Mechanism of Secretion from the Adrenal Medulla

II. Release of Catecholamines and Storage Vesicle Protein in Response to Chemical Stimulation

N. KIRSHNER,[1] H. J. SAGE, AND W. J. SMITH[2]

Departments of Biochemistry and Pathology Duke University Medical Center, Durham, North Carolina 27706

(Received December 19, 1966)

SUMMARY

The release of catecholamines and a specific protein of the storage vesicles was measured in isolated perfused bovine adrenal glands. Stimulation of catecholamine secretion by acetylcholine, nicotine, or Ba^{++} caused a parallel increase in the secretion of the storage vesicle protein. The ratio of catecholamines:vesicle protein in the perfusates was, within the limits of experimental error, the same as that in the intact gland. The release of both the vesicle protein and the catecholamines required Ca^{++} and was temperature dependent. These studies provide additional evidence to support the hypothesis that catecholamines are secreted from the medulla by a process in which the storage vesicle contents are released directly to the exterior of the cell.

INTRODUCTION

Most of the catecholamines of the adrenal medulla are stored in intracellular vesicles (1, 2). In addition to the catecholamines the vesicles contain large amounts of adenine nucleotides (mostly ATP) and protein (3). Generally, four hypotheses have been proposed to explain the process by which catecholamines are secreted in response to nerve stimulation or to perfusion with certain chemicals. (a) A change in the plasma membrane permeability of a stimulated cell results in an increased outward diffusion of "free" (cytoplasmic) catecholamines. (b) Stimulation causes an intracellular release of catecholamines from storage vesicles. The resulting increased concentration of "free" catecholamines results in increased diffusion across the plasma membrane. (c) Stimulation brings about an extrusion of the entire storage vesicle from the cell. The extruded vesicle lyses and liberates its contents extracellularly. (d) Secretion occurs by a process of exocytosis or "reverse pinocytosis" in which the vesicles fuse with the plasma membranes and liberate their contents directly to the exterior of the cell. Recent evidence has provided new insight into the secretory process. Douglas *et al.* (4, 5) have demonstrated that adenine nucleotides and catecholamines appeared in stimulated perfused glands simultaneously and in approximately the same relative amounts as found in the isolated storage vesicles. This indicates that secretion occurred by process (c) or (d). Banks and Helle (6), using immunological procedures, were able to detect the presence of a specific protein obtained from the storage vesicles (7, 8) in perfusates of stimulated glands but not in perfusates of unstim-

[1] Career Development Awardee GM-K3-15, 184 of the National Institutes of Health.

[2] During the period of this work Dr. Smith was a senior research fellow of the American Heart Association. Present address: Department of Biology, East Carolina College, Greenville, North Carolina.

ulated glands. Reported below are quantitative measurements of the release of catecholamine and the specific vesicle protein in isolated perfused adrenal glands. The similar ratio of catecholamines:protein in the perfusates and intact storage vesicles and the almost simultaneous release of catecholamines and specific protein provide additional evidence for a process in which catecholamines are secreted directly to the exterior of the cell. Preliminary reports of this work have appeared elsewhere (9, 10).

MATERIALS AND METHODS

Perfusion of adrenal glands. Adrenal glands were removed from cows 20–30 min after slaughter. The glands were cannulated through the adrenal vein, scarified by making multiple incisions 0.5–1.0 mm deep in the cortex, flushed with 20 ml of ice cold Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 2.15 mM Na_2HPO_4, 0.86 mM NaH_2PO_4, 10 mM glucose), and transported in ice to the laboratory (45–60 min). The glands were then perfused for 45 min at a flow rate of 4 ml/min (unless otherwise indicated) with Locke's solution before samples were collected. Secretion was evoked by changing to Locke's solution containing 10^{-5} g/ml of acetylcholine chloride, nicotine (free base), or 2 mM $BaCl_2$. Generally, the glands were stimulated for 2 min and 2-min samples were collected. In the early experiments, the glands were perfused at ambient room temperature. In later experiments the temperature was controlled at 30°.

At the end of the perfusion, the gland was chilled in ice and the cortex was dissected from the medulla. The medulla was blotted dry, weighed, and homogenized in 15–20 volumes of 0.25 M sucrose using a conical Potter-Elvehjem glass homogenizer. The homogenate was centrifuged at 800 *g* for 8 min and separated into a low speed sediment and low speed supernatant. The low speed supernatant was then centrifuged at 25,000 *g* for 20 min and separated into a high speed sediment, which contained the catecholamine storage vesicles, and a high speed supernatant (A). The low and high speed sediments were lysed by homogenization in 10 ml of ice cold distilled water and the volume measured. The suspensions were centrifuged at 25,000 *g* for 20 min and the resulting supernatant was decanted and saved for assay. Aliquots of the total homogenate, the low speed supernatant, and high speed supernatant (A) were diluted with 4 volumes of ice cold water and centrifuged at 25,000 *g* for 20 min. The supernatants were decanted and used for assay. Thus all results refer to those components which remained in the 25,000 *g* supernatants. In several instances, the contralateral gland of the animal was removed, kept in ice throughout the experimental period, and fractionated as above for assay.

Analytical methods. Total protein was determined either by the method of Moore and Stein (11) or by the method of Lowry *et al.* (12). The perfusates were centrifuged to remove red blood cells and other debris. Aliquots of the perfusates (0.1 ml) and of each of the fractions of the medulla (0.05 ml) were diluted to 1 ml, and the protein was precipitated by the addition of 1 ml of 0.8 N perchloric acid. The precipitates were centrifuged at 15,000 *g* for 10 min and the supernatants were decanted. The precipitates were washed once with 0.4 N perchloric acid, and the remainder of the assay was carried out on the pellets in the centrifuge tubes. For each series of assays, 30, 60, and 90 μg of bovine serum albumin and a reagent blank were carried through the entire procedure for a standard reference curve.

Catecholamines were assayed by the trihydroxyindole method (13). The perfusates were diluted 1:50, and 0.1 ml of this solution was used for assay without further purification. Protein in the medulla fractions was removed by precipitation with an equal volume of 10% trichloroacetic acid. The resulting supernatants were diluted 1:50 (homogenate, low speed sediment, high and low speed supernatants) or 1:500 (high speed sediment) with distilled water; 0.1 ml was used for assay. Because of these large dilutions, it was not necessary to purify the catecholamines on *alumina* prior to assay. Catecholamine values are expressed

as micrograms of free base. The concentrations of specific vesicle protein in perfusates and medulla fractions were determined by microcomplement fixation (14). Dopamine-β-hydroxylase was determined as described by Smith and Kirshner (8). Phenethanolamine-*N*-methyltransferase was measured by the method of Axelrod (15).

RESULTS

Release of Catecholamines and Vesicle Protein—Effect of Perfusion Rate

The secretion of catecholamines and vesicle protein at perfusion rates of 2 and 4 ml/min is shown in Fig. 1. In this experiment secretion was evoked by perfusion with acetylcholine for 4 min at each flow rate. In the 8 min following the start of stimulation, the total amounts of catecholamines and protein were 968 μg and 728 μg at the 2 ml/min perfusion rate; at the 4 ml/min perfusion rate they were 1240 μg and 952 μg, respectively. The ratio of catecholamines:vesicle protein was the same at both perfusion rates. The greater secretion at 4 ml/min may have been due either to the larger amounts of acetylcholine administered or to a better washout. Several factors, such as the decreasing response to continued injection of acetylcholine, possible re-uptake of catecholamines, efficiency of washout, and others may affect the secretion rate as measured in these experiments. Because of these multiple factors and for experimental convenience, a standard procedure using a 2-min stimulation period at a flow rate of 4 ml/min was adopted.

The data of Fig. 1B also show that the appearance of the vesicle protein in the perfusates lags behind the catecholamines.

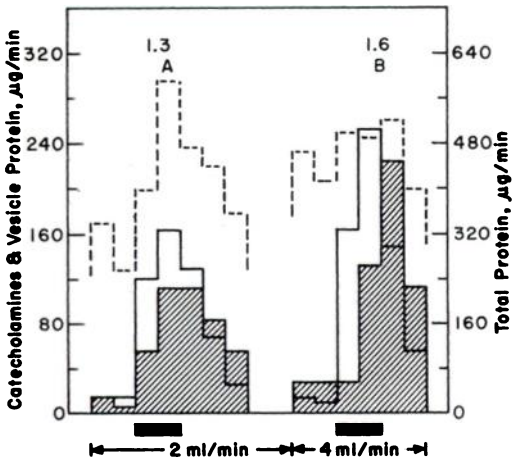

FIG. 1. *The release of catecholamines and proteins at two different perfusion rates*

Each bar represents a 2-min period. Catecholamines, open solid line bars; total protein, broken line bars; vesicle protein, shaded bars. Horizontal solid bars indicate period of perfusion with acetylcholine. The numbers above the bars are the net catecholamine:vesicle protein ratios.

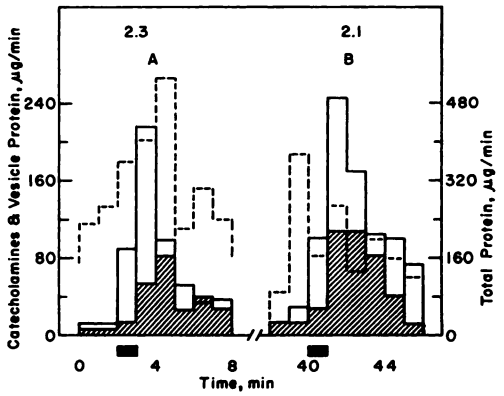

FIG. 2. *Release of catecholamines, vesicle protein, and total protein*

Each bar represents a 1-min period. The gland was perfused for 30 min with 10^{-5} M DFP between parts A and B. Catecholamines, open solid line bars; total protein, broken line bars; vesicle protein, shaded bars. Horizontal solid bars indicate period of perfusion with acetylcholine. The numbers above the bars are the net catecholamine:vesicle protein ratio.

To obtain further information on this phenomenon, a perfusion was carried out in which samples were collected at 1-min intervals following a 1-min stimulation with acetylcholine (Fig. 2A). A measurable increase of the protein in the perfusate was found within 1 min, and maximal amounts were found in 2–3 min. A marked increase of catecholamines was found within 1 min, and the peak was reached in 2

min. These data indicate that the lag is less than 1 min, but the lag time may depend to some extent on the size of the gland and the perfusion rate. The lag in the appearance of the protein is a general characteristic as can be seen from the other figures and will be discussed.

Recovery of Vesicle Protein Added to Perfusion Fluid

Purified vesicle protein in Locke's solution was perfused through the gland to determine the effectiveness of recovery. The results obtained are shown in Fig. 3. In part A, Locke's solution containing 28 μg/ml of the protein was perfused for 1 min at a flow rate of 4 ml/min. The perfusate collected during the infusion period and the subsequent 3 min contained a total of 280 μg of the antigenic protein. Correcting for the base level of 28 μg/min gave a net recovery of 168 μg which was within the limits of the experimental error of the immunological assay. These data indicate that the protein was not appreciably altered, if at all, in its passage through the gland and that no protein was lost after it had entered the vasculature. It does not rule out the possibility that some of the endogenously released protein may have been lost or altered prior to its entrance into the vasculature. However, the data in Tables 1 and 2 indicate that these losses, if any, were small. The addition of vesicle protein to the perfusion medium did not cause any change in the resting level of catecholamines.

In part B the vesicle protein in Locke's solution, 28 μg/ml, was perfused for 10 min at a flow rate of 4 ml/min and samples were collected at 1-min intervals. Within 1 min the increased concentration of vesicle protein in the perfusates was, within experimental error, the same as that in the perfusing medium. When the perfusing medium was switched to protein-free Locke's solution, the amounts of vesicle protein in the perfusates dropped to control levels within 4 min. The rapid rise of the protein in the perfusates upon changing to the protein-Locke perfusion medium and the rapid decline upon the change back to the protein-free Locke's solution indicates that the protein did not diffuse out of the vasculature to any appreciable extent. These observations also indicate that the observed lag in the appearance of the protein was probably due to a delayed entrance into the vasculature.

After 90 min of perfusion the gland was still able to respond to acetylcholine stimulation as shown by secretion of both catecholamines and vesicle protein (Fig. 3D, E).

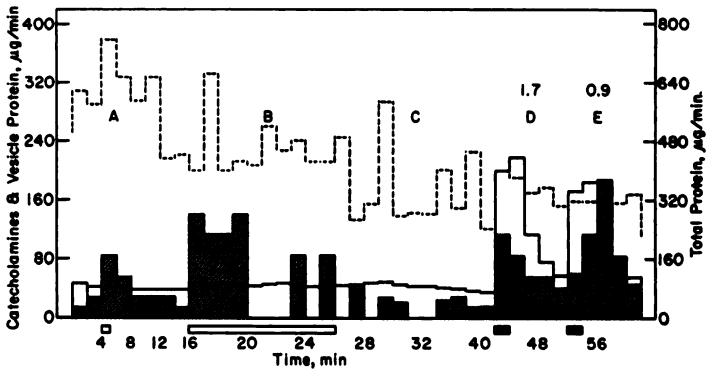

FIG. 3. *Recovery of perfused vesicle protein*

Each bar represents a 2-min sample. Catecholamines, solid line open bars; total protein, broken line bars; vesicle protein, shaded bars. Horizontal open bars represent period of perfusion with Locke-vesicle protein solution. Horizontal solid bars represent period of perfusion with Locke's solution containing acetylcholine.

Effect of Cocaine and DFP on Secretion

Cocaine enhanced the secretion of catecholamines from adrenal glands of cats when secretion was evoked by stimulation of the splanchnic nerves *in situ* (16). This enhancement was presumably due to inhibition of uptake of released catecholamines. In our studies, cocaine, added to the perfusion medium at 5×10^{-5} g/ml, appeared to inhibit secretion of both catecholamines and protein (Fig. 4). Similar

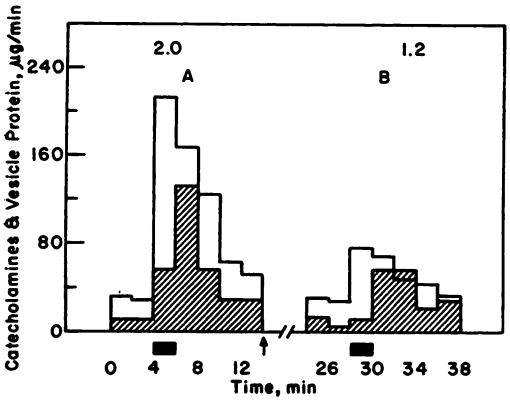

FIG. 4. *Effect of cocaine on release of catecholamines and vesicle protein*

Each bar represents a 2-min sample. Between parts A and B the gland was perfused for 15 min with Locke's solution containing 5×10^{-5} g/ml of cocaine. Catecholamines, open bars; vesicle protein, shaded bars. Solid horizontal bars indicate period of perfusion with Locke's solution containing 10^{-5} g/ml of acetylcholine.

results were obtained when cocaine was present at a concentration of 1×10^{-5} g/ml. However, the catecholamine:vesicle protein ratios in parts A and B indicate that there was no cocaine-blocked uptake of released catecholamines. If the uptake of catecholamines had been blocked, an increased catecholamine:vesicle protein ratio would have been obtained unless the uptake of the vesicle protein was also blocked.

In part B of the experiment shown in Fig. 2 the effect of diisopropylfluorophosphate (DFP) on secretion was investigated. Hokin (17) has reported that DFP inhibited a transport ATPase in kidney. The primary purpose of the experiment was to determine whether DFP would inhibit secretion, possibly by interfering with some transport process (17). At the end of part A the gland was perfused for 30 min with Locke's solution containing 10^{-5} M DFP. Two 1-min control samples were collected, followed by stimulation with acetylcholine for 1 min and collection of subsequent 1-min samples. The DFP enhanced the effect of acetylcholine in stimulating the release of catecholamines and vesicle protein, probably due to inhibition of acetylcholinesterase. The ratio of catecholamines: vesicle protein was the same as that obtained in part A. This experiment does not rule out the possibility that a transport phenomenon may be involved in the secretory process, but it does show that if a transport process is involved, it is not inhibited by DFP under the conditions of this experiment.

Ca^{++}-Requirement for Release of Catecholamines and Vesicle Protein

Douglas and Rubin (18, 19) have shown that Ca^{++} was required for acetylcholine or nicotine-evoked secretion of catecholamines in the cat adrenal, and further demonstrated that marked secretion was induced by Locke's solution containing 2.2 mM Ca^{++} alone if the gland was first perfused with Ca^{++}-free Locke's solution. To determine whether Ca^{++} was also required for the secretion of the vesicle protein, the experiments shown in Fig. 6 were performed.

The bovine adrenal gland, as previously shown (20), requires Ca^{++} ions for stimulation-secretion coupling. However, the bovine gland is less sensitive than the cat gland to the addition of Ca^{++} ions following perfusion with a Ca^{++}-free solution (Fig. 5). After perfusion of the gland for 35 minutes with Ca^{++}-free Locke's solution, only a very slight increase of catecholamines was found (Fig. 5C) when the perfusion medium was switched to Locke's solution containing 2.2 mM Ca^{++}; when the Ca^{++}-free perfusion medium was changed to Locke's solution containing 8.8 mM Ca^{++} (Fig. 6D) there was a moderate increase in catecholamine and protein release, but this increased catecholamine release was not as

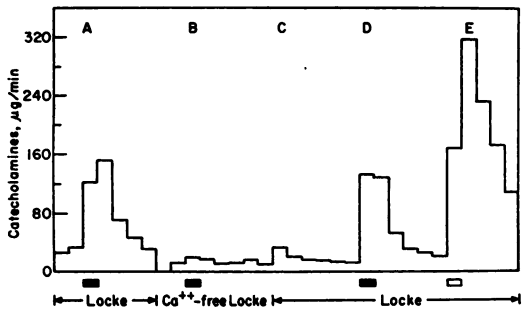

FIG. 5. *Ca^{++} requirement for the release of catecholamines from the bovine adrenal gland*

Each bar represents a 2-min sample. Between parts A and B the gland was prefused for 20 minutes with Ca^{++}-free Locke's solution. At C, perfusion with Locke's solution containing 2.2 mM Ca^{++} was reinitiated. Horizontal solid bars. stimulation period with acetylcholine; horizontal open bar, stimulation with nicotine.

striking as that observed in the cat at much lower concentrations of Ca^{++}. Figure 5, D and E, shows that the gland can fully recover its response to acetylcholine or nicotine when Ca^{++} is again present in the perfusion medium.

cant release of the vesicle protein or catecholamines. In the presence of Ca^{++}, nicotine (Fig. 8G) and acetylcholine (Fig. 6E) stimulated the release of catecholamines and vesicle protein.

Ba^{++}-Evoked Secretion of Catecholamines and Vesicle Protein

Ba^{++} is a powerful secretogogue for the release of catecholamines and adenine nucleotides from perfused adrenal glands of cats (21). When added to Locke's solution at a concentration of 2 mM and perfused through the cow adrenal gland, it stimulated a very high rate of secretion of both catecholamines and vesicle protein (Fig. 7).

Effect of Temperature on Secretion

The effect of temperature on secretion from the adrenal medulla is shown in Fig. 8. At 13° or below, acetylcholine did not detectably stimulate secretion of either catecholamines or protein. At 33° the net rate of secretion of both catecholamines and vesicle protein evoked by acetylcholine was almost three times the rate of 23°.

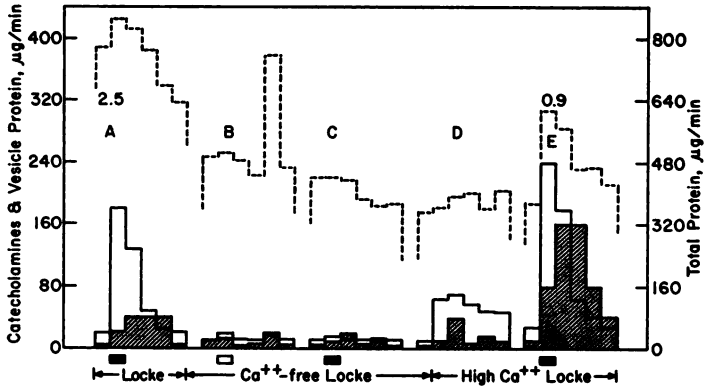

FIG. 6. *Ca^{++} requirement for release of catecholamines and vesicle protein*

Each bar represents a 2-min sample. Between each part of the experiment, the gland was perfused for 15 min with the indicated solutions. Locke's solution contained 2.2 mM Ca^{++}; high-calcium Locke's solution contained 8.8 mM Ca^{++}. Catecholamines, solid line open bars; total protein, broken line open bars; vesicle protein, shaded bars; stimulation with acetylcholine, solid horizontal bars; stimulation with nicotine, horizontal open bar.

As shown in Fig. 6 and Fig. 8, Ca^{++} ions were required for the release of catecholamines and vesicle protein. In the absence of Ca^{++}, neither acetylcholine (Fig. (6C) nor nicotine (Fig. 6B) caused signifi-

At 23° the unstimulated rates of catecholamine and vesicle protein release were 33 and 5 µg/min. After stimulation with acetylcholine the rates increased to 55 and 17 µg/min to give net increases of 22 and

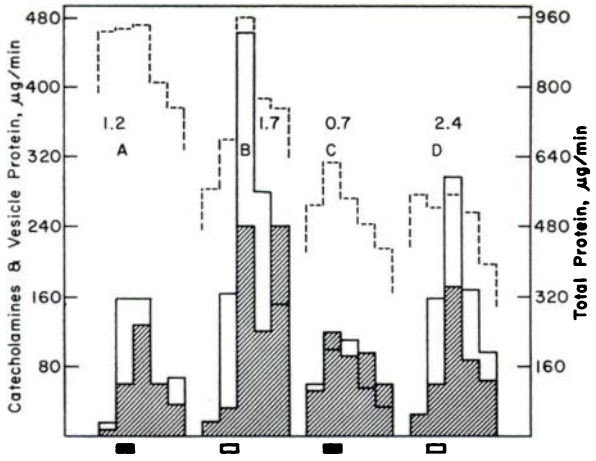

FIG. 7. *Ba^{++}-evoked release of catecholamines and vesicle protein*

Each bar represents a 2-min sample. Between each part the gland was perfused for 10 min with Locke's solution. Catecholamines, solid line open bars; total protein, broken line bars; vesicle protein, shaded bars; stimulation with acetylcholine, solid horizontal bars; stimulation with Locke's solution containing 2 mM Ba^{++}, horizontal open bars.

12 μg/min, respectively, for catecholamines and vesicle protein. At 33° the unstimulated rate of secretion was 52 and 7 μg/min, respectively, for catecholamines and vesicle protein. After stimulation, the rates increased to 112 and 39 μg/min to give net increases of 60 and 32 μg/min, respectively,

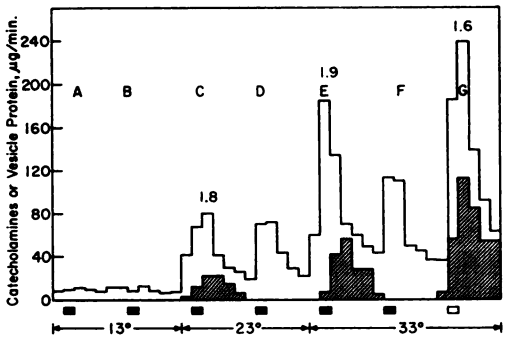

FIG. 8. *Effect of temperature on secretion of catecholamines and vesicle protein*

Each bar represents a 2-min sample. Between the temperature changes the gland was perfused for 30 min with the warmer solution. The temperature of the gland was monitored with a thermistor needle probe inserted into the cortex. Catecholamines, open bars; vesicle protein, shaded bars; stimulation period with acetylcholine, solid horizontal bars; stimulation period with nicotine, horizontal open bar.

for catecholamines and vesicle protein. If the calculation is made using the total amounts of catecholamines and vesicle protein secreted at the two temperatures instead of the rates corrected for the unstimulated release, then the Q_{10} is approximately 2 for both the catecholamines and the vesicle protein. In either case the data show that the secretion is temperature dependent and that the Q_{10} is between 2 and 3, indicating that a chemical reaction is involved in the secretion process. Because of the decreasing rate of secretion obtained upon repeated stimulation, the rate of secretion obtained at F of Fig. 8 was less than twice that obtained at C or D.

The data obtained on the release of catecholamines and vesicle protein from ten glands with two or more secretion periods from each gland are summarized in Table 1. The control levels were obtained from the 2-min sample collected immediately before changing to the perfusion medium containing the secretogogue. The rates of stimulated secretion were averaged over the 8 min immediately following the change to the Locke's solution containing the secretogogue. The net values are the differences between the control rates and the

TABLE 1
Catecholamines and vesicle protein in perfusates[a]

Gland No.	Total CA (μg/min)	Total VP (μg/min)	Total CA/VP	Net CA (μg/min)	Net VP (μg/min)	Net CA/VP
1. a Control	14	10	1.4			
AcCh	121	91	1.3	107	81	1.3
b Control	12	28	0.4			
AcCh	155	119	1.3	143	91	1.6
2. a Control	12	6	2.0			
AcCh	97	43	2.3	85	37	2.3
b Control	21	12	1.8			
AcCh	146	73	2.0	125	64	2.1
3. a Control	32	11	2.9			
AcCh	123	56	2.2	91	46	2.0
b Control	30	14	2.1			
AcCh	53	34	1.6	23	20	1.2
4. a Control	24	3	8			
AcCh	153	43	3.6	129	40	3.2
b Control	20	9	2.2			
AcCh	82	37	2.2	62	28	2.2
5. a Control	23	6	3.8			
AcCh	84	35	2.4	61	29	2.1
b Control	23	6	3.8			
AcCh	75	38	2.0	52	32	1.6
6. a Control	38	14	2.7			
AcCh	123	70	1.9	95	56	1.7
b Control	38	14	2.7			
AcCh	118	100	1.2	80	86	0.9
7. a Control	28	11	2.6			
AcCh	77	49	1.6	49	38	1.3
b Control	15	7	2.1			
Nicotine	147	91	1.6	132	84	1.6
8. a Control	20	4	5.0			
AcCh	96	35	2.7	76	31	2.5
b Control	28	10	2.8			
AcCh	110	104	1.1	82	94	0.9
9. a Control	17	8	2.1			
AcCh	104	78	1.3	87	70	1.2
b Control	19	16	1.2			
Ba^{++}	265	158	1.7	246	142	1.7
c Control	52	60	0.9			
AcCh	74	92	0.8	22	32	0.7
d Control	27	32	0.8			
Ba^{++}	180	96	1.9	153	64	2.4
10. a Control	33	5	6.6			
AcCh	55	17	3.2	22	12	1.8
b Control	52	7	7.2			
AcCh	112	39	2.9	60	32	1.9
c Control	37	7	5.1			
Nicotine	144	73	2.0	107	66	1.6
Control Average:	27 (11)	13 (12)	3.1 (2.0)			
Stimulated Aver:	118 (45)	68 (34)	1.9 (0.6)	91 (49)	55 (30)	1.7 (0.6)

[a] The figures in parentheses are standard deviations. CA, Catecholamines; VP, Vesicle Protein.

TABLE 2
Catecholamine: vesicle protein ratios in adrenal medullas

Gland No.[a]	Whole homogenate	Low speed sediment	Low speed supernatant	High speed supernatant	High speed sediment	Average	Perfusates, average
2 Perfused	1.2	1.4	1.2	0.9	1.2	1.2	2.2
3 Perfused	0.9	0.8	1.6	0.9	2.5	1.3	1.6
Control	1.2	1.2	1.2	0.9	2.1	1.3	—
4 Perfused	0.7	1.4	1.2	0.6	2.7	1.3	2.7
5 Perfused	—	0.9	1.3	1.4	0.9	1.1	2.8
Control	1.2	1.4	1.8	1.5	—	1.5	—
6 Perfused	1.3	—	1.2	—	1.4	1.3	1.3
Control	1.0	—	1.4	1.2	1.0	1.2	—
7 Perfused	0.7	—	0.9	—	1.9	1.2	1.5
8 Perfused	2.9	2.1	1.4	2.2	1.4	2.0	1.8
9 Perfused	0.8	—	0.8	1.2	0.9	0.9	1.5
10 Perfused	0.8	—	1.5	0.5	1.1	0.9	1.8
Average	1.3 (0.4)	1.3 (0.4)	1.3 (0.3)	1.1 (0.5)	1.6 (0.6)	1.3 (0.3)	1.8 (0.5)

[a] The gland numbers correspond to those in Table 1. The figures in parentheses are standard deviations.

total rates of secretion during stimulation. The average net catecholamine to protein ratio in the 23 periods of stimulation was 1.7 ± 0.6 and was not significantly different from the values of 1.9 ± 0.6 obtained for the average total secretion during the periods of stimulation.

Table 2 lists the catecholamine:specific vesicle protein ratios measured in subcellular fractions of the adrenal glands used throughout these experiments. Within the limits of experimental error the ratios were the same in all the fractions, and the average total ratio of 1.3 ± 0.3 was not significantly different from the average net ratio of 1.8 ± 0.5 found in the perfusates.

The recoveries of catecholamines, specific vesicle protein, and total protein in the various fractions of the medullas are shown in Table 3. The percentage recovered in the low speed sediment and low speed supernatant was calculated from the total amounts of the materials present in the homogenate; the recoveries in the high speed supernatant and high speed sediment were calculated from the total amounts present in the low speed supernatant. The vesicle protein in the various fractions represented the following percent of total protein: homogenate, 21 ± 5; low speed sediment, 10 ± 3; low speed supernatant, 17 ± 6; high speed supernatant, 5 ± 1; high speed sediment, 51 ± 6. Thus about one-half of the total soluble protein of the fraction containing the storage vesicles consisted of the specific antigenic protein.

Absence of Dopamine-β-hydroxylase and Phenethanolamine-N-Methylatransferase in Perfusates

Several perfusates were assayed for dopamine-β-hydroxylase and phenethanolamine-*N*-methyltransferase (PENMT) activities to obtain further information on the source of the vesicle protein. Since dopamine-β-hydroxylase activity is firmly attached to the vesicle membrane, the presence of this enzyme in the perfusates would indicate that the entire vesicle was extruded from the medulla cells. PENMT is obtained in the soluble fraction of cell homogenates and considered to be present in the cytoplasm of intact cells. Its presence in perfusates, and its increased activity in perfusates of stimulated glands, would indicate that the stimulated cells became generally more permeable to protein and would largely invalidate the contention that the specific granule protein is secreted directly to the exterior of the cell.

The presence of dopamine-β-hydroxylase in concentrated perfusates or in the sedi-

TABLE 3

Percent distribution of catecholamines, total protein, and vesicle protein in subcellular fractions of adrenal medulla homogenates[a]

Gland No.		Homogenate (mg/g)	Low speed sediment (%)	Low speed supernatant (%)	High speed supernatant (%)	High speed sediment (%)
4	CA	9.3	—	95	22	75
	TP	53.1	—	75	57	14
	VP	9.1	—	89	30	65
6P	CA	9.6	8	83	13	70
	TP	47.6	16	77	66	14
	VP	10.5	9	47	34	46
6C	CA	9.6	7	93	10	60
	TP	64.7	11	76	68	30
	VP	8.3	16	88	14	36
7	CA	6.4	7	84	21	90
	TP	47.9	15	76	70	10
	VP	9.8	4	46	30	77
11P	CA	8.9	3	89	23	57
	TP	51.6	15	60	86	23
	VP	7.0	18	91	14	53
11C	CA	9.4	3	98	26	65
	TP	50.0	18	99	76	20
	VP	9.1	8	70	20	65
14	CA	7.9	—	92	29	69
	TP	47.6	—	61	81	11
	VP	12.8	—	70	64	30
17	CA	9.1	5	91	37	61
	TP	58.6	7	78	78	15
	VP	6.4	7	188	23	61
19	CA	8.2	—	96	28	65
	TP	41.2	—	101	70	12
	VP	10.7	—	92	18	62
Average	CA	8.7 (1.1)	6 (2)	91 (5)	23 (7)	68 (9)
	TP	51.4 (6.3)	14 (4)	78 (13)	72 (9)	15 (4)
	VP	8.9 (3.1)	9 (5)	87 (40)	27 (15)	55 (14)

[a] The percent recoveries in the low speed supernatant and low speed sediment were based on the total amounts present in the homogenate; the percent recoveries in the high speed supernatant and high speed sediment were based on the amounts present in the low speed supernatant. C, Control gland; P, perfused gland; CA, catecholamines; TP, total protein; VP, specific vesicle protein; the figures in parentheses are standard deviations.

ments obtained upon centrifugation of the perfusates could not be demonstrated either enzymically or by immunoelectrophoresis (14). PENMT could not be demonstrated enzymically even when the perfusates had been concentrated tenfold. In these experiments the specific activity of PENMT in the soluble fraction of homogenates of the perfused gland was measured. From the amounts of protein present in the concentrated perfusates, we would have been easily able to detect the presence of PENMT even if its specific activity was tenfold less than that of the soluble fraction of the supernatant. In two trials no PENMT activity could be detected in the perfusates.

DISCUSSION

In evaluating the data on the concentration of the specific vesicle protein, it should be understood that the immunological assay (14) employed a twofold serial dilution having an inherent error of

−50 to +100% of the observed values. Within these limits of experimental error, the relative amounts of specific vesicle protein and catecholamines found in the perfusates was similar to that present in the intact glands.

The distribution of the vesicle protein in the various cell fractions closely paralleled the distribution of catecholamines (Table 3). In a previous report (14) it was also shown that the specific vesicle protein in intact vesicles paralleled the distribution of catecholamines after centrifugation through a sucrose density gradient. These data indicate that the specific protein and catecholamines are stored in the same vesicles in the intact cell. The extent to which catecholamines are "free" in the cytoplasm of intact cells is uncertain. At least 90% of the catecholamines can be recovered in a particulate fraction, and one could conservatively estimate that less than 1% of the catecholamines are "free" in the cytoplasm. The distribution studies of the vesicle protein reported here also indicate that most of the specific protein is within the vesicles.

The appearance of vesicle protein, adenine nucleotides (4, 5), and catecholamines in perfusates of stimulated glands in relative amounts similar to that present in intact storage vesicles strongly supports a secretory process in which the storage vesicle contents are discharged directly to the exterior of the cell. The simultaneous appearance in perfusates of both adenine nucleotides and catecholamines observed by Douglas and Poisner (4, 5) indicates that a change in the permeability of the membrane followed by diffusion of "free" catecholamines is unlikely. The presence of vesicle protein, adenine nucleotides, and catecholamines in perfusates in the same ratios as that present in intact vesicles also indicates that intracellular disruption of the storage vesicles followed by outward diffusion is unlikely, since one would not expect all three of these components to diffuse out of the cell either in equal amounts or at approximately the same rate. The failure to detect PENMT in the perfusates further indicates that the cell membrane is either impermeable to this protein or that it becomes highly selective for the permeation of the vesicle protein even though the vesicle protein has a larger Stokes[3] radius than PENMT.

The data reported here and by Douglas and Poisner (4, 5) are consistent with a process in which either the contents of the granule or the entire granule are extruded directly to the exterior of the cell. The latter seems more unlikely, and the absence of dopamine-β-hydroxylase in the perfusates also argues against this process. The time lag in the appearance of the vesicle protein in perfusates can be explained by its slower rate of diffusion through the intracellular spaces or across the endothelial lining of the capillaries. It is most interesting that Elfvin (22) has found that the capillaries of the medulla have a fenestrated appearance. The individual endothelial cells in many areas are not in close apposition but appear to be separated by a thin single-layered membrane. This seems to be a common feature of protein-secreting organs and may provide areas through which the large protein molecules can readily diffuse. Another possible explanation for the time lag of the appearance of the protein in the perfusates may be that the protein does not cross the endothelial lining of the capillaries but is eliminated through the lymphatic system of the adrenal gland.

The release of the storage vesicle protein appears to be an integral part of the secretory process, a process in which the storage vesicle contents are released in the same relative amounts as that found in the intact gland. The protein was released by the three secretogogues which released catecholamines. The release of protein and catecholamines by acetylcholine and nicotine was Ca^{++}-dependent (23) and affected to the same extent by changes in temperature. However, we have little biochemical information on the nature of this process. The temperature dependence of secretion

[3] R. Connett and N. Kirshner, unpublished observations. PENMT is retained on Sephadex G-200 and G-100 to a much larger extent than the purified vesicle protein.

indicates that chemical reactions are involved, but whether these reactions are specifically involved in the secretion process itself or whether they are required to maintain the cell in a functional state is not known. More recent observations (24) show that a source of metabolic energy that can be supplied by either glycolysis or oxidative metabolism is required for secretion, but again the nature of this requirement is not known.

ACKNOWLEDGMENT

This work was supported by grants AM-0542 and AI-06710 from the National Institutes of Health.

REFERENCES

1. H. Blaschko and A. D. Welch, *Arch. Exptl. Pathol. Pharmakol.* **219**, 27 (1953).
2. N.-A. Hillarp, S. Lagerstedt and B. Nilson, *Acta Physiol. Scand* **29**, 251 (1953).
3. N.-A. Hillarp, *in* "Adrenergic Mechanisms" (J. R. Vane, G. E. W. Wolstenholme and M. O'Connor, eds.), p. 461. Churchill, London, 1960.
4. W. W. Douglas, A. M. Poisner and R. P. Rubin, *J. Physiol.* (*London*) **179**, 130 (1965).
5. W. W. Douglas and A. M. Poisner, *J. Physiol.* (*London*) **183**, 236 (1966).
6. P. Banks and K. Helle, *J. Biochem.* **97**, 40C (1965).
7. K. B. Helle, *Mol. Pharmacol.* **2**, 298 (1966).
8. W. J. Smith and N. Kirshner, *Mol. Pharmacol.* **3**, 52 (1967).
9. N. Kirshner, H. J. Sage, W. J. Smith and A. G. Kirshner, *Federation Proc.* **25**, 735 (1966).
10. N. Kirshner, H. J. Sage, W. J. Smith and A. G. Kirshner, *Science* **154**, 529 (1966).
11. W. S. Moore and W. H. Stein, *J. Biol. Chem.* **211**, 907 (1954).
12. O. H. Lowry, N. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
13. U. S. von Euler and F. Lishajko, *Acta Physiol. Scand.* **51**, 348 (1961).
14. H. J. Sage, W. J. Smith and N. Kirshner, *Mol. Pharmacol.* **3**, 81 (1967).
15. J. Axelrod, *J. Biol. Chem.* **237**, 1657 (1962).
16. E. Marley and G. I. Prout, *J. Physiol.* (*London*) **180**, 483 (1965).
17. L. E. Hokin and A. Yoda, *Proc. Natl. Acad. Sci. U.S.* **52**, 454 (1964).
18. W. W. Douglas and R. P. Rubin, *J. Physiol.* (*London*) **159**, 40 (1961).
19. W. W. Douglas and R. P. Rubin, *J. Physiol.* (*London*) **167**, 288 (1963).
20. A. Philippu and H. J. Schumann, *Arch. Exptl. Pathol. Pharmakol.* **252**, 339 (1966).
21. W. W. Douglas and R. P. Rubin, *Nature* **203**, 305 (1964).
22. L.-G. Elfvin, *J. Ultrastruct. Res.* **12**, 687 (1965).
23. W. W. Douglas, *Pharmacol. Rev.* **18**, Pt. 1, 471 (1966).
24. N. Kirshner and W. J. Smith, *Science* **154**, 422 (1966).