PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Chromogranin A, Dopamine <latex>β</latex>-Hydroxylase and Secretion from the Adrenal Medulla

N. Kirshner and A. G. Kirshner

Phil. Trans. R. Soc. Lond. B 1971 **261**, 279-289
doi: 10.1098/rstb.1971.0057

Phil. Trans. Roy. Soc. Lond. B. **261**, 279–289 (1971) [279]
Printed in Great Britain

Chromogranin A, dopamine β-hydroxylase and secretion from the adrenal medulla

By N. Kirshner and A. G. Kirshner
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, U.S.A.

Studies of the biosynthesis, storage and secretion of catecholamines by the adrenal medulla have served as models for similar studies of the adrenergic neuron. For example, the synthesis of noradrenaline and the intracellular distribution of the biosynthetic enzymes was first described in the adrenal medulla and subsequently shown to be the same in sympathetic nerves (Blaschko 1939; Kirshner 1957, 1959; Levin, Levenberg & Kaufman 1960; Potter & Axelrod 1963; Nagatsu, Levitt & Udenfriend 1964; Stjärne & Lishajko 1966; Oka *et al.* 1967; Musacchio 1968; Laduron & Belpaire 1968). The storage vesicles of the adrenal medulla have counterparts in the synaptic vesicles (Blaschko & Welch 1953; Hillarp, Lagerstedt & Nilson 1953; von Euler & Hillarp 1956; Schümann 1958) and the incorporation of catecholamines into the storage vesicles, and the storage complex itself, seems to be similar in both tissues, (Kirshner 1962; Carlsson, Hillarp & Waldeck 1963; von Euler & Lishajko 1963; von Euler, Lishajko & Stjärne 1963; Stjärne 1964). Recently it has been demonstrated that proteins specifically localized in the storage vesicles of the adrenal medulla are also present in the storage vesicles of sympathetic nerve endings (Hopwood 1967, 1968; Geffen, Livett & Rush 1969; Banks, Helle & Major 1969; de Potter, de Schaepdryver, Moerman & Smith 1969). There are obvious differences between the two types of vesicles (Stjärne 1964; Potter 1967), but the similarities are such as to suggest that the vesicles from both tissues serve the same physiological functions—to synthesize and store adrenaline or noradrenaline and to release these compounds in response to neural stimulation.

Secretion from the adrenal medulla appears to be a good model for release of neurotransmitters at synapses in the sense that it provides and suggests experimental approaches to the problem (Geffen *et al.* 1969; de Potter *et al.* 1969). In general, the secretion of substances which are synthesized in cells and stored in subcellular organelles have many features in common (Douglas 1968; Stormorken 1969) and release of neurotransmitters at synapses may be another example of this generalized biological process. During the past few years, evidence has been presented from several laboratories that secretion from the adrenal medulla occurs by exocytosis. The simultaneous release of catecholamines, adenine nucleotides, chromogranins and soluble dopamine β-hydroxylase contained within the storage vesicles and the retention of dopamine-β-hydroxylase firmly bound to the vesicle membrane have provided critical information on this secretory process.

Soluble proteins of the catecholamine storage vesicles

The catecholamines of the adrenal medulla are stored in vesicles which can be isolated by differential centrifugation (Blaschko & Welch 1953; Hillarp *et al.* 1953), millipore filtration

(Oka, Ohuchi, Yoshida & Imaizumi 1966; Poisner & Trifaro 1967) or by density gradient centrifugation (Smith & Winkler 1967*a*; Poisner & Trifaro 1969) after homogenizing the tissue in an isotonic medium. Analysis of the storage vesicles revealed, in addition to catecholamines (0.5 mol/l), the presence of large amounts of adenine nucleotides (mainly ATP) such that the molar ratio of catecholamines:adenine nucleotides is approximately 4:1 (Hillarp 1959; Hillarp & Thieme 1959; Hillarp 1960*a*). The vesicles also contain large amounts of protein (1.8 mg protein/mg catecholamines), most of which is readily solubilized upon lysis of the vesicles in distilled water (Hillarp 1958*a*). Since the isolated vesicles maintained their catecholamine content for 2 to 3 days when kept in 0.30 mol/l sucrose near 0 °C, but readily released their content when the isotonicity was decreased, Hillarp (1958*b*) proposed that the catecholamines, ATP, and protein formed a non-diffusible, isomotically inactive complex. Our initial interests in the soluble proteins were concerned with their role in the storage of catecholamines, and studies were initiated to obtain further information on their physical and chemical properties.

Upon osmotic lysis of the chromaffin vesicles, 77 % of the total protein is found in the soluble fraction (Hillarp 1958*a*). The great majority of these proteins appears to be non-enzymic, although it is now known from work in this laboratory that a soluble dopamine β-hydroxylase (DBO) is also present and will be discussed later. Smith & Winkler (1967*b*) and Schneider, Smith & Winkler (1967) found that on starch gel electrophoresis of the soluble protein fraction there are eight proteins present which have been named the chromogranins by Blaschko *et al.* (1967). About 30 to 40 % of the soluble protein can be isolated as a homogeneous protein—chromogranin A (Helle 1966*a*; Smith & Kirshner 1967; Smith & Winkler 1967*b*) for which no biological or enzymic activity has yet been found.

Some of the chemical and physical properties of chromogranin A have been characterized by Helle (1966*a*), Smith & Winkler (1967*b*) and by our laboratory (Smith & Kirshner 1967; Kirshner & Kirshner 1969). Smith & Winkler (1967*b*) showed that the protein has a molecular mass of 77 390 by the sedimentation diffusion and approach to equilibrium methods. We find a molecular mass of 81 200 by rapid equilibrium osmometry measurements in neutral salt solns.

The amino acid analyses of chromogranin A performed by the laboratory at Oxford and our laboratory are in excellent agreement. The protein is very high in the acidic amino acids, aspartic and glutamic acids account for 32 % of the residues, whereas lysine and arginine account for only 14 %. The proline content of 9 % is also high and their effect is reflected in the optical rotatory dispersion measurements of Smith & Winkler (1967*b*) which indicate the presence of very little α-helical structure. There are four cysteine residues/80 000 molecular mass. The other chromogranins have not been characterized except to the extent that they appear to have amino acid compositions similar to that of chromogranin A (Schneider *et al.* 1967; Smith & Kirshner 1967; Strieder, Ziegler, Winkler & Smith 1968). We also found that when the protein is dissolved in 6 mol/l guanidine hydrochloride 0.1 mol/l mercaptoethanol, it dissociates into two chains of approximately 40 600 molecular mass each as measured by sedimentation equilibrium (Kirshner & Kirshner 1969). This measurement was later verified by us using gel filtration through a 6 % Agarose column equilibrated with 6 mol/l guanidine hydrochloride, 0.1 mol/l mercaptoethanol (Fish, Mann & Tanford 1969). Again, only one major peak was eluted from the column corresponding to a molecular mass of 43 000 $\pm$ 3200. Fingerprints of tryptic digests of the protein indicate that the two chains are identical (Smith & Kirshner 1967).

Chromogranin A, which has an isoelectric pH of approximately 4.5 is most compact in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

structure at low pH (4.9). At a higher pH or at low ionic strength there is partial randomization of certain susceptible regions of the protein, though the protein as a whole maintains considerable structure. Evidence for this may be found in our sedimentation velocity studies (Kirshner & Kirshner 1969) and the intrinsic viscosity studies done at Oxford. At pH 4.9 in 0.3 mol/l NaCl we find that the dependence of the sedimentation coefficient upon protein concentration is not as marked as at neutral pH. K as calculated from

$$1/s = (1 + Kc)/s^0.$$

is 17.4 ml/g. (K for globular proteins is in the range of 5 to 10 ml/g.) The value of 17.4 ml/g is in sharp contrast to the marked concentration dependence of s at neutral pH, where K is 60–74 ml/g. At a low ionic strength, K is as high as 116 ml/g (Smith & Winkler 1967*b*). The value of the intrinsic viscosity of chromogranin A in a solvent of ionic strength 0.015 is 42.4 ml/g but in $I = 0.3$, pH 5.9, the intrinsic viscosity is 18.9 ml/g (Smith & Winkler 1967*b*) and indicates the presence of considerable structure in the protein even at this pH. Although chromogranin A is thought to be involved in the formation of the storage complex within the vesicles, it binds only two to three times as much adrenaline as does serum albumin either in the presence or absence of Mg^{2+} plus ATP. This binding could account for only 1 to 2 % of the total catecholamines within the vesicles (Smith & Kirshner 1967).

Dopamine β-hydroxylase

Early studies of dopamine β-hydroxylase had shown the enzyme to be present in the particulate fraction of the storage vesicles (Kirshner 1957; Levin *et al.* 1960). Friedman & Kaufman (1965) later solubilized and purified the particulate dopamine β-hydroxylase and have reported some of its properties. It is a mixed function oxidase of molecular mass 290000 with Cu in both the Cu^+ and Cu^{2+} form in its prosthetic group. From its molecular mass and its $s_{20,w}$ value in 1 mol/l NaCl of 8.9, they calculate a frictional ratio of approximately 2. More recently it has been found that dopamine β-hydroxylase is also present in the soluble fraction of lysed storage vesicles; this will be discussed later. Preliminary studies on the physical properties of the soluble dopamine β-hydroxylase show it to be similar in size to the particulate enzyme. Its molecular mass in 0.1 mol/l NaCl is 273000 by sedimentation equilibrium measurements. It's $s_{20,w}$ value in this same solvent is 10.3, from which a frictional ratio of about 1.6 can be calculated. In 1 mol/l NaCl the $s_{20,w}$ value drops to 8.6 which is in close agreement to that reported by Friedman & Kaufman (1965), but obviously the increased salt concentration is affecting the protein structure. No information as yet has been obtained about either the amino acid composition or number of subunits of the soluble and particulate dopamine β-hydroxylase.

Secretion from the adrenal medulla

Douglas & Rubin (1961) coined the expression 'stimulus-secretion coupling' to encompass the chain of events initiated by the action of acetylcholine, or other secretagogues, on the adrenal medullary cells and which results in the secretion of adrenaline or noradrenaline. Studies by Douglas and co-workers (see Douglas 1968) demonstrated that Ca^{2+} was an essential link in this process and that 'stimulus-secretion coupling' at secretory organs was similar in several respects to 'excitation-contraction coupling' at the neuromuscular junction.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Four hypotheses, illustrated in figure 1, have been proposed for the secretion of catecholamines by the adrenal gland. (1) Interaction of acetylcholine with the cell membrane in the presence of Ca^{2+} increases the permeability of the 'free' catecholamines which diffuse out of the cell. The concentration of the 'free' pool is maintained by diffusion from the storage vesicles. (2) Stimulation causes a change in the permeability of the storage vesicle membrane resulting in an increase in the concentration of 'free' catecholamines which then diffuse out of the cell. (3) Stimulation results in extrusion of the intact storage vesicle which then disrupts outside of the cell liberating its contents. (4) Stimulation initiates a sequence of events resulting in release

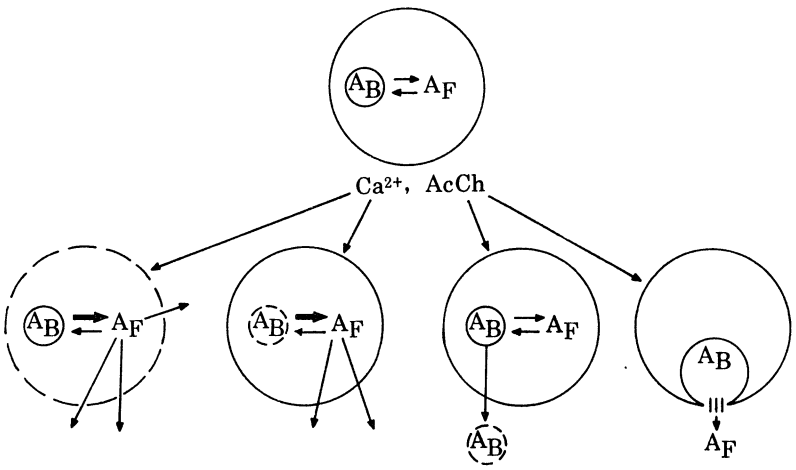

FIGURE 1. Proposed mechanism for secretion from the adrenal medulla.

of the storage vesicle content directly to the exterior of the cell with retention of vesicle membrane within the cell (exocytosis). This may occur by fusion of the vesicle and plasma membranes resulting in a direct opening to the exterior of the cell, or by adhesion of the vesicles to the plasma membrane and consequent changes in the permeability of both the vesicle and plasma membranes such that the vesicle contents can diffuse or be expelled to the exterior.

Early electron microscope studies of the adrenal medulla suggested that secretion occurred by exocytosis. However, photographs depicting storage vesicle membranes fused with plasma membranes were infrequently seen and not clearly interpretable (De Robertis & Vaz Ferreira 1957; Wetzstein 1957). The more recent work of Diner (1967) on the hamster adrenal medulla clearly shows fusion of the vesicle and plasma membranes. Since no statistical studies had been done on the number of vesicles adhering to or fused with plasma membranes from stimulated and unstimulated glands, it was not possible to determine whether the observed structures did indeed represent the process of secretion or whether they were merely random events.

Although most of the catecholamines of the adrenal medulla can be recovered in the storage vesicle fraction, it is generally accepted that there also exists a small pool of 'free' cytoplasmic catecholamines (Hillarp 1960*b*). The size of this 'free' pool in the adrenal medulla is certainly less than 10% of the total amines and probably less than 1%. It had been proposed that this extravesicular pool was the source of the amines released upon stimulation of the adrenal gland (Blaschko & Welch 1953; Schümann 1961). However, studies from several laboratories have shown that the storage vesicles are the immediate source of catecholamines released upon stimulation and that secretion occurs by exocytosis.

Douglas and co-workers (Douglas, Poisner & Rubin 1965; Douglas & Poisner 1966*a*, *b*; see

also Stjärne 1964) found that upon stimulation of perfused cat adrenal glands, adenine nucleotides and their breakdown products were released simultaneously with catecholamines. The molar ratio of catecholamines: adenine nucleotides in the perfusates was close to 4: 1—the same as the catecholamines: ATP ratio in the isolated storage vesicles. When the ATPase activity of the gland was inhibited by perfusion with EDTA and Ca^{2+}-free Locke's solution, secretion of

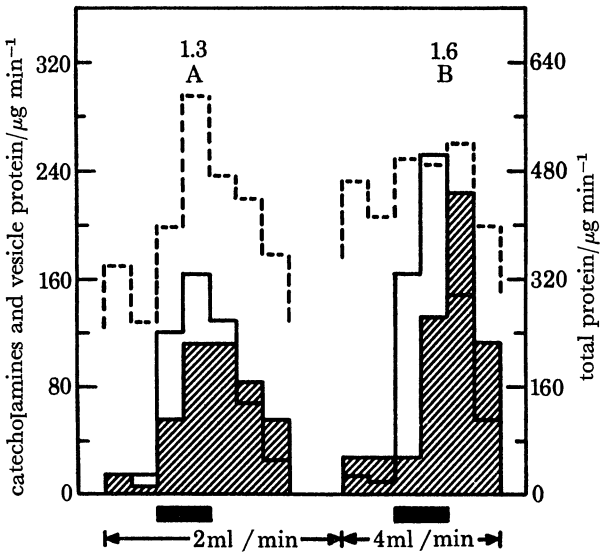

FIGURE 2. Secretion of catecholamines and chromogranin A from perfused bovine adrenal glands. Each bar represents a 2 min collection period. Catecholamines, open bars; total protein, broken line; chromogranin A (vesicle protein), shaded bars. The number above the bars are the net catecholamine: chromogranin A ratios during the 10 min period beginning with the infusion of acetylcholine (solid horizontal bar). The perfusion rates were 2 and 4 ml/min. (Kirshner *et al.* 1967).

TABLE 1. CATECHOLAMINES AND CHROMOGRANIN A IN PERFUSATES AND IN STORAGE VESICLES OF BOVINE ADRENAL GLAND

	CA μg/min	Chr. A μg/min	CA/Chr. A
control	27 ± 2.3	13 ± 2.6	3.1 ± 0.4
stimulation	117 ± 9.7	68 ± 7.2	1.9 ± 0.1
net change	91 ± 10.5	55 ± 6.4	1.7 ± 0.1
storage vesicles	—	—	1.6 ± 0.2

Values are the means ± standard error of the means for samples collected during 23 control periods and 23 periods of stimulation. Storage vesicles were obtained from 11 different glands. CA, catecholamines; Chr. A, chromogranin A (Kirshner *et al.* 1966).

catecholamines elicited by Ba^{2+} also released large amounts of ATP. Here too the molar ratio of catecholamines: total adenine nucleotides was approximately 4: 1. Similar results were obtained by Banks (1966) using perfused bovine adrenal glands, but ATP was more extensively metabolized in this tissue, and adenosine, inosine and hypoxanthine appeared in the perfusates as well as substantial amounts of AMP. Although these results could be best interpreted as release directly from the storage vesicles to the exterior of the cell, they did not eliminate the possibility that the vesicles had released their contents into the cytoplasm whence, because of the changed permeability of the plasma membrane, diffusion occurred.

More conclusive evidence that secretion occurred by release directly to the exterior of the cell was provided by the demonstrations that catecholamines and proteins specifically contained

within the storage vesicles were released in the same relative amounts as those found in the intact vesicles, but that proteins present in the cytosol were not released. Since chromogranin A has no known biological activity, antibodies specifically directed against chromogranin A were prepared (Helle 1966*b*; Sage, Smith & Kirshner 1967) and used to measure the protein. Banks & Helle (1965) were able to detect chromogranin A in perfusates of stimulated glands but not in perfusates of resting glands. By use of micro-complement fixation (Kirshner, Sage, Smith & Kirshner 1966; Kirshner, Sage & Smith 1967) it was shown that catecholamines and chromogranin A were released during stimulation of isolated perfused bovine adrenal glands with acetylcholine or nicotine in the same ratios as present in intact storage vesicles (figure 2 and table 1). In these same experiments, phenylethanolamine-*N*-methyltransferase, a cytoplasmic enzyme, could not be detected in the perfusates. In addition to chromogranin A, Schneider *et al.* (1967) demonstrated that the other soluble proteins present in the storage vesicles were also released during stimulation but that cytoplasmic proteins such as lactic dehydrogenase were not released.

The relevance of the observations made on isolated perfused adrenal glands to secretion in the living animal was established by Blaschko *et al.* (1967). They collected adrenal venous blood from anaesthetized calves during and between periods of stimulation of the splanchnic nerve and found that the ratios of catecholamines to chromogranin A during periods of stimulation were similar to those reported by Schneider *et al.* (1967) for the isolated perfused gland. In the living animal as well as in the isolated perfused gland the appearance of the protein lagged behind that of the catecholamines (figure 2). This lag has been attributed to a slower rate of diffusion of the protein through the interstitial spaces and vasculature.

The combined work cited above established that secretion occurred by release of the vesicle content directly to the exterior of the cell but could not distinguish whether secretion occurred by extrusion of the entire vesicle or whether only the contents were released and the vesicle membranes retained. Electron microscopic studies of stimulated adrenal glands showed structures which resembled empty storage vesicles to be present within the medullary cells (De Robertis & Vaz Ferreira 1957; Wetzstein 1957; D'Anzi 1969), and storage vesicles prepared from stimulated adrenals were more electron translucent than vesicles prepared from unstimulated glands (Malamed, Poisner, Trifaro & Douglas 1968). Electron micrographs did not reveal structures in the extracellular spaces resembling storage vesicle membranes. Biochemical evidence also suggested that the vesicle membranes were retained after secretion.

Perfusates of adrenal glands were examined by Trifaro, Poisner & Douglas (1967) and by Schneider *et al.* (1967) for increased amounts of typical membrane components such as cholesterol, phospholipids and fatty acids, but little or no increases were found. Poisner *et al.* (1967) found a decrease in the protein and lipid content of the storage vesicle fraction isolated in sucrose density gradients after stimulation and a slight but not quantitative increase in the lipid content of the fraction corresponding to membranes from lysed vesicles.

The observation that dopamine β-hydroxylase was associated with the storage vesicle membrane (Kirshner 1957; Levin *et al.* 1960) indicated that this enzyme could serve as a specific marker to detect vesicle membranes before and after secretion. Preliminary studies, however, revealed that the enzyme was present in the 100000*g* supernatant of tissue homogenates and, after lysis of the vesicles in the soluble fraction as well as in the sediment (Duch, Viveros & Kirshner 1968). The enzyme present in the 100000*g* supernatant fraction of tissue homogenates most likely comes from storage vesicles which are disrupted during the preparation. Failure to

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

find this soluble enzyme in earlier work was due to the presence of potent endogenous inhibitors of the enzyme which completely masked its presence (Creveling 1962; Nagatsu, Kuzuya & Kidaka 1967; Austin, Livett & Chubb 1967; Duch *et al.* 1968).

The presence of soluble dopamine β-hydroxylase within the storage vesicles provided an additional tool to test the previous observations that catecholamines and soluble proteins contained within the storage vesicles were released during secretion. Using isolated perfused bovine adrenal glands, Viveros, Arqueros & Kirshner (1968) demonstrated that soluble dopamine β-hydroxylase was released upon stimulation with acetylcholine.

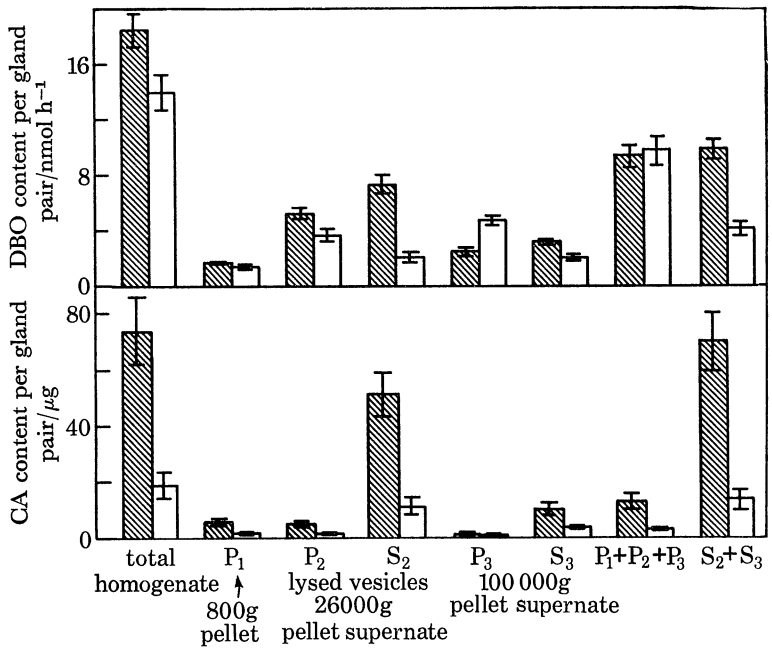

FIGURE 3. Distribution of dopamine β-hydroxylase and catecholamines in subcellular fractions of rabbit adrenals obtained from control and insulin-treated animals (Viveros *et al.* 1969*a*, 1970). ▨, control $n = 7$; □, insulin (40 i.u./kg) $n = 6$.

The fact that dopamine β-hydroxylase was present in both the particulate and soluble fractions of storage vesicles did not decrease its usefulness as a membrane marker. Analysis of rabbit adrenal glands after neurogenic stimulation induced by insulin hypoglycaemia showed decreases in the catecholamine and in the dopamine β-hydroxylase content (Viveros, Arqueros, Connett & Kirshner 1969), but the decrease in dopamine β-hydroxylase was solely due to loss of the enzyme from the soluble portion of the storage vesicles; this can be seen in figure 3 (Viveros, Arqueros & Kirshner 1969*a*, 1970). Treatment of the animals with large doses of reserpine (1 to 5 mg/kg) also caused a decrease in catecholamines and dopamine β-hydroxylase. However, if neural stimulation of the adrenal gland was blocked by administering a ganglionic blocking agent such as chlorisondamine, reserpine still caused a marked depletion of the catecholamine content but no decrease in either the soluble or particulate dopamine β-hydroxylase. Thus, the decrease in soluble enzyme required nerve stimulation and was a consequence of secretion and not due merely to depletion of the catecholamine stores. These data in combination with the previously cited electron microscope studies have established that secretion from chromaffin cells occurs by exocytosis and that the storage vesicle membranes remain within the cell after secretion.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Further insight into the secretory process was obtained upon examination of the dopamine β-hydroxylase and catecholamine content of vesicles obtained from stimulated and non-stimulated glands after purification on sucrose density gradients (Viveros, Arqueros & Kirshner 1969*b*). It had not been previously determined whether a vesicle secretes a portion of its soluble content or its total content in response to a stimulus. In rabbit adrenal storage vesicles approximately 50 % of the dopamine β-hydroxylase is tightly bound to the vesicle membrane; the

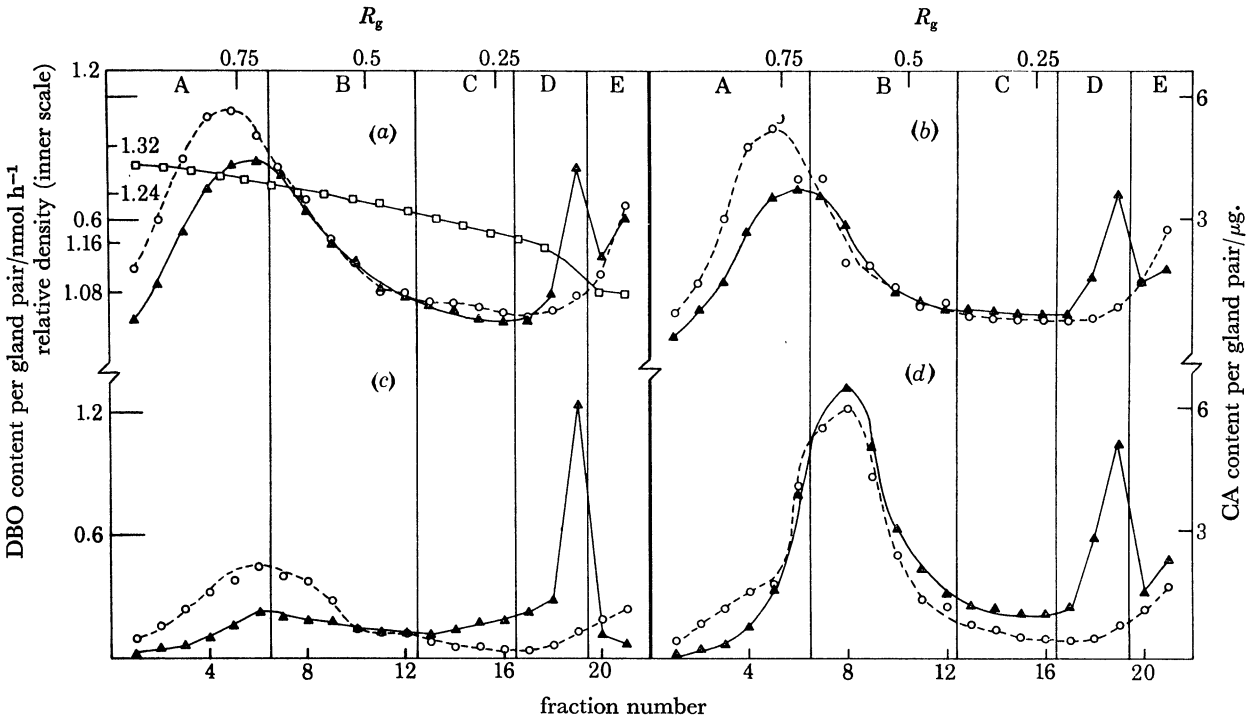

FIGURE 4. Isopycnic distribution of adrenal storage vesicles on sucrose density gradients. ○, catecholamines; ▲, dopamine β-hydroxylase; □, relative density of gradients. For details, see Viveros *et al.* (1969*b*). (*a*) control; (*b*) chlorisondamine; (*c*) insulin; (*d*) chlorisondamine + reserpine.

TABLE 2. DOPAMINE β-HYDROXYLASE, CATECHOLAMINES AND R_g OF PURIFIED STORAGE VESICLES AFTER VARIOUS DRUG TREATMENTS

	100 × DBO concentration per gland pair/nmol h^{-1}	R_g DBO	CA content per gland pair/μg	R_g CA	DBO/CA
none (7)	853 ± 88	0.74 ± 0.01	53 ± 7	0.79 ± 0.01	15 ± 1.6
insulin, 3 h (6)	116 ± 16[a]	0.72 ± 0.02	10 ± 1.2[a]	0.77 ± 0.02	12 ± 1.3
chlorisondamine (7)	600 ± 16[c]	0.74 ± 0.02	46 ± 5	0.75 ± 0.02	14 ± 1.8
chlorisondamine + reserpine (7)	537 ± 48[b]	0.64 ± 0.01[a]*	17 ± 3.5[a]*	0.68 ± 0.02[a]*	42 ± 9.6[c]*

Animals were treated with the drugs, the crude storage vesicle fraction (26000 *g* pellet) prepared, centrifuged through sucrose density gradients and assayed for CA and DBO as previously described (Viveros *et al.* 1969*b*). The figures in parentheses are the numbers of animals in each group. Data are means and standard errors of the means: *a*, P^* 0.001; *b*, P^* 0.01; *c*, P^* 0.02 compared to untreated animals. The DBO values of the chlorisondamine-treated group and the chlorisondamine plus reserpine-treated group were not significantly different from each other.

* P values were the same compared to either the untreated animals or the chlorisondamine-treated animals. R_g is defined as the distance of a peak from the top of the gradient divided by the total length of the gradient and is a relative measure of the buoyant density of that constituent.

remainder is soluble and is released together with the catecholamines and other soluble components upon neurogenic stimulation (Viveros *et al.* 1969*a*). Thus, the dopamine β-hydroxylase:catecholamine ratios in vesicles isolated from stimulated glands would increase if vesicles secreted only a portion of their content in response to a stimulus. In addition, one might expect changes in the buoyant density of the vesicles which secreted. On the other hand, if vesicles released their total content, then the dopamine β-hydroxylase:catecholamine ratios and the buoyant densities of the remaining vesicles should be the same as those from unstimulated glands. The results obtained from experiments in which storage vesicles obtained from control animals, insulin-treated animals, animals treated with chlorisondamine (a long-lasting ganglionic blocking agent) and animals treated with chlorisondamine and reserpine were centrifuged through sucrose density gradients and fractions assayed for dopamine β-hydroxylase and catecholamines are shown in figure 4 and summarized in table 2. The storage vesicles are found in segments A and B of the gradients (figure 4) and in each instance contained 75 to 80 % of the total catecholamines applied to the gradients. The data in table 2 are the total amounts of dopamine β-hydroxylase and catecholamines in segments A and B.

Insulin treatment resulted in a marked decrease of both the enzyme activity and catecholamine content of the storage vesicle fractions, but there was no change in the dopamine β-hydroxylase:catecholamine ratio and no change in the buoyant density of the vesicles. Treatment with chlorisondamine resulted in a small decrease in both the enzyme level and catecholamine content but no change in the enzyme:catecholamine ratio and no change in the buoyant density. After treatment with chlorisondamine and reserpine, there was a marked decrease in the catecholamine content, and a small decrease in the enzyme content compared to the untreated animals, but it was not significantly different from the animals treated with chlorisondamine alone. These changes resulted in a marked increase in the dopamine β-hydroxylase:catecholamine ratios as well as in a decrease in the buoyant density of the vesicles.

These data suggest that each vesicle that responds to a neurogenic stimulus releases its total content in an 'all or none' response. However, the data do not exclude the possibility that more than one stimulus is required to discharge the total content of a vesicle. For example, a vesicle may become 'fixed' into a secreting position upon a first stimulus and release a portion of its content but remain 'fixed' and respond to successive stimuli until it is completely depleted. Calculations show that the amount of catecholamines released from the cat adrenal medulla for each maximal stimulus applied to the first splanchnic nerve is of the order of one vesicle content per pulse per chromaffin cell (Kirshner & Viveros 1970). That is to say, each maximal stimulus releases sufficient acetylcholine from the splanchnic nerve endings to excite each cell to release the total content of one vesicle. Secretion from the adrenal medulla may be considered to be 'quantal' in the sense that the minimal response to an effective stimulus is the secretion of the content of one storage vesicle.

The obvious analogy that one wishes to draw is that between the apparent 'all or none' release of catecholamines from the storage vesicles of the adrenal medulla and the quantal release of neurotransmitters at the synapse.

The quantal hypothesis for the release of neurotransmitters (Del Castillo & Katz 1954) has received wide support. Not only have miniature end plate potentials been observed at the skeletal neuromuscular junction but also at central synapses and those in sympathetically innervated smooth muscle (Katz 1969). The demonstration of synaptic vesicles (Robertson

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

1956) shortly after the quantal hypothesis was proposed led to the speculation that the vesicles were the morphological quantal units (Del Castillo & Katz 1956). This view received additional support from the work of De Robertis *et al.* (1961) and Gray & Whittaker (1962) in which it was found that the synaptic vesicles contained acetylcholine and in amounts consistent with the quantal hypothesis (Whittaker & Sheridan 1965). Studies of the vesicles at the adrenergic synapse leave little doubt that they contain the major stores of noradrenaline (see Bloom & Giarman 1968) and evidence suggests that release of noradrenaline occurs from these stores during neural stimulation (Malmfors 1965; Häggendal & Malmfors 1969; Geffen *et al.* 1969; De Potter *et al.* 1969). Calculations by Folkow, Häggendal & Lisander (1967) and by Stjärne, Hedqvist & Bygdeman (1969) indicate that the synaptic vesicles at the noradrenergic synapse contain too much noradrenaline to be the quantal unit, and that, if the total contents were released similar to that in the adrenal gland, neither the rate of protein synthesis at the synapse nor the rate of migration of new vesicles down the axon would be sufficiently rapid to account for the replenishment of the noradrenaline stores during neural activity. These calculations do not argue against the quantal hypothesis but are addressed to the size of the quantal packet and to the identification or existence of a morphological quantal unit. Data on the quantitative aspects of noradrenaline release at the synapse are only approximate, and it remains to be established whether the synaptic vesicles are the immediate source from which the neurotransmitters are released and whether they are the morphological quantal units. The studies described here on secretion from the adrenal medulla provide both a model and the tools to investigate these phenomena at the sympathetic synapse.

This work was supported by a grant from the National Institutes of Health AM-05427.

References (Kirshner & Kirshner)

Austin, L., Livett, B. G. & Chubb, I. W. 1967 *Am. Heart Ass. Monograph*, **17**, 111.
Banks, P. 1966 *Biochem. J.* **101**, 536.
Banks, P. & Helle, K. 1965 *Biochem. J.* **97**, 40*C*.
Banks, P., Helle, K. B. & Major, D. 1969 *Molec. Pharmacol.* **5**, 210.
Blaschko, H. 1939 *J. Physiol.* **96**, 50*P*.
Blaschko, H., Comline, R. S., Schneider, F. H., Silver, M. & Smith, A. D. 1967 *Nature, Lond.* **215**, 58.
Blaschko, H. & Welch, A. D. 1953 *Arch. exp. Path. Pharmak.* **219**, 17.
Bloom, F. E. & Giarman, N. J. 1968 *Ann. Rev. Pharmacol.* **8**, 229.
Carlsson, A., Hillarp, N-Å. & Waldeck, B. 1963 *Acta physiol. scand.* **59**, suppl. 215, 5.
Creveling, C. R. 1962 Studies on dopamine-β-oxidase. Doctoral Thesis, The George Washington University, Washington, D.C.
D'Anzi, F. A. 1969 *Am. J. Anat.* **125**, 381.
Del Castillo, J. & Katz, B. 1954 *J. Physiol.* **124**, 560.
Del Castillo, J. & Katz, B. 1956 *Progr. Biophys.* **16**, 121.
De Potter, W. P., de Schaepdryver, A. F., Moerman, E. J. & Smith, A. D. 1969 *J. Physiol.* **204**, 102*P*.
De Robertis, E. D. P., Pellegrino De Iraldi, A., Rodriguez, G. & Gomez, C. J. 1961 *J. biophys. biochem. Cytol.* **9**, 229.
De Robertis, E. D. P. & Vaz Ferreira, A. 1957 *Expl. Cell Res.* **12**, 568.
Diner, O. 1967 *C. r. hebd. Séanc. Acad. Sci., Paris* **265**, 616.
Douglas, W. W. 1968 *Br. J. Pharmac. Chemother.* **34**, 451.
Douglas, W. W., Poisner, A. M. & Rubin, R. P. 1965 *J. Physiol.* **179**, 130.
Douglas, W. W. & Poisner, A. M. 1966*a* *J. Physiol.* **183**, 236.
Douglas, W. W. & Poisner, A. M. 1966*b* *J. Physiol.* **183**, 249.
Douglas, W. W. & Rubin, R. P. 1961 *J. Physiol.* **159**, 40.
Duch, D. S., Viveros, O. H. & Kirshner, N. 1968 *Biochem. Pharmac.* **17**, 255.
Euler, U. S. von & Hillarp, N-Å. 1956 *Nature, Lond.* **177**, 44.
Euler, U. S. von & Lishajko, F. 1963 *Acta physiol. scand.* **59**, 454.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Euler, U. S. von, Lishajko, F. & Stjärne, L. 1963 *Acta physiol. scand.* **59**, 495.
Fish, W. W., Mann, K. G. & Tanford, C. 1969 *J. biol. Chem.* **244**, 4989.
Folkow, B., Häggendal, J. & Lisander, B. 1967 *Acta physiol. scand.* **72**, *Suppl.* 307, 1–38.
Friedman, S. & Kaufman, S. 1965 *J. biol. Chem.* **240**, 4763.
Gray, E. G. & Whittaker, V. P. 1962 *J. Anat.* **96**, 79.
Geffen, L. B., Livett, B. G. & Rush, R. A. 1969 *J. Physiol.* **204**, 1.
Häggendal, J. & Malmfors, T. 1969 *Acta physiol. scand.* **75**, 33.
Helle, K. B. 1966*a* *Molec. Pharmacol.* **2**, 298.
Helle, K. B. 1966*b* *Biochim. biophys. Acta* **117**, 107.
Hillarp, N-Å. 1958*a* *Acta physiol. scand.* **42**, 321.
Hillarp, N-Å. 1958*b* *Acta physiol. scand.* **42**, 144.
Hillarp, N-Å. 1959 *Acta physiol. scand.* **47**, 271.
Hillarp, N-Å. 1960*a* In *Adrenergic mechanisms* (ed. J. R. Vane, G. E. W. Wolstenholme and M. O'Connor), pp. 481–486. London: J. A. Churchill.
Hillarp, N-Å. 1960*b* *Acta physiol. scand.* **50**, 8.
Hillarp, N-Å., Lagerstedt, S. & Nilson, B. 1953 *Acta physiol. scand.* **29**, 251.
Hillarp, N-Å. & Thieme, G. 1959 *Acta physiol. scand.* **45**, 328.
Hopwood, D. 1967 *J. Anat.* **101**, 619.
Hopwood, D. 1968 *Histochemie* **13**, 323.
Katz, B. 1969 *The release of neural transmitter substances*, Liverpool University Press.
Kirshner, A. G. & Kirshner, N. 1969 *Biochim. biophys. Acta* **181**, 219.
Kirshner, N. 1957 *J. biol. Chem.* **226**, 821.
Kirshner, N. 1959 *Pharmacol. Rev.* **11**, 350.
Kirshner, N. 1962 *J. biol. Chem.* **237**, 2311.
Kirshner, N., Sage, H. J. & Smith, W. J. 1967 *Molec. Pharmacol.* **3**, 254.
Kirshner, N., Sage, H. J., Smith, W. J. & Kirshner, A. G. 1966 *Science, N.Y.* **154**, 529.
Kirshner, N. & Viveros, O. H. 1970 In *New aspects of storage and release mechanisms of catecholamines* (Bayer Symposium) (ed. H. G. Kroneberg and H. J. Schümann), pp. 78–88. Berlin: Springer.
Laduron, P. & Belpaire, F. 1968 *Biochem. Pharmacol.* **17**, 1127.
Levin, E. Y., Levenberg, B. & Kaufman, S. 1960 *J. biol. Chem.* **235**, 2080.
Malamed, S., Poisner, A. M., Trifaro, J. M. & Douglas, W. W. 1968 *Biochem. Pharmacol.* **17**, 241.
Malmfors, T. 1965 *Acta physiol. scand.* **64**, Suppl. 248.
Musacchio, J. M. 1968 *Biochem. Pharmacol.* **17**, 1470.
Nagatsu, T., Kuzuya, H. & Kidaka, H. 1967 *Biochim. biophys. Acta* **139**, 319.
Nagatsu, T., Levitt, M. & Udenfriend, S. 1964 *J. biol. Chem.* **239**, 2910.
Oka, M., Ohuchi, T., Yoshida, H. & Imaizumi, R. 1966 *Life Sci.* **5**, 427.
Oka, M., Kajikawa, K., Ohuchi, I., Yoshida, H. & Imaizumi, R. 1967 *Life Sci.* **6**, 461.
Poisner, A. M. & Trifaro, J. M. 1967 *Molec. Pharmacol.* **3**, 561.
Poisner, A. M. & Trifaro, J. M. 1969 *Molec. Pharmacol.* **5**, 294.
Poisner, A. M., Trifaro, J. M. & Douglas, W. W. 1967 *Biochem. Pharmacol.* **16**, 2101.
Potter, L. T. 1967 *Am. Heart Ass. Monograph* **17**, 13.
Potter, L. T. & Axelrod, J. 1963 *J. Pharmacol.* **142**, 299.
Robertson, J. D. 1956 *J. biophys. biochem. Cytol.* **2**, 381.
Sage, H. J., Smith, W. J. & Kirshner, N. 1967 *Molec. Pharmacol.* **3**, 81.
Schneider, F. H., Smith, A. D. & Winkler, H. 1967 *Br. J. Pharmacol.* **31**, 94.
Schümann, H. J. 1958 *Arch. exp. Path. Pharmak.* **233**, 296.
Schümann, H. J. 1961 *Sympn dt. Ges. Endokr.* **8**, 23.
Smith, W. J. & Kirshner, N. 1967 *Molec. Pharmacol.* **3**, 52.
Smith, A. D. & Winkler, H. 1967*a* *Biochem. J.* **103**, 480.
Smith, A. D. & Winkler, H. 1967*b* *Biochem. J.* **103**, 483.
Stjärne, L. 1964 *Acta physiol. scand.* **62**, Suppl. 228.
Stjärne, L., Hedqvist, P. & Bygdeman, S. 1969 *Life Sci.* **8**, 189.
Stjärne, L. & Lishajko, F. 1966 *Br. J. Pharmacol.* **27**, 398.
Stormorken, H. 1969 *Scand. J. Hematol.* Suppl. 9.
Strieder, N., Ziegler, E., Winkler, H. & Smith, A. D. 1968 *Biochem. Pharmacol.* **17**, 1553.
Trifaro, J. M., Poisner, A. M. & Douglas, W. W. 1967 *Biochem. Pharmacol.* **16**, 2095.
Viveros, O. H., Arqueros, L., Connett, R. C. & Kirshner, N. 1969 *Molec. Pharmacol.* **5**, 69.
Viveros, O. H., Arqueros, L. & Kirshner, N. 1968 *Life Sci.* **7**, 609.
Viveros, O. H., Arqueros, L. & Kirshner, N. 1969*a* *Molec. Pharmacol.* **5**, 342.
Viveros, O. H., Arqueros, L. & Kirshner, N. 1969*b* *Science, N.Y.* **165**, 911.
Viveros, O. H., Arqueros, L. & Kirshner, N. 1971 (in preparation).
Wetzstein, R. 1957 *Z. Zellforsch.* **46**, 517.
Whittaker, V. P. & Sheridan, N. M. 1965 *J. Neurochem.* **12**, 363.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES