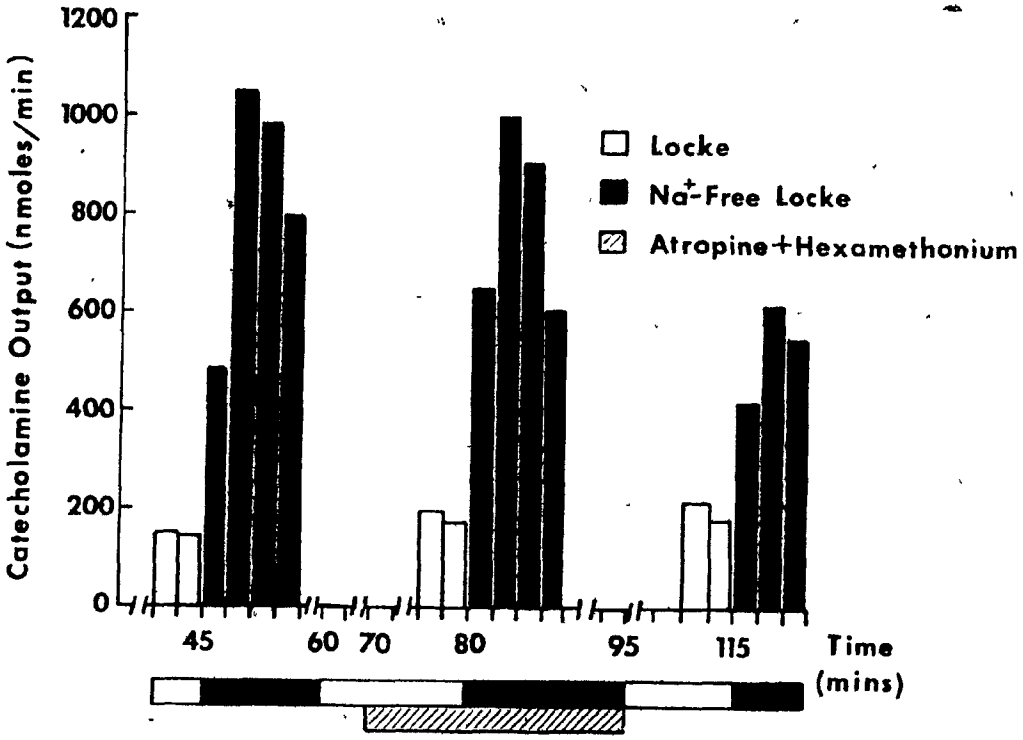


Fig. 4. The lack of effect of anticholinergic drugs on the release of catecholamines produced by the omission of Na^+ from the perfusion fluid. A bovine adrenal gland was perfused alternately with Locke solution (□) and Na^+-free Locke solution (■) for periods of 20-15 min respectively. During the second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

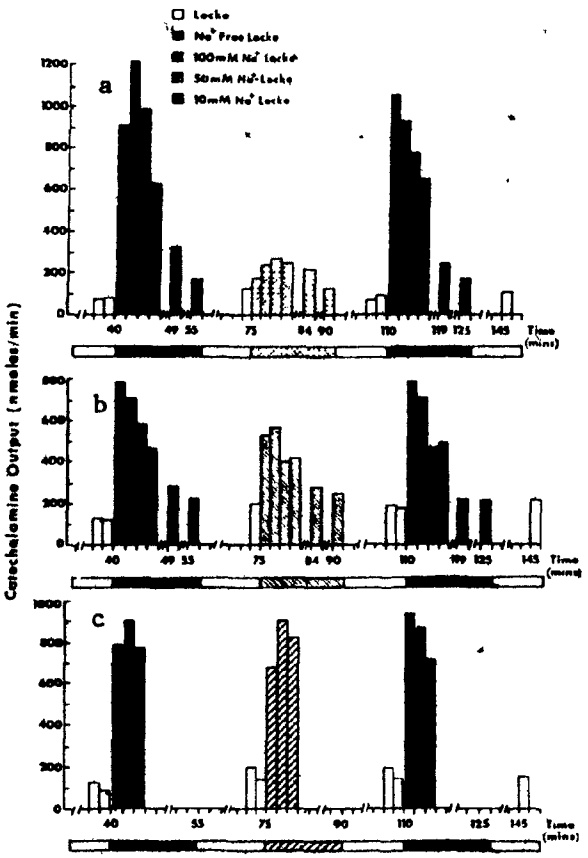
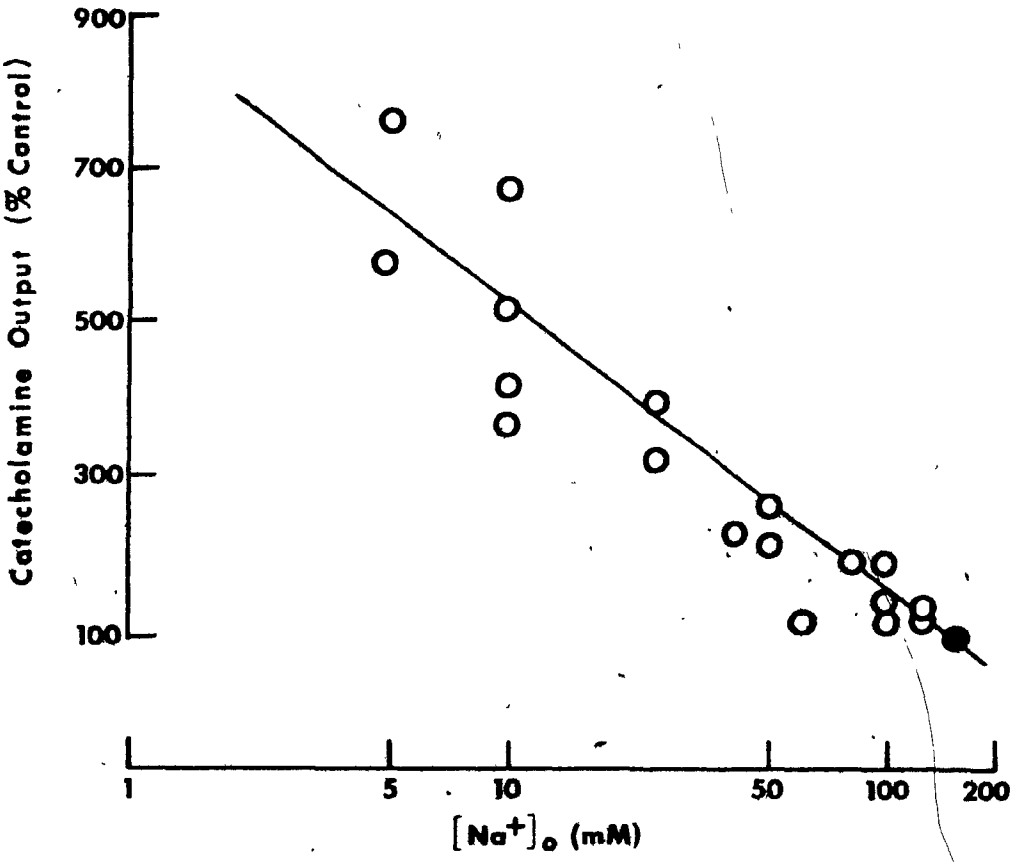


Fig. 5. A comparison of 3 different concentrations of sodium in the perfusion fluid on catecholamine output. Bovine adrenal glands (a), (b) and (c) were perfused with Locke solution and the perfusion fluids were switched three times to modified Locke solutions. All glands were perfused during the first and third stimulation periods with Na^+-free Locke solution. During the second stimulation period, glands (a), (b) and (c) were perfused with modified Locke solution containing 100, 50 and 10 mM NaCl respectively. In these solutions, NaCl was replaced by osmotically equivalent amounts of sucrose. Other conditions were as described in Fig. 1.

Fig. 6. Effect of graded substitution of Na^+ in the perfusion fluid on the output of catecholamines. Bovine adrenal glands (n = 18) were first perfused with Locke solution for 40 min. At the end of this perfusion period samples were collected for 2 min and assayed for catecholamine content. The values thus obtained were considered equal to 100% (●). After perfusion with Locke solution the glands were then perfused with modified Locke solutions (O) and samples were collected during the first 2 min of perfusion and assayed for catecholamines. The modified Locke solutions contained between 5 and 125 mM-NaCl. In these solutions NaCl was replaced by osmotically equivalent amounts of sucrose. The ordinate represents catecholamine output as the percentage of the value obtained during perfusion with Locke solution. The abscissa represents the logarithm of the extracellular concentration of Na^+ ions. The line was computer fitted (correlation coefficient = -0.90). Other conditions were as described in Fig. 1.


900
700
500
300
100
Catecholamine Output (% Control)
1
5
10
50
100
200
[Na+]o (mM)

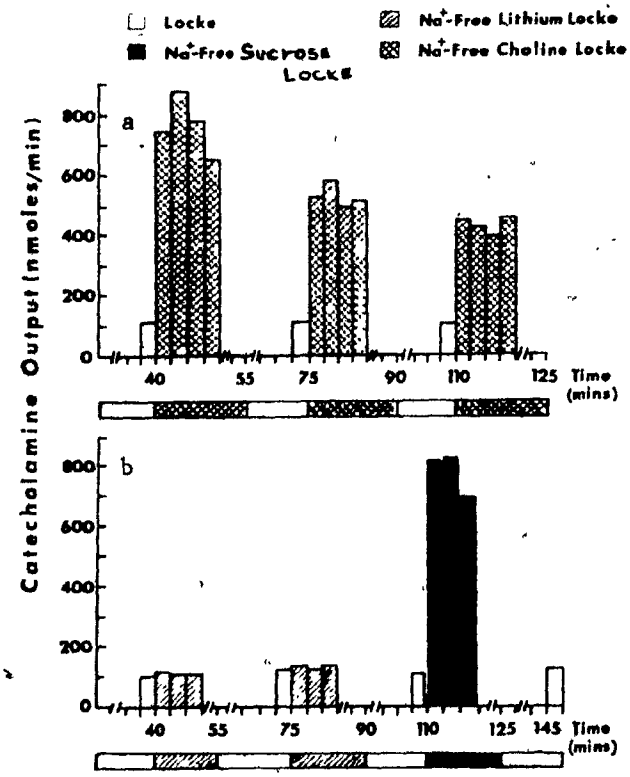


Fig. 7. Differences in catecholamine outputs obtained during the substitution of Na^+ in the perfusion fluid by osmotically equivalent amounts of sucrose, choline or Li^+. Bovine adrenal glands a and b were first perfused with Locke solution (▭). Then gland a was perfused alternately with Locke solution and Na^+-free (choline) Locke solution (▩) for periods of 20 and 15 min respectively. Gland b was perfused alternately with Locke solution and two periods of Na^+-free (Li) Locke solution (▨). The perfusion was then continued with Locke solution for 20 min followed by a 15 min period of perfusion with Na^+-free sucrose Locke solution (■). Similar results were obtained in three other experiments. Other conditions were as described in Fig. 1.

glands to Na^+-free solutions decreases the responsiveness of the gland. It was, therefore, decided to perfuse glands with Locke solution and then expose the glands on five successive occasions to Na^+-free solution, followed by successive stimulations with acetylcholine (10^{-4} M). It was observed that the first acetylcholine stimulation, that is, the sixth stimulation, (since the five previous stimulations were done by Na^+ omissions), produced a greater increase in catecholamine output than the three or four preceding stimulations done by Na^+-free solutions (Fig. 8). Similar results were obtained when the first five stimulations were done by acetylcholine. The sixth stimulation, that is, the first by Na^+ omission, produced an increase in amine output greater than the two preceding acetylcholine stimulations.

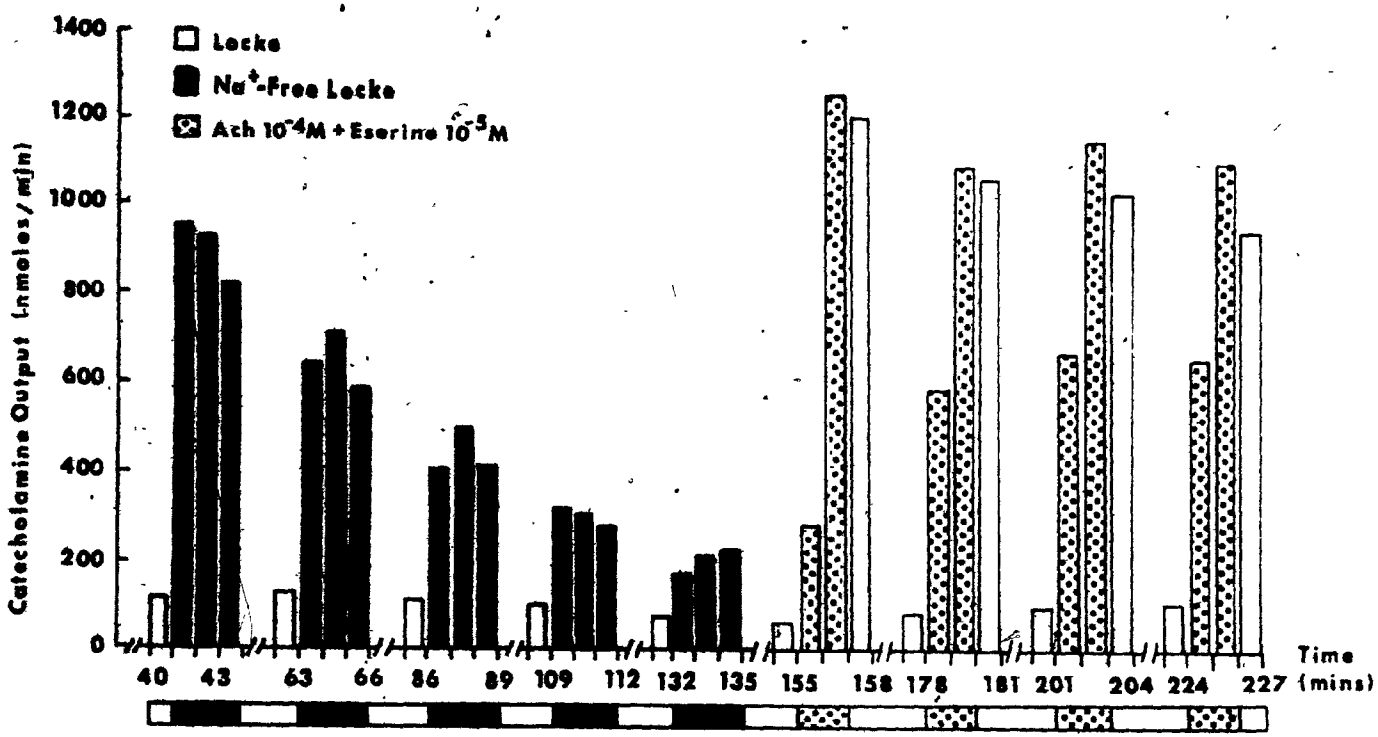
G. Effect of increasing the concentration of K^+ in the perfusion fluid on the increased output of catecholamines produced by Na^+ omission

It is well known that acetylcholine is capable of inducing release of amines during perfusion of adrenal glands with solutions containing 56 mM-K^+ (Douglas & Rubin, 1963). To see the effect of depolarizing concentrations of K^+ on the Na^+-free effect, glands were perfused with Locke solution, stimulated for a first time by Na^+-free Locke solution, and after 20 min of perfusion with normal Locke solution they were stimulated with 56 mM-K^+ medium; perfusion was switched to a 56 mM-K^+ Locke solution in which the 104 mM-Na^+ was substituted by an osmotically equivalent concentration of sucrose. The omission of Na^+ from a high K^+ solution during perfusion of the adrenal glands produced the characteristic increase in catecholamine output (Fig. 9).

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

If Na^+ is omitted from the extracellular environment there is an increase in the ratio between the intra- and extracellular concentrations

Fig. 8. Effect of successive stimulations by Na^+-free Locke solution on the response of the adrenal gland to acetylcholine. A bovine adrenal gland was perfused 5 consecutive times alternately with Locke and Na^+-free Locke solutions. This was followed by four periods of perfusion using Locke and acetylcholine-Locke solutions alternately. Similar results were obtained in four other experiments. Other conditions were as described in Fig. 1.


Locke
Na+-Free Locke
Ach 10-4M + Eserine 10-5M
Catecholamine Output (nmoles/min)
1400
1200
1000
800
600
400
200
0
40 43 63 66 86 89 109 112 132 135 155 158 178 181 201 204 224 227
Time (mins)

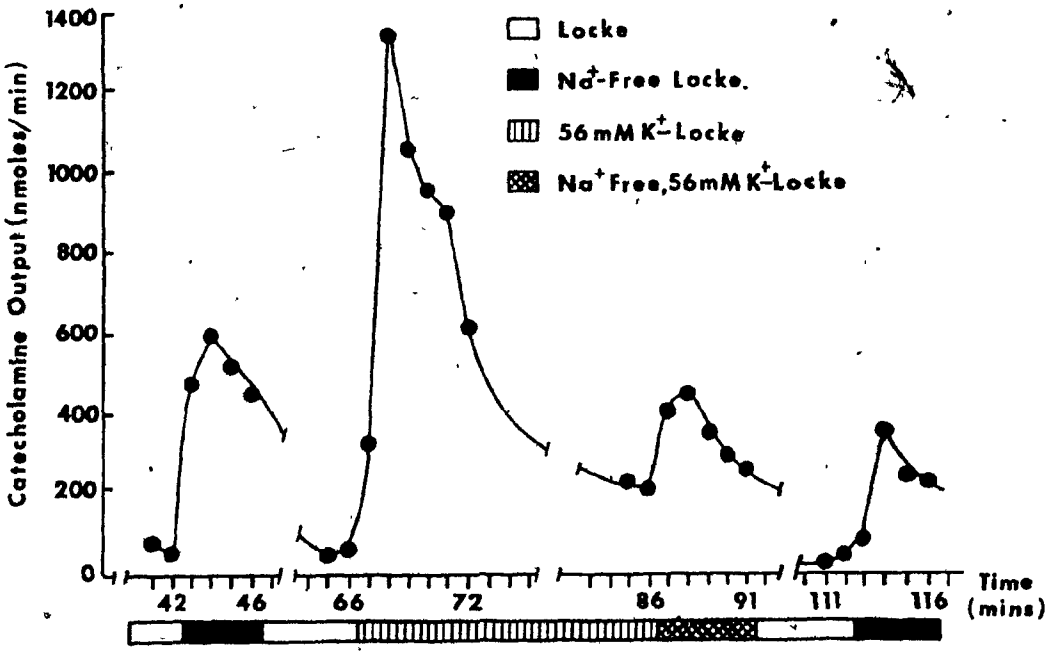


Fig. 9. Effects of Na^+-deprivation on the output of catecholamines during depolarization with KCl. A bovine adrenal gland was perfused with Locke solution for 40 min. Then perfusion was for 15 min with Na^+-free Locke solution. Perfusion was continued for a further 20 min with Locke solution followed by a 25 min period of perfusion with 56 mM-K^+-Locke solution. During the last 5 min of this perfusion period, 104 mM-NaCl was replaced by an osmotically equivalent amount of sucrose. After this period, perfusion was switched to Locke solution. This was followed by another period of perfusion with Na^+-free Locke solution. Similar results were obtained in four other experiments. Other conditions were as described in Fig. 1.

of Na^+ ($[Na^+]_i/[Na^+]_o$). This seems to be an ideal condition to increase the entry of Ca^{2+} into cells (Baker, 1970). It was, therefore, possible that the increased amine output observed during Na^+ deprivation was a result of an enhanced Ca^{2+} influx into chromaffin cells. If this were so, increasing the extracellular concentration of Mg^{2+} should partially or totally block amine released by Na^+ deprivation because Mg^{2+} has been shown to decrease the acetylcholine-evoked release of catecholamines from the adrenal medulla by competing with the Ca^{2+} entry into the cells (Douglas & Rubin, 1963). When glands were perfused with Na^+-free Locke solution in the presence of 10 mM-Mg^{2+} there was a decrease in the catecholamine output in response to Na^+ deprivation. Under these conditions, that is, in the presence of 10 mM-Mg^{2+}, the catecholamine output during the first 3 min of perfusion was 43.6 ± 6.1% (n = 4) of that obtained during the preceding stimulation in the absence of Mg^{2+} (Fig. 10). This figure is significantly different from the value 91.1 ± 11.1% (n = 13, $P < 0.005$) obtained when the second stimulation was carried out in the absence of Mg^{2+}. Increasing the concentration of Mg^{2+} in the perfusion fluid to 20 mM produced a greater blocking effect ($P < 0.001$). The results obtained in five experiments are shown in Table 1. It should also be noticed that the third stimulation, which was carried out in the absence of Mg^{2+}, produced a response of 84.7 ± 8.2%. This figure is not significantly different from the value of 73.7 ± 11%, obtained for the third response when all responses to Na^+-free Locke solution were obtained in the absence of Mg^{2+}.

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

If the blockage in Na^+-free stimulated amine release produced by

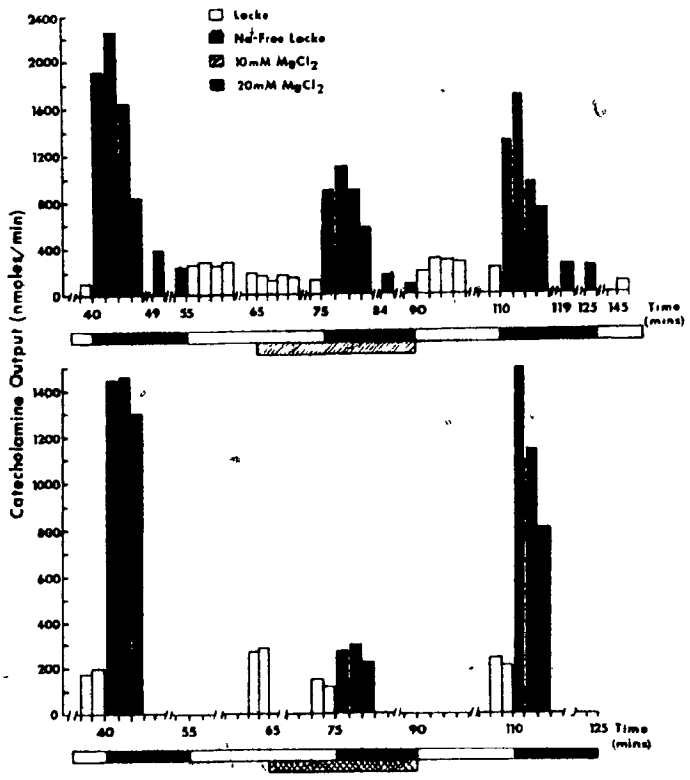


Fig. 10. Effect of Mg^{2+} on the output of catecholamines evoked by Na^+ omission. Both bovine adrenal glands (a) and (b) were perfused alternately with Locke solution and Na^+-free Locke solution for periods of 20 and 15 minutes respectively. During the second perfusion period, the Na^+-free Locke solution contained 10 and 20 mM $MgCl_2$ in glands (a) and (b) respectively. Other conditions were as described in Figure 2.

Table 1. Effect of Mg^{2+} on the output of catecholamines evoked by Na^{+} omission.

Adrenal glands were perfused during the first and third stimulation periods with Na^{+}-free Locke solution. During the 2nd stimulation period, the Na^{+}-free Locke solution contained 20 mM-$MgCl_2$.

Expt. no.	Increased catecholamine release (n-mole/min)			Ratio of 2nd to 1st stimulation (%)	Ratio of 3rd to 1st stimulation (%)
	1st stimulation	2nd stimulation	3rd stimulation		
1	+284	+11	+240	3.9	84.5
2	+331	+33	+323	9.9	97.6
3	+504	+26	+312	5.2	62.0
4	+734	+103	+788	14.0	107.4
5	+1227	+10	+883	0.8	72.0
Mean ± S.E. of mean				6.8±2.3	84.7±8.2

raising the extracellular concentration of Mg^{2+} is due to the competition between this ion and Ca^{2+}, it should be possible to obtain similar results when Ca^{2+} is omitted from the Na^{+}-free solutions. In the Ca^{2+}-free, Na^{+}-free solutions, in which Na^{+} was replaced by osmotically equivalent amounts of sucrose, there was 9.96 ± 0.9 μM (n = 5) of Ca^{2+}. This amount can easily be chelated by adding EGTA or EDTA to the solutions.

Adrenal glands were perfused with Locke solution and three successive stimulations were performed by switching to a Na^{+}-free Locke solution. The second stimulation was done in the absence of Ca^{2+} and the presence of 0.1 mM EGTA. In the absence of Ca^{2+} the amine output produced by the Na^{+}-free medium was not diminished (Fig. 11). On the contrary, there was a small increase in amine output during Ca^{2+} omission. The results of five experiments are shown in Table 2. The value of 120 ± 23% (n = 5) is not significantly different from the figure 91.1 ± 11.1% (n = 13), obtained for the second stimulation in the control experiments. Six other experiments were done in which the Ca^{2+}-free, Na^{+}-free solution contained 2.0 mM-EDTA. Under these conditions, during the second stimulation, the Na^{+}-free solution evoked release of amines was of 101 ± 24% (n = 6). Two glands were stimulated to release catecholamines by acetylcholine; perfusion was then switched to Ca^{2+}-free medium and catecholamine release by Na^{+}-free medium and by acetylcholine was tested. The results obtained with two glands were similar, and the results obtained with one of them are shown in Fig. 12. These results should be compared with those of Fig. 1 (a), and unlike those experiments, the response to acetylcholine stimulation was completely abolished. However, Na^{+} deprivation still induced catecholamine release. Furthermore, in order to see if the blockage of the acetylcholine response was

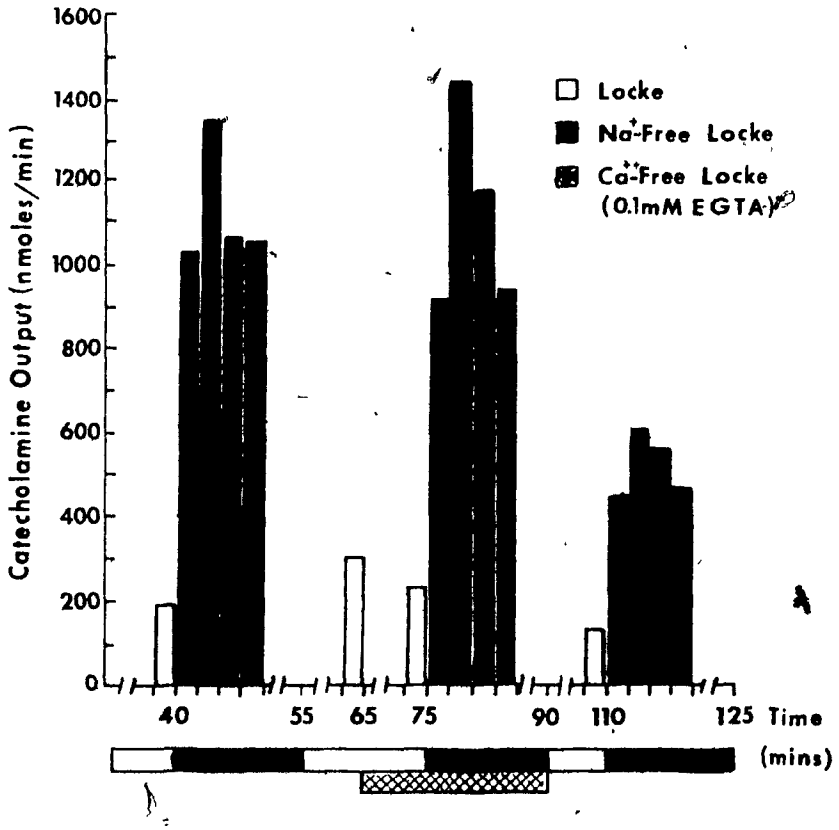


Fig. 11. Effect of Ca^{2+} omission from the extracellular environment on the release of catecholamines produced during perfusion with Na^{+}-free Locke solution. A bovine adrenal gland was perfused alternately with Locke solution (▭) and Na^{+}-free Locke solution (▬) for periods of 20 and 15 min respectively. During the second perfusion period, Ca^{2+} was omitted and 0.1 mM-EGTA was present in the Na^{+}-free Locke solution. Other conditions were as described in Fig. 1.

Table 2. Effect of Ca^{2+} omission on the release of catecholamines produced during perfusion with Na^{+}-free Locke solution. Adrenal glands were perfused during the 1st stimulation period with Na^{+}-free Locke solution containing 2.2 mM-$CaCl_2$. During the 2nd stimulation period, $CaCl_2$ was omitted and 0.1 mM EGTA was added to the Na^{+}-free medium.

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

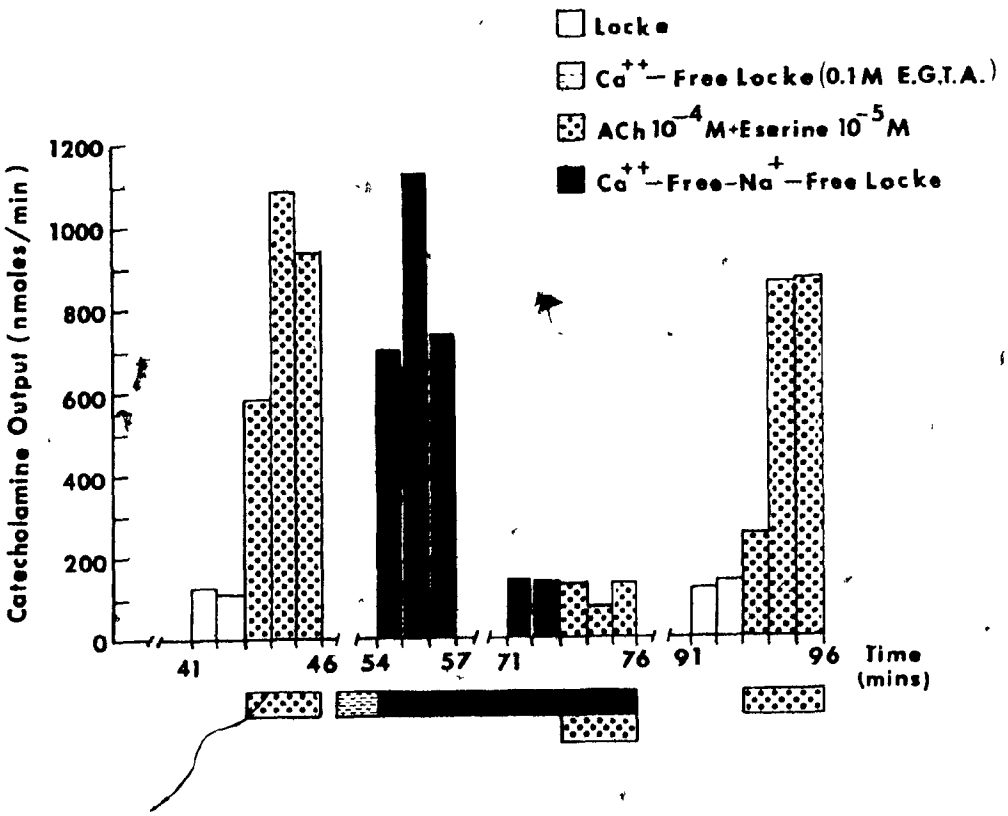


Fig. 12. Effect of Ca^{2+} and Na^{+} omission on acetylcholine-evoked release of catecholamines. A bovine adrenal gland was perfused with Locke solution and was subjected to two periods of stimulation by acetylcholine (▩). Between these two stimulation periods, the gland was first perfused with Ca^{2+} -free Locke solution (▩), and then there followed a 22 min period of perfusion with Ca^{2+}-free, Na^{+}-free Locke solution (■). During the last 3 min of this period, the gland was stimulated by acetylcholine (▩). Similar results were obtained with another gland. Other conditions were as described in Fig. 1.

due to the long exposure of the glands to Ca^{2+}-free medium, experiments were also performed in four other glands. Here, during the period between the two acetylcholine stimulations, the glands were perfused with Ca^{2+}-free Locke solution, and they were first exposed to acetylcholine for 3 min. Under these conditions the response to acetylcholine stimulation was abolished. However, switching the perfusion fluid to Ca^{2+}-free, Na^{+}-free Locke solution produced a characteristic increase in catecholamine output.

Due to these unexpected results, it was decided to repeat the experiments described in the preceding section. However, on this occasion, the effect of 20 mM-Mg^{2+} on the stimulation produced by Na^{+} omission was tested in the absence of Ca^{2+} from the perfusion medium. Here again, the Na^{+} deprivation effect on amine release was blocked by Mg^{2+}. The response in the presence of Mg^{2+} was 6.7 ± 1.8% (n = 3) of that obtained in the absence of Mg^{2+}, and this value is similar to that obtained in the presence of extracellular Ca^{2+} (see Table 1, column 4).

J. Is exocytosis the mechanism of release of catecholamines during Na^{+} omission?

The results of the experiments shown in Fig. 8 seem to suggest that the amines released during the omission of Na^{+} from the extracellular environment came from different cellular pools or different cells than those amines secreted during acetylcholine stimulation. Furthermore, unlike during acetylcholine stimulation, the release produced by Na^{+} omission occurs in the absence of extracellular Ca^{2+}, although our experiments do not rule out the possibility of mobilization of intracellular Ca^{2+} during sodium omission. Therefore, due to this discrepancy, it was decided to study the mode of secretion during Na^{+} deprivation. This was done by perfusing adrenal glands with Na^{+}-free Locke solution in the presence or absence of extracellular

Ca^{2+} and the perfusates were examined for other soluble constituents of the chromaffin granules, i.e. dopamine β-hydroxylase (DBH) and adenosine triphosphate (ATP).

(a) DBH efflux. Fig. 13 shows the results obtained with two glands: gland a was stimulated by acetylcholine whereas gland b was stimulated by Na^+ deprivation. The increase in catecholamine output produced by both forms of stimulation was associated with concomitant increases in DBH outputs. The ratios between catecholamines and DBH determined in the perfusates during either acetylcholine or Na^+ omission induced release were similar to those ratios determined in the soluble content of isolated chromaffin granules (Table 3). Na^+ deprivation also induces release of DBH in the absence of extracellular Ca^{2+} (presence of EDTA), as indicated in Table 3.

(b) ATP efflux. Under the conditions used in these experiments, that is, absence of extracellular Ca^{2+} and Mg^{2+}, extracellular ATPases should be inhibited, and if catecholamines are released by exocytosis, ATP should be recovered in the perfusates. Eighteen tests were performed on six glands. The results obtained with three of these glands are shown in Fig. 14, which shows that, during stimulation, there was a parallel increase in both catecholamine and ATP outputs. The molar ratios in the perfusates between catecholamines and ATP are shown in Fig. 15. The catecholamine ATP molar ratio in the perfusates was of 3.97 ± 0.18 ($n = 18$), a figure which is not significantly different from the value of 4.21 ± 0.44 ($n = 7$), previously reported for the soluble content of isolated chromaffin granules (Trifaro & Dworkind, 1970). In addition, perfusates were also assayed for lactate dehydrogenase (LDH), an enzyme used as a cytoplasmic marker. If stimulation by Na^+ deprivation damages or produces a non-physiological increase in the

Fig. 13. Effect of Na^+ deprivation and acetylcholine stimulation on the outputs of catecholamines, dopamine β-hydroxylase, and lactate dehydrogenase. Two bovine adrenal glands were perfused with Locke solution and stimulated five consecutive times for 6-8 min by either acetylcholine (a) or Na^+ deprivation (b). Samples of the perfusates were collected before and during the stimulations, at 2 min intervals. The Figure represents the catecholamine (□) and dopamine β-hydroxylase (⊡) outputs after combining the samples of the five stimulation periods in the following manner: samples taken during the first 2 min of the five stimulation periods were combined; similarly, the samples taken during the second 2 min of stimulation were also combined, etc. Aliquots were taken from these pooled samples and assayed for catecholamines and lactate dehydrogenase as described in the Methods section. The rest of the samples were dialysed for 48 hr against 0.5 mM phosphate buffer (pH 6.5) and later lyophilized. The resultant dried precipitates were suspended and assayed for dopamine β-hydroxylase as described in the Methods section. One unit of the DBH activity represents the formation of 10 p-mole of octopamine per hour. The Figure also shows the outputs of lactate dehydrogenase (Hatched bars, ▨), and for clarity, the units of LDH are not indicated in the graph. However, LDH outputs were similar to those indicated in Table 4.

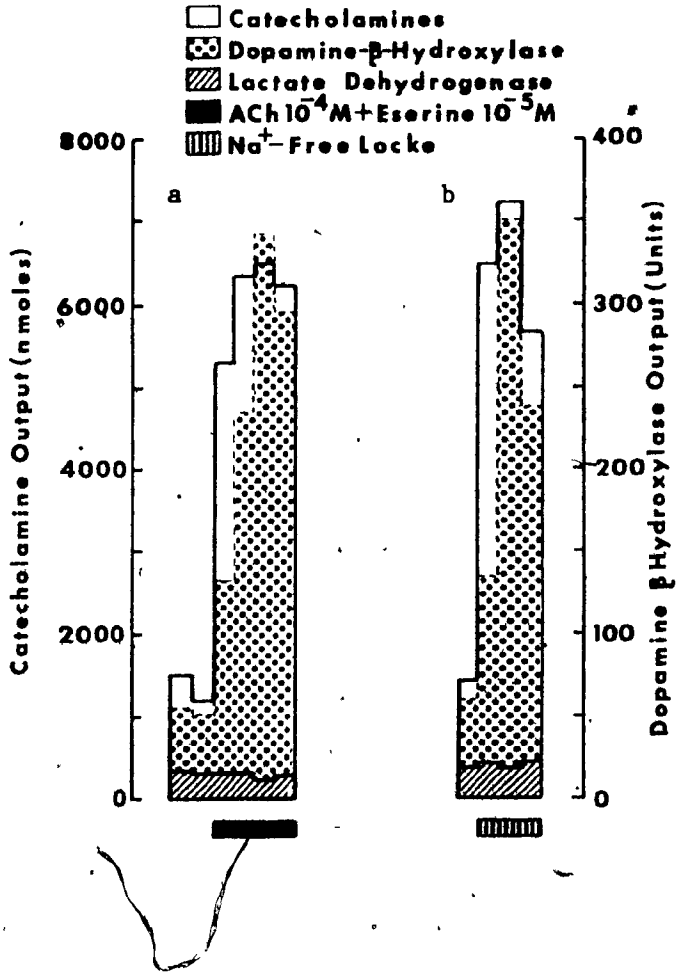

Catecholamines
Dopamine-β-Hydroxylase
Lactate Dehydrogenase
ACh 10^{-4}M+Eserine 10^{-5}M
Na^{+}-Free Locke
8000
6000
4000
2000
0
a
b
400
300
200
100
0
Catecholamine Output (n moles)
Dopamine β Hydroxylase Output (Units)

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

Condition	Ratio: Catecholamines / Dopamine β-hydroxylase (n-mole/units*)
ACh evoked release	20.6 ± 4.6 (n = 5)†
Na^+-free evoked release	22.2 ± 4.6 (n = 7)
Ca^{2+}-free, Na^+-free evoked release	26.0 ± 3.0 (n = 11)
Soluble granule contents	23.6 ± 4.5 (n = 14)

* 1 unit = 10 p-moles of octopamine formed per hour.

† n = number of tests.

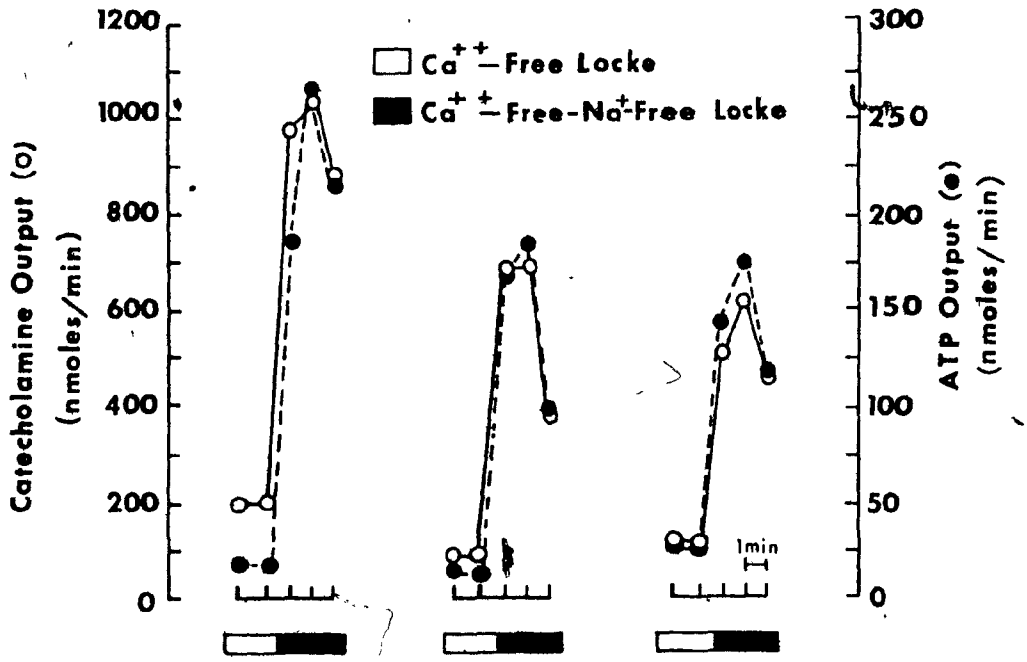


Fig. 14. Effect of Na^+ deprivation on the outputs of catecholamines and adenosine triphosphate in the absence of extracellular Ca^{2+}. Three bovine adrenal glands were perfused with Ca^{2+}-free Locke solution (□) and these followed a 3 min perfusion period with Ca^{2+}-free, Na^+-free Locke solution (■). Both solutions contained 1.0 mM-EDTA and 0.1 mM-EGTA. Samples of the perfusates were collected at 1 min intervals and they were assayed for catecholamines (○) and ATP (●) as described in the Methods. Similar results were obtained with three other glands. Other conditions were as described in Fig. 1.

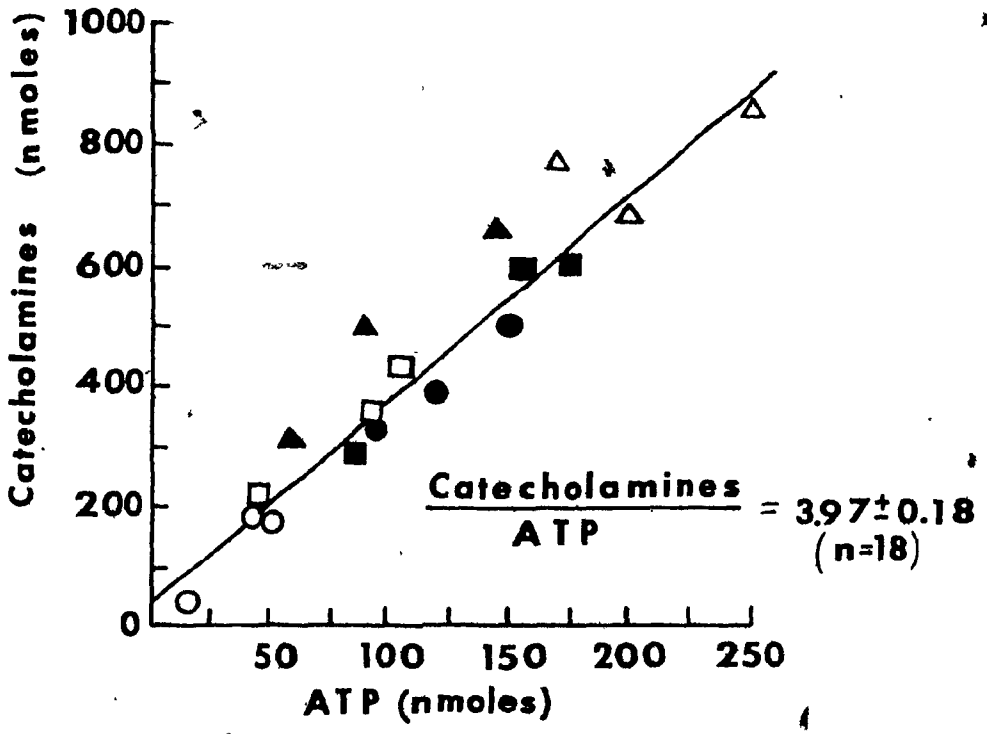


Fig. 15. Correlation of catecholamine output with ATP efflux during stimulation by Na^+ deprivation. A total of eighteen tests were performed on six glands where after perfusion with Ca^{2+}-free Locke solution, the perfusion was switched to Ca^{2+}-free, Na^+-free Locke solution. Both solutions contained 1.0 mM-EDTA and 0.1 mM-EGTA. The results of tests performed on each gland are represented by the same symbol. The line was computer fitted (correlation coefficient = 0.95).

permeability of the cell membrane which would account for the release of catecholamines together with DBH and ATP, one might expect a concomitant rise in the perfusates of the levels of this cytoplasmic enzyme. Fig. 13 and Table 4 shows that as during acetylcholine stimulation, Na^+ omission did not produce an elevation in the LDH levels of the perfusates. The LDH content of the medulla and cortex were of 4398 ± 85 (n = 5) and 5380 ± 24 (n = 5) units per gram of wet tissue respectively. The ratio of the cortex to the medulla (w:w) in the adrenal gland was of 3.51 ± 0.15 (n = 9). Because the average weight of the perfused gland was 12 ± 2 g, a gland of an average weight would contain about 61925 units of LDH. The LDH levels detected in the perfusates were of 1.9 to 5.4 units/min (Table 4). These values represent 0.003 - 0.009% of the total LDH content of the gland. Therefore, in 2 hr of perfusion, the total LDH released would be about 1% of the total content of the gland.

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

	Units*/min							
	Gland 1		Gland 2		Gland 3		Gland 4	
Condition	LDH	CA	LDH	CA	CA	CA	LDH	CA
Locke	2.9	140	2.0	106	4.3	142	5.1	246
Na^+-free Locke	3.2	614	1.9	230	4.7	812	5.2	634
Na^+-free Locke	3.2	690	1.9	458	4.4	822	5.3	988
Na^+-free Locke	3.2	576	2.0	230	4.4	602	5.1	804
Locke	3.0	131	---	---	---	---	5.4	337
Acetylcholine Locke	2.8	484	---	---	---	---	5.4	464
Acetylcholine Locke	2.8	1102	---	---	---	---	5.4	1753
Acetylcholine Locke	2.6	1271	---	---	---	---	5.4	1771

* 1 unit of lactate dehydrogenase (LDH) is the fall of 0.01 of an optical density unit per minute. 1 unit of catecholamine (CA) is 1 n-mole.

DISCUSSION

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

A. Na^{+} deprivation and the output of catecholamines

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

splanchnic nerve terminals during Na^+ deprivation was obtained in the experiments in which the increased catecholamine release was still observed when glands were perfused with a Na^+-free medium in the presence of atropine and hexamethonium.

Unlike the increase in catecholamine release in response to ouabain (Banks, 1967), Na^+ omission produces an immediate and sharp rise in the output of catecholamines, and as with the acetylcholine-evoked release, this high output of catecholamines is not maintained and declines to resting levels in 12 - 15 min. We have not investigated the reason why the response of the gland died out with time. However, it is possible that if Na^+ deprivation somehow mobilizes and increases the intracellular ionized Ca^{2+}, and if the amount of Ca^{2+} available for the release reaction is limited, and once it is used, the release response would decrease. Another possibility is that a high intracellular Ca^{2+} level is related to a higher $[Na^+]_i/[Na^+]_o$ ratio, and that the release response declines when the intracellular Na^+ is being extruded from the cell. In either case, perfusion with regular Locke solution should then restore the cellular ionic equilibrium and make the cell available again for release. The results indicate that the response to Na^+ deprivation can be obtained 7 - 10 consecutive times provided that short periods of perfusion with normal Locke solution precede the omission of Na^+ from the perfusion medium.

Our results also indicate that the release of catecholamines was proportional to the concentration of the extracellular Na^+; the lower the concentration of this ion, the greater the output of catecholamines. Decreasing the concentration of extracellular Na^+ produces an increase in the $[Na^+]_i/[Na^+]_o$ ratio, and perhaps a concomitant increase in the intracellular Ca^{2+}

levels, since it is known that extracellular Na^+ is necessary for a normal Ca^{2+} efflux (Blaustein & Hodgkin, 1969).

B. Na^+ deprivation and the acetylcholine-evoked release of catecholamines

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

C. Replacement of Na^+ by Li^+, choline or sucrose and the release of catecholamines.

Replacement of Na^+ by Li^+ failed to release catecholamines from the adrenal medulla. The effect of Na^+ deprivation on release was observed only when Na^+ was replaced by either sucrose or choline. Our results with Li^+ can be explained if we assume that the external surface of the plasma membrane of the chromaffin cell is not able to distinguish between Li^+ and Na^+ ions. This assumption is not unreasonable if we consider that Keynes &

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

D. Ca^{2+} and Mg^{2+} ions and the release of catecholamines induced by Na^+ deprivation.

Douglas & Rubin (1963, 1964) have shown that increasing the concentration of Mg^{2+} in the extracellular environment decreases or blocks the effects of acetylcholine, high K^+ and Ba^{2+} on the release of catecholamines. They interpreted their findings as a result of a blockage of the entry of Ca^{2+} or Ba^{2+} into the cells. Since our results clearly indicate that Mg^{2+}, not only in the presence but also in the absence of extracellular Ca^{2+}, blocks the release of catecholamines induced by Na^+ deprivation, here, another explanation for the effects of Mg^{2+} other than that of the competition with the entry of Ca^{2+} must be found. Possibilities include: (a) Mg^{2+} might block the combination of Ca^{2+} with intracellular receptors; (b) Mg^{2+} might also block the intracellular release or translocation of Ca^{2+}, since Mg^{2+} has been shown to be necessary for the uptake and binding of $^{45}Ca^{2+}$

into adrenal medullary microsomes (Poisner & Hava, 1970). On the contrary, Ba^{2+} interferes with Ca^{2+} binding by adrenal medullary microsomes (Poisner & Hava, 1970). These two explanations of the effect of Mg^{2+} assume that Na^{+} deprivation would hyperpolarize the cells and that this would increase the intracellular levels of Ca^{2+}. Douglas, Kanno & Sampson (1967) have shown that withdrawal of Na^{+} from the extracellular space produces hyperpolarization of the chromaffin cells. In addition, it has been suggested that hyperpolarization enhances transmitter release at other synapses (del Castillo & Katz, 1954); (c) Mg^{2+} might inhibit the Mg^{2+}-dependent ATPase of the chromaffin granules. From in vitro studies it has been suggested that the granule ATPase may play a role in the secretory process (Poisner & Trifaró, 1967; Trifaró & Poisner, 1967). The results show that concentrations of Mg^{2+} of 10 mM or more blocked catecholamine release in response to Na^{+} deprivation. The same concentration range has been shown to have an inhibitory effect on the chromaffin granule ATPase (Winkler, Hörtnagl, Hörtnagl & Smith, 1970).

E. Mechanism of release during Na^{+} deprivation

Previous studies have demonstrated that the release of catecholamines by acetylcholine from the adrenal medulla is by exocytosis. The evidence for release by exocytosis included the demonstration of simultaneous release of catecholamines with ATP (Douglas & Poisner, 1966a, b), chromogranin A (Banks & Helle, 1965), and dopamine β-hydroxylase (Viveros, Arqueros & Kirshner, 1968). On the contrary, the membrane components of the chromaffin granules are not released into the perfusate (Trifaró et al., 1967; Schneider, Smith & Winkler, 1967), and are quantitatively retained within the cell (Poisner et al., 1967; Viveros, Arqueros, Connett & Kirshner, 1969).

This process appears also to be the mechanism by which noradrenaline is released from sympathetic nerve endings (Smith & Winkler, 1972). In all these systems extracellular Ca^{2+} seems to be essential for the release process (Rubin, 1970). However, in the presence or in the absence of extracellular Ca^{2+}, the release induced by Na^{+} deprivation was apparently by exocytosis because catecholamines released by Na^{+}-free medium were accompanied by ATP and dopamine β-hydroxylase. The ratio of catecholamines to dopamine β-hydroxylase or to ATP in the perfusates was similar to that in the granules. Furthermore, the increased release of dopamine β-hydroxylase was not accompanied by a parallel increase in the output of a cytoplasmic protein of smaller molecular weight, such as lactate dehydrogenase. The unchanged efflux of lactate dehydrogenase during the release of catecholamines induced by Na^{+} omission also indicates that there was no cell damage during perfusion with the Na^{+}-free medium. While this thesis was in preparation, a study was published on the effect of Na^{+} deprivation on the content of catecholamines of cat's spleen slices (García & Kirpekar, 1973). The experimental conditions used in these experiments did not allow, as they did in our case, the study of the immediate effect of Na^{+} deprivation on the release of catecholamines. Furthermore, contrary to our findings, 25 mM-Mg^{2+} did not block the loss of catecholamines from the spleen slices during incubation in Na^{+}-free medium. The authors' conclusion was that prolonged exposure to Na^{+}-free solution might cause damage to the storage of noradrenaline in sympathetic nerves. However, they did not measure either any cytoplasmic marker, as for example, lactate dehydrogenase, or any other soluble components of the storage vesicles in order to determine if the loss of amines from the incubated slices was due to release by

exocytosis, which is the case for the adrenal medulla, as our results indicate.

F. General inferences

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

on an increased entry of Ca^{2+}. If this is true, experiments like those shown in Fig. 8 may be interpreted as catecholamines released from the same cells, but whose release is triggered by two different mechanisms of increasing Ca^{2+} levels, rather than catecholamines released from different cells or different catecholamine pools.

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

SUMMARY

SOMMAIRE

SUMMARY

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

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

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

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

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

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

7. In the presence or the absence of extracellular Ca^{2+}, the increase in the output of catecholamines induced by Na^+ deprivation

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

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

SOMMAIRE

EFFET

DES IONS SODIUM ET CALCIUM SUR LA SÉCRÉTION DES CATÉCHOLAMINES PAR LA MÉDULLOSURRÉNALE: LA PRIVATION DE SODIUM ENTRAÎNE LA SÉCRÉTION PAR EXOCYTOSE EN L'ABSENCE DE CALCIUM EXTRACELLULAIRE

1. Si l'on effectue une perfusion dans les surrénales d'un bovin à l'aide d'une solution de Locke désodée, pendant 15 à 40 mn, on ne modifie pas l'accroissement de la sécrétion des catécholamines au niveau des glandes stimulées par l'acétylcholine. Cependant, après 80 à 100 mn de perfusion avec une solution désodée, la réaction à la stimulation de l'acétylcholine se trouve diminuée ou même supprimée.

2. Immédiatement après avoir remplacé le liquide de perfusion par une solution désodée, on a constaté un fort accroissement dans la production de catécholamines (6 à 10 fois par rapport aux chiffres témoins).

3. Le remplacement graduel de Na^+ dans le liquide de perfusion augmente la sécrétion des catécholamines. Cette augmentation est fonction linéaire du logarithme de la concentration sodique extracellulaire.

4. La sécrétion de catécholamines en l'absence de Na^+ n'a pas été diminuée par la présence d'atropine et d'hexaméthonium ni par l'omission de Ca^{2+} en présence d'E.D.T.A. ou d'E.G.T.A.

5. L'excès de Mg^{2+} dans le liquide de perfusion a réduit (10 mmol - Mg^{2+}) ou inhibé (20 mmol - Mg^{2+}) l'accroissement de la production de catécholamines entraîné par la privation de Na^+ en présence ou en l'absence de Ca^{2+} extracellulaire.

6. La privation de Na^+ a provoqué une sécrétion de catécholamines, pendant la perfusion des glandes, avec dépolarisation des concentrations (56 mmol) de K^+.

7. En présence ou en l'absence de Ca^{2+} extracellulaire, l'accroissement de la production de catécholamines, entraîné par la privation de Na^+ a été accompagné d'une plus forte sécrétion de la dopamine β-hydroxylase mais pas de la déshydrogénase lactique. En outre, pendant la perfusion avec la solution désodée, la privation de Na^+ a provoqué un accroissement concomitant des sécrétions de catécholamines et de triphosphate d'adénosine.

...

2.

8. Les rapports catécholamines/dopamine β-hydroxylase et catécholamines/triphosphate d'adénosine - déterminés d'après les prélèvements effectués sur les glandes pendant la perfusion à l'aide de la solution désodée - étaient semblables à ceux que l'on trouva dans le contenu soluble des granules de chromaffine isolés. Du point de vue biochimique, ces résultats prouvent que la sécrétion des catécholamines, dans les cas de privation sodique, s'effectue par exocytose.

BIBLIOGRAPHY

Aldrich, T.B. (1901). A preliminary report on the active principle of the suprarenal gland. Amer. J. Physiol., 5: 457-461.

Anton, A.H., and Sayre, D.F. (1962). A study of the factors affecting the aluminum oxide trihydroxyindole procedure for the analysis of catecholamines. J. Pharmacol. Exp. Ther., 138; 360-371.

Axelrod, J. (1957). O-Methylation of epinephrine and other catechols in vitro and in vivo. Science, 126: 400-401.

Axelrod, J. (1959). Metabolism of epinephrine and other sympathomimetic amines. Physiol. Rev., 39: 75-76.

Axelrod, J. (1959). The metabolism of catecholamines in vitro and in vivo. Pharmacol. Rev., II: 402-408.

Axelrod, J. (1960). Adrenergic Mechanisms (J.R. Vane, G.E.W. Wolstenholme and M. O'Conn r, eds.), Little, Brown, Boston, Mass., 1960, pp. 28-30.

Axelrod, J. (1962). Purification and properties of phenylethanolamine-N-methyl transferase. J. Biol. Chem., 237: 1657.

Axelrod, J., and Tomchik, R. (1958). Enzymatic O-methylation of epinephrine and other catechols. J. Biol. Chem., 233: 702-705.

Axelrod, J., Senoh, S., and Witkop, B.B. (1958). O-Methylation of catecholamines in vivo. J. Biol. Chem., 233: 697-701.

Baker, P.F. (1970). Sodium-calcium exchange across the nerve cell membrane. In, Calcium and Cellular Function. ed. Cuthbert, A.W. Macmillan and Co. Ltd., Great Britain. pp. 96-107.

Baker, P.F. (1972). Transport and metabolism of calcium ions in nerve. Progr. Biophys. Mol. Biol., 24: 177.

Baker, P.F. (1974). Excitation-secretion coupling. In, Recent advances in physiology. ed. Linden, R.J. p. 51

Baker, P.F., and Reuter, H. (1975). Calcium Movement in Excitable Cells. Pergamon Press, London.

Banks, P. (1965). Effects of stimulation by carbachol on the metabolism of the bovine adrenal medulla. Biochem. J., 97: 555.

Banks, P. (1966). An interaction between chromaffin granules and calcium ions. Biochem. J., 101: 182.

Banks, P. (1967). The effect of ouabain on the secretion of catecholamines and on the intracellular concentration of potassium. J. Physiol., 193: 631-637.

Banks, P. (1970). Involvement of calcium in the secretion of catecholamines. In, Calcium and Cellular Function. Ed. Cuthbert, A.W. Macmillan and Co. Ltd., Great Britain. pp. 148-162.

Banks, P., Biggins, R., and Bishop, R. (1969a). Sodium ions and the secretion of catecholamines. J. Physiol. (Lond.), 200: 797-805.

Banks, P., Biggins, R., and Bishop, R. (1969b). Monovalent cations and the secretion of catecholamines. J. Physiol. (Lond.), 201: 47P-48P.

Banks, P., and Helle, K. (1965). The release of protein from the stimulated adrenal medulla. Biochem. J., 97: 40C-41C.

Benedeczky, I., Puppi, A., Tigyi, A., and Lissak, K. (1966). Various cell types in the adrenal medulla. Nature, 209: 592-594.

Bergström, S., Evler, U.S.V., and Hamberg, U. Isolation of nor-adrenaline from the adrenal gland. Acta Chem. Scand., 3: 305.

Biedl, A., and Wiesel, J. Ueber die functionelle Dedentung der Nebenorgane des Sympathicus (Inckerkandl) und der Chromaffin Zellgruppen. Pflug. Arch. ges. Physiol., 91: 434-61.

Birks, R.I., and Cohen, M.W. (1968a). The action of Na-pump inhibitors on neuromuscular transmission. Proc. Roy. Soc. (Biol.), 170: 381-399.

Birks, R.I., and Cohen, M.W. (1968b). The influence of internal sodium on the behaviour of motor nerve endings. Proc. Roy. Soc. (Biol.), 170: 401-421.

Blaschko, H. (1939). The specific action of l-dopa decarboxylase. J. Physiol., (Lond), 96: 50P-51P.

Blaschko, H. (1952). Amine oxidase and amine metabolism. Pharmacol. Rev., 4: 415-458.

Blaschko, H., Comline, R.S., Schneider, F.H., Silver, M., and Smith, A.D. (1967a). Secretion of chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. Nature (Lond.), 215: 58-59.

Blaschko, H., Firemark, H., and Smith, A.H. (1967b). Lipids of the adrenal medulla. Lysolecithin, a characteristic constituent of chromaffin granules. Biochem. J., 104: 545-549.

Blaschko, H., Richter, D., and Schlossmann, H. (1937). The oxidation of adrenaline and other amines. Biochem. J., 31: 2187-2196.

Blaschko, H., and Welch, A.D. (1953). Localization of adrenaline in cytoplasmic particles of the bovine adrenal medulla. Naunyn-Schmiedebergs Arch. Pharmacol. Exp. Pathol., 219: 17.

Blaustein, M.P., and Hodgkin, A.L. (1969). The effect oc cyanide on the efflux of calcium from squid axons. J. Physiol. (Lond.), 200: 497-527.

Brundin, T. (1965). Catecholamines in adrenals from fetal rabbits. Acta Physiol. Scand., 63: 509-510.

Bülbring, E. (1949). The methylation of noradrenaline by minced suprarenal tissue. Brit. J. Pharmacol., 4: 234-244.

Bülbring, E., and Burn, J.H. (1949). Liberation of noradrenaline from the suprarenal gland. Brit. J. Pharmacol., 4: 202-208.

Bygdeman, S., Enler, U.S.V., and Hokfelt, B. (1960). Resynthesis of adrenaline in the rabbit's adrenal medulla during insulin-induced hypoglycemia. Acta Physiol. Scand., 49: 21-28.

Cannon, W.B. (1931). Studies on the conditions of activity in endocrine organs. XXVII. Evidnece that medulli adrenal secretion is not continuous. Amer. J. Physiol., 98: 447-453.

Cannon, W.B., and Rosenblueth, A. (1937). Autonomic neuro-effector systems. New York: Macmillan.

Cochrane, D.E., and Douglas, W.W. (1974). Calcium-induced extrusion of secretory granules (exocytosis) in mast cells exposed to 48-80 or the ionophores. Proc. Natl. Acad. Sci. (USA), 194.

Coupland, R.E. (1953). On the morphology and adrenaline-noradrenaline content of chromaffin tissue. J. Endocrinol., 9: 194.

Coupland, R.E. (1956). The development and fate of the abdominal chromaffin tissue in the rabbit. J. Anat. Lond., 90: 527-537.

Coupland, R.E. (1962). Nerve endings on chromaffin cells in the rat adrenal medulla. Nature (Lond.), 194: 310.

Coupland, R.E. (1963). The innervation of the rat adrenal medulla. An electron microscopic study. J. Anat., 97: 141.

Coupland, R.E. (1965). The Natural History of the Chromaffin Cell. Longmans,

Coupland, R.E. (1965). Electron microscopic observations on the structure of the rat adrenal medulla. I. The ultrastructure and organization of chromaffin cells in the normal adrenal medulla. J. Anat., 99: 231.

Coupland, R.E., Pyper, A.S., and Hopwood, D. (1964). A method for differentiating between noradrenaline and adrenaline storing cells with light and electron microscope. Nature (Lond.), 201: 1240.

Da Prada, M., and Pletscher, A. (1970). Synthesis and storage of nucleotides in blood platelets. Life Sci., II-9: 1271-1282.

De Robertis, E.D.P., and Vaz Ferreira, A. (1957). A multivescular catecholamine-containing body of the adrenal medulla of the rabbit. Exp. Cell. Res., 12: 575-578.

De Robertis, E.D.P., and Vaz Ferreira, A. (1957). Electron microscopic study of the excretion of catechol-containing droplets in the adrenal medulla. Exp. Cell. Res., 12: 568-574.

Del Castillo, J., and Katz, B. (1954). Changes in end-plate activity produced by pre-synaptic polarization. J. Physiol., 124: 586-604.

Del Castillo, J., and Katz, B. (1955). On localization of acetylcholine receptor. J. Physiol., 128: 157-181.

Diner, O. (1967). L'expulsion des granules de la medullosurrenale chez le hamster. C.R. Acad. Sci. (Paris), 265: 616-619.

Douglas, W.W. (1968). Stimulus-secretion coupling. The concept and clues from chromaffin and other cells. Brit. J. Pharmacol., 34: 457-474.

Douglas, W.W., and Kanno, T. (1967). The effect of amethocaine on acetylcholine-induced depolarization in the adrenal chromaffin cell. Brit. J. Pharmacol. Chemother., 30: 612-619.

Douglas, W.W., Kanno, T., and Sampson, S.R. (1967a). Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: An analysis employing techniques of tissue culture. J. Physiol., 188: 107.

Douglas, W.W., Kanno, T., and Sampson, S.R. (1967). Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and on the depolarizing effect of acetylcholine. J. Physiol., 191: 107-121.

Douglas, W.W., and Poisner, A.M. (1962). On hhe mode of action of acetylcholine in evoking adrenal medullary secretion: increased uptake of calcium during the secretory response. J. Physiol., 162: 385-392.

Douglas, W.W., and Poisner, A.M. (1963). The influence of calcium on the secretory response of the submaxillary gland to acetylcholine or to noradrenaline. J. Physiol. (Lond.), 165: 528-541.

Douglas, W.W., and Poisner, A.M. (1965). Preferential release of adrenaline from the adrenal medulla by muscarine and pilocarpine. Nature, 208: 1102.

Douglas, W.W., and Poisner, A.M. (1966a). Evidence that the secreting adrenal chromaffin cell releases catecholamines directly from ATP-rich granules. J. Physiol., 183: 236-248.

Douglas, W.W., and Poisner, A.M. (1966b). On the relation between ATP splitting and secretion in the adrenal chromaffin cell: extrusion of ATP (unhydrolysed) during release of catecholamines. J. Physiol., 183: 249-256.

Douglas, W.W., and Rubin, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol., 159: 40-57.

Douglas, W.W., and Rubin, R.P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J. Physiol., 167: 288-310.

Douglas, W.W., and Rubin, R.P. (1964). Stimulant action of barium on the adrenal medulla. Nature (Lond.), 203: 305-307.

Dreyer, G.P. (1899). On secretory nerves to the suprarenal capsules. Amer. J. Physiol., 2: 203-219.

Duch, D.S., Viveros, O.H., and Kirshner, N. (1968). Endogenous inhibition on the adrenal medulla of dopamine β-hydroxylase. Biochem. Pharmacol., 17: 255-264.

Eccles, J.C. (1964). The Physiology of Synapses. Springer-Verlag. Berlin.

Elliott, T.R. (1904). On the action of adrenalin. J. Physiol. (Lond), 31: XX-XXI.

Elliott, T.R. (1905). The action of adrenalin. J. Physiol., 32: 401-467.

Eränkö, O. (1955a). Distribution of adrenaline and noradrenaline in the adrenal medulla. Nature (Lond.), 175: 88-89.

Eränkö, O. (1955b). Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. Acta Endocrinol. Copenhagen, 18: 174.

Euler, U.S.V. (1956). Noradrenaline. Thomas, Springfield, Ill.

Euler, U.S.V. (1967). Reuptake and net uptake of nor-adrenaline in adrenergic nerve granules with a note on the affinity for l- and d-isomers. Acta Physiol. Scand., 71: 151-162.

Euler, U.S.V., and Hamberg, U. (1949). l-Noradrenaline in the suprarenal medulla. Nature (Lond.), 163: 642-643.

Euler, U.S.V., and Luft, R. (1952). Effect of insulin on urinary excretion of adrenaline and noradrenaline. Metabolism, 1: 528-532.

Flatmark, T., and Terland, O. (1971). Cytochrome b561 of the bovine adrenal chromaffin granules. A high potential b-type cytochrome. Biochim. Biophys. Acta, 253: 487-491.

Folkow, B., and Euler, U.S.V. (1954). Selective activation of noradrenaline and adrenaline producing cells in the cat's adrenal gland by hypothalamic stimulation. Circ. Res., 2: 191-195.

Friedman, S., and Kaufman, S. (1965). 3,4-Dihydroxyphenylethylamine β-hydroxylase: a copper protein. J. Biol. Chem., 240: PC552-554.

García, A.G., and Kirpekar, S.M. (1973). Release of sodium from the cat spleen by Na^+ deprivation. Brit. J. Pharmacol., 47: 729-747.

Goldstein, M., Fuxe, K., and Hökfelt, T. (1972). Characterization and tissue localization of catecholamine synthesizing enzymes. Pharmacol. Rev., 24: 293-309.

Goldstein, M., Gang, H., and Joh, T.H. (1969). Subcellular distribution and properties of phenylethanolamine-N-methyl transferase (PNMT). Fed. Proc., 28: 287.

Goldstein, M., Lauber, E., and McKereghan, M.R. (1965). Studies on the purification and characterization of 3,4-dihydroxyphenylethylamine-hydroxylase. J. Biol. Chem., 240: 2066.

Goodall, McC. (1951). Studies of adrenaline and noradrenaline in mammalian heart and suprarenals. Acta Physiol. Scand., 24: Suppl. 85.

Goodall, McC., and Kirshner, N. (1957). Biosynthesis of adrenaline and noradrenaline in vitro. J. Biol. Chem., 226: 213-221.

Goodall, McC., and Kirshner, N. (1958). Biosynthesis of epinephrine and norepinephrine by sympathetic nerves and ganglia. Circ., 17: 366-371.

Gordon, R., Spector, S., Sjoerdsma, A., and Udenfriend, S. (1966). Increased synthesis of norepinephrine and epinephrine in the intact rat during exercise and exposure to cold. J. Pharmacol. Exp. Ther., 153: 440-447.

Greer, C.M., Pinkston, J.O., Baxter, J.R., and Brannon, E.S. (1938). Norepinephrine (β-3,4-dihydroxyphenyl-β-hydroxyethylamine) as a possible mediator in the sympathetic division of the autonomic nervous system. J. Pharmacol., 62: 189-227.

Gurins, E., and Delluva, A.M. (1947). The biological synthesis of radioactive adrenaline from phenylalanine. J. Biol. Chem., 170: 545-550.

Harrison, R.G., and Hoey, M.J. (1960). Adrenal Circulation. Blackwell Scientific Publications. Oxford.

Hillarp, N.Å. (1958). Enzymic synthesis involving adenosinephosphates in the adrenaline and noradrenaline containing granules of adrenal medulla. Acta Physiol. Scand., 42: 144-185.

Hillarp, N.Å. (1958). Isolation and some biochemical properties of the catecholamine granules in the cow adrenal medulla. Acta Physiol. Scand., 43: 82-96.

Hillarp, N.Å. (1958). The release of catecholamines from the amine containing granules of the adrenal medulla. Acta Physiol. Scand., 43: 292-302.

Hillarp, N.Å., Hokfelt, B. (1953). Evidence of adrenaline and noradrenaline in separate adrenal medullary cells. Acta Physiol. Scand., 30: 55-68.

Hillarp, N.Å., and Thieme, G. (1959). Nucleotides in the catecholamine granules of the adrenal medulla. Acta Physiol. Scand., 45: 328.

Holtz, P. (1939). Dopadecarboxylase. Naturwissenschaften, 27: 724-725.

Holtz, P., Credner, K., and Kroneberg, G. (1947). Uber das sympathico-mimetische pressorische Prinzip des Harns ('Urosympathin'). Naunyn-Schmiedeberg's Arch exp. Path. Pharmak., 204: 228-243.

Holtzbauer, M., and Vogt, M. (1954). The concentration of adrenaline in the peripheral blood during insulin hypoglycemia. Brit. J. Pharmacol., 9: 249-252.

Hörtnagl, H., Winkler, H., and Lochs, H. (1972). Membrane proteins of chromaffin granules, dopamine β-hydroxylase, a major constituent. Biochem. J., 129: 187-195.

Houssay, B.A., and Molinelli, E.A. (1925). Centre adrénalino-sécréteur hypothalamique. C.R. Sciences Soc. Biol. (Paris), 93: 1454.

Houssay, B.A., Wassermann, G.F., and Tramezzani, J.H. (1962). Formation et secretion differentialles d'adrenaline et de noradrenaline surrenales. Arch. int. Pharmacodyn., 140: 84.

Iversen, L.L. (1965). The uptake of catecholamines at high perfusion concentrations in the rat isolated heart: a novel catecholamine uptake process. Brit. J. Pharmacol., 25: 18-33.

Kalsner, S., and Nickerson, M. (1969b). Effects of reserpine on the disposition of sympathomimetic amines in vascular tissue. Brit. J. Pharmacol., 35: 394-405.

Kano, M. (1959). Electron microscopic study on the adrenal medulla of domestic fowl. Arch. Hist. Jap., 18: 25-26.

Kanno, T., Cochrane, D.E., and Douglas, W.W. (1973). Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. Can. J. Physiol. Pharmacol., 51: 12P-1001.

Keynes, R.D., and Swan, R.C. (1959). The permeability of frog muscle to lithium ions. J. Physiol., 147: 626-638.

Kirshner, N. (1957). Pathway of noradrenaline formation from dopa. J. Biol. Chem., 226: 821-825.

Kirshner, N. (1962). Uptake of catecholamines by a particulate fraction of the adrenal medulla. J. Biol. Chem., 237: 2311-2317.

Kirshner, N. (1974). Molecular organization of the chromaffin vesicles of the adrenal medulla. Advances in Cytopharmacology, Vol. 2. eds. Ceccarelli, B., Clementi, F., and Meldolesi, J. Raven Press, New York.

Kirshner, N., and Goodall, McC. (1957). The formation of adrenaline from noradrenaline. Biochim. Biophys. Acta, 24: 658.

Kirshner, N., Holloway, C., Smith, W.J., and Kirshner, A.G. (1966). In, Mechanisms of Release of Biogenic Amines. eds. von Euler, U.S., Rosell, S., and Uvnas, B. Pergamon Press, Oxford. pp. 109-123.

Kirshner, N., Sage, H.J., and Smith, W.J. (1967). Mechanism of secretion from the adrenal medulla. II. Release of catecholamines and storage vesicle protein in response to chemical stimulation. Mol. Pharmacol., 3: 254.

Kirshner, N., Sage, H.J., Smith, W.J., and Kirshner, A.G. (1967). Mechanism of secretion from the adrenal medulla. 2. Release of catecholamines and storage vesicle protein in response to chemical stimulation. Mol. Pharmacol., 3: 254-265.

Kohn, A. (1902). Das Chromaffine Gewebe. Ergebn. Anat. Entwgesch., 12: 253-348.

Kopin, I.J., and Gordon, E.K. (1963). Metabolism of administered and drug-released norepinephrine-7-H^3 in the rat. J. Pharmacol., 140: 207-216.

Lacy, P.E., Howell, S.L., Young, D.A., and Fink, C.J. (1968). New hypothesis of insulin secretion. Nature, 219: 1177.

Lastowecka, A., and Trifaro, J.M. (1974). The effect of sodium and calcium ions on the release of catecholamines from the adrenal medulla: Sodium deprivation induces release by exocytosis in the absence of extracellular calcium. J. Physiol., 236: 681-705.

Lee, F.L., and Trendelenburg, U. (1967). Muscarinic transmission of preganglionic impulses to the adrenal medulla of the cat. J. Pharmacol. Exp. Ther., 158: 73-79.

Lever, J.D. (1955). Electron microscopic observations on the normal and denervated adrenal medulla of the rat. Endocrinology, 57: 621.

Levin, E.Y., Levenberg, B., and Kaufman, S. (1960). The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. J. Biol. Chem., 235: 2080.

Levitt, M., Spector, S., Sjoerdsma, A., and Udenfriend, S. (1965). Elucidation of the rate-limiting step in norepinephrine biosynthesis in the perfused guinea-pig heart. J. Pharmacol. Exp. Ther., 148: 1-8.

Lifson, N., and Visscher, M.B. (1944). Osmosis in living systems. M. Physics, pp. 869-892.

Lovenberg, W., Weissbach, H., and Udenfriend, S. (1960). Aromatic L-amino acid decarboxylase. J. Biol. Chem., 237: 89.

Magoun, W.H., Ranson, B.W., and Hetherington, A. (1937). The liberation of adrenalin and sympathin induced by stimulation of the hypothalamus. Amer. J. Physiol., 119: 615.

Maizles, M. (1954). Cation transport in chicken erythrocytes. J. Physiol., 125: 263-277.

Malmejac, J. (1955). Action of adrenaline on synaptic transmission and on adrenal medullary secretion. J. Physiol. (Lond.), 130: 497-512.

Matthews, E.K., Evans, R.J., and Dean, P.M. (1972). The ionogenic nature of the secretory-granule membrane. Biochem. J., 130: 825.

Maycock, W.D.A., and Heslop. T.S. (1939). An experimental investigation of the nerve supply of the adrenal medulla of the cat. J. Anat., 73: 551.

Miledi, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. Proc. R. Soc. Lond. (Biol.), 183: 421-425.

Muller, T.W., and Kirshner, N. (1975). ATPase and phosphatidylinositol kinase activities of adrenal chromaffin vesicles. J. Neurochem., 24: 1155-1161.

Musacchio, J.M., Bhagat, B., and Jackson, C.J. (1966). The effect of disulfiram on the restoration of the response to tyramine by dopamine and α-methyl-dopa in the reserpine-treated cat. J. Pharmacol. Exp. Ther., 152: 2937.

Nagatsu, T., Levitt, M., and Udenfriend, S. (1964). Tyrosine hydroxylase: The initial step in norepinephrine biosynthesis. J. Biol. Chem., 239: 2910.

Oliver, G., and Schäfer, E.A. (1894). On the physiological action of extract of the suprarenal capsules. J. Physiol., 16: i-ivP.

Paton, W.D., Vizi, E.S., and Zar, M.A. (1971). The mechanism of acetylcholine release from parasympathetic nerves. J. Physiol. (Lond.), 215: 731-746.

Pohorecky, L.A., and Rust, J.H. (1968). Studies on the cortical control of the adrenal medulla in the rat. J. Pharmacol. Exp. Ther., 162: 227.

Poisner, A.M., and Douglas, W.W. (1966). The need for calcium in adrenomedullary secretion evoked by biogenic amines, polypeptides, and muscurinic agents. Proc. Soc. Exp. Biol. (NY), 123: 62-64.

Poisner, A.M., and Hava, M. (1970). The role of ATP and ATPase in the release of catecholamines from the adrenal medulla: 4. ATP-activated uptake of calcium by microsomes and mitochondria. Molec. Pharmacol., 6: 407-415.

Poisner, A.M., and Trifaro, J.M. (1967). The role of ATP and ATPase in the release of catecholamines from the adrenal medulla: 1. ATP-evoked release of catecholamines, ATP, and protein from isolated chromaffin granules. Molec. Pharmacol., 3: 561-571.

Poisner, A.M., Trifaro, J.M., and Douglas, W.W. (1967). The fate of the chromaffin granule during catecholamine release from the adrenal medulla: II. Loss of protein and retention of lipid in subcellular fraction. Biochem. Pharmacol., 16: 2101-2108.

Poste, G., and Allison, A.C. (1971). Membrane fusion reaction: A theory. J. Theoret. Biol., 32: 165.

Reuter, H. (1974). The exchange of calcium ions in the mammalian myocardium. Circ. Res., 34: 599.

Rubin, R.P. (1970). The role of energy metabolism in calcium-evoked secretion from the adrenal medulla. J. Physiol. (Lond.), 206: 181-192.

Rubin, R.P. (1974). Calcium and the secretory process. Plenum Press, New York and London.

Rubin, R.P., Feinstein, M.B., Jaanus, S.D., and Paimre, M. (1967). Inhibition of catecholamine secretion and calcium exchange in perfused cat adrenal glands by tetracaine and magnesium. J. Pharmacol. Exp. Ther., 155: 125-137.

Sandow, A. (1952). Excitation-contraction coupling in muscular response. Yale J. Biol. Med., 25: 176.

Schmidt, F.O. (1968). Fibrous proteins - neuronal organelles. Proc. Natl. Acad. Sci., 60: 1092.

Schneider, F.H., Smith, A.D., and Winkler, H. (1967). Secretion from the adrenal medulla: biochemical evidence for exocytosis. Brit. J. Pharmacol. Chemother., 31: 94-104.

Schumann, H.J., and Phillipu, A. (1962b). Release of catechol amines from isolated medullary granules by sympathomimetic amines. Nature (Lond.), 193: 890-891.

Shepherd, D.M., and West, G.B. (1951). Noradrenaline and the suprarenal medulla. Brit. J. Pharmacol. Chemother., 6: 655.

Smith, W.J., and Kirshner, N. (1967). A specific soluble protein from the catecholamine storage vesicles of bovine adrenal medulla. I. Purification and chemical characterization. Molec. Pharmacol., 3: 52-62.

Smith, A.D., and Winkler, H. (1967). Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. Biochem. J., 103: 483-492.

Smith, A.D., and Winkler, H. (1967). A simple method for the isolation of adrenal chromaffin granules on a large scale. Biochem. J., 103: 480-482.

Smith, A.D., and Winkler, H. (1972). Fundamental mechanism in the release of catecholamines. In, Handbook of Experimental Pharmacology, Vol. 23, eds. Blaschko, H., and Muschell, E. New York: Springer-Verlag. pp. 538-617.

Soulié, A.H. (1903). Recherches sur le développement des capsules surrénales chez les vertébrés supérieurs. J. Anat., Paris, 39: 197-293.

Spector, S., Zaltzman-Nirenberg, P., Levitt, M., and Udenfriend, S. (1963). Norepinephrine synthesis from tyrosine-C^{14} in isolated perfused guinea pig heart. Science, 139: 1299-1301.

Spiro, M.J., and Ball, E.G. (1961). Studies on the respiratory enzymes of the adrenal gland. I. The medulla. J. Biol. Chem., 236: 231-235.

Stjärne, L. (1966). Studies of noradrenaline biosynthesis in nerve tissue. Acta Physiol. Scand., 67: 441-454.

Stjärne, L., and Lishajko, F. (1966). Comparison of spontaneous loss of catecholamines and ATP in vitro from isolated bovine adrenomedullary, vesicular gland, vas deferens, and splenic nerve granules. J. Neurochem., 13: 1213-1216.

Strehler, B. (1963). Adenosine-5'-triphosphate and creatine phosphate. Determination with luciferase. In, Methods of Enzymatic Analysis. eds. Bergmeyer, A.V. Verlag Chemie, G.M.B.H., and Academic Press, New York, pp. 559-567.

Takamine, J. (1901). Adrenaline, the active principle of the suprarenal glands and its mode of preparation. Amer. J. Pharm., 73: 523-531.

Taugner, G. (1971). The membrane of catecholamine storage vesicles of adrenal medulla, catecholamine fluxes and ATPase activity. Naunyn-Schmiedeberg's Arch. Pharmakol., 270: 392-406.

Taugner, G. (1972). The membrane of catecholamine storage vesicles of adrenal medulla, uptake and release of noradrenaline in relation to the pH and the concentration and steric configuration of the amine present in the medium. Naunyn-Schmiedeberg's Arch. Pharmacol., 274 : 299-314.

Thoenen, H., Mueller, R.A., and Axelrod, J. (1969a). Increased tyrosine hydroxylase activity after drug induced alteration of sympathetic transmission. Nature (Lond.), 221: 1264.

Thoenen, H., Mueller, R.A., and Axelrod, J. (1969b). Transynaptic induction of adrenal tyrosine hydroxylase. J. Pharmacol. Exp. Ther., 169: 249-254.

Trifaró, J.M. (1970). The secretory process of the adrenal medulla. Endocrinol. Exp., 4: 225-251.

Trifaró, J.M. (1973). Phospholipids of the chromaffin granule membrane and catecholamine release. In, Frontiers in Catecholamine Research. Pergamon Press, Great Britain. pp. 501-503.

Trifaró, J.M., and Dworkind, J. (1970). A new and simple method for isolation of adrenal chromaffin granules by means of an isotonic density gradient. Anal. Biochem., 34: 403-412.

Trifaró, J.M., and Dworkind, J. (1971). Phosphorylation of membrane components of adrenal chromaffin granules by adenosine triphosphate. Molec. Pharmacol., 7: 52-65.

Trifaró, J.M., and Dworkind, J. (1975). Phosphorylation of the membrane components of chromaffin granules: Synthesis of diphosphatidylinositol and presence of phosphatidylinositol kinase in granule membranes. Can. J. Physiol. Pharmacol., 53: 479-492.

Trifaró, J.M., and Poisner, A.M. (1967). The role of ATP and ATPase in the release of catecholamines from the adrenal medulla: 2. ATP-evoked fall in optical density of isolated chromaffin granules. Molec. Pharmacol., 3: 572-580.

Trifaro, J.M., Poisner, A.M., and Douglas, W.W. (1967). The fate of the chromaffin granule during catecholamine release from the adrenal medulla. I. Unchanged efflux of phospholipid and cholesterol. Biochem. Pharmacol., 16: 2095-2100.

Udenfriend, S. (1966). Biosynthesis of the sympathetic neurotransmitter, norepinephrine. Harvey Lect., 60: 57-83.

Udenfriend, S., Cooper, J.R., Clark, C.T., and Baer, J.E. (1953). Rate of turnover of epinephrine in the adrenal medulla. Science, 117: 663-665.

Udenfriend, S., and Wyngaarden, J.B. (1956). Precursors of adrenal epinephrine and norepinephrine in vivo. Biochim. Biophys. Acta., 20: 48-52.

Viveros, O.H., Arqueros, L., Connett, R.J., and Kirshner, N. (1969). Mechanism of secretion from the adrenal medulla: III. Studies of dopamine β-hydroxylase as a marker for catecholamine storage vesicle membranes in rabbit adrenal glands. Molec. Pharmacol., 5: 60-68.

Viveros, O.H., Arqueros, L., and Kirshner, N. (1968). Release of catecholamines and dopamine β-hydroxylase from the adrenal medulla. Life Sci. Oxford, 7: 609-618.

Vizi, E.S. (1972). Stimulation by inhibition of $(Na^+-K^+-Mg^{2+})$-activated ATPase of acetylcholine release in cortical slices from rat brain. J. Physiol., 226: 65-117.

Wassermann, G., and Tremazzani, J.H. (1963). Separate distribution of adrenaline- and noradrenaline-secreting cells in the adrenals of snakes. Gen. Comp. Endocrinol., 3: 480.

Winkler, H., and Hortnagl, H. (1973). In, Third International Catecholamine Symposium. J. Neurochem. (in press).

Winkler, H., Hörtnagl, H., and Asamer, H. (1972). Membrane proteins of catecholamine-storing vesicles in adrenal medulla and sympathetic nerves. Adv. Exp. Med. Biol., 32: 69-81.

Winkler, H., Hörtnagl, H., Hörtnagl, H., and Smith, A.D. (1970). Membranes of adrenal medulla: Behaviour of insoluble proteins of chromaffin granules on gel electrophoresis. Biochem. J., 118: 303-310.

Winkler, H., Hörtnagl, H., and Schöpf, J.A. (1971). Bovine adrenal medulla: Synthesis and secretion of radioactively labelled catecholamines and chromogranins. Arch. Pharmakol., 271: 193-203.

Winkler, H., Schöpf, J.A.L., Hörtnagl, H., and Hörtnagl, H. (1972). Bovine adrenal medulla: Subcellular distribution of newly synthesized catecholamines, nucleotides and chromogranins. N.S. Arch. Pharmacol., 273 : 43-61.

Wróblewski, F., and La Due, J.S. (1955). Lactic dehydrogenrse activity in blood. Proc. Soc. Exp. Biol. Med., 90: 210-213.

Wurtman, R.J. (1965). Catecholamines. Little, Brown & Co., Boston.

Wurtman, R.J., and Axelrod, J. (1965). Adrenaline synthesis: Control by the pituitary gland and adrenal glucocorticoids. Science, 150: 1464.

Wurtman, R.J., and Axelrod, J. (1966). Control of enzymatic synthesis of adrenaline in the adrenal medulla by adrenal cortical steroids. J. Biol. Chem., 241: 2301-2305.

Wurtman, R.J., and Tramezzani, J. (1967). Distribution of the adrenaline forming enzyme in the adrenal gland of a snake - Xenodon merremii. Nature (Lond.), 215: 879-880.

Young, J.Z. (1939). Partial degeneration of the nerve supply of the adrenal. A study in autonomic innervation. J. Anat., 73: 540.

Yntema, C.L., and Hammond, W.S. (1947). The development of the autonomic nervous system. Biol. Rev., 22: 344-359.

Zuckerkandl, E. (1912). The development of the chromaffin organs and of the suprarenal glands. Keibel and Mall's Manual of Human Embryology. U.S.A. Lippincott, 2: 157-179.